PROCEEDINGS OF THE

SECOND INTERNATIONAL CONFERENCE ON AQUACULTURE NUTRITION: BIOCHEMICAL AND PHYSIOLOGICAL APPROACHES TO SHELLFISH NUTRITION

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EDITORS
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and D. CONKLIN

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PREFACE

This Second International Conference on Aquaculture Nutrition "Biochemical and Physiological Approaches to Shellfish Nutrition" in many respects can be considered a "nutritionists" nutrition conference. It was a gathering of highly respected scientists from around the world to report on and discuss their work on "real" nutrition.

Characteristically, nutrition studies on aquatic invertebrates are hampered by physical, chemical and biological instability of the feed. Extraordinary measures are required to minimize leaching and associated bacterial activity with the feed, prior to its ingestion by aquatic invertebrates.

It should be remembered that in the world of commercial intensive aquatic production systems, bacterial activities may play important roles in invertebrate nutrition. For example, it has been suggested that microbial populations in pond systems may serve as an "external" ruminant stomach for partial digestion of feed and supplements. Bacteria and algae may also enter the food web and may interact to maintain satisfactory environmental conditions.

ACKNOWLEDGMENTS

Special thanks are due to the National Office of Sea Grant, the Sea Grant Program of the University of Delaware, the U.S./Japan Aquaculture Panel, UJNR, and the World Mariculture Society for sponsoring this conference.

We express our appreciation to the honorable Spark M. Matsunaga, U.S. Senator, Hawaii for his address "Awakening the Public to Aquaculture".

We acknowledge the fine program coordination provided by Dorothy Kosey, of the Conferences and Centers Division of Continuing Education of the University of Delaware. We are indebted to Rae Clark of Delaware and Nancy Heinzel of California for their secretarial assistance and to the students and staff at the Center for Mariculture Research for their hospitality.

In many ways the quality of this proceedings is due to the extraordinary efforts of Christopher Landgon and Douglas Conklin for their unrelenting and rigorous reviews of the manuscripts. In this task they were assisted by: Mel Carriker, Chuck Epifanio, Peter Gabbott, Julius Gordon, Mike Helm, John Hilton, Dave Holland, Donal Manahan, Roger Newell, Glen Patterson, Dan Rittschof, Brenda Sanders, Sandra Shumway, Lowell Sick, Stan Slinger, Ravenna Ukeles, Mike Waldock, John Wickins, and Karl Wilbur.

Finally we are indebted to the World Mariculture Society for publishing these proceedings.

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INTRODUCTION AND WELCOME

Ellis T. Bolton

On behalf of the University of Delaware Sea Grant Program, in cooperation with the U.S./Japan Aquaculture Panel and the World Mariculture Society, my colleagues and I are pleased to welcome delegates from many nations, the speakers, and the participants to the Second International Conference on Aquaculture Nutrition. The First Aquaculture Nutrition Conference was held here just six years ago. In concluding that conference Harold Goodwin commented, "In spite of our vast ignorance, [about aquaculture nutrition] we're obviously doing something right" and suggested that we "continue to combine empiricism with definitive nutritional and physiological research". This second conference results from the fact that many scientists have anticipated and, indeed, followed Hal Goodwin's advice so that after six years it is most timely to examine what we know and where we are along the 'Biochemical and Physiological Approach to Shellfish Nutrition'.

The subject of aquaculture is large and its practice extends through millenia. Across the centuries empiricism, arts and tradition have demonstrated that aquaculture has an astonishingly high, but mostly unrealized, potential for supplementing the contribution of agriculture to man's supply of food and to help meet his need for biochemicals and energy. Yet it is only in most recent times, in about the past twenty-five years, that we find a truly burgeoning interest in applying the required scientific and technological methods towards revealing the complex framework of interrelationships by which organisms maintain themselves in aquatic environments. Today we stand poised to probe deeply into the intimate molecular and functional bases of biology so that we can better learn how organisms cope with aquatic environments for their sustenance, how they manage to exist in harmony with other forms in the aqueous continuum, and how we might take advantage of these properties to increase the productivity of natural waters and intensify cultivation under controlled conditions. The challenge is demanding. Again Goodwin puts it aptly, "Culture of any living organism begins with the environment and the aquatic environment is one of dismaying complexity. The aquatic animals we want to culture are, to a considerable extent, an extension of their environment. They are immersed in it, it flows through their gills, it either supports or stresses them, depending on its physical and chemical characteristics and their physiological response". It will take time to understand the interrelationships adequately, as it has in the agriculture counterpart, and it will take no less than the most dedicated men and women, such as those who will speak to us in this conference, to make the challenge manageable.

The science of nourishment, including the study of the nutrients that each organism must obtain from its environment lies at the very heart of the matter. Each particular kind of organism has its

distinctive needs and they can be studied separately, but there is also a far-reaching biochemical unity in nature which gives coherence to the whole subject. As a result, the bivalve nutritionist does not work in intellectual isolation from the fin-fish specialist, nor for that matter from the human nutritionist. We recognize in this common plan at the biochemical level the universal need of animals for a limited set of chemical substances including minerals, amino acids, carbohydrates, lipids, vitamins and other organic materials of diverse structure. Further, we recognize the common physical basis why this condition should be so as a result of the momentous discovery in our own time that genetic determination underlies both the nutritional needs and the processes which satisfy them. It is most fitting therefore that this conference brings together scientists at the leading edge of discovery in the nutrition of animals and that we consider fresh knowledge from phytoplankton to fishes, from chemical diffusion to process energetics, from meat to skeletal architecture, from minerals to hormones. We take pleasure in this opportunity for sharing nutritional science and inviting you to participate where the action is: in the presentations that form this program.

AWAKENING THE PUBLIC TO AQUACULTURE

Special Address by the Honorable Spark M. Matsunaga

Thank you, Mr. Bill Hougart, for your most generous introduction. Dean Gaither, Dr. Pruder, distinguished guests and delegates to the Second International Conference on Aquaculture. I am delighted and honored to join you this evening and to be able to greet you in the manner and spirit of the state I represent in the United States Congress; that is, with - ALOHA! I notice that your ranks include an important contingent from the land of my ancestors, members of the UJNR Aquaculture Panel.

You know, for the past 19 years, as a member of congress from our nation's only island state, I have flown back and forth over 2,000 miles of blue water, hundreds of times. And each time, that vast and seemingly limitless expanse has invited me, as it were, to contemplation. What an extraordinary resource, teeming with plant life and animal life, rich with minerals, holding the potential for enough power to make our petroleum problems irrelevant - and so poorly understood, so underutilized. While we struggle to meet the needs of a global population expanding geometrically, the largely overlooked aquatic resources covering 70% of the earth's surface beckon to us. And as this conference indicates, growing numbers amongst us are finally beginning to bring the scientific knowhow that is a hallmark of civilization to bear upon that part of our planet which originally gave birth to life itself. We are coming full circle, so to speak, and it is about time.

For me personally, the growing awareness of the potential offered by the cultivation of aquatic life constitutes a vindication. As some of you may know, I am regarded by many of my colleagues in the Senate as something of a visionary. As a member of the Senate Energy and Natural Resources Committee, I have consistently championed the cause of renewable energy, insisting that the United States Senate pay more attention to such seemingly exotic technologies as solar photovoltaics, ocean thermal energy conversion, biomass production, lowhead hydro and geothermal energy. I have introduced bills with names like The Advanced Storage Battery Act, The Hydrogen Economy Act, The Photovoltaic Research Act, Development and Demonstration Act, that initially caused some of my colleagues to shake their heads in puzzlement. But no longer. Renewable resources are steadily, inexorably, moving into the mainstream of energy production; and as they do, the doubters are joining the believers in welcoming an energy future opening outward rather than closing in upon itself. Such is becoming the case also, more slowly in the United States but no less inexorably, I believe, for aquaculture.

It hasn't been easy. My files are bulging with testimony, speeches, correspondence, drafts of legislation, recording my efforts on behalf of aquaculture during the past two decades. I especially recall joining with my colleague from Hawaii, Senator Daniel K. Inouye, and Senator Lloyd Bentsen of Texas, in delivering the speeches on the Senate floor that accompanied the introduction of the National Aquaculture Act of 1980. We had shepherded a similar piece of legislation through the Congress in 1978, only to have it pocket vetoed by the President, due to objections to certain financial incentives the bill contained.

But this time, we made it. The bill signed into law by the President in 1980 set in motion, among other things, an 18-month effort by an interagency task force to prepare the First National Aquaculture Development Plan, under the able direction of Bill Hougart, with a view toward bringing cohesion and direction to the government's fragmented and all-too-limited activities in this field. Aquaculture entrepeneurs and researchers had felt the need for some time. As made clear by a study on regulatory constraints prepared in association with the task force effort, and here I quote: "Serious regulatory constraints can arise merely from the absence of proper coordination and direction of existing, even well-intended legislative programs for aquaculture." Especially, I might add, when those programs involve thirteen federal agencies directly and another dozen indirectly, as had been the case in this instance.

But a new era is dawning. Only last Friday, the National Aquaculture Development Plan was presented for public review, prior to its formal submission to Congress next March. It is an admirable document. Now, at last, an operating framework has been established for a long overdue coordinated commitment to aquaculture policy and development in the United States.

I can testify to the significance of such a framework from the perspective of the first state to develop one. The Hawaii State Aquaculture Plan is a model of its kind. Out of it have grown these state-sponsored activities: a low-interest aquaculture loan program; a disease diagnosis and prevention program; free marketing and site selection assistance; a mapping project identifying suitable lands for aquaculture, funding efforts in pond management, nutrition, engineering systems, processing and handling; a prawn promotion program; a program offering free prawn larvae to small businesses desiring to enter aquaculture; an extremely significant streamlining of the state permit process from two years to as little as two months. As a result, Hawaii's nascent aquaculture industry now boasts 40 commercial aquaculture farms, producing prawns, oysters, catfish, saltwater and freshwater finfish, and marine shrimp. In addition, research centers are working with mullet, milkfish and carp, among others. And on my native island of Kauai, where, to give you some idea of how the wind is blowing, the Chairman of the County Council is a marine biologist, another is a marine biologist who is operating his own Tilapia research facility, with the announced intention of marketing commercially within two years. Even Japan Air Lines has gotten into the act, with the announcement earlier this year of special commodity rates for freshwater prawns and marine shrimp, on the Honolulu to Tokyo run. Nearly all this activity dates from the introduction of the Hawaii State Plan three years ago. Now, with an infrastructure in place and serious inquiries from prospective aquaculture farmers running at an average of seven a

week, the state believes the industry is at the takeoff point. Revenues are expected to jump from $2 million last year to $20 million in 1982.

Hawaii, of course, is a special case. Its year round tropical climate makes it especially suited for aquaculture. Its mid-ocean location is by itself a lure to aquatic-minded entrepreneurs.

Nonetheless, it was not until a plan was developed by the government and its objectives pushed by the government that aquaculture really began to take hold in Hawaii.

Hawaii thus offers a case for a plan encompassing the nation as a whole as a prerequisite to any meaningful development of aquaculture on a national scale.

Fifty disconnected state plans can't do it, no more for aquaculture than for agriculture. Iowa, for instance, has an effective state agricultural effort capable of producing results on its own. But it has been in tandem with national agricultural policies and programs that the Iowa effort has reached its current level of international repute. Indeed, in most instances, it has been national agricultural policy that has created the conditions necessary for successful state agricultural programs. So it must also be for aquaculture. My own state of Hawaii has come a long way. But it will never realize its full potential unless the federal government makes a parallel commitment.

But stating that requirement amounts also to stating the major problem facing aquaculture in America today, with or without a national plan. The key to the success of any such plan, as the case of Hawaii demonstrates, must be a perceived need. Hawaii's Aquaculture Plan is prerequisite to meaningful development, but it still would not bring a sympathetic public. A recent Department of Commerce Report on Aquaculture summed up one aspect of the problem neatly: "For the foreseeable future, marine aquaculture will produce limited quantities of high-priced seafood, and will become a major source of inexpensive animal protein only if national policies so dictate. Federal funding directly targeted at aquaculture during fiscal year 1982 probably won't exceed $10 million, and it may even be considerably less. National policymakers do not perceive a need for a major commitment to aquaculture - nor, for that matter, do the vast majority of the American people - yet.

That "yet" is what makes the difference between an aquaculture pessimist and an aquaculture optimist. It also suggests a distinction that I believe is vital to the fruitful consideration of any National Aquaculture Development Plan. There can be no doubt that the coordination achieved as a result of this plan, even with limited funding, will result in important advances in aquaculture research and development. Nothing is more important for the pursuit of an interest within the halls of government than an organized constituency. This plan organizes the budding aquaculture constituency. One of its first dividends, I would hope, would be further redirection of benefits obtainable from numerous agricultural programs toward aquaculture - research, extension services, financial assistance. That could result in obtaining major appropriations while avoiding an as - yet unsympathetic appropriation process. It also would serve to establish fish farmers on a parity with other farmers in the eyes of the federal government, which is where they belong, and which would set the stage for a steadily expanding federal commitment. Most agricultural research

programs were conceived for the purpose of encouraging new technologies. They have been successful to the point now of redundancy in many instances. The introduction of aquaculture into the center of that process would insure a position in the top most category for research funding. The benefits for aquaculture research would be considerable, to put it mildly. Other Aquaculture Development Plan dividends would come from a speed-up in information transfer for researchers and in permit clearance for entrepreneurs. Altogether, it constitutes a most promising package and Bill Hougart and his colleagues on the Interagency Task Force are to be congratulated for their superior efforts.

The distinction I want to make is between the preceding category of dividends and what I consider to be an overriding objective, namely making American policymakers and the American public aware of the need for aquaculture development, to the point where a major national commitment ensues.

Now, heretofore, among those of us who support aquaculture, that overriding objective has been pursued by what I would call a process of argument. While research and private enterprise advance incrementally, we speak out on the larger issues that might bring our cause to the attention of policymakers and the public at large.

To list a few:

(1) Indications are that the global harvest is approaching maximum sustainable yields. It has levelled off at 70 million metric tons, and the best estimates do not exceed 100 million metric tons. Important shortages are predicted for within 3 to 10 years, worldwide.

(2) Only last week, the World Food Council reported that the number of people suffering from malnutrition will double during the 1980s to one billion. The era of cheap food imports is ending for less-developed countries. They need inexpensive sources of protein, fast.

(3) Although wild harvests are stagnating, fish farming is showing important gains. Worldwide output has doubled in the past five years, to 6 million metric tons, or 10% of world fisheries production. Some countries, especially in Southeast Asia, rely on aquaculture for 40% of their total fisheries production.

(4) In 1979, the People's Republic of China obtained 1.2 million metric tons of fish and fish products through aquaculture; Japan 480,000; the Soviet Union 190,000; the United States, a mere 65,000.

(5) In order to expand production still further, Japan has launched a 7-year coastal fisheries development program which includes $333 million for aquaculture. Mexico has a $1 billion fisheries program, of which $200 million is targeted for aquaculture. Canada will spend $300 million restoring salmon stocks in British Columbia. In the United States, funds for aquaculture are a comparably inconsequential fraction of those accorded by our less financially endowed neighbors to the north and south, not to mention Japan.

(6) Marine shrimp, the most valuable U.S. fishery, is in the midst of a crisis, traceable in large measure to skyrocketing energy costs. In 1980, the dockside value of imported shrimp was $719 million, nearly double the domestic catch.

(7) Domestic oyster production is down while demand is up. Since 1950, American oyster imports have increased from .5% to 25% of the market.

(8) The American lobster is on the verge of a major decline. During the past decade, domestic landings have held steady, but fishing efforts have quadrupled. That means a major intrusion into the reproduction process that could bring catastrophic results.

(9) Although aquaculture accounts for less than 3% of domestic seafood yields in the U.S., and is operating midst a host of constraints and virtually without federal financial support, it has nonetheless recorded impressive gains in key areas. Twenty-five percent of the salmon consumed in the United States originates in hatcheries. Private aquaculture produces 40% of our oysters, 50% of our catfish and crawfish, nearly 100% of our trout.

(10) Catfish offers a telling example of the potential. The catfish aquaculture industry has captured half the domestic market in barely 20 years. (In 1955, there were less than 50 hectares of catfish ponds. By 1975, the figure was 18,809 hectares. Domestic production leaped from 9 million pounds in 1969 to 84 million in 1975.) There are now an estimated 3,000 catfish farmers in 42 states.

(11) In light of the above, it is worth keeping in mind that the United States has over 2.5 million farm ponds, covering 2.7 million acres. The potential of aquaculture as a supplementary income for farmers is enormous. It could take the peaks and valleys out of many farming operations, especially amongst small farmers. It could thus also become an important stabilizing element for rural communities while combating unemployment.

(12) The United States imports 60% of the fish products it consumes thereby adding $2 billion to our balance of payments deficit. Fish imports account for an astonishing 28% of our nonpetroleum-related imports.

(13) Fish and fish products consumption is increasing in the United States, up 30% from 1960, from 10 pounds per capita to 13 pounds.

(14) While demand has increased, the United States commercial harvest has held steady for more than a decade. It is estimated that demand will nearly double by the year 2000. Under present conditions, that demand will have to be met by imports in an increasingly competitive global market, meaning drastically higher prices and uncertain availability.

(15) Although per capita consumption of fish and fish products is increasing in the United States, we still lag behind the rest of the world. We consume 13 pounds per capita annually. The world average is 24 pounds. In Japan and Iceland, it is 79 pounds. Yet fish are very efficient converters of feed to flesh; they offer an excellent balance of essential amino acids and are high in essential minerals and water soluble vitamins; and they are, on the average, lower than any other meat in fat content - they are arguably the most complete and healthiest source of animal protein available in the world today, at a time when the cultivation of other animal protein products, notably beef, is being pushed to its limits and the cost and availability of feed grain could by itself trigger a global nutrition crisis.

My list of arguments could continue, but I believe it is enough even at this point to convince anyone of the manifold and vastly underrated significance of aquaculture in the world today. Yet as one who has put those arguments forward in Congress and other public forums, in one form or another, for nearly two decades, I must report to you that they are still not enough to establish the sense of national need that would give real impetus to National Aquaculture Development Plan. It's an old, familiar story. By their very size and complexity, issues of this scope invite a sense of helplessness among all but those directly involved. Furthermore, the general public derives limited inspiration from a largely incomprehensible research effort. That's not a judgment but a fact. Goals must therefore be translated into concrete sharply-focused targets, dramatic in concept and capable of offering high visibility and widespread appeal if a national commitment to aquaculture is to be obtained. Before leaving you tonight, I would like to offer two specific suggestions of that order.

First, I would like to suggest to you that the newly-organized National Aquaculture Constituency launch a concerted effort to integrate aquaculture products into the nations's $2 billion school lunch program, with emphasis on low-value products for which worldwide demand is highest and U.S. demand minimal due mainly to sociological factors. Catfish or mullet offer as much protein as striped bass or shrimp and at a fraction of the price. I do not doubt that their entry into the school lunch program would mean a significant increase in protein intake among those American children most likely to suffer from malnutrition, the disadvantaged poor. (And it would come at ages when the side effects of insufficient protiein can be most damaging, as you well know. And imagine the impact upon the fish farming industry! It would prove decisive, I believe, in catapulting the industry into the mainstream of food production, where it belongs.)

Now I can guess what some of you may be thinking. How does one induce American school kids to consume catfish and mullet? Frozen fish sticks, steamed fish cakes, tempura and fish patties are possibilities that should be investigated. They could be made quite tasty, certainly tastier than a lot of the fare currently being offered at taxpayer's expense. And once children developed a taste for such products, they would carry it forward into adulthood, to the considerable benefit of their personal health, and the health of the aquaculture industry.

For those not familiar with it, the USDA-Administered School Lunch Program consists primarily of block grants to states that are funnelled down to school districts, to be spent as those districts see fit so long as they meet nutritional requirements. I would suggest that the aquaculture constituency, which now has a strong base in the Agriculture Department, work to establish pilot projects in school districts, with a goal of eventual nationwide participation. This is a project in which researchers, extension workers, and entrepreneurs could all participate.

My second suggestion has to do with the nutritional requirements of developing nations. Statistics indicate that aquaculture is catching on far more rapidly in the developing world than in the United States, moreover, the American effort is targeted pretty much toward high-value fish and shellfish with relatively complex life cycles and needs, such as shrimp and oysters. The people of developing nations, on the other hand, are looking more toward freshwater fish with simple life cycles

capable of living in muddy or poorly oxygenated waters. The prime example is tilapia. Others would be mullet and milkfish. But efficient development of those relatively less complex species still requires a level of scientific and technical sophistication that is sorely lacking in the devleoping world, in the areas of genetics, nutrition, disease prevention, reproductive biology, ecology, and technology. Tilapia, carp, mullet and milkfish researchers in Hawaii tell me that the unsolicited requests for technical assistance from the developing world far exceed their limited capacities. The requests are not for handouts. They are for expertise in the science of survival.

As a first step to meeting that important global need, I will be submitting a change in wording to an amendment to Public Law 89-808, the USDA-Administered Food for Peace Program. The amendment is Section 406 and I am familiar with it because I wrote it; in fact, it is often referred to as the "Matsunaga Amendment."

Section 406 was inspired by the need to strengthen food production capabilities in the so-called "hunger-belt" of food deficient countries. Most of those countries, as you know, are in tropical and subtropical regions, which happen to be ideally suited for aquaculture. Section 406 authorizes the Secretary of Agriculture, and here I quote, "to conduct research in tropical and subtropical agriculture for improvement and development of tropical and subtropical food products for dissemination and cultivation in friendly countries." My amendment to the Matsunaga Amendment would make it clear that aquaculture research also qualifies and that special attention should be given to technical training in that field.

My suggestion to the aquaculture constituency, then, is that you support me in insisting upon the need for such programs and in working out various means to implement them, either through the establishment of institutes in the United States or research and training missions abroad - or both. By the same token, you might enlist the support of colleagues in Japan, Europe, Canada, and elsewhere in the industrialized world. This is to me an ideal, vitally-needed, desparately important North-South project. It would at once provide protein and production knowhow, survival and self-sufficiency.

FISH NUTRITION

FISH NUTRITION - RELEVANCE TO INVERTEBRATES

Colin B. Cowey[1] and Albert G.J. Tacon[2]

ABSTRACT

A number of topics in nutrition that have immediate relevance to the cultivation of invertebrates and which experience with fish suggests are susceptible to particular approaches are presented. First is optimal amino acid balance, where the evidence suggests that the pattern of essential amino acids in the product formed (usually muscle) forms a reasonable basis for the requirement pattern; additional methods (i.e. weight gain) of assessing the requirement for individual amino acids are considered. A second topic is essential fatty acid requirement, here knowledge on the synthetic capacity of the animal, readily obtained by tracer methods, can provide data on some of the characteristics of the fatty acids required; modification of the fatty acid spectrum of live foods to meet the requirements of the larvae for which they are intended is another useful procedure and one which might be extended to the study of other nutrients; various ways of assessing dietary antioxidant status based on tissue analysis and physiological tests with cells or sub-cellular organelles are discussed. Finally factors involved in food presentation are examined; the value of feeding attractants is emphasized and principles involved in resolution and identification of them in fish are stated.

KEY WORDS: Essential amino acids, optimal balance, essential fatty acids, antioxidant nutritional status, diet presentation, feeding attractant, fish.

INTRODUCTION

Detailed studies on the nutrition of several domesticated vertebrates have been carried out over the last sixty years or so. During the past twenty years similar studies have been applied to a number of fish species. The over-riding picture from these studies is that of a qualitative uniformity of requirements among vertebrates. Variations on this common theme do arise as in the requirement for

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certain species for certain vitamins (e.g. that of primates, the guinea pig and various fish for ascorbic acid); there are shades of emphasis as between carnivores and herbivores and there are obvious and important differences between animals such as ruminants, with a vast microbial input, and non-ruminants.

This qualitative uniformity appears also to extend to invertebrates for nutritional requirements of insects bear a distinct similarity to those of vertebrates (Dadd 1982). Differences between these phyla do occur - insects (and Crustacea) have an absolute requirement for sterols while vertebrates are able to synthesize them from simple carbon sources - but the basic pattern persists. Fish and aquatic invertebrates have another common characteristic, namely, life in water and this implies for marine fish a simplified mineral requirement (Sakamoto and Yone 1979). Mineral requirements of aquatic invertebrates may therefore mirror those of fish especially in species that possess a calcified exoskeleton.

The nutrition of fish has been reviewed several times, the earlier literature being covered by Halver (1972) and by Cowey and Sargent (1972). The latter authors have updated their review (Cowey and Sargent 1979) and in addition a statement of the requirements of cold water fish has also recently appeared (Anon 1981). It is not the intent of the present authors to again review this literature. Those interested in invertebrate nutrition and who see the relevance to it of fish nutrition are likely already to be acquainted with the literature. Those who do not are unlikely to be stimulated by a further review. Instead, the emphasis in this chapter is placed upon selected areas in which it is suggested advances might be made by the application of particular approaches.

These areas are quantitation of the pattern of essential amino acid requirement, assessment of essential fatty acid requirment using tracer methods to assess the synthetic capacity of the animal with regard to chain elongation and desaturation, modification of the fatty acid spectrum of live food used to culture larvae so that it meets the essential fatty acid requirements of the larvae, possible extension of this approach to other nutrients such as carotenoids and establishment of the nature of taste and olfactory attractants. These areas were selected because of their intrinsic importance and because the problems concerned seemed capable of resolution.

OPTIMAL BALANCE OF AMINO ACIDS IN THE DIET

The nutritive value of a dietary protein is governed by the extent to which its content of essential amino acids simulate the needs of the animal in question. The closer the composition of the protein to the pattern of requirement the greater will be its nutritive value. Theoretically even poor proteins (i.e. those in which there is little correspondence between amino acid composition and pattern of requirement) should meet the needs of an animal provided sufficient amounts are eaten. In practice such proteins rarely seem able to support high rates of growth in mammals and birds and there is some evidence that this is also true of fish (cf. Rumsey and Ketola 1975). Perhaps the assimilation of excessive amounts of certain amino acids may lead to a derangement of amino acid metabolism.

In young growing animals the greatest proportion of body weight gain is in the form of muscle. It is reasonable to infer therefore that the pattern of dietary essential amino acid requirement will be closely related to, or even governed by, the pattern of amino acids present in muscle protein. This proposition has been examined for birds and mammals by Boorman (1980) and Figure 1 shows the correlation he obtained between pattern of amino acids in muscle and requirements in the diet of pig and chick. The deviations from muscle pattern of lysine and sulphur amino acid requirements in the case of the chick were related to feather growth while lysine requirement of the pig may be overestimated and that of isoleucine underestimated. Boorman concluded that, "in the absence of more detailed information the pattern of essential amino acids in the product can be used for the pattern in the diet".

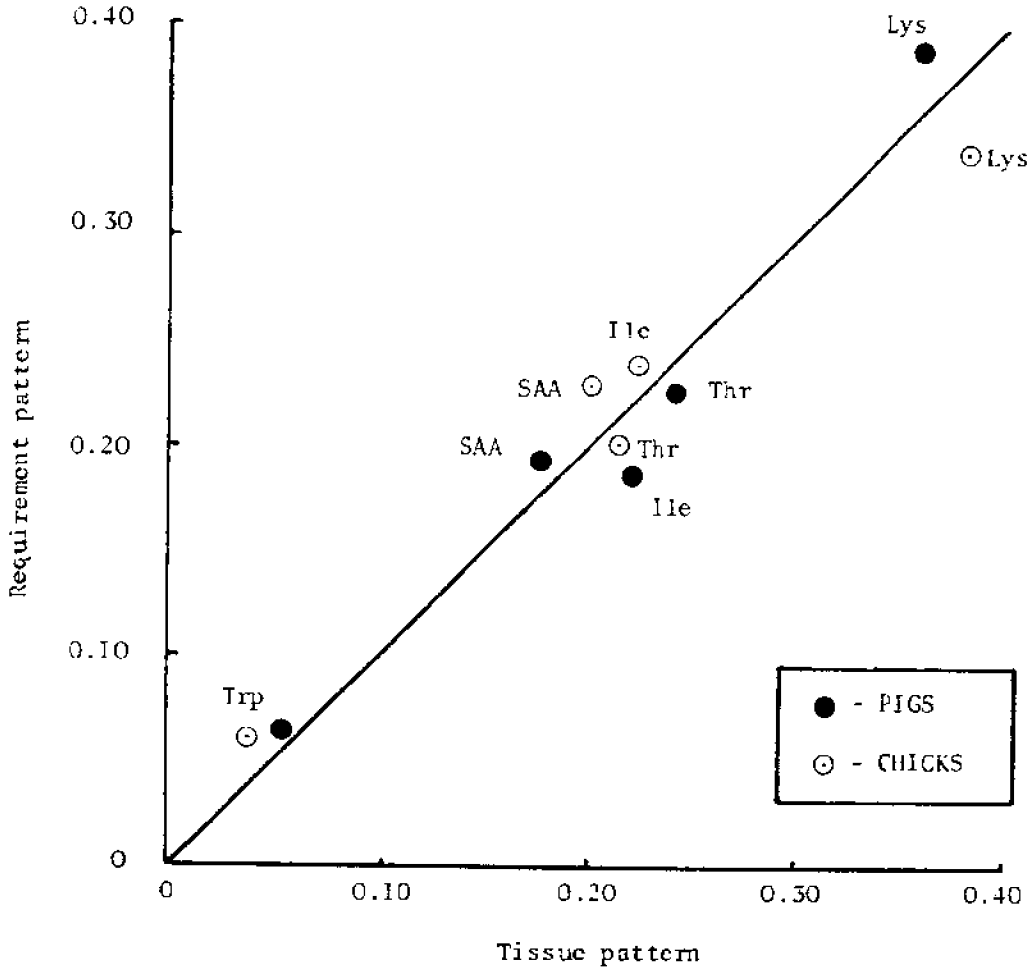

Figure 1. **Relationships between pattern of amino acid requirements found by feeding experiments for lysine (Lys), methionine plus cystine (SAA), isoleucine (Ile), threonine (Thr) and tryptophan (Trp) and the pattern of the same amino acids in tissue (muscle) of growing pigs and chicks. The level of each amino acid is represented as a fraction of the sum of all five in each pattern. The line represents coincidence of requirement and tissue patterns. (Published courtesy of Dr. K. N. Boorman and Butterworth & Co. (Publishers) Ltd.).**

A similar correlation is shown in Figure 2 for carp (Cyprinus carpio) where the values of requirement for 10 essential amino acids are those obtained by Nose (1979) from dose/response curves using amino acid test diets. Although there appears to be under-estimation of leucine and lysine requirement and some over-estimation of requirement for two or three other amino acids, the relationship obtained between pattern of essential amino acids in the muscle or whole body tissue and the requirement pattern of the same amino acids is surprisingly close. This is especially so in view of (1) the inexactness of measurements of amino acid requirement and (2) the fact that this approach to arriving at an optimal pattern for dietary essential amino acids takes no account of their metabolic fate in the body other than for protein synthesis, i.e. no allowance is made for maintenance.

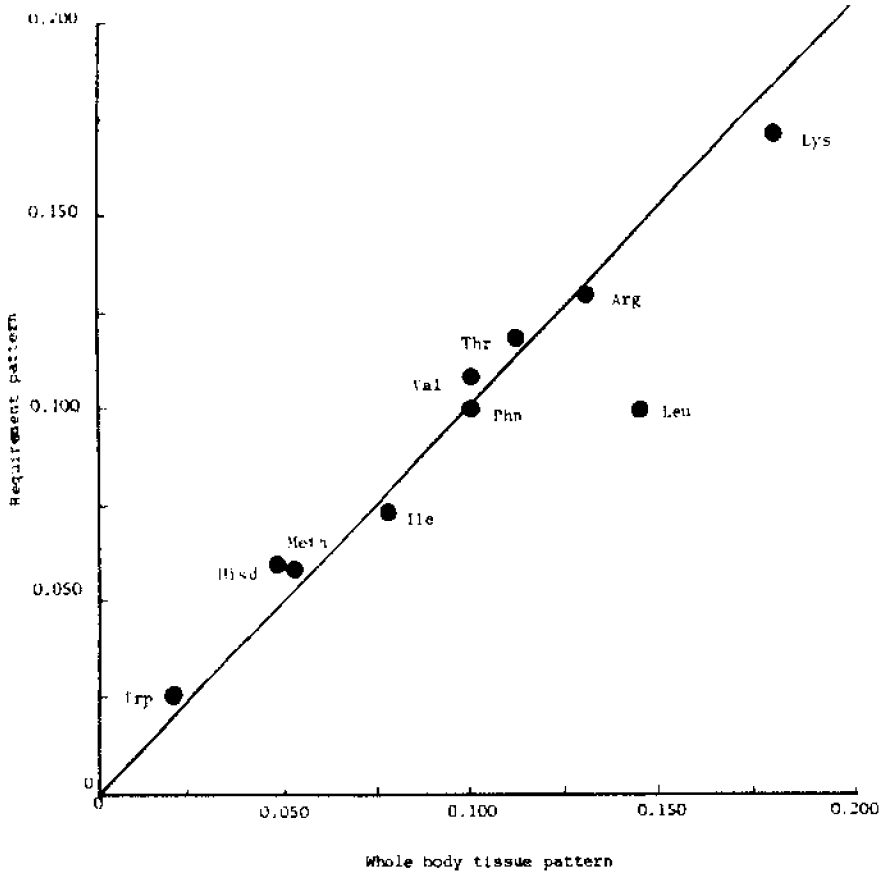

Figure 2. Relationship between pattern of requirements found by feeding experiments for ten essential amino acids as determined by Nose (1979) using amino acid test diets and the pattern of the same amino acids in the whole body of growing carp (Cyprinus carpio).

Lack of precision over measurement of amino acid requirement stems from a number of causes. Growth rate of several species given a diet in which the "protein" component is composed of free amino acids is frequently inferior to that of fish given a diet of nearly identical amino acid composition but composed of protein (e.g. Murai et al. 1981; Walton et al. 1982). Secondly, with substances that are readily soluble

in water there is an obvious danger that losses will occur as a consequence of leaching. The extent of such losses will vary considerably with feeding habits of the fish, the feeding procedures employed and the water stability of the pellets. Thirdly, interpretation of the dose/response curve itself is frequently subjective, in particular the point of intersection of the two portions (plateau and slope) of the curve cannot always be discerned exactly.

Little information is available on the requirements of fish for maintenance. Ogino and Chen (1973) evaluated maintenance requirements of carp given dietary proteins of biological values between 30 and 80 as being a constant 12% of absorbed N, that is, a relatively small proportion of utilized dietary protein. A much larger proportion of absorbed N (29% in the case of a protein of biological value 80) was however categorized as "waste energy". Presumably some of this N would be properly ascribed to maintenance, as pathways of amino acid metabolism, in addition to protein synthesis, are stimulated during rapid growth. Nevertheless it is unlikely that any one amino acid will be used very much more for maintenance purposes than are others. Fish have no special requirement for amino acids such as for feather growth in chicks and it appears unlikely that maintenance needs will greatly influence the pattern of requirements.

As the amino acid composition of muscle for different species of fish is similar (Connell and Howgate 1957) it follows from the thesis developed here that the pattern of requirement for different species will also be similar. These patterns are shown from currently available data for four species of fish in Figure 3. While apparent differences do occur between species for one or two amino acids (arginine and methionine in chinook salmon; lysine in channel catfish) there is a general similarity of requirement between all four species. Taken together with the empirical manner in which amino acid requirements are investigated and the difficulties outlined above, inherent in accurate measurements of requirement, the use of a pattern based on that of muscle protein provides a very useful first base for assessment of invertebrate requirements. This is especially so as accurate measurement of amino acid requirement in invertebrates is likely to be more hazardous than that of fish. With filter feeders, particles of large surface area to volume ratios may be exposed to leaching for unacceptably long periods of time, besides which the filter feeders may be selective of particles ingested. With carnivorous crustaceans food pellets may be torn apart and only portions of them ingested, again resulting in serious leaching of nutrients from food not immediately eaten.

Adoption of this method of arriving at a pattern of essential amino acids still leaves the desirability of measuring the requirement for one or two amino acids in order to ascertain the optimal intake of a dietary protein component of the defined pattern. For this purpose proteins either entirely, or relatively, deficient in an essential amino acid may be combined with high quality proteins in diets in such a way as to provide graded dietary concentrations of the amino acids. Thus Kaushik (1977) used combinations of zein and fishmeal to measure the arginine requirement of rainbow trout. Such mixtures may be particularly apposite in studies on invertebrates. They may also be combined with biochemical procedures aimed at establishing the limit of response in a dose-response curve.

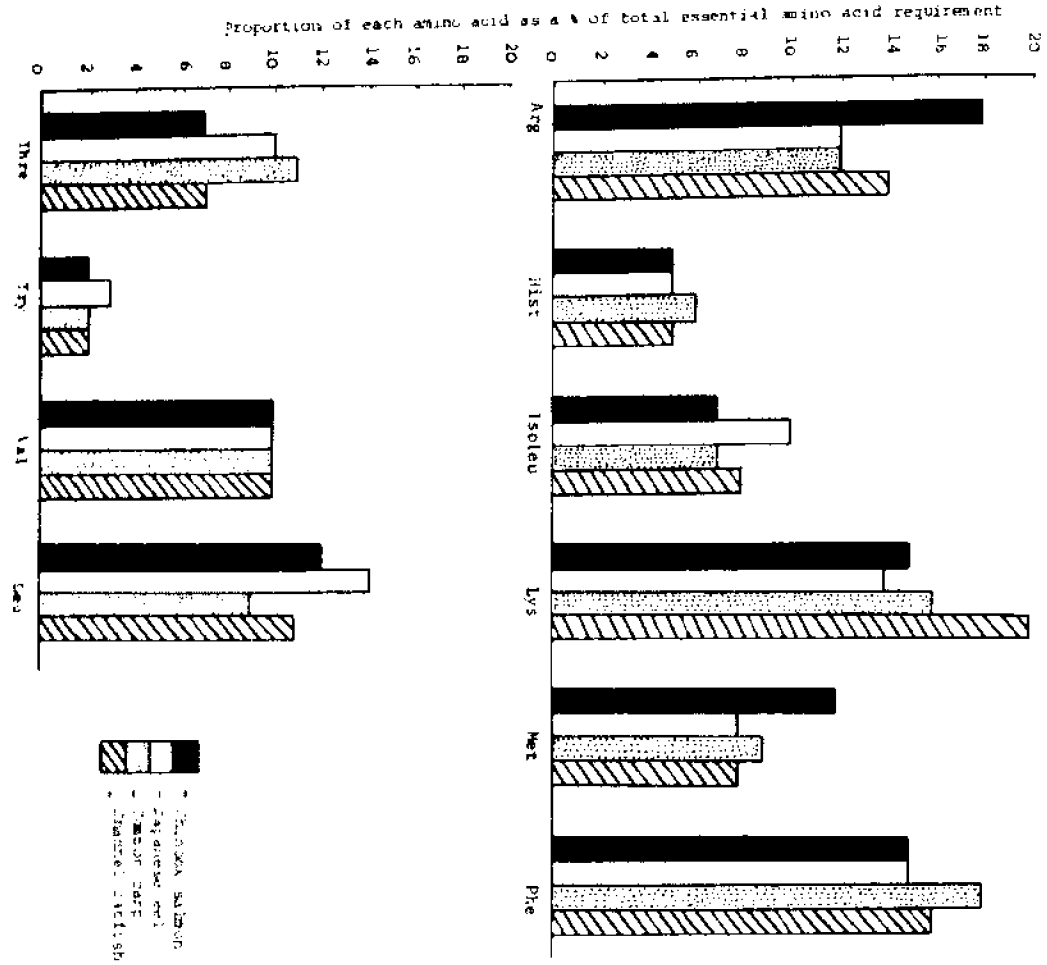

Figure 3. Relative amounts of each of ten essential amino acids required by four species of fish. The proportion of each amino acid is represented as a fraction of the sum of all ten amino acids. Data for chinook salmon (Oncorhynchus tshawytscha) are from Mertz (1969), for Japanese eel (Anguilla japonica) and for carp (Cyprinus carpio) from Nose (1979), and for channel catfish (Ictalurus punctatus) from Wilson and his colleagues (Wilson et al. 1978; Robinson et al. 1980).

One such procedure is measurement of concentrations of free amino acids in whole blood, plasma or other tissues (liver, muscle) of animals given a diet, such as that described above, in which although the amino acid component is supplied as protein, graded concentrations of the amino acid under test are present in the different treatments. The method, which has been applied to several species of fish (Kaushik 1979; Plakas et al. 1980) relies on the fact that; as the concentration of the amino acid in the diet increases; a point is reached where supply exceeds demand (requirement) and free amino acid concentrations in tissue pools increase (Wilson et al. 1978). Not all tissues and amino

acids respond in the same way (Robinson et al. 1980) and as with any method careful evaluation is necessary in applying it to invertebrates.

A possible further development of this procedure is to examine the oxidation of the radioactively labelled amino acid (administered either orally or by injection) in animals given diets containing graded levels of the amino acid under test. Again the method relies on a rapid increase in oxidation of the amino acid once demand for protein synthesis has been satisfied and again the method should be carefully evaluated for the animal and amino acid in question. Thus Cowey (1975) was not able to detect any effect of level of protein intake on oxidation [^{14}C] glutamate, leucine or phenylalanine in plaice (Pleuronectes platessa). However, oxidation of either [^{14}COOH] or [^{14}CH$_3$] methionine by rainbow trout increased with increasing concentrations of dietary methionine (Walton et al. 1982).

ESSENTIAL FATTY ACID REQUIREMENT AND ANTIOXIDANT STATUS

To some extent the essential fatty acid (EFA) requirements of fish are related to their feeding habit and position in the aquatic food chain and upon the physical and chemical nature of the environment they inhabit. In general, cold water fish are likely to be more demanding in their requirement for EFA than warm water fish if only because constraints imposed in maintaining membrane fluidity are greater at low temperatures (Hazel 1979). This may also be true of invertebrates. Similarly carnivorous fish that obtain a surplus of polyunsaturated fatty acids (PUFA) in their natural diet may possess less ability to interconvert (mainly chain elongate and desaturate) fatty acids than do herbivorous species and thus be more demanding in EFA requirements. This again may hold true for invertebrates.

Specific EFA deficiency diseases were demonstrated in rainbow trout about 10 years ago by Castell and his colleagues (see Castell 1979 for review). These diseases could be prevented by the addition of linolenic acid, 18:3(n-3) to purified diets at a level of 1%. No other fatty acids when added to purified fat-free diets possessed this capability. Dietary linolenic acid was chain elongated and further desaturated by the rainbow trout to 20:5(n-3) and 22:6(n-3) acids which were incorporated in membrane lipids. In the absence of a dietary source of 18:3(n-3), eicosatrienoic acid (20:3(n-9)) accumulated in the polar lipids of the tissues being a fatty acid which the trout could itself synthesize de novo from ordinarily available materials. Consequently Castell et al. (1972) suggested that the ratio $\frac{20:3(N-9)}{22:6(N-3)}$ in polar lipids from the liver would serve as an index of the sufficiency or otherwise of dietary linolenic acid intake in trout. If this ratio was less than 0.4 then the diet contained an adequate supply of linolenic acid (or higher acid of the (n-3) series).

Subsequently it was shown that not all species of fish were able to desaturate and chain elongate 18:3(n-3) to acids with full EFA activity (20:5(n-3) and 22:6(n-3)). Cowey et al. (1976a) were first to demonstrate that turbot (Scophthalmus maximus) is incapable of carrying out this conversion. This finding was fully confirmed (Owen et al. 1975) by experiments in which [^{14}C] oleic, linoleic and linolenic acids were fed separately to turbot and to rainbow trout. In turbot radioactivity remained almost wholly in the parent fatty acid fed with

little chain elongation and no desaturation. In rainbow trout on the other hand, extensive incorporation of radioactivity was found in 22:6(n-3) from the radioactive linolenic acid fed. Experiments (Cowey et al. 1976b) in which purified diets containing specific fatty acids were fed to turbot further confirmed the inability of these fish to convert linolenic acid to arachidonic acid. Consequently for optimal growth and freedom from pathology turbot must be given diets containing preformed (n-3) series PUFA. Moreover the fatty acid ratio suggested by Castell et al. (1972) as an index of dietary EFA sufficiency cannot be applied to turbot as they do not synthesize either of the fatty acids concerned.

Since these experiments were carried out, several other species have been shown unable to chain elongate and desaturate eighteen carbon fatty acids, thus red sea bream (Chrysophyrs major) must also be supplied with preformed (n-3) series PUFA for normal growth (Yamada et al. 1980). The use of [^{14}C] precursors to investigate fatty acid biosynthetic capacities serves to show whether eighteen carbon fatty acids are likely to meet the EFA requirements of the fish in question or not and has been extended to other species (Kanazawa et al. 1980). The method may also provide a ready answer to this question, if applied to invertebrates and indeed studies of this type on prawns have already been reported (Kanazawa et al. 1979a,b).

EFA requirement among fish species appears further complicated in that certain freshwater species including common carp (Cyprinus carpio), eel (Anguilla japonica), channel catfish (Ictalurus punctatus) and tilapia (Tilapia zilli) appear to have a requirement for both (n-3) series and (n-6) series fatty acids (Cowey and Sargent 1977; Kanazawa et al. 1979c, 1980).

The relevance of these findings to invertebrates is that a whole spectrum of EFA requirements may be present among species of interest ranging from an absolute requirement for (n-3) or (n-6) PUFA to none. If anything, marine species may be more exacting in their requirements than those of freshwater species.

The larval stages of a number of marine (and perhaps some fresh water) fish are reared, both in the laboratory and in commercial practice, for a period of time on live food organisms. These live food organisms must, of course, supply all the essential nutrients. Some time ago it became evident (Scott and Middleton 1979) that two of the most commonly used organisms, a rotifer (Brachionus plicatilis) and the brine shrimp (Artemia salina), did not always provide an adequate complement of EFA.

Methods of modifying the fatty acid profiles of these organisms so that they do supply a sufficiency of EFA to the fish larvae have been developed by Watanabe (1981). He was able to alter the PUFA content of Brachionus plicatilis in either of two ways, 1) indirectly, by feeding the rotifers on baker's yeast (Saccharomyces cerevisiae) which had been grown in a medium supplemented with fish oil or cuttlefish liver oil (both of which are rich in PUFA of the (n-3) series), 2) by feeding the rotifers directly on emulsions of lipid (of appropriate fatty acid composition) raw egg yolk and water together with baker's yeast. These methods were equally effective in ensuring that the rotifers served as a consistent and adequate source of EFA so improving the growth and survival of marine fish larvae to which they were fed. If, as seems

probable, live food organisms are to be used for the early rearing stages of larval invertebrate species, at least until a satisfactory alternative is found, then the methods developed by Watanabe (1981) are highly relevant.

Highly unsaturated fatty acids of the type described above that are necessary to meet the EFA requirements of the animal and that function within the biomembranes of the animal are particularly vulnerable to free radical attack, hydrogen abstraction and a free radical chain reaction leading to the formation of unstable hydro-peroxides that break down to toxic carbonyl. Animals are susceptible to peroxidative attacks on membrane lipids because free radicals do occur as part and parcel of their own metabolism (e.g. terminal respiration) and any uncontrolled release of free radicals would have pathological consequences. Presumably as a result of this, animals have evolved a multilevel defense system against free radical attack and the ultimate stage in this system is the free radical chain terminator α-tocopherol or vitamin E.

Fish under intensive cultivation are at risk not only from free radicals produced normally in the course of metabolism but also from the highly unsaturated marine oils (if they are not protected by synthetic or natural anti-oxidants) used as dietary sources of EFA and from fishmeal. The latter material is almost ubiquitous in commercial diets and; if improperly prepared, the relatively high levels of unsaturated lipid present in it may, as a result of the harsh preparative procedures involved, lead to the occurrence of peroxides and their unbroken products with deleterious results for fish to which it is eventually fed. Suffice it to say that mass mortalities, linked to the presence of rancid fat in the diet, are known to have occurred in commercial practice. Thus nutritional indices of the status of the fish with respect to vitamin E intake are of considerable value. A number of such indices are being applied to fish and may have equal relevance to invertebrates, they include:

1. Tissue levels of α-tocopherol and especially its concentration in liver or hepatopancreas. The concentration of α-tocoperhol in the liver of rainbow trout given purified diets without supplementary tocopherol for 16 weeks was 7.04 μg/g tissue and such a level would certainly indicate a deficiency (Cowey et al. 1981).

2. Molar ratios of PUFA to tocopherol in lipids from microsomes or mitochondria of liver as suggested by Evarts and Bieri (1974) for mammals. It will be clear from the opening paragraph of this section that PUFA and tocopherol are related (see also Watanabe et al. 1977) and that low tissue levels of the latter would diminish the protection afforded the former against peroxidative attack. The molar ratio of PUFA/tocopherol in the livers of trout given diets supplemented with 100 mg α-tocopherol/kg was 170 and values of this order would indicate a sufficient intake (Cowey et al. 1981).

3. Because of its functional role in preventing peroxidation of unsaturated fatty acids, lipids and membranous organelles from tissues of animals deficient in tocopherol will form many more hydroperoxides when incubated under controlled conditions in vitro than will lipids from healthy animals. Bieri and Anderson (1960) first exploited this relationship as a guide to tocopherol status when they measured ascorbic acid $-Fe^{3+}$ stimulation of lipid peroxidation in rat livers. This method has now been successfully applied to ascertaining of dietary tocopherol

requirements of rainbow trout (Cowey et al. 1981).

4. In mammals, measures of erythrocyte fragility (Draper and Csallany 1969) have proved a very reliable indication of tocopherol deficiency. In rainbow trout also they are a sensitive indicator of this condition and may be used to assess tocopherol status (Cowey et al. 1981). Whether any readily available cells from invertebrates are similarly sensitive remains to be seen and is an area worthy of investigation.

FACTORS RELATED TO DIET PRESENTATION

Exploitation of knowledge on nutritional requirements, demands some understanding of feeding behavior. A complete, balanced diet loses much of its value if not delivered nearly intact to the gastro-intestinal system of the fish. The particular problem of nutrient losses by leaching has already been touched on and these may be especially severe in those fish or invertebrate species which do not immediately consume their feed but instead nibble or leave some of it in the water for a considerable period of time before ingestion.

Water soluble vitamins (Murai and Andrews 1975; Hilton et al. 1977; Goldblatt et al. 1979; Slinger et al. 1979), free amino acids, and certain minerals (Goldblatt et al. 1979) are reported rapidly lost through leaching from uncoated, conventional, compounded feeds. Losses of 5-20%, 17-27% and 55-67% for pantothenic, folic and ascorbic acids respectively occurred through leaching from 1.18 - 2.36 mm crumbled trout pellets after being agitated for only 10 seconds in water (Slinger et al. 1979); concentrations of pantothenic, folic and ascorbic acids at the outset were 174, 9.6 and 441 mg/kg respectively.

By contrast little significant leaching of water soluble nutrients occurred in ethyl cellulose encapsulated crustacean ratios (Goldblatt et al. 1979) and the low losses of ascorbic acid recorded in these diets (rarely exceeding 20%) contrasts with those found by Slinger et al. (1979). The latter authors point out that measurement of leaching losses should serve as a useful indicator of safety margins. They also suggest that by using ingredients containing high levels of vitamins in practical diets, the effects of leaching losses are likely to be reduced. Those familiar with the levels of B vitamins present in commercial "vitamin free" casein (having used it for assessment of B vitamin requirement) will echo this thought.

Nowhere is nutrient leaching more of a hazard than in diets for larval fish where size of mouthparts necessitate the use of food particles with a very high surface area/volume ratio (particles of diameter 100μ used in experiments with first feeding turbot). These problems have led to the use of numerous sophisticated artificial diet preparations ranging from crumbs and flakes to micro-encapsulated preparations (Jones et al. 1976; Beck and Poston, 1980; Seidel et al. 1980). No artificial diets have yet been produced, however, which will support growth and survival of larval fish comparable to that of live food organisms. Even so the consensus is that failure of artificial diets to match live food is unlikely to be the result of nutrient deficiency. The view has been expressed that the undeveloped digestive system of the newly hatched larva and a need for exogenous enzymes that might be provided from live food (Dabrowska et al. 1979) may explain the

gap between live and artificial foods in supporting larval growth. In fact it is always likely that live food providing, for example, large amounts of soluble protein of high nutritive value at relatively high dilution will remain superior to insoluble proteins that have been subject to a degree of processing.

The manipulation of the composition of live food organisms to provide a suitable EFA complement for fish has already been mentioned. This general approach might, with profit, be extended to other nutrients to test their essentiality. For example it has been suggested that the carotenoid pigments, present naturally in significant concentrations in Artemia salina and other live food, may be nutritionally important during larval development in fish (Tacon 1981). The possible nutritive value of these substances in their active cis form might be tested by culturing Artemia salina over successive generations in the dark so producing strains free of carotenoids. Their food value for fish larvae could then be compared with normal strains Artemia salina.

Knowledge of the feeding behavior of the fish species under cultivation should be harnessed to the full to elicit a feeding response and consequent ingestion of food. For example, the feeding behavior of the Atlantic salmon (Salmo salar) differs from that of the brown trout (Salmo trutta) and rainbow trout in that the fry after yolk sac absorption do not actively swim and search for food but remain stationary on the bottom of the tank and await food particles to drift into their path. In view of the feeding behavior of Atlantic salmon fry it is essential that a slow sinking diet, of appropriate density and particle size, be supplied directly into the path or feeding station of the fry so as to elicit maximum feeding response and consumption. In addition it must be borne in mind that the sinking characteristics of particulate diets in water will vary depending on the water flow and tank design employed.

Most of the artificial food presented to fish under cultivation is very obviously different in texture and taste from that which they consume in the natural environment. As attempts are made to use greater amounts of novel proteins and proteins of vegetable origin in practical diets, the latter are likely to become even more alien and the problem of acceptability to the fish becomes greater. For visual feeders the food must be attractive enough that it is retained in the mouth and in this sense internal taste appears to control food intake. Several groups of fish also possess an external sense of taste, often on lips and barbels, sometimes over the entire body surface, and in some species, (e.g. Dover sole, Solea solea) the food is carefully sensed prior to ingestion. It may be noted parenthetically that although the terms olfaction and gustation are applied to aquatic organisms they have rather different meanings than in the terrestrial sense; volatility is not important but many fish do use chemical signals to detect food at a distance by means of olfactory and gustatory receptors. The various activators involved in feeding behavior of fish are defined by Mackie (1982).

The starting point for studies on taste attractants has been the preferred natural food of the fish in question. This may be fractionated by chemical procedures, and each fraction bioassayed for attractant activity. The active fraction is analyzed chemically and the active components in it may be identified by making a synthetic mixture and running omission tests with the bioassay. The crucial requirement

in such studies is a quantitative bioassay. If this is linked to quantitative chemical analyses realistic comparisons can be made between the stimulatory capacity of the preferred food and that of the extracted fractions and final synthetic compound or mixture of compounds.

Studies using this general approach have been applied to a limited number of species of fish by Carr (1982), Hidaka (1982) and Mackie (1982). These studies indicate that the feeding response in different species of fish is stimulated by different chemical compounds. In turbot the major stimulant was a single substance inosine (Mackie and Adron 1978), in trout it was a mixture of L-amino acids (Adron and Mackie 1978), while a mixture of L-amino acids together with glycine and betaine acted on Dover sole (Mackie et al. 1980), puffer Fugu pardalis (Hidaka 1982), pinfish (Lagodon rhomboides) and the pig fish (Orthopristis chrysopterus) (Carr 1982). However, in the pinfish, other as yet unidentified chemicals are also involved (Carr 1982). These studies provide a measure of support for the view Bardach and Villars (1974) that "biologically meaningful scents" will generally be due to mixtures of compounds rather than single substances. Carr (1982) noted that substances so far identified as feeding stimulants for fish are characterized by 1) low molecular weight (< 1000), 2) they are non-volatile, 3) they all contain nitrogen, 4) they are amphoteric.

These studies have obvious relevance to the rearing of invertebrates in captivity. It has already been shown, Carr (1978), that a mixture of amino acids and betaine elicit a feeding reponse in the shrimp Palaemonetes pugio and a number of earlier papers cited by Carr (1978) indicate a significant stimulatory role for betaine in molluscs and other crustaceans.

CONCLUSIONS

There is a close relationship between the pattern of essential amino acids in the muscle or whole body tissue of a number of fish and their requirement pattern. This should be exploited for invertebrates where measurement of requirements for individual amino acids is likely to be inexact and difficult. Changes in the concentration of an amino acid in tissues as the level of it in the diet is increased may be helpful in attempting to measure optimal intake of one or other of the amino acids within the pattern.

The ability of animals to chain elongate and desaturate eighteen carbon fatty acids is readily assessed isotopically. This will show whether certain eighteen carbon fatty acids can meet EFA requirements or whether more unsaturated longer chain acids with full EFA activity must be provided preformed in the diet. Certain live food organisms can be cultured in such a way that their content of unsaturated fatty acids will meet EFA requirements of larval fishes. This method of manipulating the composition of live food organisms may provide a useful tool in larval nutritional studies with other nutrients.

Food should be presented in a form that elicits a feeding response. This entails some knowledge of feeding behavior and an ability to modify the physical characteristics of food pellets (buoyancy characteristics). Food attractants have an obvious role in stimulating feeding. They have been useful as a research tool in fish nutrition and may yet be used in practical diets.

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QUESTIONS AND ANSWERS

QUESTION

CASTELL: (Halifax:) I'm interested in the carotenoids. If you intend using brine shrimp and grow them in the dark, so that you produce a brine shrimp that has no carotenoid, can you do that without changing any other nutrient in the animal?

TACON: I am not sure if Artemia grown for successive generations in the dark, and on a substrate devoid of cartenoids, would undergo biochemical changes other than that of carotenoid depletion.

CASTELL: You were talking before the meeting about the possible nutritional value of carotenoids to fish and live food organisms commonly used in aquaculture. Would you like to mention some of these functions to the rest of the audience?

TACON: Yes. The carotenoids are a complex group of plant synthesized polyene pigments. Evidence indicates that carotenoids may perform other biochemical functions in animal nutrition apart from their metabolic role as dietary sources of provitamin A and their behavioral association with courtship and to sexual dichromatism in fish. In particular, carotenoids have been shown to be potent inhibitors of both in vivo and in vitro photodynamic damage in plants, photosynthetic and non-photosynthetic bacteria, and model lipid membrane systems, and have been implicated in bovine fertility. In fish, carotenoids may perform a specific biological role during egg and larval development, similar to that of α-tocopherol (as a naturally occurring antioxidant). However, much further research is required before the true biological role of carotenoids in animal nutrition can be assessed. The study of carotenoids function is hampered to a large extent by their extreme susceptibility to oxidation, and their conversion from the active cis- to the inactive trans-form.

QUESTION

SIMPSON: (Rhode Island:) If you want to grow Artemia that are free of carotenoids you would have to grow them for generations on something like rice bran. However, I find your comments on possible carotenoid function very interesting. You mentioned the work of Lofthammer in regard to carotenoids acting as a vitamin in their own right. Very interestingly a paper was presented at the last carotenoid symposium in Liverpool in which a group from Israel questioned this data and showed supporting data so I do not know where that situation lies.

QUESTION

CONKLIN: (California, Davis): You indicated that microencapsulation techniques were readily available to prevent the leaching of water soluble nutrients. I'm not familiar with techniques that form capsules that retain water soluble nutrients. Could you elaborate on them?

TACON: I'm in no way an expert on microencapsulation. A person in Scotland who is doing a lot of work on this is Dr. J. W. Adron in Aberdeen. He found that depending on the microencapsulation technique employed and on the properties of the capsule used to surround the food particle (i.e. whether semi-permable or not) affected the loss by leaching of water soluble vitamins.

CONKLIN: It is my contention that we don't have a readily available microencapsulation technique to utilize. Maybe some people do, but it's not widely available and I still have a great feeling than any unknown live food factor that can't be reproduced in microencapsulated diets may be related still to significant leaching of these water soluble nutrients. Thank you.

QUESTION

KITTREDGE: (Southern California): You feel that the free amino acid content of the tissue is a reflection of their concentration in the diet. Work that's been done over the past 20 years has shown that the free amino acid pattern in almost any tissue is very tightly metabolically controlled. You can recognize the free amino acid pattern in nervous tissue or any other tissue.

TACON: To answer that, there are 3 people in the audience today who have looked at the levels of free amino acids in various tissue pools in relation to intake and their work which is very sound shows that once the requirement of amino acid has been met that the free amino acid level of that particular amino acid in a muscle or in whole blood will rise. It is at least partly a question of rate of input versus rate of removal by catabolic (oxidation) and anabolic (protein synthesis) processes. Changes in hepatic portal concentration of free amino acids relative to food intake have long been recognized.

INSECT NUTRITION

INSECT NUTRITION: RELEVANCE TO MARINE INVERTEBRATES

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ABSTRACT

Recent studies covering all essential mosquito nutrients provide a focus for this overview of insect nutrition. Essential nutrients for insects and vertebrates are for the most part qualitatively uniform within and between the taxa, suggesting that substantial uniformity probably embraces other invertebrates. Given qualitative nutrient uniformity, extreme dietetic diversity of insects may largely be accounted for by differences in optimal nutrient proportionalities, dependence on specific phagostimulants, and dependence on symbiotes for digestive assistance and scarce nutrients. This review will emphasize aspects of nutrition that set insects apart from vertebrates and are probably of most interest to other invertebrate nutritionists, especially those studying the taxonomically close Crustacea. The trace mineral status of sodium and calcium for insects contrasts with major vertebrate requirements; for calcium, this clearly relates to bonelessness, and in this context calcified crustaceans and molluscs more likely parallel vertebrates. One absolute nutritional difference between insects and vertebrates concerns the universal insect dietary requirement for sterol, biosynthesized by vertebrates but not by insects. Sterol requirements are reported for crustaceans and probably characterize the entire Arthropoda. Requirements for amino acids, nucleic acids and other growth factors limited to particular insect groups are noted, with emphasis on those such as nucleotides and ascorbic acid of known importance for crustaceans. Ascorbic acid, essential for most phytophagous insects, is possibly a distinguishing requirement of animals whose natural dietetics center on live plant tissues. The currently most active area of insect nutrition concerns lipid growth factors. Recent work on sterol and essential fatty acid metabolism and possible requirements for vitamins A, E and K are discussed.

Keywords: Insects, nutrition, synthetic diets, essential amino acids, vitamins, growth factors, sterols, polyunsaturated fatty acids, prostaglandins.

Invertebrate nutrition is heavily indebted to prior work with vertebrates, and most knowledge of insect requirements has been accrued from the application of findings first obtained using mammals. For example, the role of vitamins in rats, humans and the like was determined during the early decades of this century, and as pure vitamins became commercially available by the late 40s, they were tested as replacements for the essential but ill-defined yeast and liver extracts of early artificial insect diets. At this time it was widely thought that because many insects grow on seemingly unnutritious or narrowly specific foods, their requirements might encompass unique factors not needed by vertebrates. By and large this proved not so; although a few unique insect requirements indeed emerged, notably for sterol, qualitative requirements for the two taxa are for the most part similar.

The oddity or narrow specificity of many insect natural diets is thought to indicate one of two things: those having unlikely food, such as wood, wax or wool, generally depend on the cryptic assistance of symbiotic microorganisms for digestion or the provision of scarce nutrients; and fastidious insects limited to a specific food, such as the single plant species suitable for a monophage, are limited by behavioural rather than nutritional constraints, having evolved dependence on specific and often non-nutritive chemical stimuli from the food to trigger feeding behaviour or to determine oviposition sites for mothers of the feeding larval stages. Once these matters were appreciated by the mid-1950's, synthetic diets of more or less complete definition became commonplace for a great variety of insects and opened the way to our present extensive knowledge of the nutritional requirements of the class.

Because insects are largely terrestrial and their food a discrete component of a basically dry environment, the physical stability of artificial foods is unproblematic, and their chemical stability, if threatened with putrefaction, can be enhanced by simple aseptic techniques. Furthermore, the various non-nutritional problems that may arise from excessive bodily contact with osmotically-active or surface-active components in a dietary medium for an aquatic animal are avoided or mitigated. It is largely these features of discreteness and stability of food that determine the relative ease of nutritional study of insects as compared with other, mostly aquatic, invertebrates. Economically important aquatic molluscs and crustacea, when confined with artificial food, soon become immersed in a solution of rapidly destabilizing diet, making precise data difficult to obtain.

Given that insect nutrition is now qualitatively well understood, can it illuminate those problems that are of prime interest to the shellfish nutritionist? Insect and vertebrate nutritional requirements are basically similar, suggesting that requirements of metazoan animals in general share much in common. Nevertheless, the needs of insects do differ in some significant respects from those of vertebrates, and to the extent that insects and crustaceans are phyletic cousins, such differences suggest areas in which practitioners of crustacean nutrition may look to insects rather than vertebrates for inspiration.

With the above in mind this review of insect nutrition will mainly skim the surface of the subject so as to concentrate on those features

in which all or some insects depart from the vertebrate pattern. Also emphasized will be topics in lipid nutrition that are currently under most intensive investigation. It is perhaps also appropriate to introduce insect nutrition to dieticians and nutritionists dealing with marine invertebrates by focusing on mosquitoes, a group of insects whose larval growth stages are entirely aquatic. Among insects this is rather unusual, and the study of which posed many of the same problems facing all who contemplate rigorous nutritional work with an aquatic animal, namely, how to ensure actual feeding on a manipulable artificial diet provided to an animal immersed in water.

FEEDING BEHAVIOUR AND SYNTHETIC DIET DEVELOPMENT

Like most insects, mosquitoes obtain nutrients for growth mainly during their larval stages, their adult nutrition being limited to the satisfaction of needs for water, sugar for energy, and, in the female only, proteinaceous blood meals for development of eggs. The four morphologically similar larval stages are all filter feeders. Hence the problem of developing synthetic diets for nutritional work had much in common with the problems of those concerned with nutrition of copepods, the particle-feeding juvenile stages of the larger crustacea, and bivalves.

When feeding naturally, the rapidly beating mouthbrushes of mosquito larvae filter out particulate material of limited size (optimally 1-10 μm) from the ambient water and pass it via pharynx and short oesophagus into the straight tubular midgut running most of the length of the body. Solids in the midgut are normally moved rearwards only by the ingestion anteriorly of fresh particulate solids, which push back existing midgut contents. If particulate material of appropriate dimensions is available and filtering activity continuous, transit of solids through the gut of the largest (4th stage) larvae takes about an hour. Particulate material is ingested indiscriminately, but maintenance of filtering activity depends upon the presence of phagostimulant solutes in the ambient water. Most soluble nutrients, such as sugars and amino acids, have only a moderate effect on filtering and ingestion, but nucleic acids and their component nucleotides, especially adenylic acid, are powerful phagostimulants. Thus, in the vicinity of any source of phagostimulants such as decaying organic tissues, filtering activity and the consequent ingestion of the associated microbes, the natural food of mosquito larvae, tends to be enhanced (Dadd 1970a, b).

Given this mode of feeding, it seemed likely that a particulate component would be needed in artificial diets; the best previous mosquito diets, for Aedes aegypti, were based on proteins such as casein sedimented in a dilute solution of all other nutrients (Akov 1962). When diets with all nutrients in solution were first tried, growth and survival were low and feeding studies indicated poor ingestion (Dadd 1972). Microencapsulated diets (Jones et al. 1974) were considered but the complex problem of retaining water-soluble nutrients within microcapsules in water was avoided by finding a means to enhance the drinking by mosquito larvae of the nutrient solution in which they lived. If water is texturized with very low concentrations of certain colloids, it is ingested to the midgut as readily as particulate solids (Dadd 1975). A colloid-texturized medium (0.05% agar) was well-ingested and development times and survival of larvae approached those reared in

crude natural media (Dadd and Kleinjan 1976). With such texturized, fully defined basal media, nutrient requirements were systematically determined by suitable manipulations of individual dietary components.

GENERAL NUTRITIONAL REQUIREMENTS

Table I shows the composition of a synthetic dietary medium in which we have reared 14 sequential generations of Culex pipiens axenically and are thus fairly confident that it contains all required nutrients, though not necessarily in optimal proportions. Except for fat, the mosquito recipe includes most of the major bulk components that are to be anticipated in any animal diet: there is carbohydrate, as sugar; a mixture of amino acids replaces the proteins of most natural diets; and there is a group of major minerals. Among the minor components are several trace minerals; the lipid nutrients, cholesterol, arachidonic acid and vitamins A and E; and several water-soluble growth factors, including most B vitamins and a group of nucleic acid constituents. Three minor components of the diet fill non-nutritional functions: agarose is the texturizer that facilitates ingestion; ascorbyl palmitate is an antioxidant included to protect arachidonic acid, not to supply vitamin C, which is not required dietarily by mosquitoes though it is by many other insects; and synthetic lecithin facilitating lipid dispersion.

This recipe also provides a convenient basis for an overview of insect nutrient requirements in general, reserving features of special pertinence to other invertebrates for more detailed consideration later. In what follows, many of the general statements made will be unsupported by specific citations since they are based on results obtained with many and diverse insects and now form part of the accepted cannon of insect nutrition. The subject has been reviewed perhaps 20 times over the past three decades and individual studies that provide the basis for unreferenced generalizations can be traced by consulting the most recent of these reviews (Dadd 1970c, 1973, 1977; House 1972, 1974; Friend and Dadd 1982).

CARBOHYDRATE AND OTHER ENERGY SOURCES

C. pipens completes development with no dietary carbohydrate, but at a reduced growth rate. Many other insects, especially carnivores such as the flesh flies, can dispense with carbohydrate entirely while maintaining normal larval growth. On the other hand, carbohydrate of some sort (various sugars or polysaccharides, depending upon the insects' digestive enzyme complement) is needed in the diet of most larval phytophagous insects, usually in substantial proportions: seed and cereal feeders require carbohydrate at levels of 20 to 70% of their dietary solids, and the plantsucking aphids, which feed on phloem sap, need a diet containing about 80% by weight of all nutrient solids as sucrose. To complicate the issue, for many insects that require no carbohydrate as larvae, or may even find it detrimental, sugar is often the only nutrient required for normal adult longevity, as is the case for mosquitoes. Thus, dietary carbohydrate needs may be highly variable between species and also between the juveniles and adult stages of the same species, reflecting the chemistry of the insects' natural foods and changing at metamorphosis if life-style and food change.

Table I. Composition of Mosquito Synthetic Dietary Medium (Dadd and Kleinjan 1978)

Component	mg/100 ml
Sucrose/Glucose	750
19 L-Amino acids[a]	1390
Na and K Phosphates[b]	60
Mg sulphate	20
Trace minerals	21.5
Choline chloride	10
Water-soluble B vitamins[c]	7.1
5 Nucleotides (A,G,C,U,T)	160
Cholesterol	1
Arachidonic acid[d]	0.4
Vitamins A and E[d]	0.2
Ascorbyl palmitate[d]	0.8
Dipalmitoyl lecithin[d]	2
Agarose	50

[a]Alanine, arginine*, asparagine*, aspartic acid, cycteine, glutamic acid, glycine, histidine*, isoleucine*, leucine*, methionine*, phenylalaine*, proline*, serine, threonine*, tryptophan*, valine*, tyrosine. Asterisks indicate essential for C. pipiens.

[b]Ca gluconate 5, Na chloride 10, Fe sequestrene 2, Zn sequestrene 2, Ma sequestrene 2 and Cu sequestrene 0.5; Geigy sequestrenes are chelates with sodium EDTA.

[c]Thiamine, riboflavine, nicotinic acid/amide, pyridoxine, pantothenate, folic acid, biotin.

[d]For methods of incorporation of lipids see Dadd and Kleinjan (1978, 1979).

Mosquitoes require no bulk lipid, nor is it essential for any of the 100 or so insect species that have been reared on defined diets, though if present in the food, many undoubtedly utilize it efficiently as an energy source. Even the waxmoth, whose natural diet, beescomb, consists largely of beeswax, grows normally on an artificial diet with carbohydrate completely replacing wax as the major energy source. Insects in general thus seem to have no need of lipid beyond the quantitatively minor but very important growth factors discussed later.

Since many dipterous larvae grow best with diets lacking both carbohydrate and bulk lipid they evidently satisfy their energy needs entirely by oxidation of protein-derived amino acids. Like the mosquito, several phytophagous larval insects require substantial carbohydrate for optimal growth but nevertheless complete development at a reduced rate without it (Harvey 1974), and thus can facultatively operate an amino acid-based energy metabolism. The hide beetle, Dermestes maculatus, grows well with the triglyceride completely replacing the carbohydrate otherwise required (Applebaum et al., 1971), and thus, like the waxworm, evidently utilizes lipid and carbohydrate interchangeably. Probably most insects have some ability to induct metabolites from carbohydrate, protein and fat into the pathways of

energy production, though for most, such versatility is an adjunct to a primary dependence on carbohydrate.

INORGANIC REQUIREMENTS

Major minerals provided in the C. pipiens diet are sodium and potassium phosphates and magnesium sulphate, but there is no major source of calcium, which for insects in general is a trace requiremnt. Compared with the high vertebrate calcium requirement, its relative unimportance for insects is often ascribed to their lack of bone. Quite clearly, heavy calcification of many crustacean and molluscan exoskeletons would entail high calcium needs, setting their mineral metabolism and nutrition apart from that of insects. Providing phosphates only as potassium salts, thereby reducing sodium to trace levels, has no adverse effect on mosquito growth; in general it is difficult to demonstrate a sodium requirement in insects above the trace contaminant levels in virtually all dietary components. Except for sodium, all trace metals listed in Table 1 have been shown necessary for optimal growth of mosquito larvae (Dadd unpublished) and similar trace requirements have been determined for a few other insects. Insect mineral nutrition is a somewhat neglected area. Most synthetic diets incorporate one or other of the standard mammalian salt mixtures, though for insects these have grossly excessive amounts of calcium, sodium and chloride. The fact that such diets are nevertheless satisfactory for nearly all species studied says more about the homeostatic prowess of insects than about their mineral needs.

WATER-SOLUBLE VITAMINS

All B vitamins except B_{12} (cyanocobalamin) are probably required by all insects, although these requirements are often masked in insects harboring yeast or bacterial symbiotes. The status of B_{12} for insects is uncertain; a few studies, including one for another mosquito, Aedes aegypti (Singh and Brown 1957), have claimed it is needed for optimal growth, but in most trials it had no effect and generally is not included in synthetic diets. Cyanocobalamin does not improve the growth of C. pipiens.

Two other water-soluble growth factors not needed by mosquitoes, inositol and vitamin C, are commonly required by other insects. Both have been shown necessary for some crustaceans, and their status in insect nutrition is therefore germane to this discussion, particularly as they illustrate the parallel coevolution of specific nutrient needs with particular natural types of food.

Several insects that feed on plant materials, though by no means all, require dietary inositol, presumably for incorporation into the phosphatidylinositol component of their membrane and transport phospholipids. However, a majority of insects studied require no dietary inositol and must be able to biosynthesize it. Plant feeders that do not require it presumably have lost the biosynthetic capability because of the ample amounts of combined inositol present in plants.

A very clear relationship to phytophagy governs the status of vitamin C in insect nutrition. It is essential for nearly all species

whose natural food is living plant tissue, but is not required by any insect so far studied with a different feeding habit. For example, most leaf-feeding caterpillars, beetles and grasshoppers, and plant-sucking aphids and leafhoppers need dietary ascorbic acid, whereas caterpillars, beetle larvae and cockroach pests of stored seed and cereal products and carnivorous or saprophytic fly larvae do not require it. Histological and chemical methods have shown ascorbic acid to be present in tissues of many insects, including some that do not require it in their diet. It has been proposed (Dadd 1973), though recently questioned (Kramer and Seib in press), that an ability to biosynthesize ascorbic acid was the original insect condition and that taxa which subsequently evolved primarily as feeders on live plant tissues lost the ascorbate biosynthetic ability as a result of the very high levels always present in their natural food. This is essentially the view of ascorbate need in vertebrates; most can synthesize vitamin C from sugar, but a few taxa, thought to have a major frugivorous or vegetarian feeding habit in their recent evolutionary history, have lost certain steps of the biosynthetic pathway (Chippendale 1975). As more crustacean species become amenable to nutritional study, it will be interesting to see whether a similar pattern of essentiality linked to the ascorbic acid content of the natural diet will emerge.

A few other water-soluble materials (α-lipoic acid, glutathione, spermidine) have been claimed as essential or at least beneficial growth factors to one insect species or another (Dadd 1970a). Carnitine, chemically related to choline and biochemically involved in the fatty acid metabolism of insects as in other animals is essential for all species of the beetle family Tenebrionidae that have been studied nutritionally, but it is unnecessary for all other insects though it may spare choline in some. The tenebrionid carnitine requirement is of interest in showing that in spite of a general uniformity in qualitative nutrition, unique and specific requirements may sometimes characterize certain narrowly defined taxa.

AMINO ACIDS

All insects that have been studied require the same 10 essential amino acids originally found necessary for the rat, excluding from consideration species such as aphids in which symbiotes have been shown to supply essential amino acids lacking from the diet. For several insects additional amino acids are essential. Larvae of the flesh fly, Phormia regina, and the silkworm, Bombyx mori, require proline, and these insects also cannot complete development unless their dietary amino acid mixture includes either glutamic or aspartic acid. Although a few insect species develop normally on synthetic diets containing only the 10 rat-essential amino acids, most require in addition some non-essentials, which vary from species to species and may include glutamic acid, alanine, serine and glycine. The latter was claimed as essential for some insects in early studies, but this was probably due to imbalances in the proportions of other amino acids.

The earlier insect literature presented occasional data suggesting that a sulphur-containing amino acid, cystine, and a phenolic amino acid, tyrosine, were required nutrients for some species. Tyrosine, of great importance in the schlerotization and melanization of insect cuticle, is derived metabolically from phenylalanine, which is

essential; hence tyrosine might appear necessary with suboptimal amounts of dietary phenylalanine, which it spares. Cystine or cysteine can sometimes spare the essential sulphur-containing amino acid, methionine, and also may appear to be essential because it sometimes acts as a supplementary sulphur source in diets with inadequate sulphate.

C. pipiens not only requires dietary proline, but is unique among insects studied thus far in having an absolute requirement for asparagine (Dadd 1978), needed at much the same dietary concentration as other amino acids and thus a major structural requirement rather than a vitamin-like growth factor. Another mosquito, Aedes aegypti, requires neither asparagine nor aspartic acid (Singh and Brown 1957; Dadd 1978), and a preliminary survey of 6 other mosquito species indicates asparagine essentially for some but not others.

This unusual asparagine requirement seems unique in the animal literature, with two exceptions. Asparagine is essential for growth of certain mammalian leukemia cell lines, owing to their lack of asparagine synthetase, which in normal tissues converts aspartic acid to asparagine (Wriston and Yellin 1973). Secondly, and very pertinent to the present topic, a radioisotope study to indicate essential amino acids for the crayfish, Astacus leptodactylus, found that although aspartic acid became labelled following injection of labelled glucose, asparagine remained unlabelled, indicating essentiality (Van Marrewijk and Zandee 1975). As pointed out, these findings raised the question of asparagine essentiality for this crayfish, and suggest the possibility it may be required by other crustaceans. It is also noteworthy that recent studies indicate a requirement for dietary asparagine in pregnant and weanling rats (Newburg and Fillios 1979).

NUCLEIC ACIDS AND DERIVATIVES

As nucleic acid components are needed in synthetic diets developed for brine shrimp, certain copepods and other crustaceans, their status in insect nutrition calls for special comment even though most insects studied have no need for dietary nucleic acids and the class as a whole is evidently capable of their biosynthesis. However, it is characteristic of Diptera that an optimal larval diet must contain nucleic acids or certain precursors thereof. For many of these Diptera, nucleic acids are not essential for complete development to the adult stage, but larval growth rate is severely retarded without them, indicating rate-limiting biosynthesis. A number of Diptera, however, including the screwworm, Cochlyomyia hominivorax, certain strains of D. melanogaster and the mosquitos, C. pipiens and Aedes aegypti, have an absolute requirement for dietary nucleic acid or certain nucleotides to complete development, and in these cases one must presume the complete loss of some biosynthetic steps. Much of the early work dealt only with crude nucleic acids, and an interesting point to emerge from such studies is that whereas yeast RNA is always effective, sperm or thymus DNA generally is not. These points are well illustrated by C. pipiens, which develops very poorly and dies before adulthood without nucleic acid or with sperm DNA, but grows well to the adult stage with yeast RNA. Good development also results if sperm DNA is supplemented with uridylic acid; the supplement is a normal component of RNAs but not DNAs, being replaced in the latter by the deoxynucleotide, thymidylic acid, and this would seem to account for the general finding that DNA is

inadequate. However, if one substitutes for yeast RNA and the 4 ribonucleotides that are its main constituents (adenylic, guanylic, cytidylic and uridylic acids), development is incomplete, whereas complete development is obtained with adenylic, guanylic, cytidylic and thymidylic acids, a mixture which provides all DNA bases. These results with nucleotide mixtures indicate a need for thymidylic acid, which would seem to conflict with the results obtained with yeast RNA considered to lack thymine containing constitutents. However, crude yeast RNA contains a proportion of transfer RNA, which has small amounts of thymine in its base composition, and this is presumably why it is effective. The failure of most previous attempts to replace crude nucleic acids with nucleotides for other Diptera may be due to the fact that in no case was a thymine-containing component included.

With adequate and defined nucleotide mixtures, it became possible to specify the minimal nucleic acid constituents essential for C. pipiens, namely: the purine ribonucleotide adenylic acid (with inosinic acid and the corresponding nucleosides, adenosine and inosine, almost as effective) plus a pyrimidine ribonucleoside, either uridine or cytidine, and the pyrimidine deoxyribonucleoside, thymidine. Except for slight utilization of thymine, bases are ineffective (Dadd and Kleinjan 1977; Dadd 1979).

When requirements for nucleic acid components of C. pipiens and other Diptera are compared, no pattern covering all cases can be discerned, this may be due in part to a lack of completely adequate substitute mixtures for whole nucleic acid for all species except C. pipiens, so that individual nucleic acid components having some positive effect are necessarily acting against a very unbalanced nucleotide background. Requirements for nucleic acid constituents have been most extensively studied in numerous wild-type and auxotrophic mutants of D. melanogaster, providing several points of special interest (Sang 1978). First, the primary requirement of various wild-type strains for adenine-containing constituents of RNA can vary in magnitude with different levels of certain dietary amino acids and the vitamin, folic acid, which are, respectively, nucleotide precursor substrates and an enzyme cofactor for crucial pathways in the biosynthesis of nucleic acids; these findings support the view that although the complete biosynthetic apparatus may be present, synthesis may be reduced due to an imbalance of other dietary factors. Secondly, in a number of mutants, deficiencies of dietary nucleic acid constituents, or imbalances between them, may result in the development of melanotic tumors as well as growth reductions. Thirdly, a great number of auxotrophic mutants have been selected with specific purine and/or pyrimidine requirements. The ease with which a diversity of nucleotide-requiring mutants may be artificially obtained suggests an especially high liability in genes for nucleotide biosynthesis which, in nature, could have brought about the apparent diversity of requirements among species of Diptera.

FAT SOLUBLE VITAMINS

Artificial diets for crustaceans often include vitamins A and E. Vitamin A or its carotene precursor is undoubtedly required for visual pigment in both crustaceans and insects, and evidence from both taxa implicates vitamin E in reproductive function (Dadd 1970a).

Since vitamins A, D and E had no effect on development in early studies, insects were long believed to need no dietary fat-soluble vitamins. However, subsequent studies that examined criteria of adult performance revealed a requirement for α-tocopherol (vitamin E) in a few species to ensure fertility, of the male in one case, of the female in others. Thus, male house crickets reared without tocopherol produce non-viable sperm, whereas in a parasitoid fly, Agria affinis, a deficiency manifests itself as developmental stasis of embryos in the larviparous female. A role for tocopherol specifically as vitamin E is generally difficult to distinguish from its non-specific antioxidant function, and it is usually the latter which dictates its inclusion in diets for insects requiring essential polyunsaturated fatty acids. However, even when external protection of polyunsaturates in diet is the ostensible function of tocopherol, there is also recent evidence for endogenous stabilization of fatty acids in insect tissues (Turunen 1976). Two physiological roles are ascribed to the tocopherol with the napthoquinone core of the tocopherol molecule, whereas a growth promoting effect of the vitamin, supported equally by Vitamin K_1 (b7t not K_3 or K_5), is ascribed to the phytyl side chain common to both vitamins E and K_1 (McKFarlane 1976). This, incidentally, is the only positive evidence for claiming a Vitamin K function in any insect, though vitamins K have been examined with several species.

Whether vitamin A is of import to insects other than in vision is still equivocal, despite reports of slight growth-promoting effects for a few species. Provitamin A, carotene, is needed to maintain normal coloration in phytophagous grasshoppers and caterpillars, but only in locusts, and perhaps the fly, A. affinis, has shown that deprivation of dietary carotene or vitamin A results in adverse effects on growth, in the locusts only after two generations. Vitamins E and A are sometimes included in our diets for C. pipiens. Omission has no effect in single generation studies, but with sequential generations the treatments lacking either or both vitamins tend to die out sooner. Nevertheless, 6 sequential generations have been reared with neither vitamin present, which argues against their essentiality. Because of the well authenticated need for vitamin A in vision, small differences in growth cannot readily be ascribed to strictly nutritional causes because behavioral dysfunctions resulting from blindness might equally interfere with growth. To decide the issue will probably require a search for retinoic acid or other vitamin A metabolites in organs other than eyes.

STEROLS

The essentiality of dietary sterol for insects absolutely distinguishes the class nutritionally from vertebrates. This distinction probably holds for the whole arthropod phylum, though the evidence for this is less secure. In a broader physiological context, the insect sterol requirement assumed especial interest when insect moulting hormones, the ecdysones, were found to be steroids. A metabolic function already postulated to underlie cholesterol essentiality (see below) was then readily interpreted as the provision of ecdysone precursors. Ecdysone research received great impetus from the hope that insect pest control applications might accrue from interventions in hormone function, and sterol utilization incidentally

became one of the better understood topics of insect nutritional metabolism.

The importance of dietary sterol for insects was first recognized in the mid-1930's when blowfly larvae were shown unable to complete development with artificial diets lacking cholesterol. Excluding some symbiote-dependent insects, a need for dietary sterol has been found in every study since, and, complementing this, metabolic studies of sterol precursors known from vertebrate metabolism (acetate, mevalonate, farnesoate, squalene, etc.) indicated no incorporation into insect tissue sterols (Clayton 1964). In retrospect, it can be seen that until the essentiality of sterol had been appreciated, the development of satisfactory defined diets for insects and the consequent ability to carry out various nutritional studies was not possible.

With two exceptions discussed below, cholesterol satisfies the sterol requirement in all species studied, whether for carnivorous insects or for plant-eating insects whose natural food contains various phytosterols rather than cholesterol. Since cholesterol was thought, erroneously as we now know, to be entirely absent from plant tissues, but was usually the major sterol in plant-eating insects, it seemed that phytophagous insects must be able to convert dietary C29 and C28 phytosterols to C27 sterols, principally cholesterol but also in some cases 7-dehydrocholesterol; these, the principal sterols of vertebrates, were thought of as the typical animal sterols.

Early studies of the dietary adequacy for diverse insects of a wide range of sterols and related steroids (Clayton 1964) showed that certain dermstid beetles which feed on animal carcass materials could utilize only the C27 animal sterols, cholesterol and 7-dehydrocholesterol, whereas phytophagous, graminivorous, mycophagous and omnivorous insects could utilize a wide array of phytosterols in addition to cholesterol. These findings, supported by bioassays for cholesterol based on Dermestes growth with sterol provided solely as extracts from phytophagous insects (Levinson 1962), provided the basis for a hypothesis that all insects have a physiological need for cholesterol, obtained directly from food by carnivores, but metabolized from dietary phytosterols in insects which normally eat vegetable matter. Subsequently, as more species were examined exceptions abounded and it became evident that the initial hypothesis was an over-simplification.

Before considering our current understanding of the metabolism of food sterols into tissue sterols and ecdysteroids, the phenomenon of sterol sparing and its bearing on the physiological functions of sterols must be mentioned. Early studies had shown that some insects which developed optimally on cholesterol but poorly on other sterols could develop well with a suboptimal sterol if it was supplemented with a small amount of cholesterol that, on its own, failed to support significant growth. In such a case the suboptimal sterol was said to spare all but a small fraction of the cholesterol requirement. This phenomenon led to the postulation of at least two distinct physiological functions for sterol in insects, both of which could be compeletely met by cholesterol: a structural function, as a constituent of lipid membranes, for which the bulk of dietary sterol was utilized and which was relatively unspecific with respect to the sterol needed and so could be met by sparing sterols; and a metabolic function, requiring cholesterol specifically in relatively small amounts. With the discovery that ecdysone moulting hormones were steroids derived from

cholesterol, it seemed likely that the metabolic function represented that portion of the overall sterol requirement needed for ecdysone precursors.

The importance of the concept of sparing sterols is well exemplified by the housefly, whose larvae develop in decaying matter of both plant and animal origin. Housefly larvae grow to apparently normal adults on diets containing cholesterol or either of the plant sterols, sitosterol and campesterol, and tissue analysis reveals their body sterol to consist almost entirely of the unchanged dietary sterol. However, adult females reared as larvae on diets containing only phytosterol produce non-viable eggs unless they acquire cholesterol in their adult food. Thus, if the whole life-cycle is taken into consideration, the housefly requires cholesterol specifically, but sitosterol or campesterol act as sparing sterols satisfying all bulk requirements for the sterol structural functions of larval and pupal stages. Presumably the smaller metabolic requirement for ecdysone sythesis can be provided for by cholesterol reserves carried over from the egg.

Although, like the housefly, some phytophagous insects cannot convert phytosterols to cholesterol, the majority of those studied do so, and the metabolic pathways involved have been elucidated in considerable detail (Robbins et al. 1971; Svoboda et al. 1975, 1978). Figure 1 summarizes the principal pathways whereby common phytosterols are metabolized to cholesterol in the tobacco hornworm, Manduca sexta, and many other insects, though alternate pathways (not shown) may be more prominent in some species. Figure 1 also indicates the pathway leading from cholesterol via 7-dehydrocholesterol to α-ecdysone, the moulting hormone formed in the prothoracic gland, and thence to 20-hydroxyecdysone (β-ecdysone, ecdysterone, or crustecdysone as it was called when first found in crayfish), which is the form in which the hormone acts at responsive tissue sites. Whatever the dietary sterol, the central role of cholesterol in relation to molting hormones is clear from this scheme.

That sterol requirements and metabolism in phytophagous insects could be strikingly different first became evident when cholesterol was found to be an inadequate dietary sterol for two species. First, larvae of Drosophila pachea, in nature found only in rotting senita cactus, developed in artificial media only if provided with the senita cactus sterol, schottenol (Δ^7-stigmastenol) or related Δ^7 sterols including 7-dehydrocholesterol and lathosterol; the common plant sterols and cholesterol were totally inadequate (Heed and Kircher 1965). Secondly, larvae of an ambrosia beetle, Xyleborus ferrugineus, grew well with only cholesterol in a synthetic diet, but failed to pupate unless provided with ergosterol or 7-dehydrocholesterol (Chu et al. 1970). Ergosterol, the sole sterol of the beetle's symbiotic fungal food, is completely adequate, and cholesterol in this species acts only as a sparing sterol. In D. pachea the principal tissue sterols are lathosterol (Δ^7-cholestenol) and 7-dehydrocholesterol, and it is notable that all adequate dietary sterols for both species are characterized by the Δ^7 double bond which also characterizes the ecdysones.

Table II lists the principal host-plant and tissue sterols for a number of phytophagous insects that do not seem to form cholesterol from their dietary phytosterols, though usually at least trace amounts of tissue cholesterol can be detected. In general, the preponderance of

sterols other than cholesterol in these examples suggests that the structural functions for which most dietary sterol is required may often be better served by sterols other than cholesterol; it is difficult otherwise to account for the metabolism of host-plant phytosterols mainly to fully saturated stanols in the beetle Epilachna (Svoboda and Thompson 1974), or the accumulation and transfer of major quantities of 24-methylenecholesterol to developing larvae by worker honeybees whatever the dietary sterol supplied to the workers (Svododa et al. 1981).

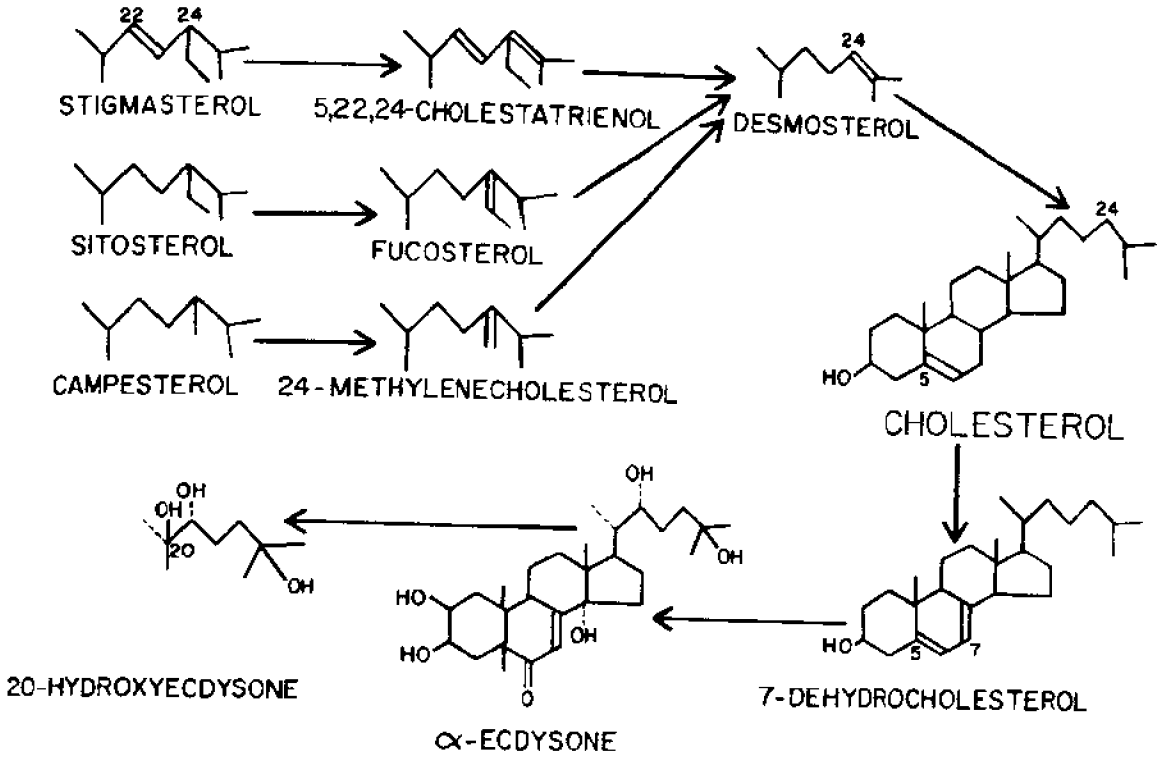

Figure 1. Metabolic pathways for conversion of phytosterols to cholesterol and the ecdysones in many phytophagous insects. Simplified from Svoboda et al. (1975, 1978) with various steps and alternate pathways omitted.

This apart, one wonders how phytophagous insects that are unable to synthesize cholesterol obtain precursors for ecdysone synthesis, presumed to be cholesterol of Δ^7-sterols such as 7-dehydrocholesterol and perhaps lathosterol. Possibly a very low and difficult to detect conversion of phytosterol to cholesterol occurs in some. Also, it is now known that traces of cholesterol occur in many plants and might be preferentially sequestered by the insect to provide the small quantity needed for ecdysone precursors, as has indeed been demonstrated in the beetle Trogoderma, and for the housefly. Most intriguingly, the situation in Oncopeltus fasciatus questions whether moulting hormones are necessarily of the ecdysone type derived from cholesterol. The ecdysteroid, makisterone, has greater moulting hormone activity than ecdysone for Oncopeltus, and has been detected in Oncopeltus eggs; makisterone is related to campesterol, a phytosterol preferentially

accumulated by Oncopeltus from its food plant, raising the possibility that it may be the natural moulting hormone in this sucking bug (Kaplanis et al. 1980).

Table II. Food and Tissue Sterols of Insects that Do Not Convert Phytosterols to Cholesterol. Sterols listed in order of decreasing proportions (Svoboda et al. 1975, 1978, 1981).

Insect	Food sterols	Insect sterols	Metabolic conversions
Epilachna varivestis Mexican bean beetle	Campesterol Stigmasterol Sitosterol trace Cholesterol	Cholestanol Phytostanols Lathosterol other Δ^7-sterols low Cholesterol	Phytosterols to stanols and Δ^7-sterols. Cholesterol sequestered.
Oncopeltus fasciatus Milkweed bug	Sitosterol Stigmasterol Campesterol trace Cholesterol	Sitosterol Campesterol Stigmasterol trace Cholesterol Makisterone	No metabolism of phytosterols. Cholesterol increased.
Apis mellifera Honeybee	Workers obtain phytosterols, 24-methylene-cholesterol and low cholesterol from pollen and other forage	In brood larvae: 24-methylene-cholesterol Sitosterol Isofucosterol low Cholesterol	Workers pass on sequestered food sterols in the special larval food.
Drosophila pachea Senita cactus fly	Schottenol Δ^7-stigmastenol	Lathosterol Δ^7-cholestenol 7-dehydrocholesterol	Δ^7-phytostenol to Δ^7-C27-stenols

For practitioners of crustacean nutrition the most suggestive point to emerge from the recently appreciated diversity of insect sterol metabolism is that although dietary cholesterol is well utilized and entirely adequate for most species, there are exceptions for which some other sterol is essential. Besides the two examples of cholesterol inadequacy mentioned above, other insects are known to grow better with sitosterol than cholesterol. The differences in these cases may be behavioral rather than metabolic, since sitosterol acts also as a phagostimulant for some insects. These findings advocate an open mind about required sterols when concocting synthetic diets for arthropods. Pertinent to this, a cautionary example is provided by recent work on the sterol requirement of mosquitoes. Being omnivorous, one would expect C. pipiens to utilize diverse phytosterols efficiently, as is the case for another mosquito, A. aegypti. Table III illustrates developmental indices (see Dadd and Kleinjan 1976) obtained with various sterols tested as part of a basal diet with or without the tiny amount of synthetic lecithin incorporated primarily to aid in the dispersion of essential fatty acids. Without lecithin, only cholesterol supported good development, while stigmasterol and ergosterol were ineffective. With lecithin in the diet, these and most other phytosterols proved as

good as cholesterol. This probably indicates that, without an emulsifier, the gut differs in its ability to absorb the various sterols dispersed in the diet. This possible action of lecithin is of potential importance with respect to the absorption of all lipid nutrients dispersed in an aqueous dietary medium for any aquatic animal.

Table III. Developmental Indices[a] and Percentages of Adults (in parentheses) for Culex pipiens Reared with Various Sterols (1 mg/100 ml) With or Without 99% Pure Synthetic Lecithin (2 mg/100 ml) in the Dietary Medium

Sterol	Without lecithin		With lecithin	
Cholesterol	3.1	(65)	3.8	(69)
Desmosterol	2.9	(67)	3.4	(69)
7-Dehydrocholesterol	2.5	(17)	3.0	(50)
Sitosterol	1.6	(24)	3.5	(53)
Campesterol	0.5	(3)	3.4	(76)
Stigmasterol	0.4	(0)	3.5	(57)
Ergosterol	0.4	(0)	3.7	(70)
Cholestanol	0.9	(17)	0.8	(20)
No sterol	0.4	(0)	0.5	(0)

[a]Value combining degree of development and rate of development: average number of molts divided by average number of days to pupation multiplied by 10 (see Dadd and Kleinjan [1976] for details).

ESSENTIAL FATTY ACIDS

Polyunsaturated fatty acids are essential for the majority of symbiote-independent species studies. Deficiencies are most dramatically evident in Lepidoptera; moth larvae reared on fat-free diet develop to the pupal stage normally, though usually at a slightly retarded rate, but at the pupal/adult molt, the emerging adult is trapped or characteristically malformed. Similar failure of adult emergence occurs in various grasshoppers, wasps and mosquitoes, but in other insects such as certain species of cockroach and beetle it may take more than one generation for fatty acid deprivation to become apparent. Because most insect nutritional studies have been carried out over only one generation, it is quite possible that some species with no apparent need for fatty acids over one life span might, nonetheless, require it if dietary deprivation were extended.

In most early studies the requirement was met equally well by the C18 polyunsaturated, linoleic (18:2ω6) and linolenic (18:3ω3) acids, all saturated and monoenoic acids tested being ineffective. Of particular interest was the finding, with lepidopterous larvae and acridid grasshoppers, that the C20/22 polyunsaturates, arachidonic (20:4ω6), eicosapentaeonic (20:5ω3) and docosahexaenoic (22:6ω3) acids, were ineffective; this would suggest that physiological functions of essential fatty acids in insects might differ basically from those of vertebrates, for which C20/22 polyunsaturates have a central physiological role and if not present in food are derived from linoleic

or linolenic acids by carbon chain elongation and further desaturation (Mead 1970).

The essentiality of polyunsaturated fatty acid was considered to reside in the insects' inability to biosynthesize fatty acids with more than one double bond. Nearly all metabolic studies seemed to confirm this (Gilbert 1967; Downer 1978), though exceptions have recently been claimed (Blomquist et al. in press). Since many insects with no apparent fatty acid requirement are also unable to synthesize polyunsaturates, this would imply that such insects had no physiological need for them, a surprising conclusion in view of the general importance of polyunsaturates in the lipid membranes of animal cells.

Essential fatty acid nutrition in insects became more complicated when it was found that some Lepidoptera required linolenic acid specifically to achieve normal adult emergence; a recent review (Dadd 1981) noted that of 18 species by then investigated, 3 required no fatty acid, 6 utilized linoleic or linolenic acids equally well, 3 utilized both, but with linolenic the more potent, 5 required linolenic acid specifically to ensure a normal pupal/adult moult, and one required both linolenic and linoleic acids. It should be emphasized that this summary refers only to the requirement for normal adult emergence; linolenic acid also affects growth positively in many of these moths, and in several, linoleic acid is needed in addition for optimal growth (Turunen 1974).

Our current studies with mosquitoes now add further complexity. Originally the basal dietary medium, which lacked fatty acid, supported normal growth to the adult stage, but emergent adults were unable to fly and soon died entrapped at the medium surface. Flying adults were first obtained using diet supplemented with mammalian serum lipoprotein, cephalin, or lecithins of animal, but not vegetable, origin. Active animal phospholipids were effectively replaced by pure arachidonic acid, though not be linoleic acid (Dadd and Kleinjan 1978, 1979). Subsequent tests of many fatty acids (Dadd 1980, 1981) gave results shown in Figure 2, in which fatty acids are grouped as active, if they supported the emergence of flying adults, semi-active, if adults stood or hopped on the medium surface on emergence. From a comparison of the carbon chain structure for active and semi-active fatty acids it appears that full activity depends upon a sequence of three cis double bonds in an interrupted methylene pattern spanning carbons 6 to 13 from the methyl termination. Additional double bonds to both sides of this grouping do not negate activity, so that full activity is found for members of both the $\omega 3$ & $\omega 6$ fatty acid families derived from linolenic and linoleic acids, respectively. Semi-active fatty acids all contain two of the triplet of three double bonds characterizing fully active acids, and can also be $\omega 3$ or $\omega 6$.

Apart from its novelty in insect nutrition, the essentiality of arachidonic acid and structurally related polyunsaturates for C. pipiens (and for several other mosquito species) is of special interest in relation to the dietary requirements, metabolism and physiological functions of essential fatty acids for vertebrates. For warm-blooded vertebrats arachidonic acid ($10{:}4\omega 6$) is of central physiological importance as a necessary constituent of cell membrane phospholipids and as a precursor for prostaglandins and related hormone-like entities with many localized regulatory functions in diverse tissues (Mead 1970; Lands et al. 1977). Arachidonic acid, and lower members of the $\omega 6$ fatty acid

family are the primary, fully effective essential fatty acids, whereas linolenic acid (18:3ω3) and higher members of the ω3 family are not completely effective in averting all symptoms of deficiency. In contrast, linolenic and the higher ω3 acids (e.g., eicosapentaenoic or 20:5ω3; docosahexaenoic or 22:6ω3) appear to be the primary essential fatty acids for many fish (Tinoco et al. 1979). In vertebrates generally, whether ω6 or ω3 specialists, the dietarily effective and physiologically important fatty acids include C20/22 polyunsaturates, which are substantially present in their fats, especially in phospholipids, whereas until recently fatty acids higher than 18:3 were rarely detected in the hundreds of analyses performed on insect lipids (Fast 1964, 1970).

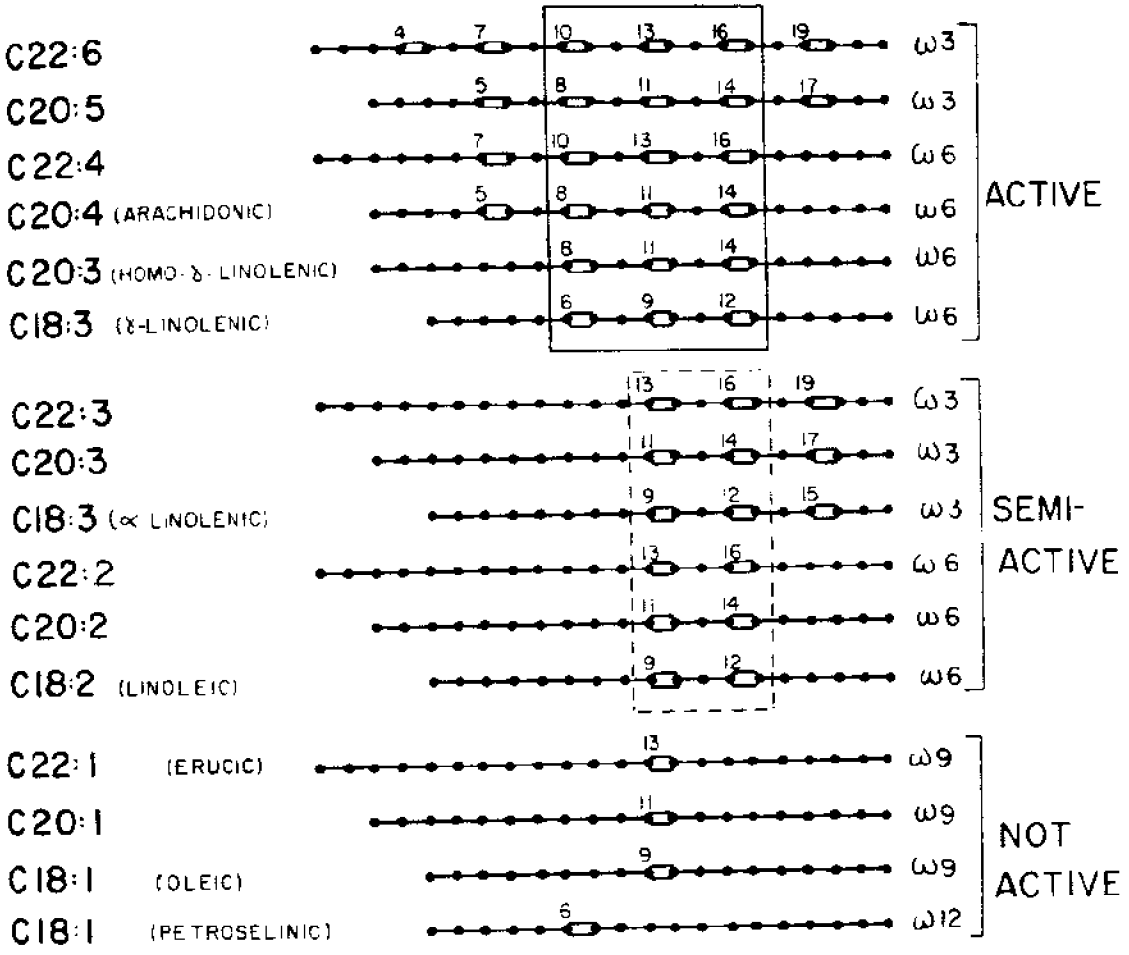

Figure 2. Carbon chain structures (methyl terminations to the left) of active, semi-active and inactive unsaturated fatty acids for Culex pipiens. C16, C18, C20 and C22 saturated fatty acids were all inactive.

Compared with the relatively well understood nutritional, metabolic, and physiological functions of the essential fatty acids in vertebrates, knowledge of essential fatty acid function for insects as a whole is fragmentary and presents certain contradictions. On the one hand, mosquito dietary requirements indicate a physiologically crucial role for C20/22 polyunsaturates, as in vertebrates; on the other hand,

dietary utilizability of C18 but not C20/22 polyunsaturated fatty acids in various other insects suggests a somewhat different physiologic role, emphasized by the lack of evidence from most insect fatty acid analyses for any conversion of C18 polyunsaturates to higher members. Further, whereas the essentiality of C20 polyunsaturates in vertebrates is accountable in part to their function as precursors of prostaglandins, until 5 years ago these substances were unknown in insects.

Recent findings, reviewed in detail elsewhere (Dadd 1981) may go some way to resolving these problems. Arachidonic acid and other polyunsaturates have now been detected in mosquito tissues, contrary to many previous analyses showing none (Stanley-Samuelson and Dadd 1981). More interestingly, a recent search for polyunsaturates in a dozen insect species of diverse taxa and feeding habit revealed their presence in all cases, usually in low or trace amounts, but often proportionally greatly enhanced in phospholipids and in specific tissues such as the central nervous system. On the basis of this and an increasing number of records of longchain polyunsaturates in the more recent insect lipid literature, I believe longchain polyunsaturates are probably universally present in insects. Their concentration in particular tissues and lipid classes indicates a functional rather than adventitious role, a view supported by the very recent detection of prostaglandins in tissues from 5 species representing 4 orders (Stanley-Samuelson and Loher in press).

I am currently examining the ability of various polyunsaturates to avert the adult malformations of essential fatty acid deficiency in the waxmoth, one of the species previously found unable to use arachidonic acid in place of linoleic or linolenic acids. Results are summarized in Table IV. Both linolenic (ω3) and linoleic (ω6) acids support the emergence of normal adults, but about 10 times the concentration of linoleic is required, so I call it weakly active. Confirming an earlier study, arachidonic acid proved completely ineffective, as did all other 6 fatty acids except linoleic. Of the ω3 acids, 20:3ω3 and 22:3ω3 are fully as active as linolenic, whereas 20:5ω3 (eicosapentaenoic) and 22:6 ω3(docosahexaenoic) are inactive. It thus appears that linolenic acid and its longer-chained trienoic analogues share a fully active structure, but activity is negated in other ω3 members containing the same structure if there are too many additional double bonds toward the carboxyl end. These results show that certain C20/22 polyunsaturates can indeed replace dietary C18 polyunsaturates, though the features accounting for activity are quite different for the waxmoth than for mosquitoes.

Table IV. Activity of Fatty Acids for Galleria mellonella with Respect to Averting Adult Malformation at the Pupal/Adult Moult

Inactive		Semi-active	
18:1ω9	oleic	18:2ω6	linoleic
18:3ω6	γ-linolenic		
20:2ω6			
20:3ω6	homo-γ-linolenic	Fully active	
20:4ω6	arachidonic	18:3ω3	α-linolenic
20:5ω3	eicosapentaenoic	20:3 3	
22:6δ3	docosahexaenoic	22:3 3	

The weak activity of linoleic acid and inactivity of all other 6 fatty acids is difficult to account for. Another lepidopteran for which linolenic acid ($\omega 3$) is the primary essential, the silkworm, is among those 6 species of insects in the prostaglandins, especially PGE_2, have now been detected, and, in this insect, shown to have a function in reproduction (Setty and Ramaiah 1980); this indicates at least a low requirement for arachidonic acid, the precursor of PGE_2, presumably derived ultimately from the linoleic acid of its food plant. Much other evidence suggests Lepidoptera in general require both linolenic and linoleic acids, and a double requirement may be widespread among insects. Perhaps the most compelling evidence for this comes from recent fatty acid analyses of retinal phospholipids of insects from three orders, including a phytophagous lepidopteran; in all cases arachidonic acid ($\omega 6$) was substantially present, while eicosapentaenoic acid ($\omega 3$) was the major fatty acid (Zinkler 1975). The possibility that both $\omega 3$ and $\omega 6$ fatty acids may be essential for vertebrates has recently been raised (Tinoco et al. 1979), and it would be of great interest to know whether fish and crustacea that seem to require primarily linolenic or other $\omega 3$ dietary fatty acids contain prostaglandins of the 2 series, as this would imply the essentiallity, in addition, of linoleic or other $\omega 6$ fatty acids.

CONCLUSIONS

So many species from the main insect orders have now been reared and studied nutritionally through a complete cycle of growth and development of holidic diets that the qualitative nutrient requirements of the class as a whole are very well understood. Contrary to some early speculations, these requirements are basically similar to those of vertebrates, and highly specialized or apparently unnutritious natural diets correlate with narrowly fastidious feeding behaviors or obligate dependence on symbiote associations. A categorical and strictly nutritional difference between the needs of insects and vertebrates is the insect requirement for dietary sterol, and because of their importance for endocrinological regulation of development, sterol requirements and metabolism are currently among the most active areas of insect nutrition. A sterol requirement is probably general among Arthropoda, and hence the extensive findings with insects have special relevance for crustacean nutritionists.

Although most nutrient needs of insects are now known, novel requirements nonetheless still come to light for particular groups of insects. Notable among those more recently uncovered are the essentiality of asparagine for some mosquitoes, and the apparently general mosquito requirement, unique among insects, for certain longchain polyunsaturated fatty acids. The recent surge of interest among insect lipid physiologists in essential polyunsaturated fatty acids and protaglandins promises to link this area of insect physiology closely to parallel interests among crustacean physiologists, and to tie both into the more extensive background of knowledge gained with vertebrates.

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CRUSTACEAN NUTRITION

FEEDING AND DIGESTION IN DECAPOD CRUSTACEANS

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ABSTRACT

The feeding mechanisms and digestive physiology of decapod crustaceans are reviewed. A variety of cephalic and thoracic appendages are utilized for food capture, but feeding is essentially either setous or raptorial. Ingestion is facilitated by mucoid secretions discharged by the oesophageal tegumental glands. Food in the stomach is reduced to a semi-fluid state through the concerted mechanical and chemical effects of the gastric mill, digestive enzymes and emulsifying agents. The stomach contents are then sorted by setous filtration processes, fluid and microparticulate material entering the hepatopancreas for the further stages of digestion, residual substances passing directly to the midgut. Within the hepatopancreatic tubules embryonic cells differentiate either into resorptive or into fibrillar cells, the latter in turn maturing to form secretory cells. Secretory cells release enzymes by either holocrine or merocrine mechanisms for the extracellular stages of digestion.. Morocrine discharge is followed by a restitution process which replenishes secretory cells. The absorption of luminar nutrients, and possible contact (membrane) and intracellular digestion, in the hepatopancreas are discussed. Nutrient absorption is also effected in the midgut and may involve enzymes secreted by the midgut diverticula. Waste materials are compacted as they pass through the mid- and hindgut, tegumental gland secretions in the latter region assisting in binding and eliminating the feces.

Keywords: Decapoda; feeding; digestion; enzymes; emulsifiers; oesophagus; stomach; hepatopancreas; midgut; hindgut.

INTRODUCTION

The class Crustacea comprises a diverse group of arthropods with a wide range of feeding methods. Primitively all groups are filter feeders, trapping small particles with setae borne on the body appendages. This method is characteristic of larvae, branchiopods, cirripedes, copepods, ostracods and shrimp-like forms in several malacostracan groups. In many more advanced Malacostraca, however, the anterior appendages are specialized for raptorial feeding, the food being seized and mechanically fragmented before it is ingested. Setous

and raptorial feeding methods are the two basic types found within Crustacea, although there are highly modified ecto- and endoparasitic forms, such as the Branchiura with piercing and sucking mouthparts or the rhizocephalan Cirripedia in which the gut is completely lost and nutrients are absorbed across the surface of ramifying outgrowths of the body wall (Cheng 1964).

Studies of feeding and digestion in Crustacea have principally been concerned with Decapoda, a predominantly marine group which accounts for some 30% of the known crustacean species and includes many forms of commercial value to man. The present paper reviews current thoughts on the digestive processes of decapod crustaceans.

FOOD AND FEEDING MECHANISMS

The scavenged diet typical of many decapod species, particularly amongst the Reptantia, is well summarized by Mackie's (1973) comment that "a wide variety of materials, ranging from smoked cormorant to canned cat food, can serve as bait" for catching lobsters and crabs. Many scavenging forms are also broadly omnivorous and readily feed upon a variety of both plant and animal material. In others, however, the diet is biased towards flesh or vegetation. Primarily herbivorous habits are in fact uncommon amongst the higher Crustacea, although found in forms such as the crab Birgus, which feeds on coconuts (Barnes 1968), and a species of Sesarma, which climbs marsh-grass to feed upon the seeds (Day 1974). Commonly scavengers are also opportunistic predators and predaceous habits are typical of many natantic decapods. Both predatory and scavenging forms, whether omnivorous, carnivorous or herbivorous, largely rely upon raptorial feeding mechanisms to obtain their food. The first three pairs of thoracic limbs (pereiopods) in decapods are typically modified for food capture. In the Natantia (prawns and shrimps) any one of all of these pairs of pereiopods may be chelate or they may be slender and lack chelipeds, whereas in the Reptantia (crabs and lobsters) the typical large crusting chela or claw is developed on the first pereiopod. Raptorial feeders either seize their food directly with the mouth parts or first grasp it with their chelae and then pass it to the oral region. The mouth parts comprise an array of paired maxillae, maxillipeds and mandibles which act together to reduce larger food fragments to a size suitable for ingestion. Amongst natantic forms the food is apparently chewed to a finely particulate size by the mandibles before it is swallowed, but in the Reptantia food is more coarsely shredded and large fragments are ingested intact. Barker and Gibson (1977) showed that in Homarus the mandibles do not actually possess a masticatory function but merely grip food material as it is torn apart by the pulling action of the third maxillipeds. The remaining maxillipeds, together with the maxillae, then direct food fragments to the mouth for swallowing.

Not all decapods employ raptorial mechanisms and many species are filter feeders. Particulate matter is collected and sifted by chitinous setae and bristles borne on a variety of appendages before it is passed to the mouth. For example, hippid mole crabs and the hermit crab Diogenes strain particles from the water with their feathery antennae, anomurans such as Pachycheles and Porcella trap plankton with their maxillipeds, galathean and porcelain crabs scrape up and filter bottom deposits with long setae on their third maxillipeds, and ghost shrimps

(Callianassa) and mud shrimp (Upogebia) use setae on their pereiopods to filter sediments and water respectively before further sifting is accomplished by their mouth parts (Barnes 1968; Schaefer 1970; Meglitsch 1972). Filter feeding is also likely to be the principal method employed by larval stages, as in Penaeus where the maximum filtering efficiency is reached at the mysis stage (Emmerson 1980). Most filter feeders are probably unselective microphagous omnivores, but some degree of food selection may take place as demonstrated for certain species of copepods and branchiopods (Meglitsch 1972; Marshall 1973).

MORPHOLOGY AND PHYSIOLOGY OF THE ALIMENTARY TRACT

The alimentary tract in decapod crustaceans is divisible into three regions; these are the foregut, or ectodermal origin and further subdivided into an oesophagus and a two-chambered stomach, the endodermal midgut with its various caecal or diverticular derivatives, and the hindgut, which is also embryologically derived from ectoderm.

Oesophagus

The oesophagus is essentially a short tubular structure whose primary function is to conduct food material from the mouth to the stomach. The oesophageal wall is folded and composed of a columnar epithelium overlain by a thick chitinous layer divided into outer homogeneous exocuticular and inner, often horizontally laminated, endocuticular zones. Erri Babu et all. (1979a) showed that in the crab Xantho these exo- and endocuticular layers are composed of elastin and collagen respectively. The thin outer epicuticle is in some species developed into spines whose purpose remains uncertain.

Embedded in the connective tissues surrounding the oesophagus are large numbers of spherical tegumental glands. Each gland is composed of conical cells arranged around a central collecting chamber, from which a slender duct leads through the epithelial and cuticular layers to open into the oesophagus. The glands have variously been described as salivary, digestive, lubricatory, or responsible for the deposition and subsequent maintenance of the oesophageal epicuticle. Studies by Barker and Gibson (1977, 1978), Shyamasundari and Hanumantha Rao (1978) and Erri Babu et al. (1979b), among others, have shown that a considerable amount of acid mucopolysaccharide material is always present in the glands, even in starved animals. Barker (1976) suggested that during feeding compression of the glands, due to contractions of the oesophageal musculature and the labrum being raised as the mandibles are opened to receive food, could result in the gland contents being expelled into the oesophageal lumen. In those Decapoda which only coarsely shred their food before ingestion, such mucoid substances would clearly facilitate both swallowing and the subsequent passage of larger food fragments along the oesophagus. In forms where the food is ingested in a finely particulate form the mucus could also entangle the particles and form a food string. The abundance of tegumental glands around the anterior portion of the oesophagus supports these suggestions.

Other substances identified in the tegumental glands: acid phosphatase, adenosine triphosphatase (Barker 1976), sialic acid, hyaluronic acid, and glycoproteins (Shyamasundari and Hanumantha Rao

(1978; Erri Babu et al. 1979b), are probably in some way involved with the synthesis of the mucopolysaccharides. Suggestions that the glands may produce and secrete amylases (Vonk 1960) are not substantiated and, although Erri Babu et al. (1979b) stated that "Due to the acidic nature of the gland secretions, they may play a role in digestion", there is no certain evidence at present that the osesophageal tegumental glands possess any digestive function. Whether or not this is true for all decapods, however, remains to be determined.

Stomach

The decapod stomach typically comprises anterior cardiac and posterior pyloric regions. Much of the cardiac portion forms a large, thin-walled and flexible chamber, but its posterior half and the pyloric region are reinforced by a complex of calcified chitinous ossicles and plates, some of which bear teeth and constitute the trituratory gastric mill. The functional morphology of both regions has been described on many occasions (e.g., Schaefer 1970; Powell 1974) and a detailed account of the gastric ossicles, musculature and neural supply is given by Maynard and Dando (1974).

The degree of development of the gastric armature depends upon the efficiency of the feeding mechanism (Reddy 1935). Thus in forms which ingest finely chewed food, the gastric mill is not especially complex, whereas in species swallowing large food fragments the mill is correspondingly well developed. Reddy concluded that the combined efficiency of the internal and external masticatory structures remained more or less constant irrespective of the species concerned.

After trituration, the particulate food matter is then sorted and filtered. In simple forms, such as penaeids, this is performed in the pyloric stomach and the two stomach regions are separated only by a constriction. In the more advanced Reptantia, however, the process is more complex and some provisional sorting is effected by setae and grooves in the cardiac region. Larger food fragments are retained for further trituration, while smaller or intermediate sized material passes through the cardio-pyloric valves into the pyloric chamber. Here the food is further reduced in size and sorted. Ultimately the smallest particles (less than 100 nm diameter, Hopkin and Nott 1980), together with the the fluid stomach contents, are directed towards the hepatopancreas, whilst the remaining material is passed via other valves into the midgut. Schaefer (1970) found that adaptations to different diets are reflected in the type and distribution of stomach setae; a finer filtration mechanism occurs in forms such ad Upogebia, which feed on very small food particles, than in species which ingest larger food fragments (Cyclograpsus).

The stomach thus possesses two main functions; it reduces ingested food to a slurry, thereby increasing the surface area of material available for enzymic action, and it sorts the triturated contents so that all but the smallest particles are prevented from entering the hepatopancreas. As food is fragmented in the stomach it becomes intimately mixed with digestive juices, secreted by the hepatopancreas, which contain a wide range of digestive enzymes (Gibson and Barker 1979) and their hydrolytic effect supplements the mechanical breakdown achieved by the gastric mill. The juice also contains emulsifying substances which play an important part in the digestion and absorption

of dietary fats. The stomach is therefore also the center for the extracellular stages in digestion. It is the hepatopancreas, however, which is regarded as the digestive organ.

Hepatopancreas

Despite views to the contrary (Van Weel 1974; Dall 1981), the term hepatopancreas is here retained for the decapod digestive organ. The anatomy of the structure and its involvement in digestion have recently been extensively reviewed by Gibson and Barker (1979).

The hepatopancreas, occupying much of the cephalothoracic space, comprises a pair of extremely well developed midgut appendages elaborated to form a large but compact complex of ducts and blind-ending tubules. Each half of the organ, usually composed of two or three lobes, variously opens into the anterior part of the midgut, at the junction between pyloric stomach and midgut, or into the pyloric stomach itself, depending upon the species. The tubules are lined by an epithelium in which four cell types can be recognized; these are widely known as the E- (embryonic or undifferentiated), F- (fibrillar), R- (resorptive or absorptive) and B- (secretory) cells. The relationships between these various cell types have attracted much attention and not a little disagreement. The current understanding of cellular differentiation in the hepatopancreatic tubules of decapods is summarized in Figure 1.

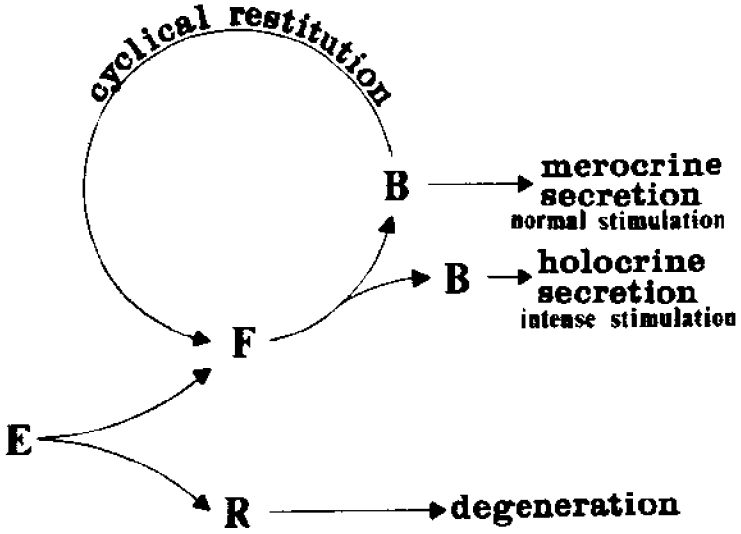

Figure 1. Diagrammatic representation of the sequence of cellular differentiation in the hepatopancreatic tubule epithelium of decapod crustaceans. The various cell relationships are discussed in the text.

Ultrastructural and histochemical studies indicate that each cell type possesses its own specific roles in the functioning of the hepatopancreas. E-cells undergo cytoplasmic differentiation as they migrate proximally down each tuble, giving rise either to F- or R-cells. Fibrillar F-cells, so called because they appear striated in

histological preparations, "synthesize digestive enzymes and sequester them in a supranuclear vacuole which enlarges by pinocytic intake of luminal nutrients and fluids" (Loizzi 1971). It is the F-cells which thus become transformed into secretory B-cells. These are the largest of the cell types and characteristically contain a single enormous vacuole which may occupy 80-90% of the total cell volume (Barker and Gibson 1977). B-cell secretion has variously been reported as holocrine, apocrine or merocrine. Loizzi (1966) concluded that liberation of the vacuolar contents occurred in either an apocrine or a merocrine fashion under normal circumstances, i.e., when an animal is fed regularly without intervening periods of starvation, but that the intense stimulation of feeding subsequent to a prolonged fast led to holocrine secretion. Barker and Gibson (1977, 1978) found that the first wave of B-cell secretion was holocrine, but that subsequent secretory phases in the digestive cycle were accomplished by other mechanisms. Unless secretion involves the total liberation of the cell (i.e., holocrine discharge), post-secretory B-cells must either ultimately degenerate and/or be voided from the epithelium or become reconstituted. Gibson and Barker (1979), in commenting that the "loss of the secretory vacuole via a merocrine mechanism would leave a structure reminiscent of a young F-cell", subscribed to earlier hypotheses that B-cell replacement occurs via a restitution process. This process depends upon a mechanism directly analogous with the way in which F-cells produce and sequester enzymes as they evolve into B-cells. In recent studies on Carcinus, however, Hopkin and Nott (1980), whilst agreeing that B-cells are derived from F-cells, do not subscribe to the B-cell restitution theory. Further, they do not agree that B-cell vacuoles form a store of digestive enzymes, suggesting instead that "these vacuoles are a means of packaging waste products of digestion for removal from the hepatopancreas" since most of the vacuoles after being liberated remain intact until voided with the feces. Hopkin (pers. comm.) suggests that B-cells are involved in digestion during the first 12 hours of the digestive cycle in Carcinus, but that this is effected in the apical channels of the cells. If Hopkin and Nott's inference is correct, the question remains as to what is the source of the extracellularly acting enzymes present in the gastric juice. It is possible that the fate of B-cells may depend upon the stage in the digestive cycle. Enzymes are released into the gastric juice from the hepatopancreas in three waves during the first 12 hours after feeding (Barker and Gibson 1977, 1978). B-cells extruded into the hepatopancreatic lumen and supposedly containing waste material, as suggested by Hopkin and Nott (1980), are found after this 12-hour period, i.e., when extracellular digestion has been accomplished and there is no further need for active enzymes in the digestive juice. B-cells may therefore possess a dual role. At first their vacuoles contain enzymes, synthesized by their F-cell precursors, available for secretion and extracellular action if the presence of a meal so demands. Once extracellular digestion has been completed, however, the synthesis of additional enzymes for this purpose, whether directly by F-cells or via the restitution mechanisms, becomes a superfluous process and the cells, by some physiological switch as yet not demonstrated, alter their role to that of waste elimination. That sequential cellular differentiation continues in starved animals and apparently produces an excess of B-cells, with intact secretory vacuoles appearing amongst the hepatopancreatic and gut lumen contents even when no food is present, may support or negate this hypothesis depending upon the view of the individual.

The fourth type of hepatopancreatic cell, the R-cell, appears to be better understood and it is generally agreed that its function is to absorb nutrients and store and metabolize lipid and carbohydrate materials. There is also evidence that the later, alkaline, phases of digestion are completed intracellularly within R-cells (Barker 1976; Barker and Gibson 1977, 1978), whilst the various enzymes located at the brush border may be involved in the uptake of nutrients from the tubule lumen through contact (membrane) digestion (Ugolev and Laey 1973) being followed by molecular transport across the microvillar membrane (Gibson and Barker 1979). Acid mucopolysaccharides at the same location may act as surface binding agents and in some way be concerned with the pinocytic or phagocytic uptake of intraluminar material (Momin and Rangneker 1974). Hopkin and Nott (1980), however, comment that because the hepatopancreatic tubules in Carcinus are lined by a peritrophic membrane, only the molecular and soluble products of digestion could come into contact with the absorptive surface of the epithelial cells. It may be, therefore, that some correlation exists between the detailed mechanism of nutrient uptake by the R-cells and the presence or absence of a peritrophic lining at the microvillous cell surface.

Van den Oord (1966) suggested that the emulsifying agents present in decapod gastric juice were of endogenous hepatopancreatic origin. However, the cells responsible for the synthesis of these emulsifying agents are not known. Gibson and Barker (1979) considered two possibilities; that they are produced by F-cells along with the digestive enzymes and secreted by mature B-cells, or that they are synthesized in the lipid-rich resorptive cells and released as the older R-cells degenerate and rupture. They concluded that the R-cell explanation appeared a more plausible one. Certainly older R-cells, especially in the proximal tubule regions, do break down and rupture and, if one accepts the premise that biological processes have evolved largely so as not to be materially wasteful, then released R-cells remnants may be presumed to serve some purpose.

During digestion, therefore, enzymes and emulsifying agents synthesized and secreted by hepatopancreatic cells pass into the pyloric, and thence to the cardiac, stomach, where they act extracellularly in conjunction with the mechanical processes of the gastric mill (Figure 2). The resultant semi-fluid mixture is subsequently sifted by stomach setae, soluble and microparticulate constituents entering the primary hepatopancreatic ducts through additional filtration devices located at their entrance. Within the hepatopancreas, digestion is completed by a combination of extra- and intracellular processes. The movement of material out of and into the hepatopancreas is effected by contractions of the musculature surrounding each tubule. These contractions are especially vigorous when a previously starved animal is first fed, and at such times the movement of the muscles is also conducive to holocrine B-cell secretion associated with this stage of the digestive cycle (Loizzi 1966).

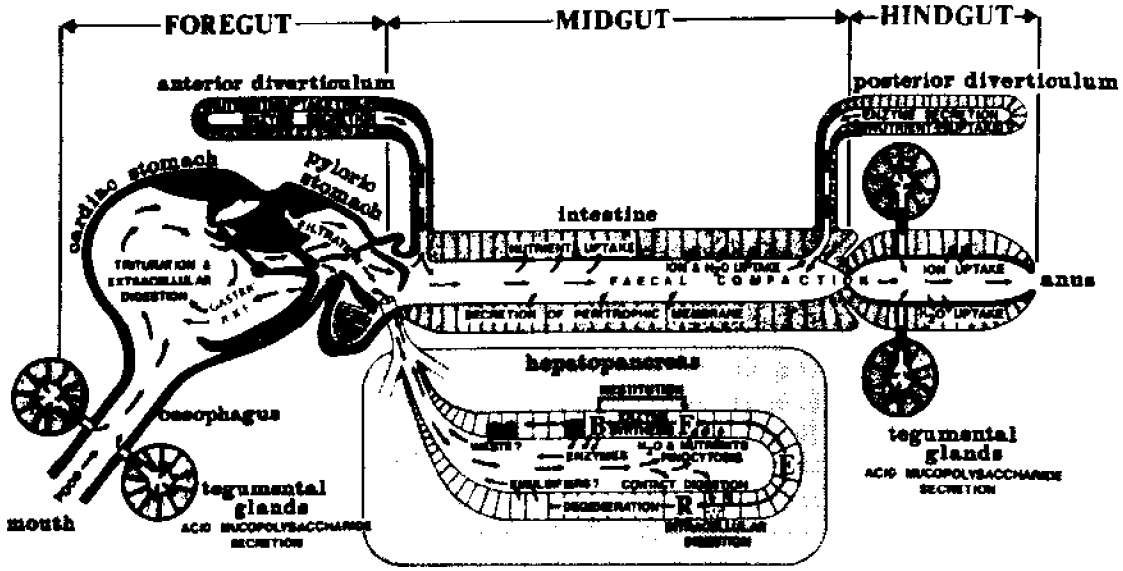

Figure 2. Schematic representation of the decapod alimentary tract and its associated structures, summarizing the current concepts of the digestive processes occurring within the various regions. For convenience the hepatopancreatic B-cell restitution phase is shown as involving F-cells; see Figure 1 and the text for an explanation of this stage in the digestive cycle.

Midgut

The endodermal midgut is short in most decapods and usually accounts for less than 50% of the post-gastric tract. In others, however, it is extremely long and in Homarus, for example, comprises nearly 85% of the intestinal length (Barker and Gibson 1977). Essentially this gut region forms a simple straight tube, enclosed by circular and longitudinal muscles, lined with a columnar epithelium which possesses a microvillous border and ultrastructurally resembles other transporting epithelia (Mykles 1979). It usually bears unbranched caeca or diverticula, arranged singly or in pairs (Smith 1978), which typically open at or near the junctions between fore- and midgut and mid- and hindgut. The caeca, which may be as long as or longer than the main midgut region, resemble the main tract histologically although their musculature is normally reduced. Histochemical investigations have identified a number of enzymes in the midgut and caecal epithelia, particularly in the distal cell cytoplasm (Barker 1976). Since these enzymes are generally not demonstrable in starved individuals Barker concluded that they were indicative of nutrient uptake from the gut lumen. Perfusion experiments by several workers (e.g. Ahearn and Maginniss 1977; Brick and Ahearn 1978; Mykles and Ahearn 1978) support this conclusion. Intracellular acid and alkaline phosphatases exhibit both a spatial and a temporal separation in the midgut wall (Barker 1976) and these enzymes in other invertebrate groups are characteristically associated with the early acidic and late alkaline stages of intracellular digestion respectively. It thus appears that some aspects of digestion are accomplished by the midgut wall. Alkaline phosphatases are known to be involved in the active phosphorylative

transfer of metabolites across epithelial membranes (e.g., see Saev 1963) and form an active zone at the microvillous surface of hepatopancreatic cells; their absence from the midgut brush border may therefore indicate that nutrient uptake for intracellular digestion here is passive rather than active.

The midgut caeca presumably serve mainly to increase the effective absorptive surface area of the midgut. Holliday et al. (1980), however, note that fluid secreted by the caeca in Cancer contains low levels of amylolytic and proteolytic activity. Since the rate of fluid production is unaffected by feeding, they conclude that any enzymic involvement in (extracellular?) digestion is minimal.

Other functions of the midgut and its caeca include the synthesis and secretion of the peritrophic membrane and the transport of ions and water (e.g., see Ahearn et al. 1977; Mykles 1979).

Hindgut

The decapod hindgut has received comparatively little attention and most work on it relates to the tegumental glands of its surrounding connective tissues. In general there appears to be an inverse relationship between the lengths of the mid- and the hindgut.

The hindgut lining is composed of a columnar epithelium overlain by a thick chitinous cuticle; in some species the cuticular surface is studded with microtrichia (Pugh 1962). Most of the genera so far investigated possess longitudinal ridges in the cuticle which protrude, to varying degrees, into the gut lumen. These ridges house longitudinal muscle bands. Circular muscles are also usually well developed around the hindgut, particularly in its more posterior regions. The lack of an anal sphincter is apparently compensated by the natural elasticity of the chitin.

The hindgut apparently serves mainly as a region for the compaction and transportation of fecal material, although water and ion movement across its wall have been demonstrated, for example, by Malley (1977). The mucopolysaccharide contents of the hindgut tegumental glands are presumed both to assist in the binding of fecal matter and to facilitate its movement (Barker 1976). Mucus discharge from the glands appears to be caused by the peristaltic contractions of the gut musculature. Enzymes identified in the glands (Barker and Gibson 1977, 1978) are believed to be concerned with the processes of mucus synthesis and are not digestive in nature.

CONCLUSION

Figure 2 summarizes our current understanding of the digestive processes occurring in decapod crustaceans. Certain aspects of these processes are both well established and widely accepted; for example, there is no disagreement as to the importance of the hepatopancreas (or whatever one chooses to call it) in digestion, nor is there any doubt that food material in the stomach is reduced to a semi-fluid state through the combined effects of mechanical and enzymic activity. Other aspects, however, are subject to much discussion and speculation, often with conflicting data. In the hepatopancreas, for example, do B-cells

switch from the secretion of digestive enzymes to the elimination of waste products at the appropriate stage in the digestive sequence? Are degenerating R-cells responsible for the release of emulsifying agents? Is nutrient uptake in the midgut effected by active transport or passive absorption? What is certain is not only that many questions remain to be answered, but that a much wider range of species, and their larval stages, need to be thoroughly investigated before more definite conclusions can be drawn. In other invertebrate groups variations in the physiology of digestion can be related, for example, to differences in diet or to variations in gut morphology. How true this is amongst decapod crustaceans remains to be determined.

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CRUSTACEAN BIOENERGETICS: THE ROLE OF ENVIRONMENTAL VARIABLES AND DIETARY LEVELS OF MACRONUTRIENTS ON ENERGETIC EFFICIENCIES

by

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ABSTRACT

Aquaculturists have been interested in optimizing the growth of various crustacean species in their attempts to develop commercially viable methods for cultivation. Crucial to optimization of crustacean growth is an understanding of energy partitioning and metabolic utilization of various dietary constituents by crustaceans, as metabolic responses are important determinants of the physiological processing of calories and nutrients. Studies dealing with the effects of diet on growth and energetic efficiencies of various crustacean species are reviewed, with particular emphasis on several species of Homarus, Macrobrachium and Penaeus.

Utilization of a dietary component for energy needs is affected by the dietary level, the crustacean's ability to catabolize that substrate and the availability of other dietary constituents as sources of energy. Protein utilization and the protein sparing action of dietary lipid and carbohydrate have been investigated for several species of crustaceans. Decreased rates of protein catabolism and increased protein efficiency ratios were observed in Homarus and Macrobrachium fed diets with lipid and/or carbohydrate levels as energy supplements. In studies with Penaeus, the rate of protein catabolism and the dietary protein levels that promoted optimum growth were affected by the total energy content of specific diets. Other dietary constituents that have been shown to have a significant impact on attempts to define energetic efficiencies include essential fatty acids and fiber (cellulose) content. The comparative bioenergetic aspects of crustacean nutrition are discussed.

Key words: Crustaceans, Bioenergetics, Energetic Efficiencies, Homarus, Macrobrachium, Penaeus, Procambarus, Protein, Lipids, Carbohydrates.

INTRODUCTION

Aquaculturists have been interested in the nutrition and growth of commercially important decapod crustaceans in their attempts to find suitable methods for commercial cultivation. The use of formulated

feeds in crustacean aquaculture may minimize the cost of feeding in commercial operations, particularly if diets were formulated from low-cost feedstuffs, while expensive components such as protein were kept at a minimum level. Crucial to the optimization of crustacean growth is an understanding of energy partitioning and metabolic utilization of various dietary constituents by crustaceans.

Metabolic responses of crustaceans to various components of their diet are important determinants of the physiological processing of calories and nutrients and may provide an index of the efficiency of energy utilization and the growth promoting potential of a specific diet. These responses may be further modified by environmental variables such as temperature, water quality, the daily ratio and frequency of feeding. Studies dealing with the effects of environmental variables and dietary levels of macronutrient constituents on growth and energetic efficiencies are reviewed, with particular emphasis given to several species of Homarus, Macrobrachium and Penaeus and the comparative aspects of crustacean bioenergetics.

GENERAL ASPECTS OF BIOENERGETICS

The bioenergetics and growth of an organism can be defined through the construction of an energy budget, where the energetic fate of food consumed is quantified in terms of caloric equivalents. A schematic diagram of the energy budget devised by Warren and Davis (1967) is presented in Fig. 1. Some of the energy value of the food consumed (Q_C) is lost through unassimilated materials (Q_U), nitrogenous waste products (Q_V), the energy demands of metabolic maintenance ($Q_S + Q_D$) and behavioral activities (Q_A); the net energy gain is channeled into growth (Q_G). In crustaceans, energy losses associated with molting (Q_M) must also be considered and include both the losses of energy reserves metabolized during molting and the shed exuvia. Although metabolic costs of the actual molting proces may be high, values for Q_M have been found to be small components of the overall energy budget of crustaceans (Logan and Epifanio 1978; Capuzzo 1979).

The energy metabolically utilized or released ($Q_S + Q_D + Q_A$) can be summarized by the symbol Q_R, and the energy lost through waste products ($Q_U + Q_V$) by the symbol Q_W. Thus, the following simple equation can be derived:

$$Q_C - Q_W = Q_R + Q_G$$

Energy Budget

Using various components of the energy budget to calculate energetic efficiencies, the following parameters can be derived (Ivlev 1945):

Gross Conversion Efficiency $K_1 = Q_G/Q_C$

and

Net Conversion Efficiency $K_2 = Q_G/(Q_C - Q_U)$.

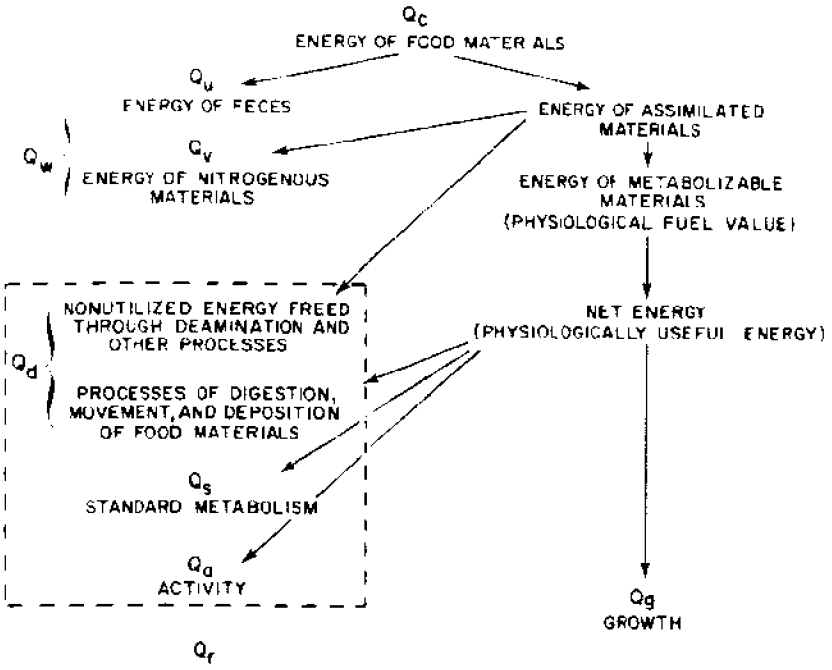

Figure 1. Schematic diagram of the energy budget devised by Warren and Davis (1967).

Estimates of K_2 are important when the assimilation efficiencies of crustaceans fed various diets differ greatly, resulting in different energy yields for metabolic processes and growth; K_2 values, however, are not routinely measured in aquaculture studies.

The consumption of food by an organism results in an increase in oxygen consumption (Q_R) and heat production; this increase is termed the specific dynamic action and is related to the catabolism of biochemical substrates. Energy lost through catabolic processes may vary with the utilization of different diets by crustaceans, thus resulting in significant differences in the channeling of energy towards growth and differences in energetic efficiencies.

From a practical standpoint the energetic efficiencies of food utilization in commercial aquaculture operations are not based on the construction of energy budgets but on the amount of feed offered and the resulting yield of new biomass. Food Conversion Rations are calculated as follows:

Food Conversion Ratio $FCR = \frac{F}{\Delta W}$

where, F = dry weight of feed offered and W = change in wet weight gain;

and

Normalized Food Conversion Ratio $FCR' = \frac{F}{(W_2 + W_D) - W_1}$

where food conversion ratios are normalized to include losses attributed to mortality and,

F = dry weight of food offered (grams),

W_1 = initial wet weight of population (grams),

W_2 = final wet weight of population (grams), and

W_D = wet weight of animals lost from the population (grams; Venkataramiah et al. 1975).

Energetic efficiencies based on values for food conversion ratios include estimates of energy losses related not only to the metabolic expenditures of an organism but also losses related to uneaten food and leaching of nutrients from unstable pelleted feeds. Although the normalized food conversion ratio is technically more accurate, aquaculturists are primarily interested in the overall efficiency of a particular feed and generally calculate food conversion ratios by the simplified version of the equation.

Values for K_1 (gross conversion effficiency) may also be calculated on a weight basis where equivalent values for caloric content of a specified feed and the organism are assumed. These values are calculated from the following equations:

$$K_1 = \frac{\Delta W}{C}$$

where, ΔW = wet weight gain (grams) and

C = dry weight food consumed (grams),

or $K = \frac{W}{R \Delta T}$ (Palaheimo and Dickie 1966),

where, W = wet weight gain (grams),

R = food rations fed per unit time (grams per day) and

ΔT = time (days).

Food conversion ratios and energetic efficiencies of decapod crustaceans are presented in Table I. Although values for FCR and K_1 varied with temperature (Bartley et al. 1980), ration size (Sedgewick, 1979a; Bartley et al. 1980) and dietary constituents (Venkataramiah et al. 1975; Balazs and Ross 1976; Colvin 1976a; Clifford and Brick 1979; Millikin et al. 1980; Read 1981), the intraspecific variation is greater than the interspecific comparison. The low values of FCR (0.3-0.5) measured for penaeids fed a diet of *Artemia* nauplii (Brand and Colvin 1977; Colvin and Brand 1977) may reflect the low wastage associated with this diet in comparison with formulated feeds, rather than improved energetic efficiencies alone. Energetic efficiencies of lobsters (*Homarus americanus*) based on the calculation of energy budgets do not reflect great differences between lobsters fed *Artemia* and those fed formulated feeds (Logan and Epifanio 1978; Capuzzo 1979; Bartley et al. 1980).

Bartley et al. (1980) found that at a constant temperature, energetic efficiencies of *H. americanus* decreased with increased daily ration size and suggested that larger ratios stimulated greater feeding activity and higher metabolic expenditures. Lower efficiencies might also result from greater wastage of larger ratios. Smaller ratios required less postabsorptive processing, resulting in higher efficiencies. Sedgwick (1979a) found that growth rate and utilization efficiency of *Penaeus merguiensis* increased with increased feeding frequency but values for FCR declined with increased body size and ratio size; relative decreases in efficiency were at a minimum when rations

were maintained near their maximum level (12 percent body weight per day). In addition dietary energy content and protein content may influence ingestion rates and conversion efficiencies, as shown by Sedgwick (1979b) for P. merguiensis and Balazs and Ross (1976) for Macrobrachium rosenbergii, presumably due to differences in palatability, energy density (kcal/gram) and feeding stimulants. Optimization of conversion ratios for crustaceans, therefore, requires an understanding of not only the nutritional quality of a specific diet but also the feeding behavior and digestive efficiency of individual species.

Table I. Food Conversion Ratios (FCR) and Energetic Efficiencies (K_1) of Decapod Crustaceans

Species	°C	Ration[a]	FCR	K_1	Diet	Reference
Homarus americanus	10	1-4	1.7-2.7	34-38	Alginate bound Artemia	Bartley et al. 1980
	15		1.0-2.1	44-89		
	20		1.2-2.0	43-79		
	25		1.5-1.7	55-63		
	20-22	5	1.9-2.2	38-42	Formulated Feeds	Capuzzo and Lancaster 1979; Capuzzo 1979
			2.1	39	Artemia	
	22	Ad lib.		18-33	Artemia	Logan and Epifanio 1978
Macrobrachium rosenbergii	22-27.6	5	1.3-1.9	-	Formulated Feeds + Algal supplement	Balazs and Ross 1976
	29.5	Ad lib.	-	4-59	Formulated Feeds	Clifford and Brick 1979
	28.5	9-12	1.5-2.5	-	Formulated Feeds	Millikin et al. 1980
	26.4	10-35	1.9-6.4	-	Supplemented Commercial Pellet	Sandifer and Joseph 1976
	30.0	Excess	1.8-3.2	-	Commercial Pellet supplemented with amino acids	Farmanfarmaian and Lauterio 1979, 1980
Penaeus aztecus	25	50-100	2.8-19.0	-	Formulated Feeds	Venkataramiah et al. 1975
P. californiensis	22	60	2.6-6.7	-	Formulated Feeds	Colvin and Brand 1977
			0.4	-	Artemia nauplii	
	22	60	2.6-4.0	-	Formulated Feeds	Brand and Colvin 1977
			0.3-0.4	-	Artemia nauplii	
P. indicus	28-30	10-15	2.4-5.7	-	Formulated Feeds	Colvin 1976a
	26	Ad lib.	1.9-3.5	-	Formulated Feeds	Read 1981
P. japonicus	23-28	2.5-5.5	1.3	-	Formulated Feeds	Deshimaru and Shigueno 1972
P. merguiensis	28	5-14	1.0-2.9	-	Commercial Pellet	Sedgwick 1979a
	28	Ad lib.	3.0-78.8	-	Formulated Feeds	Sedgwick 1979b
P. stylirostris	22	60	2.0-6.8	-	Formulated Feeds	Colvin and Brand 1977
			0.5		Artemia nauplii	
P. vannamei	22	60	1.2-3.1	-	Formulated Feeds	Colvin and Brand 1977
Procambarus clarkii	22	5	0.7-1.4	-	Formulated Feeds	Huner and Meyers 1979

[a] % of body weight fed daily.

Capuzzo (1979) compared the energy budgets of postlarval lobsters (H. americanus) fed an Artemia diet (51 percent protein, 5.1 protein: carbohydrate ratio) with the energy budgets of lobsters fed formulated feeds (varying in protein level 16.7-23.3 percent and protein: carbohydrate ratio 0.5-1.0). The best growth was measured among

lobsters fed the Artemia diet and the 23.3 percent protein diet, followed by the two lower protein diets (16.7 percent and 20.0 percent). All diets were assimilated at the same level (90 percent) but there were significant differences in other components of the energy budgets among the various groups. The greatest energy losses for all groups were due to metabolic expenditures (Q_R) and the highest metabolic rates were measured among lobsters fed the Artemia diet due to a higher specific dynamic action associated with digestion of this diet. Food consumption rates (Q_C) were reduced among lobsters fed the two lowest protein diets due to decreased pellet stability and resulted in the reduced growth rates of these groups. No difference in energy partitioning was observed among lobsters fed the formulated feeds; high gross and net conversion efficiencies were observed among all groups. Thus, the poor growth performance of some crustacean diets may be due as much to pellet stability and palatability as nutritional deficiencies.

Although little information is available on changes in bioenergetics in relation to the crustacean molt cycle, several studies have suggested an important role of nutrition in the molt cycle, particularly with regards to the storage and mobilization of reserves during molting (Passano 1960; Dall 1965; Thomas 1965; Castell and Budson 1974). Nutritional deficiencies may be reflected in inadequate energy stores, resulting in reduced growth increments, molt inhibition and poor conversion efficiencies. The relationship of diet and the bioenergetics of molting warrants further investigation.

Forster (1976) suggested that food conversion ratios of at least 2.0 were mandatory if crustacean aquaculture is to be economically feasible. From the data presented in Table I, the outlook for economically successful cultivation is quite favorable. The effect of various dietary constituents on energetic efficiencies of crustacean species is discussed in detail below.

EFFECTS OF MACRONUTRIENTS ON CRUSTACEAN BIOENERGETICS

Protein

Protein is an essential but expensive component of crustacean diets. It is necessary for tissue growth and maintenance but may also be catabolized as a source of energy by crustaceans (Wolvekamp and Waterman 1960; Cowey and Sargent 1972). Utilization of protein is affected by the nature of the dietary protein source, the level of protein intake, and the ability of an organism to utilize other dietary components as sources of energy.

Several investigators have tried to determine the suitability of various protein sources and optimum protein levels for supporting crustacean growth. The inadequacy of several commercially available protein sources and the requirement for high dietary protein levels appeared to be the result of specific amino acid deficiencies (Deshimaru and Shigueno 1972; Colvin 1976b; Boghen and Castell 1981). Farmanfarmaian and Lauterio (1979, 1980) found that supplementation of a commercial pelleted ratio with essential amino acids deficient in the pelleted feed (arginine, phenylalanine, leucine and isoleucine) resulted in improved growth and conversion efficiencies. In addition, factors other than amino acid composition such as mineral content and digestibility of various protein sources may also be important.

Reported values of optimum protein levels for penaeid species range considerably from as low as 30-40 percent (Andrews et al. 1972; Colvin 1976b; Colvin and Brand 1977; Sedgwick 1979b) to >60 percent (Deshimaru and Shigueno 1972). Other crustacean species, regardless of natural feeding habits, appear to have requirements similar to the lower values for penaeids: M. rosenbergii, 35-40 percent (Balazs and Ross 1976; Millikin et al. 1980); Procambarus clarkii, 20-30 percent (Huner and Meyers 1979); and H. americanus, 25-50 percent (Capuzzo and Lancaster 1979). It is clear from recent work, however, that optimum dietary protein levels can only be established in relation to other dietary constituents and the total energy content of the diet. Diets with low levels of non-protein energy sources may result in erroneously high protein "requirements" as much of the protein may be catabolized for energy needs.

To maximize both growth and protein efficiency ratios in crustaceans, energy sources in addition to protein must be utilized. Energy production from protein oxidation is both nutritionally and economically wasteful and the protein sparing action and utilization of other dietary constituents must be optimized. The efficiency of protein utilization is defined by the protein efficiency ratio and is calculated as follows:

$$PER = \frac{\Delta W}{P}$$

where, ΔW = wet weight gain (grams) and

P = dry weight protein (grams) fed.

If changes in biochemical composition of experimental animals are known, an index of protein utilization efficiency can be calculated as follows:

$$PUE = \frac{\Delta P_W}{P}$$

ΔPW = where dry weight protein (grams) deposited in tissue and

P = dry weight protein (grams) fed.

The partitioning of protein into catabolic and anabolic processes may be further defined by calculation of nitrogen balance:

$$N_B = N_I - (N_E + N_F)$$

where, N_B = nitrogen balance,

N_I = nitrogen intake,

N_E = nitrogen excreted from protein catabolism (both (1) NH_4^+-N and (2) amino-N as primary amines)

and N_F = unassimilated nitrogen (fecal N);

N_B/N_I - net protein utilization (NPU).

Protein efficiency ratios of decapod crustaceans are presented in Table II. Values for PER decreased with increasing dietary protein levels for each of the crustacean species studied. Capuzzo and Lancaster (1979) and Clifford and Brick (1979) attributed high values of PER at low dietary protein levels for Homarus and Macrobrachium, respectively, to the protein sparing effect of non-protein dietary

energy sources. Capuzzo and Lancaster (1979) found that lobsters fed diets with high carbohydrate levels and low protein: carbohydrate ratios, had reduced rates of protein catabolism as measured by N-excretion; O:N ratios and values for PER and PUE increased proportionately with decreased protein level. Similar metabolic responses were observed by Clifford and Brick (1979) and Millikin et al. (1980) for M. rosenbergii fed increased lipid and carbohydrate levels, but in the latter instance protein sparing at low (23-32 percent) dietary protein levels was not adequate to maintain optimum growth rates. Farmanfarmaian and Lauterio (1980) found that values for PER for M. rosenbergii increased with amino acid supplementation of a commercial pelleted feed, especially with the addition of lysine and to a lesser extent with the addition of arginine, leucine and isoleucine, indicating that limiting amino acids may affect PER values. Venkataramiah et al. (1975) found that growth rates of P. aztecus decreased with increased protein level, strongly suggesting that the channeling of protein into catabolic processes may affect energetic efficiencies. This is further supported by increased values of PER and improved growth of crustaceans fed diets with low protein: energy ratios (Gallagher et al. 1979; Sedgwick 1979b).

Table II. Protein Efficiency Ratios (PER) of Decapod Crustaceans

Species	Protein		PER	Reference
	Level (% dry wt)	Energy ratio (g protein/kcal)		
Homarus americanus	16.7	0.06	2.7	Capuzzo and Lancaster 1979
	20.0	0.07	2.3	
	23.3	0.08	2.2	
	51.0	0.16	0.9	
Macrobrachium rosenbergii	22.7	0.05	1.8	Millikin et al. 1980
	31.5	0.07	1.6	
	40.2	0.08	1.7	
	48.7	0.10	1.2	
Penaeus indicus	21.3	0.05	1.6	Colvin 1976b
	33.4	0.07	1.1	
	42.8	0.09	1.0	
	53.1	0.11	0.8	
P. merguiensis	16.6	0.04	0.9	Sedgwick 1979b
	28.0	0.06	0.8	
	39.5	0.09	0.8	
	50.9	0.11	0.4	
	28.0	0.07	0.9	
	28.0	0.07	0.8	
	28.0	0.11	0.5	

As pointed out by Sedgwick (1979b), primary energy reserves of crustaceans are in a constant state of flux, depleted by metabolic expenditures and replenished by dietary inputs. In an actively growing animal there should be an equilibrium between the amount of energy

assimilated and channeled into growth and the amount of energy utilized for metabolic maintenance. Optimum utilization of non-protein energy sources would result in diverting those sources preferentially to reserves and diverting protein maximally to growth. When non-protein energy sources are limiting in a specific diet, some dietary protein must be expended in energy production.

In summary, much of the early work regarding protein requirements of crustaceans was probably in error in suggesting that high dietary protein levels were necessary to maintain optimum growth. Recent evidence of the relationship of protein catabolism with dietary protein levels and protein sparing effect of non-protein energy sources would suggest that protein levels of 25-30 percent are sufficient for optimum growth.

Carbohydrates

If the sparing action of dietary carbohydrates is to be optimized, formulated feeds must contain carbohydrate sources that are readily and efficiently assimilated by an organism. Until recently little was known of the ability of crustaceans to assimilate and utilize various carbohydrate sources. Forster and Gabbott (1971) found that wheat starch, glycogen and dextrin were completely assimilated by the prawns Palaemon serratus and Pandalus platyceros, whereas assimilation of potato starch, both cooked and uncooked, was 93.1 percent and 86.2 percent respectively.

To compare the assimilation of different carbohydrate sources by the lobster H. americanus, Capuzzo and Lancaster (unpublished data) replaced corn starch in a formulated feed with equal amounts of either dextrin, rice starch, sucrose or wheat starch; the carbohydrate level in each diet was 23 percent. Assimilation efficiency and nitrogen balance (N_B) were determined for each group of postlarval lobsters fed the various feeds. All diets were readily consumed and assimilation efficiencies ranged from 94.2 percent to 96.8 percent. The relative percentages of protein and non-protein calories assimilated were estimated for each group and ranged from 93.6 percent to 96.5 percent for total N assimilated and 94.5 percent to 97.2 percent for non-protein calories assimilated. The highest assimilation efficiencies were measured among lobsters fed a diet containing dextrin as the carbohydrate source (#2).

Nitrogen balance of lobsters fed the various feeds is presented in Table III. Values of nitrogen ingestion (N_I) for the five groups of lobsters were not significantly different from one another. Ammonia excretion rates (N_{EI}), however, were significantly higher ($P<0.01$) among lobsters fed diets #2 (dextrin) and #4 (sucrose), suggesting that these carbohydrate sources had only a limited sparing effect on protein catabolism. The excretion of primary amines (N_{E2}) was variable among the five groups of lobsters, but was highest among lobsters fed diets #1 (corn starch) and #3 (rice starch); no excretion of primary amines was measured among lobsters fed diet #5 (wheat starch). Unassimilated nitrogen (N_F) was directly related to the assimilation efficiency of each diet. The highest values for N_B were found for diet group #1 and #5. As values for N_B reflect the catabolic and anabolic balance of protein, the positive values measured indicated that sufficient protein was being channeled into growth and anabolic processes in each of the five dietary groups.

Table III. Nitrogen Balance in Post-Larval Lobsters Fed Diets with Various Carbohydrate Sources

Parameter	Diet group #1 Corn starch	#2 Dextrin	#3 Rice starch	#4 Sucrose	#5 Wheat starch
N_I	14.7	15.1	15.3	15.7	14.5
N_A	13.9	14.6	14.5	14.9	13.8
N_{E1}	5.7	7.8	6.7	7.6	5.6
N_{E2}	1.2	0.6	1.4	0.4	-
N_F	0.8	0.5	0.8	0.8	0.7
N_B	7.0	6.2	6.4	6.9	8.2
N_B/N_I	0.48	0.41	0.42	0.44	0.57
O:N	13.0	10.4	10.8	10.8	15.6

N_I = nitrogen intake. N_A = nitrogen assimilated.

N_{E1} = ammonia excretion: measured as NH_4^+-N and converted to µg N/hour for stage VII and VIII lobsters, mean weight = 0.4 g.

N_{E2} = primary amine excretion: measured as glycine equivalents and converted to µg N/hour for stage VII and VIII lobsters, mean weight = 0.4 g.

N_F = unassimilated nitrogen.

N_B = nitrogen balance: $N_I - (N_{E(1+2)} + N_F)$.

N_B/N_I = net protein utilization (NPU).

O:N = oxygen to nitrogen ratio.

It is apparent from these findings that each of the carbohydrate sources at the specified dietary level was readily assimilated by lobsters but differences in the protein sparing effect by the various carbohydrate sources was evident as indicated by differences in both the N_B/N_I ratios and the O:N ratios. Metabolism of the simple sugars (dextrin and sucrose) presumably leads to synthesis of glycogen reserves as a long term energy store; whereas metabolism of the starches (corn and wheat) leads to utilization of the carbohydrate subunits for immediate energy needs inducing a protein sparing effect. No explanation can be given for the anomalous lack of a protein sparing effect among lobsters fed the rice starch diet.

Fair et al. (1980) found that the inclusion of cellulose fiber, presumably an undigestible dietary component, up to a level of 20 percent in an isonitrogenous series of diets stimulated growth and resulted in increased N assimilation by M. rosenbergii. Venkataramiah et al. (1975) observed a similar positive effect of fiber content on growth of P. aztecus. Fair et al. (1980) suggested two mechanisms for the increased rates of N assimilation of prawns fed higher dietary fiber levels:

(1) stimulation of microbial gut flora at higher fiber concentrations resulting in preferential protein utilization and the formation of microbial by-products that may be utilized by the prawn; and

(2) physiological factors (such as increased gut retention times) resulting in increased N assimilation.

The nutritional significance of dietary carbohydrate for crustacean species has received only limited attention but it is apparent that various carbohydrate sources may be used effectively in satisfying energy needs and improving energetic efficiencies.

Lipids

The fatty acid composition of dietary lipids is important not only in providing the fatty acids essential for growth but also in the maintenance and proper functioning of metabolic processes and the storage of lipids as energy reserves.

Crustaceans in general have only a limited capability for de novo synthesis of certain lipids, as shown by the lack of sterol synthesis (Zandee 1966a; Kanazawa et al 1971) and the lack of de novo synthesis of polyunsaturated fatty acids (PUFA) (Zandee 1966b; Kanazawa and Teshima 1977) in crustaceans examined to date. Thus, the fatty acid compositions of crustaceans maintained on formulated feeds reflect the composition of dietary lipids (Colvin 1976a; Sandifer and Joseph 1976; Bottino et al. 1980; D'Abramo et al. 1980) and demonstrate a limited capacity for biosynthetic conversion of PUFA to longer polyunsaturates of the same typed series. Inclusion of oils high in ω3 PUFA resulted in improved growth rates of H. americanus (D'Abramo et al. 1980), P. duorarum (Sick and Andrews 1973) and M. rosenbergii (Sandifer and Joseph 1976). In the latter instance improved growth and conversion efficiencies associated with the addition of shrimp head oil was not solely attributable to the calorigenic effect of a higher lipid level but to an essential requirement for ω3 fatty acids. Read (1981) also found that incorporation of linolenic (ω3) or linoleic (ω6) acids into purified diets and incorporation of natural oils high in ω3 and ω6 fatty acids into commercial rations improved growth and conversion efficiencies in P. indicus. Other penaeid species have shown similar requirements for linolenic and/or linoleic type fatty acids (Shewbart and Mies 1973; Sick and Andrews 1973; Guary et al. 1976; Kanazawa et al. 1977).

Lipid metabolism and storage is affected by both the quality and quantity of dietary lipids (Colvin 1976a; Sandifer and Joseph 1976; Bottino et al. 1980; D'Abramo et al. 1980). Understanding the effective utilization, deposition, biosynthesis and transport of lipids in crustacean species must await further investigation.

CONCLUSIONS

Optimization of growth and utilization of various dietary constituents by crustaceans cannot be viewed as a static phenomenon but as a series of dynamic processes, governed by the interrelationships of energetic pathways. To date the major achievements in crustacean nutrition include: (1) the demonstration of economically feasible energetic efficiencies using formulated feeds; (2) the identification of a protein sparing effect of dietary carbohydrates and lipids, leading to considerably lower protein requirements than originally suggested; and (3) the identification of specific lipid requirements that influence

both growth and conversion efficiencies. Establishing nutritional requirements and defining adequate dietary formulations require an understanding of the protein sparing effect of dietary constituents, the digestibility of diet components, and the biosynthetic capabilities of cultivated organisms. Our knowledge of crustacean nutrition is presently at a level that enables us to address some of the interrelationships of these processes and their importance in crustacean bioenergetics. Future studies need to address how best to maximize the sparing effect of dietary components while minimizing both protein content and wastage resulting in inefficient utilization of feeds.

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QUESTIONS AND ANSWERS

BAYER, (University of Maine): Judy, I was wondering how important you feel the contribution of the gut flora is to the assimilation process and how variable these flora are likely to be?

CAPUZZO: I'm sure that it's quite important. I don't know of any comparative studies that have looked at the role of the microbial gut flora on assimilation, but have only looked at the effect of fiber content, suggesting that microbialflora can indeed affect nutrient utilization. I'm sure this is an important aspect for the assimilation and retention of many nutrients in the gut, but I do not know of any comparative studies that have investigated differences between species, only describing the gut microbial flora and their activity in assimilating some of the nutrients.

PERSOONE (Ghent, Belgium): Judy, I was interested in your comment about fiber content. Of course, fiber content often increases the mortality and I was wondering if you think the effect might be due to retention times as you mentioned or due to increasing the absorptive digestive surfaces of food particles?

CAPUZZO: That is a possibility but it is really speculative at this point as to just what the effect of fiber content is. Some studies have shown increased mobility through the gut; other studies have shown

increased retention time. The increased surface area and increased microbial activity may both be factors affecting this and it must await further investigation.

HILTON (University of Guelph): Judy, you said there was reduced ammonia excretion in the corn and wheat starch fed lobsters, is that correct?

CAPUZZO: Yes.

HILTON: And you assume then that there was to be a decreased protein utilization in the sense of increased protein deposition as muscle. Is that correct?

CAPUZZO: Yes.

HILTON: Was this born out by the growth data or was it just a basis of decreased ammonia excretion rates, as I didn't see that on the slide?

CAPUZZO: Yes, that slide was just to show the nitrogen budget of the lobsters fed the various feeds. Growth rates were indeed higher among the corn starch and wheat starch fed animals. All of the diets had an identical protein to carbohydrate ratio. The corn starch and wheat starch fed groups grew better than the other 3 groups.

HILTON: Have you done any work on the carbohydrate metabolism in these animals looking at why digestion of some carbohydrate sources would be less efficient in terms of protein sparing?

CAPUZZO: No we haven't yet, but we do intend to in the future.

PENAEID NUTRITION

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ABSTRACT

At the outset of shellfish culture in Japan, the basic techniques for the artificial rearing of the prawn (Penaeus japonicus Bate) from hatching to commercial size were established. However, some problems such as the lack of adequate artificial diets and disease still remain. Disease incidence is related in some cases to inadequate diet. The lack of essential nutrients can result in specific deficiency diseases as well as general mortality and retardation of growth.

This paper presents an overview of the biochemical aspects of crustacean nutrition, specifically, that of the prawn (P. japonicus). Although the prawn requires, as do other animals, adequate levels of proteins, lipids, carbohydrates, minerals, and vitamins for growth, studies with the prawn also have uncovered unique aspects of metabolism, especially lipid metabolism. Based on this biochemical knowledge, artificial diets have been compounded and are used in the mass production of prawns. Recently, interest has turned to the nutritional requirements of the larvae of P. japonicus. Little information is available concerning the nutrition of these stages despite their importance in prawn culture.

KEY WORDS: Nutrition, penaeid, prawn, larvae, essential nutrients.

INTRODUCTION

Since Hudinaga (1942) succeeded in rearing the prawn (Penaeus japonicus) under artificial conditions, techniques for rearing this species from hatching to commercial size have been steadily improved in Japan and have been applied to other penaeid species in many countries. Studies on the nutritional requirements of P. japonicus juveniles were begun about 13 years ago (Kanazawa et al. 1970; Kitabayashi et al. 1971a). As a result, the specific needs for proteins, lipids, carbohydrates, minerals, and vitamins for growth and survival of P. japonicus juveniles have been identified (see New 1976a,b, 1980 for references). On the basis of this knowledge, compounded artificial diets are presently being used for the commercial production of P. japonicus as substitutes for live food such as the short-necked clam (Tapes philippinarum).

In Japan, all the processes of commercial prawn culture are carried out by the private farmers themselves. The production of larvae depends principally on live food such as diatoms and Artemia salina. This production of live feeds requires extensive labor and facilities and fluctuates with climatic conditions. As a consequence, the development of artificial diets for larval prawns is currently one of the most important research areas in the field of prawn culture. Recently, I have been attempting to prepare artificial diets for larval P. japonicus for use both as substitutes for live food and for nutritional studies.

This paper presents an overview of the biochemical aspects of penaeid nutrition, especially, that of prawn (P. japonicus) larvae and juveniles. As for general nutrition, reviews have been presented by several other workers (New 1976b; Biddle 1977; Ceccaldi 1978; Conklin 1980; Kanazawa 1980).

MICROPARTICULATE DIETS FOR LARVAL STAGES OF P. JAPONICUS

Generally, live foods such as diatoms and Artemia salina L. have been used for rearing the zoeal and mysid stages of P. japonicus. Jones et al. (1979a), Villegas and Kanazawa (1980) Kanazawa et al. (1982), and Teshima et al. (in press) have developed several types of microparticulate diets for use as live food substitutes for larval prawns. Three approaches for presenting the test diet (Table I) have been examined: nylon-protein microcapsules, carrageenan-microbound particles, and zein-microbound particles. The particulate size of these three artificial diet particles could be adjusted at will to between 10 and 150 μm. Slight aeration was used to maintain the particles in suspension. The leaching of nutrients was less for the nylon-protein capsules, although the capsules were digested poorly as compared with the carrageen and zein-microbound diets. Although all three diets had a faint white-yellowish color and were readily ingested by larvae, larvae fed more actively when the diets were stained with a red dye. All three forms were found to sustain reasonable growth and survival of P. japonicus from the zoeal to postlarval stage when compared to live foods (Figure 1). The results enabled us to study the nutritional requirements of the larval stages of P. japonicus.

PROTEINS AND ESSENTIAL AMINO ACIDS

Deshimaru and Yone (1978c) have shown that the optimum protein level for juveniles (P. japonicus) is 52-57% in the diet on the basis of weight gain and feed efficiency. In general, juvenile or adult penaeids have been shown to attain optimum growth on diets containing 30-60% protein (New 1976b). It is probable that the difference in the optimum protein levels among species is likely to be due to both differences in food habit as well as the nature of the proteins used.

Deshimaru and Kuroki (1975) prepared a diet with a crystalline amino acid mixture instead of protein to study the essential amino acid requirements of P. japonicus, and found that such a diet was unsuitable for sustaining growth and survival. Their results indicated that the prawn (P. japonicus) was incapable of efficiently utilizing free amino acids in diets. Kanazawa and Teshima (1981), therefore, investigated the incorporation of radioactive acetate into the individual amino acids

of juvenile P. japonicus in order to determine requirements for essential amino acids. The prawn was shown to require 10 amino acids, arginine, methionine, valine, threonine, isoleucine, leucine, lysine, histidine, phenylalanine, and tryptophan. These ten amino acids have also been demonstrated to be essential for Penaeus aztecus (Shewbart et al. 1972), Palaemon serratus (Cowey and Forster 1971), Homarus americanus (Gallagher 1976), Astacus astecus (Zandee 1966), and Macrobrachium ohione (Miyajima et al. 1975).

Table I. Composition of Test Diet

Ingredient	g/100 g dry diet
Glucose	5.5
Sucrose	10.0
Starch	4.0
Glucosamine-HCl	0.8
Casein (vitamin free)	50.0
Na-citrate	0.3
Na-succinate	0.3
Cholesterol	0.5
Vitamin mixture[a]	3.2
Mineral mixture[a]	8.6
DL-methionine	1.0
L-tryptophan	0.5
Pollack liver oil	7.0
Soybean lecithin	3.0
Cellulose	5.3
Total	100.0

[a]For composition of vitamin and mineral mixtures see Kanazawa et al. (1977).

CARBOHYDRATES

The addition of glucose to diets has been shown to inhibit the growth of Penaeus aztecus (Andrews et al. 1972), Penaeus duorarum (Sick and Andrews 1973), and P. japonicus (Deshimaru and Yone 1978b). Abdel-Rahman et al. (1979) also demonstrated that the addition of over 10% glucose to the diet markedly inhibited the growth of P. japonicus. However, in contrast to monosaccharides such as glucose, galactose, and fructose, disaccharides such as sucrose, maltose, and trehalose, and polysaccharides such as dextrin and starch have a high nutritive value as carbohydrate sources. Especially, high weight gains have been attained on diets containing disaccharides (Abdel-Rahman et al. 1979). In contrast to other animals, juvenile (P. japonicus) were found to contain glucose, acetyl glucosamine, and trehalose as the major carbohydrates of the body tissues (unpublished data). Also, P. japonicus converted glucose to trehalose in the hepatopancreas and muscle but not in the stomach, intestine, and blood (unpublished data) (Table II). As shown in Figure 2, when the prawn diet contained glucose, the blood glucose levels were increased after one hour and remained at a high level for over 24 hours. However, when P. japonicus

was fed a diet containing maltose as the sole carbohydrate source, the blood glucose levels were increased after 1-3 hours and then decreased to a low level after 12-24 hours (Abdel-Rahman et al. 1979). The presence of carbohydrases (Table III) in the digestive tracts and the digestibility of carbohydrates (Table IV) has also been investigated (unpublished data). Based on the results of the above investigations, it is possible that the addition of glucose to the diets may inhibit the growth of P. japonicus for the following reason. Dietary glucose is not converted to trehalose in the stomach, but it is quickly absorbed from the stomach and then released all at once into the blood. When the large quantity of glucose is absorbed, the resulting high glucose level in the blood is above that normally maintained by hormonal control and is difficult to utilize as an energy source. On the other hand, a disaccharide such as maltose is not absorbed from the stomach, but is converted to glucose in the mid-gut and then to trehalose in the hepatopancreas which is then released gradually into the blood. Dietary maltose is thus readily utilized as an energy source.

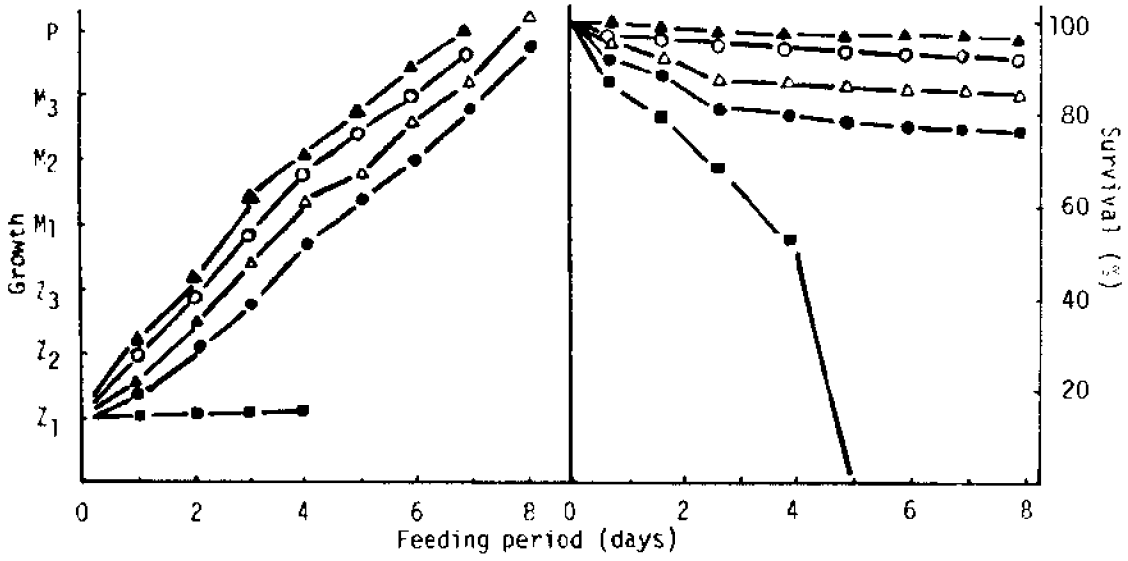

Figure 1. Growth and survival of the larvae of Penaeus japonicus fed on microparticulate diets (Kanazawa et al. 1982; Teshima et al. in press). Growth stages: Z_1, Zoea$_1$ stage; Z_2, Zoea$_2$ stage; Z_3, Zoea$_3$ stage; M_1, Mysis$_1$ stage; M_2, Mysis$_2$ stage; M_3, Mysis$_3$ stage; P, Postlarvae stage. Experimental conditions: No. of larvae--100 larvae/beaker (1000 ml); Sea water--sea water filtered through absorbent cotton; Amount of feed--0.16 mg/larva/day; Feed size at zoeal stages, 60 µm; at mysid stages, 60-125 µm; No. of feedings--2 times/day; Temperature--27°C. Food type: ○ Chaetoceros-Artemia; ● Nylon-protein microcapsules; ▲ Carrageenan microbound particles; △ Zein microbound particles; ■ No food.

Table II. In vitro Conversion of Glucose-^{14}C to Trehalose by Sliced Tissue of Adult P. japonicus. The sliced tissue (100 mg) was incubated with 0.5 μCi of glucose-U-^{14}C for one hour at 25°C. Nonradioactive glucose (2 mg) and trehalose (5 mg) were added as carriers, and the sugars were extracted from the incubation mixture. For the hemolymph, labelled glucose (0.2 μCi) was added to one ml of serum.

Tissue	Distribution of radioactivity (%)		Conversion[a] (%)
	Glucose	Trehalose	
Muscle	18.0	82.0	56.6
Hepatopancreas	72.0	28.0	17.8
Stomach	99.6	0.4	0.3
Intestine	99.1	0.9	0.8
Hemolymph	99.4	0.6	0.0

[a]Radioactivity in trehalose/radioactivity in glucose-^{14}C administered.

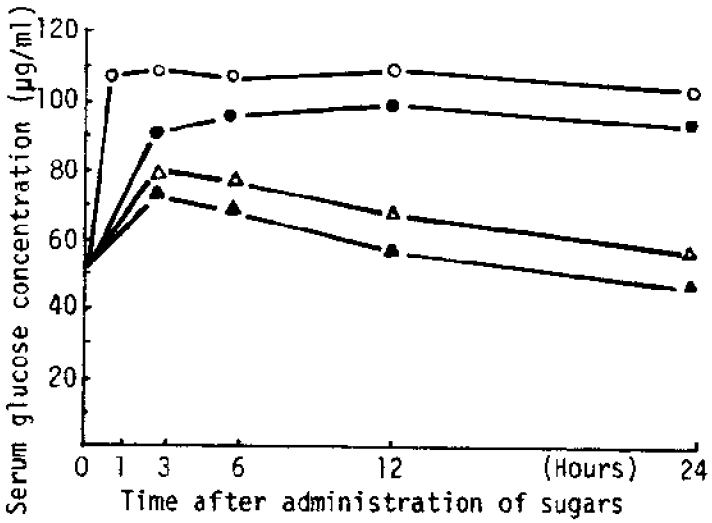

Figure 2. Variation in the serum glucose levels of juvenile P. japonicus after oral administration of 200 mg of either glucose, fructose, soluble starch, or maltose. o--Glucose, ●--Fructose, Δ--Soluble starch, ▲--Maltose.

Table III. Carbohydrases in Stomach and Mid-gut of Adult P. japonicus

Enzyme	Stomach extract	Mid-gut extract
Maltase	-	+++
Saccharase	-	++
Trehalase	-	-

Table IV. Digestibility of Carbohydrates in Adult P. japonicus

	Stomach	Mid-gut	Hind-gut
Glucose	+++	+	-
Maltose	-	+++	++
Saccharose	-	+++	++
Trehalose	-	+++	++

Crustaceans have an exoskeleton composed of chitin and it is a necessary process of growth to shed and replace the exoskeleton during molting. Recent experiments have shown that chitin, the major component of the crustacean exoskeleton, is synthesized from glucose via glucosamine in P. japonicus (unpublished data) (Figure 3). The addition of 0.52% glucosamine to the diet improved the growth of P. japonicus but the inclusion of chitin inhibited growth (Kitabayashi et al. 1971a.)

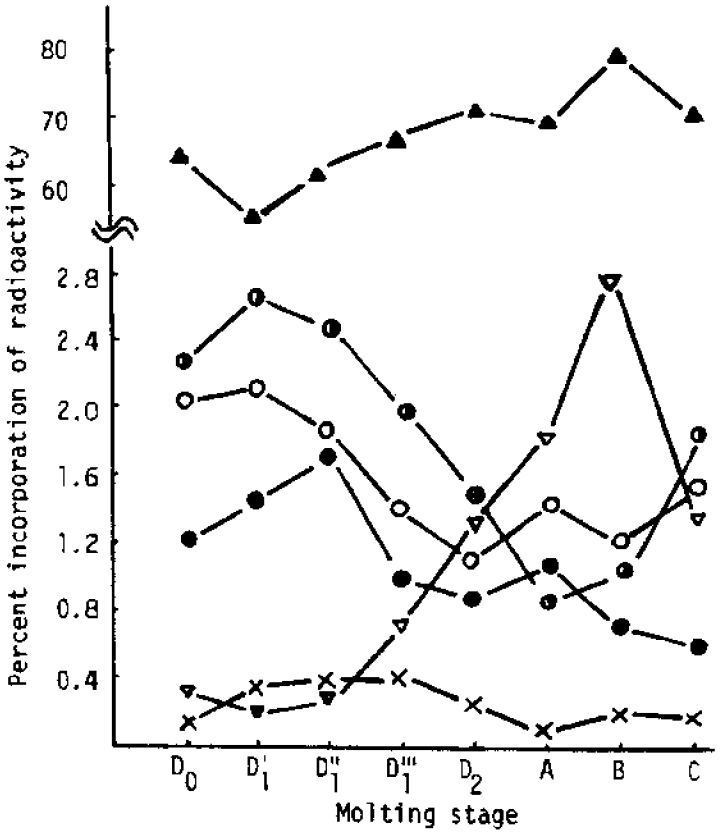

Figure 3. Radioactivity in various fractions from the juvenile prawn, Penaeus japonicus, 24 hours after injection of glucose-U-^{14}C (0.5 µCi) at various stages of the molting cycle (unpublished data). , CO_2; , Chitin; , Bound glycogen; , Acid soluble glycogen; , Protein; X, Lipid. Molt stages: A,B--Postmolt; C--Intermolt; D_0, D_1', D_1'', D_1''', D_2--Premolt.

LIPIDS

Fatty acids have been shown to play an important role not only as energy sources but also as essential nutrients in both fish and crustaceans (Teshima 1978, Yone 1978). It has also been demonstrated that crustaceans have an unique requirement for sterols and phospholipids in contrast to other aquatic animals and mammals.

Sterol Requirements

Animals are generally capable of synthesizing sterols from acetate, but crustaceans have been shown to be incapable of de novo sterol synthesis from acetate (e.g. Teshima and Kanazawa 1971). Moreover, since cholesterol is converted to sex hormones, (Kanazawa and Teshima 1971), and molting hormones (Spaziani and Kater 1973) and also utilized as a constitutent of the hypodermis (Guary and Kanazawa 1973) in several species of crustaceans, it is thought to be an essential nutrient for crustacean growth and survival. Optimum cholesterol levels in diets have been found to be about 0.5% for both juvenile (Kanazawa et al. 1971) and larval (unpublished data) P. japonicus, and also for the lobster (Castell et al. 1975). Crustaceans are capable of converting C_{28} and C_{29} sterols to cholesterol (Teshima 1971) and utilizing ergosterol and β-sitosterol to some extent for growth (Kanaqawa et al. 1971).

ESSENTIAL FATTY ACIDS

Essential Fatty Acids

Feeding experiments have shown that crustaceans, as well as fish, have a requirement for specific fatty acids (Teshima 1978). This has also been confirmed by metabolic studies using radioactive tracers. We have shown that juvenile (Kanazawa et al. 1979c) and larval (Jones et al. 1979b) P. japonicus efficiently incorporates [^{14}C] palmitic acid into saturated and monounsaturated fatty acids but little is transformed into linoleic (18:2ω6), linolenic (18:3ω3), eicosapentaenoic (20:5ω3), and docosahexaenoic (22:6ω3) acids (Table V). This suggests that 18:2 ω6, 18:3ω3, 20:5ω3, and/or 22:6ω3 are probably essential for the growth of P. japonicus. The results of feeding trials indicate that juvenile P. japonicus requires 18:2ω6, 18:3ω3, 20:5ω3, and 22:6ω3 as essential fatty acids, and that the 3 series of fatty acids is more effective than the ω6 series of fatty acids (Kanazawa et al. 1977; 1978; 1979e). The optimum dietary levels of 18:2ω6, 18:3ω3, 20:5ω3, and 22:6ω3 for juvenile P. japonicus were estimated to be 0.5-1% (Kanazawa et al. 1979a). Recently, in feeding experiments using carrageenan-microbound test diets, it was found that the larval stages of P. japonicus require the ω3 series of highly unsaturated fatty acids for their growth and survival (unpublished data) (Figure 4).

The requirements for essential fatty acids such as 18:3ω3, 20:5ω3, and 22:6ω3 vary widely among different species of crustaceans and fish. Kanazawa et al. (1979b) have investigated the capacity for conversion of 18:3ω3 to 20:5ω3 and 22:6ω3 in various species of aquatic animals, and have shown that marine fish and prawn have a lower ability for such a bioconversion than freshwater fish. These results might explain the findings that 20:5ω3 and 22:6ω3 are more effective as essential fatty

acids in the prawn and marine fish, such as the red sea bream than 18:3 $\omega 3$. It is probable that the differences in the requirements for essential fatty acids among aquatic animals are due primarily to differences in the capacity for bioconversion of exogenous 18:3ω3 to highly unsaturated fatty acids such as 20:5ω3 and 22:6ω3.

Table V. Radioactivity of the Principal Fatty Acids in the Total Lipids of Zoeal Penaeus japonicus Fed a Microencapsulated Diet Containing [1-^{14}C] Palmitic Acid (Jones et al. 1979b) and Juvenile P. japonicus Injected with [1-^{14}C] Palmitic Acid (Kanazawa et al. 1979a)

Fatty acid	Distribution of radioactivity (%)	
	Zoea[a]	Juvenile[b]
12:0	-	16.3
14:0	1.9	-
15:0	0.9	7.1
16:0	73.8	23.9
18:0	7.4	3.0
20:0	1.8	-
14:1	0.9	10.7
16:1ω7	2.7	10.9
18:1ω9	3.5	-
20:1ω9	0.3	3.4
18:2ω6	0.1	-
20:4ω6	1.5	-
18:3ω3	0.1	-
20:5ω3	0.6	0.8
22:6ω3	0.9	0.4

[a]Experimental condition of zoeal P. japonicus: Lipids were isolated from zoea 24 hours after feeding on a microencapsulated diet containing 10 µCi of [1-^{14}C] palmitic acid at 25°C.

[b]Experimental condition of juvenile P. japonicus: Five juvenile P. japonicus were injected with 2.5 µCi of [1-^{14}C] palmitic acid and maintained in the aquaria at 22-23°C. Lipids were isolated from the whole body of P. japonicus 24 hours after injection. The recovery of radioactivity in total lipids was 86.5%.

As part of the investigation into the physiological roles of 20:5 $\omega 3$ the fate of [U-^{14}C] 20:5ω3 injected into the muscle of P. japonicus has recently been examined (unpublished data). Eighty percent of the radioactivity injected into the prawn was recovered either in the lipid fraction, water-soluble fraction or as expired CO_2. As shown in Table VI, 86% of the radioactivity incorporated into lipids were associated with the phospholipid fraction.

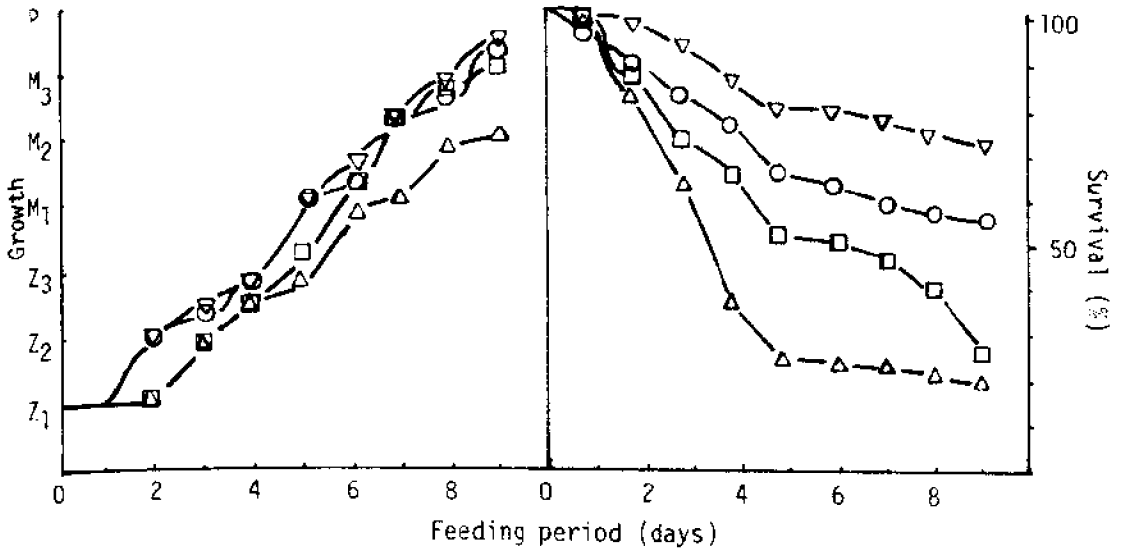

Figure 4. ω3 Polyunsaturated fatty acid requirements of prawn larvae Penaeus japonicus (unpublished data). Δ--5.0% 18:1ω9 + 3% soybean lecithin; ○--4.5% 18:1ω9 + 0.5% HUFA + 3% soybean lecithin; ▽--4.0% 18:1ω9 + 0.1% HUFA + 3% soybean lecithin; □--3.0% 18:1ω9 + 2.0% HUFA + 3% soybean lecithin. HUFA is mixture of 49% 20:5ω3 and 32% 22:6ω3. Growth stages: Z_1, $Zoea_1$ stage; Z_2, $Zoea_2$ stage; Z_3, $Zoea_3$ stage; M_1, $Mysis_1$ stage; M_2, $Mysis_2$ stage; M_3, $Mysis_3$ stage; P, Postlarvae stage. Experimental conditions: No. of larvae--100 larvae/beaker (1000 ml); Sea water--sea water filtered through absorbent cotton; Amount of feed--0.16 mg/larva/day; Feed size at zoeal stages 60 μm, at mysid stages 60-125 μm; No. of feedings--2 times/day; Temperature--27°C. Food type: Carrageenan microbound particles.

Table VI. Radioactivity Present in Each Lipid Class in the Adult Prawn (Penaeus japonicus) 24 Hours after Injection with [U-^{14}C] Eicosapentaenoic Acid (0.1 μCi) at 29°C (unpublished data)

Lipid class	Distribution of radioactivity (%)	
	Whole body	Hepatopancreas
Phospholipids	86.2	60.4
Monoglycerides	1.1	7.4
Free sterols	1.1	2.7
Diglycerides	5.2	3.2
Free fatty acids	0.5	22.5
Triglycerides	4.5	2.5
Steryl esters	1.0	0.7
Hydrocarbons	0.3	0.6

Phospholipid Requirement

To determine the reason for the superior nutritive value of shortnecked clam oil (Tapes oil) compared with pollack liver oil for the prawn, P. japonicus, Kanazawa et al. (1979d) examined the effects of several lipid fractions of Tapes oil on the growth of P. japonicus (Table VII). Juvenile P. japonicus fed on a diet supplemented with 1% Tapes lecithin grew best and a 1% Tapes cephalin was the next best phospholipid source. Conklin et al. (1980) have shown that the survival rate of juvenile lobsters was remarkably improved by the addition of 8% soybean lecithin to the diet. Recently, D'Abramo et al. (1981) have pointed out the essentiality of phosphatidylcholine for survival of the juvenile lobster Homarus americanus.

Table VII. Effect of Phospholipids on Growth of Juvenile P. japonicus[a] (Kanazawa et al. 1979d)

Dietary lipid	Weight gain (%)
7% Pollack liver oil	100
7% Pollack liver oil + 1% Tapes lecithin	160
7% Pollack liver oil + 1% Tapes cephalin	135

[a]Twenty juvenile P. japonicus of 1.0 g in body weight were maintained in a plastic aquarium (30 liters) for 45 days a5 22-25°C.

The effects of phospholipids on the growth and survival of P. japonicus larvae has also been studied by using carrageenan-microbound test diets (unpublished data). As shown in Figure 5, the P. japonicus larvae suffered 100% mortality prior to attaining the mysid state when they were fed on the diet without lecithin. But, larvae fed on the diet containing 3.0% soybean lecithin grew well, indicating the essentiality of certain phospholipids for growth and survival of the larvae from the nauplius to the post-larval stage. The effects of varius phospholipids on the growth and survival of larval prawn were further examined. The addition of either 1% soybean lecithin, bonito-egg lecithin, or soybean phosphatidylinositol markedly improved both growth and survival, whereas neither 1% dipalmytoyl-phosphatidylcholine, chicken-egg lecithin, phosphatidylethanolamine (from bovine brain), phosphatidylethanolamine (from bonito egg), phosphatidylserine (from bovine brain), sphingomyeline (from bovine brain), cytidine-51-diphosphate choline, nor taurocholic acid had a strong beneficial effect on growth and survival. I conclude the following: 1) phospholipids containing either choline or inositol exerted a positive effect on larval growth and survival; 2) phospholipids containing 18:2ω6, 18:3ω3, 20:5ω3, and 22:6ω3 in the molecule were the most effective in promoting growth and survival; 3) the effectiveness of the phospholipids seemed to be dependent on the nature of the fatty acids in the α and β positions of the phospholipid molecule; 4) the requirement for phospholipids for the larvae of P. japonicus was 0.5-1% in the diet (dry weight).

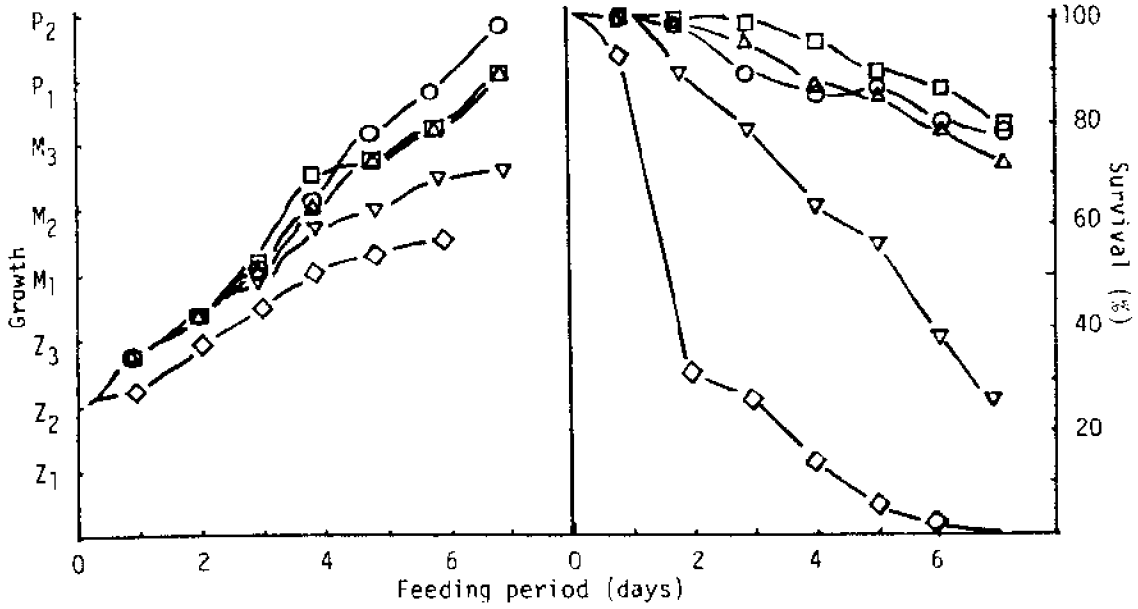

Figure 5. Lecithin requirements of larvae of the prawn Penaeus japonicus (unpublished data). ◇, Lecithin free; ○, 1.5% soybean lecithin; △, 4.5% soybean lecithin; ▽, 0.5% soybean lecithin; □, 3.0% soybean lecithin. Growth stages: Z_1, $Zoea_1$ stage; Z_2, $Zoea_2$ stage; Z_3, $Zoea_3$ stage; M_1, $Mysis_1$ stage; M_2, $Mysis_2$ stage; M_3, $Mysis_3$ stage; P, Postlarvae stage. Experimental conditions: No. of larvae--100 larvae/beaker (1000 ml); Sea water--sea water filtered through absorbent cotton; Amount of feed--0.16 mg/larva/day; Feed size at zoeal stages 60 μm, at mysid stages 60-125 μm; No. of feedings--2 times/day; Temperature--27°C. Food type: Carrageenan microbound particles.

To clarify the role of phospholipids in crustacean nutrition further, Teshima and Kanazawa (1980) have shown that the orally administrated [^{14}C] tripalmitin was incorporated into the phospholipids of the lipoproteins, HDL_2 (high density $lipoprotein_2$), HDL_3 (high density $lipoprotein_3$), and VHDL (very high density lipoproteins in P. japonicus (Figure 6). These results indicated that the transport of dietary lipids in P. japonicus was mainly carried out by phospholipids associated with high density lipoproteins, suggesting that the lipid transport mechanism of P. japonicus and probably other crustacean species is markedly different from that of mammals: in mammals, dietary lipids are transported through the lymph ducts as chylomicrons. It is proposed that the essentiality of dietary phospholipids for P. japoncius is due to both a specific requirement for phospholipids for the transport of fatty acids in the hemolymph and a slow rate of biosynthesis of these phospholipids.

MINERALS

It is suspected that crustaceans, as well as other aquatic animals, absorb minerals from the water to some extent. In the case of crustaceans, however, it is assumed they also require a dietary source of certain minerals because the exoskeleton which is rich in minerals is

lost during molting. Conklin et al. (1975) suggested that the mineralization of shell in juvenile lobsters was improved with calcium rich diets, but no significant difference in growth and survival occurred. Gallagher et al. (1978) has suggested that a calcium/phosphorous ratio of 1:2 was optimum for juvenile lobsters. Shewbart et al. (1973) has postulated that calcium, potassium, sodium and chloride requirements for Peneaus aztecus might be satisfied from sea water, however, phosphorus may be essential in the diet because it is present in large quantities in prawn but not in sea water. Deshimaru et al. (1978) has demonstrated with tracer experiments that the prawn (P. japonicus) was able to take up calcium from sea water. They have also shown that P. japonicus requires in the diet, on a dry weight basis, phosphorus (2.0%), potassium (1.0%), and trace metals (0.2%) but not calcium, magnesium, nor iron (Deshimaru and Yone 1978a). The best growth rate was achieved with diets for P. japonicus when supplementary levels of 1.04% phosphorus and 1.24% calcium were added (Kitabayashi et al. 1971a). Results of recent experiments in my laboratory indicate that the optimum levels of minerals in the diet, on a dry weight basis, of juvenile P. japonicus are as follows: calcium (1.0%), phosphorus (1.0%), magnesium (0.3%), potassium (0.9%), and copper (0.006%). A calcium/phosphorus ratio of 1:1 gave superior growth, although no significant difference was seen in the weight gains between the groups on the calcium-supplemented and calcium-free diets. The addition of 0.006% iron and 0.003% manganese to the diet inhibited growth of juvenie P. japonicus (unpublished data).

VITAMINS

The effect of vitamins on growth and survival of juvenile lobsters has been demonstrated by Conklin et al. (1980) using a purified diet. Vitamin requirements of the juvenile P. japonicus have been investigated by Kanazawa et al. (1976), Guary et al. (1976) and Deshimaru and Kuroki (1979) (Table VIII). Recently Kanazawa et al. have examined with the use of carrageenan microbound test diets, the vitamin requirements of P. japonicus larvae (unpublished data). P. japonicus larvae fed on a vitamin-free diets did not reach the postlarval stage, suffering 100% mortality in the mysid stage. Also, the survival of prawn larvae was extremely low when they were fed on diets lacking either -tocopherol, calciferol, choline, or vitamin C. Addition of vitamin C to a squid-based diet for juvenile P. japonicus accelerated growth rate, however, excess vitamin C inhibited growth. Prawn grew best at inclusion levels of 0.22% (Kitabayashi et al. 1971b). Iwata and Shigeno (1980) reported the whitening of the side-abdominal muscle of P. japonicus fed vitamin C deficient diets. However, it is not clear as to the reason of whitening induced by vitamin C deficient diets. Lightner et al. (1977) have found that Penaeus californiensis and Penaeus stylirostyis sometimes show an abnormal symptom, named "black death", with a characteristic blackening of the esophagus wall, cuticle, gastric wall, hind-gut wall, and gills. "Black death" has been recognized as a symptom of vitamin C deficiency (Margarelli et al. 1979) and it has been suggested that juvenile P. californiensis require dietary vitamin C to form adequate amounts of collagen from the unhydroxylated precursor, procollagen (Hunter et al. 1979).

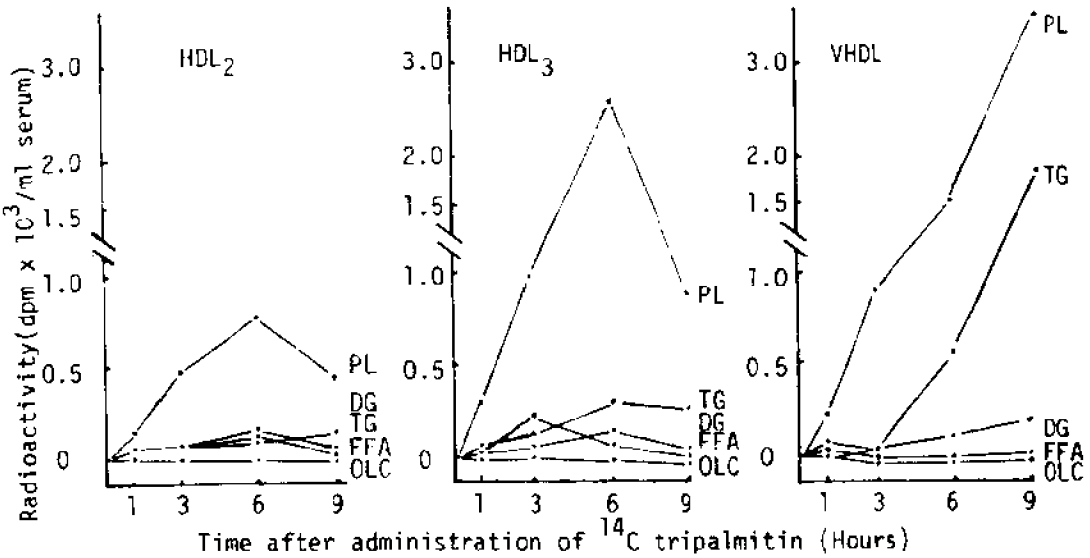

Figure 6. Incorporation of radioactivity into the lipid classes of serum lipoproteins, HDL_2, HDL_3, and VHDL of adult Penaeus japonicus, after oral administration of [^{14}C] tripalmitin (Teshima and Kanazawa 1980). Twenty-four µCi of glycerol [tri-1-^{14}C] palmitate was orally administered to 32 male P. japonicus at an intermolt period, and then 8 prawns were taken after 1, 3, 6, and 9 hours, respectively, for radioactive measurements of lipoproteins: HDL_2 (high density lipoprotein$_2$), HDL_3 (high density lipoprotein$_3$), and VHDL (very high density lipoproteins). Lipid classes constituting lipoproteins are abbreviated as follows: PL, polar lipids; DG, diglycerides; TG, triglycerides; FFA, free fatty acids; OLC, other lipid classes such as hydrocarbons, monoglycerides and steryl esters.

Table VIII. Vitamin Requirements of Juvenile Prawn Penaeus japonicus

Vitamin	Requirement (mg %)		
	Kanazawa et al. (1976)	Guary et al. (1976)	Deshimaru and Kuroki (1979)
Ascorbic acid	-	1000-2000	300
Choline	60	-	Dispensable
Inositol	200	-	400
Thiamine	-	-	6-12
Pyridoxine	-	-	12

CONCLUSION

For the larval and juvenile stages of the prawn (P. japonicus), much work has been carried out on their requirements for amino acids, sterols, fatty acids, phospholipids, minerals, and vitamins. Crustaceans require 10 amino acids, although the quantitative requirements of these amino acids have not yet been estimated. The optimum protein level in diet varies with penaeid species, dietary habits, and culture conditions as well as with the type of protein used. The author thinks that the use of plant proteins instead of expensive animal proteins is desirable in future.

The prawn (P. japonicus) has been shown to require sterols, phospholipids, and some fatty acids as essential nutrients. Cholesterol is the most capable of sustaining growth and survival to some extent. For P. japonicus, 18-2 6, 18-3 3, 20-5 3, and 22-6 3 are effective as essential fatty acids. With phospholipids, the relation between growth-promoting effects and chemical structure is still obscure.

In general, minerals and vitamins are added to artificial diets, however, there is little data on the quantitative requirements for each of the vitamins and minerals for penaeid prawns. The quantitative requirements for phosphorus, magnesium, manganese, iron, copper, thiamine, pyridoxine, inositol, choline, and ascorbic acid have been studied but the necessity for other minerals and vitamins for penaeids is unknown.

On the basis of knowledge from nutritional and biochemical studies, juvenile prawns have been successfully reared on artificial diets by private companies in Japan, the dietary value of artificial diets being almost comparable to live foods. On the other hand, the mass production of P. japonicus larvae still depends on live food, which fluctuates according to weather conditions, etc. One approach to overcome this problem is to attempt the rearing of larval prawns on micro-particulate diets. Another important problem will be the development of a diet for broodstock females in order to achieve the culture of P. japonicus over its entire life cycle on artificial diets. The author believes that knowledge of the nutrition of P. japonicus could be successfully applied to other crustacean species as well as molluscs.

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QUESTIONS AND ANSWERS - KANAZAWA

LANGDON (Delaware): Dr. Kanazawa, did the microencapsulation techniques you used alter the dietary lipid composition of your diets?

KANAZAWA: It is possible in that we do use organic solvents in making the nylon-protein microencapsulated diet. However, we have only looked at the lipids in terms of the response to the diets by the larvae.

CONKLIN (California): Dr. Kanazawa, you had reported in a study done with Dr. Jones that the nylon-protein capsule was not efficient in holding in water-soluble nutrients, yet you appear to get a fairly good growth response. Have you modified the nylon-protein capsule or are the water-soluble nutrients coming from bacterial growth on the capsule?

KANAZAWA: The response to the nylon-protein microcapsules is not always consistent. Some organic solvents are used in the preparation and there may be a problem with toxic residuals. The best of the diet types we have used for larval prawns is the carrageenan-microbound diets.

SICK (NMFS, Charleston): I'd like ask about the data you presented relating to mineral requirements. Dr. Kanazawa can you summarize in a sentence or two your results with mineral determinations?

KANAZAWA: Mineral requirements are not particularly important. Supplementing the diets with phosphorus, magnesium, potassium, and copper improves growth somewhat while iron and manganese inhibited growth slightly. Other minerals and their effect on growth have yet to be examined.

SEW-WAH TAY (M.I.T.): Zein which you used in the microbinding of diets can be toxic or indigestible for some animals. Have you done any studies on the possible toxicity of zein for the prawn?

KANAZAWA: No, we are using zein which has been heat-dried and reduced to a very fine powder. The prawn larvae appear to consume it readily and we are assuming it is easily digested.

PROTEIN AND AMINO ACID NUTRITION OF THE PRAWN

Penaeus japonicus

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ABSTRACT

The nutritive value of the short-necked clam (*Venerupis philippinarum*), considered to be an excellent food for prawn (*Penaeus japonicus*), was investigated with regard to improving the quality of compounded feeds. A review of these investigations is presented.

The soft part of fresh clam was chemically fractionated and the nutritive value of these fractions, when incorporated into a purified test diet, was assessed through feeding trials. A diet containing the clam protein fraction produced superior growth and feed efficiency in cultured prawns compared to a diet containing whole egg protein.

Whole egg protein or a casein-albumin mixture when used as the protein source for the purified diet was deficient in some essential amino acids when compared with those of the clam protein fraction. However, the addition of crystalline amino acid supplements to the diet to compensate for the deficiencies did not have any effect on prawn growth. In an experiment using radioactive arginine-[^{14}C(U)], the percentage incorporation of free arginine into prawn muscle protein was estimated to be only 0.6% after a six-day feeding trial.

By mixing purified proteins having different amino acid profiles, a diet was prepared so as to have an overall essential amino acid profile similar to that of the diet containing the clam protein fraction. Prawns which received this diet exhibited similar growth to the group fed the diet with the clam protein. On the other hand, a diet of mixed protein having a profile differing from that of the clam protein produced comparatively inferior growth in the prawn.

Keywords: Amino acids, Nutrition, *Penaeus japonicus*, Prawn, Protein.

INTRODUCTION

The prawn (*Penaeus japonicus*), known as "Kuruma Ebi" to the Japanese, is one of the most valuable aquatic animals cultured in Japan. On shrimp farms, this species is traditionally reared on the meat of fresh bivalves. However, the extensive use of this raw food results in several problems, in particular the pollution of the culture environment. Therefore, a compounded feed which can overcome the disadvantages of raw food is needed. For this reason, a series of

nutritional studies with the prawn were started in 1967 at the Kagoshima Prefecture Fisheries Experimental Station, Japan, in an attempt to produce a practical artificial diet for prawn. Feeding experiments were carried out with a pelletized diet composed of squid meal (Ommastrephes pacifica), trash shrimp meal, petroleum yeast, fish meal, whale meal (Physeter macrocephalus and Balaenoptera physalus), and soybean protein as the major ingredients. The results obtained from these studies were reviewed by Deshimaru and Shigeno (1972).

Currently, several feed manufacturers produce compounded feeds for commercial prawn culture, the formulation of which is based on those artificial diets developed at the Kagoshima Prefecture Fisheries Experimental Station. These feeds, however, are still considerably inferior in promoting prawn growth when compared to a diet of fresh short-necked clam (Venerupis philippinarum), which has been empirically established as the best of any of the raw foods used in prawn culture. In order to improve the nutritive value of compounded feeds, a further series of experiments was undertaken to clarify specific aspects of the prawn's nutritional requirements. A review of this particular series of experiments is presented with the hope that it may be of value to other investigators in the field of crustacean nutrition.

GENERAL METHODS

Prawns, less than 4g in average body weight, were obtained from a local shrimp farm in Kagoshima. They were initially fed a commercial compounded feed for one week. For each test diet, a group of twenty-five prawns were randomly selected and grown in a seventy-six liter tank with a bed of sand. Fresh sea water was continuously supplied to each tank at a rate of 700 liters per day. Each feeding experiment was conducted over a period of four weeks, except for Experiment 3 which lasted only six days. Water temperature ranged from 21 to 29°C. Other rearing procedures have been described previously (Deshimaru and Kuroki 1974a).

All the test diets were formulated based on the composition of the modified diet in Table I. They were prepared as a dry pellet (2mm in diameter, approximately 10mm in length), after adjusting the pH 6.5 to 6.7 with 25% NaOH solution.

BASELINE STUDIES

In previous studies by the author, the necessity of a number of nutrients, i.e. cholesterol, glucosamine, amino acids, minerals, carbohydrates, proteins, lipids, which were thought to be important in formulating prawn diets, were investigated and their optimum dietary levels determined (Deshimaru and Kuroki 1974b, 1974c; Delshimaru and Yone 1978a, 1978b, 1978c; Deshimaru et al. 1979). Based on the results obtained in these studies, two modified diets consisting of purified ingredients were proposed (Table I). Both modified diets seemed to satisfy the prawn's basic nutritional requirements in that mortality rates of prawns fed the two diets were low. Their nutritive values, however, in terms of growth were still lower than than of fresh clam (Table II). This result suggested that the modified diets were lacking growth factors which existed in fresh clam.

Table I. Composition of the Modified Diet for Penaeus japonicus

Ingredient	Percentage composition by dry weight	
	Diet A	Diet B
Casein-albumin (9:1) mixture	55.0	60.0
Pollack liver oil	4.0	3.0
Soybean oil	2.0	3.0
Sucrose	10.0	-
Glycogen	-	10.0
$NaH_2PO_4 \cdot 2H_2O$	10.0	10.0
KCl	2.0	2.0
Trace metals[a]	0.2	0.2
Vitamin mixture[b]	4.0	4.0
Cholesterol	1.4	2.0
Glycine	2.0	1.4
Carboxymethyl cellulose	5.0	-
Cellulose	4.4	4.4
Total	100.0	100.0

[a] $AlCl_3 \cdot 6H_2O$, 4.8; $ZnSO_4 \cdot 7H_2O$, ?; $MnSO_4 \cdot 4\text{-}6H_2O$, 21.4; CuCl, 3.0; KI, 4.6; $CoCl_2 \cdot 6H_2O$, ?; and cellulose, 43.0 mg.

[b] (Modified from Halver 1956). Thiamine-HCl, 5.0; riboflavin, 16.4; pyridoxine-HCl, 5.0; nicotinic acid, 65.8; Ca-pantothenate, 24.7; inositol, 328.8; biotin, 0.5; folic acid, 1.2; p-aminobenzoic acid, 32.9; choline chloride, 657.7; ascorbic acid, 822; α-tocopherol, 32.9; menadione, 3.3; β-carotene, 3.3; calciferol, 0.5; cyanocobalamin, 0.03; and cellulose, 700.0 mg (Deshimaru and Kuroki 1974a).

EXPERIMENT 1

In order to identify these growth factors the lyophilized soft part of fresh short-necked clam was extracted with chloroform-methanol (2:1) by the method of Bligh and Dyer (1959), and separated into three fractions, i.e. residual, methanol-soluble, and chloroform-soluble. The residual fraction was further extracted with hot 80% ethanol. The final residue from these procedures was dried to less than 10% moisture at 50°C, and was designated as the alcohol-insoluble fraction. This fraction contained 75.47% crude protein, 18.16% carbohydrates, and 5.82% ash (Table III). The methanol-soluble fraction was combined with the ethanol-soluble fraction and the solvents were evaporated off at 40°C; the residue was lyophilized to less than 10% moisture. This fraction was designated as the alcohol-soluble fraction which contained 39.56% crude protein, 0.88% crude fat, 1.34% carbohydrates, and 43.13% ash (Table III). The chloroform-soluble fraction was concentrated by evaporating off the chloroform under reduced pressure at 40°C, and was designated as the lipid fraction. This fraction, while containing primarily lipid, also included sterols at 16.2% of the fraction.

Table II. Results of Feeding Experiments Comparing the Survival and Growth of Penaeus japonicus Fed With (+) or Without (-) Clam Protein

	Diet			
	A-	A+	B-	B+
Number of prawn				
at beginning	20	20	25	25
after 4 weeks	19	18	25	23
Mean body weight (g)				
at beginning	1.38	1.34	0.85	0.85
±S.D.	±0.18	±0.21	±0.08	±0.08
after 4 weeks	2.40	3.39	1.68	2.32
±S.D.	±0.45	±0.75	±0.28	±0.37
Daily feed intake (%)[a]	6.4	4.4	7.3	3.4
Percent gain[b]	73.9	153.0	97.6	172.9
Feed efficiency (%)[c]	30.0	71.1	32.1	89.4

[a] $\frac{F}{\left(\frac{W+W_O}{2}\right)\left(\frac{N+N_O}{2}\right)} \times 100$ = daily feed intake (o/oo)

[b] $\frac{(W-W_O)}{W_O} \times 100$ = percent gain (o/oo)

[c] $\frac{(W-W_O)\left(\frac{N+N_O}{2}\right)}{F} \times 100$ = feed efficiency (o/oo)

F = total amount of feed intake during the feeding trial (dry matter, g)
D = duration of the feeding trial (days)
W_O = mean body weight of prawn at the beginning (g)
W = mean body weight of prawn at the end (g)
N_O = number of prawn at the beginning
N = number of prawn at the end

Table III. Proximate Composition (% of dry matter) of Alcohol-Insoluble and Soluble Fractions

	Fraction	
	Alcohol-insoluble	Alcohol-soluble[a]
Crude protein	75.47	39.56
Crude fat	-[b]	0.88
Carbohydrates[c]	18.16	1.34
Ash	5.82	43.13

[a] Lyophilized matter.

[b] Not found.

[c] As glucose.

These extracted fractions were then used in the formulation of four experimental diets, the composition of which are listed in Table IV. Diet No. 1 with whole egg protein was used as the basal diet. In diets Nos. 2, 3, and 4 the whole egg protein in the basal diet was substituted with either the protein containing alcohol-insoluble clam fraction, or the whole egg protein combined with the alcohol-soluble fraction, or the alcohol-insoluble fraction combined with the alcohol-soluble fraction, respectively. The amount of alcohol-soluble fraction added to diets Nos. 3 and 4 corresponded to the levels found in fresh clam.

Table IV. Composition of the Test Diets for Penaeus japonicus Incorporating Clam Fractions

Ingredient	% Composition by dry weight			
	Diet 1	Diet 2	Diet 3	Diet 4
Whole egg protein	51.1	-	38.6	-
Alcohol-insoluble fraction	-	61.6	-	48.0
Alcohol-soluble fraction	-	-	29.6	29.6
Lipid fraction	6.2	6.2	6.2	6.2
Sucrose	10.0	-	9.2	1.0
$NaH_2PO_4 \cdot 2H_2O$	8.4	7.0	8.0	7.0
KCl	2.1	1.9	0.2	-
Trace metals[a]	0.2	0.2	0.2	0.2
Vitamin mixture[a]	3.0	3.0	3.0	3.0
Glycine	1.5	1.5	-	-
Cellulose	12.5	13.6	-	-
Agar-agar	5.0	5.0	5.0	5.0
Total	100.0	100.0	100.0	100.0
Proximate composition (%)[b]				
Crude protein	47.9	48.0	47.9	47.9
Lipid	6.2	6.2	6.2	6.2
Carbohydrate	10.0	11.2	9.6	10.1
Ash	10.7	12.7	21.9	23.6
(phosphorus)	(1.7)	(1.7)	(1.7)	(1.7)
(potassium)	(1.1)	(1.1)	(1.1)	(1.1)

[a]See Table I footnotes.

[b]Proximate composition was calculated based on the analytical values for respective ingredients.

In an earlier study with P. japonicus, a superior weight gain of the prawn was reported with a diet containing short-necked clam lipid than with a diet containing pollack liver oil (Kanazawa et al. 1977). A series of nutrition studies with the prawn by the present author also revealed that the clam lipid fraction was more effective as a lipid source than a mixture of pollack liver oil and soybean oil in promoting the growth of prawn (Deshimaru, 1981). Therefore, in this experiment a mixture of pollack liver oil and soybean oil, which had been used as a lipid source for the purified test diet was substituted with the clam lipid fraction in diets 1 to 4. All the diets were formulated so as to be of a similar proximate composition to that of the basal diet.

The nutritive value of the clam protein (alcohol-insoluble fraction) was compared with that of the whole egg protein based on the results of a feeding experiment using diet Nos. 1 to 4 (Table V). The percent gain of prawn fed the diet with clam protein (diet No. 2, 111.6%) was higher than that of the prawn maintained on the diet with whole egg protein (diet No. 1, 81.0%). There was a significant difference in the growth of these diet groups at the end of four weeks of feeding, when compared using Student's "t" test ($P<0.01$). The feed efficiency of diet No. 2 (73.7%) was also superior to that of the diet containing whole egg protein (No. 1, 51.5%). When the whole egg protein or the clam protein was combined with the clam alcohol-soluble fraction (diets Nos. 3 or 4), feed efficiencies were poor (23.2 or 33.7%), although the percent weight gains (77.6 or 139.5%) were similar to those groups fed the same diets but without the addition of the clam alcohol-soluble fraction.

Table V. Comparison of Nutritive Value of Whole Egg Protein and Clam Protein (alcohol-soluble fraction) for Penaeus japonicus

	Without alcohol-soluble fraction		With alcohol-soluble fraction	
	Whole egg protein	Clam protein alcohol-insoluble fraction	Whole egg protein	Clam protein alcohol-insoluble fraction
	Diet 1	Diet 2	Diet 3	Diet 4
Number of prawn				
at beginning	25	25	25	25
after 4 weeks	25	23	24	24
Mean body weight (g)				
at beginning	0.84	0.86	0.85	0.86
±S.D.	±0.09	±0.08	±0.09	±0.07
after 4 weeks	1.52	1.82	1.51	2.06
±S.D.	±0.27	±0.35	±0.25	±0.39
"t" test ($P<0.01$)[a]	-	S	-	S
Daily feed intake (%)[b]	4.0	3.5	8.6	8.7
Percent gain[b]	81.0	111.6	77.6	139.5
Feed efficiency (%)[b]	51.5	73.7	23.2	33.7

[a]A Student's "t" test was used to determine significant difference (S) between the mean body weight of groups fed on Diets No. 1 and 2, and No. 3 and 4, at the end of four weeks of feeding.

[b]See the footnote to Table II.

These results suggested that the superior nutritive value of the clam should be attributed primarily to its protein content, and that the low nutritive value of the purified diet was due to the type of protein used.

EXPERIMENT 2

The nutritive value of a dietary protein depends on its essential amino acid balance as well as the quantity present in the diet. Phillips and Brockway (1956) suggest that dietary protein most closely approximating the amino acid profile of an animal's body protein has the highest potential nutritive value. In this experiment, the amino acids of clam protein were analyzed and its amino acid profile was compared with that of a purifed diet (diet No. 5, Table VI) containing a casein-albumin mixture as the protein source.

Table VI. Comparison of Essential Amino Acid Profiles of the Short-necked Clam (Venerupis philippinarum) and Diet 5

Amino acid	Amino acid composition (% of dry matter)		Ratio of amino acid to methionine in clam[a]	Ratio X 1.61[b]	Excess or deficient amount of amino acid in Diet 5 as compared with clam protein
	Clam	Diet 5			
Essential amino acids:					
Methionine	1.38	1.61	1.0	1.61	0.00
Threonine	2.30	2.22	1.7	2.74	-0.52
Valine	2.22	3.34	1.6	2.58	0.76
Isoleucine	2.15	2.98	1.6	2.58	0.40
Leucine	3.37	4.83	2.4	3.86	0.97
Phenylalanine	1.85	2.95	1.3	2.09	0.86
Lysine	3.51	3.81	2.5	4.03	-0.22
Histidine	0.89	1.41	0.6	0.97	0.44
Arginine	3.95	1.94	2.9	4.67	-2.73
Non-essential amino acids:					
Aspartic acid	2.56	3.96			
Serine	2.27	2.85			
Glutamic acid	8.64	13.07			
Proline	2.02	6.63			
Glycine	3.25	3.12			
Alanine	3.58	2.02			
Cystine	0.20	0.10			
Tyrosine	1.72	2.85			
Total	48.56	59.69			
Crude protein[d]	67.87	59.31			

[a]Level of each amino acid in clam protein/level of methionine in clam protein.

[b]Methionine level in Diet 5.

[c]Positive values indicate the excess amount as percent of amino acid in Diet 5. Negative values indicate the deficient amount as percent of amino acid in Diet 5.

[d]Total nitrogen x 6.25.

Acid hydrolysis of protein was conducted under reduced pressure for 24 hours at 110°C with 6N HCl in a sealed glass tube. Tryptophan was analyzed after hydrolysis for 6 hours at 120°C with 4N NaOH. The hydrolyzates were evaporated and diluted with citrate buffer solution (pH 2.2) for the amino acid analysis. The analysis was performed using a Hitacahi Liquid Chromatograph (model-034). Cystine was analyzed as

cysteic acid by the method of Moore (1963). The diet was largely deficient in arginine and somewhat deficient in threonine and lysine as compared with the amino acid profile of clam protein (Table VI).

The composition of test diets for Experiment 2 are listed in Table VII. Diet No. 5 with a casein-albumin mixture (9:1) was used as the basal diet. Diet Nos. 6, 7, and 8 were fortified with crystalline amino acids: either L-arginine, L-threonine, or L-arginine combined with L-threonine, respectively, to make up for the deficient essential amino acids in the casein-albumin mixture, as compared with those of the clam protein. All the diets were formulated to be isonitrogenous.

Table VII. Composition of the Test Diets for Penaeus japonicus Incorporating Crystalline Amino Acid Supplements

Ingredient	% Composition by dry weight			
	Diet 5	Diet 6	Diet 7	Diet 8
Casein-albumin (9:1) mixture	60.0	57.3	59.5	56.8
L-Arginine	-	2.7	-	2.7
L-Threonine	-	-	0.5	0.5
Pollack liver oil	3.0	3.0	3.0	3.0
Soybean oil	3.0	3.0	3.0	3.0
Glycogen	5.0	5.0	5.0	5.0
$NaH_2PO_4 \cdot 2H_2O$	10.0	10.0	10.0	10.0
KCl	2.0	2.0	2.0	2.0
Trace metals[a]	0.2	0.2	0.2	0.2
Vitamin mixture[a]	4.0	4.0	4.0	4.0
Cholesterol	2.0	2.0	2.0	2.0
Glycine	1.5	1.5	1.5	1.5
RNA	0.5	0.5	0.5	0.5
Cellulose	8.8	8.8	8.8	8.8
Total	100.0	100.0	100.0	100.0

[a]See Table I footnotes.

In the feeding trail the percent weight gain of prawn fed the arginine supplemented diet (No. 6) was similar (132%) to that with diet No. 5 (127%) (Figure 1). However, groups fed either the threonine or the combined arginine and threonine supplemented diet (nos. 7 and 8) grew less (107 and 110%, respectively). The highest feed efficiency was obtained with diet No. 5 (80%), while all the diets fortified with amino acids resulted in lower feed efficiencies (52 to 55%). Thus, supplementation of the diet with crystalline amino acids to make up for amino acid deficiencies in the diet did not improve its nutritive value.

EXPERIMENT 3

In connection with Experiment 2, the extent to which a dietary essential amino acid in the free form could be incorporated into muscle protein of the prawn was estimated in a feeding experiment using a diet containing radioactive arginine-[^{14}C(U)].

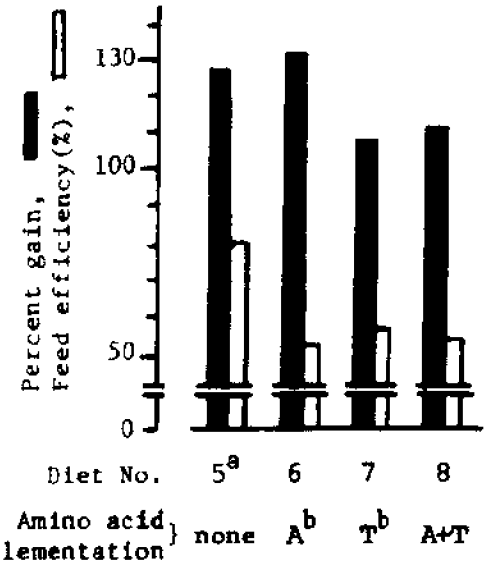

Figure 1. Effect of amino acid supplementation on the growth and feed efficiency of Penaeus japonicus. a - basal diet; b - A = arginine, T = threonine.

A diet containing L-arginine-[^{14}C(U)] (specific activity 10mCi/mmol arginine, New England Nuclear) at a total radioactivity level of 0.5 Ci per gram of diet was prepared using a modification of diet No. 5. To insure delivery, diet No. 5 was fortified with a binder, 10% w/w mannan (a type of hemicellulose), to prevent, as far as possible, leaching of the labelled arginine, and with 2% w/w hot water-soluble oyster extract as an attractant to stimulate rapid feeding by the prawns. Each day, individual prawns were fed 0.1g of the radioactive diet. Feeding took place in the evening so as to ensure that the feed would be consumed immediately. The ratio was ingested completely by the the prawn within one to two hours after presentation.

The prawn fed the diet containing radioactive arginine were sampled after day one, three, and six. Immediately after sampling, the prawns were separated into three parts: muscle, hepatopancreas, and remaining tissues. The muscle and hepatopancreas were treated with hot 80% ethanol, and fractionated into alcohol-soluble and alcohol-insoluble (residual) fractions. The alcohol of the soluble fraction was evaporated off and the residue redissolved in a citrate buffer solution (pH 2.2). The alcohol-insoluble fraction and remaining tissue portion were hydrolyzed with 6N HCl according to the procedure for the amino acid analysis as described in Experiement 2. The hydrolyzates were evaporated to dryness and the residue redissolved in a citrate buffer solution (pH 2.2). The samples with citrate buffer solution were used for the radioactivity anlysis. Radioactivity measurements of the buffered samples were made with a scintillation counter (Beckman model LS-230). A toluene solution with 0.5% w/v diphenyloxazole and 0.03% w/v dimethylphenyloxazole was used as the scintillator. The results of the experiment are summarized in Table VIII.

Table VIII. Radioactivity and Its Distribution in the Whole Body of Prawn Fed a Diet Supplemented with L-arginine [$^{14}C(U)$][a]

	Days elapsed after initial feeding					
	1		3		6	
	dpm	%	dpm	%	dpm	%
Dietary arg-^{14}C ingested by prawn	113000	100.0	339000	100.0	678000	100.0
Arg-^{14}C incorporated in whole body of prawn	12500	11.1	15670	4.6	24410	3.6
Distribution of arg-^{14}C in muscle:						
Alcohol-soluble fraction	2540	2.2	5970	1.8	7800	1.2
Alcohol-insoluble fraction in hepato-pancreas	20	<0.1	420	0.1	3760	0.6
Alcohol-soluble fraction	1080	1.0	2090	0.6	2810	0.4
Alcohol-insoluble fraction in remaining	140	0.1	490	0.1	320	<0.1
tissues	8720	7.7	6700	2.0	9720	1.4

[a]Results are based on the analysis of two prawns per sample period.

The analysis of one gram of the radioactive diet revealed 1,130,000 dpm of the radioactivity. As each prawn consumed 0.1g of the radioactive diet per day during the six day feeding experiment, the radioactivity ingested by each individual prawn was assumed to be 113,000 dpm per day. The radioactivity incorporated in the prawn whole body increased with feeding, from 12,500 dpm after one day to 15,670 dpm and 24,410 dpm at three and six days, respectively. At one day after feeding, the levels of activity distributed in the body of the prawn were 2.2% in the alcohol-soluble fraction of muscle, 1.0% in the hepatopancreatic alcohol-soluble fraction, 0.1% in the hepatopancreatic alcohol-insoluble fraction and 7.7% in the remaining tissues. At six days after feeding, the ratio in each region decreased to 1.2, 0.4, <0.1%, and 1.4%, correspondingly. In contrast, the level of activity in the muscle protein (alcohol-insoluble extraction) was <0.1% after one day of feeding, but increased slightly to 0.6% after six days of feeding. The incorporation of arginine originating from dietary protein was calculated from the amount of dietary arginine ingested and the increase in the arginine content of the prawn during the six-day feeding trial and was thus estimated to be as high as 90.5%. The assimilation of dietary free arginine into muscle protein was extremely low compared with that of protein bound arginine. These facts indicate that the prawn does not effectively utilize dietary free arginine in the synthesis of muscle protein. Thus, it was concluded that supplements of crystalline essential amino acids that are deficient in the diet will not result in an improvement of prawn growth.

EXPERIMENT 4

Experiment 1 revealed the superior nutritive value of clam protein as a dietary protein. However, attempts to improve the protein quality of deficient diets with free amino acid supplements were found to be almost completely ineffective in Experiments 2 and 3. Therefore, in the next experiment the nutritive value of a mixed protein having a similar essential amino acid profile to that of the clam protein was tested.

Whole egg protein, casein, and three purified proteins from whole P. japonicus, squid meal, and skip-jack testes meal were used as the protein sources of the test diets. The procedure outlined earlier in the fractionation of clam tissue was used in the preparation of proteins (alcohol-insoluble extraction) from P. japonicus, squid meal, and skip-jack testes meal. The amino acid compositions of the proteins are summarized in Table IX.

Table IX. Amino Acid Compositions (% of dry matter) of Whole Egg Protein, Casein, and Purified Proteins from prawn (P. japonicus), Squid, and Skip-jack Testes Meals

Amino acids	Whole egg protein	Casein, vitamin free	Purified protein		
			Prawn	Squid	Testes
Aspartic acid	8.61	7.17	11.16	9.69	7.16
Threonine	3.73	4.09	3.62	3.84	3.61
Serine	7.09	5.96	4.23	4.47	4.28
Glutamic acid	11.88	24.32	17.19	14.03	10.18
Proline	3.37	10.57	2.84	5.45	3.62
Glycine	2.71	1.86	4.09	10.46	3.59
Alanine	4.93	2.81	5.56	5.32	4.92
Cystine	1.94	0.43	1.16	0.69	0.91
Valine	3.86	5.66	2.87	2.49	3.09
Methionine	3.01	2.67	2.92	2.61	2.06
Isoleucine	3.46	4.49	2.89	2.72	2.56
Leucine	6.47	8.70	7.04	5.37	5.56
Tyrosine	3.16	5.66	3.62	2.74	3.08
Phenylalanine	4.15	5.18	3.90	3.01	3.12
Lysine	5.45	7.15	7.24	4.29	5.22
Histidine	1.71	2.73	1.66	1.45	1.72
Arginine	5.45	3.42	7.46	6.99	7.98
Tryptophan	0.79	0.92	0.52	1.03	0.67
Total	81.77	103.79	89.97	86.65	73.33
Crude protein[a]	95.21	92.50	94.36	92.87	96.47

[a]Total nitrogen x 6.25 (%).

The compositions of the test diets for Experiment 4 are listed in Table X. Diet No. 9 with the clam protein was used as the basal diet. Diet Nos. 10 and 11 were required so as to have an essential amino acid profile similar to or differing from that of the clam protein, by mixing the several purified proteins, each having different amino acid profiles. All the diets were isonitrogenous and isocaloric.

Table X. Composition of the Test Diets for Penaeus japonicus Incorporating Isolated Protein Fractions

Ingredient	Diet 9	Diet 10[a]	Diet 11[a]
Protein source:			
Clam protein fraction	75.3	-	-
Whole egg protein	-	-	6.1
P. japonicus protein fraction	-	7.1	6.1
Squid protein fraction	-	-	6.1
Testes protein fraction	-	34.5	6.1
Casein	-	17.9	36.4
Clam lipid fraction	6.2	6.2	6.2
Glycogen	-	10.0	10.0
$NaH_2PO_4 \cdot 2H_2O$	6.9	8.4	8.4
KCl	1.9	2.1	2.1
Trace metals[b]	0.2	0.2	0.2
Vitamin mixture[b]	3.0	3.0	3.0
Glycine	1.5	1.5	1.5
Cellulose	-	4.1	2.8
Agar-agar	5.0	5.0	5.0
Total	100.0	100.0	100.0

[a]Amino acid composition is listed in Table IX.

[b]See Table I footnotes.

All the proteins contained more than 92% crude protein and 82% amino acids, except for the testes protein which was slightly low in amino acid content (73%). The three purified proteins were rich in arginine as compared with whole egg protein and casein. When the essential amino acid profile of whole egg protein was compared with that of the clam protein, the whole egg protein was found to be deficient in lysine and arginine, and to a lesser extent in threonine, leucine, and histidine. The essential amino acid profiles of diets Nos. 10 and 11, which were made up with several proteins, are compared with the amino acid profile of the clam protein in Table XI.

Diet No. 10 contained sufficient amounts of essential amino acids, except for lysine which was present in slightly lower amounts than in the clam protein. Diet No. 11 was deficient in threonine, lysine, and particularly arginine - a profile differing significantly from that of the clam protein.

The results of the feeding experiment are shown in Table XII. Diet No. 10 produced the highest percent gain in the weight of prawn (210.6%). Statistically, this percentage gain was equal to that produced by diet No. 9, the basal diet containing the clam protein (200.0%). On the other hand, prawns fed diet No. 11 exhibited inferior growth (164.6%). Diet Nos. 9 and 10, which produced superior growth, also resulted in higher feed efficiencies (67.4 and 38.0% respectively) than that of diet No. 11 (21.1%).

Table XI. Amino Acid Composition (% of dry matter) of Diets 10 and 11, and a Comparison of Essential Amino Acid Profiles for Clam Protein and the Proteins of Diets 10 and 11

Amino acid	Clam protein content	Clam protein Ratio[b]	Protein source of Diet 10[a] in 59.5 g[c]	Protein source of Diet 10[a] Ratio x 1.40[d]	Protein source of Diet 10[a] Difference[e]	Protein source of Diet 11[a] in 60.8 g[c]	Protein source of Diet 11[a] Ratio x 1.62[d]	Protein source of Diet 11[a] Difference[e]
Essential amino acids:								
Methionine	1.70	1.0	1.40	1.40	0.00	1.62	1.62	0.00
Threonine	2.81	1.7	2.24	2.24	0.00	2.39	2.59	-0.00
Valine	2.18	1.3	2.28	1.82	0.46	2.82	2.11	0.71
Isoleucine	2.00	1.2	1.89	1.68	0.21	2.35	1.94	0.41
Leucine	4.01	2.3	3.97	3.22	0.75	4.66	3.73	0.93
Phenylalanine	2.13	1.2	2.28	1.68	0.60	2.75	1.94	0.81
Lysine	4.68	2.7	3.59	3.78	-0.19	3.96	4.37	-0.41
Histidine	1.27	0.7	1.20	0.98	0.22	1.39	1.13	0.26
Arginine	4.50	2.6	3.90	3.64	0.26	2.94	4.21	-1.27
Tryptophan	0.51	0.3	0.43	0.42	0.01	0.52	0.49	0.03
Non-essential amino acids:								
Aspartic acid	6.78		4.55			4.84		
Serine	3.15		2.84			3.39		
Glutamic acid	8.97		9.08			12.11		
Proline	2.23		3.34			4.78		
Glycine	2.90		1.86			1.95		
Alanine	3.07		2.59			2.29		
Cystine	1.58		0.48			0.44		
Tyrosine	2.10		2.33			2.38		
Total			50.25			58.03		
Crude protein[f]			56.54			56.69		

[a]See Table X.

[b]Relative level of each amino acid to methionine content in clam protein.

[c]Amount of the protein added to the diet.

[d]Level of methionine in the protein.

[e]Positive values indicate the excess amount (%) of amino acids in the protein source compared with those of clam protein. Likewise, negative values indicate deficient amounts of amino acids.

[f]Total nitrogen x 6.25.

Table XII. Result of Feeding Experiments Comparing the Survival and Growth of Penaeus japonicus Fed Diets 9, 10 and 11

	Diet 9	Diet 10	Diet 11
Number of prawn			
at beginning	25	25	25
after 4 weeks	22	22	24
Mean body weight (g)			
at beginning	0.64	0.66	0.65
±S.D.	±0.08	±0.07	±0.07
after 4 weeks	1.92	2.05	1.72
±S.D.	±0.65	±0.48	±0.31
"t" test ($P<0.05$)[a]	-	NS	NS
Daily feed intake[b] (%)	5.3	9.6	15.3
Percent gain[b]	200.0	210.6	164.6
Feed efficiency[b] (%)	67.4	38.0	21.1

[a]Student's "t" test was used to determine significant differences (S) between the mean body weight of groups fed the basal diet (No. 9) and test diets (No. 10 or 11) at the end of four weeks of feeding. No significant difference was shown as NS.

[b]See Table II footnotes.

CONCLUSIONS

The nutritive value of protein from the short-necked clam (V. philippinarum), considered an excellent food for the culture of P. japonicus was examined in relation to its amino acid composition in a series of feeding trials.

The effect of a diet containing clam protein on the growth and feed efficiency of prawn was far superior to that of a diet made up of either whole egg protein or a casein-albumin mixture. Amino acid analysis revealed that the whole egg protein and the casein-albumin mixture were deficient in some essential amino acids as compared with the clam protein. However, supplementing the deficient diets with crystalline amino acids did not enhance the growth and feed efficiency of the prawn. In earlier studies, the effect of crystalline amino acids on prawn growth was examined by replacing the protein portion of a purified test diet (a casein-albumin mixture) with an amino acid mixture. Regardless of the amino acid composition, the amino acid test diet resulted in very poor growth of prawn (Deshimaru and Kuroki, 1974c). Likewise, casein hydrolyzates which were prepared by treatment with various types of protease were also ineffective in promoting prawn growth (Deshimaru and Kuroki, 1975). The results obtained from both the previous and present studies suggest that the nutritive values of free amino acids and/or peptides, as a dietary nitrogen source, are far inferior to that of intact protein in sustaining prawn growth.

To determine the incorporation of dietary free amino acids into prawn muscle protein, a feeding experiment was conducted using a diet

containing radioactive arginine. The percentage incorporation of free radioactive arginine into the muscle protein was 0.6%, which was strikingly lower than that of protein bound arginine (90.5%). Thus, it was concluded from the two experiments in the present study using crystalline amino acids that essential amino acid deficiencies in the diet could not be compensated for by the use of supplements of free amino acids.

In earlier studies, the prawn's absorption efficiencies of dietary amino acids were determined in feeding trials with either an amino acid test diet or a casein-albumin test diet. The amino acids of both diets were assimilated mainly in the hepatopancreatic region of the alimentary tract and the total amino acid absorption was similar for the two groups (Deshimaru, 1976a). The changes in the concentrations of free amino acids in the hepatopancreas, plasma, and muscle of prawns receiving the amino acid test diet were compared with those of prawns fed on the casein-albumin test diet. All the free essential amino acids in the hepatopancreas of prawns fed the casein-albumin test diet attained maximum concentration levels 3 hours after feeding and then declined significantly 6 hours after feeding to below pre-feeding levels. This may be attributed to the use of essential amino acids from the hepatopancreas in the synthesis of body protein. Twenty-four hours after feeding, the levels of these amino acids had returned to pre-feeding levels. This pattern of change in essential amino acid concentration after feeding was with the casein-albumin test diet. On the other hand, levels of essential amino acids in the hepatopancreas of prawns which received the amino acid test diet reached peak concentration levels after 6 hours, decreasing to pre-feeding levels after 12 hours from feeding without ever dropping below pre-feeding levels. The concentrations of individual essential amino acids at their maximum levels, however, did not show significant difference between the two dietary groups (Deshimaru, 1976b). These results suggest that the inability of free amino acids to substitute for dietary proteins in supporting normal prawn growth is probably due to differences in the rate of absorption.

In the present series of experiments, a diet was prepared so as to have an essential amino acid profile similar to that of clam protein, by compounding several proteins each having different amino acid profiles. Prawns fed on the compounded protein diet exhibited growth rates that were similar to that of prawns fed on the diet containing clam protein. A diet prepared with mixed proteins having an essential amino acid profile differing from that of the clam protein resulted in inferior growth of prawn. This experiment indicates that the essential amino acid composition of dietary protein is an important factor affecting the nutritive value of artificial diets for prawn. Prawn growth rates, equal to that produced by using the diet with clam protein can be obtained by using several proteins in combination so as to produce an overall essential amino acid profile matching that of clam protein.

These results indicate that when compounding a practical artificial diet for the prawn, an attempt should be made to simulate, as closely as possible, the amino acid profile of the best natural food. However, the amino acids must be supplied in a protein bound form to maximize their incorporation and thereby increase feed efficiency

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QUESTIONS AND ANSWERS - DESHIMARU

FARMANFARMIAN (Rutgers): Dr. Deshimaru, what was the approximate percentage of the three free amino acid supplements: arginine, threonine, and arginine plus threonine, compared to the amount of those same amino acids contained in the protein portion of the diets in experiment 2?

DESHIMARU: Free arginine was added at the level of about 2% of the total diet which contained approximately 50% protein. As arginine represents approximately 4% of the protein, the free and bound amounts of arginine were roughly equal.

FARMANFARMIAN: In my own studies with Macrobrachium, when the level of protein in the diet is 50% the unlimited availability of all amino acids obviates any positive growth increment or any additional measure, such as PER (protein efficiency ratio), that we might use to see the effect of supplementation. We only see an effect on growth when the total amount of dietary protein is limited and also the amino acid which is deficient is limited. Such is the case with Macrobrachium, where the total protein was 20% and arginine or lysine were present at 5% of the protein or 1% of the total diet. I suggest the two species could be compared and I believe that, if these studies were done at a lower protein level, we would see an effect on both growth and PER with direct supplementation of free amino acid. This assumes of course that they are bound in the diet and are ingested by the animal. I haven't worked with other binders but alginate is certainly excellent; it retains 80% of the free amino acids in the pellet for 6 hours even when the animal is feeding and thus removing some of the external surface.

DESHIMARU: True, I used a rather high level of protein and amino acid supplements. Perhaps, if I conducted experiments at the lower levels you suggest different results might be obtained but with the data presently available no effect of supplemental free amino acids is evident.

MAUGLE (Rhode Island): Dr. Deshimaru, would you explain why you used the amino acid profile of the clam rather than the shrimp's own amino acid profile as your reference?

DESHIMARU: The clam is an excellent food for the shrimp, and the essential amino acid profiles of the two are similar. This indicated that the essential amino acid profile of the clam might be useful in satisfying the amino acid requirement of the shrimp.

MAUGLE: One other thing. Could you explain the lack of response to the alcohol-soluble fraction?

DESHIMARU: The clam extract fraction probably contains free amino acids and other types of nitrogenous components but these are already available in the diet. Thus, the extract had little effect on the prawn's growth.

HERNANDOREMA (France): Dr. Deshimaru, what is the role of RNA (ribonucleic acid) in the prawn diets? I noticed you included RNA in some of your diets but not others.

DESHIMARU: Dietary RNA seems to stimulate the feeding of the prawn but it does not appear to be of benefit in terms of improving feed efficiency.

HERNANDOREMA: Dr. Deshimaru, in those diets where you used RNA it appeared to be at a constant RNA: protein ratio. Have you tried varying this ratio?

DESHIMARU: No, I haven't yet carried out any experiments varying that ratio.

FATTY ACID METABOLISM IN CRUSTACEANS

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ABSTRACT

The ability to synthesize saturated fatty acids from acetate was shown to be present in the few species of marine crustaceans studied to date. The delta-9-desaturase system, which converts saturates to monoenes, was also demonstrated in several crustaceans. The de novo synthesis of fatty acids of the linoleic and linolenic series was either extremely limited or non-existent in crustaceans. Fatty acids of either of these two series obtained from dietary sources could, however, be chain elongated and further desaturated. Thus, the fatty acid pattern of crustacean lipids could be expected to represent a combination of those fatty acids synthesized de novo and those obtained from diets.

The fatty acid composition of crustacean lipids has been shown to be affected by many factors. Variation between different species is probably due to a combination of genetics, diet, environmental temperature and other biotic and abiotic factors. Even within single species, fatty acid patterns may be significantly altered by one or more of these factors. The fatty acids vary among different tissues and organs and even among different lipid classes of any tissue or organ. In spite of this complex fatty acid composition picture, there are a number of consistent patterns that might be valuable in predicting essential fatty acid (EFA) requirements of crustacean species of interest to aquaculture.

Marine crustaceans tend to have higher levels of linolenic series fatty acids and higher amounts of 20 and 22 carbon polyunsaturated fatty acids (PUFA) than freshwater crustaceans. The freshwater species tend to have higher levels of linoleic type fatty acids. Thus, one might predict that linolenic series fatty acids will have a greater EFA value to marine crustaceans, while fresh water species might require more linoleic series fatty acids or a mixture of both. Depending on the rate at which they are able to desaturate and elongate fatty acids, some marine crustaceans might require eicosapentenoic or docosahexenoic acid to satisfy their EFA requirement. Similarly, coldwater crustaceans might be expected, based on differences in fatty acid patterns, to require more linolenic series fatty acid than warmwater species.

Relatively little research on EFA requirements of crustaceans has been done. Linolenic acid has been shown to have greater EFA value than

linoleic acid for a few marine crustaceans. The 20 and 22 carbon linolenic series fatty acids were superior to linoleic acid. Some warmwater crustaceans were shown to do better with a mixture of linoleic and linolenic acids than either alone. The experimental protocol for determining EFA requirements is available. Many important species of crustacean have yet to be examined and this should be a very fertile area in the next few years of aquaculture nutrition research.

KEY WORDS: Crustacean, lipid, essential fatty acid, metabolism, linolenic, linoleic, temperature, salinity, diet.

INTRODUCTION

It is well known that lipids are the most energy rich of the nutrient classes, providing approximately 9 Cal/g. compared with 4-5 Cal/g for carbohydrates and proteins. The catabolism and subsequent release of energy from fatty acids in crustaceans will not, however, be discussed in this review. Instead this review will focus on the nutritional and anabolic aspects of crustacean fatty acid metabolism. The principal components of most lipids are fatty acids. Since the time of Burr and Burr (1929, 1930) it has been known that animals have an essential dietary requirement for specific types of fatty acids. The essential fatty acid (EFA) requirement of homeothermic animals was generally satisfied by fatty acids of the linoleic acid series (Holman 1968). Fish, poikilothermic animals, have been shown to have varying requirements for either linoleic, linolenic or mixtures of fatty acids from both series, depending on factors such as temperature, salinity, genetic variation and other environmental and biotic factors (Castell 1979; Castell et al. 1981).

The hypothesis that crustaceans also have varying EFA requirements will be examined in this review. The interactions between de novo synthesis and fatty acids of dietary origin in the lipids of various aquatic crustaceans will also be investigated.

FATTY ACID GROUPS

To simplify understanding, fatty acids may be divided into three different groups as follows:

1. Fatty acids that can be synthesized de novo from acetate. This group includes all even carbon number, straight-chain, saturated fatty acids up to 20, 22 or 24 carbons. The most abundant is usually 16:0[1], with lesser amounts of 14:0, 18:0, and 20:0.

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[1] A shorthand designation for identifying fatty acids. The first number is the number of carbons in the molecule. The number following the colon is the number of double bonds. The n-x (which is replacing the earlier omega designation in much of the recent literature) indicates the position of the last double bond, where x is the number of carbons from the methyl end to the double bond. Substituting the total number of carbons in the fatty

Animals usually have a delta-9-desaturase enzyme system which permits them to convert saturated fatty acids to monoenoic fatty acids.

For example:

14:0 ----------> 14:1n-5
16:0 ----------> 16:1n-7
18:0 ----------> 18-1n-9

The most abundant crustacean monoene is 18:1n-9 followed by 16:1n-7. It is usually possible to chain elongate or shorten these monoenes. This retains the (n-x) component and thus a number of chain length variations arising from a specific monoene are possible, for example: 18:1n-9 to 16:1n-9 or 20:1n-9. It is also common for animals to be able to add more double bonds in a methylene interrupted configuration, toward the carboxy, but not toward the methyl end of monoenoic fatty acids. Thus, a typical symptom of EFA deficiency in rats is the production of 20:3n-9 from 18:1n-9 (Holman 1968).

2. Unusual fatty acids that exist in the lipids of many crustaceans. These include:
   a) Odd chain fatty acids such as 13:0, 15:0, 17:0, 19:0, 15:1 and 17:1.
   b) Branched chain fatty acids of both the iso and the anti-iso configuration.
   c) Non-methylene interrupted fatty acids where two or more double bonds are present but separated by more than three carbons.
   d) Cyclopropanoic and cyclopropenoic fatty acids.

The amount of these unusual fatty acids in crustacean lipids is usually small. There are exceptions: for instance, the amphipod, Pontoporeia femorata contains almost equal amounts of both odd and even chained fatty acids (Paradis and Ackman 1976). Although there is some dispute, it is probable that all of these unusual fatty acids are of dietary origin in crustaceans, having been generated earlier in the food web by microorganisms. This group of fatty acids will not be discussed further in this report, as they have not been shown to have any nutritional value beyond their caloric content. The greatest interest in these unusual fatty acids would be in tracing feeding patterns through the aquatic food web.

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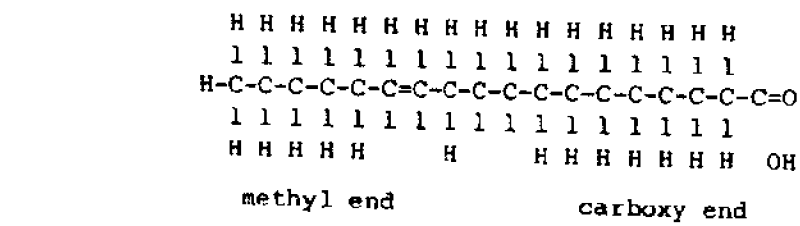
acid for n gives the position of this double bond from the carboxy end.
For example: linoleic acid or 18-2n-6 is



3. Essential fatty acids (EFA) that are the primary focus of this review. There are two families, or series, of fatty acids (Figure 1) which cannot be synthesized de novo by crustaceans (Kayama et al. 1980). As pointed out earlier, animals can add double bonds toward the carboxy but not toward the methyl end of a fatty acid. The characteristic of each of the families of fatty acids in this group is the length of the methyl end counting from the 1st double bond. Those with a 6 carbon end are in the linoleic family and have the greatest EFA value to homeothermic animals such as rats and men (Holman 1968). Those with a 3 carbon end are in the linolenic family and have greater EFA value to cold-water fish (Castell 1979).

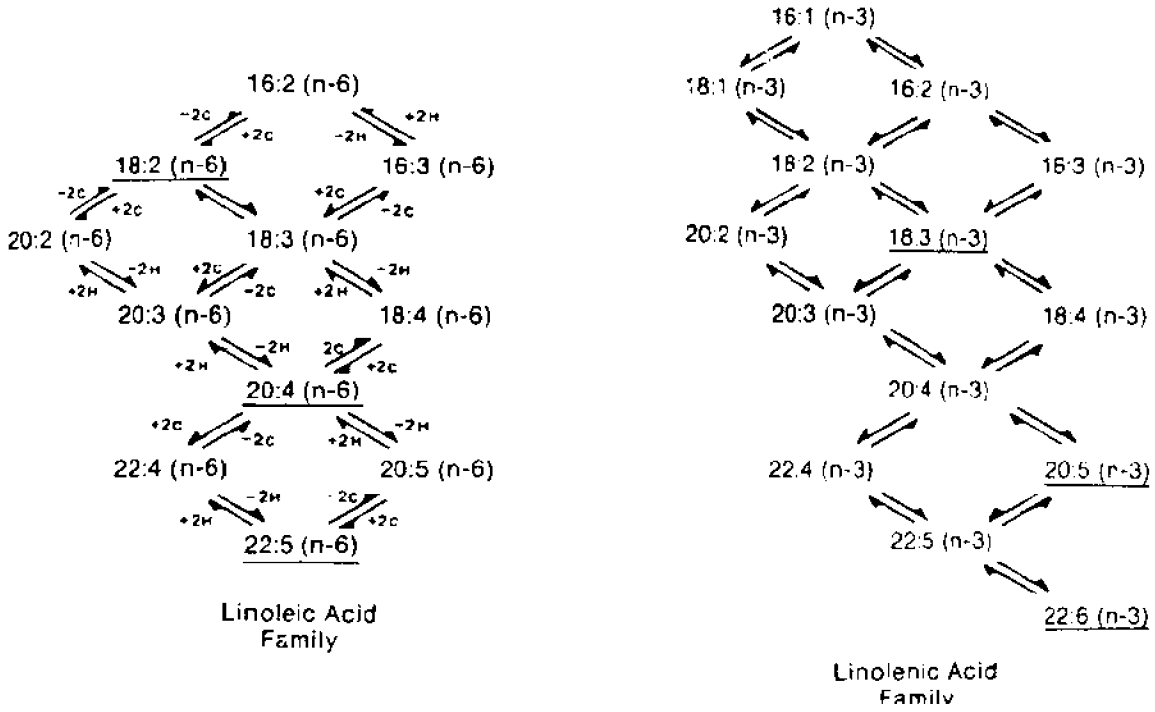


Figure 1. Reversible desaturation-elongation pathways for linoleic and linolenic acids. The fatty acids that are underlined are the representatives of those families of fatty acids most often found in marine organisms in significant quantities.

Just as most animals can lengthen or shorten, and add or subtract double bonds to de novo synthesized fatty acids, most have some degree of ability to do the same with dietary EFAs.

FATTY ACID SYNTHESIS IN CRUSTACEANS

The de novo synthesis of fatty acids from C^{14} labeled acetate has been demonstrated in the crayfish Astacus astacus (Zandee 1966), the lobster, Homarus gammarus (Zandee 1967), the prawn, Penaeus japonicus (Kanazawa and Teshima 1977) and P. monodon and P. merguiensis (Kanazawa

et al. 1979b). The major portion of the labeled carbon was always found in the saturated and monoenoic fatty acids. The small amounts of radioactivity found in the n-6 and n-3 polyunsaturated fatty acids (PUFA) probably resulted from the addition of acetate units to the carboxy end of EFA of dietary origin.

The conversion of C^{14} palmitic and stearic acids to the corresponding monoenoic fatty acids has also been demonstrated in the prawn, Penaeus japonicus (Kanazawa et al. 1979a), the gammarid, Gammarus roeselii (Herodek 1970) and the mysid, Gnathophausia sp. (Morris and Sargent 1973). Again the small amount of radioactivity found in 20:5n-3, 22:5n-3 and 22:6n-3 (Kanazawa et al. 1979a) was probably the result of incorporation of C^{14} acetate removed from palmitic acid and used to elongate shorter n-3 fatty acids of dietary orgin. On the other hand, the label in 12:0 could not have come from simple shortening of 16:0, as the labeled carbon was in the one position and would be lost upon shortening. The possibility that an acetate unit has been added or removed at the carboxy end of a fatty acid must always be considered when interpreting radio tracer data on fatty acid metabolism. Morris and Sargent (1973) found that some oceanic crustaceans were also able to convert C^{14} labeled fatty acids into fatty alcohols for incorporation into wax esters.

The ability to elongate and desaturate fatty acids of the linoleic and linolenic series has been demonstrated in a number of crustaceans. Kayama et al. (1963) found that brine shrimp (Artemia salina) were able to synthesize considerable quantities of 20:5n-3 when fed on a diatom (chactoceros simplex), which contained 18:3n-3 but no 20:5n-3. Kanazawa et al. (1979f) compared the rate of conversion of C^{14} labeled linolenic acid to 20:5n-3 or 22:6n-3 by a number of fish and crustaceans. They noted that the fish with a very slow rate of conversion were those which required dietary 20:5n-3 or 22:6n-3 to satisfy their EFA requirement, while the fast converters could be satisfied with dietary 18:3n-3. It would be logical to expect that a similar range of relative ability to elongate and desaturate might exist and affect nutritional requirements of crustaceans.

FACTORS AFFECTING THE FATTY ACID COMPOSITION

There are a number of factors in addition to the de novo synthesis and conversions of dietary fatty acids that affect the fatty acid patterns of crustacean lipids. Castell (1979) noted that valuable inferences about EFA requirements of different fish could be drawn from the differences in the fatty acid patterns of their lipids. Some of the factors affecting the fatty acid composition of crustacean lipids and the possibility that similar differences in EFA requirements exist among the various species of crustaceans will now be examined.

Salinity

The fatty acid patterns among crustaceans in freshwater and marine environments tend to be similar to those for finfish (Castell 1979). The sums of saturated and monoenoic fatty acids (those that can be synthesized de novo), and of the n-6 and n-3 acids and of the three major chain lengths, and the ratio of n-6/n-3 are presented in Table I for a number of freshwater and marine crustaceans as well as for one

Table I. Summary of Trends in Fatty Acid Composition of Marine and Freshwater Crustaceans

Species	Sums							Ratio	Reference
	Sat	Mono	n-6	n-3	18C	20C	22C	n-6/n-3	
Fresh Water									
Crawfish									
Astacus astacus	41.9	39.5	18.9	1.0	47.9	4.8	1.0	18.90	Collatz 1969
Orconectes limosus	35.1	24.8	22.9	10.5	30.3	23.4	7.7	2.18	Collatz 1969
Orconectes rusticus	31.8	36.6	13.1	16.3	44.5	16.2	-	0.80	Wolfe et al. 1965
Shrimp									
Macrobrachium rosenbergii	24.4	20.7	32.2	12.7	60.0	9.4	3.2	2.53	Ackman 1980
Palaemon paucidens	17.4	40.5	8.3	31.5	31.1	28.4	8.8	0.26	Teshima et al. 1976
Brackish Waters									
Crab									
Helice tridens	28.0	27.8	11.6	29.2	27.3	28.7	9.3	0.40	Teshima et al. 1976
Marine Species									
Prawn									
Penaeus japonicus	26.9	25.1	17.4	42.3	35.8	25.7	14.8	0.41	Teshima et al. 1976
Penaeus setiferus	43.0	19.8	9.5	23.9	11.7	22.1	13.4	0.40	Bottino et al. 1980
Penaeus indicus	27.0	25.5	8.8	26.2	22.0	22.8	12.5	0.34	Colvin 1976
Penaeus aztecus	29.9	28.9	8.4	30.3	25.4	26.8	13.8	0.27	Bottino et al. 1979
Penaeus merguiensis	27.5	25.9	6.5	20.1	28.0	17.7	14.6	0.23	Clarke and Wickins 1980
Shrimp									
Pandalus borealis	31.4	50.5	6.2	11.9	32.3	11.3	4.9	0.52	Hayashi 1976
Pandalus borealis	21.5	45.8	3.5	25.8	25.5	22.3	10.6	0.14	Ackman and Eaton 1967
Pandalus nipponesis	28.0	54.9	4.1	12.9	30.3	19.9	3.8	0.32	Ackman and Eaton 1967
Pandalus hypsinotus	28.5	49.3	3.5	18.1	34.7	16.8	7.3	0.19	Ackman and Eaton 1967
Crab									
Chionectes opilio	17.8	30.7	4.7	45.0	25.1	37.1	14.3	0.10	Addison et al. 1970
Euphausia									
Meganyctiphanes norvegica	22.7	52.3	2.5	20.3	18.3	22.8	22.5	0.12	Ackman and Eaton 1967
Euphausia krohnii	28.4	22.1	6.4	42.4	19.6	22.9	24.3	0.15	Morris 1973
Systellaspis debilis	25.2	41.3	4.1	28.9	35.7	18.4	18.1	0.14	Morris 1973
Acanthephyra purpurea	27.7	32.2	3.8	36.1	27.6	20.3	21.9	0.11	Morris 1973
Heterocarpus grimaldii	24.3	49.7	5.3	20.5	36.0	16.1	11.9	0.26	Morris 1973
Neomysis integer	33.5	17.2	3.0	46.3	15.7	26.2	22.8	0.06	Morris 1973

brackish water species. The linoleic acid series tends to be the predominant EFA group in freshwater species while the linolenic series predominates in the marine crustaceans. The marine species also have a definite trend to higher proportions of longer chain (20 and 22 carbon) fatty acids. The pattern for saturated and monoenoic fatty acids appears to vary considerably between species and does not show any apparent relationship to the salinity.

Lipid Class

Within any one species the fatty acid pattern associated with each lipid class may vary considerably. The triglyceride fatty acids tend to reflect the dietary lipids while the phospholipid or membrane lipid tends to be a more stable characteristic of each species. Table II presents a summary of fatty acid patterns for triglyceride, phospholipid and in some cases sterol or wax esters from several freshwater and marine crustaceans. The same general patterns noted in Table 1 for total lipid fatty acids with regard to n-6 and n-3 EFA and chain lengths are still observed in the phospholipids in Table II.

In the case of the Pandalus montagia males, (Clarke 1979) the location of capture appeared to affect the content of triglycerides more than phospholipid. It is probable that the diet of P. montagia at the mouth of the Crouch River contained higher levels of both n-6 and n-3, 20 and 22 carbon fatty acids than at Lynn Wall, about 100 km further north in England on the North Sea. Other factors such as temperature or molt stage, also may have had an effect on the fatty acid composition of this crustacean.

Even among the phospholipids it can be seen that fatty acid composition varies between molecular species. In Orconectus limosus the fatty acid pattern of phosphatidylcholine is not the same as that of total phospholipid (Table II). Similarly the sterol and wax esters have their own distinct fatty acid pattern different from the other lipid classes within each species of crustacean.

Tissues and Organs

Not only is the fatty acid composition of the various lipid classes different for a given species but these patterns also vary from one tissue or organ to another within any given crustacean. Table III gives an example of the variation in fatty acid pattern of total lipid from several tissues and organs of the Horseshoe crab, Xiphosura polyphemus (van der Horst et al. 1973). In addition to the variation in total saturates (32-46%), monoenes (20-36%) total n-6 (7.3-15.5%) and n-3 (10-25%) there are a few other interesting differences. The gonad lipid, for example, has 4-5 times as much of both 20:3n-6 and 20:3n-3 as any of the other tissues and the gill has much more 22:4n-6 than most of the other tissues except the egg and to a lesser extent the hepatopancreas. The possible role of the gonadal eicosatrienoic acids in prostaglandin synthesis will be discussed later in this paper in connection with some C^{14} radio tracer work carried out with lobsters.

Table II. Summary of Trends in Fatty Acid Composition of Different Lipid Classes of Marine and Freshwater Crustaceans

Species	Lipid class	Sums							Ratio n-6/n-3	Reference
		Sat	Mono	n-6	n-3	18C	20C	22C		
					Fresh Water					
Orconectes limosus										
Neutral lipid	(NL)	26.7	21.4	38.2	10.7	51.1	12.7	5.5	3.57	Collatz 1969
Phospholipid	(PL)	30.1	27.9	29.1	13.2	37.6	35.7	-	2.20	
Phosphatidylcholine	(PC)	26.1	34.3	28.8	9.4	33.2	29.1	-	3.06	
Orconectes rusticus										
Triglyceride	(TG)	35.6	37.8	10.4	8.6	37.9	5.5	-	1.21	Wolfe et al. 1965
	PL	27.3	26.5	7.7	28.9	37.0	29.7	4.0	0.27	
Cholesterol ester	(CE)	37.5	44.1	2.0	6.4	44.3	4.5	-		
Acanthodiaptomus pacificus	TG	37.2	33.7	7.6	21.6	27.3	11.6	8.8	0.35	Takahashi and
	PL	58.0	27.2	4.9	9.9	22.8	5.4	5.4	0.49	Yamada 1976
Sterol ester	(SE)	33.8	27.3	8.1	28.2	20.5	20.6	10.5	0.29	
Moina sp.	TG	34.3	58.8	1.6	1.9	14.8	-	-	0.84	Takahashi and
	PL	21.1	61.6	9.9	5.5	42.0	1.8	-	1.80	Yamada 1976
	SE	40.6	46.4	3.6	6.3	20.5	6.7	0	0.59	
					Brackish and Marine Species					
Pandalus montagui Crouch River	TG	23.1	40.8	7.4	26.9	18.3	25.3	11.1	0.28	Clarke 1979
	PL	23.3	24.4	6.8	44.2	21.5	32.5	18.9	0.15	
Pandalus montagui Lynn Wall	TG	38.4	39.4	3.9	11.0	27.2	10.4	5.8	0.35	Clarke 1979
	PL	27.1	30.1	5.2	33.9	26.0	23.4	16.3	0.15	
Meganyctiphanes norvegica	TG	29.1	41.8	1.9	18.9	26.1	17.4	16.4	0.10	Ackman 1980
	PL	21.2	21.8	3.3	46.0	22.6	18.7	29.6	0.07	
Euphausia superba	TG	25.7	23.9	3.6	30.2	24.2	18.4	10.0	0.12	Ackman 1980
	PL	25.8	18.3	4.0	33.6	21.5	17.2	15.2	0.12	
Euphausia pacifica	TG	35.0	26.8	4.5	16.8	27.3	9.4	4.8	0.27	Ackman 1980
	PL	23.2	20.1	6.0	44.2	23.1	21.8	22.9	0.14	

Table II (continued)

Species	Lipid class	Sums							Ratio n-6/n-3	Reference
		Sat	Mono	n-6	n-3	18C	20C	22C		
Penaeus merguiensis	TG	36.8	32.8	8.2	18.8	22.4	17.2	12.4	0.44	Clarke and
	PL	30.8	26.3	9.3	31.9	28.9	22.8	17.5	0.29	Wickens 1980
Calanus plumchrus	TG	43.2	39.7	2.0	15.2	26.8	12.9	6.9	0.13	Clarke and
	PL	52.6	36.2	1.3	7.9	33.6	4.4	3.4	0.16	Wickins 1980
Wax ester	(WE)	41.5	24.7	5.2	27.6	20.7	20.0	8.9	0.19	
Parathemisto japonica	TG	38.3	41.9	2.7	16.6	27.3	16.8	7.4	0.16	Takahashi and
	PL	55.8	31.0	4.2	9.1	27.5	11.0	5.3	0.46	Yamada 1976
	WE	45.6	35.6	1.7	17.1	20.6	16.6	8.0	0.09	
Hyperia sp.	TG	37.8	31.9	3.9	25.7	20.8	19.2	13.8	0.15	Takahashi and
	PL	46.0	34.9	4.6	14.4	31.8	10.0	8.1	0.32	Yamada 1976
	WE	14.4	72.7	1.9	11.1	26.5	32.6	15.9	0.17	
Thysanoessa inermis	TG	48.6	42.9	1.2	7.4	30.5	7.3	2.8	0.16	Takahashi and
	PL	59.0	35.8	0.7	4.4	30.3	4.1	1.8	0.16	Yamada 1976
	WE	31.3	49.2	1.4	18.2	40.1	13.4	6.4	0.07	

Table III. Fatty Acid Patterns of Total Lipids of Various Tissues of the Horseshoe Crab, Xiphosura polyphemus (van der Horst et al. 1973)

Fatty acid	Hepato-pancreas	Gonad	Heart	Muscle	Gill	Carapace	Egg
14:0	2.1	2.6	1.6	1.2	2.1	2.1	2.1
16:0	7.6	8.0	6.7	7.4	5.6	12.3	10.4
18:0	9.7	6.8	10.9	18.2	9.8	11.8	8.7
20:0	2.0	1.5	1.1	0.1	0.2	1.6	0.6
22:0	0.6	0.4	0.2	0.1	0.5	0.3	0.3
SUM Sat	37.1	37.2	32.6	41.4	34.8	46.8	36.0
16:1	3.0	1.9	2.4	1.7	2.2	2.1	2.8
18:1	15.4	17.6	19.3	30.0	14.9	18.9	17.3
20:1	5.2	5.1	3.4	4.4	2.3	4.3	3.6
22:1	0.9	0.5	0.3	0.2	0.3	0.3	0.3
SUM Monenes	25.7	25.9	26.5	36.9	20.7	26.8	25.2
18:2n-6	0.9	1.3	0.9	1.0	1.1	1.5	0.8
18:3n-6	0.8	0.9	0.8	1.2	0.7	0.4	0.6
20:2n-6	0.8	0.3	1.1	0.4	0.8	0.8	0.7
20:3n-6	0.6	2.7	0.6	0.5	0.5	0.6	0.4
20:4n-6	5.7	4.6	8.3	5.2	9.6	3.9	6.8
22:4n-6	0.9	0.2	0.7	0.2	2.8	0.1	1.8
SUM n-6	9.7	10.0	12.4	8.5	15.5	7.3	11.1
18:3n-3	0.9	0.4	0.7	1.2	0.8	0.9	1.6
18:4n-3	0.1	0.1	0.1	0.2	0.2	0.2	0.1
20:3n-3	0.2	0.9	0.3	0.3	0.2	0.3	0.1
20:5n-3	15.0	12.8	16.6	6.4	13.2	7.1	10.5
22:4n-3	0.6	0.5	0.6	0.5	3.9	3.6	4.7
22:5n-3	2.2	1.9	1.3	0.4	1.1	0.7	1.0
22:6n-3	3.3	4.7	2.5	0.4	1.4	1.2	2.0
SUM n-3	24.7	24.0	25.1	10.0	25.4	16.3	25.0

Season

The fatty acid pattern of Pandalus montagia captured at two different locations at the same time of year varied considerably (Table II). However, Bottino et al. (1980) found that the fatty acid patterns of three species of shrimp (Penaeus setiferus, P. aztecus and P. duorarum) collected at the same time of year differed very little from each other. There was a seasonal variation irrespective of the species. Saturated fatty acids increased during the warm season and decreased in the cold months, while the opposite occurred with the monoenoic and polyenoic fatty acids. Guary et al. (1975) showed a similar seasonal variation in the fatty acid pattern of Penaeus japonicus. They also reported a seasonal variation in the total lipid content with peak values in October and November for female and male prawn, respectively. The seasonal fluctuations in fatty acid patterns of crustacean lipids are probably caused by a combinatin of several factors, including temperature, diet, age, reproduction and molt cycle.

Temperature

It has been well documented for finfish that saturated fatty acids vary directly and some monoenoic and polyunsaturated fatty acids inversely with temperature (Kayama et al. 1963; Reiser et al. 1963; Johnston and Roots 1964; Knipprath and Mead 1966; Kemp an Smith 1970; Leslie and Buckley 1976; Leger et al. 1977). As one might have predicted crustacean lipids exhibit a similar response to changes in environmental temperature (Farkas and Herodesk 1964; Martin and Ceccaldi 1977). Both desaturation and chain elongation increase with decrease in temperature (Table IV).

Table IV. Effect of Culture Temperature on the Fatty Acid Composition of Palaemon serratus (Martin and Ceccaldi 1977)

Temp.	Sums							Ratio
	Sat	Mono	n-6	n-3	18	20	22	n-6/n-3
25	34.3	36.7	5.2	21.6	45.0	15.0	4.1	0.24
15	37.2	37.1	6.7	17.6	39.9	10.8	5.4	0.38
9	26.7	31.8	11.4	28.5	39.5	20.4	8.5	0.40

Diet

When Penaeus setiferus were fed a diet high in 18:2n-6 and low in 20 and 22 carbon PUFA the influence of dietary lipids on fatty acid composition of this shrimp was apparent after one month (Table V. Bottino et al. 1980). Even after 3 months, however, the specific retention of n-3 fatty acids was clear as the shrimp n-6/n-3 ratio was 1:4 compared with 4:5 in the dietary lipid. Although 20:4n-6, 20:5n-3 and 22:6n-3 were higher in P. setiferus at the beginning of the experiment, elongation and desaturation activity was evident from the relatively high levels of these fatty acids compared with dietary levels even after three months of feeding.

Molt cycle

A crustacean must periodically molt or cast off the old shell and grow a new one to permit growth. This discontinuous growth pattern results in fluctuations in the rate of de novo synthesis of lipids from C^{14}-acetate (Patrois et al. 1978). Maximum lipid synthesis in Penaeus japonicus occurred in B and early C (postmolt and early intermolt stages, respectively) with synthesis reaching a minimum at D prior to the next molt. Patrois et al. (1978) also noted that the distribution of radioactive lipids varied with tissue type and lipid class over the molt cycle. Kanazawa et al. (1976) and Ando et al. (1977) analyzed lipid content of various tissues and found highest total lipid levels at pre-molt stage D in most tissues of Penaeus japonicus. The fatty acid pattern of the several lipid classes was also found to vary over the molt cycle in Palaemon paucidens (Teshima and Kanazawa 1976) and Penaeus japonicus (Teshima et al. 1977). In Palaemon paucidens the saturates 16:0 and 18:0 increased at molt stage D while 20:4n-6, 20:5n-3 and

22:6n-3 decreased in the phospholipid fraction. The triglyceride showed no marked variation in fatty acid composition during molt cycle. The fatty acid pattern of the phospholipids of Penaeus japonicus was more constant with only a slight increase in 18:1 and a decrease in 20:5 and 22:6 at molt stage C. This prawn did display a significant fluctuation in triglyceride fatty acids. The triglyceride level of 22:6n-3 was extremely high at molt stage C while 20:3n-3 was very high following ecdysis, at molt stage A_2, and showed a steady decrease to premolt, molt stages D_{3-4}. Molt stage also resulted in changes in the fatty acid pattern of sterol esters, free fatty acids, mono- and diglycerides.

Table V. Effect of Diet on Fatty Acid Composition of Penaeus setiferus (Bottino et al. 1980)

Fatty acid	Composition of P. setiferus lipid after 0 months	1 month	3 months	Diet
14:0	0.6	0.5	0.5	1.6
16:0	14.8	13.4	15.0	15.5
18:0	11.2	8.7	10.0	7.9
SUM	26.6	22.6	25.6	25.0
16:1	5.1	2.3	2.2	1.7
18:1	13.1	22.9	20.0	28.4
SUM	18.2	25.2	22.2	30.1
18:2n-6	2.3	18.1	14.1	32.3
20:4n-6	11.6	9.4	10.3	0.7
SUM	13.9	27.5	24.4	33.0
18:3n-3	2.8	2.1	1.3	4.4
20:5n-3	10.4	8.7	9.7	2.6
22:6n-3	11.3	6.3	6.9	0.3
SUM	24.5	17.1	17.9	7.3
n-6/n-3	.57	1.61	1.36	4.5

Age, Reproduction and Sex

In the carp (Shimma et al. 1977), it was found that decreased levels of 22:6n-3 reduced hatchability. Castell (1979) quoted several reports of higher concentrations of n-3 fatty acids in fish roe than those in the total adult fish lipids. The ovaries of three species of crustaceans exhibited the same trend (Table VI). Middleditch et al. (1980) noted that shrimp Penaeus setiferus fed a diet low in 20 and 22 PUFA would not produce eggs. Feeding supplements of annelid worms rich in these fatty acids resulted in successful ovarian development and spawning.

Morris (1973) compared the fatty acid composition of juveniles and mature males and females of several species of marine crustaceans. The young generally had higher levels of PUFA than the adults. The males tended to have higher levels of PUFA than the females, while the females had higher levels of saturates or monoenes. It is possible that some of the females sacrificed their PUFA to produce healthy eggs and larvae.

Table VI. Comparisons of Fatty Acid Patterns of Total Adult Lipids with Ovary Lipids of Several Crustaceans (Hayashi 1976)

Fatty acid	Pandalus borealis		Heptacarpus pandaloides		Spirontacaris sp.	
	Whole	Ovary	Whole	Ovary	Whole	Ovary
14:0	4.8	3.9	4.7	4.0	8.1	5.1
15:0	0.6	1.0	1.6	1.0	0.6	0.7
16:0	33.7	16.9	23.2	17.8	20.2	16.2
17:0	1.1	1.5	1.8	1.4	0.3	1.0
18:0	1.8	1.5	2.4	2.0	1.4	0.6
19:0	-	-	-	-	0.4	0.5
20:0	-	0.9	1.1	0.4	-	-
SUM	42.0	25.7	34.8	26.6	31.0	24.1
14:1	0.9	1.1	2.1	1.4	0.5	0.2
16:1	12.3	16.7	10.0	13.3	19.5	23.6
17:1	.8	2.1	2.2	2.0	1.0	1.8
18:1	29.1	31.0	28.2	34.0	30.0	28.7
19:1	0.5	1.5	1.3	0.9	tr	tr
20:1	3.8	4.1	3.9	3.6	2.5	0.2
22:1	2.0	2.2	2.1	1.7	0.5	-
SUM	51.0	59.5	50.3	57.8	54.1	54.6
18:2n-6	0.8	1.5	2.3	1.7	0.9	1.6
20:4n-6	0.3	1.0	1.3	1.0	2.6	2.7
SUM	1.1	2.5	3.6	2.7	3.5	4.3
18:3n-3	tr	tr	tr	tr	0.1	0.2
18:4n-3	tr	-	0.9	tr	1.6	0.2
20:5n-3	5.5	10.7	8.4	11.0	7.2	13.4
22:5n-3	tr	tr				
22:6n-3	0.3	1.5	1.9	1.9	2.4	3.2
SUM	5.8	12.2	11.2	12.9	11.3	17.0

ESSENTIAL FATTY ACID REQUIREMENTS OF CRUSTACEANS

It should be clear that the fatty acid picture in crustaceans is not simple. The fatty acid composition of each lipid class is affected by de novo synthesis ability, by dietary lipid composition, by ability to alter (lengthen, shorten, desaturate and saturate) dietary fatty acids and by the relative rate of catabolism of each fatty acid group. The fatty acid patterns change with season, probably due to a combination of changes in diet, temperatures and reproduction and molt cycle. The salinity appears to exert an influence on the fatty acid pattern as well. Moreover, the fatty acid patterns differ between different tissues and organs of each species of crustacean, and each lipid class of each tissue has its own different fatty acid composition. Nevertheless, in spite of this, it may be possible to draw certain inferences about dietary lipid requirements of crustaceans from the foregoing discussions.

As the differences in fatty acid compositions of fish cultured at different temperatures and salinities were correlated with differences

in dietary fatty acid requirements (Castell 1979 and Castell et al. 1981), it would be reasonable to expect that the wide range of fatty acid patterns of crustaceans is also indicative of differences in dietary fatty acid requirements. Some indication of EFA requirements can be gained from feeding trials using natural oils high in n-6 or n-3 fatty acids and observing effects on growth, survival and other health parameters. The only reliable proof of EFA requirements, however, is that obtained with semi-purified or purified diets with purified saturates, or n-6, or n-3 unsaturated fatty acids.

Colvin (1976) fed groups of Peaneus indicus diets supplemented with linseed, sunflower, soy or peanut oil. It appeared that peanut oil, low in 18:2n-6 and with no 18:3n-3, gave best growth. In these test diets, however, prawn and fish meal contributed 3-4% lipid. Individual growth variance was so large that the dietary differences were not significant. High levels of either 18:2n-6 or 18:3n-3 from these vegetable oils appeared to depress growth of P. indicus. Shewbart and Mies (1973) found that supplements of 1% linolenic acid added to a commercial marine chow resulted in increased growth of P. aztecus.

Guary et al. (1976) found that vegetable oils, high in 18:3n-3, gave better growth of Penaeus japonicus than those high in 18:2n-6. Marine oils from sardine or clam, high in 20 and 22 carbon n-3 fatty acids, resulted in better growth and survival than any of the vegetable oils. Kanazawa et al. (1977a) reported similar superior results with pollock residual oil and clam oil compared to soybean oil in P. japonicus diets. The superiority of oils of marine origin over vegetable oils in crustacean diets was also demonstrated for crab, Carcinus maenas (Ponat and Adelung 1980), freshwater shrimp, Macrobrachium rosenbergii (Joseph and Williams 1975; Sandifer and Joseph 1976) and lobster, Homarus americanus (Castell and Covey 1976). Deshimaru et al. (1979) found that a dietary mixture of pollock liver oil and linseed oil gave superior growth and feed efficiency in P. japonicus than did diets with either oil alone. The natural oils used in these feeding trials contained a mixture of many different fatty acids as well as other lipid components such as sterols, pigments, phospholipids, vitamins, etc. Thus, it is difficult to make definite conclusions about EFA requirements based on such feeding trials. It has been shown that crustaceans require cholesterol and also that dietary lecithin supplementation enhanced growth and survival (Kanazawa 1976).

In a series of experiments (Kanazawa et al. 1977a, 1977b, 1978, 1979c, 1979d, 1979e, Kayama et al. 1980), it was whown that both 18:2n-6 and 18:3n-3 improve growth of Penaeus japonicus compared with diets containing no fat or only oleic acid. The nutritive value of linolenic was higher than that of linoleic acid. The prawn, although able to elongate and desaturate these fatty acids, did not seem to be able to produce enough long chain n-3 fatty acids as both 20:3n-3 and 22:6n-3 proved to be superior to 18:3n-3 as dietary supplements. Even after the prawn were fed diets with n-3 as the only supplemented PUFA for about two months they retained significant levels of n-6 fatty acids, especially in the polar or phospholipids.

Martin (1980) found that 18:2n-6 and 18:3n-3 added together in the diet of Palaemon serratus gave better growth than either acid added alone. Adding cod liver oil resulted in better growth than any mixture of 18:2n-6 or 18-3n-3, suggesting the P. serratus was also not able to elongate and desaturate these fatty acids fast enough to satisfy the

requirement for long chain PUFA. Read (1981) found with Penaeus indicus that no mixture of 18:2n-6 plus 18:3n-3 tested was better than 18:3n-3 alone, but that supplementing 18:3n-3 and 18:2n-6 with dietary 20:5n-3 and 22:6n-3 gave superior growth and survival compared with supplementing a low fat diet with these long chain n-3 fatty acids alone. Similarly he found that a mixture of sunflower oil and fish oil was superior to either oil alone. Since the fatty acid patterns of Penaeus indicus and P. japonicus (Table 1) are similar, one is tempted to speculate that P. japonicus would also benefit from some level of n-6 supplementation in addition to the 20:5n-3 and 22:6n-3. There is clearly much research to be done in the field of EFA requirements of crustaceans. Even with Penaeus japonicus, the species on which most work has been done, the question still remains whether some level of n-6 supplementation is essential for long term growth and survival. The fatty acid patterns of crustaceans vary from seawater to freshwater, from cold temperatures to warm temperatures, from one tissue to another, etc. It is very probably that these differences reflect real differences in EFA requirements.

In experiments with lobster, Homarus americanus, Castell and Boghen (1979) found that dietary supplements of 18:3n-3 or 22:6n-3, in the absence of dietary n-6 acids, reduced the desaturation of 16:0 and 18:0 to 16:1n-7 and 18:1n-9 and resulted in unusually high levels of 16:1n-3 and 20:3n-3. When 18:2n-6 was added to the diet with 18:3n-3 the de novo synthesis of 16 and 18 carbon monoenoic fatty acids was enhanced and the levels of 16:1n-3 and 20:3n-3 were greatly reduced in the lobster lipids. Surprisingly, the level of 20:4n-6 in the lobster lipid remained relatively unaffected by the presence or absence of either n-6 or n-3 fatty acids in the diet. At the time they published this work the authors had no explanation for the production of unusually high levels of 16:1n-3 and 20:3n-3.

The relatively high levels of 20:3n-6 and 20:3n-3 in the gonad of the Horseshoe crab, compared with other tissue lipids (table III, van der Horst et al. 1973), was mentioned earlier. It is possible that these fatty acids play an important role in crustacean reproductive organ physiology. Cormier[1] and Castell (unpublished results) found that the rate of prostaglandin synthesis by lobster Homarus americanus) testicular tissue was five times greater when C^{14}-20:3n-6 was the precursor than when C^{14} labelled 20:4n-6 was used. The production of high levels of 20:3n-3 in the absence of dietary n-6, as noted above for H. americanus, might be an attempt to produce a substitute for an essential precursor for prostaglandin synthesis, similar to the production of 20:3n-9 in EFA-deficient rats.

Although 16:1n-7 and 18:1n-9 can normally be synthesized by crustaceans from 16:0 and 18:0 and are thus not essential in the diet, they may play a very important physiological role. When dietary n-3 lipid somehow blocked the delta-9-desaturase enzyme system, the lobsters appeared to be trying to satisfy their monoene requirement by saturating and chain shortening dietary 18:3n-3 or 22:6n-3 to 16:1n-3. These conclusions are subject to some criticism especially since the diets

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1 Research conducted by Mr. R. Cormier as part of his BSc. honors thesis, University of Moncton, Moncton, New Brunswick. 1977.

used by Castell and Boghen (1979) were limiting in one or more essential amino acids as well. They do, however, point out areas for interesting and fertile research in the future. It is clear that the EFA requirement of any organism will be the sum of the EFA requirements for synthesis of each important lipid class of each tissue and organ that makes up that organism. It is affected by changes in the organism such as growth, maturation, reproduction and molting and by environmental parameters such as salinity and temperature. Although the EFA requirements of very few crustaceans are known, meaningful inferences might be drawn from the fatty acid composition of each species grown in its natural habitat.

DEDICATION

This paper is dedicated to Dr. Aiko Kanazawa of Kagoshima University, Tokyo, Japan who did much of the research work reviewed in this paper and to Prof. R. O. Sinnhuber, my former major professor, who led me into the lipid nutrition field and who is retiring this year from the Department of Food Science and Technology, Oregon State University.

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QUESTIONS AND ANSWERS - CASTELL

CONKLIN (California, Davis): Given that long chain polyunsaturated fatty acids are important in the diet of many aquatic animals, are we always going to be stuck with marine sources for these or do we have alternatives?

CASTELL: Well, I'm not sure that the long chain unsaturates are required for the lobster, at least. The lobsters seem pretty adept at flipping up and down through desaturation and chain elongation. When we fed 18:3n-3 they were able to whip it up to 20:5n-3 and down to 16:1n-3, so that with the lobster we may not have to have the long chain polyunsaturated fatty acids. There are lots of fish in the ocean so that I don't think that we have to worry about the limitation of fish oils at this point.

COLVIN (Univ. of Arizona): Some of the papers that I've read seem to indicate that at least the peneaids don't have the ability for this elongation and desaturation.

CASTELL: In regard to the peneaid shrimp and their ability to desaturate in chain elongate, I think Dr. Kanazawa has shown fairly conclusively that Penaeus japonicus, does have the ability to chain elongate and desaturate although not as great an ability as for instance rainbow trout. Some of the other studies may be misleading, often the author has used linolenic acid in the diet and said there is no evidence of chain elongation or desaturation. What happens is you start out and the animal in nature has relatively high levels of 20:5n-3 and 22:6n-7. After about one month the levels of 20:5n-3 and 22:6n-3 have gone down and the level of 18:3n-3 has gone up. After 3 or 4 months, however, you find that the level of 20:5n-3 and 22:6n-3 may have leveled off. The animal is still growing. Either, they are chain elongating or it (the 20:5n-3 and 22:6n-3) is coming by magic. If the animal has increased in total weight and the total lipid is increased and the percent of 20:5n-3 and 22:6n-3 has stayed the same then the total amount has increased. Shrimp may, as Dr. Kanazawa has shown, have a reduced ability to chain elongate and desaturate. However, in some of the papers where the author concluded that there was no desaturation ability, I think, if you really look at the data, that there's fairly good evidence that they are chain elongating and desaturating.

COLVIN: I really should revise my statement and say limited ability is what I've seen. The question is not its ability but rather is the capability at a high enough level to support what we presume to be a requirement.

CASTELL: I think the evidence in terms of actual feeding is that fish oils or lipids with high levels of 20:5n-3 and 22:6n-3 give better growth than the linolenic acid. This agrees with what you're saying that not only do they have a requirement for short chain n-3 fatty acids but they also benefit from the long chain polyunsaturated fatty acids. They're partly sparing their limiting ability to desaturate and chaining elongate.

CONKLIN: I was also wondering about contamination of fish oils with some of the chlorinated hydrocarbons.

CASTELL: That fits in well with some of our current work. Dr. Ackman of NSTU and I are sharing a student who's looking at steam stripping and pesticides, PBC's and those sort of things out of fish oils. If it works well these fish oils can be used as a source of essential fatty acids for fish feeds without concern for pollutants.

COLVIN: In my work with penaeids, we encountered a severe contamination problem with PCB's in some of our marine oil. Most of the samples of the fish oils that we were using coming from the Atlantic were pretty heavily loaded with PCB's. We examined some fish oil samples produced from Pacific species and there we found not PCB's but DDT derivatives. So they're everywhere. The DDT derivatives don't seem to be as bad at least with the penaeid species that we've worked with. We did go ahead and try some steam stripping and our experience would suggest that it's not worth the effort. I'd like to hear your comments on that though.

CASTELL: I haven't seen any evidence in the work I've done with lobsters of detrimental effects with the fish oils that we use. The work that Dr. Ackman's student is doing may show us a little bit more on the effect of steam stripping. We may find the same result that you have, that it didn't make any difference.

COLVIN: I'd like to make one more comment about steam stripping that you may want to guard against. We had a commercial firm do some steam stripping for us. While we started with menhaden oil, when it came back it looked more like soybean oil in terms of its fatty acid composition. They completely stripped all of the highly unsaturated long chain fatty acids out of it.

THE ROLE OF MICRONUTRIENTS IN THE BIOSYNTHESIS OF THE CRUSTACEAN EXOSKELETON

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ABSTRACT

Exoskeleton biosynthesis is reviewed from the standpoint of identifying possible nutrient demands of this process in crustaceans; specifically examined are the possible roles of selected micronutrients - vitamins C, A, D, E, and K. The functions of these in vertebrate systems are discussed as well as the possibility that they function in crustacean exoskeleton biosynthesis and molting.

Key words: Nutrition, Crustacea, exoskeleton, ascorbic acid, vitamin C, fat-soluble vitamins, vitamins A, D, E, K.

INTRODUCTION

The rigid body-covering of crustaceans provides them not only with a name but also an integument and skeleton. Although this common characteristic of all arthropods has been studied extensively (for review see Richards 1951; Dennell 1960; Travis 1960a; Hackman 1971; and Nelville 1975), one aspect not well examined is the relationship between the exoskeleton and specific nutrients in the diet. A primary reason for this omission has been the lack of the required tools, i.e., reliable crustacean test diets. As suitable diets are being developed, a review of the available information on crustacean exoskeleton biosynthesis and promising research directions is appropriate. Specifically of interest, along with the major nutrient classes of proteins, carbohydrates, and lipids, are vitamin C, and vitamin D, as well as the other fat-soluble vitamins A, E, and K.

The examination of this particular group of micronutrients reflects the tendency of comparative animal nutritionists to view the nutritional physiology of a relatively small taxonomic group of animals, the white rat and its vertebrate relatives, as representative of the norm. While historically vitamin C and the fat-soluble vitamins were sometimes considered to be required only by vertebrates, the more usual approach has been to also include these vitamins in invertebrate test diets. This assumption will be examined, with regard to the synthesis of the crustacean integument, in an attempt to identify possible differences in nutritional physiology between crustaceans and vertebrates.

GENERAL STRUCTURE OF THE INTEGUMENT

The crustacean integument (see Figure 1) is made up of two basic parts: a single layer of epidermal cells, and the cuticle, a non-living portion that has been secreted externally by the epidermis. This cuticle is composed principally of layered chitin impregnated with protein that has been variously modified with lipids and mineral salts, depending on species, body location and depth. This range in composition of the cuticle layers has lead to a confusing assortment of classification schemes and terms, the most common of which are summarized by Aiken (1980). The following discussion will use the terminology originated by Richards (1951) i.e. epicuticle, exocuticle, endocuticle, and membranous layer.

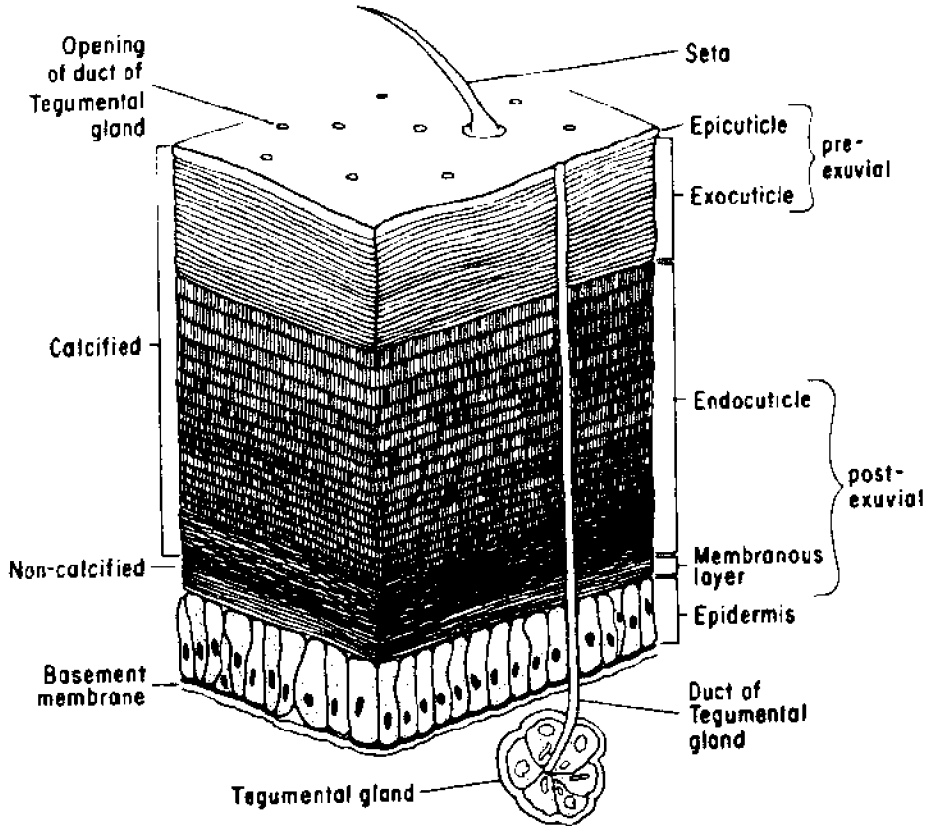

Figure 1. The crustacean exoskeleton in cross section (adapted from Aiken 1980).

In most crustaceans, the outer most component of the integument, the epicuticle, consists of two layers: a lipid outer layer and a lipoprotein inner layer. Some calcium is found in the epicuticle, typically in scattered aggregates (Digby 1968; Hegdahl et al. 1977b). Permeability of the cuticle seems related to the composition and presence or absence of this epicuticle (Yonge 1936; Mary and Krishman 1974). The epicuticle may also protect the underlying chitinous regions from bacterial attack (Fisher et al. 1978).

The bulk of the cuticle is often referred to as the procuticle and has three zones: the exocuticle, endocuticle, and membranous layer. The exocuticle is composed of laminar sheets of chitin fibrils enveloped in a matrix of protein that may be sclerotized or tanned. The exocuticle is formed prior to ecdysis, but calcification of this layer does not occur until later. Following ecdysis, the exocuticle is calcified rapidly and serves as the only cuticle until the endocuticle and the membranous layer are formed medically. Like the exocuticle, the endocuticle consists of chitin and protein laid down in laminae parallel to the surface. As laminae are formed, calcite crystals are deposited generally parallel to the chitin microfibrils (Hegdahl et al. 1977a). The membranous layer is a similar chitin-protein layered complex but is not calcified.

MINERALS

With the exception of the membranous layer, the cuticle of most crustaceans is heavily mineralized, primarily with calcium carbonate with minor amounts of magnesium, phosphorus, and sulfur. The amount of calcium (up to 99% of the total inorganic component of the exoskeleton) can vary tremendously depending on species, location on the body, and stage of the molt cycle (for extensive reviews, see Richards 1951; Travis 1960a, 1963, 1965; Dall 1965a, b; Greenaway 1974a, b, c; Welinder 1974, 1975a, b; Huner et al. 1976, 1979a, b).

Compared with other nutrient groups, mineral requirements of crustaceans have received little attention. Early work with shrimp (for review see New 1976) indicated a need for dietary calcium and phosphorus for growth. A ratio close to 1:1, calcium to phosphorus, was considered appropriate, with the total amount of dietary minerals being as high as 19.5%. Gallagher and co-workers (1978) suggested a ratio of 1:2 as optimum for juvenile American lobsters. Sedgwick (1980) found that the addition of a 7.0% mineral mix promoted growth if a vitamin mix was included in the diet but that minerals were toxic in the absence of vitamins.

Passano (1960) suggested dietary calcium would not be a requirement for marine crustaceans because it is so abundant in sea water. Recently Deshimaru and Yone (1978) reported growth of the prawn, Penaeus japonicus, was not inhibited by a lack of supplemental calcium, although additions of phosphorus (2.0%), potassium (1.0%), and trace metals (0.2%) improved growth. Deshimaru et al. (1978) later demonstrated the prawn could obtain adequate calcium from the surrounding sea water in the absence of a dietary source. Fresh water, of course, has a lower calcium content but should still provide adequate amounts in most cases (Rawson 1939). The work by Mills and Lake (1976) and Mills et al. (1976) suggests that exceptionally low calcium concentrations in fresh water can be compensated for by reduced exoskeleton mineralization, although there must be a minimum requirement (Greenaway 1974c). Thus, while the information available is still limited and much of the early work on crustacean mineral requirements may have been flawed by the lack of adequate test diets, it would appear that crustaceans meet most, if not all, of their demands for calcium from the surrounding aqueous medium, particularly in the marine environment. Crustacean requirements for other minerals, such as phosphorus, that are not readily available from the water, probably have to be satisfied by dietary intake.

VITAMIN D

Vitamin D, typically included in crustacean diets (see Table I), and is of interest because of its recognized importance in vertebrate bone mineralization and its possible analogous role in the mineralization of the crustacean exoskeleton. It is important to note, however, that in higher vertebrates vitamin D is often considered more as a hormone than a vitamin. Rather than serving as a catalyst for enzymatic activity, vitamin D regulates cell function by interacting directly with the genome. It is, therefore, appropriate to briefly review the current model for steroid hormonal action.

Table I. Vitamin Requirements of the Chick and Dietary Vitamin Levels for Trout and Crustaceans (mg or [IU]/100 g dry diet)

	Vitamin				
	C	A	D	E	K
Chick[a]	NR[b]	150 [IU]	20 [IU]	1 [IU]	0.05
Trout[c]	10	200 [IU]	100 [IU][d]	3	8
Shrimp[e]	25	200 [IU]	12.5 [IU]	1.25	0.12
Shrimp[f]	2,000	9.6 (β-carotene)	1.2	20	4
Shrimp[g]	875	3.5 (β-carotene)	0.5	35	3.5
Crab[h]	200	1.2 (β-carotene)	0.6	20	4
Lobster[i]	500	65,000 [IU]	32,500 [IU]	100	-

[a]National Research Council 1977. [b]NR - not required. [c]National Research Council 1973. [d]Barnett et al. 1979. [e]Deshimaru and Shigeno 1972. [f]Kanazawa et al. 1977. [g]Sedgewick 1980. [h]Ponat and Adelung 1980. [i]D'Abramo et al. 1981.

In vertebrates, steroid hormones are synthesized in endocrine tissues, released into the blood stream, and transported bound to specific proteins. Upon reaching the target tissues, the hormones are concentrated within the cells by the action of intracellular receptor proteins that selectively bind the hormone. The hormones then regulate the functions of the target cells by affecting gene expression and the subsequent synthesis of proteins. Regulation of a hormonal system can be complex, involving feedback inhibition of precursor synthesis and of the active metabolite as well as interactions with other hormones. Vitamin D metabolism and function in higher vertebrates clearly fits this steroid hormone model. The only significant difference is that the precursor molecule cannot be synthesized de novo.

Although not every effect of vitamin D is explained through hormonal action, the steroid model has been useful in examining vitamin D's role in higher vertebrates. According to Deluca (1979) providing there is an adequate exposure to sunlight or UV irradiation, the epidermis of mammals can synthesize enough cholecalciferol from dietary

sources of 7-dehydrocholesterol that dietary input is unnecessary. In mammals, cholecalciferol, either from the diet or from epidermal synthesis, is rapidly cleared from the blood by the liver, which hydroxylates it to form 25-hydroxycholecalciferol. This step is self-regulating by a negative feedback process. The 25-hydroxycholecalciferol is transported by a specific serum protein to the kidney, where it is further hydroxylated to form 1, 25-dihydroxycholecalciferol. Although the transport mechanism for 1, 25-dihydroxycholecalciferol has yet to be identified, it is assumed also to include a specific transport protein. The active metabolite, 1, 25-dihydroxycholecalciferol, is concentrated by and acts at the site of various target tissues, principally the kidney, gut, and bone, by altering protein synthesis so as to affect the calcium flux of the cells. Although the molecular mechanism is not always completely understood, the result is an increase or decrease in calcium movement in each of the target tissues so as to maintain physiologically normal calcium levels in the blood. In bone, for example, the metabolite's action mobilizes calcium into the circulatory system when blood calcium levels are low. In maintaining blood calcium levels, 1, 25-dihydroxycholecalciferol also interacts with two other hormones, the parathyroid hormone and calcitonin. These two hormones also act directly in regulating the production of the active metabolite in the kidney; the parathyroid hormone promotes the production of, 25-dihydroxycholecalciferol while calcitonin inhibits the production.

Although limited evidence indicates a vitamin D dietary requirement in crustaceans (Conklin 1980; Kanazawa this volume), there is no real indication that crustaceans regulate serum calcium levels in an analagous fashion to that found in vertebrates. Injections of mammalian parathyroid extracts stimulate the movement of minerals from the crustacean exoskeleton to the hemolymph (McWhinnie et al. 1969). However, under more normal physiological conditions, studies with crustaceans indicate hemolymph calcium concentrations are not maintained through use of the shell calcium. For example, no movement of shell calcium was found in the lobster Homarus americanus, after hemolymph calcium levels were halved (Hayes et al. 1962).

Although the mineralized cuticle serves admirably as both a skeleton and a protective integument, its rigid structure does present problems with respect to the animals growth. Arthropods, of course, have resolved this difficulty by molting and the most dramatic movement of calcium from the crustacean exoskeleton occurs in preparation for this process. Both Travis (1955b) and Dall (1965a) have suggested that the resorptive processes occurring in preparation for molting appear to be primarily for the conservation of organic constituents, not calcium. However, the existence in many crustaceans of various mineral storage mechanisms, such as gastroliths, seems to indicate regulation and thus a possible regulatory role for vitamin D. Gastroliths are paired mineralized structures formed between the epidermal and cuticular lining of the stomach and have the typical cuticular structure (Travis 1963). In species with gastroliths, minerals are deposited in these structures as the exoskeletal mineral content is decreased during premolt (Travis 1960b; McWhinnie 1962). At ecdysis, the gastroliths are shed with the cuticular lining into the stomach, and upon digestion the stores become available to the animal.

Several problems arise in attempts to assign gastrolith and mineral regulation to a vitamin D system. First, gastroliths are not found in all crustaceans, and their presence is not restricted to species inhabiting a specific environment with respect to calcium availability. Thus, gastroliths appear superfluous particularly for marine species, which presumably can meet their calcium requirements from the surrounding medium. Second, most evidence indicates the molt and the formation of the gastroliths are both regulated by an ecdysteroid hormone, probably 10-hydroxyecdysone (Kleinholz and Keller 1979). To further postulate the involvement of another hormonal regulatory agent such as vitamin D appears unnecessary and thus unattractive. Third, while the involvement of a parathyroid-like hormone in gastrolith calcium mobilization was postulated by McWhinnie and co-workers (1969), no further evidence has been reported. It should also be noted that McWhinnie and co-workers, however, used parathyroid gland extracts and not the purified hormone. Finally, gastrolith stores contain only a small portion both of the calcium that is resorbed from the integument before molting (McWhinnie 1962) and of the calcium required following the molt (Travis, 1960b). The calcium stored in the gastroliths is probably advantageous only in reducing the time necessary to establish minimum rigidity for consumption of the old exoskeleton and for beginning feeding. The gastroliths, then, are considered highly specialized adaptations, and their presence does not indicate calcium conservation in a closely regulated hormonal system.

The actual mechanism by which calcium is transported to and from the exoskeleton has always intrigued crustacean biologists. Simkiss (1976), in reviewing the mineralization of organic matrices in invertebrates, points out the inadequacy of existing models and the complexities involving both the intracellular and extracellular phases of this process. Although the recent model proposed by Roer (1980) does not include the mechanism of calcium deposition in the organic matrix of the exoskeleton, it is attractive in its simplicity with regard to the two-way transport required at different stages of the molt cycle. Roer's model suggests the exoskeleton epidermis is continually actively transporting calcium out of the cells and that it is the morphology of the epidermal cells that dictates the direction of the net calcium movement. As the model presumes the epidermal cells are connected by intercellular junctions that restrict any paracellular calcium movement, molt-related changes in the morphology of these cells results in the transport of calcium. During postmolt the squamous cells of the epidermis elaborate numerous protoplasmic projections that extend through the pore canals of the cuticle. This comparatively large surface area on the external side of the epidermis results in a net calcium movement to the exoskeleton. At the onset of premolt, the cell extensions are lost and the cells become more columnar in appearance when the old cuticle and its pore canals are separated from the epidermis. These changes reverse the surface ratio on the two sides of the intercellular junctions, thus reversing the direction of net calcium movement. Although structural changes occur in the intestinal epithelium of higher vertebrates in response to vitamin D (Bikle et al. 1981), there is no evidence of vitamin D's involvement in the changes in the structure of crustacean epidermis. As stated earlier, these molt related cellular changes in crustaceans are presently assumed to be regulated by the molting hormone, 20-hydroxyecdysone.

VITAMIN A

Morphological changes in epithelial cells of higher vertebrates are known to occur in response to vitamin A. Vitamin A profoundly affects cell division and differentiation of epithelial cells. Deprivation of this vitamin, therefore, leads first to retardation and ultimately to cessation of growth. As with vitamin D, vitamin A in higher vertebrates appears to act primarily as a hormone rather than as an enzymatic co-factor (for reveiw see Ganguly et al. 1980). In arthropods no clear evidence demonstrates an analogous role for vitamin A in growth (see Dadd this volume).

Based on work done with insects (for references see Dadd 1977a), vitamin A probably has a role in the crustacean visual cycle (Fisher and Kon 1959) that is the same as that in the vertebrate visual cycle. An important difference between vertebrates and invertebrates, however, is in the vitamin's distribution. In crustaceans, vitamin A is concentrated in the eye (Fisher and Kon 1959), but in higher vertebrates, "the amount of the vitamin involved in the visual cycle is only a very small fraction of the total amount of the vitamin in the rest of the animal body" (Ganguly et al. 1980).

PROTEINS

The chemical composition of the cuticle is predominated by chitin and protein, the relative amounts and ratios of which vary considerably among species. The amount of protein present appears inversely related to the degree of calcification (Travis 1960a). Amino acid analysis has revealed different protein compositions of the cuticle depending on species, region of the cuticle, and solvent extractions (Travis et al. 1967; Sameshima et al. 1973; Hackman 1974; Welinder 1974, 1975a). Although the information available on cuticle protein composition is still limited, it does not appear likely that modifications in the amino acid dietary ratios will be needed in order to satisfy a unique amino acid requirement for cuticle formation.

VITAMIN K

One amino acid absent from the crustacean exoskeleton is γ-carboxyglutamic acid (King 1978). This amino acid, which was discovered originally in the vertebrate blood clotting factor prothrombin, appears to be an important component of bone proteins and is thought to have a role in calcium binding. Work with vertebrate systems indicates that the formation of γ-carboxyglutamic acid is vitamin K dependent (Stenflo and Suttie 1977). In a limited survey of mineralized tissues in vertebrates and invertebrates, King (1978) found γ-carboxyglutamic acid only in the vertebrates. The apparent absence of this amino acid in invertebrates and the fact that vertebrate plasma coagulation factors do not affect arthropod hemolymph coagulation (Durliat and Vranckx 1981) suggests these vitamin K dependent activities do not play a role in the physiology of crustaceans.

VITAMIN C

Penaeid shrimp have been found to have a vitamin C, or ascorbic acid, requirement for growth (Kitabayaski et al. 1971b; Deshimaru and Kuroki 1976; Guary et al. 1976; Lightner et al. 1977). Lack of ascorbic acid in the diet of Penaeus californiensis and P. stylirostris results in a high incidence of "black death," a deficiency disease of shrimp characterized by reduced growth rates, poor feed conversion ratios, decreased resistance to stress and reduced rates of wound repair (Lightner et al. 1979). In vertebrates, an ascorbic acid deficiency results in impaired collagen formation (Barnes and Kodicek 1972), and results reported for crustaceans by Lightner and co-workers (1979) parallel the effect found in vertebrates. In ascorbic acid-deficient shrimp, Lightner et al. (1979) recorded a decrease in the amount of hydroxyproline, which was assumed to reflect a decrease in the collagen content. Collagen, or collagen-like proteins, depending on the definition used (Adam 1978), are present in crustaceans and may be an important element of integument structure and function, particularly in the specialized case of wound repair.

Ascorbic acid may also be important in other aspects of the crustacean exoskeleton formation and function. In vertebrates, ascorbic acid is involved with alkaline phosphatase activity; for example, catfish grown on diets without ascorbic acid supplementation were found to have low serum alkaline phosphatase activity (Wilson and Poe 1973). Gould and Shwachman (1942) found that morphological changes in guinea pig osteoblasts, as a result of an ascorbic acid deficiency, were correlated with decreased alkaline phosphatase levels. Although similar studies have yet to be done on crustaceans, Travis (1955a) found extensive changes in both the morphology and presumed alkaline phosphatase activity in epidermal cells of the spiny lobster just preceding ecdysis.

Ascorbic acid may be involved in the sclerotization or tanning of the crustacean epicuticle. The insect cuticle, which is not particularly calcified, is hardened primarily by tanning (Nelville 1975), a cross-linking of cuticle proteins by quinones. The quinones are formed through the oxidation of various phenols; a reaction catalyzed by the enzyme tyrosinase. Dadd (1960) first suggested that ascorbic acid had a role in the regulation of the enzymes involved in insect sclerotization, and recent work by Navon (1978) with the Egyptian cotton leafworm clearly supports Dadd's speculations. An ascorbic acid deficiency in this insect leads to the premature hardening of the cuticle, causing impaired ecdysis and mouth-part function and leading to death by starvation. Navon's studies and other studies with insects (Chippendale 1975; Kramer et al. 1978) indicate that various derivatives of vitamin C and related compounds, while not as effective as ascorbic acid, do have a hierarchy of effectiveness similar to that found in vertebrates. In crustaceans, sclerotization (see Yamaoka and Sheer 1971) of the epicuticle also is catalyzed by tyrosinase (Stevenson and Schneider 1962) and most likely is also affected by ascorbic acid.

Another possible vitamin C function was suggested by Guary and co-workers (1975), who found that the ascorbic acid levels of juvenile shrimp Palaemon serratus diminished immediately before and after molting, when the animals were not feeding. These decreases were suggested to be the result of vitamin C metabolism during the synthesis

of chitin. Although no direct evidence supports this suggestion, Guary and co-workers cite evidence of Antonowicy and Kodicek (1968) that vitamin C has an essential role in the analogous vertebrate synthesis of mucopolysaccharides.

CARBOHYDRATES

Based on a relationship between the crustacean molt cycle and glycogen levels of the hepatopancreas and epidermis, researchers have postulated that the precursor of chitin is glucose derived from stored glycogen (Passano 1960; Travis 1960a, 1963). Stevenson and Hettick (1980) have suggested the pathway shown in Fig. 2 for the synthesis of chitin; identification of enzymes for each of these steps has yet to be demonstrated in individual species, therefore this pathway represents a composite picture.

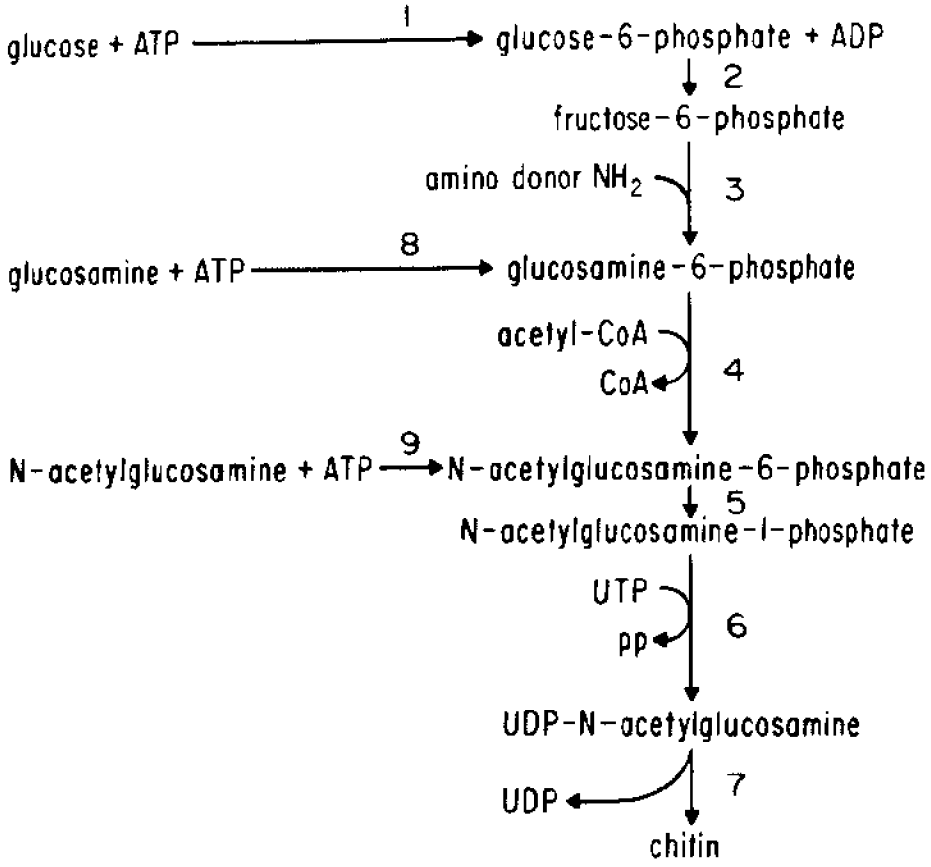

Figure 2. Suggested metabolic pathway for the synthesis of chitin in arthropods (adapted from Pahlic and Stevenson 1978; Stevenson and Hettick 1980). Individual enzymatic steps 1-6 have been demonstrated in the locust (Candy and Kilby 1962); 1,3,5,6,7, 8 and 9 in the crawfish (Lang 1971; Speck et al. 1972; Gwinn and Stevenson 1973b; Pahlic and Stevenson 1978), and step 7 in brine shrimp and a crab (Carey 1965; Hohnke 1971).

This scheme also provides a pathway for the direct utilization of intermediate products, glucosamine and N-acetylglucosamine, arising from the breakdown of the old cuticle at molt. The findings of Hornung and Stevenson (1971) and Gwinn and Stevenson (1973a) compared the incorporation of N-acetylglucosamine with glucose in chitin synthesis support this purposed recycling. Gwinn and Stevenson (1973a) indicated that over half of the postmolt chitin in crayfish consisted of recycled components of the former cuticle. The utilization of the chitin breakdown product, N-acetylglucosamine, may partly explain the benefits of including shrimp by-product meals in crustacean diets (New 1976). A stimulatory effect on growth also has been noted in shrimp with inclusion of glucosamine in the diet (Kitabayashi et al. 1971a), though glucosamine is not an indispensable nutrient (Deshimaru and Kuroki 1976) as indicated by the above pathway.

LIPIDS

Lipids have a central role in the biology of crustaceans, not only as energy sources but also as structural elements. Although information on the lipid composition of the crustacean exoskeleton is not extensive, it is known that the lipid content is low (approximately 1% or less) (Allen 1971; Ando et al. 1977; van den Horst et al. 1973). Cuticle lipids, as mentioned earlier, are thought to help control water permeability and perhaps help protect the chitinous regions from bacterial attack.

Cholesterol and polyunsaturated fatty acids are critical structural elements of cell membranes and are thus important for elaboration of the crustacean epidermis. In this respect, it is surprising that arthropods seem to be generally incapable of synthesizing cholesterol or polyunsaturated fatty acids (Dadd 1977a, b). Sterols, especially cholesterol, seem essential for membrane structure throughout the animal kingdom (Nes 1974). In crustaceans, the extensive morphological changes in the epidermal cells associated with molting must require cholesterol for membrane synthesis. Indeed, Guary and Kanazawa (1973) found that after being injected with labelled cholesterol, the epidermis of Penaeus japonicus had a high radioactive count.

Recently, several groups of investigators have shown phospholipids are a necessary crustacean dietary factor (Kanazawa et al. 1979; Conklin et al. 1980), and an phospholipid involvement in exoskeleton formation has been noted in juvenile lobsters (Homarus americanus) (Conklin et al. 1980). In lobsters a lack of dietary phospholipids commonly resulted in death due to the animal's inability to extricate itself at molt from the old exuvium. Also, lobsters fed phospholipid-deficient diets had abnormal calcium deposits embedded on the inner surface of their shed exoskeleton (Bowser and Rosemark 1981). Although the mechanisms of these exoskeletal abnormalities remain unknown, recent work has led to several possibilities. Phospholipids may be directly involved in membrane formation and there is also the possibility that they affect other nutrient levels.

One of the roles phospholipids apparently play in crustaceans is in the transport of fat-soluble nutrients through the hemolymph. D'Abramo et al. (1982) has shown that a dietary phospholipid deficiency in homarid lobsters reduces cholesterol transport in the hemolymph. Since crustaceans cannot synthesize this steroid, the drop in hemolymph

cholesterol could either affect epidermal membrane synthesis directly or indirectly by limiting the synthesis of the molting hormone for which cholesterol is a precursor. The phospholipid transport system may also carry fat-soluble vitamins. A further complication is the possibility that phospholipids in the pelletized diet reduce the rapid leaching of water-soluble vitamins and thus increase the available level of these vitamins in the diet (Castell personal communication).

The effectiveness of phospholipids in preventing molt related deaths is dependent on its specific constituents (D'Abramo et al. 1981). For the lobster, lecithin is the most effective phospholipid, but both lecithins and cephalins promoted growth in the prawn Penaeus japonicus (Kanazawa et al. 1979). In both animals, however, the effectiveness of the phospholipids seems also to depend on the constituent fatty acids, in particular the presence of polyunsaturated fatty acids.

VITAMIN E

The importance of polyunsaturated fatty acids in the diet of crustaceans is well established and reflects these animals' limited biosynthetic abilities (see Castell this volume). In vertebrates the amount of dietary vitamin E required is related to the amount of polyunsaturated fatty acids present (Harris and Embree 1963). Indeed the primary function of vitamin E in animals is thought to be prevention of the oxidation of fatty acids located within cellular membranes (Dam 1962). Crustaceans, with their apparent dependence on polyunsaturated fatty acids, undoubtedly will be found also to require vitamin E. Other possible biological functions such as co-factor interaction in enzymatic activities have yet to be clearly demonstrated in vertebrates or crustaceans.

CONCLUSIONS

With the limited data presently available, the critical nutrient requirements for crustacean exoskeleton biosynthesis remain unknown. Enough evidence suggest, however, that attempts to use the well-known nutritional requirements of higher vertebrates as a direct model for understanding invertebrate nutrient physiology will be misleading. A better approach will be to look closely at the specific role that each nutrient has in the physiology of these animals.

The apparent evolution of a hormonal function for some of the fat-soluble vitamins such as vitamin D and perhaps vitamin A may reflect highly specialized adaptations unique to higher vertebrates. Dacke (1979) has argued that the calcium-regulating system of higher vertebrates, which involves vitamin D and other hormones, is associated with the specific needs of bony terrestrial animals to compensate for the lack of a surrounding calcium-rich medium. Limited evidence suggests that utilization of vitamin K by vertebrates may have a similar association with calcium. Unfortunately, our knowledge of the nutritional physiology of some of the lower vertebrates, such as fish, that remain in a calcium-rich environment is still rudimentary. An interesting recent paper by George and co-workers (1981) demonstrated a vitamin D requirement for trout but found no involvement of vitamin D with the skeletal system. The researchers did, however, demonstrate

changes in the cellular morphology of muscle cells, which they speculate are related to intracellular calcium movement.

For other nutrients such as vitamin C and vitamin E that have more general cellular functions in vertebrates, the use of comparative models may be helpful. Although some evidence suggests the involvement of vitamin C in crustacean exoskeleton synthesis, whether the majority of crustaceans require the vitamin in their diet or can synthesize it, is unknown.

Although caution is stressed in the interpretation of comparative nutritional data between widely divergent groups, this should not be taken to the extreme. Most vitamins are involved in a variety of ways in the nutritional physiology of animals, even though some functions are more critical than others, depending on the species. Thus, a similar set of nutrient requirements ultimately may be demonstrated, even though divergent animal groups may evolve unique systems for the utilization of these nutrients.

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FARMANFARMIAN (Rutgers University): For what it might be worth let me give you a little information on work in my laboratory with Macrobrachium and dietary supplementation with calcium. One's first thoughts observing a crustacean voraciously consuming its exuvia is that there might be a calcium deficiency. To examine this possibility calcium supplements of 1, 2, and 3% were added to a repelletized commercial marine ratio we have used. In no case, over an approximately 14 weeks growth trial, was growth stimulated significantly and there was no change in the appetite for the exuvia.

CONKLIN: Undoubtedly, the various minerals deposited in the exoskeleton matrix are important elements of this structure and, because of its abundance, calcium is typically the one examined. Most of the reported work on crustaceans seems to support your results; a dietary source of calcium is not necessary for crustaceans. Presumably, the requirement for calcium is being met by uptake from the water. I do feel, however, that the focus on calcium has obscured the necessity of examining the dietary need for other important minerals such as phosphorus and sulfur.

ARTEMIA NUTRITION

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ABSTRACT

Using axenic culture techniques and artificial media, efforts have been made to understand the effects of the quality and quantity of dietary purines on Artemia growth and morphogenesis. An inability to synthesize the purine ring makes Artemia (Utah strain) an ideal organism for fundamental research on the significance of purine requirements. Additionally, the existence of dietarily inducible morphological changes in Artemia offers the unique advantage of being able to visualize metabolic relationships. Results of fundamental research on Artemia nutrition can be of interest to aquaculture for several reasons. 1) Artemia strains differ not only in nutritional requirements but also in their value as food for predators. Problems are sometimes encountered in the use of live Artemia as a diet for organisms of higher trophic levels. Both unfed Artemia nauplii and larvae reared on various diets have been used in the culture of marine animals without regard to the role of nucleic acids and nucleic acid components. As the nutritional value of brine shrimp cannot be divorced from the fulfillment of its nutritional requirements, this subject deserves further investigation. 2) Appropriate use of dietary purines and pyrimidines could help in the formulation of ideally balanced artificial diets promoting rapid growth of invertebrate animals. 3) Findings related to morphogenesis in Artemia may have practical application for the aquaculture of other crustaceans and can precede the achievement of the ultimate goal of fundamental research which is to understand development at the molecular level.

KEY WORDS: Artemia, nutrition, purine requirement, aquaculture, artificial diets.

INTRODUCTION

As stressed by Dr. Provasoli (1975) at the First International Conference on Aquaculture Nutrition, development of successful techniques for invertebrate culture represents a severe challenge due to the paucity of fundamental research on invertebrate nutrition.

My work with Artemia has been mainly concerned with understanding the influence of the dietary purine requirements on growth and morphogenesis. Basic nutritional needs, including the purine

requirement, of the Utah strain of Artemia had previously been established through the pioneering work of Provasoli and his co-workers, who developed the axenic culture techniques (Provasoli and Shiraishi 1959) and later an artificial media (Provasoli and D'Agostino 1969) which facilitated nutrient manipulation. Thus, the problem has not been that of identifying a unique requirement but that of understanding how different qualitative and quantitative supplies of dietary purines modify Artemia's metabolism and specifically the response at the morphological level to an unbalanced purine/pyrimidine ratio. Some comparative data suggest this response may represent fundamental processes in developmental biology, and while it is still beyond the present level of knowledge to extrapolate from the results with Artemia to generalizations, a few speculations will be discussed.

The Utah strain of Artemia offers a unique advantage for this work in that various nutritional conditions result in the induction of morphogenetic effects which allows one to visualize metabolism at the level of morphogenesis, thus demonstrating the existence of metabolic relationships between nutrients. A summary of the relationships between dietary factors: the purine concentration, the pyrimidine concentration, the quantity of protein, the effect of supplementation with lecithin, and the morphological response of Artemia is presented in Table I.

Table I. Summary of the Relationships Between Dietary Factors and Induction of Abdominal Gonopodes of Artemia (Utah strain)

Purine	Cytidylic acid			Albumin		Lecithin		Appendicular response
	none 0 mg%	norm 20 mg%	high 100 mg%	low 5 mg%	norm 20 mg%	none 0 mg%	high 4 mg%	
low ≤ 20 mg%[a]		+			+	+		gonopodes
≤ 20 mg%[b]	+				+	+		normal
≤ 20 mg%[b]		+		+		+		normal
= 40 mg%[b]		+			+	+		normal
= 40 mg%[b]		+			+		+	gonopodes
norm = 60 mg%[b]		+			+		+	normal
= 60 mg%[b]			+		+	+		gonopodes
= 60 mg%[b]			+	+		+		normal
high = 100 mg%[b]			+		+	+		normal

[a]Adenylic acid, 20 mg%; inosinic acid, 20 mg%; guanylic acid, 10 mg%; adenosine, 10 mg%; inosine, 10 mg% (or adenine, 5 mg% + guanine, 5 mg%).

[b]Adenylic acid (at concentrations stated).

In spite of the effort to replace live diets such as Artemia with artificial diets, aquaculture is still heavily dependent on this organism. Kinne (1977) states, "More than 85% of the marine animals cultivated thus far have been offered Artemia as a food source." Now an appropriate question is, "How do these findings gathered with Artemia fed on defined diets under axenic conditions apply to general shellfish nutrition?" Since the nutritional value of Artemia for predators cannot be divorced from the fulfillment of its own nutritional requirements, an

understanding of Artemia nutrition has some possible implications for aquaculture nutrition and this will be the primary focus of the present paper.

THE PURINE REQUIREMENT

Fundmental research on the role of dietary purines is scarce not only for invertebrates but for general animal nutrition as well. Most of our knowledge of conventional nutrition comes from studies with the rat, mouse and chick, however, a common feature of vertebrate nutrition is the absence of a requirement for dietary purines and pyrimidines (Hutner and Provasoli 1965). Thus, studies on purine requirements have been mainly restricted to cells in culture. Additionally, exacting studies on the purine and pyrimidine requirements of animals have been seriously hampered by the unavailability of defined media for axenic culture. This is probably why these nutritional requirements have received little attention at the organismic level. While information is limited, it does not appear that except for a few mutant auxotrophs, most, but not all organisms thus far studied, as well as most cells in culture, have the ability to develop on defined media without purine and pyrimidine supplementation and hence must be capable of de novo synthesis. However, for maximum growth in many organisms, the utilization of dietary sources of purines and pyrimidines through known re-cycling pathways may be of equal importance to those derived from de novo synthesis. This is related in part to the relatively large expenditure of energy, in terms of ATP consumption, required for de novo synthesis of purines (Kelley 1972). For example, bacteria can grow on minimal media but their generation time is much shorter when building blocks such as purines and pyrimidines are supplied. Synthetic media defined for marine ciliates contain purines and pyrimidines (Provasoli 1977). Drosophila's growth rate can be substantially improved by addition of RNA as was first shown by Schultz et al. (1946).

Artemia from Utah is incapable of synthesizing the purine ring (Clegg et al. 1967; Warner and McClean 1968). This incapacity makes Artemia an ideal organism for fundamental research on the significance of the dietary requirement for purines and its interactions with folic acid, pyrimidines and amino acids. It is not known yet if this incapacity is shared by other Artemia strains. However, as the effect of salinity on Artemia morphogenesis is strain dependent (Gilchrist 1960) and the effect of salinity is apparently mediated through the metabolism of purines (Hernandorena 1974a), it is likely that the other Artemia strains are capable of de novo purine synthesis. The media defined for the parthenogenetic Sete (France) strain of Artemia contains purines and pyrimidines (Provasoli and Pintner 1980) but this does not necessarily mean that this strain is incapable of de novo synthesis, since a requirement can arise because of a limited capacity to synthesize nucleotides. For example, the nauplii of a tetraploid parthenogenetic strain, used for comparative studies, will survive but are growth limited in a medium lacking a purine source (unpublished data).

It is not known if the incapacity to synthesize the purine ring is shared by other crustaceans. The medium defined by Conklin and Provasoli (1977) for Moina macrocopa contains purines and pyrimidines found to be essential for fertility although adults were obtained from

an original inoculum cultured in a purine-free medium. It is probable that even if crustaceans, like most organisms, are able to synthesize the purine and pyrimidine rings, they will also utilize dietary sources thus sparing precursors, cofactors and energy. Since the amount of DNA per cell and the base ratios of DNA vary from species to species, it is also likely that the optimal dietary levels will be different. For example, in certain crabs 30% of total DNA is made of "dAT" DNA and in cirriped DNA the total amount of the bases guanine and cytosine is greater than 50% (Smith 1964). Artemia has a usual guanine plus cytosine DNA content of 40% (Antonov et al. 1963; Smith 1964).

RELEVANCE TO AQUACULTURE

Results of fundamental research on Artemia nutrition can be of interest in solving several problems presently encountered by aquaculturists.

Live Diets

Different Artemia strains - bisexual, parthenogenetic, diploid, or polyploid - unfed nauplii, and larvae reared on natural or artificial diets, are used in aquaculture. They have been fed to animals without any assessment of the nutritional effects of nucleic acids and nucleic acid components. The subject deserves further investigation.

The nutritional value of algae for herbivores can be altered by changing their culture conditions (D'Agostino and Provasoli 1968; D'Agostino and Provasoli 1970; Provasoli et al. 1970; Moal et al. 1978; Scott 1980). Various Artemia strains have different nutritional requirements as revealed by the differences observed in the ability of algal species to satisfy their nutritional requirements (D'Agostino and Provasoli 1968; D'Agostino 1980); for example, the tetraploid strain of Camacchio (Italy) cannot be grown on the artificial medium which supports good growth of a diploid strain of Artemis (Utah strain) (Provasoli and Pintner 1980). As high nucleic acid synthetic rates associated with polyploidy could be expected to impose a greater burden on the biosynthetic systems, the influence of polyploidy on the purine and pyrimidine requirements was examined using a tetraploid strain from Spain kindly supplied by D'Amat. The tetraploid strain was found to need the same quantitative relationships betwen the purine and pyrimidine nucleotides as the Utah strain (Hernandorena 1979a), but the optimal ranges were much higher for the tetraploid than for the bisexual diploid Utah strain of Artemia (unpublished results) or for the parthenogenetic diploid Sete strain of Artemia (Provasoli and Pintner 1980). Differences in the ability to satisfy these specific requirements of Artemia could be an important factor in explaining the observed variation in the nutritional value of different algae species for Artemia (Provasoli et al. 1959; Reeve 1963; Walne 1967; Sick 1976).

Fast growing organisms are engaged in more protein synthesis than slow growing organisms and consequently probably differ in their ribosomal RNA content. The interdependence of nucleic acids and protein synthesis means that the protein content of live diets cannot be divorced from their nucleic acid concentrations. Therefore, the nutritional value of algal food for Artemia cannot be analyzed solely in terms of protein: carbohydrate: lipid ratios. These ratios measured

for different algal species (Parsons et al. 1961) do not provide any useful guidelines in explaining the observed nutritional values of algae for Artemia (D'Agostino 1980). Intriguingly, the DNA content, as a percentage of the total organic carbon, ranges from 0.5 to 2.5% in laboratory cultured unicellular algae with higher levels being observed for Dunaliella (Holm-Hansen et al. 1968), which is a good algal food species for Artemia.

A similar argument can be developed to explain the variation in nutritional values of Artemia strains for predators. This variation has been well documented at the first International Symposium on the Brine Shrimp. Attempts have been made to relate the food value of selected strains to individual strain characteristics. However, with one exception concerning the essential fatty acid content (Fujita et al. 1980), no convincing explanation has been presented to correlate differences observed in the nutritional values of different strains with either their amino acid composition (Seidel et al. 1980), carotenoid content (Soejima et al. 1980), chlorinated hydrocarbon and heavy metal content (Olney et al. 1980), or lipid content and gross fatty acid composition (Schawer et al. 1980). Certain fish are known to contain 25 times as much DNA per nucleus as any mammal. If synthesis of nucleic acids is rate limited in the predator species, the well known polyploidy of parthenogenetic Artemia strains might be responsible for their different nutritional value. This hypothesis could be systematically checked using various polyploid strains.

The nauplii of the Utah strain of Artemia have been shown to be of suspect quality in supporting survival and growth of crustacean larvae (Little 1969; Reeve 1969; Bookhout and Costlow 1970; Wickins 1972; Roberts 1974; Johns et al. 1980) and of fish larvae (Shelbourne 1968; Klein-MacPhee et al. 1980). Although this has been suggested to be due to high pesticide content, an alternative or additional cause could be associated with the absolute requirement of the Utah strain for a purine source. Paralysis, lack of coordination and difficulties in the withdrawal of exoskeleton, described in crustacean larvae fed Utah strain nauplii, are also symptoms described for Artemia larvae reared in a medium lacking folic acid (Hernandorena 1970). Since the synthesis of purines is a primary factor in determining the quantitative folic acid requirement (Hutner et al. 1959), crustacea fed purine deficient Utah strain nauplii could develop a folic acid deficiency. The fact that the poor nutritional value of Utah strain nauplii can be corrected by feeding them on algae argues against the implication of pesticides and in favor of a nutritional deficiency (Wickins 1972). It is also interesting to note that problems such as high mortality and abnormal development encountered by animals fed Artemia nauplii from Utah arise at specific developmental stages, as in the case in Artemia where the deletion of both folic acid and thymidine from the medium delays metamorphosis (Hernandorena 1979b). It should be interesting to compare the effect of folic acid supplements with that of purines supplements in relieving the detrimental effect on crustacean and fish culture, when Utah strain nauplii are used as a food.

The rate of ammonia excretion which is also strain dependent (Bellini and de Vincintis 1960) is another point that should be considered when analyzing the culture value of different Artemia strains, since the ammonia excreted by Artemia can seriously contaminate culture tanks (Moffet and Fisher 1978). The determination of the

relative contribution to ammonia production of anabolic and catabolic deamination reactions of the purine ring is an essential prerequisite to an understanding of the metabolic significance of ammonia excretion. Artemia appears to offer unique opportunities to unravel this problem. For the Utah strain of Artemia, the rate of ammonia excretion increases when the dietary adenylic acid supply is increased, but the excretion rate is much higher when the purine requirement is met by adenylic acid or by adenosine rather than by inosinic acid, inosine or guanylic acid (Hernandorena and Kaushik 1981 and unpublished data).

Artificial Diets

Even if the problems encountered with the different nutritional values of Artemia strains are solved by the controlled production of cysts, thus providing aquaculturists with a ready supply of a nutritionally valuable strain, the ultimate aim of aquaculturists will be to replace live diets such as Artemia by artificial diets.

When considering the formulae of the artificial diets available for the culture of marine animals, it appears that the protein requirements of decapods and fishes are considerable. As stressed by Cowey and Sargent (1972) protein oxidation is nutritionally and economically wasteful and efforts should be made to direct metabolism towards oxidation of lipids and carbohydrates. Dietary purines and pyrimidines might help in the formulation of ideally balanced diets, because the albumin requirement of Artemia is strictly related not only to the starch concentration, as shown by Provasoli and D'Agostino (1969), but also to the purine to pyrimidine ratio of the diet.

The relationship between the requirements for nucleic acids and protein appears to be complex in Artemia. In the artificial medium defined for Artemia the starch: albumin ratio is 5 to 1. Starch is an essential component of the diet. When starch is deleted from the diet, Artemia larvae immediately show an increase in amylase activity. Mortality increases rapidly if the duration of the deletion period is prolonged for more than three days. On the other hand, larvae can survive long periods of albumin deletion (Samain and Hernandorena unpublished data). Similarly, when faced with a purine deletion, Artemia nauplii and larvae survive for extended periods (Hernandorena 1974b). Interestingly, the protein concentration of young adults transferred from a standard medium to a medium lacking adenylic acid continues to increase for a while and then decreases sharply thereafter (unpublished results). This is probably related to the ability of cells to maintain a normal adenylate energy charge (a measure of the total metabolic energy stored in the adenine nucleotide pool) in spite of adenine nucleotide level decreasing to 30% of normal (Swedes et al. 1975). The metabolic ability of the Utah strain larvae to compensate for a decrease in the purine concentration of their diets depends on external temperature and salinity (Hernandorena 1974a, 1976a) but is limited by an albumin-rich diet (Hernandorena 1974a) or a lecithin-rich diet (Hernandorena 1979a). This metabolic adaptation is apparent at the morphological level by reduced abdominal length and by the induction of supernumerary gonopodes on abdominal segments.

The metabolic ability of Artemia to compensate for an increase in the purine concentration of its diet by increased pyrimidine biosynthesis also appears to be limited. The Utah strain can synthesize

the pyrimidine ring (Warner and McClean 1968) but apparently at a limiting rate under our experimental conditions since growth rate and survival increase with increasing the pyrimidine dietary supply. The effect of pyrimidine deletion on survival depends not only on the quantity (Hernandorena 1979a) but also on the quality of dietary purines (Hernandorena 1980a). Further, it also depends on both the quantity and quality of dietary albumin (Hernandorena 1974a). The factor limiting pyrimidine biosynthesis is apparently not aspartic acid (unpublished results). I found that when the quality of albumin is altered by different heat processing, no morphogenetic action is induced by a reduced purine to pyrimidine ratio (unpublished result). Specific amino acids may modify the activity of xanthine dehydrogenase by increasing the sensitivity to allopurinol, a xanthine dehydrogenase inhibitor (Hernandorena 1981a). Additionally, since allopurinol administration results in a melanogenetic effect (Hernandorena 1970) I suspect pterin metabolism as well as purine catabolism to be involved.

With diets containing the same starch to albumin ratio, Artemia larvae can be forced to grow faster by increasing the adenylic acid concentration of the diet. This faster growth results in a reduced protein synthesis if the cytidylic acid supply is limited or they can be forced to increase protein synthesis by increasing the cytidylic acid supply (Hernandorena 1981b). So it appears that the metabolic fate of nutrients at a given dietary starch to albumin ratio will depend on the purine to pyrimidine ratio, and this will affect the biochemical composition of Artemia.

In the artificial medium defined for Artemia, the nucleotides are supplied at high concentration levels in the liquid phase because of the limited osmotrophy of Artemia. When supplied as particles, nutrients are 60 times more effective than as solutes (Provasoli and D'Agostino 1969). If corrected by this factor, the 60:20 adenylic acid to albumin ratio of the artificial medium becomes 1:20 and is comparable to the 1:14 RNA to protein ratio defined for Drosophila. Attempts to introduce RNA in the diet of Penaeus japonicus at the lower 1:120 RNA to protein ratio did not result in any apparent beneficial effect (Kanazawa et al 1970). This result should not discourage further investigation using different ratios.

Developmental Control

The practical application to aquaculture of findings related to Artemia morphogenesis could precede the achievement of the ultimate goal of research which is to understand development at the molecular level. While the use of hormones in cultured animals leads to a number of concerns about effects on the consumer, dietary manipulations are possible which would achieve the same production goals. When Artemia purine and pyrimidine requirements are met, folic acid deletion prevents sexual maturity. Interestingly, giant individuals incapable of reproduction are induced in suctorian popultions by manipulations of dietary guanylic acid (Palincsar 1959). It may be possible to take advantage of this type of nutritional effect to prevent the conversion of nutrients into the production of gametes in cultured invertebrates. Sexual maturation could be restricted at will to animals meant for reproduction while production animals would continue to channel nutrients only into growth processes.

Commercial interest in shrimp, prawn and lobster aquaculture,

should also stimulate fundamental research on the purine and pyrimidine requirements of crustaceans, with the aim of producing at will the elongation of abdominal length, which is easily achieved during the anamorphic development of Artemia, and should be tried during the metamorphic development of decapod Crustacea.

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SICK (NMFS, Charleston): I was interested in your comment on possible folic acid deficiencies in crustaceans. In some studies carried out in my laboratory, low levels of folates have been noted in the prawn, apparently due to some nutritional deficiency. Specifically do you know of any evidence for or against the ability of crustaceans to synthesize tetrahydrofolic acid?

HERNANDORENA: Only that folic acid is an essential nutrient for Artemia, at least based on work done in my laboratory. The nauplii apparently receive a bit from the mother and as a result can complete a few molts in a medium lacking folic acid. The maternal supplies, however, are not enough for complete development. This is particularly true in a medium lacking the pyrimidine deoxyribonucleoside thymidine which appears to spare folic acid in Artemia. In other organisms it is usually purines which spare folic acid but this is not the case in Artemia.

CONTE (Oregon State University): Dr. Hernandorena, your work on nucleic acids is excellent. I was wondering what you feel is the control point for purine and pyrimidine metabolism. Drs. Clegg, Warner and Finamore have done extensive work looking at the metabolism of nucleic acids in the cysts in conjunction with emergence. They, however, have not gone beyond the naupliar stage as you have done. Work done in my laboratory indicates that a lot of nucleic acid metabolism is very much dependent on ATP levels.

HERNANDORENA: Yes, the problem is although it has been demonstrated that the Utah strain of Artemia cannot synthesize the purine ring they, apparently, based on radiolabelling experiments, quickly incorporate precursors into pyrimidines. However, the present media I use seems to be lacking something, and it is not aspartic acid, so I have to add a pyrimidine in spite of the fact they can synthesize it. That is something I have not solved. Perhaps the rate of synthesis is not sufficient for growth.

CONTE: So you're thinking the control is at a point beyond aspartic acid in the condensation step.

HERNANDORENA: I've tried to increase the aspartic acid concentration of the medium but found no improvement in media where there was no pyrimidine.

PERSOONE (Ghent, Belgium): I was interested in your alternative hypothesis for the poor growth and high mortality associated with the Utah strain of Artemia. In recent research in my laboratory at Ghent, the Utah strain seems to be growing faster than the California strain from San Francisco thus making it a likely candidate for use in aquaculture. I was wondering, after hearing the data of Ken Simpson, if there is a relationship between the pesticides and purine metabolism?

HERNANDORENA: You will have to answer that question, not me. I haven't done any work with pesticides.

SIMPSON (Rhode Island): It's my opinion from comparative studies that the pesticide story has been over extended. I think with regard to pesticides and the Utah strain of Artemia, it is not as bad as some

others and yet the others support better survival and growth. What has not been established is the role of fatty acids in the Utah strain which is consistently poor. This would be also corrected by feeding algae with the right fatty acids.

HERNANDORENA: The two ideas are certainly not exclusive.

SIMPSON: Just a point of information for Dr. Persoone. I was at Salt Lake and talked to the people collecting cysts. They are presently collecting them from a different location than in 1975 when the catastrophic problems occurred with the Utah strain. Also the lake has changed in terms of salinity and other things so the habitat is markedly different from what it was in 1975.

ZOOPLANKTON AS A FOOD SOURCE

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ABSTRACT

While thousands of zooplankton species could potentially serve as food for larval stages of cultured fish and crustaceans, the aquaculturists practical choice of a reproducible live food ratio is quite limited. In practice, rotifers and brine shrimp are the most commonly used zooplankton for these critical larval stages. The reasons for the popularity of these organisms lies in the fact that they are easily obtained or cultured, are of appropriate size, and have been shown to support a wide spectrum of larval forms. Recently, aquaculturists have turned their attention to the quality of the rotifers and brine shrimp that are being fed. A number of papers have reported the success of rearing a larval fish or shellfish on one batch of rotifer or brine shrimp, but not on another. It has been well documented that the nutrition and environment of the zooplankton has a profound effect on the predatory organism. The brine shrimp has been shown to have a limited capacity to biosynthesize long-chain fatty acids and, thus, its nutrition takes on added significance. Also, Artemia have been shown to readily bioaccumulate chlorinated hydrocarbons from the water and pass these on to the predator.

An Artemia reference sample is now available, so that one can compare the growth and survival of experimental animals fed the reference brine shrimp versus those fed an acquired batch of Artemia.

KEY WORDS: zooplankton, rotifers, Artemia, copepods, Brachionus.

INTRODUCTION

The word, plankton, is derived from a Greek word meaning, to wander. Zooplankton, referring specifically to microscopic animal life floating or drifting in water, serves as the primary source of food for larval stages of many aquatic species of economic importance. The ususal list of zooplankton organisms includes protozoans, rotifers,

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brachiopods, copepods and invertebrate larvae. Thus, thousands of animals could serve as potential food for cultured fish and shellfish.

Since the culture of laboratory animals requires a stable, reproducible ration, the number of zooplankton species of potential use to culturists rapidly shrinks to a few standard organisms. In actual practice, aquaculturists depend heavily on rotifers and brine shrimp to support critical phases of larval development, though neither of these organisms is found in significant numbers in the sea. The reason for the popularity of these organisms lies in the fact that both are easily cultured or obtained, and have been shown to support a very wide variety of larval forms. Comprehensive bibliographies are available on both Artemia (Sorgeloos et al. 1980a) and Brachionus (Solangi and Ogle 1977). The treatise edited by Kinne (1977) should be consulted for the cultivation of zooplankton in general.

The present paper will focus on the nutritional aspects of zooplankton (proteins, lipids, etc.), and some non-nutritional factors (size, contamination, etc.). The ultimate goal of an aquacultural feed is to provide for rapid growth and high survival of the cultured organism. It is also important, for consumer acceptance, that the aquaculturally produced animals resemble the wild organism in such characteristics as color, taste, texture, and size.

What has been "discovered" recently is that Artemia, rotifers, etc. are living organisms and can vary with environmental conditions. Thus, in describing the diet of an animal, to say, "Artemia was fed", is not equivalent to saying, "casein was fed." Examples will be given in this paper to show that some sources of rotifers, Artemia, etc. support good survival and growth whereas others cause 100% mortality of larvae. The effect is obvious -- the cause is slowly being understood.

SIZE

The suitability of zooplankton for early life stages may be directly related to size. The live food may be of good nutritional value but not practical because it is too large to be ingested by larvae. Sulkin and Epifanio (1975) compared rotifers, Artemia nauplii, and sea urchin gastrulae as food for the first and second zoeal stages of the blue crab (Callinectes sapidus). The rotifers used in this experiment ranged in size between 45 and 180 μm, whereas the "recently hatched" Artemia nauplii were 250 μm in width. These authors stated that 110 μm was the maximum size for the food of the early blue crab larvae.

Brachionus rotifer strains vary in size. A Japanese strain ranges in size from 74-124 μm (Ito 1960), and a California strain from 66-182 μm (Theilacker and McMaster 1971). The biometrics of Artemia strains from different geographic origins have been reported by Vanhaecke and Sorgeloos (1980). It was found that there was a large variation between strains and little difference within strains. Beck and Bengtson (in press) reported the effect of size in eight geographical strains of brine shrimp ranging from 433 $\pm$ 21 μm to 515 $\pm$ 37 μm in length. A direct relationship was found between increased brine shrimp size and mortality of Atlantic sliverside (Menidia menidia) larvae fed the various strains (Figure 1).

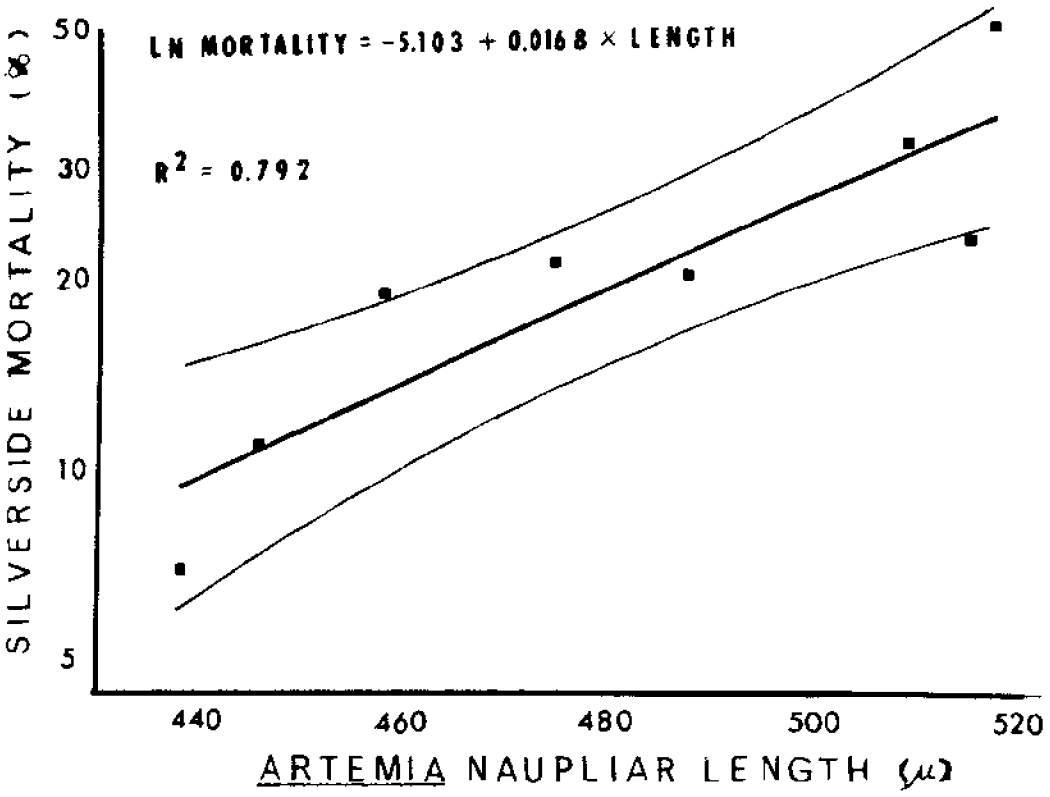

Figure 1. Natural logarithm of mortality of Atlantic silversides during the first five days of an experiment as a function of mean length of eight strains of Artemia nauplii fed to the fish. Bold, straight line is the regression line; thinner, curved lines represent 95% confidence limits.

Data regarding size and suitability of a live organism as a first food are primarily from studies with fish. For example, several types of live organisms have been cultured as first food for small larval fish. Trochophore larvae of oysters (Crassostrea virginica) were used to rear turbot, Scophthalmus maximus (Howell 1973). Veliger larvae of opistobranch molluscs (90-140 μm) have been used successfully to feed northern anchovy, Engraulis mordax, in combination with a naked dinoflagellate, Gymnodinium splendens (Lasker et al. 1970). The anchovy larvae selected prey in the size range 50-90 μm when first feeding.

Copepods were fed to first feeding, scaled sardines, Harengula pensacolae, and bay anchovy, Anchoa mitchilli. Scaled sardines, which began feeding at 4.5 mm total length, selected copepodites averaging 63 μm width while bay anchovy, which began feeding at 3.4 mm total length, selected copepodites averaging 58 μm in width (Detwyler and Houde 1970). Houde (1973) stated that, according to his experience, most fish larvae, including those with relatively large mouths, began feeding on organisms in the 50-100 μm width range. Copepod nauplii and molluscan larvae fall within this range as does the rotifer, Brachionus plicatilis, one of the most important food organisms used in culturing small-mouthed larval fish.

Most decapod crustacean larvae can be reared on Artemia nauplii for their complete development (Rice and Williamson 1970; Provenzano and Goy

1976). Some examples are the American lobster, Homarus americanus (Carlberg and Van Olst 1975), stone crab, Menippe mercenaria (Mootz and Epifano 1974), grass shrimp, Palaemonetes pugio (Provenzano and Goy 1976), and Cancer irroratus (Sastry 1970). The exceptions appear to be blue crab, Callinectes sapidus, mentioned previously (Sulkin and Epifanio 1975), and penaeid shrimp which are fed various species of algae (Hudinga and Miyamura 1962; Griffith et al. 1973). Mock et al. (1980) used active dry baker's yeast as a replacement for algae with some success for feeding Penaeus stylirostris. When the shrimp metamorphosed to mysis I stage, they were fed frozen Artemia nauplii and then switched to live Artemia nauplii at the end of mysis II stage.

PROTEIN

It is generally assumed that a live food should provide protein levels and amino acid profiles similar to that found in the cultured or wild organism. A second approach is to pattern the amino acid profiles after the food considered best for that organism. In a series of elegant experiments based on this concept, Deshimaru and Yone (1978) reported on an "artificial clam" diet for the prawn, Penaeus japonicus. The optimum dietary level of protein was in the range of 52-57%. Other penaeid shrimp have been reported to require a diet containing less than 40% total protein, e.g. Penaeus setiferus (Andrews et al. 1972). P. aztecus (Venkataramiah et al. 1975), and P. indicus (Colvin 1976).

Potential shrimp feed shown to contain approximately 50% protein include rotifers cultured on algae (Scott and Baynes 1978), brine shrimp nauplii (Schauer et al. 1979), and brine shrimp adults (Deshimaru and Shigeno 1972; Gallagher and Brown 1975).

Although more is known about the amino acid requirements for fresh water and marine fish than shellfish (cf. Cowey 1979), some basic work has been accomplished in prawn nutrition. Deshimaru and Shigeno (1972) found that diets similar to P. japonicus in amino acid profiles, such as Artemia, squid, and short neck clams supported good growth. As part of the same study, white fish meal-based diets were found to give poor growth. These authors concluded that a protein source lacking sufficient levels of basic amino acids (e.g. lysine, arginine and histidine) supported inefficient growth. They listed fish meal as a poor source of the basic amino acids.

Table I lists the amino acid profiles of several zooplankton species. Brachionus, Acartia, Tigriopus, Moina and Penaeus show very similar amino acid profiles. Artemia nauplii amino acid patterns show the greatest variation. Seidel et al. (1980a) compared the amino acid profiles and also found some variation between the nauplii from Shark Bay, Australia, Macau, Brazil, San Pablo Bay, California, the Great Salt Lake, Utah and Margherita di Savoia, Italy. These differences in profiles among strains were less, however, than those reported in Table I, possibly showing variation in laboratory techniques. Seidel et al. (1980a) concluded that all five geographical strains could meet the essential amino acid requirements as established for Chinook salmon. Gallagher and Brown (1975) concluded that "the brine shrimp values compare favorably with those for both casein and egg albumin." On the basis of PER and NPU values and protein digestibility, Watanabe et al. (1978a), concluded that Brachionus, Artemia, Tigriopus, Moina and

Daphnia are superior food protein sources for fish.

Diet appears to have little effect on the amino acid composition of the live food (Watanabe et al. 1978a; Claus et al. 1979), and in turn, amino acid composition of the predator (Seidel et al. 1980b).

Table I. Amino Acid Composition of Zooplankton as Compared with *Penaeus japonicus* (g/100 g crude protein)

Amino acid	*Artemia salina*			*Brachionus*[d] *plicatilis*	*Arcartia*[d] *clausi*	*Tigriopus*[d] *japonicus*	*Moina*[d] spp.	*Penaeus*[e] *japonicus*
	#1[a]	#2[b]	#3[c]					
Isoleucine	2.6	5.6	5.3	3.4	3.5	2.5	2.5	3.3
Leucine	6.1	8.9	8.0	6.1	5.5	5.0	6.0	5.5
Methionine	0.9	2.2	2.7	0.8	1.5	1.1	1.0	2.0
Cystine	0.4	-	2.2	0.6	0.8	0.7	0.6	0.8
Phenylalanine	3.2	5.1	4.7	3.9	3.7	3.5	3.6	3.3
Tyrosine	3.7	10.5	4.5	3.1	3.6	4.0	3.3	2.9
Threonine	1.7	5.2	4.6	3.2	4.2	3.8	3.8	3.0
Tryptophan	1.0	-	1.0	1.2	1.1	1.1	1.2	-
Valine	3.2	5.3	5.4	4.2	4.5	3.3	3.2	3.3
Lysine	6.1	11.7	7.6	6.1	5.4	5.7	5.8	5.8
Arginine	5.0	11.5	6.5	4.6	4.3	5.2	5.1	5.6
Histidine	1.3	4.9	1.8	1.5	1.9	1.6	1.6	1.6
Alanine	4.1	4.6	6.9	3.8	5.4	4.9	4.9	4.1
Aspartic acid	7.5	11.0	9.2	8.0	9.0	9.0	8.3	8.0
Glutamic acid	8.8	13.1	14.2	9.8	9.5	10.8	9.8	11.7
Glycine	3.4	6.0	5.3	3.1	4.6	4.5	3.7	3.4
Proline	4.7	5.7	5.2	6.7	4.6	4.8	4.2	2.6
Serine	4.6	4.5	4.8	4.0	3.3	4.3	4.0	3.0
Total	74.1		99.9	74.1	76.4	75.8	72.6	69.9

[a]Newly-hatched; Watanabe et al. 1978a.

[b]Seidel et al. 1980b; Instar I stage, Macau, Brazil strain; expressed as % amino acids; tryptophan destroyed.

[c]Gallagher and Brown 1975.

[d]Watanabe et al. 1978a.

[e]Deshimaru and Shigeno 1972.

LIPIDS

The lipid content of a food is an important factor in the value of the food. In practice, the term lipid denotes any material extracted by an organic solvent such as chloroform. Thus, crude lipid would include glycerides, steroids, waxes, carotenoids, phospholipids, etc. Triglycerides, waxes and phospholipids constitute the major source of metabolizable energy as well as a source of essential fatty acids.

Waxes are esters of long chain alcohols and fatty acids. They have been shown to be a common storage lipid for copepods in temperate and polar environments where lipid storage is more pronounced (Lee and Hirota 1973). As a reserve energy source, the waxes may represent a level of greater than 50% of the total lipid present (Lee 1975). Copepods from tropical and subtropical areas were characterized by having fewer storage lipids, including waxes. It appears that the waxes represent a *de novo* synthesis by the arctic copepods (Sargent and Lee 1975).

While wax-containing copepods are abundant, little is known about the assimilation of the waxes by predators. It has been reported that trout and herring metabolize dietary wax esters and deposit triacylglycerols in their bodies (Sargent et al. 1979). Sargent et al. (1979) and Patton and Benson (1975) conclude that "wax esters are very efficiently assimilated in the marine food-chain."

As with proteins, more is known about the essential fatty acid requirements of fish than shellfish. Nevertheless, a very similar picture is emerging in regard to the need for these fatty acids in the diet. Unlike protein/amino acid patterns, the fatty acid profiles found in the cultured organism often closely resemble the phytoplankton it feeds upon. Thus, the fatty acid profile of the rotifer, Artemia, or copepod may be significantly changed by diet and could be of good or poor nutritional value.

Generally, Artemia cysts are purchased whereas rotifers are cultured in the laboratory. The Artemia cysts would presumably resemble the adult Artemia in fatty acid composition while the rotifer would resemble the culture diet. The need for essential fatty acids in the diet of rotifers has become apparent and a similar picture for Artemia has emerged.

Scott and Middleton (1979) cultured Brachionus plicatilis on several different unicellular algae at three different temperatures. The rotifers generally had fatty acid profiles like their algal food. The Dunaliella-fed rotifers were high in 18:3ω3 and low in 20:5ω3 and 22:6ω3. The Isochrysis, and especially the Pavlova and Phaeodactylum-fed rotifers contained less 18:3ω3 and more 20:5ω3 and 22:6ω3. The turbot larvae fed the Dunaliella-fed rotifers exhibited stunted growth and high mortalities. Turbot larvae fed Dunaliella-fed rotifers plus other algae-fed rotifers showed good survival and growth, revealing an apparent nutritional problem rather than a toxic effect.

A very similar picture has emerged from the work on rotifers in Japan (c.f. Fujita 1979). Because of the great demand for rotifers, baker's yeast was substituted for Chlorella as a rotifer feed and the result was high mortalities in sea bream (Watanabe et al. 1979; Kitajima et al. 1980a), and ayu, Plecoglossus altivelis (Kitajima et al. 1980b). A number of experiments were reported with a so-called ω-yeast. The ω-yeast was prepared by culturing baker's yeast in a medium supplemented with fish oils. The resulting rotifers were found to have a higher level of 3 HUFA's (highly unsaturated fatty acid), and to support good survival and growth of red sea bream (Kitajima et al. 1980a), and P. altivelis (Kitajima et al. 1980b).

Watanabe et al. (1978b) analyzed the fatty acid content of brine shrimp from San Francisco, South America and Canada. These authors classified Artemia on the basis of their fatty acid profiles. They grouped those with high 18:3ω3 levels as being suitable for fresh water fish only, and those with high 20:5ω3 as being most suitable for marine fish. In a later report, Watanabe et al. (1980) classified Artemia cysts from San Francisco, China, Canada and West Germany into the same two categories. The San Francisco strain was shown to fall into both classes, depending on the year of harvest. An Artemia with a high 18:3 ω3and low 20:5 3 content was consistently found, by these authors, not to support growth and survival of sea bream larvae.

Schauer et al. (1980) reported on the fatty acid profiles of three San Francisco Bay area *Artemia* collections, and on strains from Australia, Brazil, Italy and Utah (Table II). As can be seen, San Pablo Bay (SPB 1628) and Utah (1977) are high in 18:3ω3 and low in 20:5ω3. One of the San Francisco collections (SF 313) was found to be intermediate between the San Pablo Bay (SPB 1628) and Utah, and the other strains. The most significant variation was in the survival and growth of various fish and shellfish larvae on the various strains. This will be discussed in detail below.

Table II. Fatty Acid (as fatty acid methyl esters [FAME]) Composition of Newly Hatched *Artemia* (from Schauer et al. 1980)

FAME	Australia	Brazil	California			Italy	Utah
			SF (lot #313)	SF (lot #321)	SPB (lot #1628)		
14:0	1.34	1.57	0.99	1.57	0.43	1.53	0.93
14:1	2.23	0.81	1.27	0.74	2.26	3.30	1.45
15:0	0.34	0.67	0.16	0.58	0.25	0.11	0.11
15:1	0.15	0.24	0.20	0.13	0.46	0.54	0.37
16:0	13.45	15.42	10.33	12.13	7.79	15.23	11.78
16:1	9.97	10.79	13.27	19.52	5.24	10.38	5.64
16:2ω7	---[a]	---	---	---	1.51	2.94	---
16:3ω4/17:1ω8	3.87	3.88	2.09	2.32	2.44	3.28	2.90
18:0	3.07	2.79	6.83	2.90	3.08	3.17	4.07
18:1ω9	28.23	35.86	26.97	31.20	29.15	29.05	28.58
18:2ω6	5.78	9.59	9.35	3.69	4.60	6.79	4.60
18:3ω3	14.77	4.87	17.33	5.16	33.59	6.35	31.46
18:4ω3	4.37	0.96	3.26	1.28	4.88	1.01	3.10
20:1ω9	0.37	0.52	0.41	0.35	0.35	0.42	0.37
20:2ω6/ω9	0.12	0.06	0.06	---	0.24	0.20	0.09
20:3ω6	0.79	2.76	1.01	2.23	0.05	1.47	0.48
20:3ω3/20:4ω6	---	---	1.48	2.69	1.48	---	---
20:5ω3	10.50	8.98	4.06	12.44	1.68	13.63	3.55
22:6ω3	0.26	0.06	---	---	---	---	---

[a]--- indicates the fatty acid was not found.

Moreno et al. (1979a) suggests that phytoplankton are able to perform *de novo* synthesis of all their fatty acids. These authors showed that the diatom, *Phaeodactylum tricornutum*, transformed radioactive staturated and unsaturated precursors into 20:5ω3 and 22:6ω3 fatty acids. Phytoplankton that have high concentrations of 20:5 3 and 22:6ω3 fatty acids would contribute them to the marine food web. Many marine species have been shown to require these polyunsaturated fatty acids in their diet. This requirement is largely based upon the inability of the organism to biosynthesize the ω3 20- and 22-carbon fatty acids from 18-carbon analogs.

The calanoid copepod, *Paracalanus parva* was shown by Moreno et al. (1979b) to be able to form ω9 fatty acids and synthesize long chain fatty acids. *P. parva* was shown to be incapable of synthesis of linoleic or linolenic acids. Schauer (personal communication) incorporated 1-^{14}C 18:3ω3 into a rice bran diet for *Artemia*. The *Artemia* were then fed to rainbow trout and winter flounder (*Pseudopleuronectes americanus*). As noted in Table III, 1-^{14}C 18:3ω3

was the only ω3 fatty acid in the diet. Artemia were shown to form 20:5 ω3and 22:6ω3 fatty acids from 18:3ω3. While 22:6ω3 is not commonly isolated from nauplii, we have found it in adult Artemia. These data show that Artemia can make HUFA's from 18:3ω3, probably in sufficient amounts to satisfy their needs. The results shown in Table II reflect a combination of fatty acid profiles resulting from biosynthesis and the particular diet available at the time to the Artemia in that geographical region. The ability of some marine crustaceans to biosynthesize 20:5ω3 and 22:6ω3 from palmitate was also reported by Morris and Sargent (1973). Table III further shows that rainbow trout were able to form 20:5ω3 and 22:6ω3, whereas winter flounder contained approximately the same amounts of these fatty acids at 24 hours as were in the Artemia.

Table III. Percent Distribution of 1-^{14}C 18:3ω3 in a Short Food Chain (from Schauer 1982)

Number double bonds	Labeled rice bran Diet #1[a]	Artemia fed labeled Diet #1[b]	Rainbow trout fed labeled Artemia[c]	Winter flounder fed labeled Artemia[c]
0 + 1	0.21	8.80	8.29	8.07
2	0.04	3.26	19.2	24.2
3	98.5	68.2	23.1	20.5
4		2.50	5.50	30.4
5	1.24	6.93	12.5	7.02
6		10.30	31.4	9.80

[a] 1-^{14}C 18:3ω3 was the only ω3 fatty acid in the diet.

[b] Artemia were fed the labeled diet for 7 days.

[c] Rainbow trout and winter flounder were fed the labeled Artemia for 3 days and starved for 3 days subsequent to the last feeding.

Kanazawa et al. (1979) injected 1-^{14}C 18:3ω3 fatty acids into serveral different marine species in order to compare their ability to form 20:5ω3 and 22:6ω3 fatty acids. Table IV shows the conversion relative to rainbow trout. A number of authors have shown the ability of the freshwater fish to convert 18:3ω3 to HUFA's (cf Kanazawa et al. 1979, rainbow trout). Kanazawa et al. (1979) concluded that the marine animals in their study were incapable of synthesizing enough 20:5ω3 and 22:6ω3 from 18:3ω3 to satisfy their dietary requirements. Penaeus japonicus was listed as moderate in this biosynthetic capacity.

Table V shows the fatty acid profiles of San Pablo and Brazil Artemia nauplii which were cultured in ponds at SEAFDEC (South East Asia Fisheries Development Center), Philippines. The harvested adult profiles were similar to each other, very different from the parent cysts, and probably resembled the phytoplankton in the pond.

Sorgeloos et al. (1980b) fed defatted rice bran to Artemia and reported a good protein recovery in the Artemia. However, the fatty acid profile showed very low levels of HUFA's. Thus, if the adult

Artemia is to be fed to marine species, biomass is not as important a consideration as fatty acid profiles, and the warnings of Watanabe et al. (1980) need to be heeded.

Table IV. Percentage Incorporation of [1-^{14}C] Linolenic Acid into the ω3 Pentaenes and Hexaenes of Lipids from Aquatic Animals Injected with [1-^{14}C] Linolenic Acid (from Kanazawa et al. 1979)

Animal	Radioactive ω3 pentaenes and hexaenes formed from [1-^{14}C] linolenic acid		
	Radioactivity (dpm x 10^4)	% Incorporation[a]	Relative % incorporation to rainbow trout
Rainbow trout	128.4	12.7	100
Ayu	6.5	4.5	36
Eel	11.3	2.5	20
Red sea bream	5.0	1.9	15
Rockfish	19.6	0.9	7
Globefish	25.8	1.6	13
Prawn	18.5	2.6	20

[a]% Incorporation of injected [1-^{14}C] linolenic acid to ω3 pentaenes and hexaenes comprising 20:5ω3, 22:5ω3, and 22:6ω3.

Table V. Fatty Acid (as fatty acid methyl esters [FAME]) Profiles of Artemia Nauplii and Adults Cultured in SEAFDEC Ponds (unpublished data)

FAME	B-Nauplii[a]	B-Adults[c]		SPB-Nauplii (lot #1628)	SPB-Adults[c]	
		6/30/80[d]	7/2/80[d]		6/30/80[d]	7/2/80[d]
14:0	2.04	3.80	3.06	0.65	3.67	3.07
16:0	16.35	21.39	18.19	9.80	18.28	18.26
16:1	12.88	12.80	11.19	6.49	11.08	13.04
18:0	2.34	9.00	7.87	2.38	8.15	
18:1ω9	33.50	32.68	34.95	27.43	32.11	40.70
18:2ω6	9.17	9.07	8.95	5.30	10.74	9.24
18:3ω3	4.39	9.23	10.99	31.85	13.18	14.04
20:3ω6	2.30	0.69	1.15	0.04	0.98	0.08
20:5ω3	8.35	1.27	3.01	1.66	0.11	0.67

[a]Eggs from Macau, Brazil.

[b]Eggs from San Pablo Bay, California, USA.

[c]Adults cultured from Brazil (B) or San Pablo Bay (SPB) cysts.

[d]Collection date.

CAROTENOIDS

Zooplankton such as the copepods and Artemia are pigmented with carotenoids. These lipids may be deposited in the predator or, in some cases, assimilated as vitamin A precursors. Astaxanthin is the major crustacean pigment, although canthaxanthin is the major carotenoid present in a variety of geographical strains of Artemia (Soejima et al. 1980). When incorporated into their diet, a number of carotenoids, ordinarily not isolated from Artemia, were found (Soejima et al. 1980). Table VI shows that effect of feeding Artemia a diet of Spirulina, containing 25% β-carotine and 65% zeaxanthin.

Table VI. The Amounts of β-Carotene, Echineone, Canthaxanthin and Zeaxanthin Present in Artemia after Feeding with Spirulina Pigments (from Soejima et al. 1980)

Feeding program	Concentrations of carotenoids present (µg/g fresh wt)				Unknown
	β-Carotene	Echinenone	Canthaxanthin	Zeaxanthin	
Group 1	0.11	5.87			
Group 4	0.71	3.16	6.97	2.71	0.13
Group 4'	0.19	1.48	6.58	1.99	

Group 1: Rice bran only (12 days).

Group 4: Rice bran + pigments of Spirulina (6 days).

Group 4': Same as Group 4, then rice bran only (6 days).

Colorless lobsters, crabs, prawn, and other crustaceans can be produced by feeding a carotenoid-free diet. Nevertheless, consumer acceptance is based partly on color of the marketed food product. For example, when purchasing Penaeus japonicus live, Japanese consumers demand a red, not a blue-colored prawn. The feeding of pigmented zooplankton has been shown to be effective in providing proper pigmentation (c.f. Sargent et al. 1979; Simpson et al. 1981).

MINERALS

Several authors have reported the mineral composition of zooplankton (Gallagher and Brown 1975; Watanabe et al. 1978c; Olney et al. 1980). Watanabe et al. (1978c) fed Tigriopus, Moina and rotifers several live diets and found no significant differences in the mineral content of the cultured organisms. Table VII shows the mineral composition of Artemia cysts and nauplii taken from five geographical locations. The twelve metals were measured by atomic absorption and neutron activation analysis. While large variations were seen in the metal content of the cysts, the nauplii showed lower levels and less variation between strains.

Table VII. Metals (µg/g dry wt) in Artemia Cysts (C) and Nauplii (N) from Five Locations (from Olney et al. 1980)

	Brazil		Australia[a]	Italy		Utah		California	
	C	N	C	C	N	C	N	C	N
Fe	804.	62.	820.	1860.	70.	800.	47.	1380.	46.
Zn	59.	89.	144.	125.	104.	81.	102.	78.	98.
Pb	8.9		2.1	17.	3.0	5.1	6.2	6.6	3.8
Cu	4.7	6.3	4.6	14.	9.2	42.	8.2	10.2	10.8
Cd	0.02	0.15	0.15	0.09	0.12	0.28	0.14	0.26	0.10
Cr		1.4	1.8	3.7	0.66	2.0	1.1	4.4	0.48
Ni	0.23	0.29	0.50	4.0	0.09	2.9	0.70	6.6	0.12
Co	0.35	0.38	0.20	1.6	0.14	0.34		1.9	
Sc	0.02		0.01	0.41		0.03		0.31	
Sb	0.53		0.53						
Se	1.4		1.2	1.2	1.0	1.5	2.0	2.3	0.83
Rb	6.5	12.			7.5	23.	23.	9.1	6.5

[a]Nauplii from Australia not analyzed (insufficient sample).

Watanabe et al. (1978c) compared the mineral content of a single strain of Artemia hatched in three different locations, and reported similar levels of the minerals tested. Watanabe et al. (1978c) reported a slight seasonal variation in the mineral content of Arcatia clausi. Watanabe et al. (1978c) concluded that "minerals are not the principal factors in the dietary value of living feeds." Olney et al. (1980) further concluded that the differences in survival and growth obtained by feeding certain geographical strains to several fishes and invertebrates was not related to the mineral content analyzed.

VITAMINS

Data on the vitamin content of zooplankton appears to be limited to essentially a single report on brine shrimp by Gallagher and Brown (19759. These data are represented in Table VIII.

CONTAMINANTS

Since the publication of Bookhout and Costlow (1970), attention has been drawn to the fact that live food can carry adventitious materials to the predator. Most commonly noted, perhaps, is the role of the persistent chlorinated hydrocarbons. Less attention has been given to heavy metals, biotoxins of microorganisms that might be carried by the zooplankton.

The paper by Bookhout and Costlow (1970) compared the ability of Artemia nauplii from Utah and San Francisco to support growth and survival of four crab species. It was consistently found that the Utah Artemia were not as good as those from San Francisco. It was reported that the Utah Artemia had three times the level of DDT (dichlorodiphenyltrichoroethane) as that contained in the San Francisco strain; the higher level of DDT was suggested as the cause of larval crab mortalities. A short time after these data appeared, Wickens (1972) reported lower levels of DDT in the Utah Artemia.

Table VIII. Vitamin Composition of the Brine Shrimp *Artemia* as Compared to Minimum Dietary Requirement (MDR) of Salmonids

Constituent	mg/g dry wt	
	Artemia[a]	Salmonid MDR[b]
Biotin	0.001	0.00005
Choline chloride	6.10	0.05
Folic acid	0.001	0.001
Niacin	0.130	0.001
Pantothenic acid	0.068	0.01
Pyridoxine HCl	0.008	0.005
Inositol	1.20	0.25
Riboflavine	0.017	0.005
Thiamine	0.027[c]	0.001
Vitamin B_{12}	0.003	-
Vitamin A	14.6 IU/g[c]	2.5
Vitamin C	0.049[c]	0.050

[a]Gallagher and Brown (1975).

[b]Ketola (1976).

[c]M. Gallagher, personal communication (1982).

In 1980, Olney et al. analyzed the chlorinated hydrocarbon content of five geographical strains of *Artemia*. It can be seen from Table IX that there is much variation between sample sites (Australia, Brazil, Italy, Utah, California) and some variation between San Francisco Bay and San Pablo Bay. The five strains of *Artemia* were tested on winter flounder (Klein-MacPhee et al. 1980) and Atlantic silversides (*Menidia menidia*) (Beck et al. 1980). In both cases, Utah and San Pablo Bay *Artemia* gave poor results. Olney et al. (1980) reported that these fish accumulated some chlordane, dieldrin, DDT and PCB's, (polychlorinated biphenyls) and suggested that these could be the cause of poor results for San Pablo *Artemia*. However, in Table IX the contamination and accumulation data for Utah are not high and would seem to offer no explanation for the poor survival with the Utah diet.

In an attempt to delineate the possible causative role of chlordane and dieldrin, McLean (1980) contaminated *Artemia* with these two compounds at physiological levels. In a preliminary experiment, defatted rice bran was contaminated with the pesticides and fed to *Artemia*. The *Artemia* produced by this procedure showed a low bioaccumulation factor. Rearing the nauplii in water contaminated with the pesticides resulted in higher tissue residues in a shorter time, thus providing suitably-sized nauplii for feeding. The treatments included controls and various combinations of low contamination (0.1 ppb) and high contamination (1.0 ppb) of chlordane and dieldrin. Table X shows that *Artemia* readily bioconcentrate these two pesticides. When *Artemia* from Brazil, with various levels of contamination, were fed to winter flounder, significant changes in fish length were noted that were directly related to the chlorinated hydrocarbon content. Although no mortalities were noted, it should be pointed out that due to the size of the contaminated *Artemia* nauplii, post-metamorphic winter flounder were used in the experiment.

Table IX. Chlorinated Hydrocarbons (ng/g wet wt) in Artemia Nauplii (A) and the Mud Crab, Rhithropanopeus harrisii (R) (from Olney et al. 1980)

Hydrocarbons	Brazil		Australia		Italy[a]	Utah		California[b]			
								SPB		SFB (lot #313)	SFB (lot #321)
	A	R	A	R	A	A	R	A	R	A	A
HCB	0.1	0.7	0.2	---[c]	4.4	0.4	1.2	0.5	0.6	0.5	0.7
α-BHC	1.1	1.2	0.6	5.2	5.2	5.9	2.3	3.1	1.3	2.8	1.4
γ-BHC	0.8	0.2	---	1.8	5.0	1.9	0.6	3.2	0.3	1.0	2.8
c-chlordane	0.1	0.1	---	1.3	2.2	0.8	0.9	14.0	3.5	1.3	6.6
t-nonachlor	<.1	---	---	---	---	1.2	---	5.5	---	1.4	8.3
dieldrin	0.2	---	0.1	---	---	0.7	0.8	1.4	1.8	0.2	1.2
op-DDE	0.4	---	---	---	3.9	0.7	---	5.3	6.3	0.9	6.7
pp-DDE	1.2	2.4	1.3	6.1	100.0	2.2	4.0	18.0	19.0	2.0	10.0
pp-DDD	0.4	---	---	---	74.0	3.0	2.1	13.0	6.5	3.8	22.0
op-DDT	0.4	---	0.2	1.5	60.0	0.2	---	1.0	---	---	2.0
pp-DDT	1.9	1.0	4.3	2.4	188.0	1.2	1.1	4.6	1.8	1.9	2.0
Σ DDT	4.3	3.4	5.8	10.0	422.0	7.3	7.2	42.0	33.6	8.7	43.0
PCB (1016)	5.3	21.0	2.0	29.0	5.3	3.2	30.0	14.0	16.0	6.4	18.0
PCB (1254)	1.6	14.0	0.9	20.0	19.0	6.5	20.0	29.0	33.0	3.5	23.0
PCB (1260)	---	3.2	---	2.0	7.5	4.9	6.1	22.0	25.0	3.5	2.8
Σ PCB	6.9	38.2	2.9	51.0	32.0	15.0	56.1	66.0	74.0	13.0	43.0
% Survival		77%		61%	75%(R)		0%		0%		

[a]No crabs available for Italy.

[b]San Pablo Bay, Calif. USA (SPB); San Francisco Bay, Calif. USA (SFB).

[c]--- indicates the hydrocarbon was not found.

[d]Johns et al. (1980); 1st crab stage.

Table X. Pesticide Concentrations (ng/g), Bioconcentration Factors (BCF) and Percent Uptake of the Pesticides in the Artemia (A) Treatment Groups (from McLean 1980)

	Treatment group								
	A-1,2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10
Treatment[a]	Cont.	LC	HC	LD	HD	LC/LD	LC/HD	HC/LD	HC/HD
Pesticide conc. in water (ng/ml)									
cis-chlordane	0	0.1	1.0	0	0	0.1	0.1	1.0	1.0
dieldrin	0	0	0	0.1	1.0	0.1	1.0	0.1	1.0
ng Exposure									
cis-chlordane	0	50	500	0	0	50	50	500	500
dieldrin	0	0	0	50	500	50	500	50	500
Pesticide conc. in Artemia sp. (ng/g)									
cis-chlordane	0	10.8[b]	92.7	0	0	9.65	7.44	78.2	79.6
		2.45[c]	6.80			1.72	0.91	7.47	11.9
dieldrin	0	0	0	12.0	170.0	13.4	115.0	9.70	131.0
				0.74	63.5	1.99	52.1	0.50	37.9
BCF of Artemia sp.									
cis-chlordane	0	108.0	92.7	0	0	96.5	74.4	78.2	79.6
dieldrin	0	0	0	120.0	171.0	134.0	115.0	96.9	131.0
Percent uptake									
cis-chlordane	0	21.7	18.5	0	0	19.3	14.9	15.6	15.9
dieldrin	0	0	0	24.1	34.2	26.8	23.1	19.4	26.2

[a]Low contamination (0.1 ppb) of cis-chlordane (LC); high contamination (1.0 ppb) of cis-chlordane (HC); low contamination (0.1 ppb) of dieldrin (LD); high contamination (1.0 ppb) of dieldrin (HD).

[b]Means of the two analytical replicates.

[c]Standard deviation of the two means.

In a similar study (Johns et al. in press), contaminated Artemia were fed to Rhithropanopeus harrisii larvae. No adverse effect on growth or survival in the mud crabs was noted. These results suggest that the contamination levels reported by Olney et al. (1980) and Bookhout and Costlow (1970) do not totally explain the adverse effect noted with certain Artemia predators.

Schauer et al. (1980) suggested that a poor fatty acid profile and a "high" level of contamination may give the poor survival and growth noted in these studies. The controlled feeding of a poor fatty acid profile and contaminated Artemia to either crab or winter flounder larvae has not been reported.

It should be noted that an analysis of the chlorinated hydrocarbons in Utah Artemia (1965-1978) has shown a steady improvement (Seidel, personal communication) (Table XI), although the 1977 and 1978 lots of Artemia from Utah gave poor results with R. harrisii larvae (Johns et al. 1980).

Table XI. Temporal Study of Chlorinated Hydrocarbons in Utah Artemia Nauplii (from C. R. Seidel, personal communication)

	1965	1966	1970	1977	1978
HCB	0.32	0.32	0.62	0.73	0.49
Σ PCB	58.1	44.2	29.8	19.6	7.25
Σ DDT	96.8	51.5	27.3	22.9	11.6
γ-BHC	1.51	0.68	0.34	0.65	0.41
Σ Chlordanes	4.1	4.3	8.2	4.2	4.5

The results of the various feeding trials of the five geographical strains showed some variation as to the ranking of the Artemia cysts. The results are tabulated in Table XII. It should be stressed that these results apply to only the particular batches tested, as we have noted significant variations between batches from the same source.

ENZYMES

It is a common experience among aquaculturists that there are critical stages of larval fish growth which, to date, are supplied only by live food. Artificial diets have been constructed which are similar in nutrients and size (microcapsules) yet seem to fall short of giving the survival and growth of the live diet (cf. Seidel et al. 1980b). An obvious problem with the artificial diet is that of the leaching of nutrients and subsequent uncontrolled bacterial growth. This problem will be eventually addressed through improved microencapsulation techniques. A second possible advantage of the live diet is that the live food contains exogenous digestive enzymes which may supplement and/or activate endogenous enzymes of the larval species (Dabrowski and Glogowski, 1977).

Table XII. Summary of the Biological Response of Several Fish and Shellfish to Five Geographical Strains of *Artemia*. (+ = good survival, - = poor survival, ± = good survival but poor growth)

	Brazil	Australia	Utah	SPB	Italy
Cancer irroratus[a]	+	+	-	-	+
Rhithropanopeus harrisii[a]	+	+	-	-	+
Mysidopsis bahia[b]	+	+	+	±	+
Menidia menidia[c]	+	+	+	±	+
Pseudopleuronectes americanus[d]	+	+	-	-	+

[a]From Johns et al. (1980). [b]From Johns and Walton (1979).

[c]From Beck et al. (1980). [d]Klein-MacPhee et al. (1980).

Samain et al. (1980) determined the levels of trypsin and amylase in *Artemia* during the first 24 days after hatching. The *Artemia* were fed on various phytoplankton in these studies. While the amylase and trypsin levels were seen to rise between day 1 and day 2, these levels were low in comparison to the later specific activities of these enzymes. Moreover, the activities were reported to be controlled by the food levels and the food composition. Thus, the non-feeding instar I through *Artemia* nauplius III stage was shown to be low in these two enzymes. Benijts et al. (1975) showed that the instar I stage of *Artemia* was superior to later stages as a feed from a caloric point of view and Clauret al. (1979) reported the same results from their growth experiments.

Seidel et al. (1980b) compared the growth and survival of Atlantic silverside larvae on a live 3-day-old brine shrimp diet, an artificial diet, and a combination of the two diets. While the artificial diet alone gave poor results, a combination of *Artemia* on one day and the artificial diet for the next 7 days gave nearly comparable results to a 100% *Artemia* diet. These results would tend to rule out the digestive enzymes of brine shrimp as its main advantage as food for Atlantic silverside larvae. In the same study, Seidel et al. (1980b) observed that freeze-dried *Artemia* did not support fish growth and survival as well as live *Artemia*. Grabner et al. (1981) studied the suitability of freeze-dried and frozen zooplankton for larval fish. The two processes (freezing and freeze-drying) had little effect on the enzymes tested but did damage the cells, causing significant leaching of enzymes into the medium. In addition, free amino acids were found to be readily lost and one would expect a simultaneous loss of water soluble vitamins, had they been measured.

Studies on the role of exogenous digestive enzymes supplied from a live food source, in the digestion processes of predators are underway in our laboratory at this time.

CONCLUSIONS

The value of zooplankton in the nutrition of larval forms of shellfish and finfish has been well established. In the last decade, we have become aware of the fact that there are significant physicial, chemical and biological variations between, and among strains of zooplankton. These variations are of genetic and environmental origin. While the protein level is generously similar, large variations have been found in the size, fatty acid profiles and contamination of the cultured zooplankton. Nevertheless, the literature still contains references that state only that "a given zooplankton was fed," as though it were a defined diet.

The analysis of the available Artemia strains for selected chemical, biochemical, morphological, and genetic characteristics, and the cultivation response of a predator is clearly beyond the capability of a single laboratory. In 1977, the International Study of Artemia (ISA) group was formed with the purpose of characterizing five geographical strains of Artemia. Much of the research cited in this paper has been drawn from approximately 30 ISA papers. The complete analysis of a single lot of Artemia has allowed comparisons of data to be made which are not possible with much of the literature on zooplankton. The ISA group's effort could serve as a model for a multidisciplinary approach to aquaculture.

A workshop was held during the International Symposium on the Brine Shrimp, Artemia (Corpus Christi, TX, 1979), in which characterization of the strains of Artemia for aquaculture was discussed (Simpson et al. 1980). As a result of the workshop, an Artemia reference sample was made available. This reference lot would serve as an internal control for aquaculturists (cf. Sorgeloos, 1980). The reference cysts have been characterized and found to be suitable as food for marine fish and invertebrates. They are now available from the Artemia Reference Center, Ghent, Belgium. It is hoped that the same standardization can be achieved in the culture of other zooplankton which are fed to aquaculture species.

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QUESTION AND ANSWERS

Editors Note: There was an exchange between Dr. Simpson, Dr. Conte and Dr. Persoone that was unclear from our transcripts.

CASTELL (Halifax): I have a couple of things I would like to point out. In the slides you presented, the Penaeus japonicus which received carotenoid pigments in their diets appeared larger than others. Was there a difference in growth?

SIMPSON: The prawns that were photographed were a random selection from short term feeding trials. There should be no difference in growth.

CASTELL: I am pleased that you pointed out the importance of a standard reference brine shrimp. I think that an awful lot of nutrition work reported in the literature of aquaculture needs standard reference diets. I would like to recommend the report of the working group on standardization of methodology for fish nutrition that was published by FAO. It is a joint work of the IUNS, ICES and IFAC with contributions from the Nutrition Task Force of the World Mariculture Society.

SIMPSON: I have seen it and it is an excellent report.

CRUSTACEAN/MOLLUSCAN NUTRITION

STEROL METABOLISM

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ABSTRACT

This paper presents an overview of sterol metabolism in both crustaceans and molluscs. The author will survey information available from biochemical studies and relate this to the results of feeding experiments, focusing, where possible, on important aquaculture species. Crustacea generally do not have the capacity for de novo sterol synthesis but are able to dealkylate dietary C_{28} and C_{29} sterols to cholesterol. The inclusion of cholesterol in the diet is shown to improve the growth and survival of Penaeus japonicus and Homarus americanus juveniles. The addition of cholesterol to the diet is also shown to improve the survival of P. japonicus larvae. Molluscan sterol metabolism differs from class to class. Gastropods synthesize cholesterol and dealkylate some phytosterols to cholesterol. In contrast, although pelecypods possess the ability to dealkylate some C_{28} and C_{29} sterols to cholesterol, they are capable of only limited sterol synthesis and therefore presumably require sterols for growth. However, feeding experiments using defined, artificial diets have yet to be carried out with pelecypods.

Key Words: Sterols; metabolism; crustaceans; molluscs.

INTRODUCTION

During the past decade, the sterol composition of various phyla of marine invertebrates including shellfish has been re-examined using improved separation and analytical techniques. As a result, the sterol composition of marine invertebrates has been shown to be more complex than suspected (Goad 1978). However, information on the sterol metabolism of shellfish and other marine invertebrates is relatively limited and fragmentary. This paper gives an overview of sterol metabolism in both crustaceans and molluscs from the viewpoint of nutrition.

CRUSTACEANS

Mammals synthesize cholesterol from acetate, mevalonate, squalene, lanosterol, etc. (Nes and McKean 1977). Cholesterol is the exclusively major sterol in crustaceans (Goad 1978) as in mammals (Nes and McKean

1977), but crustaceans are incapable of de novo sterol synthesis (Teshima 1978). For example, no incorporation of [^{14}C] acetate into sterols of the prawn (Penaeus japonicus), crab (Portunus trituberculatus), and spiny lobster (Panulirus japonicus) was found, although fatty acids were labelled (Teshima and Kanazawa 1971). Similar results have been found in a variety of crustaceans (See Teshima 1978 for review). It is therefore likely that crustaceans in general are incapable of de novo sterol synthesis.

Requirement for Cholesterol

Since crustaceans lack the ability for de novo sterol synthesis, they are thought to require sterols as indispensable nutrients for growth. This was confirmed for the prawn (P. japonicus) by feeding trials with a purified diet (Kanazawa et al. 1971). The prawn fed on a sterol-free diet had poor growth and survival rates, but they grew well on a diet containing 0.5% cholesterol. Other workers have also demonstrated the necessity of dietary cholesterol for good growth of P. japonicus (Shudo et al. 1971; Deshimaru and Kuroki 1974), juvenile lobsters (Homarus americanus) (Castell et al. 1975), and the free-swimming crustaceans, Artemia salina, Daphnia magna, and Moina macrocopa (Provasoli 1975). Interestingly, Castell and Covey (1976) have pointed out that a supplement of cholesterol to the diets did not improve growth of adult lobster (H. americanus), 300-600 g in body weight, in contrast to the reported requirements of juveniles (Castell et al. 1975). Castell and Covey's (1976) findings suggest that the cholesterol requirement of lobsters is probably variable with age.

An optimum dietary level of 0.5% cholesterol has been found for P. japonicus (Kanazawa et al. 1971) and H. americanus juveniles (Castell et al. 1975). This value is similar to the level of cholesterol required for insects (Clayton 1964). Other workers, however, have obtained the best growth of P. japonicus with diets containing 0.2% (Shudo et al. 1971) and 2.0% (Deshimaru and Kuroki 1974) cholesterol. These conflicting results with P. japonicus could be due to differences in the composition of the test diets used and also to the probable contamination of dietary ingredients with cholesterol (Shudo et al. 1971).

Quite recently, Teshima et al. (unpublished data) have shown by using purified diets that the survival of P. japonicus larvae was improved by the addition of either 1.0% or 5.0% cholesterol to a sterol-free diet (Figure 1). Interestingly, the addition of 5.0% cholesterol showed an inferior survival rate compared to that with 1.0% cholesterol.

A dietary supplement of phospholipid has been shown to improve the growth of P. japonicus juveniles (Kanazawa et al. 1979) and larvae (Kanazawa, these proceedings). D'Abramo et al. (1981) have also demonstrated that phosphatidylcholine was essential for the survival of juvenile lobsters (H. americanus). Since lipoproteins rich in phospholipids have been found to be involved in lipid transport in P. japonicus (Teshima 1980), it is possible that some phospholipids play an important role in the transport of cholesterol.

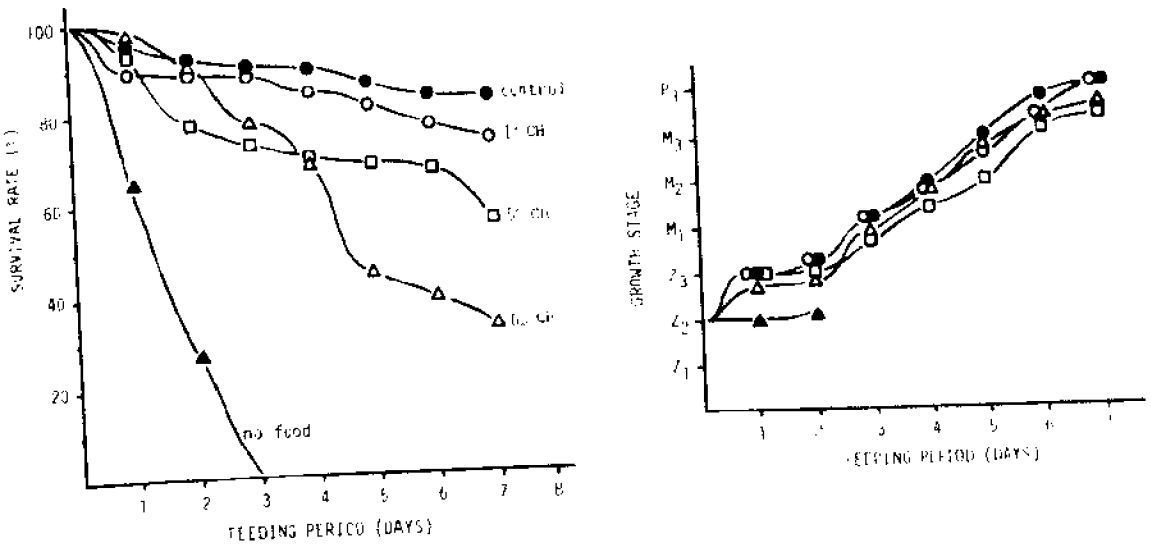

Figure 1. Effects of dietary cholesterol on the growth and survival of Penaeus japonicus larvae (unpublished data). The developmental stages are as follows: Z_1, zoea$_1$; Z_2, zoea$_2$; Z_3, zoea$_3$; M_1, mysis$_1$; M_2, mysis$_2$; M_3, mysis$_3$; P_1, postlarva$_1$. CH indicates cholesterol. The test diets contained kappa-carrageenan (5 g) and other ingredients (100 g); casein 50 g, glucose 5.5 g, sucrose 10.0 g, α-starch 4.0 g, glucosamine HCl 0.8 g, sodium citrate 0.3 g, sodium succinate 0.3 g, minerals 8.5 g, vitamins 3.2 g, pollack liver oil (PLO) 6.0 g, soybean lecithin (SBL) 3.0 g, cholesterol (0, 1.0, or 5.0 g), and cellulose powder (8.4, 7.4, or 3.4 g). Since mixture of PLO and SBL contained 0.5% cholesterol, the basal diet (0.0% CH) contains 0.045% cholesterol in the dietary ingredients (carrageenan not included).

Groups of 100 larvae received a test diet (0.16 mg/larva/day) and were cultured in one-liter beakers with aeration at 27-28°C. The sea water (specific gravity, 1.026) was filtered through a column packed with cotton. The control group was fed on Chaetoceros gracilis (5-10 x 10^4 cells/ml) during zoeal stages and then Artemia salina nauplii (50 individuals/ml) during mysid stages. The survival rates were significantly ($P < 0.05$) different between 5.0% CH group and 1.0% CH or 0% CH group.

Dietary Value of Phytosterol

Cholesterol is often not the predominant sterol of the live food organisms used for crustacean culture. Kanazawa et al. (1971) have shown that survival rates of the prawn (P. japonicus) fed diets containing ergosterol, stigmasterol, or β-sitosterol as substitutes for cholesterol were good, but growth with diets containing these sterols was inferior to that with diets containing cholesterol. This suggests that P. japonicus and probably other crustaceans can utilize some C_{28} and C_{29} sterols by conversion of these sterols to cholesterol. The above assumption has been substantiated with several crustacean species by tracer experiments using radioisotope labelled sterols (see Teshima

1972, 1978 for references). In these experiments, we have shown that Artemia salina converted radioactive ergosterol, 24-methylcholestrol, and brassicasterol to cholesterol. Furthermore, we have revealed the formation of cholesterol from ergosterol by the crab (P. trituberculatus), from β-sitosterol by P. japonicus and P. trituberculatus, and from desmosterol by P. japonicus, the shrimp (Palaemon serratus) and the crab (Sesarma dehaani). From these results, I assume that dealkylation of C_{28} and C_{29} sterols in crustaceans probably proceeds via desmosterol (Figure 2) in a similar sequence to that found in insects (Svoboda et al. 1978). Thus, most crustaceans are likely to dealkylate some phytosterols to cholesterol which is the most effective sterol for their growth and survival.

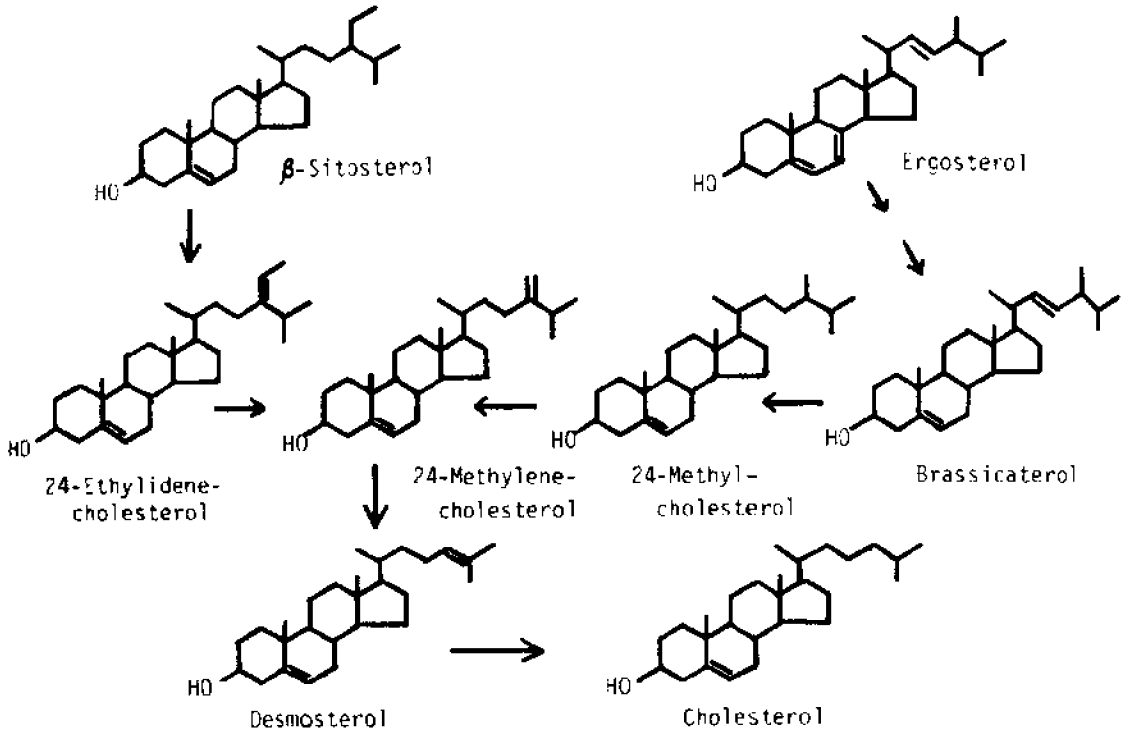

Figure 2. Proposed mechanism for C-24 alkylation of C_{28} and C_{29} sterols in crustaceans (Teshima 1978). C_{27} sterols: cholesterol and desmosterol. C_{28} sterols: ergosterol, brassicasterol, 24-methylcholesterol, and 24-methylenecholesterol. C_{29} sterols: β-sitosterol and 24-ethylidenecholesterol.

Absorption of Dietary Sterols

Since the alimentary tract of mammals absorbs only limited amounts (about 5%) of dietary phytosterols, such as β-sitosterol, and about half of the dietary cholesterol from food (Sugano 1976, 1981), the question arises as to whether dietary sterols, especially C_{28} and C_{29} sterols, are effectively absorbed and assimilated by crustaceans. The prawn (P. japonicus) has been shown to be capable of absorbing both cholesterol (82.6% absorption) and phytosterols such as ergosterol, 24-methylenecholesterol, brassicasterol, fucosterol, and β-sitosterol (77.3 to 98.8% absorption) (Teshima et al. 1974) indicating crustaceans can absorb both cholestrol and phytosterols more effectively than mammals.

For what is known to date, the process of absorption of dietary sterols in crustaceans also differs from that of mammals. The gastric juice of the crab (Cancer pagurus) does not contain bile salts (Van den Oord et al. 1964) but instead fatty acylsarcosyltaurines (Van den Oord et al. 1965) which act as emulsifiers and are formed endogenously from acetate (Van den Oord 1966). Holwerda and Vonk (1973) have also isolated fatty acylsarcosyltaurines from the digestive juices of the crayfish (Astacus leptodactylus) and the lobster (Homarus vulgaris). Lester et al. (1975) have demonstrated that the solubilization of cholesterol was enhanced by N-(N-dodecanoylsarcosyl)taurine, which is representative of the surface active agents synthesized by the crustacean hepatopancreas and secreted into the intestine.

Fate and Physiological Role of Cholesterol

As mentioned above, the sterol requirement of crustaceans is quite unique. However, little is known of the physiological role of sterols in crustaceans. The information available (Teshima 1978) indicates that some species of prawns, crabs, and lobsters convert exogenous cholesterol to cholesteryl esters, steroid hormones (progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone), and molting hormones (ecdysterone and ecdysone). However, we have shown that most exogenous cholesterol remained unchanged as free sterol for 7-10 days after injection or feeding in the spiny lobster (P. japonica) (Teshima 1972) and the crab (S. dehaani) (Teshima et al. 1976). The cholesterol content has also been shown to remain relatively constant during the molting cycle of the shrimp (P. serratus) (Teshima et al. 1975) and during the developmental process of the crab (Callinectes sapidus) (Whitney 1969). There is abundant evidence that free cholesterol is necessary for maintenance of membranous structures in mammals (Nes 1974) and insects (Svoboda et al. 1978). Cholesterol is assumed to be important as a constituent of membranes of cellular and subcellular structures in crustaceans too. However, further studies must be carried out in order to clarify all the functions of cholesterol in crustaceans.

MOLLUSCS

The sterol composition of molluscs varies from class to class (Idler and Wiseman 1972). As a generalization, chitons, the most primitive molluscs belonging to class Amphineura, contain Δ^7-sterols, mainly 7-cholestenol, in contrast to other classes of molluscs which predominantly possess Δ^5-sterols. Gastropods and cephalopods, except for a few species such as the triton (Charonia tritonis) (Teshima et al. 1979a), contain primarily cholesterol with only small amounts of C_{28} and C_{29} sterols. Pelecypods contain complex mixtures of C_{26}, C_{27}, C_{28}, C_{29}, and C_{30} sterols together with cholesterol, and in some species C_{28} and C_{29} sterols are more predominant than cholesterol (Idler and Wiseman 1972).

As compared with our knowledge of Molluscan sterol composition, the information on sterol metabolism in molluscs is still fragmentary and sometimes contradictory (Goad 1978). Figure 3 shows simplified pathways for de novo sterol synthesis and for transformation of dietary sources of sterols in molluscs on the basis of data available to date.

Sterol Metabolism in Chitons and Gastropods

Chitons and gastropods are generally considered to synthesize 7-cholestenol and cholesterol, respectively, from precursors such as acetate, mevalonate etc. (Figure 3). Teshima and Kanazawa (1973a) demonstrated that the chiton (Liolophura japonica) synthesized 7-cholestenol but not cholesterol from [^{14}C] mevalonate. In contrast, Voogt and Van Rheenan (1974) have observed a significant incorporation of radioactivity into 7-cholestenol and to a lesser extent into cholesterol in the chiton (Lepidochitonia cinerea) after administration of either radioactive acetate or mevalonate. Cholesterol, however, is a minor sterol in the tissues of chitons (Teshima and Kanazawa 1973a; Voogt and van Rheenen 1974) and is likely to be mainly derived from dietary sources rather than from de novo synthesis. Teshima and Kanazawa (1978) have demonstrated that L. japonica is able to convert exogenuous [^{14}C] cholesterol to 7-cholestenol in a similar way to that found in starfish (Goad 1978).

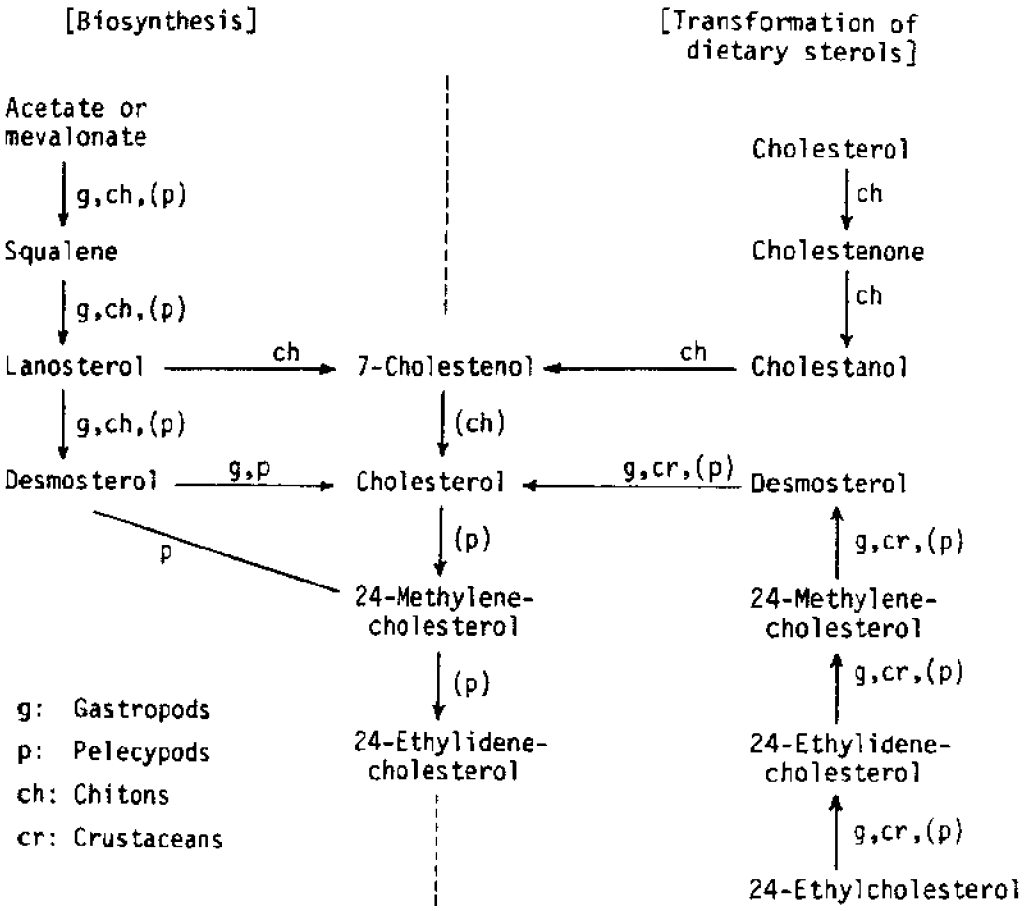

Figure 3. Simplified pathways for both de novo sterol synthesis and transformation of dietary sterols in crustaceans and molluscs. Letters in parentheses imply that data available are conflicting.

Many gastropod species have been shown to possess the ability for de novo synthesis of cholesterol. In general, cholesterol biosynthesis seems to proceed at a rapid rate in gastropods (Goad 1978) except for in the whelk (Buccinum undatum) (Voogt 1972). Gastropods are also probably capable of dealkylating some phytosterols to cholesterol. Collington-Thiennot et al. (1973) have shown that the archaeogastropod Patella vulgata was able to convert [^{3}H]β-sitosterol and [^{3}H]fucosterol to desmosterol and cholesterol. Later, Khalil and Idler (1976) have shown the bioconversion of [^{3}H]-sitosterol and [^{3}H] 24-methylene-cholesterol to cholesterol in the whelk (B. undatum). Teshima et al. (1979b) have also observed the dealkylation of [^{3}H] β-sitosterol to cholesterol in the snail (Omphalius pfeifferi). Gastropods possibly meet their requirements of cholesterol by both de novo synthesis and dealkylation of exogenous phytosterols. It is possible that most gastropods do not require a dietary source of cholesterol for growth, although no feeding experiments have been conducted to test this.

Sterol-Synthesizing Ability of Pelecypods and Cephalopods

In contrast to gastropods and chitins, pelecypods and cephalopods seem to poorly incorporate radioisotope labelled acetate and mevalonate into sterols. However, the information on the sterol-synthesizing abilities of pelecypods and cephalopods is contradictory even for closely related species. For example, some workers have indicated de novo sterol synthesis in the mussels, Mytilus californianus (Fagerlund and Idler 1960) and Mytilus edulis (Teshima and Kanazawa 1973b), the octopus (Octopus vulgaris) (Voogt 1973), and the squid (Sepia officinalis) (Voogt 1973). In contrast, other workers have failed to show the incorporation of radioactive acetate and/or mevalonate into the sterols of M. edulis (Walton and Pennock 1972) and the cephalopods, S. officinalis (Zandee 1967) and Eledon aldovandi (Voogt 1973).

It is not easy to give a satisfactory explanation for the conflicting results on sterol biosynthesis in pelecypods and cephalopods. Both classes of molluscs appear to have the capacity for limited sterol synthesis. Under some conditions, however, de novo sterol synthesis could be suppressed by regulatory enzymes such as hydroxymethyl glutaryl-CoA reductase, because the rate of sterol synthesis in some molluscs may be dependent on dietary sterol intake (Goad 1978; Voogt 1973).

C-24 Alkylation and Dealkylation of Sterols by Pelecypods

There is evidence to suggest that some pelecypods are capable of de novo synthesis of C-24 alkylated sterols from precursors. We have shown that the mussel (M. edulis) incorporated radioactive mevalonate and desmosterol into 24-methylenecholesterol in addition to C_{27} sterols such as cholesterol, desmosterol, and 22-dehydrocholesterol (Teshima and Kanazawa 1973b; Teshima et al. 1979b). Recently, Teshima and Patterson (1981) have also demonstrated the formation of 24-methylenecholesterol and 24-ethylidenecholestrol from radioactive mevalonate and lanosterol in the oyster (Crassostrea virginica). Further evidence for sterol C-24 alkylation has been found in the scallop (Saxidomus giganteus) (Fagerlund and Idler 1961) and in the eulamellibranchs, Anodonta cygnea, Cyprina islandica, Cardium edulis, and Mya arenaria (Voogt 1975).

Saliot and Barbier (1973) have demonstrated that the oyster (Ostrea gryphea) dealkylated [^{3}H] fucosterol to desmosterol and cholesterol. In contrast, we have found that the mussel (M. edulis) synthesized both cholesterol and some C_{28} and C_{29} sterols de novo but was incapable of dealkylating β-sitosterol to cholesterol, and we concluded that the variety of sterols found in M. edulis was probably a reflection of its complex dietary habit and incapability of sterol C-24 dealkylation (Teshima et al. 1975b). Although the occurrence of both C-24 alkylation and deaklylation in oysters is puzzling, the contribution of gut flora together with other factors such as dietary status may be responsible for the conflicting results.

Sterol Requirements of Pelecypods

As mentioned above, biochemical studies have suggested that the sterol metabolism in pelecypods proceeds at a very slow rate. Tamura et al. (1964) and Berenberg and Patterson (1981) have suggested that the sterols of the oyster (C. virginica) are of dietary origin on the basis of sterol analysis of cultured oysters. Recently, Trider and Castell (1980) have studied the effects of dietary lipids and cholesterol on the growth of the oyster (C. virginica) using artificial diets, and have suggested that the oyster requires low levels of cholesterol (0.1-0.2%) for growth. Interestingly, the addition of 1.0% cholesterol to the diet inhibited growth. However, Trider and Castell's work may be criticized since the tested artificial diets were inadequate and did not support an increase in oyster tissue weight. Furthermore, the contribution of sterols from both the lipid portion of the artificial diet and contaminating microorganisms should be considered in evaluating their results, as sterol-free lipids were not used and the seawater was only filtered down to 10 μm.

With respect to the sterol requirements of molluscs, no other feeding trials using artificial diets have been reported.

CONCLUSION

Crustaceans are incapable of synthesizing sterols from either acetate or mevalonate and require a dietary source of sterol for growth and survival. The prawn (P. japonicus) efficiently absorbs cholesterol and phytosterols from food in the gut and also dealkylates some phytosterols to cholesterol, the sterol with the highest nutritive value. Available evidence suggests that the dealkylation of C_{28} and C_{29} sterols to cholesterol in crustaceans takes place in a similar way to that established for insects.

The sterol metabolism of molluscs differs between classes. Chitons can synthesize 7-cholestenol from low molecular weight precursors and also transform exogenous cholesterol to 7-cholestenol. Gastropods synthesize cholesterol de novo and can also dealkylate phytosterols to cholesterol. Pelecypods are capable of limited synthesis of cholesterol and some C_{28} and C_{29} sterols. However, sterol metabolism in pelecypods seems to proceed at a slow rate. Accordingly, pelecypods are likely to require dietary sources of sterols for growth and survival.

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THE POTENTIAL FOR HORMONE MANIPULATION IN SHELLFISH AQUACULTURE

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ABSTRACT

Two procedures in limited use today in aquaculture involve the alteration of an organism's hormonal balance to benefit the mass culturing of the species. These are (1) the unilateral removal of an eyestalk from mature female prawns which reduces the inhibitory hormone and allows the stimulatory hormone to begin or enhance ovarian development and (2) the treatment of mature male or female bivalves with a weak solution of hydrogen peroxide which mimics the afferent signals causing a neuromuscular response in the ovotestes resulting in spawning.

There are other producers which could be of value in aquaculture if additional research resources were allocated to their development: (1) somatic growth stimulation - the removal of one or both eyestalks with the injection of a synthetic molting hormone into late juvenile crustaceans; (2) ovarian growth stimulation - the characterization and synthesis of the ovarian stimulating hormones from gastropods and cephalopods; (3) sex ratio alteration - the characterization and synthesis of the androgenic hormone from crustaceans and the cerebral ganglion hormone from gastropods; (4) induction of settling and metamorphosis - the use of plant products such as the biologically active α-aminobutyric acid (GABA).

KEYWORDS: invertebrate endocrinology, hormone therapy, eyestalk removal, androgenic hormone, ovarian stimulating hormone, molting hormone.

INTRODUCTION

There is a substantial body of knowledge in invertebrate endocrinology (Golding 1974; Boer and Joosse 1975; Highnam and Hill 1977; Barrington 1979; Goldsworthy et al. 1981) which may be of value in resolving problems in the cultivation of selected shellfish species (Reay 1979; Kinne 1980; Kutty 1980). Most of these problems are nutritional and/or environmental but some may partially be solved by altering the physiology of the organism through the manipulation of the endocrine system.

Aquaculturists have only to look to agriculturists to see how helpful hormone therapy or manipulations can be to the economic success of farming. Animal hormones are used in agriculture to synchronize ovulation as well as gestation in cattle, sheep, and swine; to increase the ratio of protein to body weight in cattle; and to initiate the lactation process, thus bypassing pregnancy in cattle (McCann 1974; Barrington 1975). These procedures have shown a clear advantage in the cost-benefit analyses of livestock operations.

In the cultivation of fish, hormones are playing an ever-increasing role (Stickney 1979). The induction of spawning in carp follows the injection of a synthesized steroid (Jalabent et al. 1977). Increased protein synthesis and improved food conversion in trout can be achieved by the administration of oral steroids (Matty and Cheema 1978) and significant body weight increase can follow the administration of insulin (Ablett et al. 1981). Sex ratios in salmon and trout can be significantly altered from the 1:1 expected ratio by treatment with androgens and/or estrogens (Goetz et al. 1979).

These examples of hormone therapy are now possible because of years of basic biological investigations. From these studies has come the confirmation that certain endocrinological principles exist in the animal kingdom (Barrington 1979), and in specific cases intervention in the hormone balance has proven beneficial to agriculture and to the fish hatchery industry (Stickney 1979). There is every reason to suspect that similar manipulations can be employed in the culturing of invertebrate species. Very few therapies are in use for shellfish; others are the subject of intense research, but the potential for future economic gain through hormone therapy is enormous.

In each physiological process the endocrine system plays an integrating role both in responding to the external environmental conditions and in programming the organism's long-term response so that growth will proceed and reproduction will be successfully completed (Aiken 1969; Giese and Pearse 1974; Goldsworthy et al. 1981). A major objective in aquaculture research is to control the entire life cycle in order that a constant and predictable supply of eggs and larvae of the chosen species will be available for cultivation. To accomplish this, the aquaculturalist must provide satisfactory conditions in the rearing area in order to ensure growth and reproduction. If such conditions are too difficult or expensive, the effect of the available environmental regime may be modified by a hormone treatment appropriate for the induction of growth or reproductive development. Such control over the organism is precisely why agriculturalists raising cattle, swine, or poultry have, for many years, been interested in the vertebrate's endocrine system and why aquaculturalists have recently become similarly interested in endocrinology.

It is my purpose to examine briefly four physiological processes in both the Crustacea and Mollusca: somatic growth and development, sex differentiation, gonadal development, and spawning. In each of these areas, I will review the generalized endocrine mechanisms and comment on how hormones are now being manipulated or possibly could be manipulated to facilitate the cultivation of the organism. Many of the mechanisms are still hypothetical, and their experimental proof has yet to be recorded. The emphasis of this paper is on the potential for aquacultural utilization of chemical regulators, not on the endocrine mechanisms themselves.

Somatic Growth and Development - There are two major hormones which control growth, the postulated molt-inhibiting hormone (MIH), presumably produced and released in the eyestalk, and the molting hormone (MH), synthesized and released to the blood from the thoracic ecdysial glands (Kleinholz 1976; Aiken 1980). The MH is believed to express its potentiality when the balance between the levels of the two hormones swings in its favor. Until that occurs, the inhibiting hormone retards the initiation of the molt cycle. If growth in body size and weight results from a series of molts when diet and environment are satisfactory, it is only logical to suggest that if the frequency of molts can be increased, then under similar conditions the size of the animal would increase more rapidly - a goal which is compatible with those of aquaculture. This assumption has been tested directly and indirectly with the injection of a synthetic molting hormone separately and in conjunction with the surgical removal of the eyestalks. Limited success has been obtained with the American lobster, Homarus americanus (Rao et al. 1973; Aiken and Waddy 1976). The reported heavy mortality may have been the result of a poor hormone delivery system, the inherent toxicity of the hormone, or the ablation technique used. The removal of eyestalks need not be an all-or-nothing procedure; unilateral removal should be tested in conjunction with hormone injections which vary in concentration, time of injection, and frequency of injection.

The ablation technique may find its greatest utility in producing at least one molt in commercially captured lobsters before they are sold to consumers. This final molt has been reported to produce a 50% increase in weight which could be sufficient to justify the cost of the procedure (Castell et al. 1977). However, the quality of the weight gain must be determined since it could largely be due to increased water uptake and not to muscle growth.

Sex differentiation - It has been shown for fish that when young coho salmon are immersed in or fed solutions of an estradiol or a testosterone, the developing young will demonstrate altered sex characteristics (Goetz et al. 1979; Hopkins et al. 1979). This is of interest to the cultured fish industry since a single sex population of some species might have high aquacultural potential simply because of better economics in nutrient conversion or in customer preference. Crustacea are similar to vertebrates in that the structural and functional expression of sexual differentiation is under hormonal control (Yamamoto 1975). The androgenic gland and its product, the androgenic hormone (AH), is believed to determine male sexual characteristics (Charniaux-Cotton 1960). In genetic females, the androgenic gland is not developed; in genetic males, the gland develops. If the gland is removed from young male Macrobrachium rosenbergii, female characteristics will develop with the eventual production of oocytes (Nagamine et al. 1980a). If the gland from a young male is transplanted into a young female, the germ cells will produce spermatocytes rather than oocytes (Nagamine et al. 1980b).

Conceivably, if AH was available commercially, it could be used in the cultivation of a marketable species with the production of only males or an intersexual population. The degree of maleness would possibly be a function of the hormone concentration and therapy duration, and the degree of maleness or femaleness would be chosen on the basis of its market appeal. The marketed mature organisms need not

be fertile adults since they would not be part of the brood-stock.

Gonadal development - In juvenile Crustacea, cyclic somatic growth and development generally take precedence over gonadal development; and in mature individuals, the reverse is apparent. Evidence for this comes from experiments where eyestalks are removed; when this occurs in juveniles, they prepare to molt and in mature organisms they prepare for spawning (Adiyodi and Adiyodi 1970; Webb 1977). The results of such experiments suggest that there is a gonadal - inhibiting hormone (GIH) as well as a molt-inhibiting hormone (MIH) released from the eyestalk sinus gland. Similar experiments also suggest that there is an ovarian-stimulating hormone (OSH) released into the blood after the GIH is removed. These two (GIH, OSH) are probably the major hormones governing gonadal development (Aiken and Waddy 1980).

Some of the most exciting current research involves unilateral eyestalk ablation of sexually mature female prawns. Arnstein and Beard (1975) found that with Penaeus orientalis the removal of only one eyestalk was sufficient to induce gonadal development whereas high mortality resulted from the removal of both eyestalks. Santiago (1977), reporting on his studies in the Philippines, and Aquacop (1977), reporting on work in Polynesia, were among the first to successfully obtain viable eggs from Penaeus monodon through a unilateral ablation procedure. The F_1 progeny in both latter studies appeared to be normal and fully capable of growing to maturity. These two reports are among the first to record successful completion of the life cycle of P. monodon in captivity; this experiment has been repeated by Primavera (1978), and Beard and Wickins (1980).

Lumare (1979) used similar techniques with Penaeus kerathurus in Italy and obtained spawning in 94% of the females treated. The average period of time between ablation and spawning for females treated in November was 69 days but was only 10 days in females treated in May or June. Similar results have recently been obtained with P. setiferus (Lawrence et al. 1980) and P. plebejus (Kelemec and Smith 1980). Further research on penaeids must be pursued vigorously to determine the optimal time and environmental conditions for this procedure.

The procedure of unilateral eyestalk removal or enucleation is successful because apparently the concentration in the blood of GIH hormone is reduced to a level low enough to allow OSH to promote maturation and the subsequent stage of spawning. In comparison with bilateral surgery, the unilateral procedure has less impact on feeding and social behavior of the animal, and there is also a reduced chance of blood loss and infection. The economic benefits of this procedure which would provide an adequate and continuous supply of uniformly aged spawners to an aquaculture industry are obvious.

Spawning - At present, it is not possible to distinguish in Crustacea between the hormonal control mechanisms for gonadal development and those for spawning. The control mechanism for the release of the mature ova from the female may involve the presence of a stimulatory hormone as in echinoderms and molluscs, or the absence of an inhibitory hormone as in echinoderms and polychaetes, or involve a simple neuromuscular reflex with a stretch receptor initiating the action, a mechanism common in insects (Goldsworthy et al. 1981). Thermal shocks have been used indirectly to induce spawning in mature Penaeus (Lumare 1976). Such procedures do not support either a hormonal

or neuromuscular mechanism but simply show that an environmental shock can mimic or trigger a physiological afferent signal to the neuroendocrine system which then initiates the spawning process.

Being able to predict when spawning will occur and having control over that reproductive process through the interplay of inhibitory or stimulatory synthetic hormones would be of great value to an aquaculture operation. Until we identify, characterize, and synthesize the crustacean hormones and describe more completely the physiology of reproduction in these shellfish, it seems likely that the mechanism controlling gonadal development in some way also controls spawning.

MOLLUSCA

Somatic growth and development - Experimentally verifiable data concerning the hormonal control of growth and development among molluscs are available but limited. In the commercially important bivalves growth is a gradual, temperature-dependent, non-cyclical process which eliminates the necessity for hormonal regulation of a complex biological cycle, as in arthropods. An example of our limited knowledge is illustrated by the fresh-water snail Lymnaea stagnalis where one or more neurosecretions controlling somatic growth are believed to originte in cells imbedded within the cerebral ganglion (Geraerts 1976). That location presents a difficult task to endocrinologists who would like to remove the hormone source for experimental studies without sacrificing the animal as can be done so simply by cutting off eyestalks in crustaceans.

Recent studies of Morse et al. (1979, 1980) have shown that α-aminobutyric acid (GABA), produced by the crustose coralline red algae, serves in nature as a very potent and specific inducer of behavioral and developmental metamorphosis in red abalone (Haliotis rufescens) larvae. Without this amino acid, or one of its homologs, laboratory cultures the larvae do not settle nor do they enter metamorphosis. This is an elegant example of the control of animal behavior and subsequent morphological development, not by an intrinsically produced hormone diffusing through the bloodstream, but by an extrinsically produced, biologically active compound diffusing through the external environment. There are interesting evolutionary facets to this finding since GABA is also a biologically active compound in animals, and a neurotransmitter in vertebrate and invertebrate nervous systems. The potential of such a compound in the mass culturing of marine gastropods is clearly evident.

Speaking as in invertebrate physiologist familiar with the problems facing aquaculturalists, I do not believe that the unraveling of the hormonal control of growth is where research resources should be directed. The development of adequate nutrition for the veligers and juvenile bivalves in a closed system is vastly more important, and that effort will bring greater returns. Endocrinology research will follow where adequate nutrition is available to support an experimental population.

Sex differentiation - In isolation, the gonads of many gastropods differentiate into ovaries, as the presence of a hormone released from the cerebral ganglion is required for testes formation (Choquet 1965). This is similar to the crustaceans where the androgenic gland

synthesizes and releases a masculinizing factor, and in its absence the gonads become ovaries. Very little work has been done on the control of sex differentiation of bivalves. Possibly the only paper is that of Mori et al. (1969) in which they showed that estradiol-17β was active in reversing the sex of a significant number of oysters from male to female. If this is an effective hormonal control mechanism for bivalves, then there may be the opportunity to influence sex ratios in molluscs under closed culture conditions. The synthesized steroid hormone could be incorporated into food or into the circulating water.

As was stated in the discussion on crustaceans, the question would then become one of economics. Would there be greater appeal to the culturist for all males, all females, or for intersexed organisms, and would the benefits outweigh the cost of production? I believe that there is a good probability that the answer to the latter question would be affirmative. This is an ideal area for industry to support research in molluscan reproductive endocrinology.

Gonadal developement - Once sex has been determined in the developing gastropods, the production of large numbers of oocytes and their maturation is under the control of a hormone produced by the dorsal bodies. When these bodies of neurosecretory cells are removed, vitellogensis is inhibited; when they are reimplanted, yolk deposition returns to normal (Joosse 1972). A similar pattern exists in the cephalopods where a hormone from the optic glands influences yolk deposition by acting on the follicle cells which surround the oocytes (Wells and Wells 1975). Unfortunately, there is no information concerning the hormonal control of gonadal development in the economically important bivalves; we must assume that the suggested mechanisms for gastropods and cephalopods are appropriate also for the bivalve molluscs. If this vitellogenic hormone, which is functionally analogous to the ovarian-stimulating hormone of crustaceans, were available, it could be tested for its effects in speeding the maturation process of female bivalves of the brood-stock.

Spawning - In contrast, to the lack of information on the endocrinology of spawning in Crustacea, there has been some very exciting work conducted on molluscs. A water extract of the bag-cells of Aplysia californica, an opisthobranch, will produce spawning when injected into another sea hare. These cells are neurosecretory, located in a peripheral ganglion, and the extracted hormone is almost certainly a peptide (Arch et al. 1976; Dudek et al. 1980). The primary function of the hormone is to cause the contraction of muscles surrounding the ovotestis, forcing the ripe oocytes and sperm to the outside. Stimulli received during copulation are the signals which cause the hormone to be released into the blood. In a similar study, Gwyther and Munro (1981) induced spawning in the giant (Tridacna maxima) with water extracts of freeze-dried gonads. Most likely, they preserved a biologically active peptide or protein which acted as a prostaglandin.

Morse et al. (1977) reported on the use of a solution of hydrogen peroxide to initiate the intrinsic neurophysiological response necessary to stimulate a number of cultured species to spawn successfully. This procedure seems to be simpler, less expensive, faster, and more reliable than any other means for the induction of spawning in both males and females. In a similar vein, abalone can be made to spawn by bathing the mature adults for an hour in U.V. irradiated sea water which produces H_2O_2 (Kan-no 1976). In such cases, the exact mechanism for the induction

of spawning is not known, but a prostaglandin-like compound may be involved. The application of this hydrogen peroxide procedure to shellfish mariculture could be exceedingly beneficial. This would allow the selected breeders to spawn together to produce a high percentage of fertilized zygotes.

CONCLUSIONS

There are many opportunities for improving the efficiency of shellfish aquaculture through the manipulation of the organism's endocrine balance. In this paper, I have indicated some procedures that are in limited use and have identified others that need additional research. Table 1 summarizes the procedures for the four physiological systems and the two taxonomic groups. The ability to alter a species' hormonal balance and thereby its growth, development and reproduction through external manipulations can provide an advantage to the aquaculturist. As this meeting has shown, there will be a continuing association between the culturists and the physiologists in reaching common objectives.

Table 1. Examples of Hormonal Manipulations in Shellfish Which Are in Limited Use or Are Being Studied

	Crustacea	Mollusca
Somatic growth	Eyestalk Removal (MIH) → MH Injections → Additional Molt	GABA → Settling, Metamorphosis
Sex differentiation	AH → Maleness	Estradiol → Feminization
Gonadal development	Unilateral Eyestalk Removal (GIH) → Vitellogenesis	Optic Gland Hormone → Vitellogenesis
Spawning	———	H_2O_2 → Release Bag-Cell Hormone → Release

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QUESTIONS AND ANSWERS

ELLIS (Ohio): I was wondering if the removal of one eyestalk in a crustacean might lead to compensatory hypertrophy of the other remaining eyestalk as you might find in a lateral ovariectomized rat?

TOMBES: Some increase in gland size may occur, but I am not aware of any data to support that idea. The expected response in crustaceans is the regeneration of the removed eyestalk and associated endocrine tissues.

ARSANOFF (Halifax): In response to the first question, I would think that hypertrophy would occur simply because there is a need for the various neural hormones that the x-organ and sinus gland produces.

TOMBES: You may be correct.

ARSANOFF: I would like to make a comment in regard to control mechanisms for the x-organ sinus gland complex. You just mentioned the eyestalk but what controls the production of the molt-inhibiting hormone and the other neural hormones? Within axons that extend from the x-organ to the sinus gland are synaptic contacts containing biogenic amines. These, in turn, control the release and possible production of the neural hormones. Therefore, the control mechanisms which I think are most important utilize biogenic amines extensively.

KITTREDGE (U. of So. CA): Steve Coon, a graduate student with the University of Maryland, recently told me about using the biogenic amine DOPA to induce a fixed action potential in bivalve larvae to hasten their settling. I think that is an example of what you just said.

ARSANOFF: All I wanted to mention was the connection between the neurotransmitters of the nervous system and the endocrine system.

TOMBES: I could say a lot about that. We used to ignore the biogenic amines in a discussion of endocrine mechanisms but now we know just how important they are to the various control mechanisms.

ARSANOFF: That is exactly correct.

MOLLUSCAN NUTRITION

ASPECTS OF BIVALVE FEEDING AND DIGESTION RELEVANT TO AQUACULTURE NUTRITION

by

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ABSTRACT

Bivalves have been reported to be continuous, rhythmic, irregular or opportunistic in feeding behavior. Feeding stimuli may include tidal flow, the presence of food, and the condition of the digestive system. Mechanical stimuli appear to predominate over chemical ones. A variety of pallial, ciliary organs with putative mechanosensory function are revealed by the scanning electron microscope. At present the feeding behavior of bivalves must be assessed species by species.

Within the category of commercially significant suspension feeders there is a range of discrimination of food particle size and density, Ostreidae, Mytilidae and Pectinidae being more selective than burrowing clams. Three functionally distinct stomach types occur in this category. Consequently all such suspension feeders under aquaculture may have specific requirements of food particle quality as well as feeding regime.

Studies of gastric digestive processes reveal in addition to style ammylase, enzymes such as trypsin, cathepsin B, exopeptidases, esterases and phosphatases. These are secreted in response to ingestion of food. Gastric enzyme levels, which are low in comparison with intracellular levels, are sufficient to lyse cell membranes, penetrate cell walls, and partially solubilise the dietary organisms, but digestion is largely completed intracellularly. The bivalve mid-gut possesses membrane-associated esterases, phosphatases and amino-peptidases, indicating a coupled digestive-absorptive process. Digestion may follow a rhythmic tidal or diurnal cycle, though not all digestive tissues are completely in phase. Some diverticular autophagy occurs during starvation; cathepsin D increases and lipid depots are mobilized.

Bacteria associated with bivalves may contribute to nutrition as food, as providers of soluble nutritive molecules for absorption by the gills or the gut, or as contributors to digestion. While the crystalline style has some antibiotic qualities, some bacteria, especially *Cristispira*, abound in the bivalve gut, and intracellular bacteria have been located.

Two bivalve genera prove the rules for the rest, *Tridacna* and

Solemya. Tridacna gigas is nutritionally dependent on its symbiotic zooxanthellae, but also feeds on suspended phytoplankton, reef detritus and coral exudates. Solemya reidi is a gutless protobranch. The clarification of its nutritive economy sheds some light on non-digestive nutritive processes in bivalves with normal alimentary tracts.

KEY WORDS: bivalve feeding; digestion; particle-retention; mantle cavity; stomach; crystalline style; digestive diverticula; bacteria; symbioses.

INTRODUCTION

In the aquaculture of bivalves it is necessary to know the food preference and feeding behavior of the organisms as well as knowing how the alimentary system utilizes the food. Since the beginning of the century our knowledge of bivalves in their natural environment has been growing. However, in order to meet the requirements of aquaculture in controlled conditions, more detailed knowledge of how bivalves function as individuals and as species is required. The fundamental causes and relationships within the alimentary system need to be understood, so that satisfaction of basic needs can be planned, and the unexpected dealt with before it becomes unmanageable. This paper will attempt to describe the fundamental elements of alimentation in the Bivalvia, and especially in those species which are commercially important.

FEEDING BEHAVIOR

The original portrayal of bivalves as continuous feeders set forth by C.M. Yonge in his seminal work on Mya arenaria and Ostrea edulis was based partly on observation, and partly inferred from the characteristics of the crystalline style (Yonge 1923, 1926). It was, of course, recognized that the continuity was interrupted in intertidal animals by the ebb and flow of the tide. One extreme case, Lasaea rubra, which is submerged in seawater for only brief periods, and possesses as a consequence a distinct digestive cycle, was originally thought to be exceptional (Ballantine and Morton 1956). Subsequent work by B.S. Morton (1970) on Cardium edule suggested that a discontinuous, rhythmic feeding behavior resulted in a distinctive four-phase digestive cycle, and that this cyclic activity might be more typical than exceptional, a view that was supported by Purchon (1971). Discontinuity in feeding behavior and variability in feeding rate has now been confirmed for many species of bivalve, with differences of opinion being expressed with regard to the periodicity of the activity, whether it be endogenously or exogenously rhythmic, or whether it be irregular and opportunistic (Morton 1973; Langton and Gabbott 1974; Mathers 1976; Mathers et al. 1979; Palmer 1980a; Robinson and Langton 1980). Palmer (1980b) has suggested that the function of discontinuity in feeding behavior is the provision of a continuity in the digestive process. Robinson and Langton (1980) suggest that efficiency of digestion is the goal of modifications of feeding behavior. The only safe conclusion is that feeding is not universally continuous, nor of constant rate. Therefore, in the design of feeding regimes for bivalves in aquaculture, each species must be individually assessed in order to maximize food utilization, providing food when the animals are in a receptive

condition and minimizing waste, particularly since the latter problem can be compounded by contamination of the system with undesirable microorganisms. Such regimes must also take the consequent digestive processes into consideration. These consequences are discussed below.

There are a number of possible conditions for the initiation of feeding behavior. The first and most obvious is a deprived condition, due to tidal exposure or to the lack of food. Commencement of respiratory activity after a period of valve closure brings suspended material to the filtration organs. The material might provide a mechanical or a chemical stimulus detected by the gills or labial palps, resulting in more rapid feeding or rejection of the material as pseudofeces. Despite isolated reports concerning the ability of pallial or organs to discriminate on the basis of taste (e.g., Loosanoff 1949), there is little evidence to support the argument that pallial chemosensation is the primary factor in the acceptance or rejection of filtered particles. The osphradium, an organ present in many bivalves, and associated with chemoreception in Gastropoda, has never been shown to possess this faculty in Bivalvia (Bayne et al. 1976). Kraemer (1981) studying the osphradium of fresh water bivalves suggests the functions of the organ may be related to exhalant current flow, adduction of the valves, and it may also be light sensitive. A variety of pallial responses to mechanical stimuli have been demonstrated. Bernard (1974) has suggested that distasteful chemicals, such as hydrogen sulphide associated with the sulphur bacterium Chromatium warmingii, might result in a generalized rejectory mucous secretion, but only if these are in dense suspensions. The carnivorous Cuspidariidae possess a number of siphonal mechanoreceptors, ciliated organs which are responsive to low-frequency vibrations, including the turbulence created by the passage of large particles in their vicinity (Reid and Reid 1974; Reid and Crosby 1980). Similar, though smaller organs have been found on the mantle edges and siphonal tips of all bivalve species investigated by the author, using a scanning electron microscope. A variety of ciliated organs are also found on the inner surfaces of the inhalant siphon of many bivalves. These may have the function of detecting potential food or general turbidity in the respiratory currents. The role of particulate organic material in the diet and the relationship between feeding rates and density of suspended material is discussed by Newell in these proceedings.

PALLIAL ACTIVITY

Despite fifty years of study, the function of the pallial organs in bivalves is still not fully understood, especially with regard to the mechanism and biochemistry of mucous secretion. Orton (1912) originally suggested that mucous secretion was a local response to particles coming in contact with the gill filaments. MacGinitie (1941) postulated that a general mucus sheet secreted by the gills and hypobranchial gland was moved down the gill surface to form food strings at the marginal food grooves. Pasteels (1968) suggested that mucocytes in the ctenidia respond with a thick, local secretion to particles of unsuitable size and density resulting in their rejection as pseudofeces, while a thin continuous mucous layer secreted by other epithelial cells of the filament surfaces constituted the food collecting layer. In Solemya reidi a copious mucus secretion from the hypobranchial gland contributes

to the formation of pseudofeces (Reid 1981). Bernard (1974), studying Crassostrea gigas, agreed with Pasteel's hypothesis, noting that physical contact between dense particle and single gill filament was not an effective stimulus, but contact with two or more filaments stimulated a heavy mucous secretion.

With regard to the size range of accepted particles, the upper limits can be determined by an examination of gastric contents. The largest dense particles, 300 - 400 μm in diameter, are found in clams burrowing in sand, e.g., Tivela stultorum (Coe 1947) and Macoma secta (Reid and Reid 1969). I have found that other eulamellibranchiate clams (e.g., Tresus capax and Tridacna gigas) accept dense particles in the same upper size range. There is, however, a considerable range in the upper size limits of dense particles that will be ingested. Macoma calcarea rarely accepts dense particles larger than 10 μm (Reid and Reid 1969). Bernard (1974) calculated that in Crassostrea gigas the preliminary rejection of dense particles more than 14 μm in diamater is largely a consequence of their settlement in response to gill filament stimulation.

The efficiency with which smaller particles are retained by the gills differs from one species to another. Vahl (1972a) found that Mytilus edulis retained 80% of 2 μm particles but below that size there was a rapid decrease in efficiency or retention. In Chlamys opercularis 70% of 7 μm particles were retained, but smaller particles were largely lost (Vahl 1972b). Hughes (1969) found that Scrobicularia retained 100% of 4 μm particles. Haven and Morales-Almo (1970) found that Crassostrea virginica retained 100% of 3 μm particles but that there was also significant retention in the 1-3 μm size range. Foster-Smith (1975a) found that Cerastoderma edule, Venerupis pullastra, and Mytilus edulis showed no size selection for graphite particles in the tested range of ca 5 μm - 120 μm. He also found no discrimination between alumina particles (ca 7.5 μm - 31.5 μm) and the alga Phaeodactylum tricornutum (ca 29 μm) despite the fact that the alumina particles and Phaeodactylum cells were of different density and chemical composition. A more comprehensive bibliography on particle size retention and selection has been provided by Bayne et al. (1976).

Once the food strings have been formed in the marginal and dorsal food grooves of the gills they are passed anteriorly to the oral groove which leads to the mouth and is bounded dorsally and ventrally by the labial palps (Fig. 1). A sorting function is usually attributed to the labial palps of bivalves. This may be correct with respect to experimental conditions where the animal has been dissected, the labial palps separated, and particles dropped onto the inner palp surfaces. The relevance of such observations to natural conditions is debatable. Some bivalves, especially the nucleid protobranchs, are able to use their palp lamellae as collecting and sorting organs under natural conditions (Stasek 1961). However, in most bivalves the bulk of material which comes into contact with the palps is in the form of mucous-bound food strings from the ctenidia, and a number of authors have concluded that the most significant function of the palps is the diversion of excess food strings from the mouth region to the mantle surface as pseudofeces (Gilmour 1964; Bernard 1974; Reid and Porteous 1980). Bernard (1974) observed, by means of a cystoscope inserted between the valves of an undissected Crassostrea, that the palps are always closely appressed, and has concluded that the major function is

the regulation of the amount of mucous-bound food entering the oral groove. Foster-Smith, who has revived MacGinitie's valve-window technique (MacGinitie 1941; Foster-Smith 1975b and 1978) has added another dimension to studies of the functional morphology of the bivalve pallium, by observing changes in activity related to changes in turbidity. In very turbid conditions, where thick mucous-bound food strings are formed, there is a high degree of indiscriminate rejection from ctenidia and palps. Alternatively, if turbidity is low all particles within the acceptable size range are accepted. The rejectory behavior of the labial palps can become acceptance behavior by muscular alteration of the disposition of rejection and acceptance tracts (Foster-Smith 1978).

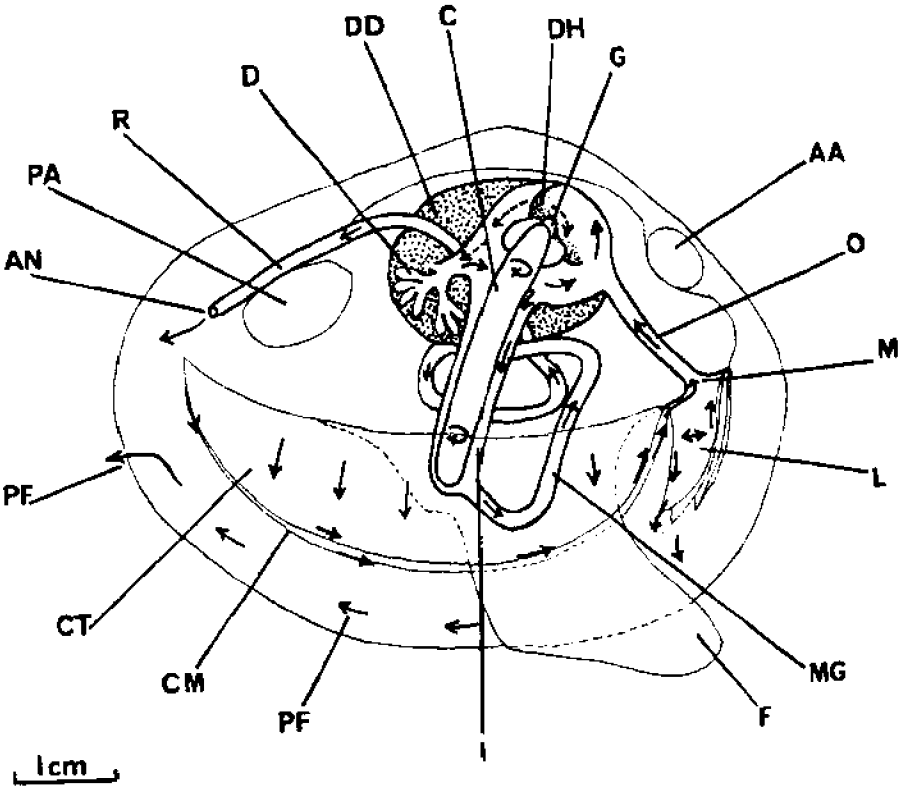

Figure 1. The bivalve alimentary system. The dimensions of the digestive tract have been exaggerated, and the gill ciliary currents simplified, for the sake of clarity. AA = anterior adductor muscle; AN = anus; C = crystalline style; CM = marginal groove of ctenidium; CT = ctenidium; D = digestive tubule; DD = digestive diverticula; DH = dorsal hood; F = foot; G = gastric shield; I = intestinal groove; L = labial palp; M = mouth; MG = mid gut; O = oesophagus; PA = posterior adductor muscle; PF = movement of pseudofaeces on mantle; R = rectum.

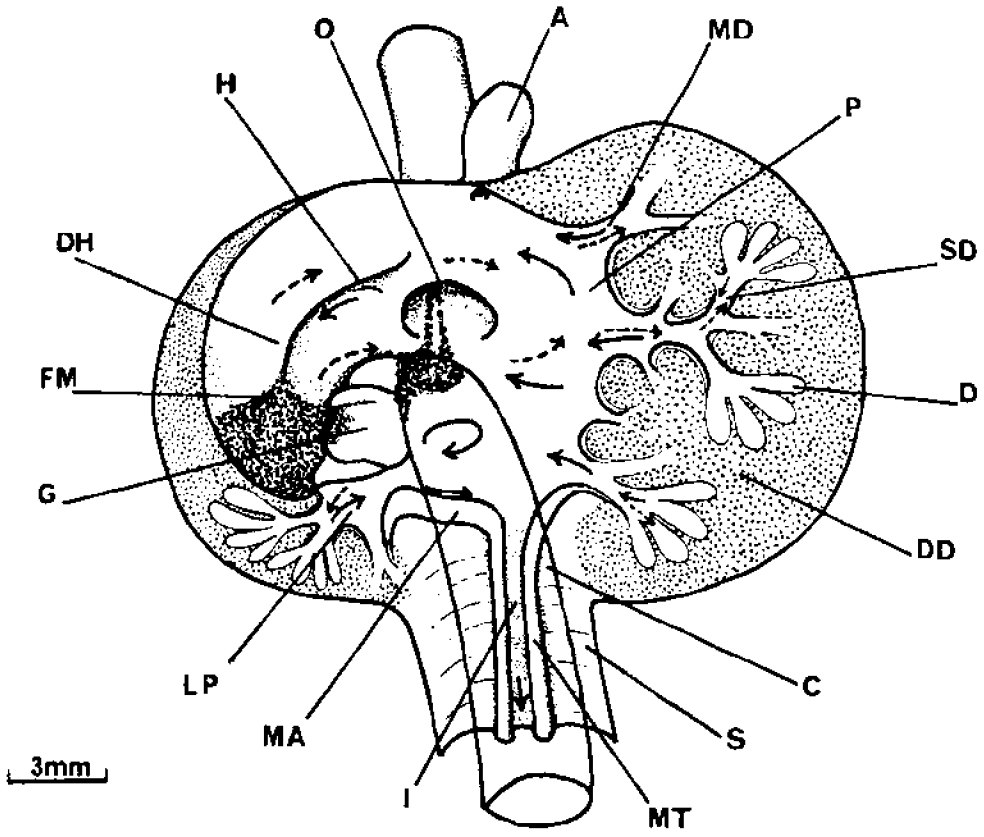

Figure 2. The bivalve stomach. This generalized diagram displays a stomach as if the dorsal wall had been removed and the dorsal hood area spread out slightly. Arrows indicate direct ciliary currents; broken arrows indicate counter currents. A = appendix; C = crystalline style; D = digestive tubule; DD = digestive diverticula; DH = dorsal hood; FM = food-mass; G = gastric shield; H = hood groove; I = intestinal groove; LP = left pouch; MA = major typhlosole; MD = main duct; MT = minor typhlosole; O = oesophagus; P = posterior sorting area; S = style sac; SD = secondary duct.

GASTRIC FUNCTIONAL MORPHOLOGY

The gastric sorting functions of bivalves are the most complex in the animal kingdom. Purchon has described five basic stomach types of which three are found in commercially significant bivalves, namely types III, IV and V (Purchon 1957, 1958, 1960). The softening of the mucous food strings due to the pH of the gastric juice, and their disintegration caused by mechanical contact with the head of the crystalline style which rotates against the gastric shield, releases small particles. These are kept in suspension by a turbulent ciliary circulation of the gastric juice (Figure 2). Larger mucous masses and large dense particles come in contact with the stomach walls by gravity of centrifugal force and are subjected to a variety of sorting mechanisms (Reid 1965). The lightest masses are recycled back to the dorsal hood. In type III stomachs (e.g., Mytilidae, Ostreidae) a sorting caecum removes small dense particles. The sorting function of

the caecum is therefore separate from the gastric digestive and absorptive functions. In type IV stomachs (e.g., Pectinidae) a recycling process retains all but the very small dense particles and therefore increases the effect of gastric digestion on ingested food, to the extent that large dense indigestible particles cannot be easily rejected, and are stored in a gastric appendix. From this sac they are possibly rejected periodically by convulsive muscular movements of the adjacent adductor muscle. In the type V stomach (e.g. Mactridae, Veneridae) there is a combined sorting and absorptive caecum, the duct caecum, which removes small dense particles and permits the uptake of gastric fluids by the ducts of the digestive diverticula by a ciliary counter-current (Owen 1955; Reid 1965). The large posterior sorting area of the type IV stomach has wide rejectory grooves and large dense particles released from incoming food strings are immediately rejected. In order that gastric function can continue the stomach must not become clogged with food. Excess particulate material may be indiscriminately eliminated by means of a muscular widening of the rejectory grooves and a peristaltic movement of the whole stomach. Several observers have noted that undigested and sometimes live algal cells may be found in the feces under such conditions (Bayne et al. 1976). Consequently gastric function must be considered in the planning of optimal feeding levels in maricultural systems.

The crystalline style has dominated most discussions of gastric digestion. The functional role of this unique structure is not altogether clear. Its role as a releaser of α-amylase is well known. The suggestion that the pH of the crystalline style buffers the gastric pH at a stable level (Yonge 1925) has been shown to be in error by Mathers (1974) who demonstrated that in Ostrea edulis gastric pH lies between pH 5.8 and 7.2, and in Crassostrea gigas between pH 5.9 and 6.9. Less well known are the antibiotic and emulsification qualities of the crystalline style (Kristensen 1972a). The digestive role of the crystalline style may have been exaggerated in some species (Kristensen 1972b). Low levels of phosphatase, esterase and proteinase activity have been found in some crystalline styles, probably originating from the gastric juice (Reid 1966). There is a correlation between style consistency and presumably ultrastructure, and the type of material found in the stomach. The crystalline styles of bivalves which ingest sand grains are stiffer and harder than those of other bivalves. This suggests that the physical integrity of the structure is important. Therefore, the mechanical role of mixing the stomach contents, or even trituration of incoming food strings as Yonge (1923) originally suggested may be more important than is generally appreciated. The crystalline style is susceptible to digestion by proteinases, and the more flaccid crystalline styles found in oysters are more easily digested than the harder crystalline styles of some clams (Reid and Sweeney 1980). Judd (1979) has suggested that Cristispira bacteria may digest style carbohydrate. The process is also influenced by pH effects (Mathers 1974) and the rate of secretion of the crystalline style.

The full range of digestive enzymes found in the stomachs of bivalves is given in Table I. Levels of enzyme activity differ according to the feeding type, but any generalization on this matter is fraught with exceptions. Amylolytic activity is lower in the stomach than in the digestive diverticula in some species (Kristensen 1972b) and higher in others (Wojtowicz 1972). Proteolytic activity is generally lower in the stomach than in the digestive diverticula with the

exception of the carnivorous septibranchs (Reid 1977, 1978). The gastric enzymes, such as phosphatases, esterases, cathepsin B, trypsin, and a number of exopeptidases, may emanate from the gastric epithelia, the style sac and the digestive diverticula. Esterases, acid and alkaline phosphatases are found in the A and B cells of the style sac. In the C cells alkaline phosphatase is found. These tissues may be the source of some style enzymes (Reid 1966). Gastric enzymes are secreted in response to the ingestion of food (Mathers 1973a; Reid 1978). For example, in Tresus capax the secretion of proteinases keeps pace with the rapid increase in volume of the gastric juice, so that within an hour of the commencement of feeding, after a hiatus caused by tidal exposure, an eightfold increase in total enzyme activity occurs (Reid 1978). Such a direct response to the ingestion of food argues against the view that gastric enzymes originate from mature digestive cells in the digestive diverticula by "leakage" (Ballantine and Morton 1956), or as "waste" enzymes (Morton 1977) or as exocytotic by-products of digestive cells (Palmer 1979). Moreover, the complement of gastric enzymes is different from the active enzymes of the digestive diverticula both in terms of enzyme type and electrophoretic mobility. Some diverticular enzymes, such as chymotrypsin, and a molecular form of amylase, together with some esterases, are not found in the stomachs of bivalves. The absence of trypsin from the digestive diverticular extracts does not negate its possible origin there. There is some evidence that trypsin originates in the digestive diverticula as trypsinogen (Reid and Rauchert 1976). I have also circumstantial evidence that in Crassostrea gigas the diverticular cells in the disintegrating phase (type III), which Palmer (1979) and Robinson and Langton (1980) have proposed as the source of gastric enzymes, are characterized by high levels of low pH (ca pH 3) proteolytic activity which would be ineffective at gastric pH levels. These disintegrating digestive cells are therefore not good candidates as the general source of gastric enzymes. Owens (1970) suggested that digestive tubule cells in their earlier stage of development, i.e., as basophilic cells were actually secretory. Palmer (1979), noting that secretion from these cells has never been positively demonstrated, concluded that these cells are immature digestive cells which retain the primary lysosomes for intracellular digestion. Frankboner (pers. comm.), on the basis of transmission electron microscope studies of Tridacna gigas considers the diverticular duct epithelia to be the most likely source of gastric enzymes. These are certainly histo-enzymologically active for esterases, phosphatases, and leucine aminopeptidases (Reid 1966; Mathers 1973a; Palmer 1979). The overall picture is that the complement of gastric enzymes is appropriate to the digestion of the carbohydrate and proteinaceous components of the cell walls of diatoms, lysis of cell membranes of food material and solubilization of cell contents. The mechanical action of the crystalline style and inorganic particles aid in these processes. Naked flagellates and bacteria are rendered structurally unrecognizable within minutes of ingestion. Partially digested food remains in suspension as gastric juice flows into the ducts of the digestive diverticula, and is pinocytosed by the digestive cells. While particulate matter can be phagocytosed by the digestive cells it is doubtful that this occurs with many large particles or intact food organisms (Owen 1970; Palmer 1979).

Table I. Digestive Enzymes Found in Bivalve Stomachs

Enzyme	Activity reported in pH range	Origin	Selected references
Trypsin (endopeptidase)	5.5-8.0	Diverticular duct epithelia? Digestive cells? Gastric epithelia?	Reid and Rauchert 1970, 1976 Reid 1977, 1978 Kozlovskaya and Vaskovsky 1970
Cathepsin B; (endopeptidase)	4.5-7.0	as for trypsin	as for trypsin
Cathepsin A (exopeptidase)	3.5	Distintegrating digestive cells?	as for trypsin
Cathepsin C (exopeptidase)	3.5-4.5	as for cathepsin A	as for trypsin
Carboxypeptidase A (exopeptidase)	5-7.5	as for trypsin	as for trypsin
Carboxypeptidase B (exopeptidase)	5-7.5	as for trypsin	as for trypsin
Leucine aminopeptidase (exopeptidase)	7-8	Ciliated epithelia	Mathers 1973a; Palmer 1979; Reid 1966; as for trypsin
"True lipase" (glycerol ester hydrolase)	6-7.5	Ciliated epithelia? Digestive cells?	Yonge 1926; Mansour-Bek 1944, 1948; George 1952; Reid 1966
Arylesterase	6-7.5	Ciliated epithelia	Mathers 1973a; Palmer 1979; Reid 1966
Alkaline phosphatase	8-9	Ciliated epithelia; C cells of style sac	as for arylesterase
Acid phosphatase	5-6	B cells of style sac; Crystalline style	as for arylesterase
α-Amylase	5.5-7.1	C cells of style sac; Crystalline style	Yonge 1923; Kristensen 1972b Langton and Gabbot 1974 Wojtowicz 1972
True cellulase (C_1)	5.5-6	Digestive diverticula Crystalline style	Payne et al. 1972 Koopmans 1970 Stone and Morton 1958 Owen 1974 (review)
Poly-β-glucosidase	5.5	Digestive diverticula? Crystalline style	Crosby and Reid 1971 and as for true cellulase
Cellobiase	5.6	As for cellulase and poly-β-glucosidase	As for cellulase and poly-β-glucosidase
Chitinase	5.6-7.6	Digestive diverticula? Crystalline style	Kristensen 1972b; Jeuniaux 1963; Wojtowicz 1972
Chitobiase	5.6	as for chitinase	as for chitinase
Laminarinase	4.3-7.6	Digestive diverticula? Crystalline style	Kristensen 1972b; Wojtowicz 1972; Mathers 1973b
Alginase	4.2-7.3	Digestive diverticula? Crystalline style	Kristensen 1972b; Franssen and Jeuniaux 1965
α-glucosidase (maltase)	4.3-7.6	Digestive diverticula?	Yonge 1926; Kristensen 1972b; Mathers 1973b
α-galactosidase	5-7	Digestive diverticula	Mathers 1973b; Wojtowicz 1972

INTRACELLULAR DIGESTION AND DIGESTION CYCLES

Intracellular digestion is one of a number of important functions of the bivalve digestive diverticula, which also include food-storage, excretion and acid/base control. A complete list of diverticular digestive enzymes is given in Table II. Starch, disaccharides and other algal sugars can be digested. A true cellulase is rare in bivalves, but poly-β-glucosidases and cellobiases are widely distributed. This subject has been reviewed by Owen (1974). Proteins are digested by the typical range of lysosomal proteinases together with alkaline exopeptidases and a chymotryptic enzyme in some species.

Table II. Digestive Enzymes in the Digestive Diverticula

Enzyme	Activity reported in pH range	Selected references
Chymotrypsin (endopeptidase)	7-8	Reid 1966; Reid and Rauchert 1970, 1976
Leucine aminopeptidase	7-8	Reid and Rauchert 1976
Carboxypeptidases A, B	5-7.5	as for leucine aminopeptidase
Cathepsin B_1	4-5.7	Rosen 1949; Kamat 1957; Reid and Rauchert 1976
Cathepsin D (endopeptidase)	2-3	Reid and Rauchert 1976
Cathepsin A, C	3.5-4.5	Reid and Rauchert 1976
True lipase	6-7.5	Yonge 1926' Mansour-Bek 1946, 1948; George 1952
Arylesterase	6.5-7.5	Reid 1966; Mathers 1973a; Palmer 1979
Carboxylesterase	6.5-7.5	Reid 1966
Alkaline phosphatase	8-9	Reid 1966; Mathers 1973a; Palmer 1979
Acid phosphatase	5-6	as for alkaline phosphatase; Owen 1972
Carbohydrases (all of those listed in Table I	see Table I	see Table I

Studies of the putative digestive cycle in bivalves have generally focused more on changes in pH and the crystalline style, together with the histological condition of the digestive cells than on the more central question of enzyme activity, with the exception of Langton and Gabbott's (1974) and Langton's (1977) studies of amylases in Ostrea edulis and Mytilus edulis. Histochemical studies by Mathers (1973) and

Palmer (1979) are also relevant to this problem. I have made preliminary studies of protein digestion in Tresus capax and Crassostrea gigas in relation to the tidal cycle. In Tresus gastric proteolysis levels increased with the volume of the gastric juice after exposure by the ebb tide. Diverticular proteolysis began to rise within three hours of the commencement of feeding and within six hours reached a plateau of activity that remained constant whilst the animal was covered by the tide. This study was carried out under diurnal tidal conditions when there was a single ebb in daylight.

In Crassostrea gigas, studied under semi-diurnal tidal conditions, samples of ten specimens were taken at four hour intervals over 48 hours. Average gastric volumes were largest during the daylight high tide, and lowest during the ebb. At the nocturnal high tide there was considerable individual variation resulting in a low average volume. The range of gastric proteolytic activity at the gastric pH (6.0 - 6.5) was small, the maximum values being found at the high tide. Highest digestive diverticular proteolytic activity at pH 6.0 - 7.5 was found about 1 hour after the daylight high tide and there was no discernable peak associated with the nocturnal high tide. At pH 3 the highest activity in the digestive diverticula was found at night, developing 6 to 8 hours after the peak of pH 6.0 - 7.5 activity.

My interpretation of these observations is that under the conditions of this study the oysters were feeding during both light and darkness, and that the greater daylight feeding activity established the overall pattern of intracellular protein digestion, which commenced in the pH 6.0 - 7.5 range in the day and was completed at pH 3 at night. In addition, the individual variability at any sampling time indicates that not all of the digestive cells were in the same physiological phase at the same time.

Morton (1978) discovered that C. gigas had a diurnal 24-hour cycle, with feeding activity greatest in daylight. He related this to the inequality of the semi-diurnal tides during the time of his study, and suggested that equal semi-diurnal tides would establish a 12-hour cycle. Morton also found that "in general" all of the digestive tubules behaved synchronously. My observations indicate that a 24-hour cycle persists even when the semi-diurnal tides are of equal height. I would hypothesize that nocturnal feeding establishes a secondary physiological cycle which in most individuals is masked by the dominant daylight cycle; however in a few individuals the nocturnal cycle may be dominant. A study combining histological and enzymological observations is required to test this.

The tridacnids show a typical daylight filtering rhythmicity since during the night they appear to go into a torpor (Morton 1978) which I can personally confirm for Tridacna gigas. I have also found that pH 3 proteolysis is at its peak during the later part of the torpid period in this species. However, my collaborator, P. Fankboner, has been unable to find any histological homogeneity in diverticular tissue, from the same specimens, that would support the hypothesis that all of the digestive cells were in phase with one another. McQuiston (1969) found that in Laseae rubra two histological phases could be found at any time in the digestive diverticula. Mathers (1976) and Mathers et al. (1979) reported similar heterogeneity in Pecten maximus, Chlamys varia and Venerupis decussata. Robinson and Langton (1980) have reviewed the literature on this topic, and reported on a subtidal population of

Mercenaria mercenaria. They have found considerable histological variation within the digestive diverticula, regardless of the state of the tide. Although the cells were not in the same phase there were four peaks of absorptive activity that corresponded to variations of environmental levels of food organisms.

It is difficult to generalize on the topic of digestive cycles, significant though it may be for aquaculture. With regard to the histological condition, and by inference the digestive condition of the digestive diverticula, the weight of evidence suggests heterogeneity in subtidal populations at all states of the tide, with minor peaks of digestive activity correlated with food availability. In intertidal populations tidal movements impose a degree of digestive rhythmicity, though conflicting opinions have been expressed concerning the homogeneity of the digestive cell condition resulting from tidal rhythmicity. There is general agreement with Owen (1972) that prolonged exposure will bring the digestive cells into a 'holding phase'. Most authors admit to the possibility of circadian rhythmicity in some species, but the general opinion is veering back towards a modification of the traditional view of continuous feeding and digestion in bivalves, the modification being that feeding may be variable to take best advantage of food when available and to ensure a steady level of ingestion. It is difficult, however, to abstract anything of practical value from this generalization. Different species have been shown to differ in their behavior according to environmental conditions. Once more, each species in aquaculture must be characterized with these cautions in mind.

In addition to their other functions, the bivalves' digestive diverticula store energy in the form of lipid, and this lipid is mobilized during times of low food availability. The digestive diverticula cells may also be partly mobilized by autophagy during times of starvation. Cathepsin D levels remain high in Tresus when the food supply is poor in winter, suggesting autophagic lysosomal activity (Reid and Rauchert 1976).

In the mid-gut of bivalves a number of gastric enzymes are found, indicating that digestion continues in this region. Associated with the intestinal epithelia are esterases, phosphatases and leucine aminopeptidases (Reid 1966; Mathers 1973a). The distribution of the exopeptidase is similar to that found in vertebrates, and indicates a coupled digestion/absorption mechanism, i.e., as terminal amino acids are enzymatically released from the dietary protein they are transferred to an amino acid uptake site on the epithelial membrane. Digestion and absorption must therefore be added to the intestine's other function of fecal consolidation.

Thompson and Bayne (1972) identified in the feces of Mytilus edulis two distinct components, one consisting of rejecta from the gastric sorting processes, and the other consisting of exocytotic material from the digestive diverticula. The proportions of the two components were assumed to be correlated with the digestive capacity of the digestive diverticula and the concentration of food in the stomach.

BACTERIA AND BIVALVE NUTRITION

The role of bacteria in the nutritive economy of bivalves has come under closer scrutiny in the last decade. Previously bacteria had been recognized as important dietary constituents for detritus feeders (Braefield and Newell 1961), and possibly in suspension-feeders (Zobell and Feltham 1938). Braefield and Newell postulated that down to certain limits, the more finely divided detrital material provided a better physical substrate for associated bacteria. However, some bivalve species have modified their pallial behavior to take advantage of the epiflora and epifauna of large substrate particles such as sand grains. Braefield and Newell argued that the nutritive potential of bacteria associated with detritus was more important than that of the detrital material itself. Detritus-feeders have been regarded as recipients of a continuously available diet of low nutrient level, in comparison to the discontinuous food availability for suspension feeders (Levinton 1972). It must also be remembered that some detritus feeders are facultative suspension feeders, and even those that cannot alter their feeding behavior to take advantage of suspended food must benefit from that food when it comes close to the substrate/water interface, or when it is deposited at that interface. Bacteria are known to be the major dietary constituent of the Galapagos Rift bivalves (Corliss et al. 1979). Furthermore, bacteria associated with the ctenidia have been found in two species of Solemya: S. velum (Cavanaugh et al. 1981) and S. panamensis (Felbeck et al. 1981). These bacteria possess enzymes of the Calvin-Benson cycle together with sulphide oxidation enzymes, indicating their ability to fix carbon dioxide using the energy of hydrogen sulphide. Masses of bacteria have been found to constitute the "gland" of Deshayes in the shipworm Bankia australis (Popham and Dickson 1973) and it was suggested that these ctenidial bacteria make up for the nutrient deficiencies of the shipworm's wood diet by fixing nitrogen (Trytek and Allen 1980). Another role for bacteria in the microenvironment of the burrow or mantle cavity might be the preliminary extraorganismic digestion of organic material, the hydrolysates of which could be used by the bivalve as food. A number of bacterial species of the spirochaete Cristispira from the bivalve gut have been described (Berkeley 1959; Tall and Nauman 1981). The contribution of bacteria to cellulose digestion in Scrobicularia plana has been assessed by Payne et al. (1972), and they concluded that none of the isolated bacteria had cellulolytic activity and therefore such an ability must be endogenous to the bivalve. There has been an active debate concerning the role of bacteria in shipworms, reviewed by Morton (1978) and by Mann at the Symposium on Marine Biodeterioration (1981). There is a consensus that in the wood-digesting caecum of teredinids, bacteria do not produce the cellulases (Mann 1981). However, Popham (pers. comm.) has detected intracellular bacteria in vesicles which arise from the digestive tubles in Bankia rochi. These bacteria have not yet been characterized.

EXCEPTIONS THAT PROVE THE RULE

The shipworms and the carnivorous bivalves are of no direct interest to aquaculturists, but they do demonstrate the adaptational plasticity of the Bivalvia, and indicate qualities that the more 'law-abiding' bivalves may possess to a lesser degree. Two other bivalve types of personal interest to the author prove the rules for the

rest. Solemya reidi (Bernard 1980) and possibly other members of this genus are completely gutless (Reid and Bernard 1980). Deprived of normal alimentation, they must depend on other sources of food. Members of the genus have already been shown to possess chemosynthetic symbionts (Felbeck et al. 1981). I have detected a rapid uptake of dissolved organic molecules by the mantle and gill epithelia of S. reidi, and there remains the possibility of complex synergy between bacteria in the mantle cavity and burrow. Such studies may have relevance to the nutrition of better known bivalves.

Tridacna gigas is partially dependent on the symbiotic zooxanthellae held in the enlarged mantle edges. This bivalve now seems less exceptional in the light of recent findings concerning bivalves and symbiotic microorganisms. Tridacna gigas also feeds on phytoplankton, reef detritus and coral zooxanthellae released from heat-stressed corals, and possibly dissolved organic exudates from reef corals (Fankboner and Reid 1981). Since this organism has potential for aquaculture or at least 'domestication' these environmental factors should be taken into account.

In conclusion, the exceptional may inform us about the 'normal' in bivalve biology. Indeed the 'normal' may be so exceptional that every species must be assessed individually for nutritive and digestive characteristics. The expansion of our knowledge of these matters in the recent years continues and each of the studies that I have discussed provides us with new directions of interest.

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PRUDER (Delaware): Dr. Reid we've been growing bivalve molluscs particularly Crossostrea virginica in a controlled-environment system for almost ten years. During that time we've experimented with many different feeding regimes. The engineering considerations in a controlled-environment would seem to prefer a system which could be fed sequentially or pulse fed as compared to attempting to maintain a fairly low concentration of algal cells in water. We've had considerable experience and success supporting very rapid growth of the eastern oyster with pulse feeding techniques. The initial concentration of algae to which the animal is exposed can be as high as 2 million cells per ml of a combination of Tahitian Isochrysis and Thalassiosira pseudonana. If the animal is continually exposed to such high algal concentrations it will shut down and eventually perish. But if the animal biomass to seawater volume ratio is such that the animal exposed to the very high concentration can reduce the concentration down to low levels in a matter of 3, 4, or 5 hours by feeding, the process works. We have in a poster presented in this meeting showing results of experiments with silt and algae where we demonstrate once again that the pulse feeding technique works. Do you see how pulse feeding fits into a digestive pattern? There are other options, not just high algal concentration or low algal concentration. Could you give some explanation of why pulse feeding seems to be effective?

REID: For one thing in nature Crassostrea virginica will "pulse feed" by choice to some extent. It will show 2 to 3 peaks of intense feeding activity over 24 hours. This is on the basis of Robert Palmer's 1980 paper. Secondly, cultured bivalves may to some extent be like Clever Hans, the counting horse, in the sense that they're being trained to a certain feeding regime that in a way that the culturists don't consciously realize. I think it may be possible to bring all bivalves under culture into a feeding regime that suits both the bivalves and the culturists. That would be highly desirable if it's true, but it's only speculative.

NEWELL (Maryland): Would Dr. Reid like to comment on the recent work of Kiorboe et al. (1980)* which indicated that the mussel Mytilus edulis is capable of preferentially ingesting algae from a homogenous suspension of algae and silt particles? The pseudofeces were observed to have reduced algal content, on a dry weight basis, indicating that they may be used as a means of voiding the silt. My recent research also indicates that the oyster Crassostrea virginica can preferentially ingest the organic component of natural seston and reject inorganic or non-nutritive particles as pseudofeces.

REID: Were the particles you were feeding the same size?

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* Kiorboe,, T. F. Mohlenberg and O. Nohr. 1980. Feeding, particle selection and carbon absorption in Mytilus edulis in different mixtures of algae and resuspended bottom material. Ophelia 19:193-205.

NEWELL: They appear from Coulter Counter analysis to be in the same size but we don't know if they are of the same density. We do not understand the mechanism of this selection but the labial palps have frequently been identified as an organ which may sort and select food particles.

REID: Obviously this subject is complex and requires further research. Bernard's observation that Crassostrea gigas will "reject" inorganic particles, because they settle out before reaching the sorting mechanism may be relevant here. During the last ten years we have moved out of the classical mold of studying molluscs. Instead of making a few observations for a few species and generalizing we have the same species being examined by two different sets of workers with contradictory results. To what extent the wishes of the researcher enter into this, I do not know.

McCORMICK (California): Dr. Reid over the recent years people have been rethinking bivalve nutrition. Early work in the 30s from Zobell looked at bacteria uptake, then in the 60s and 70s work from Winter looked at silt, and we see it at this symposium here, the importance of silt. Can you comment on what you feel the relative importance of diatoms vs. flagellates vs. bacteria vs. silt are?

REID: I can try. For one thing the digestive enzyme component of the stomach is fairly suitable for getting into diatom cells. It's not a matter of digesting the diatoms, it's a matter of lysing the cell walls sufficiently by digesting the carbohydrate and protein components. Naked flagellates are also lysed. Although the enzyme levels in the bivalve's stomach are generally quite low they are sufficient for lysis, to allow partial solubilization. The presence of mineral particles of the same sizes as the food may be significant in mechanical terms in helping break diatom frustules and in helping simply to reduce the particle size. I would guess from the silt experiments that have been described in the poster session there's no question of associated bacteria with that silt. There is no silicase in bivalves; silt cannot be digested, so there must be some other role for it, if you take the causal view that is, and the mechanical role is the only one that's left. I find it very intriguing.

MOLLUSCAN BIOENERGETICS - A SYNOPSIS

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ABSTRACT

An understanding of the energy and nutrient flow through the individual, population, or community is one of the cornerstones of modern production ecology. Energy budgeting techniques are also being increasingly used by marine physiologists to quantify the degree to which various physiological adaptations and environmental perturbations alter an animal's growth rate and fecundity. Although these techniques have been extensively applied to terrestrial animal husbandry, they have not yet been widely adopted in mariculture.

This general review considers the application of bioenergetics to bivalve molluscs. Emphasis is placed on some adaptations that require further elucidation or are important for mariculturists to consider, such as optimum methods of providing the ration and methods for assessing growth.

INTRODUCTION

One of the major emphases in coastal marine zoology, until about ten years ago, was the explanation of animal distributional patterns in terms of a species tolerance to an array of environmental variables, e.g. temperature, desiccation, etc. Numerous such studies (reviewed by Newell 1979) demonstrated that the majority of littoral animals exist well within their zones of physiological tolerance. Recently, there has been increasing recognition that less tangible factors than had previously been studied, e.g. temporal and spatial variations in food availability, differential partitioning of energy to growth and reproduction, are of paramount importance in governing the distribution and abundance of species within its zone of physiological tolerance

(Bayne et al. 1976b; Newell 1979). The complex interactions between such variables may be elucidated by measuring all factors in terms of energy units and combining the various energy gains and losses into an overall index of energy balance.

The primary components of an individual's energy budget are identified in the following "balanced energy equation" (Warren and Davis 1967) written using IBP terminology (Crisp 1971).

$$P + G = C - (R + F + U)$$

where P = Somatic growth
G = Germinal growth } = "Scope for Growth (Warren and Davis 1967)

C = Energy value of food consumed

R = Energy metabolically utilized

F = Energy value of feces

U = Energy value of material that is absorbed but later excreted as urine or other exudates

Units = Energy units (Joules)

The magnitude of a positive energy balance indicates the quantity of energy available for assimilation into either somatic or germinal tissue or to be sequestered in a nutrient reserve. Conversely, if there is a negative energy balance the organism can only survive under such conditions whilst it has a nutrient reserve to utilize. Thus, the index of energy balance, or "Scope for Growth" (Bayne et al. 1976c; 1979), is one sensitive method for assessing and quantifying the adaptability of an organism to a particular environment, including maricultural facilities.

In this paper I have not attempted to give an exhaustive review of the very extensive literature covering all aspects of molluscan bioenergetics as the field has already been adequately reviewed (Bayne et al. 1976a, b, c; Conover 1978; Widdows 1978a, b; Winter 1978; Newell 1979, 1980a, b). Instead, by concentrating on bivalve species which are both well studied and commercially important, I intend to give a broad overview of how a bioenergetic approach may be used to direct and focus research towards elucidating the key components of an organism's biology. I also hope to show that the efficiency of mariculture projects may be increased by the introduction of even a small number of the techniques currently avaliable in bioenergetics.

FOOD CONSUMPTION AND ABSORPTION

The majority of bivalve species are sessile suspension feeders, and most can partially adjust their filtration rate in response to the quantity and "food quality" of the seston. In general, however, there is a rapid rise in pumping rate to a plateau level, with increases in particle concentration, from a certain minimum level necessary to supply oxygenated water to the gills. A point is reached, though, where any further increase in particle concentration causes the pumping rate to decline from this maximum plateau level (Winter 1978; Widdows et al. 1979). Superimposed on such short term variations in filtration rate are seasonal changes which may be a response to both changes in water

temperature and food availability as well as cyclical changes in physiological condition, associated with the energy demands of gametogenesis (Bayne and Widdows 1978; Newell and Bayne 1980). The consumed ration is, therefore, dependent both on the food concentration and the animals' feeding behavior.

In bivalves, the digestive process is complex because as the rate of consumption increases the digestive gland becomes full and excess food then by-passes the gland and is voided as intestinal feces, together with feces from the digestive gland (Thompson and Bayne 1972; Widdows et al. 1979). When ingestion rates are below the level which results in intestinal fecal production, absorption efficiency, defined as the energy uptake from the food in the digestive system (Crisp 1971), is at a maximum. With increasing ingestion, and hence the ejection of undigested food as intestinal feces, absorption efficiency declines. A point is ultimately reached above which excess material is not ingested but is rejected as pseudofeces (Thompson and Bayne 1972; Winter 1978). Any further increase in consumption above this level does not further reduce the absorption efficiency of the material in the digestive system. However, such a consideration of absorption efficiency neglects the energy lost in pseudofecal production. Therefore, when constructing the energy budget of an animal, the energy absorbed should be expressed as a percentage of the total energy consumed, not just of the energy ingested. In practice this is achieved by pooling both pseudofeces and feces and analyzing them using the ratio method of Conover (1966). Widdows (1978a) found that for M. edulis, absorption efficiency declined with increasing food concentration and also with decreasing body size for animals feeding at the same food concentration. Such a result was to be expected as in both cases undigested food (both intestinal feces and pseudofeces) comprised an increasing proportion of the ejecta.

The production of pseudofeces assumes critical importance in closed or semi-closed mariculture systems because frequently one of the largest operating costs is the provision of a unicellular algal food. There have been numerous studies designed to determine the most efficient means of feeding algae to bivalves in order to minimize wastage. Many are based on the results of Morton (1969, 1970, 1973) who found that for intertidal bivalves there is a cyclical relationship between feeding and both extracellular and intracellular digestion. Epifanio and Ewart (1977) found that for continuously submerged oysters (Crassostrea virginica) there were also periods of feeding activity and quiescence, although there was no apparent tidal or diurnal rhythm (Palmer 1980). Epifanio and Ewart (1977) concluded that when rearing bivalves it would be advantageous to add food discontinuously. This would entrain the feeding and digestive activity of the animals to the feeding regime and hence prevent excess food being lost during periods of non-feeding.

Similarly, Langton and McKay (1974, 1976) found higher growth rates when the same total amount of algae was fed to the oyster Crassostrea gigas discontinuously, compared with a continuous feeding regime. This result is expected because the continuous addition of algae would have resulted in a low food concentration that may not have fully stimulated the oyster's feeding activity, whilst the discontinuous feeding regime would have resulted in much higher initial concentrations. This conclusion is supported by the results of Winter and Langton (1976) who found that for maximum growth in the mussel, M. edulis, food should be continuously replenished so that a constant optimum level is maintained.

However, before discontinuous feeding regimes are widely adopted, a complex series of economic and energy budget factors must be considered. In one scenario, if optimum ration levels were continuously maintained, animals would ingest a maximum ration with little or no pseudofecal production. Any synchrony between individual's feeding and digestive cycles in the continuously immersed animals would be lost within two weeks of being placed in such conditions (Langton and Gabbott 1974) and thus periods when all animals were not feeding would be unlikely to occur. In such conditions growth would be maximized. If it is necessary to reduce the algal ration, energy budgeting can be used to determine the least harmful way of achieving this. One alternative might be to adopt a discontinuous feeding regime where the daily feeding periods are balanced between economic restraints and achieving maximum growth. However, a more effective solution might be the maintenance of optimum ration levels during the seasonal periods of physiologically and genetically entrained maximum growth and a reduction in ration during the quiescent periods.

In addition to an animal's energy intake, it is also important to consider its requirement for specific nutrients, e.g. essential amino acids, lipids, etc. For example, Russell-Hunter (1970) has shown that the reproductive capacity of the marsh snail, Melampus bidentatus, is limited at certain times by lack of organic nitrogenous compounds. Unfortunately, as yet, very little research effort has been directed towards this type of study in bivalves, due to the technical complexities of measuring the various rate functions in terms of the selected nutrient. However, the development of artificial diets suitable for filter feeding bivalves (see Langdon - these proceedings), will allow experimental manipulation of the ration. This should yield the sort of detailed information concerning nutritional requirements that is already available for crustaceans (see Kanazawa - these proceedings).

INFLUENCE OF INORGANIC PARTICLES ON FEEDING AND GROWTH

The negative influence of high levels of particulate inorganic material (PIM) on the filtration rate of bivalves has been extensively studied (Foster-Smith 1975; Moore 1977; Widdows et al. 1979). However, there is some evidence that low levels of PIM added to an algal diet can enhance feeding and growth (Loosanoff 1962; Kiorboe et al. 1981). Winter (1976) found that mussels continuously fed a mixture of Dunaliella marina (40×10^6 cells ℓ^{-1}) and 12.5 mg dry wt ℓ^{-1} of PIM (produced by wet oxidizing natural sediment with H_2O_2) had a 32% greater dry weight after 26 days, compared with control mussels fed the same concentration of algae alone. Winter (1976) demonstrated that this enchanced growth was due to the stimulatory effect of low levels of PIM on filtration rate, and a reduction in pseudofecal production, which resulted in an increased rate of ingestion. Experiments by Ali (1980) confirmed the stimulatory effects of PIM added to the algal food, on the growth of the oyster, C. virginica.

Murken (1976) fed M. edulis a mixture PIM, the dissolved organic fraction (DOF) from fish waste, and algae and found that growth was improved compared with that of mussels fed on a mixture of just algae and DOF. Further experiments are required to see if the enhanced growth was mainly due to the stimulatory effect of increased particle

concentration on ingestion rate or if the bivalves were utilizing the DOF, or both. The active absorption, by aquatic molluscs, of dissolved amino acids (see Stephens - these proceedings) has clearly been demonstrated (Wright and Stephens 1977, 1978; Manahan et al. 1981). This may supply a proportion of the animal's energy requirements but perhaps more importantly specific nutrient requirements at certain critical periods in their life cycle.

The PIM filtered from suspension does not necessarily "dilute" the organic material ingested, as postulated by Widdows et al. (1979). Instead, recent research indicates that both the mussel M. edulis (Kiorboe et al. 1980) and the oyster, C. virginica (Newell, unpublished data) preferentially ingest algae and reject the PIM in the pseudofeces. C. virginica can also preferentially ingest the organic matter from natural seston which contains not only algae but bacteria, detritus, etc. (Newell, unpublished data).

There are many possible explanations for the effects that a PIM supplement to the algal diet can have on bivalve growth, including a) addition of bacteria or organic matter which enhances food levels; b) provision of a large surface area, for either the absorption of DOF which is thus made available to the digestive processes (Murken 1976), or the removal of inhibitory metabolites (Moore 1977); c) addition of an unknown growth factor(s) (Winter 1976); d) improvement of digestion efficiency by the mechanical grinding action of the inorganic particles in the gut (Murken 1976); and e) a stimulatory effect on filtration rate and hence an increase in the amount of material ingested (Winter 1976). Further investigation is required to determine which of these mechanisms is responsible for the enhanced growth. However, the last explanation seems perhaps the most plausible and is very similar to the effect found for crustaceans when a "bulking" fiber agent is added to their diet (Capuzzo - these proceedings).

RESPIRATION

Bivalves under normoxic conditions (i.e. active animal, freely ventilating its mantle cavity in fully aerated seawater) use approximately 65% of the energy of metabolic substrates to phosphorylate ADP to ATP by the classical tricarboxylic acid cycle (Calow 1978), with the remainder of the energy being lost as heat. In order to determine the amount of energy respired either the metabolic and mechanical heat output or the animal's oxygen demand must be measured. The latter measurement can then be converted to energy units using standard coefficients (Elliot and Davison 1975). Due to the relative ease and accuracy of measuring oxygen uptake, compared with heat output, it is almost exclusively used to quantify metabolic energy demands of aquatic molluscs (Crisp 1971).

In some circumstances, e.g. during periods of shell closure, a bivalve is deprived of oxygen. In such situations ATP is generated by perhaps as many as four anaerobic pathways, which may be differentially utilized, both temporally and spatially, within the animal's tissue (Kluytmans et al. 1980; Zandee et al. 1980; Zurburg and Ebberink 1980). These pathways regenerate ATP by incomplete chemical oxidation of predominately glycogen, resulting in the accumulation of a succession of fermentation end-products, including succinate, amino acids and volatile fatty acids.

Anaerobic metabolic pathways have frequently been considered simply as useful adaptations that enable a "facultatively anaerobic" animal to supply its ATP requirments under hypoxic or anoxic conditions (Pamatmat 1979). Also, as the respiratory substrates are incompletely oxidized, anaerobsis has been considered energetically inefficient, with ATP yields from glycogen being 6-8 times less than if an equimolar amount had been aerobically catabolized (de Zwaan 1977). However, the energy of the anaerobic end-products is not necessarily lost to the animal, as succinate and propionate can be converted to oxaloacetate, which can be used during aerobic respiration in the tricarboxylic acid cycle (de Zwaan 1977). Recent research (Henry et al. 1980) also indicates that in some bivalves, anaerobic end-products are required during normoxic conditions for the production of intracellular osmolytes. There is also some evidence that indicates that even in the presence of high oxygen levels the metabolism of certain "deep seated" tissues, e.g. the adductor muscles, may be partialy anaerobic (Chaplin and Loxton 1976; Hammen 1976; de Zwaan 1977; Booth and Mangun 1978). Few bioenergetic studies have considered the possibility that measuring oxygen demand under normoxic conditions may underestimate the total catabolism of energy reserves due to sustained anaerobic metabolism.

Hammen (1979) measured the oxygen demand and heat output under aerobic conditions of a group of mussels, Mytilus edulis and found that the total metabolic heat output was 1.59 J $hour^{-1}$ g^{-1} wet tissue, but their oxygen demand of 0.06 ml O_2 $hour^{-1}$ g^{-1} was equivalent to a metabolic heat output of only 1.21 J $hour^{-1}g^{-1}$ or, approximately 77% of that measured by calorimetry. A similar result was again reported for Mytilus edulis by Hammen (1980). Although such calorimetry experiments provide reliable estimates of heat output during anaerobiosis it was not until the work of Famme et al. (1981) that long term measurements of the heat output and oxygen consumption of M. edulis were made simultaneously, at a number of controlled oxygen tensions. Their results showed that under normoxic conditions anaerobic metabolism accounts for less than 5% of the total metabolism, which is probably associated with the energy demands of "deep seated" tissue. Although these experiments need to be performed on other species it does indicate, contrary to the work of Hammen (1979, 1980), that in M. edulis measuring oxygen consumption is an acceptably accurate technique for quantifying metabolic energy demand. However, at oxygen tensions as low as about 50% of saturation the oxygen consumption of M. edulis is reduced to 50% of its normoxic level but its total metabolism is not altered (Famme et al. 1981). This difference must, therefore, be sustained by anaerobic metabolic pathways. These results indicate that in maricultural facilities it is important that saturated oxygen tension levels are maintained either by aeration or by avoiding overcrowding so that the animals can sustain aerobic metabolism.

A bivalve's metabolism varies in response to short-term environmental perturbations such as temperature and food availability (reviewed by Bayne et al. 1979b; Newell 1979). The actual response elicited is dependent both on the species and its physiological condition. When an animal is subject to an abrupt increase in ambient temperature in conditions of high food availability, it may adopt an "exploitative" strategy. This involves an increase in feeding rate causing both the rate of consumption and metabolic costs to increase, with perhaps a reduction in absorption efficiency, but the net result is an increased scope for growth (Widdows and Bayne 1971; Newell 1979,

1980a,b). Conversely, in food limited situations a more "conservationist" strategy may be adopted in which activity is curtailed, thereby reducing metabolic energy demands to a "standard" level (Thompson and Bayne 1972), absorption efficiencies may be higher and hence the energy gain from the reduced ration is maximized.

Many commercial mariculture projects benefit from the faster "opportunistic" growth rates associated with artificially elevated temperatures. However, prior to investment in such projects it is vital to determine, using a regular sampling program, that the ambient food levels are high enough to maintain a positive annual scope for growth (Malouf and Breese 1977).

GENETIC INFLUENCES ON METABOLISM

It has frequently been found in breeding experiments that organisms which are heterozygous at a large number of gene loci grow faster than more homozygous individuals (Wilkins 1981). Zouros et al. (1980) found that for the oyster C. virginica, there was a positive relationship between the degree of heterozygosity at four to seven gene loci and the weight of a one year old animal. There was no clear explanation of this result until Koehn and Shumway (1982) demonstrated that the standard metabolism of more heterozygous oysters consumed less energy, and they suggested that this was possibly because metabolic pathways dependent on heterozygous enzymes are, on average, more efficient than homozygous ones. Koehn and Shumway (1982) postulated that reduced metabolic cost results in more energy being available for growth. This genetic component of an animal's metabolism and growth has frequently not been given sufficient consideration by physiologists and requires further study.

EXCRETA

Excreta, a term properly applied to the loss of energy in the form of nitrogenous waste compounds, is one of the least studied aspects of molluscan bioenergetics. I also include under this term exudates such as mucous (but not germinal products) (Crisp 1971).

The two main nitrogenous compounds excreted by bivalve molluscs are ammonia nitrogen (NH_4.N) and amino nitrogen (amino-N). Bayne et al. (1976b) concluded in a review of data from 8 bivalve species that NH_4.N accounted for about 65% and amino-N for about 28% of the total nitrogen excreted and the balance was made up by either urea or uric acid. Excretion of NH_4.N may be variable due to increased reliance on protein as a respiratory substrate during periods of stress, e.g. in M. edulis during and just after maximum reproductive condition (Bayne 1973; Widdow 1978a). Amino-N loss may partly be due to 1) the passive diffusion of amino acids from the body tissues (Hammen 1968); 2) an active excretory process associated with protein catabolism; and 3) osmotic adjustment to a decline in ambient salinity.

Amino-N excretion has been found to increase from a baseline value of approximately 11% of the energy of routine metabolism to a value as high as 63% when mussels are placed under environmental stress (Bayne 1973). This may perhaps be due to increased protein catabolism or to a

decreased stability of biological membranes. Amino acid excretion is an activity of great complexity considering the ability of bivalves to absorb amino acids actively from water (reviewd by Stephens - these proceedings). More research is required concerning the environmental and physiological conditions that effect the absorption or excretion of amino acids in cultured bivalves so that excretion of nitrogen is minimized whilst absorption is maximized.

The energy loss in the mucous voided with the feces and pseudofeces is not normally determined separately in energetic studes, but is included in the energy of the egesta. However, in species which produce copious pseudofeces under certain feeding conditions, e.g. Crassostrea virginica, it may be a significant loss.

PHYSIOLOGICAL INTERGRATIONS

The measurement of the physiological factors necessary to construct the complete energy budget for many molluscs may be made using standard techniques (Bayne et al. 1977, 1979). Once the appropriate interrelationships between the various functions, e.g. food concentration and feeding rate, variations in metabolic rate with season, etc. have been elucidated for a species, a computer based simulation model, can then be used to predict "scope for growth" values. A typical application could be the assessment of the maricultural potential for a species in a variety of localities. The only additional data required for each locality would be detailed information over an annual cycle of environmental factors, e.g. seston and total particulate organic matter levels, temperature, salinity, etc. The accuracy of using estimated "scope for growth" values based on simulation models to predict the actual growth of a species in the field has been tested by Newell (1977) and Bayne and Worrall (1980). The results obtained by Bayne and Worrall (1980) (Figure 1) indicate close agreement between the predicted and measured growth of Mytilus edulis.

Such techniques may be too sophisticated for maricultural facilities but at least frequent determinations of ambient food levels and absorption efficiencies should be made. The concentration of the added ration can thus be routinely regulated so that high absorption efficiencies are maintained. Widdows (1978a) found that the absorption efficiency of M. edulis was unaffected by temperatures between 5°C and 25°C in the laboratory. However, Bayne and Widdows 1978 and Newell (unpublished data) found that in field populations of M. edulis, the absorption efficiency ranged from about 70% in the summer to a winter minimum of about 5-10%, a result which may have been partly due to seasonal variation in food digestibility. However, it seems that absorption efficiency values between 60-70% can reasonably be expected when feeding bivalves on cultured algae (Winter 1978).

Information on the efficiency with which the cultured animals are utilizing the ration for growth is frequently reuired both to allow continuous adjustment of the ration to an optimum level and also to compare the performance of different groups of animals. Two useful, non-dimensional ratios are the "Gross Growth Efficiency" (K_1) and "Net Growth Efficiency" (K_2). These are defined as the efficiency with which an animal uses the energy in the consumed and assimilated ratio, respectively, for somatic and germinal growth (Klekowski and Duncan 1975).

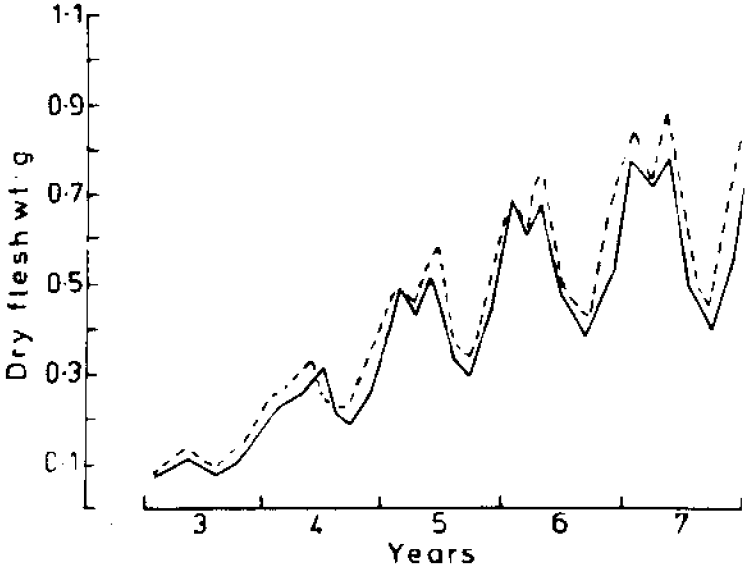

Figure 1. Comparison of the growth of a population of Mytilus edulis as calculated from age-class analysis (broken line) and from physiological estimates of the scope for growth (solid line) from Bayne and Worrall (1980).

Gross Growth Efficiency (K_1) = $\frac{P+G}{C}$

Net Growth Efficiency (K) = $\frac{P+G}{(C-F-U)}$

Where P = Somatic Growth; G = Germinal Growth; C = Ration Consumed; F = Energy Lost in the Feces; U = Energy Excreted

Perhaps the most extensive use of K_1 for the description of the interrelationships between ration, metabolism and growth in bivalves has been by Thompson and Bayne (1974) and Widdows (1978b). Thompson and Bayne (1974) found for M. edulis (Figure 2) a rapid increase in K_1 from a negative value occurring at low food rations to a point (C_m) where K is zero, which is when the absorbed ration is equal to the metabolic energy demands; thus C_m is a measure of the maintenance ration. Growth efficiency increases further with ration size until a maximum point is attained at the optimum ration level (C_{opt}) for that sized animal. Any further increase in ration results in a decline in growth efficiency. Actual values for K_1 for M. edulis range from about 45% for animals between 0.1-0.2 g dry weight to below 10% for animals over 1 g dry weight (Jorgensen 1976; Bayne et al. 1976c; Widdows 1978b).

ENERGY PARTITIONING

The discussion so far has been mainly concerned with the energy available for somatic and germinal growth and not how this energy is partitioned between these two types of growth. The majority of bioenergetic studies have not been able to quantify the differential allocation of energy because this varies not only between species but is also dependent on the individual's age, size, history and environmental

conditions. This is further complicated by the use of a nutrient reserve in many species to supply energy for gametogenesis and maintenance metabolism during periods of reduced food ingestion.

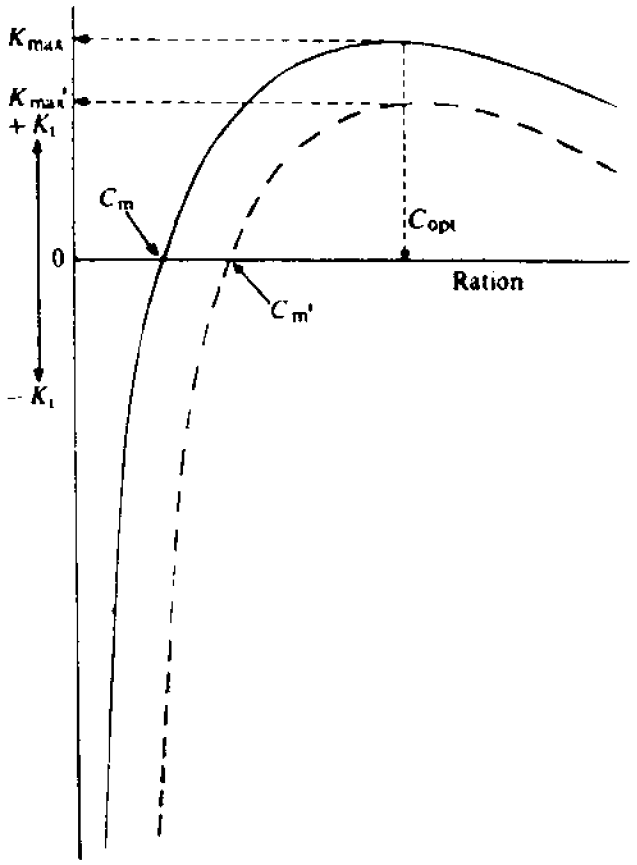

Figure 2. Generalized curve for growth efficiency (K_1) of Mytilus edulis as related to ingested ration. The solid line represents the control condition and the dashed line a decrease in growth efficiency due to increased energy demands of respiration. Where C_m and $C_m{}^1$ are the maintenance rations and C_{opt} is the optimum ration (from Thompson and Bayne 1974).

The somatic growth of a bivalve, above a certain species specific minimum size (e.g. approximately 10 mm for Mytilus edulis (Theisen 1973) can be described by a logarithmic curve, that is, a continuously decreasing weight specific growth rate. This is due, in part, to a decreasing efficiency of feeding, and an increasing metabolic maintenance cost, as the animal increases in size. This was demonstrated by Vahl (1973) for the cockle, Cardium edule, where the exponent (b) relating metabolism (R) to body weight (W) in the allometric equation

$$R = aW^b$$

was 0.77 whereas that for feeding rate was only 0.58. Also, an

increasing proportion of the scope for growth of larger animals is channeled into reproduction such that at some maximum size somatic growth virtually ceases. This is clearly illustrated (Figure 3) in the energy budget of the oyster Ostrea edulis (Rodhouse 1978). The amount of energy allocated to somatic growth in oysters under 5 years (equivalent to an animal of about 50 mm shell height) is higher than that allocated to germinal production but in older animals somatic growth virtually ceases.

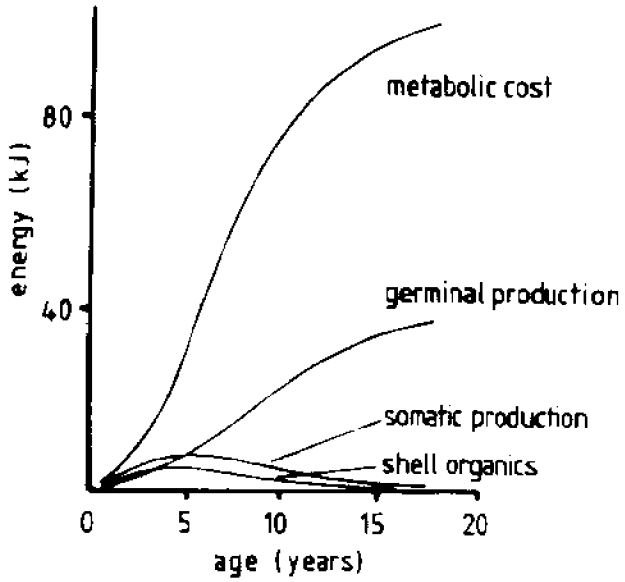

Figure 3. Age dependent metabolic costs and annual production of germinal tissue, somatic tissue, and shell organics in terms of the energy expended for the oyster Ostrea edulis (after Rodhouse 1978).

A significant proportion of the absorbed energy is not used for either somatic or germinal growth but is incorporated into the matrix of the shell. The mean shell energy content, expressed as a percentage of the total tissue energy count, is 35.3% for a number of different bivalve species (Table 1). There is wide variation in these percentages because of different shell thicknesses e.g. for the thin shelled Scrobicularia plana the dry tissue weight is equivalent to 28.1% of the shell weight whereas for the heavy shelled Crassostrea virginica the tissue is only 2.6% of the shell weight. Also, as the tissue weight varies with the seasonal changes in reproductive condition the relationship between the energy content of the tissue and the shell is not constant. These values do not indicate that a third of the absorbed energy is incorporated into the shell matrix each year, as the shell has been gradually built up over the animal's entire life, whereas of the body tissues, only the somatic portion accretes and the energy allocated to germinal production is largely lost as gametes each year. Little is known of the energetic cost to the animal of producing the shell as it is simply considered part of the metabolic demand. However, there are differences of up to 45% in the shell weight of similar sized mussels originating from different habitats (Meyers 1980). It would be of

interest to know if a reduction in shell weight significantly improves an animal's scope for growth. Furthermore, for certain bivalves, e.g. the mytilids, the production of byssus threads is also a drain on the absorbed ratio which can represent up to 8-15% of the total tissue energy content (Griffiths and King 1979).

Table I. The Energy Content of Various Bivalve Shells, Expressed as a Percentage of Their Tissue Energy Content, Calculated Using the Information Supplied in Each Reference

Species (Reference)	Shell			Tissue		Energy content of shell as % of tissue energy content
	Length (mm)	Dry wt (g)	Energy content (KJ)	Dry wt (g)	Energy content (KJ)	
Scrobicularia plana (Hughes 1970)	50	3.2	0.27	0.9	18.8	1.4
Crassostrea virginica (Dame 1972)	8.0 g[a]	5.76	1.19	0.15	3.18	37.4
Mytilus edulis (Jørgensen 1976)	35-40	1.3	4.3%[b]	0.28	NG	20.0
Geukensia demissa (Jørgensen 1976)	60	9.3	5.4%[b]	1.15	NG	43.6
	75	15.3	6.0%	1.81		50.7
Ostrea edulis (Rodhouse 1978)	100	NG	46.4	3.63	78.9	58.8
					Mean	35.3

[a]Whole wet weight of an oyster.

[b]Values are % weight loss on ignition at 450°C.

NG = not given.

The majority of energy converted to germinal growth is liberated as gametes and is therefore not reflected as in increase in marketable meat, unless the animal is harvested when gravid. Currently attempts are being made to produce sterile triploid oysters (Hidu et al. 1981) which would possibly grow faster than normal diploid animals. More of such research is required on the breeding of specialized strains of bivalves, especially suited to mariculture, which incorporate both the genetic advantages of heterzygosity (Koehn and Shumway 1982) and triploidy.

In certain situations, e.g. with low pollution levels or environmental changes leading to reduced food availability, bivalves may experience stress which is not severe enough to cause adult mortality. Instead, it may have a subtle but deleterious effect on the animals' energy budget (Bayne et al. 1979). Bivalves fed below the maintenance ration seem to produce fewer eggs, which weigh less than normal eggs because of a reduction in their nutrient content (Sastry 1975; Bayne et al. 1978). This means that when the eggs are spawned and fertilized the resulting larvae with lowered nutrient reserves may have a reduced probability of successfully attaining metamorphosis (Bayne et al. 1975). In some species, starvation may also result in the absorption and

utilization of the gonads for metabolic maintenance (Bayne et al. 1978). These findings indicate that endogenous mechanisms that regulate energy partitioning between metabolic processes and somatic or germinal growth are extremely complex and incorporate feedback loops which are dependent on the animal's physiological and reproductive condition. It is perhaps only by studying such control mechanisms that we can fully interpret the mollusc's responses to changing environmental conditions.

CONCLUSIONS

1) Energy budgets are useful techniques for integrating a number of measurements so as to provide a single index (the Scope for Growth) of an animal's ability to survive, grow and reproduce in a particular environmental regime.

2) Although the concept of energy flow is useful, there is a need to trace individual nutrients through the animal both in the laboratory and in the environment.

3) Bioenergetic techniques should be applied to mariculture so as to provide information on optimum feeding levels and growth potentials.

4) Although a large number of bioenergetic techniques have been developed and applied effectively there are still areas of ignorance. These mainly concern the loss of energy in the excreta and the mechanisms, presumably hormonal or neurosectory, that govern the differential allocation of energy between growth and reproduction in bivalves.

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WEINER (U. of Maryland): Available energy is also a very important consideration. Has anyone looked at adenylate energy charge, that is E. C. = (ATP + 1/2ADP)/(ATP + ADP + AMP), under, for example, various dietary regimes, oxygen levels or stages in the life cycle of the animal?

NEWELL: Yes, a group of scientists (Rainer et al. 1979) have used the energy charge as a means of assessing the severity of an environmental stress on molluscs. Although they obtained good measurements of all of the adenine nucleotides they concluded that the use of adenylate energy charge does not provide an absolute measure of stress in molluscs. I am not aware of any extensive monitoring of energy charge on a seasonal basis but I agree that it could provide a useful index of the animal's metabolism.

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PHYTOPLANKTON AS A FOOD SOURCE FOR BIVALVE LARVAE

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ABSTRACT

Although the nutritional requirements of bivalves are not entirely known, it is generally accepted that bivalve molluscs are dependent on phytoplanton as their principal food source. Suitability of a phytoplankton species used in an algal diet for cultured bivalves may be characterized by its digestibility, chemical composition, non-toxicity and size. In this paper, the results of our recent work and that of other investigators on the amino acid, fatty acid and carbohydrate compositions of algal species used as food for oyster larvae are reviewed and discussed.

The nutritional value of an algal species has not been found to be correlated with its total lipid or carbohydrate content. Higher concentrations of total protein within the algal cells appear related to good food quality in the algal species we studied. In general, the amino acid, fatty acid and carbohydrate compositions of algae are qualitatively similar but there are quantitative differences which appear related in part to their suitability as food. There is also evidence indicating that 20:5ω3 and 22:6ω3 fatty acids are essential for oysters and that the quantity of total ω6 fatty acids in the diet may affect growth and metamorphosis of oyster larvae.

KEYWORDS: Oyster larvae, nutrition, carbohydrates, fatty acids, amino acids, algal composition.

INTRODUCTION

The search for algal diets that promote high survival and rapid growth of cultivated bivalves has been a subject of investigation for at least the last five decades. Currently, phytoplankton species which have been successfully isolated, cultured and utilized as food sources at various marine laboratories and hatcheries include the following: Pyramimonas virginica (Pennick), Pseudoisochrysis paradoxa (F. Ott, nom. nud.), Pavlova (Monochrysis) lutheri (Droop) Green, Isochrysis galbana (Parke), Thalassiosira pseudonana (Hustedt) Hasle and Heimdal.,

Dunaliella sp., Cyclotella sp., Skeletonema costatum (Greville) Cleve, Phaeodactylum tricornutum (Bohlin), Chaetoceros sp., Chaetoceros sp., Chaetoceros calcitrans (Paulsen) Takano, Tetraselmis suecica (Butcher), Chlorella sp. In general, bivalve molluscs fed on diets consisting of more than one species of these algae grow faster and are more vigorous than those fed on single species diets.

The criteria required for a phytoplankton species to be a potential food source for bivalves are: 1) the algal cell must be within an acceptable size range, 2) non-toxicity, 3) possession of a digestible cell wall, and 4) adequate nutritonal value. These criteria will be discussed in detail in this paper.

PHYSICAL AND TOXICITY EFFECTS OF ALGAE ON FOOD VALUE

It is widely accepted that phytoplankton constitute the principal food of bivalve larvae, but not all phytoplankton species are equally suitable as dietary components. The "good" food value of some algae is thought to be related to their small cell size, absence of a thick cell wall and the production of little or no toxic metabolites (Ukeles 1970; Owen 1974). Both filtration efficiency and ingestion are affected by cell size (Hughes 1969; Haven and Morales-Almo 1970); e.g. adult Crassostrea virginica (Gmelin) have been reported to filter particles from 3 to 12 μm with the same relative efficiency and those from 1 to 3 μmwith efficiencies reduced by 1/3 to 1/2 (Haven and Morales-Almo 1970). Similar work has not been carried out with oyster larvae; however, veliger larvae of Mytilus edulis (L.) are reported to clear particles in the range from 1 to 9 μm, with those in the range of 2.5-3.5 μm being cleared at the fastest rate (Riisgard et al., 1980). The lower limit for C. virginica larvae in the range of 1 to 2 μm since Nannochloris oculata (Droop) is in this size range and has been used successfully as a larval food (Dupuy 1975). The suitable size range of food particles may differ between species as well as between life history stages of the same species.

The composition and/or thickness of the cell wall is regarded as one of the most important factors affecting digestibility. Walne (1974) measured larval growth of Ostrea edulis (L.) fed several species of Chlorella and Coccomyxa and reported that some species of these two genera supported little or no growth. He suggested that the thick cell walls of these species could not be digested by the larvae. Babinchak and Ukeles (1979) observed ingestion, lysis and digestion or rejection of two algae, P. lutheri and Chlorella autotrophica (Shihara and Krauss) by larvae of C. virginica using epifluorescence microscopy. They found that larvae fed C. autotrophica did not grow. Although C. autotrophica was readily ingested by the larvae, no lysis or digestion was observed in the gut. The diatom P. tricornutum has also been found to be a poor food for bivalves (Epifanio and Mootz 1976; Epifanio et al. 1981). We have found P. tricornutum to be unsatisfactory as a food supplement for ripening adult oysters for spawning at the Virginia Institute of Marine Science (VIMS). Epifanio and his colleagues (Epifanio et al. 1981; Epifanio 1982) suggested that the nutritional inadequacy of P. tricornutum is possibly due to either it's indigestibility or to its lack of tryptophan (Tables VI and VIII).

Some chlorophyte species such as Chlorella sp., Chlamydomonas sp., Stichococcus sp. and one chrysophyte Prymnesium parvum (Carter) have been found to be toxic to oyster and clam larvae (Davis and Guillard 1958). The toxicity of these algal species may be due to the production of toxic metabolites, or to the presence of certain toxic species of bacteria in the algal culture. The poor food value of some species such as Chlorella sp. has been suggested to be caused by their non-motility, which results in a reduction in available food due to cell settlement. This hypothesis has been rejected by Babinchak and Ukeles (1979, see above). However, some investigators have reported that a mixed algal diet containing Chlorella sp. can serve as an adequate food for some species of bivalve larvae (Davis 1953; Dupuy et al. 1977).

ALGAL DIETS FOR CULTURED BIVALVES

Martin (1927, 1928) demonstrated that oysters are capable of growing on a diet consisting solely of cultured algae. Cole (1937, 1938) first raised bivalve larvae on a diet of small naked flagellates and Bruce (1940) cultured larvae of O. edulis on a large scale using an algal diet. Later studies on the use of phytoplankton by C. virginica larvae were carried out by Davis (1950, 1953).

Dean (1957) compared the food value of the diatom, Skeletonema costatum to that of a mixture of algae consisting mainly of Cryptomonas sp. and Chlamydomonas sp. He found that the mixture was a better diet than Skeletonema alone. Since then there have been many investigations on the nutritional value of various algal diets for bivalves (e.g. Davis and Guillard 1958; Loosanoff and Davis 1963; Walne 1963).

Walne (1970) reported studies evaluating 25 species of algae as food for several juvenile bivalve species. He found that the growth rates of juvenile bivalves depended on the species of algae and the concentration of algal cells in the water. Furthermore, the relationship between the quantity of algae fed and larval concentration was critical to the growth and survival of the larvae. The optimal food concentration also varies with the stage of the larvae's life history (Schulte 1975; Dupuy et al. 1977); thus optimal larval and food concentrations must be ascertained for each species and life history stage cultured.

Feeding experiments with larvae and juvenile bivalves indicate that those fed on a diet consisting of more than one species of alga usually grow faster and are more vigorous than those fed only one species (Walne, 1970, 1974; Loosanoff and Murray 1974; Epifanio and Mootz 1976; Helm 1977; Mann and Ryther 1977; Epifanio 1979). The standard (CCP) diet for oyster larval culture at VIMS is a combination of three algal species, Chlorella sp., P. virginica and P. paradoxa (Dupuy et al. 1977). It is likely that a mixed algal diet provides the bivalve with a better balance of nutrients such as amino acids, fatty acids and sugars as well as micronutrients, e.g. vitamins and minerals. Thus some nutrient component deficient in one species may be made up by its presence in another species. This is illustrated in the section below on amino acid composition and Table VIII.

Lipid and fatty acid composition

The fatty acid composition of Chlorella sp. P. virginica and P. paradoxa vary only to a small degree with the age of the algal culture where conditions of light, salinity and temperature remain constant (Table I). These variations form no consistent pattern and do not appear to affect food quality. When these data from VIMS (Chu and Dupuy 1980) are compared to those from other laboratories, a greater variation in fatty acid composition is observed (see Table II), i.e. there are marked differences in total saturated and polyethylenic fatty acids for the algal species P. virginica, P. lutheri and I. galbana according to the source of the analysis. The ranges of variation for total saturated fatty acids and total polyethylenic fatty acids for the above three algal species are 26-45% and 31-46% respectively for P. virginica, 21-64% and 8-51% for P. lutheri, and 19-61% and 12-53% for I. galbana (Table II). These ranges of values are likely to be caused by different culture conditions, however, one should not exclude the possibility that variation in analytical techniques may also be responsible. It is also interesting to note that the total polyethylenic fatty acid content was comparatively lower for the algal species grown in our laboratory than for algae grown in other laboratories while the percentage of total saturated fatty acid was higher (Table II).

The nutritional value of lipids and fatty acids for bivalves has been examined by serveral groups of investigators (Millar and Scott 1967; Helm et al. 1973; Holland and Spencer 1973; Holland 1978; Waldock and Nascimento 1979; Chu and Dupuy 1980; Swift et al. 1980; Langdon and Waldock 1981). Researchers have demonstrated the importance of lipid in larval growth and development of oysters. Creekman (1977) reported that the lipid content of the egg and that of the resulting larvae of C. virginica were correlated and that a greater larval lipid content significantly increased larval growth, vigor, set and successful metamorphosis. Similarly, Helm et al. (1973) reported that the viability of O. edulis larvae was related to their lipid content, particularly the neutral lipid at the time of liberation. Holland and Spencer (1973) also indicated that accumulation of neutral lipid, from 8.8 to 23.2% occurred during development of O. edulis larvae. Collyer (1957) indicated that initial larval glycogen content was not related to larval viability. These results support the findings that lipid supplies most of the energy requirements of larvae during periods of both growth and starvation (Millar and Scott 1967; Holland 1978).

The growth rates of oyster larvae and spat have not been found to be directly related to the quantity of total lipid present in the algal diet (Table IV) but Waldock and Nascimento (1979) reported that the growth rate of Crassostrea gigas (Thunberg) larvae was correlated with the algae's neutral lipid content. Furthermore, they found that the fatty acid composition of the neutral lipid of the C. gigas larvae was similar to the fatty acid composition of the algal food, but the fatty acid composition of the phospholipid fraction was less dependent on diet composition (Table III).

In most cases, 14:0 and 16:0 are the dominant saturated fatty acids of algal foods and the range of total saturated fatty acids is 15-64% (for 37 of 38 species for which there are reported data). Bivalve adults and larvae contain 20-50% saturated fatty acids (Gardner and

Riley 1972; Ackman et al. 1974; Watanabe and Ackman 1974; Langdon and Waldock 1981; Chu and Webb, unpublished data). This is also true for most other marine animal lipids for which there are data (Ackman 1980).

Table I. Changes of Weight Percentage of Some Fatty Acid Components in Algae during Batch Culture (from Chu and Dupuy 1980)

Algal species / Fatty acid component	Age of culture (days) 5	10	15	20
Chlorella sp.				
12:0	7.82	3.23	2.75	2.75
16:1	2.70	3.65	5.25	0.0
18:1	9.19	7.57	7.20	15.0
Total C 18	33.18	37.22	30.1	42.4
20:5ω3	0.17	0.17	0.10	0.50
22:6ω3	trace	trace	0.60	0.0
Total ω3	15.49	23.89	20.80	15.20
Total ω6	8.62	11.43	8.90	14.20
ω6/ω3	0.56	0.48	0.43	0.93
Pyramimonas virginica				
12:0	2.40	3.60	3.45	8.70
16:1	2.60	2.00	2.50	5.75
18:1	3.10	3.10	3.90	15.70
Total C 18	21.30	21.05	22.40	26.10
20:5ω3	1.55	0.0	2.50	0.0
22:6ω3	2.60	3.05	2.75	1.40
Total ω3	23.15	27.05	28.10	13.48
Total ω6	6.85	8.65	5.65	10.10
ω6/ω3	0.30	0.32	0.20	0.75
Pseudoisochrysis paradoxa				
18:1	16.90	18.13	18.20	21.25
Total C 18	24.87	27.59	26.35	30.60
20:5ω3	0.20	0.40	0.05	0.30
22:6ω3	1.87	3.10	2.20	2.05
Total ω3	8.18	8.80	7.85	8.25
Total ω6	3.94	5.13	3.35	4.50
ω6/ω3	0.48	0.59	0.43	0.55

Langdon and Waldock (1981) have shown the essentiality of ω3 fatty acids for the spat of *C. gigas*. They reported that a deficiency of both the fatty acids 20:5ω3 and 22:6ω3 in *D. tertiolecta* limited the growth of *C. gigas* spat; supplementation of the *D. tertiolecta* algal diet with encapsulted 22:6ω3 improved growth. Growth was satisfactory on a diet of *T. suecica* which contained 20:5ω3 but not 22:6ω3. These data were interpreted to indicate essentiality for long chain polyunsaturated ω3 fatty acids but no specific requirement for either 20:5ω3 or 22:6ω3 for *C. gigas*. It should be noted, however, that *P. lutheri* contained both 20:5ω3 and 22:6ω3 in proportions similar to the oyster tissue and yet was a poorer food than *T. suecica* (Langdon and Waldock 1981); this reinforces the idea that factors other than fatty acid composition may also influence algal food quality (see Introduction).

Table II. Fatty Acid Composition of Some Algal Species Used as Food for Larvae, Spat and Adult Oysters. The data from Chu and Dupuy (1980) are means of the data from 4, 10, 15, and 20 day old batch cultures.

Fatty acids	Chlorella sp. (1)	Pyramimonas virginica (1)	Pyramimonas virginica (2)	Pseudoisochrysis paradoxa (1)	Pavlova lutheri (3)	Pavlova lutheri (4)	Pavlova lutheri (5)	Pavlova lutheri (6)	Isochrysis galbana (2)	Isochrysis galbana (6)	Isochrysis galbana (7)	Chaetoceros calcitrans (2)	Dunaliella tertiolecta (3)	Tetraselmis suecica (3)
Saturated														
8:0	-	-	-	-	-	-	-	-	-	1.47	-	-	-	-
10:0	-	-	-	-	-	-	-	4.11	-	4.02	-	-	-	-
12:0	4.14	4.54	-	2.61	-	0.20	0.60	13.07	-	11.14	0.60	-	-	-
13:0	1.02	1.09	-	0.61	-	-	0.20	2.16	-	3.10	0.20	-	-	-
14:0	2.78	3.46	1.50	23.74	11.20	11.20	9.20	14.85	7.80	13.30	10.60	1.30	1.9	5.20
15:0	0.22	0.59	0.50	0.32	0.30	0.20	0.40	1.12	1.60	1.23	1.30	0.30	0.80	0.40
16:0	30.09	29.51	19.90	17.46	22.80	15.10	10.10	17.83	8.80	16.41	22.00	6.40	16.0	25.40
17:0+16:2	3.26	2.33	-	0.71	-	-	0.40	1.87	-	1.66	-	-	-	-
18:0	2.16	3.71	3.6	1.23	1.50	-	0.40	5.88	-	4.21	2.30	2.20	0.70	1.80
19:0	-	-	-	-	-	-	-	-	0.70	-	-	-	-	-
20:0	-	-	-	-	-	-	-	1.77	-	2.16	-	-	-	-
22:0	-	-	-	-	-	-	-	2.85	-	2.38	-	-	-	-
Total	43.69	45.08	25.50	46.65	35.80	26.50	21.30	64.39	18.90	61.08	37.00	10.20	19.40	32.80
Monoethylenic														
14:1	0.34	3.70	1.40	0.97	-	0.20	-	0.98	0.20	1.32	-	1.90	0.4	trace
16:1	2.88	3.21	1.50	3.92	25.80	25.4	20.20	6.68	0.80	6.48	15.70	15.40	4.60	3.80
18:1	9.74	6.45	9.60	18.62	5.80	3.40	5.70	7.66	10.40	7.78	-	11.40	7.00	22.7
20:1	0.61	trace	0.90	0.88	-	0.60	-	0.84	-	0.60	13.50	0.90	-	2.85
22:1	6.40	0.13	0.60	0.26	-	-	-	0.84	1.00	0.81	1.20	0.90	-	-
Total	14.93	13.49	14.00	24.34	31.60	29.60	25.90	17.00	12.40	16.99	30.40	30.50	12.00	29.35
Polyethylenic														
16:2ω7	-	-	-	-	-	0.20	1.30	-	-	-	-	-	-	-
16:2ω6	-	-	1.5	-	0.50	-	-	-	-	-	-	5.80	-	-
16:2ω4	-	-	-	-	-	1.7	4.60	-	-	-	-	-	-	-
16:3ω6	-	-	0.4	-	trace	-	-	-	1.20	-	-	4.70	1.70	trace
16:3ω4	-	-	-	-	-	0.5	14.80	-	-	-	-	-	-	-
16:3ω3	4.37	8.47	1.10	1.42	trace	0.1	-	-	trace	-	0.40	7.50	1.80	0.60
16:4ω1	-	-	-	-	-	0.3	1.50	-	-	-	-	-	-	-
16:4ω3	-	-	3.80	-	trace	-	-	-	-	-	-	1.90	12.4	11.00
18:2ω6	9.94	2.18	2.60	3.59	2.80	0.70	1.60	1.40	4.30	1.31	2.30	0.20	8.80	1.20
18:3ω6	0.67	-	0.90	-	trace	0.10	-	-	0.20	-	0.20	1.40	3.50	trace
18:3ω3	13.05	5.28	10.60	2.06	0.60	0.20	-	1.07	11.0	2.05	0.40	3.00	31.10	9.80
18:4ω3	0.68	5.10	4.40	1.86	3.60	4.00	0.60	-	9.20	-	8.00	0.50	0.50	3.50
20:2ω6	0.58	2.24	-	0.58	trace	0.30	-	-	-	-	-	-	0.30	trace
20:3ω6	-	-	-	-	-	0.10	1.70	-	-	-	-	-	-	-
20:3ω3	0.20	0.20	-	0.29	-	0.10	-	-	-	-	-	-	-	-
20:4ω6	0.60	3.40	-	0.14	0.70	0.40	-	-	0.20	-	0.10	-	-	0.70
20:4ω3	0.85	-	1.60	0.07	-	0.10	0.10	4.86	0.50	5.48	-	-	-	0.60
20:5ω3	0.23	2.03	1.30	0.18	13.80	16.30	18.00	trace	3.60	0.87	7.20	15.40	-	7.70
22:2ω6	-	-	0.40	-	-	0.50	-	-	-	-	-	-	-	-
22:4ω6	-	-	0.50	-	-		-	-	-	-	-	-	-	-
22:4ω3	0.17	-	-	-	-		-	-	-	-	-	-	-	-
22:5ω6	0.35	-	1.8	-	1.10	1.20	-	-	4.30	-	-	3.30	-	-
22:5ω3	0.25	0.78	1.4	0.37	trace	0.40	0.5	0.51	trace	1.04	-	-	-	-
22:6ω3	0.60	2.45	13.70	2.31	7.90	13.10	3.30	-	18.90	1.08	4.30	1.90	-	-
Total	29.63	30.76	46.00	12.52	31.00	40.20	50.50	7.84	53.40	11.83	22.90	45.60	60.01	35.10

References: (1) Chu and Dupuy 1980; (2) Waldock and Nascimento 1979; (3) Langdon and Waldock 1981; (4) Ackman et al. 1968; (5) Chuecas and Riley 1969; (6) Chu and Webb, unpublished data; (7) Watanabe and Ackman 1974.

Table III. The Percentage Fatty Acid Composition of the Neutral Lipids and Phospholipids of Larvae of Crassostrea gigas Cultured on Different Algal Diets and the Total Fatty Acid Composition of the Algal Diets (from Waldock and Nascimento 1979)

Fatty acid	Lipids of larvae fed on:				Algal lipids		
	Chaetoceros calcitrans		Pyramimonas virginica	Isochrysis galbana	C. calcitrans	P. virginica	I. galbana
	Phospho-lipid	Neutral	Neutral	Neutral	Total	Total	Total
14:0	0.8	3.9	0.8	3.9	1.3	1.5	7.8
14:1	trace	trace	0.3	0.3	1.9	1.4	0.2
15:0	0.3	0.2	0.5	0.3	0.3	0.5	1.6
16:0	17.1	8.1	18.2	12.1	6.4	19.9	8.8
16:1ω9	trace	trace	0.8	0.3	trace	trace	trace
16:1ω7	1.4	12.65	1.1	0.7	15.4	1.5	0.8
16:2ω6	1.0	3.05	1.1	0.4	5.8	1.0	trace
16:3ω6	0.3	1.95	0.6	0.3	4.7	0.4	1.2
16:3ω3	1.2	3.85	0.9	0.4	7.5	1.1	trace
16:4ω3	1.6	0.2	1.9	trace	1.9	3.8	trace
17:0	trace	trace	trace	trace	trace	trace	trace
18:0	4.7	2.0	4.4	2.2	2.2	3.6	0.7
18:1ω9	1.2	1.3	3.1	11.7	0.8	2.5	7.2
18:1ω7	11.1	15.7	9.6	5.2	10.6	7.1	3.2
18:1ω5	trace	trace	trace	trace	trace	trace	trace
18:2ω6	0.5	0.55	1.5	6.5	0.2	2.6	4.3
18:3ω6	1.8	1.00	0.5	trace	1.4	0.9	0.2
18:3ω3	0.4	2.35	7.5	10.6	3.0	10.6	11.0
18:4ω3	trace	0.8	3.9	8.9	0.5	4.4	9.2
unknown	-	-	-	-	-	-	8.7
19:0	1.9	2.0	trace	trace	trace	trace	trace
20:1ω9	6.4	0.15	0.9	0.6	0.9	0.9	-
20:1ω7	trace	2.65	2.7	1.4	-	-	-
20:1ω5	-	trace	trace	-	-	-	-
20:2ω6	0.6	0.2	trace	0.4	trace	trace	trace
20:2ω9a	-	trace	0.5	-	-	-	-
20:3ω6	-	0.1	0.3	-	trace	-	-
20:4ω6	-	0.85	0.5	0.8	trace	-	0.2
20:4ω3	-	0.15	3.4	0.4	-	1.6	0.5
20:5ω3	14.3	20.45	4.1	3.2	15.4	1.3	3.6
22:1ω13+11	-	-	-	trace	-	-	-
22:1ω9	trace	0.15	0.3	trace	-	-	-
22:1ω7	trace	0.4	0.3	trace	0.9	0.6	1.0
22:1ω5	-	0.4	0.3	0.5	-	-	-
22:2ω6	-	trace	trace	trace	trace	0.4	0.2
unknown	9.5	1.4	0.7	trace	-	-	-
22:3ω3	1.6	-	-	0.4	-	trace	-
22:4ω6	0.8	0.5	1.3	0.6	-	0.5	trace
22:5ω6	2.2	1.85	0.8	3.7	3.3	1.8	4.3
22:5ω3	0.5	0.5	1.8	0.3	-	1.4	trace
22:6ω3	-	-	-	-	1.9	13.7	18.9
24:1ω9	-	-	-	-	-	0.5	0.2
unknown	-	-	-	-	1.4	2.6	1.1
total ω6	7.2	10.05	6.5	6.2	16.5	8.10	10.3
total ω3	19.6	28.3	23.5	24.21	32.1	40.20	43.3
ω6:ω3	0.37	0.35	0.27	0.27	0.51	0.20	0.23

Table IV. Lipid and Fatty Acid Content of Algal Species Utilized as Food for Larvae and Spat of Crassostrea virginica or Crassostrea gigas

Algal diet	Food value	Total fatty acids		Total lipid		Total		ω6:ω3 ratio	Presence of fatty acid	
		% dry wt	µg x 10^{-7} per cell	µg x 10^{-7} per cell	µg/mg dry wt	ω3 wt %	ω6 wt %		22:5ω3	22:6ω3
I. galbana	moderate[1]	12.5[1]	0.36[1]	72.36[4]	174[3]	43.3[1]	10.3[1]	0.24[1]	+1	+1
						10.52[4]	1.31[4]	0.12[4]	+4	+4
P. virginica	moderate[1] good[5]	4.2[1]	0.15[1]	15.22[2]	-	40.2[1]	8.1[1]	0.20[1]	+1	+1
						26.2[2]	7.15[2]	0.27[2]	+2	+2
C. calcitrans	good[1,3]	9.7[1]	0.21[1]	-	-	32.1[1]	16.5[1]	0.51[1]	+1	+1
P. paradoxa	good[5]	-	-	22.91[2]	-	8.38[2]	4.29[2]	0.51[2]	+2	+2
Chlorella sp.	good[5]	-	-	1.48[2]	-	22.35[2]	10.19[2]	0.46[2]	+2	+2
P. lutheri	moderate[3]	-	-	59.85[4]	-	6.44[4]	1.40[4]	0.22[4]	+4	-4
						25.90[3]	5.10[3]	0.20[3]	+3	+3
T. suecica	good[3]	-	-	-	61.8[3]	33.2[3]	2.1[3]	0.06[3]	+3	-3
D. tertiolecta	poor[3]	-	-	-	167[3]	45.8[3]	15.8[3]	0.35[3]	-3	-3

References: 1) Waldock and Nascimento 1979; 2) Chu and Dupuy 1980; 3) Langdon and Waldock 1981; 4) Chu and Webb, unpublished data; 5) Dupuy et al. 1977.

Both ω6 and ω3 fatty acids have been shown to be essential for many animals. Some mammalian species such as the rat have a high requirement for ω6 fatty acids and a low requirement for ω3 fatty acids (Tinoco et al. 1978). For the rainbow trout, Salmo gairdneri (L.), and the prawn, Penaeus japonicus (Bate), the situation is reversed and ω3 fatty acids are more important (Yu and Sinnhuber 1972; Watanabe et al. 1974; Kanazawa et al. 1977). German carp, Cyprinus carpio (L.), apparently require both ω6 and ω3 fatty acids in the diet (Watanabe et al. 1975). Oyster larvae may also require both ω3 and to a lesser extent ω6 fatty acids; this possible dual requirement has been suggested for adult C. virginica (Trider and Castell 1980). Most algae (four of five species) which have been reported to be "good" foods for oyster larvae have high rations of ω6 to ω3 fatty acids (approximately 1:2 to 1:3) compared to a ratio of 1:5 in the other algal species regarded as "moderate" foods (Table IV). D. tertiolecta has a high ratio of ω6 to ω3 but may be a poor food as a result of deficiencies of 20:5ω3 and 22:6ω3 fatty acids (Langdon and Waldock 1981).

Carbohydrate Composition

A summary of carbohydrate weight percent composition of 8 algal species which are used as food for bivalves is shown in Table V. Results of our investigation (Chu et al. 1982) agree with those of other investigators (Parsons et al. 1961; Handa and Yanagi 1969). The principal sugars present are glucose, mannose, ribose, xylose, rhamnose and galactose. Glucose and mannose are the major sugars in the 5 algal species (P. virginica, P. paradoxa, Chlorella sp., I. galbana and P. lutheri) that we studied and in the 3 diatoms (Chaetoceros sp., Phaeodactylum tricornutum and Skeletonema costatum) examined by Handa and Yanagi (1969). Parsons and his colleagues (1961) reported that glucose was the principal sugar in 11 species of phytoplankton analyzed, but galactose was the next most abundant in 9 of the species. The

patterns of algal carbohydrate composition among the five algal species we studied were similar (Chu et al. 1982) and no major differences, qualitative or quantitative, in composition were found. There was no direct relationship between the nutritional value of an algal species and its carbohydrate content.

Table V. The Monosaccharide (including those hydrolyzed from polysaccharide) Weight Percentage Composition of Several Algal Species Used as Food for Bivalves. Only the principal sugars are tabulated. P. paradoxa = Pseudoisochrysis paradoxa.

Sugar	Pyramimonas virginica	P. paradoxa	Chlorella sp.	Pavlova lutheri		Isochrysis galbana	Chaetoceros sp.		Skeletonema costatum		Phaeodactylum tricornutum	
	(1)	(1)	(1)	(1)	(2)	(1)	(2)	(3)	(2)	(3)	(2)	(3)
Glycerol	trace	1.01	trace	1.76	-	0.27	-	-	-	-	-	-
Rhamnose	1.83	4.38	1.43	1.41	-	1.39	-	1.90	-	5.10	-	6.70
Ribose	1.30	4.28	5.00	5.27	1.30	1.77	0.71	-	1.20	0.60	0.72	2.80
Fucose	2.14	4.91	5.42	3.81	-	2.48	-	0.90	-	5.80	-	2.30
Fructose	-	1.15	2.24	0.60	trace	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	3.30	-	3.10
Xylose	-	-	-	-	3.5	-	0.40	4.60	0.4	4.30	0.70	10.90
Ribitol/xylitol	-	5.70	4.18	2.08	-	0.80	-	-	-	-	-	-
Mannose	11.05	30.19	18.32	15.20	-	13.87	0.79	27.2	0.87	30.30	3.70	33.20
Galactose	2.19	-	-	-	4.40	0.59	1.5	9.10	1.80	2.00	2.70	5.50
Glucose	80.37	48.93	63.50	67.15	22.10	77.40	3.3	54.50	16.40	48.60	10.70	35.10
Manitol	2.60	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	0.89	-	-	-	-	-	-	-	-	-	-
µg x 10^{-9}/cell	5.03	2.45	0.72	6.08	-	9.23	-	-	-	-	-	-
µg x 10^{-6}/µg wet algae	19.71	20.66	24.61	26.71	-	61.88	-	-	-	-	-	-
% of total carbohydrate	-	-	-	-	3.6	-	22.80	-	9.6	-	2.5	-

(1) Chu et al. 1982. The tabulated values are the means of individual monosaccharides hydrolyzed from polysaccharide (percent weight composition), total per cell (µg x 10^{-9}/cell) and total per µg wet algae (µg x 10^{-6}) of 4, 10, 15 and 21 day old algal cultures.

(2) Parsons et al. 1961. The tabulated values are percentage dry weight of cells.

(3) Handa and Yanagi 1969. The tabulated values are monosaccharide weight percentage composition of the whole algal cells.

The widely used algal food species, P. lutheri and I. galbana, both have carbohydrate concentrations (expressed in Table V as total monosaccharide content) that are higher than that of either P. virginica, P. paradoxa or Chlorella sp. which are currently used in the standard diet (CCP diet) in our laboratory. However, a mixture of these latter three species of algae results in faster growth, earlier setting and a higher percentage of setting success for C. virginica larvae (Windsor 1977) than with a mixture of P. lutheri and I. galbana. Parsons et al. (1961) suggested that the higher percentage of glucose in the readily hydrolyzable fraction of P. lutheri was possibly responsible for the high nutritional value of this alga but our results do not support this interpretation in reference to oyster larval nutrition. In our analyses (Chu et al. 1982), the weight percent of glucose in both P. virginica and Chlorella sp. were similar to those of P. lutheri and I. galbana. Chaetoceros sp. has also been found to be "good" food for larvae and spat of C. gigas (Walne 1970, 1974; Waldock and Nascimento 1979; Langdon and Waldock 1981) although the weight percent of glucose in this diatom species was either one of the lowest or intermediate of those algal species examined, depending upon the analysis (Table V). Only P. tricornutum, which was reported for other reasons to be an inadequate food for bivalves (Epifanio and Mootz 1976) had much lower

Table VI. The Amino Acid Molar Percentage Composition of Algal Species Used as Food for Bivalves. Species Pp = Pseudoisochrysis paradoxa; species Pv = Pyramimonas virginica. The data are recalculated from the listed references. + indicates detected but not determined.

	Pavlova lutheri				Isochrysis galbana		Pp	Pv	Chlorella sp.	Nannochloris oculata	Phaeodactylum tricornutum				Tetraselmis suecica			Dunaliella tertiolecta
	(1)	(2)	(3)	(4)	(1)	(5)	(1)	(1)	(1)	(1)	(1)	(2)	(3)	(4)	(1)	(5)	(6)	(1)
Cysteic acid	1.29	+	.00	.00	1.29	.00	.08	.08	.04	.25	1.05	+	.00	.00	.20	.00	1.17	.65
Taurine	.67	.00	.00	.00	.62	.00	.75	.75	.04	.15	.53	.00	.00	.00	.41	.00	.00	.14
Aspartic acid	8.38	17.07	9.36	9.33	8.36	9.49	9.74	9.76	9.24	9.08	9.28	10.13	9.53	9.90	8.18	9.34	9.24	7.51
Threonine	4.97	3.58	5.12	4.89	5.08	3.91	5.81	5.81	5.44	5.29	5.42	5.95	6.24	5.21	5.00	3.76	6.41	5.27
Serine	5.83	+	4.32	5.16	6.20	5.37	5.80	5.81	5.32	6.00	6.69	+	4.14	6.39	5.93	5.09	6.04	7.78
Glutamic acid	9.13	3.98	9.53	10.64	9.37	10.59	12.11	12.13	15.10	12.44	10.43	8.83	12.27	11.22	12.03	11.41	9.61	11.06
Proline	4.52	+	5.97	4.19	4.51	5.75	4.87	4.88	5.19	5.27	2.46	+	5.45	6.62	6.82	3.99	3.45	4.26
Glycine	9.45	5.80	8.47	5.77	9.33	10.62	9.70	9.72	9.23	9.65	9.77	10.67	9.55	9.40	9.68	12.25	10.47	9.57
Alanine	11.82	17.54	15.30	8.41	11.63	11.84	11.60	11.62	10.97	11.58	10.25	11.63	16.25	9.95	10.16	12.14	13.43	12.59
Valine	6.33	2.50	4.10	6.65	5.81	7.64	6.40	6.41	6.24	5.53	6.16	4.38	5.56	8.08	4.41	7.53	6.53	5.56
Cystine	.00	.00	.00	1.47	.00	.41	.51	.51	.40	.46	.00	.00	.00	.79	1.31	.21	.00	.05
Methionine	1.57	+	2.78	2.71	.91	1.89	.61	.61	.22	.29	1.60	.00	1.89	1.81	.26	1.67	2.59	.88
Isoleucine	4.14	2.70	4.55	4.37	3.81	5.05	3.97	3.98	4.08	3.69	4.82	4.38	4.39	4.68	2.73	4.55	4.19	3.43
Leucine	10.14	.00	8.91	10.01	9.64	9.17	8.76	8.77	8.30	8.86	8.69	.00	7.32	8.01	7.14	9.19	8.63	8.48
Tyrosine	1.57	+	2.44	4.54	2.34	2.10	2.51	2.51	2.47	2.48	2.18	.00	2.62	2.53	2.12	2.20	2.22	2.58
Phenylalanine	4.53	1.01	5.11	5.27	4.58	4.08	3.88	3.89	4.12	4.08	5.01	7.53	6.59	4.26	3.54	4.44	4.07	3.87
Ornithine	.13	.00	.49	.00	.37	.00	.14	.14	.13	.89	1.46	.00	.00	.00	.62	.00	.00	1.42
Lysine	5.93	6.61	6.39	6.82	5.55	4.95	4.83	4.84	5.63	5.31	5.49	2.46	4.29	5.84	4.84	5.61	5.91	6.01
Tryptophan	.00	.00	.00	2.03	.01	.18	1.53	1.53	1.23	1.76	.00	.00	.00	.00	1.12	.30	.00	.05
Histidine	.01	.00	1.42	2.10	1.97	1.66	1.57	1.58	1.59	1.70	1.43	.00	.58	1.54	1.62	1.76	1.73	1.84
Arginine	5.44	.00	5.74	5.65	5.17	5.28	4.38	4.39	4.26	4.07	4.67	.00	3.31	3.79	5.57	4.57	4.31	4.37
γ-Aminobutyric acid	.00	.00	.00	.00	2.62	.00	.07	.07	.69	1.16	.00	.00	.00	.00	2.31	.00	.00	.00
Total	95.84	60.80	100.00	100.00	99.18	100.00	99.61	99.77	99.93	100.00	97.38	65.96	100.00	100.00	95.99	100.00	100.00	97.39

References: (1) Webb, Chu and Dupuy, unpublished. (2) Parsons et al. 1961. (3) Chau et al. 1967. (4) Cowey and Corner 1966. (5) Epifanio 1979. (6) Walne 1970.

concentrations of glucose (Table V). Furthermore, it is possible that carbohydrates, which have been reported to have high nutritional value for juvenile and adult oysters (Haven 1965; Ingole 1967; Dunathan et al. 1969; Castell and Trider 1974; Flaak and Epifanio 1978) may not be as important as lipid in determining food quality of algae for the larval stages (Millar and Scott 1967; Helm et al. 1973; Holland and Spencer 1973; Holland 1978; Waldock and Nascimento 1979; Chu and Dupuy 1980).

Protein and Amino acid composition

The literature suggests that the amino acid composition of phytoplankton protein is remarkably similar regardless of species (Table VI) and that differences in nutritional quality of phytoplankton species are unlikely to be due to variation in amino acid composition (e.g. Parsons et al. 1961; Cowey and Corner 1966; Chau et al. 1967). However the amount of protein per unit volume of algal cell differs considerably among the species we analyzed (Table VII). *P. virginica* and *Nannochloris oculata* (Droop) contain more than the average protein per unit cell volume whereas *P. lutheri* and *I. galbana* contain less than the average. These data seem to be correlated with the findings of Windsor (1977) who concluded *P. virginica* was the key component of the CCP diet for *C. virginica* larvae while *P. lutheri* and *I. galbana* were less satisfactory.

Table VII. Amino Acid Content per Unit Cell Volume for Algal Species Used as Foods for Bivalves. Free amino acids = FAA; bound amino acids = BAA. Mean BAA/μm^3 is 0.283 fmol.

Species	Cell volume (μm^3)	FAA/cell (fmol)	BAA/cell (fmol)	fmol BAA/μm^3	Deviation from mean fmol BAA/μm^3 for all species
Chlorella sp.	4.87	0.21	1.71	0.35	+0.067
N. oculata	5.58	0.26	2.77	0.50	+0.22
P. virginica	33.5	0.84	18.3	0.55	+0.27
P. paradoxa	47.7	0.81	9.88	0.21	-0.073
I. galbana	57.8	-	4.48	0.08	-0.20
P. lutheri	73.5	-	2.80	0.04	-0.24
T. suecica	390	20.2	96.7	0.25	-0.033

The conclusion that variations in amino acid composition of algal species is unimportant in nutritional quality is based on subjective inspection of the data rather than rigorous quantitative evaluation. One approach to such an evaluation is to compare the amino acid composition of the bivalve species to that of the same amino acids in the food source (Phillips and Brockway 1956). The nonessential amino acids need not be considered since they can be synthesized by the bivalve. Therefore, it is necessary to determine which amino acids are essential as well as the animal's overall amino acid composition. These data are unavailable for most bivalve species but Harrison, (1975) has determined them for the mussel, *Mytilus californianus* (Conrad) (Table VIII).

Table VIII. A Comparison of the Essential Amino Acids for *Mytilus californianus* and the Same Amino Acids in Algal Diets. Amino acid values are molar percent composition for *M. californianus* tissue and index of adequacy ([% composition in algae/% composition in mussel] x 100) for algal diets, i.e., numbers less than 100 indicate deficiency. Algal compositions are means from Table VI with the data of Parsons et al. (1961) omitted due to its incompleteness. *Mytilus* data from Harrison (1975).

Essential amino acid	Molar % composition of mussel tissue	Adequacy index										
		Pavlova lutheri	*Isochrysis galbana*	50% *P. lutheri* 50% *I. galbana*	*Pseudoisochrysis paradoxa*	*Pyramimonas virginica*	*Chlorella* sp.	CPP diet[a]	*Nannochloris oculata*	*Phaeodactylum tricornutum*	*Tetraselmis suecica*	*Dunaliella tertiolecta*
Threonine	14.45	60.94	61.59	61.27	77.13	81.77	77.13	79.36	75.67	78.44	72.45	78.25
Proline	4.22	200.86	240.78	220.82	236.17	234.99	252.40	241.02	258.65	231.04	233.58	216.84
Valine	11.21	90.17	118.70	104.44	100.23	116.29	114.18	112.60	102.02	118.90	113.81	106.45
Methionine	4.62	97.79	60.02	78.90	20.87	26.93	9.61	20.03	13.09	76.73	67.46	40.71
Isoleucine	8.94	88.85	98.06	93.46	88.15	90.44	93.68	91.09	85.36	104.86	88.62	82.40
Leucine	15.84	111.20	117.51	114.36	109.43	112.59	107.50	110.31	115.69	101.94	108.86	114.88
Tyrosine	6.76	84.67	64.95	74.81	69.59	75.49	74.85	74.18	75.97	73.33	66.79	81.84
Phenylalanine	6.37	154.91	134.56	144.74	118.60	123.97	132.61	125.85	132.49	167.74	130.61	130.38
Lysine	12.41	97.06	83.75	90.40	80.58	79.28	92.93	84.08	88.57	84.86	91.08	103.87
Tryptophan	.55	221.56	35.23	128.39	537.84	569.60	463.78	528.42	665.63	.00	180.25	17.87
Histidine	2.96	77.79	121.32	99.56	121.15	108.23	109.86	111.19	119.19	80.81	119.19	133.74
Arginine	11.67	97.55	88.61	92.08	108.75	76.40	74.82	81.91	72.19	68.14	85.53	80.36
No. of AA with	90	4	6	3	5	5	4	5	6	7	5	6
rating below	80	2	4	3	3	4	4	3	4	5	3	3

[a]A combination of 33.3% *Chlorella* sp., 18.7% *P. virginica* and 48% *P. paradoxa* as recommended for oyster larvae by Dupuy et al. 1977.

Protein utilization for growth is most efficient when the food protein contains the essential amino acids in the same proportions as in the tissue being formed. A specific essential amino acid may be considered limiting when its proportional occurrence in the food is less than the proportional amount needed for growth. When one essential amino acid is limiting, all other essential amino acids found in greater amounts in the food will be under-utilized for growth and either used as an energy source or not assimilated. We calculated adequacy indices ([% composition of the essential amino acid in algae / % composition of the same essential amino acid in animal tissue] x 100; Table VIII) for a number of algal species potentially utilized as food for M. californianus, the bivalve for which the most complete tissue amino acid and essential amino acid data are available. The adequacy indices of the limiting essential amino acids for the algal species listed range from zero for tryptophan in P. tricornutum to sixty-seven for tyrosine in T. suecica. An index of 100 indicates the same proportion of essential amino acid in the food as in the M. californianus tissue. We ranked these algal species in descending order of predicted protein quality: T. suecica, > P. lutheri, > I. galbana, > P. virginica, > P. paradoxa, > D. tertiolecta, > N. oculata > Chlorella sp. > P. tricornutum. This ranking agrees with some reported relative food values for the algal species; for example, Walne (1970) reported that Tetraselmis species, including T. suecica are an outstanding food source for juveniles of O. edulis and C. gigas and Langdon and Waldock (1981) reported the same order in food value for T. suecica, P. lutheri and D. tertiolecta as suggested above on the basis of protein quality. P. tricornutum is also known to be a poor bivalve food (Walne 1970; Epifanio and Mootz 1976). The above ranking, however, is at odds with the recommended CPP diet of Windsor (1977) and Dupuy et al. (1977). The poor protein quality of this diet, according to the above adequacy index criteria, may be offset by having more protein per unit of algal cell volume, e.g. P. virginica (Table VI), than do higher ranking algal species. Under these circumstances limiting amino acids may be in adequate supply and the excess amounts of the other amino acids may be utilized for other purposes apart from growth.

Another approach to ranking either single or mixed species algal diets is to calculate the number of essential amino acids with adequacy indices below a predefined value; thus a small number indicates a better amino acid balance. For example, at the 90 level a combination of P. lutheri and I. galbana results in an improved amino acid balance compared with either species alone (Table VIII). With this means of evaluation, the values for the mixed species CPP diet recommended by Dupuy et al. (1977) are only equal or less than the various individual species of the diet and no improvement in amino acid balance is evident. However, it must be remembered that other factors apart from amino acid composition affect food quality.

CONCLUSIONS

Phytoplankton are regarded as the principal food source for bivalve larvae in natural conditions. To date, no food other than unicellular algae has been found to be entirely satisfactory for cultured bivalves. Some algae appear more nutritious than others. The nutritional inadequacy of algae may be due to one or more of the following factors:

improper size, indigestibility, deficiency in essential nutrients and toxicity.

Animals fed on mixed algal diets usually grow better and are more vigorous than those fed on single species diets. This is probably because a mixed diet provides a better balance of nutrients,e.g. a 1:1 mixture of P. lutheri and I. galbana provides a better balanced protein than does either algal species individually.

It seems likely that dietary lipid composition is more critical than protein or carbohydrate composition in determining the growth and development of bivalve larvae. Evidence indicates that 20:5 3 and 22:6 3 fatty acids may be essential for growth and development of C. gigas juveniles and that these essential fatty acids may be deficient in some algal foods. For oyster larvae, the w6 family of fatty acids may also be of nutritional importance. The protein content and amino acid composition of the algal cells may also affect larval growth.

Much more work is needed to determine the essential nutrients for growth of bivalves and their availability in algal species. The development of a satisfactory artificial diet will greatly facilitate these objectives.

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QUESTIONS AND ANSWERS

WALSH (Aquaculture Research Corp.): At ARC we use only diatoms for growing Mercenaria and the diatoms that we use are Thalassiosira (3H) and Skeletonemia species. This past summer we raised C. virginica entirely on these diatoms and were able to set clams in the normal time frame of 15 and 20 days. We put the spat into our recirculating system, which is also fed only Thalassiosira and Skeletonema, and were able to produce 25 mm oysters in less than 75 days. I was also interested in microencapsulation and I checked with Biogene, Inc. who have developed a patented technique for microencapsulation. The first question asked was "what do you want to put in the capsule?" I said that I knew that 3H or Sheletonema was good and that's what I wanted to encapsulate. From a commercial aquaculture point of view the amount of algae needed to raise the larvae is insignificant compared to the amount of algae you need to raise the post-set. So whereas microencapsulation may be of interest for study with larvae it is of little consequence when it comes to raising the larvae on commercial scale. Microencapsulated feed for juveniles is not competitive with the mass algal culture facilities that we currently run.

WEBB: It seems to me it might be commercially feasible for the early stages of the larvae where you would get a large yield for a relatively small amount of microcapsules, but there are other uses for these capsules. One, it is a good research technique for getting radioactive labelled substrates into the organism to trace pathways and figure out which are essential amino acids and the like. It might also be used to provide drugs if there is a health problem of the bivalve. It might be commercially feasible to do that.

WALSH: Agreed, again if there was an organism that I'd like to see studied for microencapsulation it would be 3H. I'd love to know in detail what it is because apparently 3H comes as close to perfection has larval and a post-set food for Mercenaria, and I would dare say for C. virginica, that you can currently find among the species of algae that are cultured. Another important aspect about 3H is that it can be mass cultured consistent by in reproducible manner on a commercial scale whereas these flagellates are notoriously difficult to mass culture.

WEBB: I don't know that I necessarily agree with you that flagellates are so difficult to culture compared to diatoms. I have been arguing with John Ryther for a number of years about how important diatoms are as food organisms. I think diatoms have been greatly overrated in the world's oceans as to how much of the primary production

that they actually do. And that's no doubt because they were easy to catch and look at and people thought they were doing 80 or 90% of the action when in fact they are probably doing 10 or 15% of the action, because the flagellates and little creatures that were really doing the dirty work never got caught and never got looked at and for the large part they're still not being looked at and somebody out there is eating them. Our own experience at V.I.M.S. was that diatoms did not make very good food for our oyster larvae, but of course genetics may enter into this. There is no doubt that there is a great deal of difference in oyster larvae and a great deal of difference in diatoms that are used. So I think this is an open question. I try to keep an open mind about diatoms.

PHYTOPLANKTON AND YEAST AS FOODS FOR JUVENILE BIVALVES: A REVIEW OF RESEARCH AT THE UNIVERSITY OF DELAWARE

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ABSTRACT

This paper is a synthesis of results of recent nutritional studies conducted in support of engineering work on the development of a controlled system for the culture of bivalves. Two lines of investigation are discussed, these concern the quantitative and qualitative aspects of an adequate ration for bivalves. Results of our experiments have shown that the efficiency of utilization of a given ration is a function of temperature; large rations can only be utilized at high temperature. Qualitiative differences in diets are best explained by differential digestibility of the food particles, and there is little correlation between chemical composition (gross proximate, amino acid, fatty acid) of a diet and its value as a food for bivalves. Synergistic nutritional effects of some dietary components may be due to improved balance of micronutrients or fatty acids.

KEY WORDS: Bivalves, diet, ration, nutrition.

INTRODUCTION

In the 1950s and 60s with the development of simple techniques for the culture of marine algae, significant work was undertaken on the qualitative aspects of the nutrition of bivalve larvae (see Ukeles 1971 for review), but it was not until 1970 that Walne published the first major work on the relative value of discrete algal species as food for post-larval bivalves. This was followed by work in my own laboratory wherein we investigated long-term growth of bivalves that were fed various combinations of algal species. These and other qualitative studies, together with some rather crude investigations of the biochemical and energetic bases for the observed qualitative differences, were the subject of a review that was written for the First International Conference on Aquaculture Nutrition (Epifanio 1976).

In the present paper I review the results of studies conducted in my laboratory since that time. This work has been mainly concerned with the quantification of adequate rations for bivalves and with the study of the qualitative differences in the food value of diets observed in our early experiments.

DETERMINATION OF RATION SIZE

Determination of the amount of food that an organism requires for maximum growth is obviously fundamental to the development of any system of animal husbandry. In the case of bivalves there have been numerous studies of the rates at which various species filter particles from suspension, but little of this has resulted in an estimation of ration size (see Winter 1978 for a review).

In order to assess maximum ration for Crassostrea virginica (Gmelin), we held groups of oysters in known concentrations of algal cells and quantified the amount of algae cleared from suspension during a 24 hour period (Epifanio and Ewart 1977). The concentration of cells in suspension was monitored hourly and the number of cells cleared by the oysters was replaced, i.e. the concentration of algae in suspension remained relatively constant over the 24 hour period. Suitable controls were maintained to account for algal growth and settling, and results indicated that the total number of cells removed from suspension was related to the size of the algal cells while the amount of algae filtered, in terms of dry weight, was a characteristic of the particular species of algae. In all cases, there appeared to be a maximum quantity of material that could be filtered regardless of how concentrated the suspension was. The oysters achieved this by increasing their filtration rate at low algal concentrations and decreasing it at higher concentrations. Based on this information, we were able to determine the daily ingestion (disregarding the small amounts of pseudofeces produced) of algal cells, in terms of dry weight, and to fit this to an equation that predicted daily ingestion for an oyster of any weight. In a later study using an automatic turbidostat, we refined our estimates to show that under some conditions oysters may ingest more food than predicted by our 1977 work (Higgins 1980). A particular case of this is discussed in the next section.

TEMPERATURE EFFECTS ON UTILIZATION OF RATION

Temperature is probably the most important factor affecting the magnitude of the optimal ratio for poikilothermic organisms. In order to test the effect of temperature on the quantity of food required by oysters, Muller (1978) measured growth and ingestion in groups of C. virginica at each of nine combinations of ration and temperature. The design of the experiment was based on the maximum weight-specific ration that was determined in our earlier work (Epifanio and Ewart 1977). The ration defined by that study was termed mid-level in Muller's experiment, and this was compared to a low ration (0.25 x mid-level) and a high ration (4 x mid-level). Temperatures were 18, 23, and 28°C. Algal concentrations varied from 1.8 mg dry weight ℓ^{-1} (low ration) to 30.2 mg ℓ^{-1} (high ration).

Results showed that the amount of food cleared from suspension was a function of ration rather than temperature (Table I). Oysters cleared approximately 16 times more food from suspension at the highest ratio than at the lowest, but there was virtually no difference in clearance over a 10°C range in temperature for a given ration. Growth, however, was highly dependent on both ration and temperature. At low ration and high temperature (28°C), growth of meat was inhibited and that of shell

favored. In contrast, growth of meat and shell appeared more highly coupled at the mid and high rations regardless of temperature. With the mid-ration there was little effect of temperature on growth of meat or shell. But a high ration, both meat and shell increased with increasing temperature. Growth at high temperature - high ration was by far the greatest of any combination tested and this was largely attributable to an accelerated growth of meat.

Table I. Crassostrea virginica. Quantity of algae removed from suspension (R) and growth of meat (P_m) and shell (P_s) of juvenile oysters fed the diatom Thalassiosira pseudonana at nine combinations of temperature and ration for 28 days. All values are in mg ash-free dry weight. Mid-level ration = 11.1 mg/g oyster (total wet weight). Mean live weight of oysters at the beginning of the experiment was 0.12 ± 0.02 g. Data from Muller (1978).

Parameter	Temp. (°C)	Ration		
		Low	Mid	High
R	28	407.8	1635.0	6531.0
	23	407.9	1634.0	7484.0
	18	408.2	1629.0	6435.0
P_m	28	12.0	81.5	254.1
	23	12.3	70.8	81.7
	18	23.0	82.2	38.0
P_s	28	15.1	43.0	50.1
	23	15.9	44.5	26.5
	18	9.9	40.3	11.1
$P_m:P_s$	28	0.8	1.9	5.1
	23	0.8	1.6	3.1
	18	2.3	2.0	3.4

It is not clear why shell growth was favored at higher temperature when the oysters were offered the low ration, but the increased growth of soft tissue at high temperature and high ration may have been due to increased gonadogenesis. In any event, this increased proportion of meat growth appears to be a function of ration as the ratio of meat to shell growth was consistently higher at high ration regardless of temperature.

Oysters under conditions of low ration removed all of the food material from suspension each day, and growth was limited by the amount of food available to them. The mid-ration was four times greater than the low ration, and predictably the oysters removed four times as much food from suspension resulting in approximately four times as much growth. But results of our earlier work (Epifanio and Ewart, 1977) suggested that increasing the ration beyond the mid-level should not result in increased ingestion. This was not the case, however, as an additional fourfold increase in ration resulted in a commensurate increase in the amount of food filtered from suspension. At high

temperature (28°C) this resulted in a substantial increase in growth of soft tissue, while at mid-temperature, growth was not very different from that in the groups offered the mid-ration. At low temperature, the high ration actually inhibited growth, and total growth at that condition was only slightly greater than growth in groups offered the lowest ration at that temperature. Clearly, oysters are able to remove more cells from suspension than predicted by our earlier work, but at mid and low temperatures the material may be wasted as pseudofeces or passed through the gut without being digested (Winter and Langton 1976). However, it is not clear why growth was inhibited when oysters were fed the high ration at low temperature. Such uncertainty aside, the important conclusion for aquaculture is that there is a strong interactive effect of temperature and ration size on growth of bivalves.

FOOD QUALITY

Theories explaining the observed qualitative differences in the food value of algal species include hypothesized algal exudates that inhibit feeding of bivalves, indigestibility of the algal cells, and the lack of some chemical component necessary for the growth of bivalves. Of course these theories are not mutually exclusive and there is evidence in the literature documenting the incidence of each (Epifanio 1976; Langdon and Waldock 1981). In our laboratory we have mainly concentrated on: 1) explaining the poor food value of Phaeodactylum tricornutum (Bohlin); 2) investigating the non-additive aspects of diets consisting of several species of algae; and 3) determining the food value of non-algal diets.

The nutritional value of Phaeodactylum

The earliest studies at Delaware indicated that the diatom Phaeodactylum tricornutum was an inadequate food for bivalves (Epifanio and Mootz 1976). Nevertheless, the fact that P. tricornutum is extremely well adapted for culture in large outdoor impoundments (Mann and Ryther 1977) led to the continuation of investigations of the food value of the species. One of our initial hypotheses was that P. tricornutum excreted some compound or compounds that inhibited filtration of bivalves. This hypothesis was tested in two experiments. In the first, Marinucci (1975) investigated the effects of filtrates of cultures of P. tricornutum on rates of filtration and respiration of hard clam (Mercenaira mercenaria (L.)) larvae. Results indicated that filtrates from standard laboratory cultures of P. tricornutum had relatively little effect on respiration or filtration when compared with filtrates from the golden brown flagellate, Isochrysis galbana (Parke) which increased both respiration and filtration, or the green flagellate, Dunaliella tertiolecta Butcher, which increased respiration while greatly decreasing filtration.

In the second experiment Gorham (1978) considered the effects of filtrates of algal cultures of different ages on feeding activities. Specifically, P. tricornutum, I. galbana, and the centric diatom, Thalassiosira pseudonana were cultured at either 20 or 25°C, and filtrates were taken from cultures in either the logarithmic or stationary phase of growth. There was some indication in this experiment that metabolites produced by P. tricornutum caused mild

inhibition of filtration in juvenile oysters, but there appeared to be a stronger inhibiting effect of the algal culture medium (f/2, Guillard and Ryther 1962), itself, and Gorham was unable to draw definite conclusions. In combination, the results of the two investigations do not support the hypothesis that excretory products of P. tricornutum are the main causative factors of the poor food value of the species.

Other possible explanations involve problems in digestion of the cells, or, alternatively, a lack of specific growth-promoting factors in the chemical composition of the alga. In order to test the hypothesis that the food value of P. tricornutum is related to chemical composition we conducted an experiment in which groups of oysters were fed equal rations of diets consisting of varying proportions of P. tricornutum and T. pseudonana (Epifanio et al. 1981). As anticipated from our earlier work with effects of metabolites of P. tricornutum on filtration, there was little difference in the weight-specific ingestion among the groups fed the different diets. However, growth was inversely proportional to the amount of P. tricornutum in the diet (Table II). We were unable to explain these results in terms of either the gross composition or fatty acid composition of the diets, but we did show that P. tricornutum is lacking or at least low in the amino acid tryptophan. However, this amino acid is absent or found in very small quantities in many diatoms, regardless of their value as foods for bivalves, so we were unable to attribute the poor food value of P. tricornutum to an amino acid deficiency. Additionally, that fact that P. tricornutum appears to have no nutritional value to oysters, even when T. pseudonana made up a large portion of the diet, suggests that the poor food value of P. tricornutum is not related to the absence of some micronutrient, e.g. a specific vitamin or other growth-promoting factor. If a deficiency of such a micronutrient were the culprit, I would expect that the micronutrient present in the T. pseudonana would have allowed at least some utilization of P. tricornutum in growth. (This is based on the assumption that the micronutrient is non-limiting in T. pseudonana.)

Table II. Crassostrea virginica. Mean dry weight of food filtered each week from suspension and dry weight of soft tissues of juvenile oysters fed experimental diets for 5 weeks. Diet 1 = Thalassiosira pseudonana. Diet 2 = 75% T. pseudonana + 25% Phaeodactylum tricornutum. Diet 3 = 50% T. pseudonana + 50% P. tricornutum. Diet 4 = 25% T. pseudonana + 75% P. tricornutum. Diet 5 = P. tricornutum. Dry weight of oyster soft tissue at initiation of the experiment was 30.4 ± 5.0 mg (from Epifanio et al. 1981).

Diet	Food filtered (mg/g dry wt soft tissue)	Final dry tissue wt (mg) after 5 weeks
1	19.9 ± 5.6	46.8 ± 11.3
2	18.6 ± 6.3	40.9 ± 13.2
3	21.2 ± 2.5	34.6 ± 13.4
4	20.9 ± 3.9	29.9 ± 9.7
5	23.9 ± 5.8	29.9 ± 6.4

Our third hypothesis simply held that P. tricornutum is indigestible or at best poorly digestible by bivalves. Valenti (1978) tested this idea in an experiment where he measured absorption of nitrogen by oysters fed isonitrogeneous rations of either I. galbana or P. tricornutum. Efficiency of assimilation in the groups fed I. galbana was greater than 70% while the oysters fed P. tricornutum were unable to absorb any nitrogen from that diet. This suggests that oysters are unable to digest P. tricornutum; this hypothesis is further supported by our own microscopic observations of feces of oysters fed P. tricornutum and the results of Bayne and Scullard (1977) who observed no specific dynamic action in mussels fed P. tricornutum. But in contrast Langfoss and Maurer (1975) in a study employing bomb calorimetry found that oysters absorb P. tricornutum at an efficiency in excess of 60%. Further complicating the picture, is the study of Mann and Ryther (1977) that reports relatively rapid growth of several species of bivalve fed from large, outdoor cultures consisting primarily of P. tricornutum.

While our understanding of the food value of Phaeodactylum tricornutum is far from clear, we can conclude that the gross chemical composition of this species is such that it should support growth of bivalves, but that at least some bivalves appear unable to digest or physically disrupt the cells to gain access to the nutrients inside. This is somewhat surprising as the cell wall of P. tricornutum is not heavily mineralized nor does it appear to be constructed of unusually refractory material (Parke and Dixon 1976). Nevertheless, the conclusion that P. tricornutum is relatively indigestible is consistent with the results of each of the several lines of research conducted in my laboratory over the past several years.

Multi-species diets

Early work with larvae and juveniles indicated that diets consisting of more than one species of algae promoted superior growth (see Epifanio 1976 for a review). This was generally attributed to the vague concept of a better balanced diet. However, it was not at all certain whether this meant better balance in terms of gross components such as proteins, carbohydrates, and lipids or in terms of individual dietary components such as amino acids, fatty acids, and vitamins. Our investigation of this concept began with an experiment in which groups of juvenile oysters (C. virginica) were fed diets composed of 15 combinations of four species of algae (Epifanio 1979a). The experiment was designed such that the relative proportions of each algal species making up a given diet were always equal in terms of dry weight and the oysters in each group were always offered the same weight-specific ration (Epifanio and Ewart 1977).

In spite of the fact that the gross compositions of the diets differed markedly, there was no correlation between the size of the bivalves and either the protein, lipid, or carbohydrate content of the diets after an experimental period of six weeks (Table III). These results were in contrast to those of an earlier study where growth of juvenile oysters varied with the amount of carbohydrate in the diet (Flaak and Epifanio 1978); but in that investigation only one algal species was involved and differences in growth of the oysters were not great. In comparisons of two or more algal species, it seems that characteristics other than gross composition are more important.

Table III. A--Gross Proximate Composition of Algal Diets Fed Juvenile Oysters (*Crassostrea virginica*) During a Six-Week Experimental Period. C = *Carteria chui*, P = *Platymonas suecica*, I = *Isochrysis galbana*, T = *Thalassiosira pseudonana*.
B--Correlation Between Gross Chemical Composition of Diets and Growth of Hard and Soft Tissues (dry weight). (From Epifanio 1979a)

A

Diet	Protein	Lipid	Carbohydrate	Ash
C	50.5	12.8	30.3	6.4
P	29.8	3.0	51.5	16.7
I	60.1	17.2	15.6	7.1
T	40.7	11.7	26.5	21.1
CP	40.1	7.4	40.9	11.6
CT	55.3	15.0	23.0	6.7
CT	45.6	12.2	28.4	13.8
PI	45.0	9.6	33.5	11.9
PT	35.3	6.8	39.0	18.9
IT	50.4	14.4	21.1	14.1
CPI	46.8	10.7	32.4	10.1
PIT	43.5	10.3	31.2	15.0
CIT	50.4	13.9	21.1	11.6
CPT	40.3	8.8	36.2	14.7
CPIT	45.3	10.9	31.0	12.8

B

Species	Growth parameter	Nutrient	Coefficient of correlation (r)
C. virginica	Dry weight	Protein	0.085
C. virginica	Dry weight	Lipid	0.102
C. virginica	Dry weight	Carbohydrate	-0.384
C. virginica	Dry weight	Ash	0.353
C. virginica	Shell weight	Protein	0.158
C. virginica	Shell weight	Lipid	0.293
C. virginica	Shell weight	Carbohydrate	-0.372
C. virginica	Shell weight	Ash	0.162

Another interesting result of the 1979 study was the non-additive nutritional interactions observed among the algal species in a number of the diets. For example, diets consisting of combinations of *Platymonas suecica* Kylin or *Carteria chui* Pringsheim with either *Isochrysis galbana* or *Thalassiosira pseudonana* yielded growth similar to that with sole-component diets of *I. galbana* or *T. pseudonana*. This growth was much greater than that with either *C. chui* or *P. suecica* alone. One possible explanation of these results is that *C. chui* and *P. suecica* are deficient in some growth-promoting micronutrient found in relatively high quantities in *I. galbana* and *T. pseudonana*. If this factor acted so that a threshold quantity were necessary for growth, but that additional quantities did not increase growth, e.g. in the manner of vitamins, then non-additive effects might be expected in diets combining a deficient alga with a replete alga.

An alternative explanation attributes the results to differences in the digestibility of the algae. For example, if the cell wall of P. suecica were relatively indigestible, it would reasonably take longer to digest a given quantity of cells of that species than a similar quantity of a digestible species such as I. galbana. If the rate of digestion of P. suecica were very slow, an oyster's stomach could quickly reach maximum capacity with the associated shunting of partially digested food to the midgut without it entering the digestive diverticula. However, if P. suecica were fed in combination with a more digestible alga, the effective ration of P. suecica would be reduced and the rate of ingestion might not exceed the rate of digestion. P. suecica cells would then be totally digested and more food material would be available for growth.

However, the results of the study also showed that combinations of certain species of algae, e.g. I. galbana and T. pseudonana, yielded much greater growth than diets consisting solely of either species. This synergistic effect was not explained by our digestibility hypothesis nor was it explained by the gross compositions of the algae. Possibly, the answer lies in a fuller knowledge of the micronutrient or fatty acid compositions of the algae in question.

In a subsequent study we tested the digestibility hypothesis by measuring efficiencies of absorption and growth of juvenile oysters fed equal rations of three species of algae, singly and in combination (Romberger and Epifanio 1981). Growth responses (Table IV) were similar to those in the earlier study (Epifanio 1979a). The lack of growth of oysters fed P. suecica was clearly a consequence of the poor digestion and absorption of this alga (Table V). The intermediate growth response of oysters fed the diet consisting of P. suecica and I. galbana was less easily explained on the basis of absorption as oysters absorbed the diet at 45% efficiency[1] which is approximately the average of the efficiencies of the two component algae when fed singly. It appeared, then, that the absorption of this diet was additive while growth was non-additive. This paradox was explained by the fact that ingestion of the diet was greater than that of either of the sole component diets, P. suecica or I. galbana (Table V). Hence, even though the P. suecica in the two-component diet appeared to present digestive problems to the oysters, the increased ingestion of the diet resulted in non-additive growth. It was not clear why ingestion of the diet was enhanced, but ingestion of every two-species diet used in the experiment was greater than that of any single-species diet.

In contrast to the above, the efficiency of absorption of the diet consisting of P. suecica and T. pseudonana was greater than the average of the efficiencies of the component species fed singly and was only slightly different from that of T. pseudonana. It appears, then, that the combination of an easily digestible algal species with a relatively indigestible species does result in more effective digestion of the refractory species for at least some combinations of species. However, a combination of other species, e.g. T. pseudonana and I. galbana, yields synergistic growth responses that cannot be explained in terms of absorption or ingestion. In this experiment, the efficiency of

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[1] Absorption Efficiency = (Food Absorbed - Food Ingested) x 100

absorption of the diet consisting of I. galbana and T. pseudonana was essentially the same as that of either of the component species, and ingestion was not high enough to account for the increased growth yielded by the diet. But, the net growth efficiency of oysters fed this diet was very high, indicating that a large proportion of the absorbed food material was converted to growth (Table IV). This could only be explained in terms of the compositional quality of the diets, and since our earlier studies had shown no correlation between growth and gross composition, the synergistic interaction of the algal components seemed most likely related to the availability and balance of fatty acids (Langdon and Waldock 1981) or micronutrients such as vitamins and minerals.

Table IV. Crassostrea virginica. Comparison of Mean Dry weights of Juvenile Oysters Fed Each of Six Experimental Algal Diets for 5 Weeks. Each group contained 21 oysters. Student-Newman-Keuls test indicated no significant ($\alpha = 0.05$) difference in mean size of oysters within a particular growth-response designation. Growth response A is least growth and C is most growth. Sac = group sacrificed at initiation of experiment. P = Platymonas suecica, I = Isochrysis galbana, T = Thalassiosira pseudonana. (Modified from Romberger and Epifanio 1981).

Growth response	Diet	Dry weight (mg)	
		Soft tissue	Shell organic material
A	P	55.0	56.1
	Sac	62.6	53.4
B	PI	85.1	68.4
	PT	93.9	68.1
	I	97.8	70.0
	T	100.8	66.6
C	TI	123.7	79.5

Table V. Crassostrea virginica. Efficiency of Gorwth of Juvenile Oysters Fed Each of Six Experimental Algal Diets for 5 Weeks. Each group contained 21 oysters. Diets are defined in Table III. (Modified from Romberger and Epifanio 1981)

Diet	Food filtered from suspension (g)	Pseudo-feces (g)	Ingestion (g)	Feces (g)	Absorbed food (g)	Growth (g)	Growth efficiency	
							Gross	Net
P	4.09	1.27	2.82	2.64	0.18	Negative	Negative	Negative
PI	5.54	1.57	3.97	2.18	1.79	0.75	18.9	41.9
PT	5.50	1.17	4.33	1.81	2.52	0.92	21.2	36.5
T	4.18	0.51	3.67	1.01	2.66	1.08	28.1	38.7
I	4.89	1.34	3.55	0.94	2.61	1.09	29.0	39.5
TI	6.03	1.48	4.55	1.37	3.18	1.74	38.2	54.7

Non-algal diets

Our studies of non-algal diets have included various formulated feeds, several types of starch, and ground whole cereals (Epifanio, unpublished data). None of these has supported growth of bivalves, and it was only recently that we tested a promising non-algal food, the torulan yeast Candida utilis (Henneberg). That experiment (Epifanio 1979b) consisted of feeding diets containing varying proportions of yeast and the alga Thalassiosira pseudonana to juveniles of four species of bivalve, Argopecten irradians Lamark, Crassostrea virginica (Gmelin), Mercenaria mercenaria (L.), and Mytilus edulis L. Results showed unimpeded growth of three of the species on diets consisting of up to 50% yeast, but C virginica appeared unable to utilize the yeast, as growth of that species was inversely proportional to the amount of yeast in the diet (Table VI). This was not related to gross compositional differences in the diets (see Epifanio 1979b for data), and it was hypothesized that the oysters might be less able than the other species to digest the yeast.

Table VI. Comparison of Mean Initial and Final Dry Weight (mg) of the Soft Tissue of Bivalves Fed Experimental Diets for 28 Days. Variation is expressed as ± one standard deviation. A = 100% algae. B = 75% algae + 25% yeast. C = 50% algae + 50% yeast. D = 25% algae + 75% yeast. E = 100% yeast. (From Epifanio 1979b)

	Initial weight	Final weight				
		A	B	C	D	E
Argopecten irradians	54.5± 9.6	228.9±51.7	193.9±81.9	259.5±44.1	162.2±44.3	69.1± 9.4
Crassostrea virginica	24.6±10.0	49.4±16.5	43.9±22.6	35.8±13.3	27.9± 6.0	21.2± 7.1
Mercenaria mercenaria	10.0± 1.7	23.2± 6.9	21.8± 9.3	21.4± 4.2	17.5± 4.3	11.0± 3.3
Mytilus edulis	65.3±14.6	168.1±39.1	146.1±22.3	144.9±13.1	87.3±15.5	55.3±15.4

The fact that diets containing more than 50% yeast resulted in reduced growth of A. irradians, M. edulis, and M. mercenaria was likewise not explained by compositional differences. However, this result is possibly explained by the digestibility hypothesis discussed above (Epifanio 1979a). For example, results of a study of Alatalo (1980) indicated that juvenile M. mercenaria absorb very little organic material from diets consisting solely of yeast, but show a gross growth efficiency of nearly 20% when fed diets consisting of 50% yeast and 50% Thalassiosira pseudonana. Conceivably the increased food value of the mixed algal-yeast diet is explained by the same argument presented in the preceding section for the non-additive characteristics of the mixed algal diets.

CONCLUSIONS

The results of our studies suggest that the differing nutritional values of algal diets can often be explained by the relative digestibility of the algal components. Oysters appear to be totally incapable of utilizing certain algal species because they are unable to digest the cell walls. There is good evidence, for example, that oysters have great difficulty in digesting Phaeodactylum tricornutum. Paradoxically, some algal species that are worthless to oysters when fed as the sole component of a diet appear to support growth when combined in a diet with another species of algae. This can be explained by an argument that takes into account the time that it takes for extracellular digestion of an algal cell wall, the rate of ingestion, and the residence time of food in the stomach. However, with a wide range of values, there appears to be little correlation between the gross composition of algal or non-algal diets and their nutritional worth to bivalves. But studies of efficiency of absorption and growth do suggest that the algal components of a diet may interact synergistically due to an improved balance of fatty acids or micronutrients.

The types of investigations conducted in my laboratory over the past several years have allowed us to improve our understanding of bivalve nutrition, but future advances will require a more biochemical approach. A recent study by Langdon and Waldock (1981) wherein they rectified a fatty acid deficiency in a particular algal diet by addition of encapsulated fatty acid is a good example of the type of work that can be conducted at present. Nevertheless, it is difficult to conceive of rapid development in the field in the absence of a defined dietary formula that will support growth of bivalves. Without such an experimental diet whose detailed composition can be easily manipulated, I foresee slow progress in our understanding of bivalve nutrition at the biochemical level.

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NEW TECHNIQUES AND THEIR APPLICATION TO STUDIES OF BIVALVE NUTRITION[1]

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ABSTRACT

Little is known of the nutritional requirements of bivalve molluscs mainly because there are great difficulties in the development and testing of artificial diets for these animals. Two newly developed techniques are discussed which have helped to overcome some of these difficulties. Firstly, it has been possible to obtain axenic larvae of Crassostrea for nutrition studies, without the use of antibiotics. The results of some preliminary feeding experiments with axenic larvae are discussed. Secondly, microencapsulation techniques have been developed that enable the nutritionist to encapsulate the major dietary components such as proteins, carbohydrates, lipids and water soluble vitamins. The results of experiments with oysters fed on encapsulated and non-encapsulated nutrients are discussed.

KEY WORDS: Axenic culture, oyster larvae, oyster spat, microcapsules, artificial diets, vitamins, fatty acids.

INTRODUCTION

Despite the fact that marine bivalves have been artificially cultured for many decades little is known of their nutritional requirements or indeed of the nutritional requirements of molluscs in general. This is due to the failure to develop satisfactory artificial diets for bivalve molluscs and to the difficulties of controlling culture conditions during feeding experiments. Bacterial activity is enhanced by the addition of organic nutrients to seawater and this has been one of the main difficulties in the development of artificial diets for bivalves grown under non-axenic conditions (Loosanoff and Davis 1963). Bacteria may both be directly harmful to the animals, and may also degrade and clump food particles (Masson 1977). Millar and Scott (1967) have successfully obtained axenic Ostrea edulis larvae using

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[1] Part of this work was carried out at the M.A.F.F. Fisheries Laboratory, Conwy, U.K. and at the N.E.R.C. Unit of Marine Invertebrate Biology, Marine Science Laboratories, Menai Bridge, U.K.

antibiotic washes and have grown the axenic larvae to metamorphosis on the alga Pavlova lutheri but their techniques have not been adopted by other workers. This may have been due to the increased difficulty in sterilizing bivalve larvae with antibiotics because of the development of resistant bacterial strains (Helm and Millican 1977). In this paper, a simple and reliable method for obtaining axenic Crassostrea gigas larvae is described together with some preliminary feeding and growth experiments with axenic larvae fed on artificial diets.

In addition to the development of axenic culture techniques, a second major step in overcoming problems of diet presentation, such as particle breakdown and leaching, has been the development of microencapsulation techniques. Various microcapsule types will be discussed, together with the results of feeding experiments with microencapsulated nutrients fed to juvenile oysters.

AXENIC GROWTH EXPERIMENTS WITH C. GIGAS LARVAE FED ON ARTIFICIAL DIETS

Obtaining axenic C. gigas larvae

Ripe adult C. gigas were carefully opened by cutting through the adductor muscle, and their sex determined by removing a small piece of the gonad and examining it under a microscope. A ripe male and female were then selected and the body tissue carefully excised from the shells. Working inside a glass fronted glove box, the surface of the gonad was wiped with a sterile swab that had been dipped in 5000 ppm hypochlorite solution and a small incision was then made in the wall of the gonad with a sterile Pasteur pipette. The eggs and sperm were collected separately in flasks containing 100 ml of autoclaved seawater at 25°C and 28 °/oo salinity.

The concentration of the eggs was determined by aseptically removing 0.1 ml of the egg stock suspension and counting the number of eggs present using a binocular microscope. Eggs were transferred aseptically from the stock suspension to other flasks containing 100 mls of sterile seawater, so that a final concentration of 50 eggs ml^{-1} was obtained. Several drops of sperm suspension were then added to each flask. If more than 50% of the eggs appeared to be dividing after 3 hours, fertilization was regarded as having been successful and the flasks were placed in an incubator at 25°C and left for 18-24 hours.

After incubation, the concentration of larvae in the flasks was assessed by removing 1 or 2 ml of the culture and counting the number of larvae present, under a binocular microscope. A few ml of the larval suspension from each flask were also added to a x1 and x 10 dilution of Droop's E6 sterility test medium (Droop 1969) and incubated at 25°C for 1 month. The larvae were either used immediately or stored for 4 to 6 days at 15°C until required. During this storage period, most of the contaminated cultures could be identified by means of the sterility test. Over a period of two years work more than 80% of over 100 batches of larvae obtained in this way were found to be axenic.

Results of preliminary growth experiments with C. gigas larvae fed on artificial diets

Axenic C. gigas larvae were cultured on a wide range of artificial diets for periods of 6 to 8 days in 100 ml flat bottomed flasks which were agitated on an orbital shaker (75 to 100 revs. min^{-1}). The type of diet used for the growth studies was similar to that of Provasoli and D'Agostino (1969) who successfully cultured Artemia salina on a biphasic diet of dissolved nutrients and co-precipitated particles under axenic conditions. Conklin and Provasoli (1977, 1978) also succeeded in culturing the freshwater cladoceran Moina macrocopa using similar techniques.

The composition of the best artificial diet tested is given in Table I. The co-precipitated particle was prepared by homogenizing the heat precipitated sterile nutrient mixture using a Teflon coated tissue grinder. Particle size ranged from 2 to 20 μm, with a mean diameter of 2.5 μm. The particles were therefore small enough to ingested by the larvae. The composition of the particulate phase was based on that of Pavlova lutheri as reported by Parsons et al. (1961). P. lutheri is a mediocre algal food for C. gigas (Walne 1970; Millican and Helm 1973).

The composition of the water-soluble phase of the diet was based on that of tissue culture medium (TCM) 199 and prepared according to Morgan et al. (1955). It was found, however, that an amino acid mixture based on the composition of Isochrysis galbana (after Chau et al. 1967) supported better larval growth than the TCM 199 amino acid mixture. Furthermore, the concentration of dissolved nutrients recommended for the tissue culture medium proved too high for bivalve larvae and best growth occurred at one hundreth of this concentration (Table II). The dissolved nutrient concentrations of the artificial diet were still much higher, however, than those occurring naturally in seawater (Table II).

Larval growth was further improved by adding a 1% v/v solution of bovine amniotic fluid (Gibco Laboratories) to the defined artificial diet of Table I. It is possible that the amniotic fluid supplied the larvae with vitamin B_{12} or other micronutrients that were absent from the defined diet. The growth of larvae fed on the artificial diet enriched with 1% v/v amniotic fluid is given in Table III. Most of the artificially fed larvae reached the umbone stage (prodissoconch II stage, Carriker and Palmer 1979) within 6 days, but little further growth occurred and after 8 days, tissue wastage became increasingly apparent. Clearly the diet was either deficient in certain essential micronutrients or the form of presentation was unsatisfactory for the efficient utilization of the diet by the larvae.

There is a possibility that bivalve larvae are unable to meet all their vitamin requirements by absorption of vitamins from the freely dissolved state, even though the concentration of vitamins in the growth medium was high compared with naturally occurring levels. There have been, to my knowledge, no reported studies on the uptake of freely dissolved vitamins by bivalves. This would be an interesting area of research, especially as there is increasing evidence to suggest that bivalves can readily absorb amino acids, sugars, and fatty acids from solution (see Stewart 1979; Stephens, this volume).

Table I. Composition of the Defined Components of the Artificial Diet Tested with Axenic Crassostrea gigas Larvae

Co-precipitated particle	Fed at a concentration of 100 particles μl^{-1}
Component	Composition, ratio by wt
egg albumin (2x crystallized, Sigma)	200
soluble starch	150
oyster lipid extract	50
phosphorus	10
RNA (yeast)	1
DNA (salmon sperm)	1
Dissolved Nutrients	
Amino acids (based on composition of Isochrysis galbana)	mg l^{-1}
Alanine	0.97 (L)
2-amino-iso-butyric acid	0.05 (DL)
2-amino-n-butyric acid	0.13 (DL)
Arginine (Cl)	0.57 (L)
Aspartic acid	0.99 (L)
Cysteine (Cl)	0.05 (L)
Glutamic acid (H_2O)	0.84 (L)
Glycine	0.63 (L)
Histidine (Cl)	0.19 (L)
iso-Leucine	0.33 (L)
Leucine	1.02 (L)
Lysine (Cl)	0.73 (L)
Methionine	0.32 (L)
Ornithine	0.04 (DL)
Phenylalanine	0.44 (L)
Proline	0.67 (L)
Serine	0.60 (L)
Threonine	0.50 (L)
Tryptophan	0.04 (L)
Tyrosine (Na_2 salt)	0.21 (L)
Valine	0.68 (L)
(Total amino acid concentration = 10 mg l^{-1})	
Glucose	10
Water-soluble vitamins (based on 1/100 concentration TCM 199)	$\mu g\ l^{-1}$
Thiamine (HCl)	0.10
Nicotinic acid	0.25
Nicotinamide	0.25
Ca pantothenate	0.10
Riboflavin	0.10
Pyridoxine (2HCl)	0.25
Pyridoxal (HCl)	0.25
p-Aminobenzoic acid	0.50
Biotin	0.10
Choline chloride	5.00
Inositol	0.50
Folic acid	0.10
Ascorbic acid	0.50
(Total water-soluble vitamin concentration = 8 $\mu g\ l^{-1}$)	

Table I (continued)

Fat-soluble vitamins and sterols (solubilized in Tween 80, based on TCM 199)	$\mu g\ l^{-1}$
Calciferol	1.00
Menadione	0.10
Vitamin A (acetate)	1.40
α-Tocopherol phosphate (Na salt)	0.10
Cholesterol	2.00
Tween 80	200.00
(Total concentration = 204.60 $\mu g\ l^{-1}$)	
Purines and pyrimidines	
Adenine sulphate	100.00
Adenosinetriphosphate (Na salt)	10.00
Adenylic acid	2.00
Guanine (HCl)	3.00
Hypoxanthine	3.00
Thymine	3.00
Uracil	3.00
Xanthine	3.00
(Total concentration = 127.00 $\mu g\ l^{-1}$)	
Other components	
Deoxyribose	5.00
Glutathione	0.50
Ribose	5.00

(Total concentration of defined dissolved nutrients = 20.35 $mg\ l^{-1}$)

The morphology of the digestive system of C. virginica larvae has been described in some detail (see, for example, Elston 1980). Little is known, however, of the ability of oyster larvae to digest non-living, particulate, organic matter. It is possible that material such as the co-precipitated particle used in the growth studies is not utilized because of its poor digestibility. Feeding experiments using co-precipitated, radio-isotope labelled proteins or labelled starch would be useful in examining this. Such studies would also help in understanding the role of non-living, particulate, organic material in the nutrition of bivalves under natural conditions.

MICROENCAPSULATION OF NUTRIENTS FED TO CRASSOSTREA JUVENILES IN NON-AXENIC CONDITIONS

Nylon-protein walled capsules

Nylon-protein walled capsules are formed by interfacial polymerisation (see Chang 1966; Chang et al. 1972). The aqueous diet, containing haemoglobin and diaminohexane, is emulsified in an organic solution of sebacoyl chloride. The sebacoyl chloride reacts with both the diaminohexane to form nylon 610 and the free amino groups of the

haemoglobin to form a nylon-protein, crosslinked wall (Figure 1). Chang's method has been modified in order to prepare capsules in the 5 to 20 μm size range which are ingestible by juvenile Mytilus edulis and Crassostrea gigas (see Gabbott et al. 1976; Langdon 1977).

Table II. Comparison of the Concentration of Nutrients in the Best Tested Artificial Diet for Axenic Crassostrea gigas Larvae with Those of Seawater and with Those Recommended for TCM 199 (Langdon 1980)

Component	Seawater	Artificial diet	TCM 199
Particulates (mg l^{-1})	1 - 14[a] (mean 3.2)	5	none
Total dissolved organic matter (mg C l^{-1})	1 - 31[a] (mean 10)	48[b]	8[c]
Carbohydrates (μg l^{-1})	200 - 800[d]	1×10^4	1×10^6
Amino acids (μg l^{-1})	0.5 - 10	1×10^4	1×10^6
Biotin (μg l^{-1})	$2\text{-}5 \times 10^{-3}$	1×10^{-1}	10
Thiamine (μg l^{-1})	$8\text{-}100 \times 10^{-3}$	1×10^{-1}	10

[a]Data for the Conwy estuary, Gwynedd, North Wales, Britain (M.A.F.F. Conwy, 1978). Particulate fraction defined as the fraction remaining on GF/C filters after filtration of the seawater sample. The DOM data refer to the DOM levels present in the filtrate.

[b]Includes both the 1% v/v bovine amniotic fluid and the dissolved defined nutrients.

[c]Based on an estimate that 40% of the total organic weight of the dissolved nutrients is carbon.

[d]Data from Williams (1975) refer to oceanic DOM levels--coastal values will be higher and more variable.

Table III. Growth of Crassostrea gigas Larvae Fed on the Best Artificial Diet (Table I) with 1% v/v Bovine Amniotic Fluid Added (Langdon 1980). Larvae were grown in 25 ppt, autoclaved seawater at 26 to 28°C in 100 ml flat bottomed flasks agitated on an orbital shaker (75 to 100 rev min^{-1}). Algae were fed at an initial concentration of 100 Chaetoceros calcitrans cells μl^{-1} under non-axenic conditions.

	Mean shell length ± s.d. (μm)		
	Starved	Artificial diet	Algal diet
After 6 days (mean of 10 trials)	79.9 ± 0.8	96.0 ± 3.0	113.6 ± 8.0

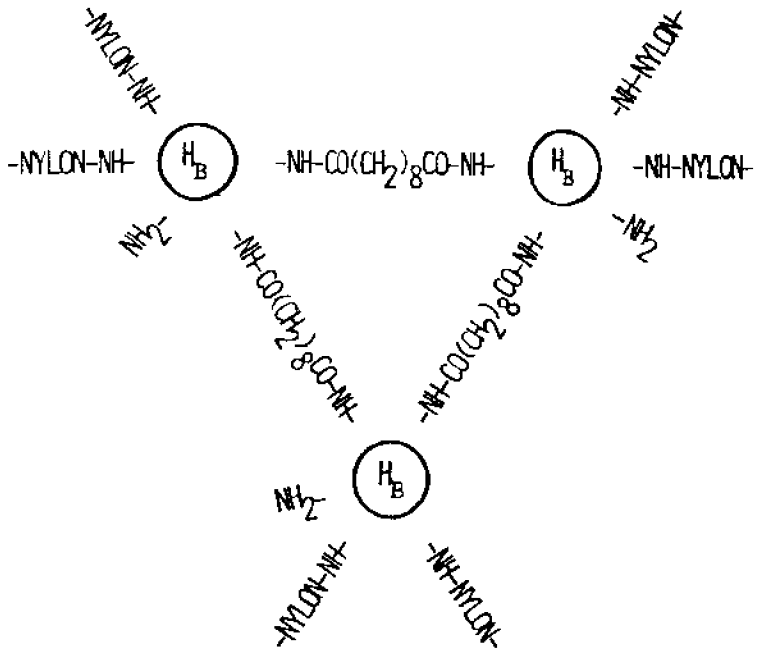

Figure 1. Haemoglobin cross-linked nylon-chains. (After Chang 1972.)

Gabbott et al. (1976) reported limited growth, in terms of wet weight, of C. gigas juveniles fed on nylon-protein capsules containing a diet of 10% w/v haemoglobin, 7.5% w/v rice starch, 2.5% w/v soluble starch and 0.2% w/v cholesterol. This work was repeated by Langdon (1977) and similar results were found (Table IV). The fact that juveniles fed on capsules grew more than the starved controls suggests that they were able to digest and assimilate the encapsulated diet. Furthermore, fecal examinations of juveniles fed on nylon-protein encapsulated graphite particles indicated that they were able to break down the capsule walls, since liberated graphite particles were observed in the feces. No measurements of changes in the organic weight of the oysters were made, however, and so it is not certain as to what extent the increase in wet weight was due to tissue growth as opposed to shell growth.

The nylon-protein encapsulated diet fed to the oyster juveniles was deficient in micronutrients such as fatty acids and vitamins. An attempt was made to supply these nutrients by supplementing the capsules with partial rations of algae. The growth of oysters fed on the mixed diets was largely determined, however, by the level of algae present (Table V) and the capsules had little beneficial effect (Langdon 1977). The reason for this result is not certain, but it is probable that the nutrients supplied by the capsules were not growth limiting in the mixed algal-capsule rations and that oyster growth was limited by the levels of other essential nutrients that were present only in the algae.

Vitamins and other low molecular weight, water soluble nutrients cannot be readily encapsulated using the nylon-protein walled capsules since the capsule wall is semipermeable and the nutrients will rapidly leach out. Furthermore, lipids tend to be extracted from the diet during the encapsulation process. Jones et al (1979), for example, noted that 50% of the radioactive labelled palmitic acid (1-^{14}C) was

lost from an artificial diet during the nylon-protein encapsulation process. In order to overcome these problems capsule types have been developed and tested with Crassostrea spat, that are designed to encapsulate dietary lipids and/or water-soluble vitamins.

Table IV. Growth of Crassostrea gigas Juveniles Fed on a Nylon-Protein Encapsulated Diet[a] (Langdon 1977)

Treatment	% Increase in total wet wt after 13 days
1) Tetraselmis suecica at 25 cells μl^{-1}	31.7
2) Starved	3.7
3) Nylon protein capsules at 5 μl^{-1}	7.7
4) Nylon protein capsules at 15 μl^{-1}	9.6
5) Nylon protein capsules at 30 μl^{-1}	14.3

[a]Conditions: 20 juvenile oysters for each treatment (initial wet weight 10.7 mg per spat) were grown in stirred, 3-liter beakers filled with filtered seawater (25 ppt) at 18-20°C. Beaker water was changed every other day. The encapsulated diet consisted of 10% haemoglobin, 7.5% rice starch grains, 2.5% hydrolyzed potato starch and 0.2% cholesterol.

Table V. The Growth of Crassostrea gigas Juveniles Fed on a Nylon-Protein Encapsulated Diet With and Without Supplements of the Alga Tetraselmis suecica[a] (Langdon 1977)

	% Increase in total weight in 13 days			
	No algae	1/6 Ration	1/3 Ration	Full ration
No capsules	3.7	12.3	23.5	31.7
5 capsules μl^{-1}	7.7	18.8	23.4	
15 capsules μl^{-1}	9.6	16.7	22.5	
30 capsules μl^{-1}	14.3	13.8	12.2	

[a]Conditions: 20 juvenile oysters for each treatment (initial wet weight 10.6 mg per spat) were grown in stirred, 3-liter beakers filled with filtered seawater (25 ppt) at 18-20°C. Beaker water was changed every other day. The full ration of Tetraselmis suecica was made up with 25 cells μl^{-1}. The encapsulated diet consisted of 10% haemoglobin, 7.5% rice starch grains, 2.5% hydrolyzed potato starch and 0.2% cholesterol.

Gelatin-acacia walled capsules

Gelatin-acacia capsules are suitable for the encapsulation of lipids and lipid soluble dietary components. The method of preparation is based on that of Green and Schleicher (1957) with slight modifications (see Langdon and Waldock 1981). The lipid is emulsified in a mixture of gelatin and acacia. The pH of the mixture is then adjusted, causing the gelatin and acacia to co-acervate and form the capsule walls. Very small capsules (mean diameter 2-3μm) that are

ingestible by larvae and juvenile oysters can easily be prepared.

Gelatin-acacia walled capsules have been used by Langdon and Waldock (1981) to investigate the essential fatty acid requirements of C. gigas juveniles. Fecal examinations, together with fatty acid analysis of the capsule-fed animals, have demonstrated that oysters can breakdown the capsule wall and utilize the capsule contents. The growth of juvenile C. gigas fed on Dunaliella tertiolecta, an algal species deficient in the long chain polyunsaturated fatty acids 20:5ω3 and 22:6 ω3 was improved by supplementing the alga with gelatin-acacia encapsulated 22:6ω3 (Table VI). Similar levels of encapsulated triolein supplements did not improve oyster growth. The positive effect of the 22-6ω3 supplement was therefore not simply due to an increase in the calorific content of the diet, but was due to its specific nutritional quality.

Table VI. Growth and Level of the Fatty Acid 22:6ω3 in Crassostrea gigas Juveniles Grown for 14 Days on Dunaliella tertiolecta With and Without Supplements of Gelatin-Acacia Encapsulated 22:6ω3[a] (Langdon and Waldock 1981)

Diet	% Wet wt increase	% Level of 22:6ω3 in fatty acids	
		Triacylglycerol	Phospholipid
Initial spat	-	4.5	7.7
Starved	9.6	nd	nd
Hatchery fed[b]	106.3	9.0	10.7
D. tertiolecta (D) at 25 cells μl^{-1}	58.6	1.7	7.4
D + 20% triolein[c]	55.4	0.4	4.4
D + 20% 22:6ω3	78.7	28.1	16.6

[a]Conditions: 120 juvenile oysters for each treatment (initial wet weight 2.5 mg per spat) were grown in 3 liters of diatomaceous earth filtered seawater (particles >1 μm removed) at 25 ppt and 24±1°C. The cultures were aerated (100 ml min^{-1}) and the water changed every other day.

[b]Hatchery fed oyster spat were fed on a mixed algal diet of Tetraselmis suecica, Isochrysis galbana and Chaetoceros calcitrans.

[c]Level of lipid supplementation is expressed as a percentage of the dry weight of the algal diet.

Lipid walled capsules

Hiestand et al. (1970) have described the preparation of capsules that have a lipid wall with an aqueous center (Figure 2). This type of capsule is of great potential use in presenting filter-feeders with low molecular weight, water soluble substances such as vitamins, trace metals, amino acids, sugars and nucleic acids.

Lipid walled capsules have been used to supply water soluble vitamins to juvenile C. virginica in a series of growth experiments. The method of preparation was as follows: 1 volume of the vitamin mix, dissolved in an aqueous solution of 10% w/v gum acacia in 1M $CaCl_2$, was

emulsified in 4 parts of menhaden oil containing 10% w/v ethyl cellulose. The primary emulsion was then cooled to 5°C to harden the menhaden oil. One volume of the emulsion was then emulsified in 4 volumes of the aqueous 10% w/v acacia/1M $CaCl_2$ solution. The secondary emulsion was made up of droplets of the menhaden oil with droplets of the aqueous vitamin solution trapped inside (Figure 2). The capsule suspension was then washed free of $CaCl_2$ and non-encapsulated vitamins by dialysis at 5°C.

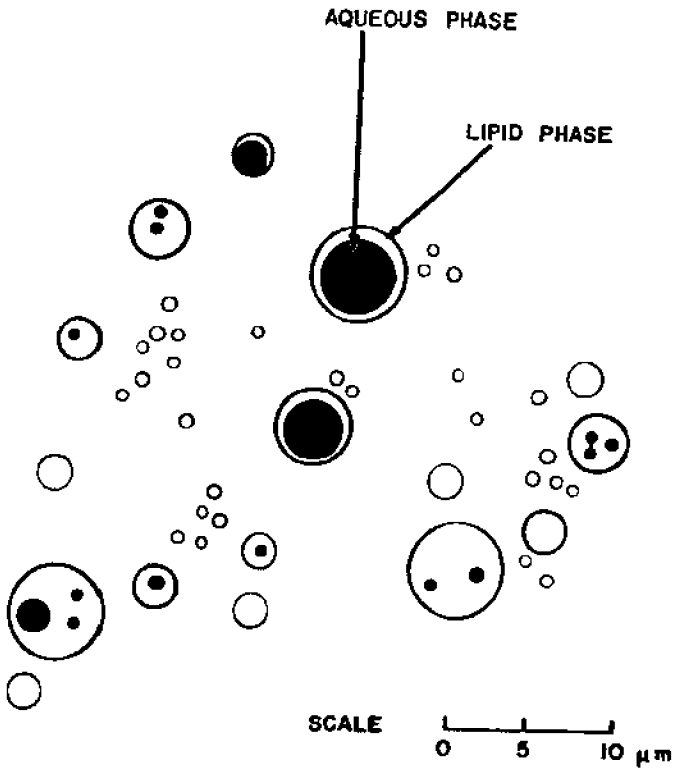

Figure 2. Lipid walled capsules.

Using ^{14}C-labelled glycine, the leakage characteristics of the menhaden walled capsules have been determined (Table VII). Only slight leakage (2.5% of the encapsulated ^{14}C-glycine) occurs over a 100 hour period at 5°C. Capsules used for feeding experiments were stored for a maximum period of 72 hours at 5°C before use. It should be noted that dialysis did not remove the acacia (molecular weight > 50,000 Daltons) from the capsule suspension and that the acacia may help stabilize the capsule walls during storage. There is no data presently available on the rates of leakage of glycine from capsules suspended in the culture medium, nor is there data on the rates of leakage of vitamins from the capsules. Fecal examinations of C. virginica spat fed on menhaden walled capsules containing water soluble dyes (phenol red, alcian blue) indicate that the spat are able to break down the capsule wall in the gut and liberate the contents.

Table VII. Characteristics of Menhaden Oil Walled Capsules Prepared with Encapsulated ^{14}C-labelled Glycine[a]

% of ^{14}C-glycine encapsulated[b]		28.2%
Volume of trapped aqueous solution per mg lipid[c]		9.1×10^{-2} µl
% of encapsulated ^{14}C-glycine lost during storage for 100 hours[d]	--at 5°C	2.5%
	--at 27°C	11.2%

[a]The menhaden oil contained 10% w/v ethyl cellulose which acted as a wall-stabilizing agent. Approximately 4.69 µCi of ^{14}C-glycine (specific activity 117.0 m Ci $mmol^{-1}$) were added to an aqueous solution of 10% w/v acacia and 1M $CaCl_2$ and encapsulated as described.

[b]The capsules were successively dialyzed to remove non-encapsulated glycine and the percentage lost and remaining in the capsules calculated.

[c]The trapped volume was calculated assuming that the concentration of ^{14}C-labelled glycine inside the capsule was equal to the concentration of ^{14}C-glycine in the solution used in the preparation of the capsules, i.e., no dilution had occurred.

[d]Aliquots of the washed capsules were dialyzed against seawater and the percentage loss of ^{14}C-glycine from the capsules calculated.

Using menhaden encapsulated vitamins, artificial diets have been developed for juvenile C. virginica (Table VIII) which supported sustained, modest growth over experimental periods of three weeks (Table IX). However, oysters fed on an algal ration had growth rates that were about 5 times greater than those fed on the artificial diet.

A further experiment was carried out to examine the effects of adding vitamins in an encapsulated form as opposed to being added in the freely dissolved state (Table IX). Spat fed on the diet with the encapsulated vitamins grew better than spat fed on diets with freely dissolved vitamins, even though the concentrations of freely dissolved vitamins were greater in some of the treatments. The difference in the dry weights of spat fed on the diet with encapsulated vitamins was significantly greater (Students' t-test, $p(t)>0.99$) than that of spat fed on the diets with freely dissolved vitamins. The encapsulation technique improved the ability of C. virginica to utilize the vitamins presented in the diet.

CONCLUSIONS

It is now possible to carry out controlled, axenic, nutrition studies with oysters and other bivalve molluscs (which can be artificially fertilized) using the techniques described in this paper. Axenic bivalve juveniles can be obtained by growing axenic larvae to metamorphosis on available bacteria-free cultures of algae. Alternative methods of obtaining axenic juveniles, such as using microencapsulated antibiotics to eliminate the gut flora, should be tested. Care should be taken, however, that the antibiotics do not directly affect their metabolism and/or nutritional rquirements.

Table VIII. Composition of an Artificial Diet for Crassostrea virginica Juveniles

1) Particles (2 to 20 µm diameter)
Fed at 2.65 mg, dry wt l^{-1}

Component	% Composition by wt
Carboxymethyl cellulose	1.2
Haemoglobin (crude preparation)	10.0
Soluble potato starch	10.0
Rice starch grains	10.0
RNA (yeast extract)	1.0
DNA (salmon sperm)	1.0
Phosphorus (as phosphate)	0.5
Water	66.3

2) Menhaden-walled vitamin capsules[a] (2 to 15 µm diameter)

3) Kaolin at 20 mg l^{-1}

4) Guillard's f trace metal mix[b] dissolved in seawater

[a]Menhaden oil contained 10% w/v ethylcellulose, plus a fat soluble vitamin mix (per gram lipid): vitamin A 60 IU, vitamin D 36 IU, vitamin E 20 IU, vitamin K 48 µg, lipoic acid 30 µg. Weight of encapsulated vitamins in 25 capsules μl^{-1} per liter (µg). Ascorbic acid 4.600, p-aminobenzoic acid 0.162, biotin 0.065, Ca pantothenate 0.065, choline chloride 3.237, folic acid 0.065, inositol 0.322, nicotinamide 0.162, nicotinic acid 0.162, pyridoxine (2HCl) 0.065, pyridoxal (HCl) 0.162, riboflavin 0.065, and thiamine (HCl) 0.065, plus phenol red 0.540. Weight of encapsulated B_{12} in 5 capsules μl^{-1} per liter = 0.0015 µg.

[b]From Guillard (1975): $NaNO_3$ 75 mg l^{-1}, $NaH_2PO_4 \cdot H_2O$ 5 mg l^{-1}, $Na_2SiO_3 \cdot 9H_2O$ 30 mg l^{-1}, Na_2EDTA 4.36 mg l^{-1}, $FeCl_3 \cdot 6H_2O$ 3.15 mg l^{-1}, $CuSO_4 \cdot 5H_2O$ 0.01 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.022 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 0.01 mg l^{-1}, $MnCl_2 \cdot 4H_2O$ 0.18 mg l^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.006 mg l^{-1}.

Attempts to develop a defined artificial diet for axenic C. gigas larvae have not been entirely successful in that the growth of larvae was limited. However, more is now known about the optimum concentrations of some of the dissolved nutrients for bivalve larvae as well as the most suitable conditions and techniques for maintaining axenicity. One of the possible reasons for the failure of oyster larvae to grow on the artificial diets is the inability of larvae to meet their requirements for vitamins by absorption from the culture medium. The use of newly developed lipid walled capsules, for the encapsulation of the vitamins, may help overcome this problem.

It is now technically possible to encapsulate all the potentially important constituents of artificial diets, namely, high molecular weight proteins and starches within nylon-protein walled capsules; lipids and lipid soluble components within gelatin-acacia walled capsules and low molecular weight water soluble components such as amino acids, minerals and vitamins within lipid walled capsules. Such

capsules are especially useful for nutrition studies with filter-feeders since particle break-down and nutrient leaching are major problems with micro-sized particles. Instability and leaching are also problems with feeds for large particle feeders such as shrimp and lobsters and encapsulation techniques may prove equally useful in this area.

Table IX. The Growth of Crassostrea virginica Juveniles Fed on Artificial Diets With and Without Encapsulated Vitamins

Treatment	Growth in a 21 day period		
	% Change in dry organic wt/spat[a]	% Change in total dry wt/spat[a]	% Change in total wet wt/spat[b]
1. Diet given in Table VIII with encapsulated vitamins (vitamin content = 9.2 µg l^{-1})	+ 89.3	+ 189.5	+ 138
2. Diet given in Table VIII, except that water soluble vitamins were freely dissolved in seawater Vitamin concentration at:			
7 µg l^{-1}	no data	+ 112.6	+ 82
35 µg l^{-1}	no data	+ 100.0	+ 64
71 µg l^{-1}	no data	+ 118.2	+ 78
355 µg l^{-1}	no data	+ 102.8	+ 68
3. Starved controls	- 17.9	+ 25.4	+ 21
4. Algal fed controls[c]	+ 494.6	+ 784.6	+ 651

[a]% Change in dry weight was calculated from the weight of a representative sample of 100 animals from the experimental population, which was analyzed at the beginning of the experiment.

[b]Wet weight changes were calculated from the wet weight of animals of each treatment at the beginning and end of the experiment.

[c]The algal ration was calculated on the basis of 100 Thalassiosira pseudonana cells μl^{-1}, per 100 mg of spat wet weight, per liter, each day (= 1.32 mg algal dry wt l^{-1}).

Using lipid walled capsules, an artificial diet has been developed for C. virginica juveniles, fed under non-axenic conditions. The diet supported sustained, modest growth over an experimental period of 3 weeks, but the growth rate was only 1/5 that of spat fed on a full algal ration. Attempts are presently being made to improve the artificial diet so that it supports better growth rates. Attention will also be given to defining the essential nutrients in the diet under conditions where bacteria can be eliminated or at least where their effect on the results of the nutrition studies can be controlled.

We are now entering a new and exciting period of bivalve nutrition research. It will take a great deal of effort and experience before bivalve nutrition is understood as well as that of fish or crustacea but

at least a number of useful techniques for this research are now available.

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QUESTIONS AND ANSWERS

HAYWORTH (Edinburgh, Scotland): I would like to comment on the number of problems you've had with the nylon-protein walled capsules in that you found their walls highly permeable and you couldn't incorporate polyunsaturated fatty acids very efficiently. Recent techniques have been developed by Mars Ltd. which allow large amounts of polyunsaturated fatty acids to be included in the nylon-protein walled capsules and the loss of amino acids and vitamins has been greatly reduced by a secondary coating on the outside wall of the capsule.

LANGDON: Dr. Dave Jones of Menai Bridge, U.K. looked at the encapsulation of fatty acids and lipids using the nylon-protein capsule. He found that about 50% of labelled palmitic acid was lost. I'm pleased to hear that there are newly developed techniques in the manufacturing of these capsules which overcome this problem and I am very interested to learn about the improvements in reducing wall permeability.

SICK (NMFS -Charleston, S.C.): Chris, using your very generously shared method for preparing the lipid walled capsules, Al Fortner and I have encapsulated some labelled glycine and found that leakage was very high. We found that by using hydrogenated lipid as a wall material, together with cholesterol as a strengthening agent, the capsules retained about 70% of the labelled glycine.

LANGDON: By using hydrogenated oils, you presumably lower the melting point of the wall and it becomes hard at room temperature. Are these capsules with hardened walls digestible?

SICK: Yes, we have fed clams on encapsulated labelled glycine and have traced the glycine into various tissues, including the epithelium of the digestive track, and it seems to be incorporated, although I don't know what the absorption efficiency is.

GASTROPOD NUTRITION

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ABSTRACT

Aspects of gastropod nutrition considered in this review are the use of artificial diets in nutritional studies, phagostimulatory properties of dietary components, and the role of intestinal microorganisms in supplying essential nutrients. Application of artificial diets in nutritional studies or in rearing-enchancement experiments with gastropod molluscs has progressed from early use of chemically undefined diets for culturing snails to later, more defined diets, including some holidic diets. Diffusion of nutrients from chemically defined diets in seawater is discussed from the standpoint of involvement in distance perception and recognition of foodstuffs, and phagostimulatory properties of the soluble substances. Certain amino acids, such as L-glutamic and L-aspartic acids, are implicated in food-finding and food-recognition by marine invertebrate herbivores. Knowledge of phagostimulation is useful in preparing better diets and necessary in interpreting effects in nutrient-deletion experiments. The nutritional roles of intestinal bacteria are considered in the context of current approaches to the study of gastropod nutrition. Use of antibiotics to rid animals of gut microflora may hamper interpretation of the role that these microflora play in their host's nutrition, through known deleterious effects of various commonly used antibiotics. Future development in nutritional studies of gastropods should emphasize greater definition of diets, clearer identification of phagostimulants, and better understanding of the role that intestinal microorganisms play in the nutrition of their hosts.

KEYWORDS: artificial diets, nutrition, phagostimulation, intestinal microorganisms, antibiotics, gastropods, molluscs.

INTRODUCTION

If we define nutrition as the qualitative and quantitative determination of requirements for chemical substances in the diet of an animal necessary for its continued well-being, then of all the diverse groups of molluscs only gastropod molluscs are well studied. In addition, of the non-bivalve classes of molluscs, only the Gastropoda has any extensive mariculture potentital. Even so, in terms of world mariculture potential, gastropod molluscs are economically less

important than are other shellfish such as bivalves and crustaceans. In fact, of the commercially important marine gastropods, only abalone are cultured to any significant extent, and these usually to a size just sufficiently large to be released into the sea (Shibui 1978). The queen conch Strombus gigas and other conchs are harvested in several countries bordering the Caribbean Sea, but the fisheries are small and apparently diminishing, and prospects for extensive culture are slight. In the same way, a culture program exists for Murex trunculus in Tunisia, but has little large-scale potential (Bardach et al. 1972). Escargot (Helix spp.) are profitably harvested in France and other European countries and can be cultured to marketable size, but the overall harvest is small compared to various shellfish and is insignificant on a world scale. A number of unrelated marine species of gastropods, including various limpets and winkles (Littorina spp.), are locally eaten but the fisheries are comparatively small and presumably also have small potentials for mariculture. As a consequence of all this, dietary studies on the nutrition of gastropods are few in number and are confined to a few select taxonomic groups.

It will not be the purpose of this study to give a complete review of work done on gastropod nutrition; this is beyond the scope of this enterprise and interest. Rather, I hope to discuss those dietary studies carried out in controlled laboratory situations, with special attention being paid to those using artificial diets. For reasons of brevity I will not be considering studies on nutrition of gastropods where natural foods are involved, unless these are correlated specifically with phagostimulatory or other nutritional components of the diet. Other excluded topics are those strictly related to biochemical or physiological events unless these, in turn, are correlated with physical or chemical manipulations of artificial diets. Of special interest will be 1) the recognition by animals of pre- and post-ingestive phagostimulatory cues, 2) the role played by intestinal microorganisms in providing essential nutrients, and 3) the effects of deletions and augmentations of dietary components.

ARTIFICIAL DIETS IN NUTRITIONAL STUDIES OF GASTROPODS

From the time when dietary studies on gastropod nutrition commenced about 35 years ago, progress has been slow, and we are still a long way from realizing what Dougherty (1959) defines as the ultimate goal in nutritional studies: that of axenic (or pure) culture of a snail species through many generations on a chemically defined diet. The reasons for this are many, but it ultimately stems from our attention to the preparation of fast, effective and inexpensive diets for application to commercial mariculture, rather than to the more theoretical and more difficult elaboration of chemically defined diets for nutritional studies. For this reason, most artificial diets used in nutritional studies of gastropods have been compounded from mixtures of whole proteins, fats, and other natural materials, with augmentations of selected chemicals such as vitamins and amino acids. Problems besetting dietary studies of gastropods are no different from those confronted in nutritional studies of other aquatic animals using artificial diets, and relate mainly to aspects of preparation, palatability, leaching of nutrients, and determination of suitable nutritional components.

The first use of artificial diets for culturing aquatic snails appears to have been by Noland and Carriker (1946) using Lymnaea stagnalis appressa, and by Standen (1951) and later by Chernin (1957a, 1959), and Chernin and Schork (1959) using Australorbis glabratus (the latter an intermediate host of schistosomiasis). Details on preparation and composition of these diets and other diets used in the study of gastropod nutrition are given in Table I. Noland and Carriker (1946) used a combination of whole lettuce, wheat cereal cooked with milk, and mineral salts with no special binding agent to rear the freshwater snail L. stagnalis appressa through twenty successive generations. The binding stability of Standen's (1951) diet was increased by bathing the sodium alginate preparation with 2% $CaCl_2$ solution, which reduced leaching and provided a flat, thin sheet on which the young snails could feed. Chernin (1957a, 1959) hatched Australorbis glabratus into a sterile salt/antibiotic solution and used a compounded diet of dried autoclaved brewer's yeast and Escherichia coli. The author's claim for axenic culture of Australorbis in these papers is questionable as living E. coli were provided as a food source and no tests for gut microflora were reported; although in another study the living component of the diet was changed to formalin-killed E. coli (Chernin and Schork 1959). Streptomycin sulphate was found to inhibit growth of Australorbis in all these studies, a point to be considered later in this paper.

In studies on the abalone Haliotis discus, Ogino and Ohta (1963) and Ogino and Kato (1964) used artificial diets compounded from white fish meal, starch, soybean oil, cellulose, vitamins, yeast and minerals bound in a calcium alginate gel. Good growth of juvenile abalone was obtained on the artificial diets, in some cases surpassing that obtained with dry preparations of the animal's natural food, the brown alga Undaria pinnatifida. Fastest growth occurred at the highest levels of crude protein tested (43-44% of the dry diet), a result consistent with two different-sized starting classes of the young abalone (Ogino and Kato 1964). Sagara and Sakai (1974) had some success growing young (0.20-0.26g) Haliotis discus and H. sieboldii on artificial diets composed mainly of either white fish meal, marine yeast, or "alcholic active sludge", combined in a calcium alginate gel with starch and brewer's yeast, but growth was less on these than on a compounded diet of Chlorella, starch, and brewer's yeast. Good success in growing juvenile Haliotis tuberculata was obtained by Koike et al. (1979) with an artificial diet compounded largely of natural or extracted vegetable products (details of composition not provided), but less so with a diet modified from that of Ogino and Kato (1964), replacing the fish meal with dried Tetraselmis suecica (Table I). A common characteristic of these studies on haliotids has been the use of compounded preparations of largely untreated foods to provide a transitional diet until the animals are large enough to eat macroalgae.

Members of the sea hare genus Aplysia have been the principal subjects in my own studies on growth, nutrition, and feeding preferences of marine invertebrate herbivores. Sea hares have voracious appetites, are relatively fast-growing, and have broad feeding preferences. Three major questions have directed my work: 1) what constitutes a nutritionally good seaweed for sea hares? 2) what factors govern choice of food? 3) are the factors governing food choice the same as those important in nutrition? Earlier studies on the nutrition of these animals showed that the growth-promoting qualities of various seaweeds could not be convincingly correlated with such nutritional

Table I. Characteristics of Artificial Diets Used in the Study of Gastropod Nutrition

Species	Binding Agent: Type	Binding Agent: % of dry wt	Nutrient Composition (in brackets: % dry wt): Main Carbohydrate source	Main Amino acid source	Main Fatty acid source	Vitamins	Minerals	Other	Physical appearance of diet	Reference
Lymnaea stagnalis appressa			prepared food: wheat cereal cooked with milk; whole lettuce				unspecified		granular suspension	Noland & Carriker 1946
Australorbis glabratus	sodium alginate + 2% $CaCl_2$ solution	28.5	powdered dry lettuce (28.5)	dried milk (14.5)				"Bemax" (28.5)	thin sheets	Standen 1951
Australorbis glabratus			prepared foods: dried autoclaved brewer's yeast; living Escherichia coli/formalin-killed E. coli					penicillin G & streptomycin sulfate in handling solution	suspension	Chernin 1957a, 1959; Chernin & Schork 1959
Haliotis discus	sodium alginate + $CaCl_2$	20-60	pre-digested starch (5-15); cellulose (0-5)	white fish meal (20-60)		"Panvitan" plus others (1.9 per 100g diet)			flat gel on glass plates	Ogino & Ohta 1963
Haliotis discus	sodium alginate +$CaCl_2$	20	dextrin (12-64); cellulose (1-2)	defatted white fish meal(10-60); dried brewer's yeast (2)	soybean oil (1)	"Panvitan" (2) plus others in some experiments	(0-1)		flat gel on glass plates	Ogino & Kato 1964
Nassarius reticulatus veligers			cornflour/drinking chocolate/"Liquifry" diatoms, other plant cells)				(dried	50 I.U. penicillin G & 0.05g streptomycin sulphate.liter^{-1} of seawater	suspensions	Mapstone 1970
Arion ater	agar	13	starch (51.5) sucrose (4.5)	Casein (17)	olive oil (6)	ascorbic acid (2); choline chloride (0.4); vitamin mixture (8 vitamins; approx 0.1)	4 mineral salts (4.5)	aureomycin (0.1); potassium hydroxide (0.9)	agar block	Wright 1973, Ridgway & Wright 1975
Haliotis discus H. sieboldii	sodium alginate + 5% $CaCl_2$	20	starch (15) brewer's yeast (5)	white fish meal, marine yeast, Chlorella, or alcoholic active sludge (60)					2 mm flat gel on glass plate	Sagara & Sakai 1974
Haliotis tuberculata	sodium alginate	20	dextrin (15), Tetraselmis suecica (60) dried yeast (2)			unspecified (2)	unspecified (1)			Koike et al. 1979
Aplysia dactylomela	agar	27.6	starch, cellulose, sugars (56.2)	20 amino acids (4.2)	4 fatty acids (2.5)	AIN vitamin mixture 76[TM] and ascorbic acid (0.2)	14 mineral salts (5.8)	cholesterol (2.5); extract of Ulva fasciata (1.0)	circular wafers (8.5X0.4cm)	Carefoot 1979, 1980
Aplysia kurodai	agar	27.6	starch, cellulose, sugars (57.2)	20 amino acids (4.2)	4 fatty acids (2.5)	AIN vitamin mixture 76[TM] and ascorbic acid (0.2)	14 mineral salts (5.8)	cholesterol (2.5)	circular wafers (8.5X0.4cm)	Carefoot 1981
Aplysia dactylomela	agar	27.6	starch, cellulose, sugars (52.4)	20 amino acids (8.3)	4 fatty acids (2.5)	14 vitamins (0.2)	14 mineral salts (5.8)	cholesterol (2.5); streptomycin sulphate and penicillin G (0.7)	circular wafers (8.5x0.4 cm)	Carefoot 1982a

characteristics as absorption of calories, nitrogen, carbohydrates, or specific amino acids, nor with texture of the seaweed or amounts eaten (Carefoot 1967, 1970). Temporal and geographical differences in the nutritional content of seaweeds made long-term studies and other comparisons difficult, and suggested that a better approach would be to use artificial diets. This, in theory, would permit deletion and augmentation experiments to be carried out with full knowledge of all other nutritional components. In practice, tests on several different formulations of chemically defined diets showed that leaching was a major problem, even with coatings on the nutrients or with precipitated "skins" on the binding gels. A surface or gel that was too impermeable was even less desirable, as some diffusion was necessary for location, recognition, and enhancement of food palatability for the animals. An illustration of how quickly some nutrients, in this case a selection of amino acids, diffuse from 4% dry weight agar gel is given in Figure 1. Note that over 50% of the amino acids diffuse from the agar gels in just a few hours, and the rates are about the same whether an amino acid is tested alone or whether all amino acids are tested together with other nutrients. Surface "skins" of calcium alginate or chitosan acetate markedly reduced diffusion rates but were found to be unsuitable in the dietary studies as they repelled the sea hares. Notwithstanding the fact that many of the amino acids chosen in the experiment shown in Figure 1 are highly diffusible (as compared with less soluble ones such as tyrosine and cystine), the results do indicate the need for attention to possible large diffusion losses especially when working entirely with chemical nutrients in an agar matrix.

Initially in the development of chemically defined diets for Aplysia, palatability was of chief concern, and the sea hares were stimulated to eat only by incorporating 1% dry weight of a distilled-water extract of the green alga Ulva fasciata in the diet (Carefoot 1979, 1980). This diet was eaten well and promoted good growth of the tropical Aplysia dactylomela, but with the Ulva extract it was not useful for defined nutritional studies. By removing this extract of Ulva from the diet and substituting carbohydrates of known phagostimulatory properties such as starch and glucose (Carefoot 1982b), the chemically defined artificial diet provided the means to assess amino acid requirements in the Japanese Aplysia kurodai through deletion techniques (Carefoot 1981). Unexpectedly, animals maintained on diets deficient in single amino acids showed no significant weight loss (including spawn production as part of growth), and indicated an apparent lack of reliance on the normally recognized complement of ten "essential" amino acids, as determined for white rats, in the diet. Of the several ways in which deficiencies of "essential" amino acids could be met from non-dietary sources, assuming the requirements of sea hares for amino acids to be similar to those of other animals, the most interesting and provocative was that of the possible contribution by intestinal microorganisms. This subject will be considered in detail later.

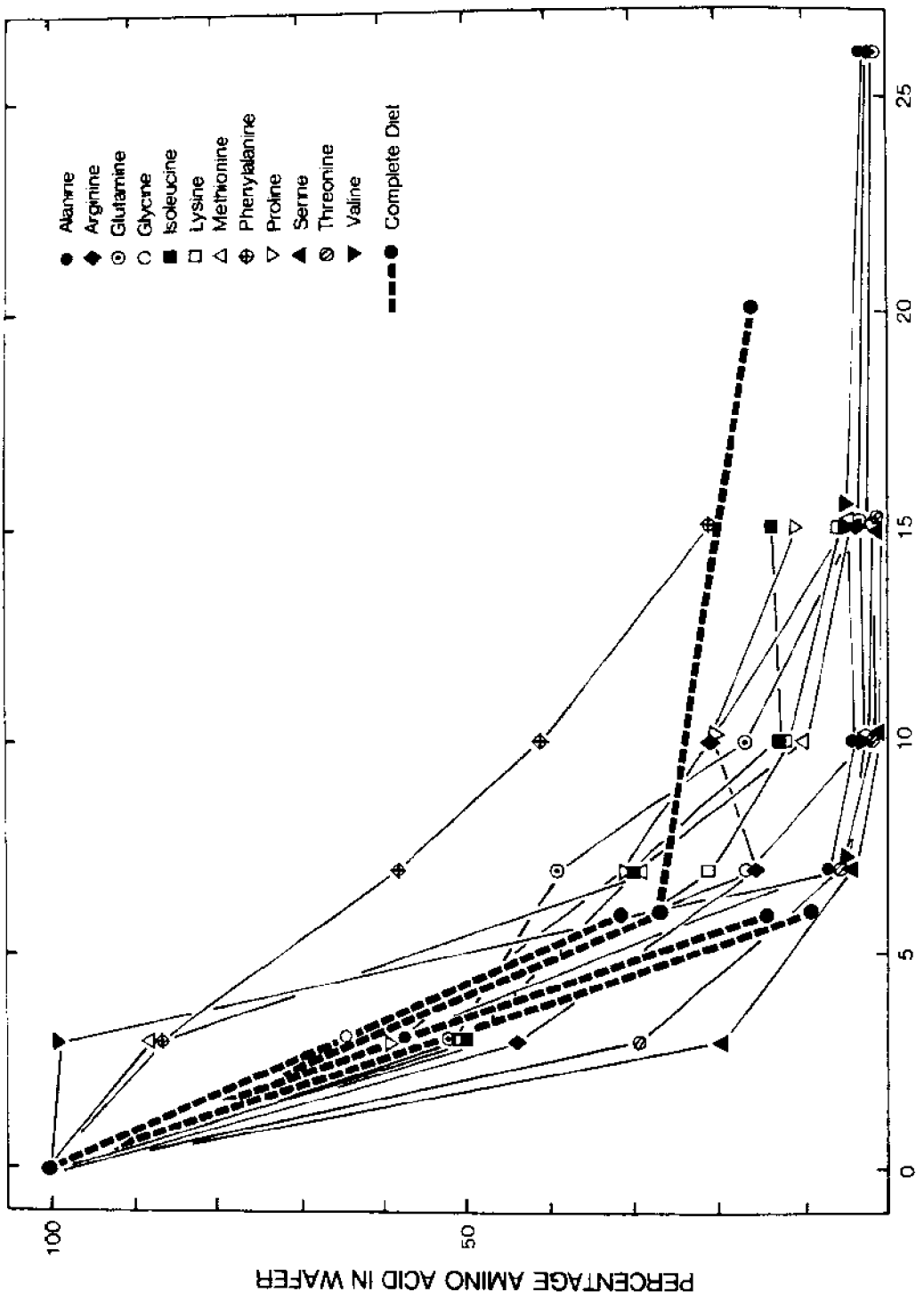

Figure 1. The leaching of amino acids from agar gels in seawater.

NUTRITIONAL STUDIES ON LARVAL AND TERRESTRIAL GASTROPODS

Little is known about the nutritional requirements of the veliger larval stage of marine gastropods. I could find no evidence in the literature that microencapsulation techniques, as have been used so effectively by Jones and his co-workers with filter-feeding invertebrates including the larvae of the prawn Penaeus japonicus (Jones et al. 1979), have been applied to nutritional studies on gastropod veligers. Clearly this is an area for further work. Mapstone (1970) was able to grow the larvae of Nassarius reticulatus on three artificial foods (cornflour, drinking chocolate, and "Liquifry" - the latter consisting mainly of dried pulverized diatoms), but even the best growth (on cornflour) was less than that on a natural algal diet (Cricosphaera sp.) and no artificial food sustained the larvae beyond 8-10 days. Bacterial contamination was also a problem with the diet of drinking chocolate, despite frequent changes of the medium and addition of antibiotics.

Terrestrial snails would seem to have been likely candidates for nutritional studies using artificial diets, based on the comparative ease of preparation and presentation of the diets in a non-aqueous medium, but such studies are few. Nutritional studies of escargot (Helix spp.) have largely emphasized growth and reproductive rates of animals fed on natural plant foods. Despite the obvious potential for mass culture in controlled situations, husbandry of escargot still relies heavily on field enclosures with natural herbage as food. Terrestrial slugs have received some attention, largely through the dietary studies of Wright (1973), and Ridgway and Wright (1975). These authors used a partially defined diet to rear Arion ater to maturity, but while growth and survival of slugs on the diet were adequate, reproductive performance was poor. An assessment of B-vitamin requirements of A. ater using deletion techniques with the diet showed that a deficiency of pantothenic acid rapidly reduced growth and resulted in poor survival, whereas deficiencies of riboflavin, thiamine, nicotinamide, folic acid, and pyridoxine caused reduced growth rate in later stages but not in earlier stages (Ridgway and Wright 1975). This study is one of only a few which has used deletion methods with artificial diets to assess nutritional requirements of a gastropod mollusc.

PHAGOSTIMULATORY COMPONENTS OF DIETS

As pointed out by Dadd (1960) in his considerations of nutritional studies of insects, it is important in dietary studies using deletion techniques with artificial diets to know whether a particular component of a diet being omitted is itself phagostimulatory. In such cases, poor growth may result from diminished food intake rather than from the omission of an essential nutrient. The absence, then, of good data on rates of food consumption makes interpretation of experimental results difficult. We also know very little about chemosensory feeding attractants for gastropods and even less about phagostimulants (see Carefoot 1982b).

Some early work in this area has identified the natural phagostimulatory properties of a crude water-extract of seaweed for the herbivorous Aplysia juliana (Frings and Frings 1965), and this has been

later confirmed for other species of sea hares (Jahan-Parwar 1972; Susswein et al. 1976; Carefoot 1980). Additionally, it is known that components of slime trails and water-borne metabolic exudates of snails can impart information of a chemosensory or chemotactile nature to other snails about recently eaten foods. None of the active components of these natural extracts, secretions, or excretions, has yet been positively identified.

On the other hand, there is an extensive literature on chemoreception in gastropods and other marine invertebrates which indicates high sensitivity to certain amino acids, sugars, and other materials (see Kohn 1961, Laverack 1968, and Bardach 1975 for reviews), and the question arises as to whether some of these chemicals may be used as feeding cues by gastropods. For example, certain amino acids, particularly L-glutamic and l-aspartic acids, have been implicated in long-distance location of foods for a number of marine invertebrates, including the gastropods Buccinum undatum (Bailey and Laverack 1963, 1966) and Aplysia californica (Jahan-Parwar 1972, 1975). Recent studies on Aplysia kurodai and A. dactylomela have also shown that L-glutamic and L-aspartic acids are more highly phagostimulatory than other amino acids or various sugars and fatty acids, and are equalled only by starch (Carefoot 1982b). In addition, tests of attractiveness of various fractions of lettuce homogenates to Biomphalaria glabrata, a vector of schistosomiasis, showed that free amino acids, specifically L-glutamic acid and L-proline, were the primary stimulants (Uhazy et al. 1978). It may seem unlikely that one, or even a few substances, would act in isolation from the host of chemical materials diffusing from an actively growing plant, as a recognition cue for an invertebrate herbivore. However, all of these studies point to possibly important food-locatory and food-recognition roles for certain amino acids. Thus, if L-glutamic and L-aspartic acids were influential firstly, in guiding sea hares to their seaweed foods, and secondly, in stimulating them to eat, we might predict high diffusion rates of L-glutamic and L-aspartic acids from marine algae as well as high levels of these amino acids in the free-state form in the algal tissues. We might also predict the highest levels of these amino acids in the most favored foods of sea hares. While we know little about natural rates of diffusion of amino acids from seaweeds, there are abundant data which indicate that the concentrations of free amino acids in marine algae are generally high and it is probable that these amino acids are lost by diffusion. Table II is a summary of data from the literature for concentrations and ranks of concentration of free and bound forms of L-glutamic and L-aspartic acids in several orders of seaweeds. Note that the concentration of free L-glutamic acid ranks highest in several large orders of seaweeds, including the Ulvales, Cladophorales, and Gigartinales, and second-highest in the Ceramiales. These four orders include many of the preferred foods of sea hares and other marine invertebrate herbivores, notably the green algae Ulva spp., Enteromorpha spp., and Cladophora spp., and the red algae Plocamium spp. and Laurencia spp. L-aspartic acid ranks second-highest in the Gigartinales and Cladophorales, but eighth-highest in the Ulvales. Concentrations of bound L-glutamic acid are highest in the Gigartinales, and second and third highest in the Cladophorales and Ulvales, respectively. Concentrations of bound L-aspartic acid are highest in the Cladophorales, but fourth highest in both Gigartinales and Ulvales. Levels of bound amino acids as opposed to free amino acids, would be unlikely to be important as recognition

cues for distance-location of suitable foods, although they could be important in contact chemoreception. Also, levels of L-glutamic or L-aspartic acids, or for that matter, other "non-essential" amino acids, would not likely be as important in post-ingestive cueing of diet suitability as would levels of various "essential" amino acids. Recognition and subsequent memory of such post-ingestive cues by animals, particularly in diets containing an imbalance of amino acids, are well known for vertebrates (Rogers and Leung 1977), but are less well known for even well studied invertebrates such as insects, and to the best of my knowledge have not been tested for except in a general way in other invertebrates (Susswein and Kupferman 1975: Croll and Chase 1980). Certainly, a knowledge of phagostimulatory properties of components of artificial diets will be useful in preparing such diets for nutritional studies, and may be necessary in order to understand fully the effects of manipulating levels of various dietary components on the nutrition of animals.

Table II. Concentrations and Ranks of Concentrations (highest to lowest for 18 amino acids) of Free and Bound L-Glutamic and L-Aspartic Acids in Several Orders of Seaweeds. Data abstracted from over 300 published amino acid analyses of species of marine algae.

Class	Order	Free					Bound				
		Glutamic acid			Aspartic acid		Glutamic acid			Aspartic acid	
		N[a]	Rank	mg % dry wt[b]	Rank	mg % dry wt	N	Rank	% dry wt	Rank	% dry wt
Rhodophyceae	Ceramiales	21	2	233	4	102	4	3	1.23	6	1.08
	Gigartinales	13	1	219	2	112	3	1	1.38	4	1.15
	Rhodymeniales	10	1	123	2	92	-[c]	-	-	-	-
Chlorophyceae	Caulerpales	10	1	1	-	0	13	3	2.29	6	1.83
	Cladophorales	2	1	5	2	4	2	2	0.93	1	1.03
	Ulvales	14	1	71	8	25	11	3	1.83	4	1.78
Phaeophyceae	Dictyotales	27	3	3	1	8	27	2	1.81	6	1.54
	Laminariales	3	2	172	11	50	-	-	-	-	-

[a] N indicates the number of analyses done on species in a given Order.

[b] mg % dry weight indicates number of mg (100 g dry alga^{-1}).

[c] A dash (-) indicates no data available.

THE ROLE OF INTESTINAL MICROORGANISMS IN THE NUTRITION OF GASTROPOD MOLLUSCS

The presence of symbiotic microorganisms in the alimentary tracts of insects has long been recognized as a complication in interpreting the nutritional requirements of the host animals. Whether associated microorganisms are always involved in an insect's nutrition or whether they are mainly important when an insect feeds on a nutritionally

inadequate foodstuff is still under debate (see House 1974), but it is clear that in many instances they do make important and necessary contributions, and in view of this it is surprising that so little attention has been paid to this problem in nutritional studies of other invertebrates. This is especially true in nutritional studies using chemically defined diets, where the effect of manipulation of levels of minor but perhaps indispensable components such as certain amino acids and vitamins can be obscured or rendered meaningless if it can later be shown through axenic techniques that symbionts were indeed providing sufficient quantities of these nutrients. Two recent examples serve to illustrate this. Firstly, my own study on Aplysia kurodai (Carefoot 1981), in which animals maintained on chemically defined diets which were deficient in a single amino acid showed an apparent lack of reliance on the normally recognized complement of ten essential amino acids (as required for the white rat); and secondly, Stahl and Ahearn's (1978) study on juvenile Macrobrachium rosenbergii in which animals fed on diets that were each deficient in one of the four "essential" amino acids arginine, lysine, methionine, and tryptophan, also showed a seeming lack of requirement for these amino acids. These later authors suggested that gut bacteria may have been involved but could not rule out cannibalism and bacterial coatings on exposed surfaces in the aquaria as alternative sources for the missing amino acids.

Several studies on marine invertebrates have demonstrated that symbiotic intestinal bacteria aid in the digestion of food in the guts of their host organisms. One of the earliest of these, by Lasker and Giese (1954), showed that large numbers of bacteria in the gut of the sea urchin Strongylocentrotus purpuratus could digest agar and a red alga Iridophycus flaccidum. Later, Galli and Giese (1959) showed that bacteria isolated from the gut of the herbivorous marine snail Tegula funebralis could cause cellular breakdown of several seaweeds, and a few bacterial strains could digest agar; however, these active strains appeared to be present in only low numbers and so the exact role of the major complement of gut bacteria in this snail was unclear. Terrestrial snails also harbor their own special intestinal microflora, and these symbionts are known in Helix pomatia and other snails and slugs to provide useful cellulose- and chitin-digesting enzymes (Florkin and Lozet 1949; Jeuniaux 1950, 1954; Evans and Jones 1962). Prim and Lawrence (1975) further demonstrated that bacteria isolated from the guts of echinoids could physically degrade a variety of marine plants and that bacteria from the gut of the echinoid Lytechinus variegatus could utilize a number of carbohydrate storage products from plants, including sugars, laminaran, carrageenan, starch, and agar, but not cellulose or chitin. These latter authors suggested that the bacterial isolates were nutritionally "opportunistic", each able to utilize a variety of polysaccharides, but proposed that their contribution to the overall nutrition of their hosts may in fact be small because of lack of a space in the gut of the animals for harboring large numbers of microorganisms, and rapid turnover of gut contents when algal food is abundant. For these reasons such bacteria should not be considered true symbionts even though their metabolic activities, however transient, may play important or even crucial roles in the nutrition of their hosts.

That gut bacteria in a marine invertebrate herbivore may provide required amino acids for their host is strongly suggested by the recent work of Fong and Mann (1980). These authors fed sea urchins

(Stronglyocentrotus droebachiensis) on small agar discs containing an homogenate of kelp and finely powdered U-^{14}C-cellulose or U-^{14}C-glucose, and found after analysis of gonadal protein that all amino acids were radioactively labelled. Bacteria were implicated by the fact that in animals treated for two days with antibiotics and then injected with labelled glucose, no radioactivity was detected in a number of amino acids of the gonad including the ten recognized as being essential for the white rat. The assumption here is that the antibiotic treatment (50 mg penicillin G and 40 mg streptomycin sulphate.liter^{-1} of seawater medium bathing the animals) affected only the gut microflora (numbers of gut bacteria decreased from 2.5 x 10^8.ml^{-1} of gut fluid to 1 x 10^3.ml^{-1} after two days exposure) and not the sea urchins. This is an important point and its implications in nutritional studies may be significant, especially when antibiotics are used in in vivo studies.

In conclusion, there is increasing evidence to suggest that symbiotic intestinal bacteria participate in the nutrition of their marine invertebrate hosts, and marine gastropods are unlikely to be an exception. This participation may take two forms: indirect, in which the digestive action of bacterial enzymes breaks down structural polysaccharides of marine plants such as cellulose and alginic acid, thus releasing cell contents for further bacterial or host-produced enzymatic digestion; or direct, in which hydrolytic bacterial enzymes act on specific carbohydrate storage products in plants or animals, such as starch, glycogen, laminaran, and various sugars, or on intercellular polysaccharides such as agar and carrageenan. While it is unlikely that intestinal bacteria would play as important a role in the nutrition of marine snails as do the chemotrophic H_2S-utilizing bacteria in the pouch-like trophosomes of deep-sea, hydrothermal, vent-inhabiting pogonophorans (which have not true gut), or the bacteria associated with certain gutless bivalves (Hochachka and Somero 1982), their potential in this regard should not be underestimated.

USE OF ANTIBIOTICS IN NUTRITIONAL STUDIES

Most work on the nutritional importance of intestinal bacteria has involved in vitro assays using bacterial isolates. Those few in vivo studies using antibiotics to kill or debilitate gut bacteria (for example, Allen and Kilgore's 1975 investigation of the amino acid requirements of Haliotis rufescens) risk the "Catch-22" criticism that antibiotic treatment affects not only the bacteria but also the health of the host organism itself. Such a criticism is not without foundation. Several studies on aquatic gastropods and crustaceans have demonstrated the deleterious effects of certain commonly used antibiotics, including streptomycin, in relatively small dosages. Growth and reproduction in the snail Australorbis glabratus, for example, were inhibited either partly or totally at streptomycin levels of 100 mg.liter^{-1} and toxicity effects lingered for some weeks after initial exposure (Chernin 1957b, 1959; Cherni and Schork 1959). Thus, where cultures were not axenic, it is not known whether these effects were due to a direct impairment of the health of the snail, or to secondary causes resulting from the effects of the antibiotic on bacteria in the gut. At higher concentrations, both penicillin (at 10,000 units.ml^{-1}) and streptomycin (at 5 mg.ml^{-1}) cause death in A. glabratus, and the effects of these antibiotics are mild compared to the

effects of others (Seneca and Bergendahl 1955). Finally, at levels of 200 and 300 mg.liter^{-1}, respectively, penicillin and streptomycin were shown to cause aberrant behavior, inhibit growth, and sometimes eventually kill larvae and adults of Artemia salina (D'Agostino 1975). Given that an antibiotic may be toxic to an organism at some high concentration, it may therefore be difficult in axenic studies where antibiotics are applied, to separate the direct and indirect effects of the drugs. As an example, in a study on the role of intestinal bacteria in the nutrition of the sea hare Aplysia juliana, Vitalis (1981) used a seawater mixture of 10 mg.liter^{-1} each of streptomycin sulphate and penicillin G to suppress the activities of gut microflora, and showed significant reductions in rates of growth of treated animals as compared with untreated animals. Yet, counts of gut bacteria showed an overall reduction of only about one order of magnitude in numbers as a result of antibiotic treatment. Unless activity of the bacteria were severely curtailed this reduction would not seem sufficient to explain the large differences in growth responses under the two treatments.

Incorporation of antibiotics directly into the food of an animal to suppress the activities of gut bacteria would seem to be a better approach than simply bathing the animals in an antibiotic-containing medium. This method has worked successfully for insects (see House 1974) and has been attempted for various gastropods, but in the latter instances with questionable success. Wright (1973), and Ridgway and Wright (1975), for example, added aureomycin to artificial diets for Arion ater to suppress the activities of intestinal and skin microflora (0.09% dry weight of diet), and found that deficiencies of several B-vitamins in the diets caused no apparent reductions in growth in the young stages - the suggestion being that bacteria may have provided sufficient of the missing vitamins. The authors reported no counts of bacteria to support their interpretation of the data. A similar study with Aplysia dactylomela, using chemically defined diets with a deficiency of a single "essential" amino acid, also provided somewhat conflicting results (Carefoot 1982a). Thus, inclusion of 500 μg each of streptomycin sulphate and penicillin G per ml of set agar in the artificial diet (0.7% dry weight of diet) did not significantly diminish numbers of bacteria in the gizzard of A. dactylomela (bacterial numbers were 1.5-6 x 10^8.ml^{-1} of gizzard fluid in antibiotic treated animals, 2 x 10^8.ml^{-1} in untreated animals), yet animals treated with antibiotics over a 20-day period steadily lost weight on a diet deficient in arginine. When this arginine-deficient diet was replaced after 20 days with a diet complete in all nutrients normal growth was restored. This suggests that bacteria may have been involved in supplying this amino acid, and thus may have only been debilitated rather than killed by the antibiotic treatment. These studies on Aplysia are continuing.

CONCLUSIONS AND FUTURE DEVELOPMENTS

The present paper has dealt with several aspects of the study of gastropod nutrition which I consider not only interesting but important, namely the strict use of chemically defined diets rather than partially defined diets to study nutritional requirements, the identification of phagostimulatory properties of chemical components of a diet, and the role of bacteria in the nutrition of marine invertebrates. Research leading to adequate artificial diets for gastropod molluscs has been

slow, partly due to an interest in culturing only a few gastropod species. However, problems of formulation, preparation, and stability of diets, appear to be no different from those encountered in studies of other shellfish. Leaching problems could be overcome by microencapsulation techniques, but this may prove too expensive and time-consuming. Chemically defined diets, although a requisite for studies of nutritional requirements, are unlikely to find general acceptance for husbandry purposes. Such diets are too expensive, hard to prepare, and unstable. Requirements for diets for mariculture or other husbandry purposes may continue to be best met by basal diets compounded from natural substances with suitable additions of essential nutrients and/or phagostimulants. Even so, our understanding of gastropod nutrition is far behind that of crustacean nutrition.

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QUESTIONS AND ANSWERS

WEINER (Univ. of Maryland): Have you identified any particular species of bacteria or group of karyotes typical of the gut of Haliotis

CAREFOOT: Aplysia is the animal we've been working on. We've been concentrating mainly on bacteria and no other prokaryotes and the answer is no. It's extremely difficult to become a taxonomist of marine

bacteria and the best we can say is that they are gram-negative. If you've ever seen a list of these you probably know that you can catagorize in terms of external characteristics whether they are fuzzy or hairy, creamy or smooth, how fast they grow, their color - those kinds of characteristics. I'm sure there will be a number of microbiologists here that will take me to task for that, but I think the microbiologists would agree that it is a big task to attempt to identify these bacteria.

WEINER: I am a microbiologist by training and I concur it is a large task, but an important one.

KITTREDGE (Univ. of So. California): Aplysia is perhaps the most beautiful example of a marine organism that has developed a high tolerance for compounds that normally are feeding inhibitors. They feed on Laurencia and a number of red algae that contain huge amounts of highly halogenated terpenoids, and thus they have available a food source that other herbivores don't feed on. These highly halogenated compounds at the same time are rather strong antibiotics and I wondered if you had a chance to look at the gut contents of normally feeding Aplysia dactylomela?

CAREFOOT: Only that one set of data that I showed you where I had them feeding on a mixture of red algae. That's a very good point, of course, and for those of you who don't know Aplysia I can mention that they use some of these halogenated materials in their aplysiopurpurin -- a secretion which we don't know the function of. Presumably it is defensive. That would be an excellent study. The whole topic is wide open: Do numbers of bacteria change? Do numbers and types of bacteria change on different diets? These animals undergo coprophagy . Is this important in the nutrition of the animal, as well? It's a point that we've considered, but have not done.

SICK (NMFS-Charleston, S.C.): I was interested in your dissolution of primary amines from your formula feeds. I have just put out a paper with Al Fortner on dissolution of primary amines from formula diets and encapsulated diets. The thing that intrigued me about this was that it's very difficult to explain massive dissolutions and I agree with your results. In our case we went back and partitioned between bound and free in the original source and found that most of the dissolution, of course, came from the free. The problem is in most feeds you wouldn't expect that much free amino acids. Most of it is going to be bound. The question is, what was the source of protein in your diet and how do you explain the dissolution that you observed?

CAREFOOT: I had no protein source in the diets; I used amino acids.

SICK: So these were in crystalline form?

CAREFOOT: Yes.

SICK: In our case, by the way, just to finish the comment, our source was fish-meal. I wasn't familiar with this, but it turns out that in the fish-meal processing they put back a lot of solubles from another part of the processing of fish-meal. Apparently that's a problem that you do have in dealing with fish meal protein source.

DISSOLVED ORGANIC MATERIAL AND THE NUTRITION OF MARINE BIVALVES

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KEY WORDS: Amino acid transport, marine bivalves, transport kinetics, nutrition.

UNUSUAL ABBREVIATIONS: FAA = free amino acids, DOM = dissolved organic material; HPLC = high performance liquid chromatography, TCA = trichloracetic acid.

INTRODUCTION

The organic material in solution in sea water represents a vast resource of chemical potential energy. Its mass is exceeded only by that of the organic material found in the form of fossil fuels; it outweighs all living things on earth (see Table I). Our information concerning the source, composition and fate of this material is very incomplete. Wangersky (1978) provides the most recent comprehensive review of these topics.

Table I. Organic Carbon in the Biosphere in Gigatons (10^{15} g)[a]

Dissolved in sea water	1,596
Total plant biomass	828
Total annual primary production	78
Marine plant biomass	1.7

[a]Estimates from Woodwell et al. (1978).

This paper will deal with one fraction of dissolved organic material (DOM), namely the free amino acids (FAA) dissolved in marine waters. This restriction takes cognizance of the fact that our ability to identify and quantitatively determine FAA in sea water is advanced compared to available analytical procedures for other categories of compounds. For this reason, it is possible to be more precise about FAA than any other category of DOM. Also, the turnover rates of FAA are very rapid compared to that of DOM as a whole (Williams et al. 1969).

This suggests that FAA may play a biologically important role. Finally, amino nitrogen is a strict requirement for metazoan nutrition which also makes the fate of FAA a matter of interest.

The concentration of DOM encountered in near-shore marine waters is low; values in the range of 2-6 mg C/ℓ are typically reported based on wet combustion analyses. Concentrations of FAA are lower still; current reports and personal observations indicate a range of approximately 20-200 μg/ℓ (Mopper and Lindroth 1982). Thus FAA represent a small fraction of the total DOM.

Attention should be drawn to the fact that particulate sources of organic carbon are also a small fraction of the total organic material in seawater. Even in relatively productive inshore waters, plankton rarely exceeds a concentration of 100 μg C/1 (Jorgensen 1966). Thus the organic carbon content of FAA in near-shore marine water is broadly comparable to that present as plankton.

The possibility that DOM might contribute to the nutrition of marine animals was suggested more than a century ago (see Jorgensen 1976). However, the hypothesis is usually associated with the name of Pütter (1909). Krogh (1931) published a brilliant and influential review of the field. He concluded that "... there is no convincing evidence that any animal takes up dissolved organic substances from natural water in any significant amount". This settled the issue for most marine biologists at that time. However, this view must now be revised. More recent investigators have had the tremendous advantage of access to analytical techniques of great power and sensitivity. It is now possible to demonstrate net entry of FAA into marine animals from natural sources at rates which are significant when compared with their metabolic requirements.

ENTRY OF FAA INTO MARINE BIVALVES

Stephens and Schinske (1957) demonstrated net entry of glycine from ambient sea water into the slipper limpet, Crepidula fornicata, and the mussel, Mytilus edulis. They later studied ten other phyla of marine invertebrates (Stephens and Schinske 1961) and included data on net entry of several amino acids (arginine, glutamate, methionine, phenylalanine and tyrosine as well as glycine) in other bivalve genera (Pecten, Mercenaria, Spisula, Yoldia). Limited sensitivity of the ninhydrin procedure employed in this work required the use of relatively high concentrations of substrate. In Spisula, the concentrations of glycine employed ranged from 20 μM (1.5 mg/ℓ) to 2 mM (150 mg/ℓ); for comparison, Manahan et al. (1982) report a naturally occurring glycine concentration of approximately 15 μg/ℓ. Stephens and Schinske (1961) also provided evidence that glycine was removed during passage of water through the mantle cavity of Spisula by analysis of samples drawn from the excurrent siphon compared with samples from the incurrent margin of the clam.

Subsequently, Stephens (1960, 1962) introduced the use of ^{14}C-labelled substrates and demonstrated that the influx of labelled substrate occurred directly across the body wall. The process showed saturation kinetics indicating carrier-mediated transport was involved. In ensuing years the use of radioisotope-labelled compounds was exploited in several laboratories to study uptake processes in a variety

of marine invertebrates. Stephens (1968, 1981) provides a general review while Stewart (1979) reviews work on marine bivalves. Péquignat (1973) used autoradiography to demonstrate that the gill was the primary site of entry of labelled glucose in bivalves. In the 1970's Bamford, Stewart, Wright and other investigators studied the kinetics of entry of labelled substrates into isolated gills or gill fragments of several bivalve species (cf. Stewart 1979). However, the use of isolated gill preparations for the study of entry of labelled substrates into bivalves was later found to be potentially misleading despite its apparent simplicity and directness. Isolation of gills typically produces cessation of lateral ciliary activity (Wright and Stephens 1978). As a result, water is held as an unstirred layer between the gill filaments and gill lamellae. The presence of this layer influences the observed kinetics of influx in ways which lead to gross errors in estimating constants in the equation which describes substrate entry. This trapped layer also introduced a non-saturable component into observed kinetics of entry which has been improperly interpreted as evidence of passive diffusion. These matters have been recently reviewed (Stephens 1981) but a brief discussion of transport kinetics is required at this point to explain these concerns further.

TRANSPORT KINETICS

Figure 1 is an idealized representation of typical results obtained when rate of entry of a ^{14}C-labelled substrate is measured and plotted as a function of substrate concentration. As shown in the figure, investigators have typically separatd the observed total influx into two components and proceeded to demonstrate a good fit between the experimental data and the following equation:

$$J^i = K[S] + (J^i_{max}[S])/(K_t + [S]) \quad \text{(Equation 1)}$$

where J^i is influx, $[S]$ is substrate concentration and the other symbols represent constants. Interpretation of $K[S]$ in Equation 1 as an indication of passive or diffusional entry of substrate is almost certainly incorrect. The concentration of total FAA in the cells of marine invertebrates, including bivalves, ranges from 0.2-0.5 M (approximately 10-50 g/ℓ). External concentrations of FAA are extremely low (cf. foregoing discussion). Thus there is a large outwardly directed concentration gradient of FAA across the epithelial membrane of the gill. If $K[S]$ is interpreted as indicating diffusion, K has a sufficiently high value that the loss of FAA to the environment would be expected to be very large. This hypothetical passive efflux of FAA would be incompatible with maintenance of the concentrated intracellular pool of FAA and has not been observed to occur in intact Mytilus edulis (Manahan et al. 1982).

Wright and Stephens (1977) showed that the apparent passive glycine influx into the mussel, Mytilus californianus, was an artifact due to the presence of labelled substrate in the intercellular spaces of the isolated gill preparation employed. The effective intercellular space was increased as a result of cessation of lateral ciliary activity. This space was measured separately and raw data relating entry of labelled substrate to substrate concentration was corrected to take account of the radioactivity associated with the intercellular space.

When this was done, there was no passive or "diffusional" component and the corrected data were fully described by the following equation:

$$J^i = (J^i_{max} S)/(K_t + S) \quad \text{(Equation 2)}$$

We may conclude that there is no measurable passive entry of FAA into the gills of Mytilus californianus. Furthermore, it is incumbent on investigators to provide independent evidence for the occurrence of diffusion in addition to reporting data such as those schematized in Figure 1 before concluding that passive entry contributes to observed influx.

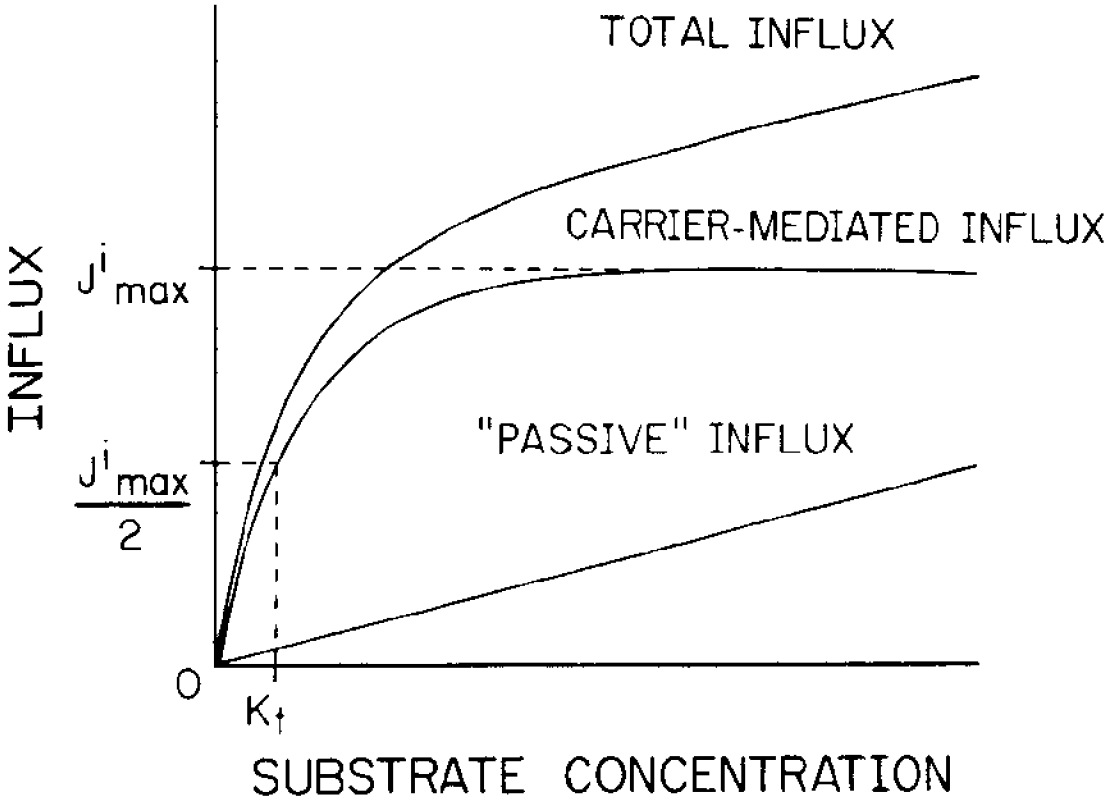

Figure 1. Diagrammatic representation of typical data relating substrate concentration (S) and rate of influx of substrate (J^i). The curve labelled "total influx" is a plot of the experimental measurements. The "passive influx" is estimated graphically and subtracted from total influx to give the curve labelled "carrier-mediated influx." J^i_{max} and K_t, the maximum rate of influx and the concentration at which the rate of influx is half-maximal, are labelled; they are derived from the carrier-mediated component of the raw data.

Equation 2 is the equation of a rectangular hyperbola. It states that the rate of influx (J^i) increases with concentration ([S]) provided [S] is low (of the same order of magnitude as K_t or lower). At high [S] ([S]>K_t), J^i approaches J^i_{max} or the maximum rate of influx. "J^i_{max}" and "K_t" are constants for a given set of conditions (e.g. temperature, salinity) and for a particular substrate and organism. The equation is

formally identical to the Michaelis-Menten equation describing the relation between reaction velocity (v) and substrate concentration [S] for an enzyme-catalyzed reaction.

"K_t" in Equation 2 is the concentration at which the carrier mediating influx is half-saturated, i.e. the concentration at which J^i is equal to 1/2 J^i_{max}. Methods for determining K_t and J^i_{max} from experimental data are given in elementary text sources and the interpretation of these constants is also discussed (see, for example, Neame and Richards 1972). Put in non-mathematical terms, "J^i_{max}" represents the capacity of the carrier to mediate influx while "K_t" represents the apparent affinity of the carrier for the substrate. Both characteristics of the transport system are functionally important.

In the present context, I wish to draw attention to the relation between observed K_t and the concentration of substrate normally available to the transport system. Stewart (1979) summarizes the work of several investigators studying the kinetics of influx of several amino acid substrates using isolated gills. He tabulates the observed K_t's and reports a range of 9-304 μM. The lowest value of 9 μM is reported for aspartate and should be treated with caution since the influx of this substrate is unusually low. We have previously noted that the normal range of FAA in near-shore waters is approximately 20-200 $\mu g/\ell$ or approximately 0.2-2 μM. Therefore, individual amino acids are normally available in submicromolar concentrations or at levels which are two orders of magnitude lower than the K_t's listed by Stewart (1979).

At concentrations far below the K_t, little transport can occur because the substrate concentration is low compared to the apparent affinity of the carrier. Figure 2 presents the resulting difficulty graphically. Two curves relating influx and substrate concentration are presented. The upper curve is drawn on the assumption that the K_t of the transport system is 1.0 μM; the lower curve assumes a K_t of 100 μM. In both cases, J^i_{max} is assumed to be 1.0 in arbitrary units. The level of available substrate is assumed to be 1.0 μM (indicated in the figure as the shaded area). With a K_t of 1.0 μM, J^i will be 0.5 units by definition of K_t. With a K_t of 100 μM, J^i will be 0.0099 units (calculated from Equation 2) or less than one fiftieth of that of the higher affinity system. Thus, if the K_t's reported in the work employing isolated gill preparations are accepted as correct, no significant influx of FAA in the gill of bivalves can occur from the concentrations available in natural waters.

To state the problem in another way, Mytilus californianus has been shown to exhibit one of the highest rates of maximum influx of FAA per unit weight of tissue among marine invertebrates studied (Wright and Stephens 1977). However, if the K_t for glycine influx determined from isolated gill preparations is accepted as correct, less than one percent of that transport capacity is functional at concentrations present in the natural habitat and the contribution of FAA as a result of influx via such transport is negligibly small when compared with the metabolic requirements of the animals. Wright and Stephens (1978) addressed this problem by devising a system for studying the kinetics of FAA influx in intact, normally pumping animals. They observed K_t's for entry of ^{14}C-labelled glycine of 2-3 μM. Thus, the transport system as observed in normally pumping animals proved to be well adapted to provide substantial influx of FAA at naturally occurring ambient substrate concentrations.

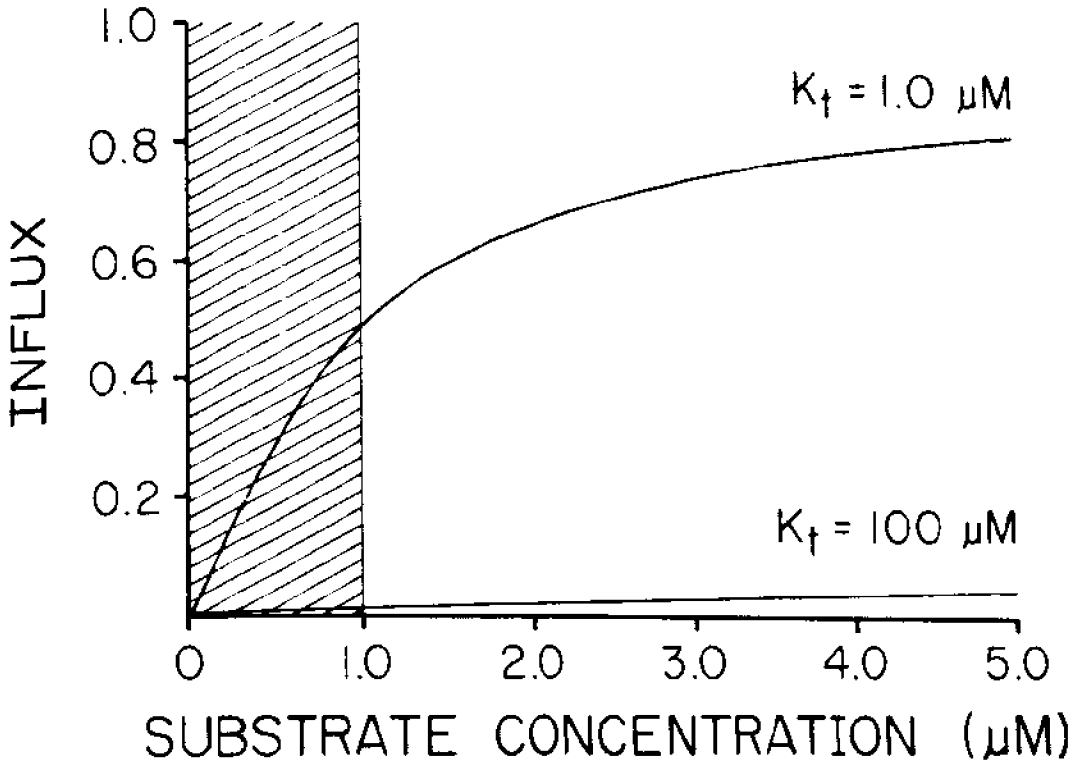

Figure 2. Curves drawn from the following kinetic assumptions: the upper curve assumes a K_t of 1.0 µM and a J^i_{max} of 1.0 (arbitrary units); the lower curve assumes a K_t of 100 µM and the same J^i_{max}. Points are calculated from Equation 2 in the text. Substrate concentration is 1.0 µM as indicated by the shaded area. Note that the transport system with the lower K_t results in an influx at 1.0 µM which is approximately 50 times greater than that of the system with the higher K_t.

Wright et al. (1980) resolved the issue of the apparent discrepancy of estimates of kinetic parameters for FAA transport in M. californianus studied with isolated gill preparations as compared to results obtained with normally pumping animals. These investigators showed that isolation of the gill disrupts normal water circulation and results in a thicker diffusional barrier or "unstirred layer" between the bulk medium and the transport surface. This introduces a major error into kinetic studies employing isolated gills and leads to a gross overestimate of K_t.

Two conclusions follow from this discussion of transport kinetics as it applies to bivalves. First, marine bivalves which have been carefully studied appear to have efficient transport systems which are well adapted for the uptake of amino acids occurring at submicromolar concentrations in the natural environment. Second, extreme caution is necessary in interpreting the values of kinetic constants for uptake of FAA which are reported in the literature.

Demonstration of entry of a ^{14}C-labelled substrate is not equivalent to demonstration of net influx of that substrate. Exchange diffusion of a specific substrate mediated by a membrane carrier shows saturation kinetics in experiments where labelled substrate is supplied in the medium and entry is studied as a function of ambient concentration. That is, data obtained by use of the radiochemical techniques which most investigators have employed do not permit us to distinguish between net influx and exchange diffusion. This point was emphasized by Johannes et al. (1969). These investigators showed that efflux of FAA from an ectocommensal flatworm, Bdelloura candida, as determined by direct chemical analysis of the appearance of FAA in the medium, exceeded influx of radio labelled FAA in their experiments. This led them to suggest that previous reports of influx of FAA should be viewed as exchange diffusion rather than net influx of substrate pending further evidence.

As noted above, early work had to employ direct chemical analysis of the disappearance of FAA to demonstrate influx of FAA into bivalves (Stephens and Schinske 1961). However, relatively high concentrations were necessarily employed because of the limited sensitivity of colorimetric techniques of analysis avaliable. In 1972, Udenfriend et al. introduced a fluorometric technique for analysis of primary amines based on a new reagent, fluorescamine. This allowed rapid determination of total primary amines at submicromolar levels. Stephens (1975) used this procedure to study net flux of FAA in marine annelids. Influx of ^{14}C-labelled amino acid substrates proved to be closely paralleled by net reduction of total primary amine in the medium as determined fluorometrically. This approach was used by Wright and Stephens (1977, 1978) to show that removal of ^{14}C-glycine from the medium by intact Mytilus calfornianus was accompanied by removal of primary amines from the medium as determined fluorometrically. These data clearly exclude simple exchange diffusion as a tenable hypothesis to account for the observed influx of labelled FAA.

The removal of primary amines as determined fluorometrically parallels disappearance of labelled substrated as stated above. However, the rate of disappearance of primary amines is slightly slower than the rate of disappearance of labelled substrate at all concentrations; this divergence becomes quite apparent at micromolar levels of substrate. As noted, fluorescamine reacts generally with primary amines. The data obtained by simultaneous monitoring of disappearance by fluorometry and radiochemical techniques has therefore been interpreted as evidence for leakage of unknown primary amine(s) accompanying the net influx of the amino acid substrate supplied (reviewed and discussed in Stephens 1981).

Fluorescamine reacts generally with primary amines, but the products differ widely in their specific fluorescence. Thus it can only be employed legitimately as an analytical procedure in circumstances where the nature of the primary amine compounds present in the medium can be specified. This is possible in experiments where a known substrate is supplied in the medium as in the case of the work of Wright and Stephens (1977, 1978). However, as time passes, amines added to the medium by the animal react with fluorescamine and the composition of the medium becomes increasingly undefined. The fact that fluorescamine

reacts generally with primary amines makes it unsuitable for determination of net influx of FAA from natural water samples since these contain a complex mixture of undefined compounds capable of reacting with the reagent. Therefore, conclusions about removal of FAA from natural waters based on such an approach must be viewed as tentative.

HPLC ANALYSIS OF NET INFLUX IN BIVALVES

Lindroth and Mopper (1979) and Gardner and Miller (1980) have reported sensitive procedures for separation and quantitation of free amino acids in sea water employing high performance liquid chromatography (HPLC). Both procedures are based on the preparation of fluorescent derivatives of FAA using the reagent, o-phthaldialdehyde (OPA). These fluorescent amino acid derivatives are then separated using HPLC and detected in the effluent stream from the chromatograph column using a fluorometer. Amino acids are identified by time of elution and quantified by measurement of peak area.

Several major advantages make the HPLC procedure attractive for studies of net entry of FAA into marine invertebrates. Derivatization with OPA is very simple. Desalting of samples is not required. Extremely small volumes of sample can be used (5-500 μl). The technique is sufficiently sensitive so that picomole quantities of individual amino acids can be detected. This allows quantitative estimation of nM concentrations of individual amino acids, i.e. at levels which are comparable to those present in near-shore marine waters.

Recent work in our laboratory (Manahan et al. 1982) was undertaken using the isocratic HPLC procedure described by Gardner and Miller (op. cit.) for amino acid analysis. Entry of amino acids into Mytilus edulis from natural water samples and from sea water solutions of known amino acids was examined. Intact animals were used and samples were taken during normal pumping activity.

The following procedure was followed to check on the reliability of the sampling technique for obtaining natural water samples from the habitat. A liter of sea water was taken from Newport Bay, California, about six inches from the site of a large population of mussels from which a few animals had been removed and brought into the laboratory on the preceding day. A small sample of sea water was also collected from the same location at the same time and was immediately aseptically filtered through a 0.2 μm Nucleopore filter to remove microorganisms. The larger sample was filtered aseptically through a 0.2 μm Nucleopore filter promptly upon returning to the laboratory, about twenty minutes after collection. No difference in concentration or qualitative composition of the FAA in the two samples were observed by HPLC chromatography. Thus, microbial activity had not significantly modified the composition of water samples during the period which elapsed between collection and filtration in the laboratory.

A mussel, 6-8 cm in length, was placed in a container with 400 ml of the aseptically filtered sea water sample; the animal began normal pumping activity within a few minutes. Small (2 ml) water samples were then drawn periodically from the incurrent margin of the animal. At the same time, small (2 ml) samples were taken from the excurrent siphon using a plastic cannula inserted into the excurrent opening. This

procedure allowed withdrawal of samples of water after passage through the mantle cavity of the animal without mixing with the surrounding ambient sea water and thus made possible the direct comparison of water before and after a single passage across the gill (see Wright and Stephens 1978 for a detailed account of this procedure).

Figure 3 shows HPLC chromatograms of sea water taken from the natural habitat of Mytilus edulis and filtered as described. The two samples were taken essentially simultaneously from the incurrent margin of the animal (upper chromatogram) and from the excurrent siphon (lower chromatogram). The three amino acids aspartate (Asp), serine (Ser) and glycine (Gly) are labelled on the chromatograms. In this case, quantitative estimation of the areas under the three designated peaks by a digital electronic integrator indicated that 63%, 84% and 72% of Asp, Ser and Gly respectively were removed by the animal during a single passage of water through the mantle cavity.

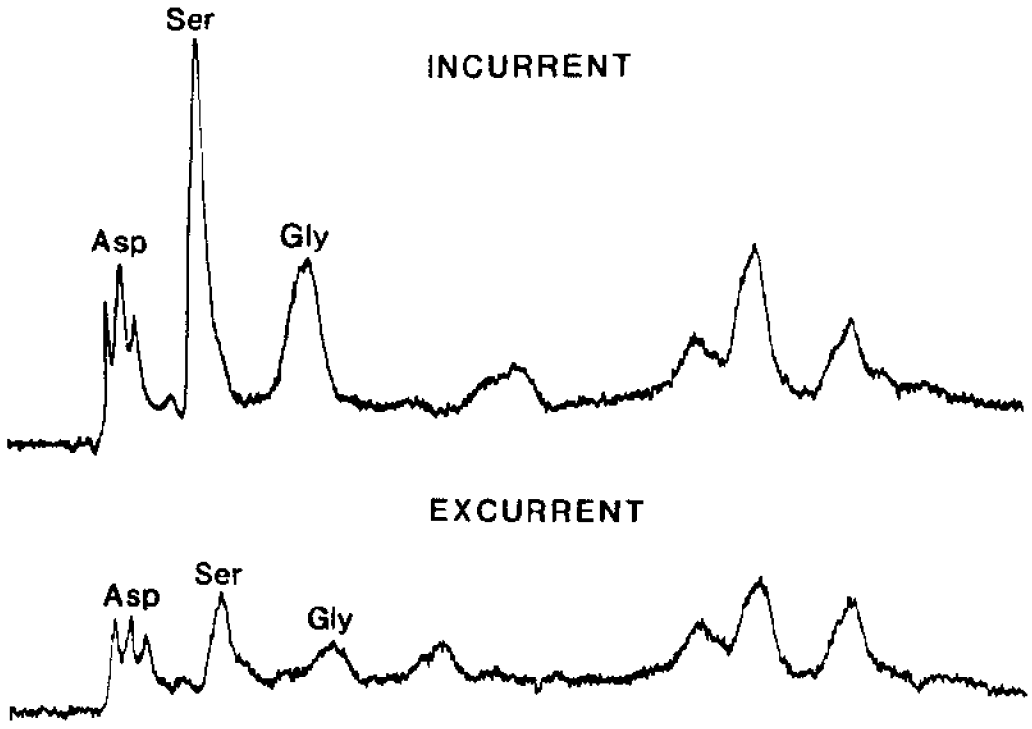

Figure 3. The upper HPLC amino acid chromatogram is of 0.2 ml of sea water taken from the immeidate environment of Mytilus edulis. The lower chromatogram is 0.2 ml of the same water taken from the excurrent siphon of an actively pumping animal. Samples were taken essentially simultaneously. The amino acids aspartate (Asp), serine (Ser) and glycine (Gly) are identified. Other peaks are unidentified components present in the natural sea water.

We also addressed the problem of the reported efflux of "unknown primary amines" which apparently accompanies influx of FAA in mussels. A solution of Asp, Ser and Gly in artificial sea water was used to simplify the experimental system and to permit detection of small amounts of OPA-reactive material liberated into the medium. Each amino acid was supplied at a concentration of 0.25 μM; 0.25 μM gamma-amino butyric acid (GABA) was included in this "artificial solution" since experiments had shown that GABA is removed from solution by mussels slowly compared to other FAA. Its presence thus served as a control for any unperceived experimental effects which may have been mistakenly interpreted as influx.

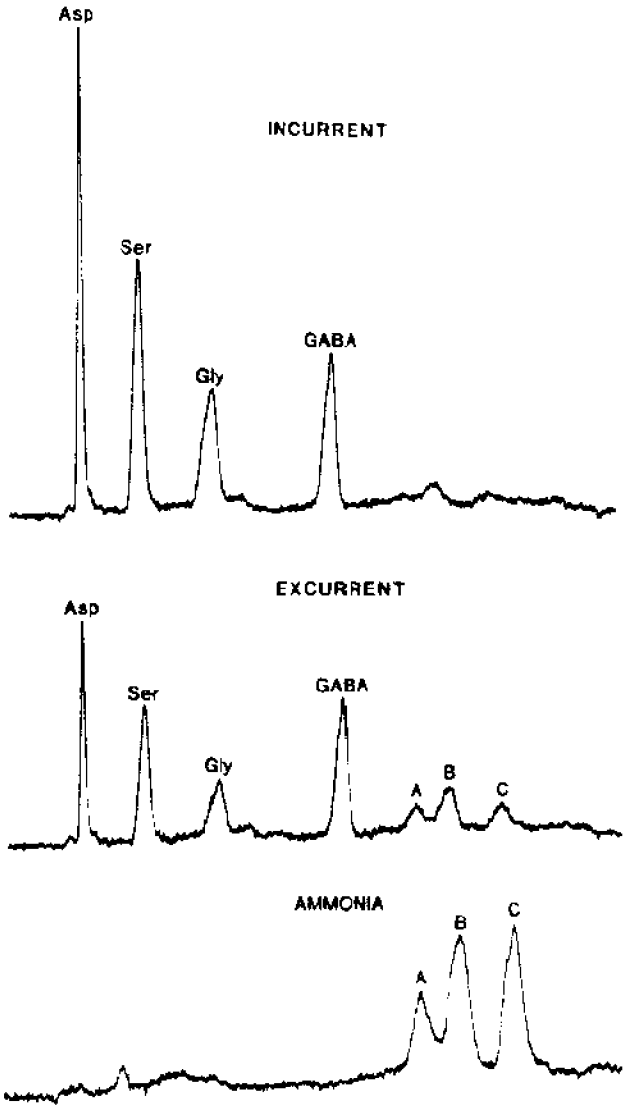

Figure 4. The top HPLC amino acid chromatogram is of 0.2 ml of an artificial sea water solution containing 0.25 µM Asp, Ser, Gly and GABA (gamma-amino butyric acid). The center chromatogram is of 0.2 ml of the same solution taken from the excurrent siphon of an actively pumping animal. Peaks labelled A, B and C appear during passage of water through the mantle cavity. The bottom chromatogram is of 4.0 nmoles of NH_4Cl in 0.2 ml of sea water.

The top chromatogram in Figure 4 is of the "artificial solution" in which a mussel was placed; beneath it is a chromatogram of a sample drawn from the excurrent siphon of the animal as soon as it began pumping. The two samples were taken essentially simultaneously. These measurements were repeated several times. The peaks labelled A, B and C in Figure 4 were consistent features of samples from the excurrent siphon. When animals were permitted to reduce the concentration of FAA to very low levels over a 45-minute period, chromatograms of the ambient medium showed a steady increase in height of peaks A, B and C. As a control, animals were placed in artificial sea water free of organic substrate and were permitted to pump for 45 minutes. Peaks A, B and C appeared and were comparable to those found in experiments with added FAA.

The bottom chromatogram in Figure 4 is of artificial sea water containing 4 nmoles of NH_4Cl. As shown in the Figure, three peaks were obtained which had the same elution times as peaks A, B and C. The reaction of ammonia with OPA may produce several products (Manahan et al. 1982). Thus we conclude that the ammonia liberated by the mussels in our experiments represents the "unknown primary amines" reported by investigators using less specific procedures to study entry of FAA. Of course this does not exclude the possibility that other OPA- or fluorescamine-positive materials may be liberated into the medium by other organisms or by Mytilus edulis under different conditions.

SIGNIFICANCE OF INFLUX OF FAA IN ADULT BIVALVES

Wright and Stephens (1978) showed that the efficiency of removal of glycine in a single passage of water through the mantle cavity of Modiolus demissus and Mytilus californianus depended on the pumping rate as well as the ambient substrate concentration. They estimated that approximately 40% of the reduced carbon required to account for oxygen consumption in M. californianus could be supplied by ambient FAA at 1.0 μM provided influx of the amino acid mixture was comparable to that of glycine. Jorgensen (1976) estimated that 30% of the reduced carbon requirement of Mytilus edulis could be supplied by influx from 1.0 μM alanine.

These estimates suggest that the input of organic material via gill transport of solutes from the medium is sufficiently large to qualify as a significant nutritional supplement. Qualitatively, a continuous supply of amino nitrogen as a supplement to other food sources is of biological significance. It should be noted that there is evidence that some bivalves possess a metabolism which is partly anaerobic, even in well aerated water (Hammen 1979). If this proves to be true for a wide range of species, the use of oxygen consumption as a measure of metabolic energy requirements should be re-examined. We should also draw attention to the uncertainty concerning levels of FAA present in the immediate habitat of bivalves. We do know that there are temporal fluctuations in concentrations of FAA (Mopper and Lindroth 1982) and marked differences related to microhabitat (Stephens 1981).

There is litle information on which to base an estimate of the importance of the influx of amino acids to the total nitrogen input of bivalves. However, using influx rates from Wright and Stephens (1978) and rate of nitrogen loss for animals in March (Bayne and Scullard

1977), Mytilus edulis would acquire about 28 μg N/hour by amino acid uptake from 1 μM FAA in sea water while losing approximately 32 μg N/hour as ammonia and amino nitrogen. Nitrogen excretion is variable seasonally and depends on nutritional state, reproductive state and temperature. However, this approximate calculation suggests that the contribution of influx of FAA to nitrogen balance may be quite significant at times.

Metabolism and incorporation of FAA into more complex molecules have been reported in bivalves. However, the subject has received relatively little attention so that a detailed review is not warranted. In general, it appears that FAA removed from the ambient sea water enters the active amino acid pools, first in the gill and later throughout the soft tissues; after entry it participates fully in the metabolism of the animal.

UPTAKE OF FAA BY BIVALVE LARVAE

Until very recently, a little work concerning bivalve larvae has been published. Crane et al. (1957) report uptake of ^{14}C-glucose by the larvae of Mactra (Spisula); Fankboner and deBurgh (1977) report uptake of a chemically undefined algal exudate by larvae of Crassostrea gigas. Rice et al. (1980) studied the entry of glycine and alanine into larvae and juveniles of the European flat oyster, Ostrea edulis, using both radiochemical and fluorescamine procedures. They were able to demonstrate net influx of these amino acids.

Major advances in our knowledge are provided by the work of Manahan and Crisp (in press). This work is based on radiochemical techniques and thus does not directly address the question of net entry of FAA from solution. However, by extension from the work with adult bivalves discussed in preceding sections of this paper as well as the work of Rice et al. (1980), it is reasonable to assume that subsequent work with fluorometric and HPLC techniques will produce a comparable picture with respect to net entry of FAA into bivalve larvae. The remainder of this section will summarize the work of Manahan and Crisp (op. cit.).

Autoradiography was carried out using larvae of Crassostrea gigas, Ostrea edulis and Mytilus edulis. The main site of amino acid uptake in veligers is the velum. Fixation of larvae in glutaraldehyde to cross-link free amino acids showed substantial radioactivity in the velum after a one-minute exposure to ^{3}H-labelled glycine. Fixation in Carnoy's, which does not cross-link amino acids, did not show radioactivity in the velum indicating that incorporation into larger molecules had not yet occurred. After a period of 10-100 minutes, radioactivity began to appear in deeper tissues and in Carnoy-fixed material indicating progressive assimilation of the labelled substrate. Uptake via the digestive tract was negligible. At settling, the velum is resorbed and the gill is not yet fully developed. However, the developing gill buds become active sites for entry of amino acids during early settlement stages.

Manahan and Crisp also showed that the transport systems for uptake of FAA are activated approximately one hour after fertilization. Once activated, they continue through all stages of the life cycle from early cleavage through adulthood.

These authors emphasize the potential importance of FAA as a nutritional supplement in larval and early settlement stages. In general, energy reserves provided for larval development by the parent adult are minimal (Crisp 1974). In early development, all bivalve larvae are incapable of utiizing particulate food. Typically, metamorphosis in bivalves involves shift from the velum to the gill as a feeding organ; during this transition, particulate food is not utilized. These arguments for the potential importance of FAA as a nutritional supplement are further supported by the very rapid rates of uptake and utilization of FAA by larvae. In C. gigas, 100 minutes after exposure to ^{14}C-glycine, 47% of the glycine taken up has been incorporated into protein, 38% has been oxidized, 2% is present in the lipid fraction and the remainder is in TCA-soluble compounds.

Rates of glycine entry are approximately an order of magnitude greater per unit wet weight than those reported for adult bivalves. This may be a consequence of the large surface presented by the larvae relative to its size. The rate of incorporation of FAA into protein is unusually high and indicates very rapid assimilation of substrate compared to that found in adult bivalves (cf. Stewart 1977).

In estimating the potential importance of uptake of FAA as a contribution to the nutritional requirements of bivalve larvae, Manahan and Crisp use their data on glycine uptake to compare influx of this substrate to the total energy requirements of the larvae and to the rates of protein synthesis during early larval growth. The contribution to total energy requirements proves to be rather small for two reasons. First, 70% of FAA uptake has been shown to support growth so only 30% is construed by Manahan and Crisp as contributing to the support of oxygen consumption. Second, glycine provides the smallest amount of reduced carbon per mole of any of the amino acids. For these reasons, their calculation that influx of glycine for an ambient concentration of 6 μM contributes approximately only 3% of the energy requirements of C. gigas pediveligers is not surprising. On the other hand, since glycine is incorporated into protein so rapidly after entry, Manahan and Crisp estimate that glycine influx can contribute approximately 9.5% to the protein synthesis required for growth.

COMPETITION OF BIVALVES WITH MICROORGANISMS FOR FAA

Marine biologists often view microorganisms and particularly bacteria as the principal heterotrophic agents in the turnover of DOM in the planktonic community (Williams 1975). Bacteria have also been suggested as the agents responsible for the apparent uptake of FAA by marine invertebrates (Sepers 1977; Siebers 1979). Several characteristics of bacteria appear to support this hypothesis. They are rapidly adaptable to changes in substrate availability and composition. Their very high surface-volume ratio is favorable for any surface limited process such as nutrient uptake. They have very low K_t's; a representative value for amino acid transport taken from Seper's review, (op. cit.) is 3×10^{-8} M.

Recent work on uptake of amino acids by adult bivalves suggests that they compete with bacteria very effectively. Wright and Stephens (1978) demonstrated that removal of 30-95% of glycine from a 1 μM solution occurred during passage of water through the mantle cavity of

mussels. This occurs during the few seconds required to replace the water during normal pumping activity; no substrate is removed if the animal is not pumping. Scanning electron microscopy and transmission electron microscopy do not provide any evidence for bacteria associated with the gills of M. edulis (Manahan et al. 1982) comparable to the intracellular bacteria reported to be present in some bivalves (Felbeck et al. 1981). Thus microorganisms play no significant role in uptake of FAA by adult Mytilus.

Manahan and Crisp (op. cit.) discuss the relative ability of bivalve larvae and bacteria to obtain glycine from solution. They point out that while the K 's of bacterial transport systems are very low, so is the J^{i}_{max} of these systems. Using the data summarized by Sepers (1977) they estimate a K_t of 3×10^{-8} M and a J^{i}_{max} of 10^{-12} moles/hour for a mass of bacteria equal to the weight of the velum of one M. edulis larvae. The larval velum has a measured K_t of 3×10^{-6} M and a J^{i}_{max} of 10^{-11} moles/hour. Figure 5 is a plot of the influx of glycine into equivalent weights of bacteria and larvae over the concentration range $0\text{-}2 \times 10^{-6}$ M. Note that larvae outcompete bacteria from FAA resources at concentrations greater than 0.3×10^{-6} M.

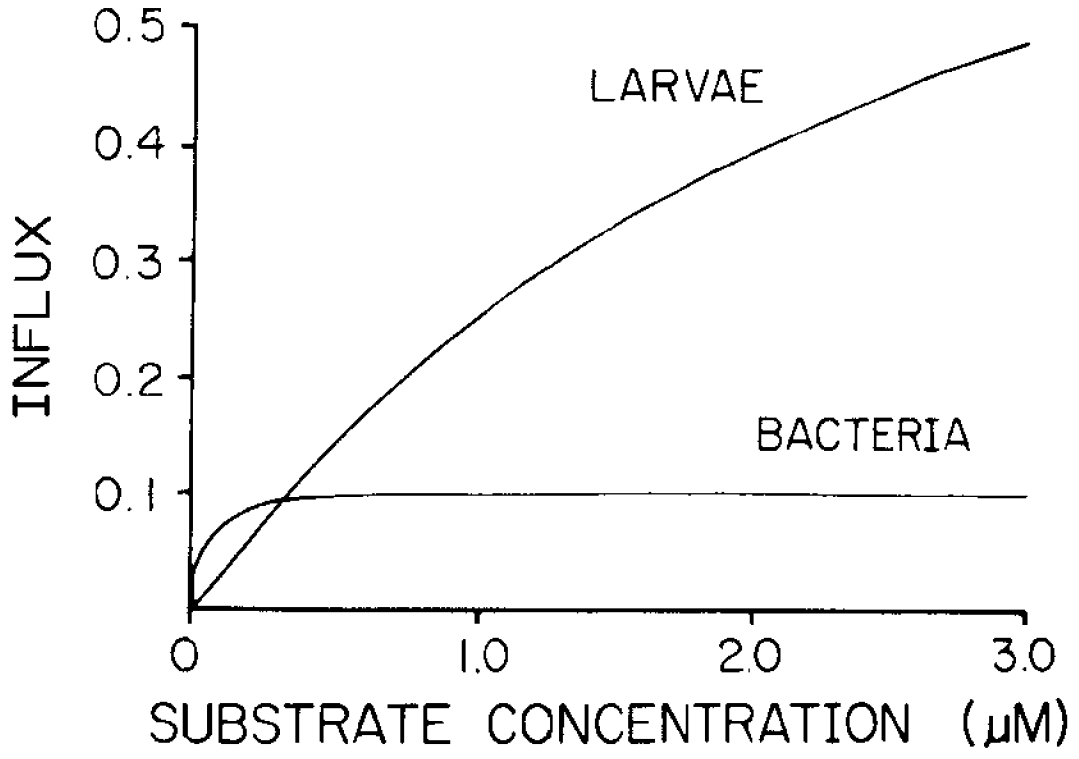

Figure 5. These theoretical curves compare expected influx of FAA into a natural bacterial population and into Mytilus edulis. The comparison is on an equal weight basis, 1.0 ml of sea water assumed to contain bacteria approximately equal in weight to one larval velum (redrawn from Manahan and Crisp, in press).

Manahan and Crisp (op. cit.) also report observations in which uptake of labelled glycine into larvae of M. edulis was directly compared with uptake by the natural population of marine bacteria in the seawater of the Menai Straits, Wales, U.K. After exposure, they separated larvae and bacteria and found the ^{14}C-label mainly present in the larvae. Population levels of bacteria were estimated by direct counting of samples stained with acridine orange. Neither bacterial growth nor a decrease in the bacterial population due to ingestion of bacteria by the larvae could be detected in comparing samples taken before and after experiments were conducted. Thus entry of substrate into the larvae did not appear to occur by ingestion of bacteria. They also state that examination by scanning electron microscopy failed to reveal any bacteria associated with the velum, which is the major site of amino acid uptake in larvae.

We may conclude from the studies cited above that both adult and larval bivalves are able to compete very effectively with naturally occurring microbial populations for available FAA in sea water.

Finally, mention should be made of a development which promises an exciting breakthrough in this area. Langdon (1980, these proceedings) has succeeded in producing axenic suspensions of C. gigas larvae. Our laboratory is currently working on the uptake of FAA using axenic suspension of C. gigas larvae obtained following Langdon's procedure. The availability of this experimental material permits, 1) investigation of the competition of larvae and natural bacterial populations for FAA under strictly controlled conditions, 2) observations on influx and net entry of FAA in the complete absence of bacteria, 3) precise quatification of the contribution of FAA inputs to the energy requirements and to protein synthesis in larvae, 4) precise determination of the metabolic fate of known substrates so that, for example, essential amino acid requirements can be determined. The combination of modern analytical procedures such as HPLC and the availability of axenic material for experimentation should lead to a dramatic improvement in our understanding of the role of DOM in larval nutrition in the near future.

IMPLICTIONS FOR MARICULTURE

Provasoli and co-workers have suggested that the concept of "biphasic diets" applies to the culture of all metazoans (e.g. Conklin and Provasaoli 1978). This phrase is intended to direct attention to the importance of both the particulate and the dissolved phases of a culture medium.

Crustaceans such as Artemia, Daphnia and Moina which served as the experimental material for much of the work of Provasoli and his collaborators are known to be incapable of transepidermal uptake of FAA (Anderson and Stephens 1969). Thus the dissolved components of the biphasic diets developed for the culture of these organisms must be supplied in high concentrations since they must enter via the gut.

Work cited and discussed in the preceding sections of this paper shows that marine bivalves readily remove dissolved substrates from solution at low concentrations, both as larvae and adults. FAA are present in the natural environment at significant concentrations. FAA

removed from solution apparently contributes both to energy requirements and to protein synthesis. In Langdon's work studying growth of axenic suspensions of C. gigas larvae on artificial diets (op. cit.), larvae grew better when supplied with both an artificial particulate diet and tissue culture medium than larvae supplied with only one or the other of these food sources. This suggests that the necessarily biphasic nature of food resources available to bivalves may be significant for commercial culture of these organisms.

Manahan and Stephens (in press) have shown that major changes in the concentration of FAA can be inadvertently produced in commercial culture of oyster larvae by addition of algae intended to serve only as a particulate food source. It seems quite likely that monitoring and adjusting the levels of specific FAA in larval and juvenile cultures may help to reduce mortality and improve growth by controlling the supply of specific dietary components. Precise determination of nutritional needs such as essential amino acid requirements, should provide a basis for the development of an adequate defined artificial diet. Measurements of natural levels and determination of the qualitative composition of DOM in sea water may also assist in site selection for mariculture operations.

CONCLUSIONS

1. Rapid net influx of FAA into adult bivalves from sources present in the immediate natural habitat has been unambiguously demonstrated. As a corollary, one may conclude that adult bivalves possess transport systems for FAA which are effectively adapted to their habitat.

2. Transport systems for FAA in marine bivalves are activated shortly after fertilization and remain functional throughout their life cycle. Radiochemical evidence strongly suggests that larvae also have access to natural resources of FAA.

3. Both adult and larval marine bivalves are capable of competing effectively with bacteria normally present in their habitat for available FAA.

4. FAA removed from dilute solution in sea water appear to contribute significantly to the energy requirements and to the amino acid requirements for protein synthesis of adult and larval bivalves.

5. The natural diet of marine bivalves is biphasic, including both particulate and dissolved components. Further study of the role of dissolved nutrients may contribute significantly to bivalve mariculture.

ACKNOWLEDGEMENTS

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LOPEZ (State Univ. of N. Y.): Dr. Stephens, would you comment on investigations that have looked at the effect of DOM uptake on either survival, growth or fecundity of marine organisms.

STEPHENS: The only studies I know which deal with bivalves are those of Dr. Langdon which were presented at this symposium yesterday. He found that larvae of C. gigas did not survive when provided with soluble nutrients. However, he did find that larvae provided with dissolved nutrients survived longer. He also found that supplementation of an artificial particulate diet with dissolved nutrients had a positive effect though, again, the larvae did not survive.

This allows me to re-emphasize Provasoli's concept of "biphasic diets"; I am convinced of the correctness of his perception that both particulate and dissolved phases of food resources are important. I believe this is particularly the case for soft-bodied marine organisms such as bivalves. However, the current state of investigations in this field do not allow us to speak of immediate practical applications. I think we are now in a position to explore the contribution of DOM much more effectively than has been the case in the past.

FATTY ACID METABOLISM OF BIVALVES

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ABSTRACT

Well-fed, mature, marine bivalves in their normal milieu possess 20-25% triglyceride in their lipids. This triglyceride serves as a temporary store for dietary and surplus fatty acids, although some saturated and monoethylenic fatty acids may be modified to suit triglyceride structural needs. Thus 16:1ω7, plentiful in marine phytoplankters, is often extended to 18:1ω7. The phospholipids are formed from fatty acids of the triglyceride acid pool, which in the wild usually includes ample preformed 20:5ω3 and 22:6ω3, but only minor amounts of 18:2ω6 and 18:3ω3 are found in either of these lipid classes. In four out of five averaged lots of marine phytoplankter fatty acids the ratio of ω6 to ω3 acids was very close to 1:5. Phospholipids of two out of three oyster species (Crassostrea virginica and Crassostrea gigas) had similar ω6 to ω3 ratios of 1:4 and a third (Ostrea edulis had a ratio of 1:1.5, whereas in three other bivalve species the ratios were markedly different (Arctica islandica 1:16; Mytilus edulis 1:14; Pectan maximus 1:20). More information on both individual phospholipids and species is needed, especially to clarify the role of non-methylene-interrupted dienoic fatty acids in bivalve nutrition and lipid biochemistry.

KEYWORDS: mollusc, bivalve, lipid, fatty acid, triglyceride, phospholipid.

INTRODUCTION

The bivalves, with the exception of terrified scallops, remain essentially in one location and in this situation have had to adapt their cellular functions to utilize the fatty acids available, primarily those derived directly from phytoplankters. The nutritional role of bacteria and of their fatty acids is not included in this study. It has also been necessary to exclude "dissolved" fatty acids (Fevrier 1976; Stewart 1979; Erhardt et al. 1980; Goutx and Saliot 1980) and those of "particulate" matter (Erhardt et al. 1980; Goutx and Saliot 1980), including those merely absorbed on inorganic particles (Barcelona and

Atwood 1979). The general functional roles of lipids in molluscs has recently been reviewed by Hoskin (1978).

Table I shows that in terms of exogenous fatty acids, bivalves are presented with a mixture of about 20-40 percent saturated acids, 10-20 percent monoethylenic acids, and 20-40 percent polyunsaturated acids, including C_{20} and C_{22} highly unsaturated fatty acids which are more commonly associated with marine animals. These averages approximate to the fatty acid composition of some of the food mixtures found to support good growth of juveniles of the European oyster (Ostrea edulis) (Helm 1977; Walne 1979). A review of the literature strongly suggests that the importance of pre-formed long-chain, highly-unsaturated fatty acids in bivalve nutrition has been underestimated.

Table I. Percentages (w/w) of Selected Fatty Acids Averaged from Individual Analyses of Total Phytoplankter Lipids

Fatty acid	Ackman et al. (1970)[a]	Waldock and Nascimento (1979)[b]	Chu and Dupuy (1980)[c]	Volkman et al. (1981)[d]	Langdon and Waldock (1981)[e]
14:0	6.8	3.5	10.3	17.3	6.1
16:0	22.3	11.7	26.2	17.6	21.4
18:0	1.2	2.2	2.1	0.8	1.3
Total	30.3	17.4	38.6	35.7	28.8
16:1	16.9	5.9	10.4	2.2	10.5
18:1	4.1	10.5	13.1	10.4	11.8
20:1	0.3	0.6	0.4	-	1.0
Total	21.3	17.0	23.9	12.6	23.3
18:2ω6	3.4	2.4	4.0	5.7	4.3
18:3ω3	7.9	8.3	3.5	8.1	13.8
18:4ω3	6.4	4.7	2.3	13.6	2.5
20:4ω6	2.4	0.1	2.0	-	0.5
20:4ω3	0.1	0.7	0.2	-	0.2
20:5ω3	10.7	6.8	4.6	2.6	7.2
22:4ω6	ND	0.2	-	-	
22:5ω6	0.2	3.1	0.5	-	0.4
22:5ω3	0.1	0.5	0.2	0.1	-
22:6ω3	4.9	11.5	1.6	9.7	2.6
Total	36.1	38.3	18.9	39.8	31.5
Total ω6	6.0	5.8	6.5	5.7	5.2
Total ω3	30.1	32.5	12.4	34.1	26.3

[a]Twelve species selected from data of Ackman et al. (1968) for inclusion in Ackman et al. (1970).

[b]Three species.

[c]Five species including data for two from the laboratory of J. Joseph (NMFS, NOAA, Charleston, S.C.).

[d]Four coccolithophorids; in three, 18:5ω3 was included at 10.1, 7.3 and 3.3%.

[e]Three species.

Growth studies involving fatty acids and juvenile bivalves have tended to be simple and to ignore most principles of animal nutrition. Algal species fed are usually selected, from those known to be assimilated by bivalves, on the basis of radical differences in fatty acid composition (e.g. Watanabe and Ackman 1974; Langdon and Waldock 1981). Under these circumstances any growth differences observed are attributed to the presence or absence of one fatty acid, or group of fatty acids, while the effects of proteins, carbohydrates, sterols etc. are usually ignored. Nevertheless, it seems that certain fatty acids could be important positive growth factors (Epifanio 1979). To put the role of the dietary fatty acids into perspective, one must consider the availability, assimilation, distribution, and biochemical modification of phytoplankter fatty acids as food for bivalves.

In this brief review an attempt will be made to cover these points, but factors other than fatty acids must necessarily be left for other authors.

INFLUENCE OF LIPID CLASSES

In bivalves, as in other animals, lipids serve as functional components in cells and membranes, but an energy reserve function is less likely than in higher animals (Allen 1976). Thus to appreciate the fate of dietary fatty acids in the bivalve it is not sufficient to just look at total fatty acids, but it is first necessary to consider the lipid recovery methods and the type of lipid recovered. Up to the 1960's most fatty acid comparisons were based on total extracted "lipid". This sometimes included extracts from dried samples in which the polyunsaturated acids had oxidized. Simple extraction with diethyl ether was also common, although this tended to extract mostly neutral lipids and to result in poor recovery of the phospholipids, important in cell membrane structure and functions. The biphasic solvent extraction method described by Bligh and Dyer (1959) eliminated lipid extraction and recovery problems, but total lipid recovery did not necessarily clarify the function of fatty acids. For example Cerma et al. (1970) examined the fatty acids of total lipids of 12 shellfish from the Adriatic Sea with considerable care but without contributing much to our understanding of fatty acid metabilism in molluscs.

Also, despite the fact that differences in the fatty acids of "neutral" lipids (i.e. triglycerides) and "polar" lipids (i.e. mostly phospholipids) were realized by progressive scientists, the lipid class separations were laborious and little had been done by the time of a thorough review of mollusc lipids by Voogt (1972), or even for the 1978 review by Hoskin. Gardner and Riley (1972) were, however, among the first to clarify the role of the two basic lipid classes in molluscs, and to note that because of the varying proportions of triglyceride and phospholipid, each with a different fatty acid composition, analyses of bivalve total fatty acids can convey only a limited amount of information on fatty acid assimilation and function. Before proceeding to discuss the lipid class data of Tables II and III in detail it is worth looking briefly at the role of these classes in bivalves.

In most animals, depot fats (usually triglycerides) are morphologically recognizable entities, although small amounts of triglycerides are also found in cell membranes. In bivalves obvious and

Table II. Percentages (w/w) of Selected Saturated and Monoethylenic Fatty Acids of Different Lipid Classes of Some Bivalve Species. ND = not determined, PL = phospholipids, TG = triglycerides and SE = sterol esters.

Fatty acid	Ackman et al. (1974) *Arctica islandica*						Langdon and Waldock (1981) *Crassostrea gigas* spat						Watanabe and Ackman (1974)				Gardner and Riley (1972)					
	Flesh			Hepatopancreas									*Crassostrea virginica*		*Ostrea edulis*		*Mytilus edulis*			*Pecten maximus*		
	PL	TG	SE	PL	TG	SE	PL[a]	TG[a]	PL[b]	TG[b]	PL[c]	TG[c]	PL	TG	PL	TG	PL	TG	SE	PL	TG	SE
14:0	0.4	3.5	4.2	1.4	4.6	7.8	0.7	4.8	1.1	1.1	2.1	2.4	2.0	4.4	6.0	8.1	1.9	3.0	1.5	4.4	4.0	4.6
16:0	12.9	32.4	18.6	22.5	20.0	20.3	7.7	18.6	14.4	20.2	13.2	20.1	26.7	34.8	31.5	42.0	10.7	13.6	11.5	19.0	11.1	10.8
18:0	6.3	7.9	7.8	11.6	5.0	7.8	2.2	1.4	3.5	7.9	5.6	4.7	6.2	2.2	12.7	7.5	10.0	4.7	5.9	4.0	5.0	6.2
Total	19.6	43.8	30.6	35.5	29.6	35.9	10.6	24.8	19.0	29.2	20.9	27.2	34.9	41.4	50.2	57.6	22.6	21.3	18.9	27.4	20.1	21.6
16:1ω9	0.2	0.8	2.4	ND	0.5	4.7	trace	0.3	0.7	0.3	0.4	2.0	0.6	1.3	1.5	1.7	4.8	8.3	6.7	3.4	7.6	5.9
16:1ω7	1.7	12.6	2.7	1.2	14.4	4.4	0.5	0.7	1.2	2.2	1.0	trace	1.8	4.0	2.5	5.9						
18:1ω13	2.1	0.8	1.1	1.9	0.9	0.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND						
18:1ω9	1.9	3.7	6.9	3.2	2.7	9.8	5.9	10.0	3.2	13.1	3.8	24.6	3.7	4.8	2.7	5.4	6.8	7.4	9.2	4.8	7.8	5.6
18:1ω7	2.9	8.3	5.3	5.2	10.5	3.9	0.6	0.8	10.0	0.3	3.4	trace	3.1	5.6	1.3	5.9						
20:1ω11	0.3	0.4	0.8	0.3	0.4	0.5	1.6	4.8	1.5	0.9	3.7	5.6	1.9	0.8	1.0	0.6	6.2[d]	8.8[d]	6.1[d]	3.3[d]	2.6[d]	3.8[d]
20:1ω9	1.1	1.7	4.0	1.6	1.6	2.2							1.5	0.5	0.8	0.4						
20:1ω7	1.5	5.3	18.8	2.6	3.4	6.4	6.9	6.1	4.1	1.8	4.5	6.9	4.9	1.5	2.2	2.6						
Total	11.7	33.6	42.0	16.0	34.4	32.2	15.5	22.7	20.7	18.6	16.8	39.1	17.5	18.5	12.0	22.5	17.8	24.5	22.0	11.5	18.0	15.3

[a]Hatchery reared (cultured in coarse-filtered seawater supplemented with a mixed algal diet.

[b]Reared on *Dunaliella tertiolecta* (Butcher).

[c]Reared on *Tetraselmis suecica* (Butcher)

[d]May contain NMID (cf. Table III).

Table III. Percentages (w/w) of Selected Polyunsaturated Fatty Acids of Different Lipid Classes of Some Bivalve Species. NMID refers to "non-methylene-interrupted" diethylenic fatty acids. ND = not determined, PL = phospholipids, TG = triglycerides, SE = sterol esters.

Fatty acid	Ackman et al. (1974) *Arctica islandica*						Langdon and Waldock (1981) *Crassostrea gigas* spat						Watanabe and Ackman (1974)				Gardner and Riley (1972)					
	Flesh			Hepatopancreas									*Crassostrea virginica*		*Ostrea edulis*		*Mytilus edulis*			*Pecten maximus*		
	PL	TG	SE	PL	TG	SE	PL[a]	TG[a]	PL[b]	TG[b]	PL[c]	TG[c]	PL	TG	PL	TG	PL	TG	SE	PL	TG	SE
18:2ω6	0.3	0.4	0.9	0.5	1.0	0.1	trace	0.3	0.5	1.4	0.2	trace	0.9	2.6	0.9	1.3	1.3	2.7	2.0	0.7	1.6	1.6
18:3ω3	1.3	1.3	0.3	2.1	0.8	1.5	5.3	1.9	9.8	15.8	3.8	7.9	3.8	2.9	4.9	1.1	2.2	0.3	2.5	0.9	0.1	trace
18:4ω3	ND	0.7	0.3	0.4	2.8	0.9	0.4	trace	0.6	trace	1.4	1.4	1.1	3.0	1.0	0.6	4.8	6.1	4.4	3.2	5.8	4.6
20:4ω6	2.1	0.2	ND	0.8	0.3	0.3	6.3	3.1	6.3	2.3	5.6	1.8	4.6	1.1	2.6	0.5	0.6	-	trace	-	-	1.6
20:4ω3	2.9	ND	ND	3.5	0.2	1.4	0.8	1.0	1.4	1.0	0.5	trace	0.1	0.6	trace	0.1	4.2	4.2	4.1	1.4	1.1	2.7
20:5ω3	15.3	5.5	0.8	9.6	20.1	0.9	11.4	7.0	4.2	1.1	11.2	5.4	8.1	9.1	6.9	2.2	9.6	12.9	7.9	12.2	9.5	2.4
22:4ω6	0.6	ND	ND	0.1	ND	ND	0.5	0.5	ND	trace	0.8	ND	0.1	trace	1.1	ND	-	-	trace	0.7	-	trace
22:5ω6[d]	0.1	ND	ND	0.1	ND	ND	2.4	2.2	1.9	1.7	1.2	0.5	0.3	0.1	trace	ND	-	-	-	-	-	-
22:5ω3	2.2	0.4	ND	1.0	ND	1.0	1.8	0.5	trace	trace	2.2	ND	0.4	0.1	0.3	trace	2.5	1.5	trace	2.5	1.5	trace
22:6ω3	23.9	2.8	ND	8.4	ND	0.8	15.8	10.5	7.4	1.7	4.7	0.5	10.3	4.7	2.3	0.9	3.2	5.9	3.2	8.4	7.9	1.6
Total	48.7	11.3	2.3	26.5	25.2	6.9	44.7	27.0	32.1	25.0	31.6	17.5	29.7	24.2	20.0	6.7	28.4	33.5	24.1	30.0	27.5	14.5
NMID	11.5	3.4	10.3	10.1	2.1	3.2	ND	ND	ND	ND	ND	ND	7.4	3.0	ND	ND	ND	ND	ND	ND	ND	ND

[a]Hatchery reared (cultured in coarse-filtered seawater supplemented with a mixed algal diet).

[b]Reared on *Dunaliella tertiolecta* (Butcher).

[c]Reared on *Tetraselmis suecica* (Butcher).

[d]Data reported by Gardner and Riley (1972) is here reported as 22:5ω3.

discrete depot fat deposits do not occur, although fat globules have been reported in mantle vesicular cells of Crassostrea virginica (Galtsoff 1964). In Crassostrea gigas nearly 40% of the lipid of the mantle margin and gonad was present as triglyceride (Allen and Conley 1982). Otherwise, it is usually reported (Barber and Blake 1981) that lipid is stored, at least temporarily, in the digestive diverticula of molluscs, for example in the sub-Antartic limpet Nacella (Patinigera) macquariensis (Simpson 1982). It is believed that in bivalves such as the Iceland scallop Chlamys islandica (Sundet and Vahl 1981), the Atlantic deep-sea scallop Placopectan magellanicus (Robinson et al. 1981), or the scallop Chlamys hericia (Vassallo 1973), as well as in C. gigas (Allen and Conley 1982), modest reserves of lipid are transferred to the gonad at maturation (cf Barber and Blake 1981). In the Japanese prickly scallop Chlamys nipponensis the lipid content (wet weight) of visceral organs was 4.6%, while that of the remaining soft parts was only 1.5% (Hayashi and Yamada 1973). It has been reported for a variety of other types of marine shellfish that visceral lipids are invariably at least twice the percentage found in the remaining parts of the animal body (Kochi 1975).

Most questions on the proportions of different lipid classes in the various organs of bivalves remain unanswered, but Allen and Conley (1982) showed that in contrast to the approximately 50:10:40 proportions of phospholipid: sterol: triglyceride in C. gigas mantle, the gill and adductor muscle both had proportions of approximately 80:12:8. The digestive diverticula differed from both with proportions of 78:7:13. Langdon and Waldock (1981) found that starved, hatchery-reared and dextrin-fed C. gigas spat had high proportions of phospholipid relative to triglyceride (Table II). Ratios of up to 8:1 were observed, and it is possible that the low levels of triglyceride indicated a poor nutritional state (Swift et al. 1980). With most of the other diets tested with C. gigas spat (Table II) the ratio of phospholipid to triglyceride approached the value of 1:1 which was also reported in wild but well-fed O. edulis and C. virginica adults (Watanabe and Ackman 1974), in wild Arctica islandica (Ackman et al. 1974) and Mesodesma mactroides (De Moreno et al. 1976), and also in several of the species studied by Gardner and Riley (1972).

It can be assumed that triglycerides have consistent fatty acid patterns throughout the bivalve body, but the distribution of fatty acids in the phospholipids may depend on the function associated with the organ, or on the relative proportions of the two major phospholipids, respectively phosphatidylethanolamine and phosphatidylcholine. Given these possibilities, it is not surprising to find conflicting reports on fatty acid compositions of organs. Thus Hayashi and Yamada (1973) reported minimal fatty acid differences between viscera and the remainder of the body soft parts in one species, whereas Kochi (1975) working with several species found that visceral fatty acids differed in consistent patterns from those of muscle lipids. Moreover, if phospholipids are the "vital" membrane component, as is commonly accepted, it is curious to find a wide variation in the levels of total saturated fatty acids in this class of lipids (Table II) from different species.

Despite our incomplete knowledge of the lipid and fatty acid biochemistry of even the most common bivalves, an overall picture can be obtained by considering the different basic types of fatty acids in detail.

SATURATED ACIDS

The saturated fatty acids found in bivalves always include several branched-chain fatty acids which can be classified into two groups. One group is made up of the three major isoprenoid fatty acids, especially 4,8,12-trimethyltridecanoic acid derived from the degradation of phytol (Ackman et al. 1971). The second group consists of the iso and anteiso fatty acids, predominantly C_{15} and C_{17}, with some iso C_{16} and C_{18} fatty acids. The branched-chain fatty acids of the second group can be accumulated in marine invertebrates from bacterial lipids (Hayashi and Takagi 1977) but are also normal minor metabolites in most animals and can be derived from amino acid precursors which are freely available in marine molluscs (Ivanovici et al. 1981). Although of biochemical interest and possibly key elements in understanding the role of bacteria in bivalve nutrition, branched-chain fatty acids are not reported by many authors and therefore will not be discussed in this review.

Table I shows that 16:0 (palmitic acid) is freely available in algal diets, and is usually accompanied by approximately half as much 14:0 (myristic acid). Some workers also report 12:0 (lauric acid) at 1-5%, but it is obvious that it and 18:0 (stearic acid) are minor components of most algal lipids. It is probable that 14:0 and 16:0 are equally well assimilated by bivalves, but they are also synthesized de novo, or elongated as required, by most animals (Sprecher and James 1979). It is not surprising that bivalve lipids (Table II) contain 20-50% saturated acids, since this is true of most marine animal lipids (Ackman 1980), and 16:0 is invariably the major saturated fatty acid at all evolutionary and trophic levels.

Tetraselmis suecica and Dunaliella tertiolecta were employed by Langdon and Waldock (1981) in a feeding study with juvenile C. gigas. The total saturated fatty acids (14:0 + 16:0 + 18:0 only) in the algae were respectively 32.4 and 18.6%. Despite this nearly two-fold difference, the total saturated fatty acids in the phospholipid fraction from lipids of juveniles fed on T. suecica and D. tertiolecta were very similar at 20.9 and 19.0% respectively (Table II). The corresponding triglycerides of the same juveniles had respective total saturated fatty acids of 27.2 and 29.2%. Although the totals for saturated fatty acids of phospholipid and triglyceride lipid classes differ, the similarity within each lipid class supports the view that bivalve fatty acid compositions tend to be species-oriented rather than diet-oriented (Watanabe and Ackman 1974).

UNSATURATED ACIDS

Both monoethylenic and saturated fatty acids may be assimilated from the diet or biosynthesized de novo by most animals (Ackman et al. 1980). The desaturation process in animals operates on the carbons in the $\Delta 9, \Delta 10$ positions counted from the carboxyl group (Sprecher and James 1979). Accordingly, direct desaturation of readily available saturated acids leads to the conversions 16:0→Δ9, Δ10-16:1 (palmitoleic acid) and 18:0→Δ9, Δ10-18:1 (oleic acid), but if chain elongation from C_{16} to C_{18} takes place then $\Delta 9, \Delta 10$-16:1→Δ11, Δ12-18:1. It is preferable to use a shorthand notation for describing unsaturated fatty acids based on the number of carbon atoms from the ethylenic bond closest to the methyl or ω end of the chain (the ω methyl group carbon counts as 1). Thus 16:1ω7 +

2C→18:1ω7 clearly indicates the process of chain elongation with retention of ω structure.

The amount of 16:1 provided by the algae (Table I) in the wild appears to be of the same order of magnitude as in the mollusc triglycerides (Table II). Most algal 16:1 is ω7 in structure, but algal 18:1 can be 18:1ω9 or 18:1ω7, or a mixture (Ackman et al. 1968). Table II shows that 16:1ω7 in bivalves, which could be of either exogenous or endogenous origin, dominates the 16:1 isomers except in triglycerides from C. gigas spat reared on T. suecica. In the sterol esters of A. islandica the levels of 16:1ω7 are approximately equal to the levels of 16:1ω9. The latter appears to be associated with the higher proportions of 18:1ω9 found in sterol esters than in the fatty acids of other lipid classes. Overall the data in Table II indicates that in bivalves total 16:1 is more important in the triglyceride fraction than in the phospholipid fraction.

In feeding experiments with juvenile C. gigas, Langdon and Waldock (1981) found that the 16:1ω7 isomer, which was present in high levels (25.8%) in Pavlova lutheri, appeared in the spat triglycerides at a level of only 4% of the total fatty acids, with 16:1ω9 also present only in trace amounts (Table IV). However the level of 18:1ω7 in the triglycerides of the Pavlova fed spat was conspicuously elevated to 14.7%, indicating that algal 16:1ω7 had been partially deposited unchanged in the triglycerides and partially elongated to 18:1ω7. With either T. suecica or D. tertiolecta as dietary sources of fatty acids, the 18:1ω9 isomer was abundant in both the algae and in the C. gigas spat triglycerides. It is remarkable, as shown in Table IV, that 18:1ω7 was expectedly important in the phospholipids of the juveniles reared on the latter two algae, as well as in those reared on P. lutheri. It is also apparent that in all cases chain elongation of 18:1ω7 to 20:1ω7 took place in the juveniles. High levels of 20:1ω7 have also been reported for other bivalve species (Table II).

Most animals synthesize triglycerides with a particular pattern of fatty acids attached to the glycerol molecule (Brockerhoff 1966), but it is unknown whether this also occurs in bivalves. Brockerhoff (1966) has indicated that for the monoethylenic fatty acids, the chain length may be important in determining the position on the glycerol molecule at which a particular fatty acid is attached. Bivalves have considerable flexibility in their biochemical pathways for lipid synthesis (Zandee et al. 1980), and it may be more economical in terms of energy expenditure to change exogenous 16:1 7 to 18:1 7 than to synthesize 18:1 9 de novo. There may also be different fatty acid pools at different steps in the lipid assembly process.

The fatty acids of phospholipids show approximately the same 18:1 isomer ratios as the corresponding triglycerides (Tables II and IV), indicating that in most bivalves the 18:1 acids are probably drawn from the same fatty acid pool. Waldock and Nascimento (1979) found that the 18:1ω9 to 18:1ω7 ratio of 13:1 in Chaetoceros calcitrans was slightly altered to 9:1 in the phospholipids of C. gigas larvae, and the chain extension process then resulted in the radically different proportions of 1.9% and 6.4% for 20:1ω9 and 20:1ω7 (erroneously published as percentages for 19:0 and 20:1ω9). The emphasis on elongation of monoethylenic ω7 isomers (Table IV), especially for the 18:1→20:1 step, probably has implications in the formation of the non-

methylene-interrupted dienoic acids NMIDs (see below) because the C_{20} NMIDs are dominated by ω7 structures.

Table IV. Comparison of Weight Percentages of the Important C_{16} and C_{18} Monoethylenic Fatty Acids in Total Lipids of Pavlova lutheri, Tetraselmis suecica and Dunaliella tertiolecta with Triglyceride and Phospholipid Fatty Acids of Experimentally Fed Crassostrea gigas Spat (from Langdon and Waldock 1981)

	P. lutheri	T. suecica	D. tertiolecta
16:1ω9 (algae)	-	1.8	0.6
spat triglyceride	trace	2.0	0.3
spat phospholipid	trace[a]	0.4	0.7
18:1ω9 (algae)	3.3	22.7	6.6
spat triglyceride	2.4	24.6	13.1
spat phospholipid	2.2[a]	3.8	3.2
20:1ω11/ω9 (algae)	-	(2.85)[b]	-
spat triglyceride	6.5	5.6	0.9
spat phospholipid	2.6	3.7	1.5
16:1ω7 (algae)	25.8	2.0	3.6
spat triglyceride	4.0	trace	2.2
spat phospholipid	2.2[a]	1.0	1.2
18:1ω7 (algae)	2.5	trace	0.4
spat triglyceride	14.7	trace	0.3
spat phospholipid	7.2[a]	3.4	10.0
20:1ω7 (algae)	-	(2.85)[b]	-
spat triglyceride	2.6	6.9	1.8
spat phospholipid	5.7	4.5	4.1

[a]Total lipid (29% triglyceride, 71% phospholipid) as phospholipid data not given.

[b]Total 20:1.

POLYUNSATURATED ACIDS

Terrestrial herbivorous animals subsist almost exclusively on a diet of plants rich in C_{18} fatty acids. There has therefore been intensive study in animals of the chain elongation of 18:2 6 (linoleic acid) to 20:4ω6 (arachidonic acid), and of 18:3ω3 (linolenic acid) to 20:5ω3 and 22:6ω3 (Holman 1981). Although many widely-studied green algae, for example Chlorella sp., are commonly rich in C_{18} fatty acids (Ackman 1981), bivalves are exposed in the wild to a wider spectrum of fatty acids from other types of phytoplankters including preformed 20:5 ω3and 22:6ω3 (Table I). Unfortunately the pioneering studies on the biochemistry of polyunsaturated fatty acids in molluscs were caried out with the land snail Cepea nemoralis by Van der Horst, Oudejans, Voogt, Zandee and others (loc. cit.); conveniently summarized by Hoskin (1978) and Zandee et al. (1980). This terrestrial snail feeds on vegetation

rich in C_{18} acids and necessarily the experimental work on fatty acid metabolism was based on radioactively labelled 18:2ω6 and 18:3ω3 introduced to the snails. It was found that a proportion of both these labelled C_{18} fatty acids was elongated to more highly unsaturated C_{20} and C_{22} fatty acids. However, part was deposited without alteration and part was also catabolized for energy.

In the first decades of this century a distinquished scientist remarked that scientific research could cease, since everything which could be discovered had been discovered. Something of the same attitude crept into marine invertebrate fatty acid biochemistry in the 1970's. Following the work with C. nemoralis scientists expected to explain all nutritional studies with fatty acids and molluscs solely in terms of this C_{18} C_{20} C_{22} desaturation/chain elongation process. Recent phytoplankton fatty acids studies (Table I) suggest on the contrary, that natural bivalve populations can receive all necessary 20:5ω3, 22:5 ω3and 22:6ω3 from their normal diet. Since 18:2ω6 and 18:3ω3 do not accumulate in proportion to availability the surplus may simply be utilized for energy. As the evidence for these views is largely circumstantial it must be gone into with some detail.

A case in point is the rearing by Landon and Waldock (1981) of juvenile C. gigas on D. tertiolecta, which had 8.8% 18:2 6 and 31.1% 18:3ω3, but no 20:5ω3 or 22:6ω3. Despite a high lipid content in the alga, growth was poor until a supplement of 22:6ω3 was fed. Supplemental triolein had no effect at similar levels. In a similar feeding with T. suecica, which had 7.7% 20:5ω3 but no 22:6ω3, greater growth occurred than with D. tertiolecta plus 22:6ω3, and adding 22:6ω3 to the T. suecica fed spat resulted in very little extra growth. These experiments suggest that chain elongation of 18:2ω6 or 18:3ω3 is not sufficient for maximum growth, and that either 20:5ω3 or 22:6ω3 is "essential" for growth. In higher animals some interconversion of these two fatty acids occurs, and it is probable that mixtures of 20:5ω3 and 22:6ω3 are optimal for bivalves.

The ten algal polyethylenic fatty acids of Table I do not include some that are potential intermediates in elongation and desaturation of fatty acids. Thus 18:3ω6, 20:2ω6 and 20:3ω3 are omitted simply because they do not figure prominently in bivalve analyses. Similarly, of the ten bivalve polyethylenic fatty acids shown in Table III, only two (20:5 ω3and 22:6ω3) are quantitatively important, and the qualitative and quantitative significance of the others seems to depend on the experiment and/or analytical group.

It is now recognized that in mammals there is often competition for elongation/desaturation enzymes between 18:2ω6 and 18:3ω3 fatty acids and their homologues (Holman 1979). For example a Δ6 and then a Δ5 desaturase are required for the conversions 18:2ω6 → 20:4ω6 and 18:3ω3 →20:5ω3. Probably the respective enzymes are identical. The fatty acids of phospholipids can presumably be derived if necessary, directly and unchanged, from the pool of fatty acids stored as triglycerides. In most of the cases compared in Table III there is a virtual absence of 18:4ω3 in the phospholipids relative to the triglycerides. This may follow from the selective association of enzymes and phospholipds as only one of these enzymes, the Δ5 desaturase, is required to convert 18:4ω3 → 20:5ω3, a fatty acid perhaps most common at the 2-position in phospholipids (Brockerhoff 1966). The moderate (1-6%) proportions of 20:4ω6 in the bivalve phospholipids (Table III) seem to be higher than

those of the algae (Table I). Comparison of the proportions of 20:4ω6 in the phospholipids and triglycerides (Table III) shows the converse of those for 18:4ω3 proportions, with 20:4ω6 being enriched in phospholipids relative to triglyceride levels. Although this acid could also be formed in bivalve cells from triglyceride 18:2ω6 if specifically required, selective withdrawal of 20:4ω6 from the triglyceride fatty acid pool is a reasonable alternative.

The turnover of fatty acids in the phospholipids of the land snail C. nemoralis is rapid (Van der Horst et al. 1973), and the same may be presumed for bivalves. This activity must be directed towards either degradation for energy, or for elongation of 18:3ω3 to 20:5ω3 and 22:6 ω3. A comparison of Tables I and III gives the firm impression that in a mixed diet preformed 20:5ω3 and 22:6ω3 are freely available. It is probable that the proportions of polyunsaturated fatty acids in bivalve triglycerides are an integration of past exposures to dietary fatty acids. This would be less apparent in the land snail triglycerides (Van der Horst and Oudejans 1976) where only C_{18} fatty acids are present in the diet. Waldock and Nascimento (1979) found that the proportions of 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 fatty acids in the triglycerides of C. gigas larvae were almost exactly similar in proportions to the fatty acids of the respective algal diets (Table V). Watanabe and Ackman (1974) found evidence for the retention of the algal polyunsaturated fatty acid 18:4ω3 in the triglycerides of wild adult C. virginica, (3.0% of fatty acids) but not in the triglyceride of wild O. edulis (0.6%) from the same habitat. With a single feeding of Isochrysis galbana containing 8% of this fatty acid there was no change in the fatty acids of C. virginica triglycerides, although phospholipid 20:5ω3 increased slightly, from 8.1 to 9.8%, but the O. edulis triglycerides showed a doubling in 18:4ω3 from 0.6 to 1.2%, and more importantly by a tripling in the percentage of 20:5ω3 from 2.2 to 6.2%. This suggests that wild adult C. virginica and O. edulis dispose of the dietary fatty acid 18:4ω3 in qualitatively similar but quantitatively dissimilar ways.

It is accepted that fatty acids of the linoleic (ω6) series and linoleic (ω3) series cannot be interconverted in animals. In Table III the ratios of ω6 to ω3 fatty acids in the flesh and hepatopancreas phospholipids of Arctica islandica are 1:15 and 1:17 respectively. For the phospholipids of M. edulis and P. maximus the ratios of ω6 to ω3 fatty acids are 1:14 and 1:20 respectively, which are similar to the A. islandica value of 1:15 for flesh phospholipids. This similarity indicates a common pathway and conversion in phospholipids. The ratio of ω6 to ω3 fatty acids in phospholipids of wild C. virginica and O. edulis differ somewhat from each other (1:4 and 1:15 respectively) but the ratio present in wild Canadian C. virginica is identical to that in hatchery reared U.K. C. gigas spat which is 1:4 (Table III). The oysters thus form one group and the other three species fall into another. There is a suggestion of evolutionary differences here, or of dietary factors which equally may reflect different ecological niches and functions for phospholipids in membrane physiology. The ω6 to ω3 ratios in the phospholipids of oysters seem to resemble in magnitude those of the dietary lipids averaged in Table I. It is not unreasonable to assume that primitive multi-celled organisms would utilize the most readily available sources of fatty acids for membrane phospholipids which would enable them to function in their special milieu, bearing in mind that the membrane fatty acids of the algal cells of the diet

fulfilled many of the same biochemical and physiological needs.

Table V. Comparison of Weight Percentages of Some C_{18}, C_{20}, and C_{22} Fatty Acids in Total Lipid of Chaetoceros calcitrans, Pyramimonas virginica and Isochrysis galbana with Triglyceride Fatty Acids of Experimentally Fed Crassostrea gigas (from Waldock and Nascimento 1979)

		C. calcitrans	P. virginica	I. galbana
18:2ω6	(algae)	0.2	2.6	4.3
	(larvae)	0.7	1.5	6.5
18:3ω3	(algae)	3.0	10.6	11.0
	(larvae)	2.9	7.5	10.6
18:4ω3	(algae)	0.5	4.4	9.2
	(larvae)	1.2	3.9	8.9
20:4ω6	(algae)	trace	-	0.2
	(larvae)	0.5	0.5	0.8
20:5ω3	(algae)	15.4	1.3	3.6
	(larvae)	22.0	4.1	3.2
22:6ω3	(algae)	1.9	13.7	18.9
	(larvae)	1.6	11.8	18.4

In a complex study Pollero and Brenner (1981) showed that the 6 fatty acids of the bivalve Diplodom patagonicus from a mountain lake were reduced by half when a sample of the population was transplanted to the marine environment of the Rio de la Plata. In the latter milieu, the total fatty acids were similar to those of Diplodom variabilis, a species occurring naturally in the Rio de la Plata. Thus the ω6 to ω3 ratios were 1:0.4 - 1:0.5 in the lake and 1:0.9 in the Rio de la Plata for D. patagonicus, and 1:08 for D. variabilis. They concluded that dietary fatty acids were more important in affecting the fatty acid composition of D. patagonicus than habitat. The change in salinity could have been equally important.

NON-METHYLENE-INTERRUPTED FATTY ACIDS

The original report on these unusual acids (Ackman and Hooper 1973) and later surveys (Paradis and Ackman 1975; 1977) showed that most bivalve species possessed both 20:2 NMID and 22:2 NMID fatty acids. Several isomers were present but the structures suggested that most commonly a desaturase acted on a fatty acid chain at carbons Δ5 and Δ6, and with a Δ13 ethylenic bond present would give a structure such as Δ5, Δ13-20:2. Possibly, the reason that the Δ11 position in 18:1 isomers is more important and the Δ13 in 20:2 is a consequence of chain elongation. Other isomers reported had different structures or the Δ5 ethylenic bond became Δ7 by chain elongation. In the freshwater mollusc

D. patagonicus mentioned above, 22:2 NMID but not 20:2 NMID fatty acids were present (Pollero et al. 1981). These shellfish were especially rich in ω6 acids and elongation of 18:2ω6 to 20:4ω6 and also to 22:4ω6 could be quite active to provide substitutes for 22:5ω3 or 22:6ω3 fatty acids. This unusual composition supports the theory that the enzyme in the mollusc which desaturated the ω6 acid Δ8, Δ11, Δ14-20:3 (dihomo-gamma-linolenic acid) to Δ5, Δ8, Δ11, Δ14-20:4 (arachidonic acid) by acting on the Δ5 and Δ6 carbons did not discriminate strongly against fatty acids of ω7 structure. The 20:1ω7 thus became Δ5, Δ13-20:2 and was then chain elongated to Δ7, Δ15-22:2. This result was also observed in the transplanted D. patagonicus (Pollero and Brenner 1981). The related marine species D. variabilis had lower levels of 22:2 NMID and twice the content of ω3 fatty acids such as 20:5ω3 and 22:6ω3. If these were supplied by the marine phytoplankters and less 20:4ω6 or 22:4ω6 was required for membrane lipids then the desaturase acting on C_{20} fatty acids at the Δ5 and Δ6 carbons would be relatively inactive as less 20:4 ω6 would be biosynthesized and less chain extension of C_{20} fatty acids to C_{22} fatty acids would be likely.

A detailed investigation of major tissues and organs of the hardshell clam Mercenaria mercenaria (Klingensmith 1982) showed the largest concentration of NMID fatty acids in the gill and mantle. The isomers of the 22:2 NMID fatty acids were Δ7, Δ15 and Δ7, Δ13 which were previously reported in the oyster C. virginica (Paradis and Ackman 1975). The 20:2 NMID fatty acids were present at much lower levels and the fatty acids identified were Δ7, Δ11 as the major isomer with much less Δ7, Δ13 and 5, Δ11. The Δ5, Δ13 isomer previously reported in C. virginica and more recently in the sea urchin Stronglyocentrotus droebachiensis (Takagi et al. 1980), was not present. No Δ3, Δ11-20:2 NMID, another isomer which has been reported by Kochi (1976), was found in the study of S. droebachiensis.

At present there is little to add to previous speculation on the role of these fatty acids. For example Ackman and Hooper (1973) suggested an accidental origin due to lack of specificity of fatty acid biochemistry. The almost ubiquitous occurrence of NMID in molluscs (Johns et al. 1980) indicates an unknown but defined role in bivalves as well as other molluscs. They were less evident in O. edulis than in C. virginica reared in the same habitat (Watanabe and Ackman 1974), confirming that species to species variations are likely.

CONCLUSIONS

The successful use of mixed fatty acids of animal origin for feeding oysters (Trider and Castell 1980; Langdon and Waldock 1981) supports the concept that a diet of mixed fatty acids of either plant or animal origin is advantageous for growth. The optimum mixture may vary somewhat from species to species and there may be rare instances of an alga being an ideal food e.g. Chaetoceros armatum for the Pacific razor clam Siliqua patula (Lewin et al. 1979). However, in general, bivalves clearly tend to take a dietary fatty acid and break it down for energy or simply store it in triglyceride form, or transfer it to, or modify it for, the phospholipids. With the advantages of contemporary technology future work on fatty acids of all types should be directed to the analysis of both specific anatomical parts and to particular lipids, including individual phospholipids. Unfortunately, the laboratories

with the best analytical technology are usually those with little concern for environmental and dietary factors in the samples. Until this situation is rectified we can expect little real progress in the study of fatty acids in the many bivalves of commercial and scientific importance.

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QUESTIONS AND ANSWERS

LANGDON (Delaware): Have you looked at the fatty acid composition of different organs of molluscs, say the adductor muscle in comparison with the hepatopancreas?

ACKMAN: No, well only that we took out the hepatopancreas and called everything else "flesh". If you've been reading my manuscript which was sent to you, you will find that the conclusion is we need more detailed analyses on different parts of the anatomy. I'm sorry to say that the Fisheries Research Board and its successor, the Federal Government Lab, did not share my enthusiasm for this kind of work and it was only done sporadically. Certainly you are quite right, we need to have analyses of different organs and different lipid classes for all fatty acids, but it's a tremendous job even for one mollusc species.

CARRIKER (Delaware): I was fascinated in your comment about the phagocytes, would you like to say a little bit more about that?

ACKMAN: Well, I'm an organic chemist at this point, I think! I don't know how many of you know about dimethyl-beta-propiothitin. It's a common constituent of certain classes of algae, and also some macrophytes. The unicellular algae are eaten by certain small "butterfly" molluscs called pterapods, which in turn are eaten by cod fish, which then smell like an oil refinery from dimethyl sulfide produced by the breakdown of thetin. That is why I was given the chance to look at the problem. After all, anything that smells bad is a good job for an organic chemist. The biochemists were all too pure to look at these things and I went into some really fascinating research. One of the sort of things you had to come up with was how does the stuff accumulate? It should be broken down by the pterapods and disposed of and it isn't. The reason is, I think, that it also yields acrylic acid as soon as the first 2 or 3 molecules are turned loose inside the

phagocyte's cell. I think everything just stops there because acrylic acid is a very powerful biostat. This is another thing that somebody should be taking a look at some time. It did lead me to read up a bit on phagocytosis. If you have forgotten, you basic body defenses depend heavily on phagocytosis. Therefore, it must be a very old evolutionary thing that developed long, long ago and of course we may have modified it as we went along.

EFFECTS OF THE AMBIENT ENVIRONMENT ON METABOLIC REGULATION OF SHELL BIOSYNTHESIS IN MARINE BIVALVE MOLLUSKS

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ABSTRACT

Pertinent literature relating effects of the ambient environment on mantle tissue metabolism of marine bivalves and their combined effects on bivalve shell biosynthesis are discussed. Several previously published models for shell synthesis are described and their possible relation to mantle tissue metabolism are presented. Although there is no experimental evidence directly relating nutritional factors to shell formation, several aspects of nutrition that may have an indirect influence on rate and quality of shell synthesis in marine bivalve mollusks are also discussed.

Results from original research directly relating calcium concentration and salinity in ambient culture media to amino acid fluxes in mantle and extrapallial fluid are presented. Increases in both ambient salinity and calcium concentrations resulted in increases in concentrations of several selected free amino acids in mantle tissue and excretion of glycine from mantle into ambient culture media. Results of this investigation plus components of previously proposed models are combined to formulate a new model. Through the proposed model, specific mechanisms are invoked to explain how the chemistry of the ambient environment and the nitrogen metabolism of the mantle tissue (including effects of nutritional factors) may regulate molluscan shell biosynthesis.

KEYWORDS: Shell biosynthesis, mollusks, metabolism.

INTRODUCTION

Molluscan shell biosynthesis, simply conceived, is presently thought to occur through mineralization of an organic matrix. While several models have been proposed to explain chemical, biochemical and physiological processes involved in such mineralization, the two most plausible mechanisms are either, 1) the organic matrix serving as a template for calcareous deposition (Glimcher 1960; Wilbur, 1964) or, 2) an organic matrix merely providing a compartment for the growth of calcareous crystals (Bevelander and Nakahara 1969). Regardless of the exact mechanism involved in shell mineralization, certain elements of skeletal formation are common to most biological systems and therefore probably occur in marine mollusks. For example, biosynthesis of molluscan shell probably includes: 1) deposition of an organic matrix, 2) sequestering of calcium and associated mineral elements, 3) sclerotization of an organic matrix, 4) at least partial control of chemical processes by enzymatic, hormonal, and metabolic mechanisms, particularly at the mantle edge, 5) expenditure of energy by the mantle tissue and 6) a significant influence of the ambient environment. While there is substantial experimental evidence to provide details for many components of these outline processes of skeletal formation, several of these have not been observed or investigated in marine, bivalve mollusks.

The primary objective of this paper is to review current concepts of molluscan shell synthesis, particularly relating effects of the environment and mantle tissue metabolism to shell formation. This review does not include a thorough coverage of all literature pertaining to shell synthesis but has relied on several recent summaries (e.g. Wilbur 1964, 1972; Wilbur and Simkiss, 1968; Watabe and Wilbur 1976). Conclusions from this view are supported with results from some original research. In addition, a model of shell biosynthesis is formulated to suggest concepts that will require examination in future experiments. Energetics, the role of organics other than proteinaceous matter, minerals and trace metals other than calcium, and control by enzymatic and hormonal processes have been excluded.

MODELS OF SHELL BIOSYNTHESIS

Organic matrix

Although too extensive to reiterate in this brief review, several models or partial models have been proposed to couple observed phenomena associated with shell synthesis with probable biological and physio-chemical processes. As summarized by Simkiss (1976), all models of molluscan shell synthesis must consider certain postulates including: 1) a mineral saturated medium adjoining the surface to be calcified, 2) a basic pH environment, 3) predeposition of an organic matrix, and 4) a mechanism for producing a crystalline structure in the mineralized substrate.

Several theories and conceptual models have been developed in attempts to incorporate the postulates discussed above, as reviewed by Wilbur (1976). Bevelander and Nakahara (1969) suggested that in a super-saturated mineralized medium, such as the extrapallial fluid of marine bivalve mollusks, a protein matrix could provide nucleation sites

for crystalline growth. Bevelander and Nakahara (1969) also observed that an organic matrix is deposited prior to shell mineralization in mollusks. Based on limited experimentation, Waddell (1972) advocated that nucleation for crystal growth could occur within protein matrices. The formation of calcium carbonate crystalline structures has also been observed to develop in the presence of acid mucopolysaccharides (Abolins-Krogis 1973), although Kobayashi (1971) could not verify this association. Kitano et al. (1969) reported the importance of specific organic substances including citrate, malate and pyruvate in the crystal formation of carbonates in supersaturated carbonate fluids simulative of fluids in marine bivalves.

Considering that a proteinaceaous matrix could serve as both a site for nucleation as well as form compartments for calcium carbonate crystal formation, Glimcher (1960) proposed that an organic matrix acts as a prerequisite template for the orderly mineralization of molluscan shell. Subsequently, Weiner and Hood (1975) observed that the digested glycoprotein matrix of oyster (Crassostrea virginica) and clam (Mercenaria mercenaria) shell consisted of a series of alternating units of aspartic acid and either glycine or serine. They further observed that the distance between repeating aspartic acid residues averaged 6.95 A° and thereby closely coincided with distances between calcium molecules in aragonitic and calcitic crystals which average approximately 6.0 A°.

Several variations in the glycine-aspartic-serine model proposed by Weiner and Hood (1975) have been reported by other investigators. Although an arrangment of aspartic acid residues was recently reported from the soluble shell fraction of Nautilus repertus (Weiner and Traub 1981), and in Nautilus sp., such residues may contain growing inorganic crystals (Weiner and Traub 1981) rather than bound calcium in a template type of organization as found in other bivalves (Weiner and Hood 1975). Although Crenshaw (1972a) did not find aspartic acid-glycine-serine residues in an analysis of the isolated glycoprotein of Mercenaria mercenaria shell, high concentrations of aspartic acid and glycine were reported. This result was based on a different methodology from that used by Weiner and Hood (1975). Since Weiner and Hood (1975) used a mild hydrolysis leaving protein on both sides of aspartic acid, their results would yield a more accurate description of total shell protein than those of Crenshaw (1972a). From an investigation of eleven species of brachiopoda, Jope (1973) found that the soluble protein of shell matrix contained relatively high concentrations of glycine, aspartic acid and proline. Furthermore, the protein fraction consisted of one principal polypeptide chain with several smaller chains, each having histidine at the N-terminal end.

Weiner and Hood (1975) and Weiner and Traub (1981) concluded that the negative carboxyl sites of aspartic acid could serve for shell mineralization. Since extrapallial fluid normally has a basic pH, except during extended periods of shell closing (Crenshaw and Neff 1969; Lutz and Rhoads 1977; Gordon and Carriker 1978), a negative charge on the carboxyl end of amino acid moieties could exist. However, even though there has been support for a carboxyl calcium binding theory in shell formation (Wilbur and Simkiss 1968), amino acid residues in the organic matrix prior to mineralization may be present in an amide, non-charged, form (Wilbur 1976). Sulfhydryl groups of sulfur containing amino acids may also bind calcium in the extrapallial fluid. However,

because of the very low concentrations of sulfur-containing amino acids found in the soluble protein fraction of most molluscan bivalve shells, relative to acidic amino acids (e.g. Jope 1967; Weiner and Hood 1975), the negative carboxyl groups of acidic amino acids would probably be more effective in binding calcium during shell synthesis than would sulfhydryl groups.

Inorganic constituents

Regardless of which of the models proposed to date most accurately depicts the organic and inorganic processes of molluscan shell formation, both calcium and carbonate groups must be readily available. Simkiss (1976) reported that the extrapallial solution must have calcium carbonate concentrations in excess of its solubility product for mineralization to occur. Wheeler (1975) has proposed a limited model to account for bicarbonate availability and function in the premineralized organic environment of the extrapallial fluid. The functioning of a biochemical bicarbonate pump (Keynes 1969) could adequately explain sufficient bicarbonate concentrations in the extrapallial fluid to account for mineralization. According to Wheeler (1975), the influx of bicarbonate, from the ambient environment and mantle tissue, during the mineralization process, could react with protons to form carbon dioxide followed by the removal of carbon dioxide by diffusion into the mantle. As proposed by Wheeler (1975), this diffusion of carbon dioxide would allow carbonic anhydrase to continue to catalyze the conversion of bicarbonate to carbon dioxide and thereby allow the mineralization of an organic substrate to continue.

The hardening or sclerotization of any protein matrix involves cross linking of various polymers and has been described by several investigators (e.g. Stevenson 1969; Welinder et al. 1976; Waite and Anderson 1980). In general, the initial chemical process is the syntheis of a protein matrix. Following protein synthesis, one of the next steps is the conversion of aromatic amino acids and phenols to quinone, the sclerotizating agent. Quinone is specifically formed from diphenol by phenol oxidase. Gordon and Carriker (1980) reported that phenol oxidase and tyrosyl hydroxyl groups were found in areas of active shell formation in the clam Mercenaria mercenaria. Furthermore, polyphenols and active phenol oxidase have recently been identified in shell proliferation areas of the mussel, Mytilus edulis (Bubel 1973) and the larval stages of the oyster Crassostrea virginica (Tomaszewski 1981). Sclerotization occurs when O-quinone reacts, without enzymatic mediation, with either N-terminal lysyl groups, or sulfhydryl groups attached to amino groups to form N-catechol polymers. These polymers are subsequently oxidized to form N-quinone protein. Further reaction of the quinoid protein with other lysyl, phenolic, or sulfhydryl terminal groups results in a relatively inert and cross-linked rigid protein matrix.

An alternative mechanism for achieving sclerotized shell protein may involve an auto-tanning process without the necessity of quinone precursors (Cranfield 1973, 1974). DOPA (dihydroxy-phenylalanine) residues are thought to be oxidized to DOPA quinone residues which bind readily with nucleophilic groups of neighboring protein molecules to form a rigid protein matrix (Waite and Anderson 1980; Waite and Tanzer 1981). Waite and Tanzer (1981) further observed that in salt solutions, protein bound DOPA residues were not as soluble as free DOPA residues.

EFFECTS OF AMBIENT ENVIRONMENTAL CHEMISTRY ON SHELL BIOSYNTHESIS

While specific mechanisms for incorporating raw materials into molluscan shell and the significance of certain chemicals may not be well understood, the effect of concentration and availability of some ambient chemicals on the composition of shell has been observed (Carriker et al. 1980). They reported a significant increase in concentrations of cadmium, copper, iron, magnesium, manganese, strontium, and zinc in the shell and soft tissues of oysters (Crassostrea virginica) grown in seawater enriched in these cations. Since the distribution of many of these elements appeared to be selective within the foliated and prismatic layers of oyster shell and concentrations of these cations increased in shell in response to respective increases in ambient concentrations, quantities and availability of specific ions or complexes in the ambient environment may have a significant effect on shell structure (Carriker et al. 1980). However, the bioavailability of most minerals, particularly calcium and mangesium, probably seldom, if ever, limits shell formation in marine mollusks in their natural environment (Greenaway 1971: Shumway 1977; Conger et al. 1978; Sick et al. 1979).

Although there is no direct evidence that trace metals accumulated from the ambient environment are essential for shell synthesis, many trace metals are accumulated at relatively high rates by bivalves (see review by Forstner and Wittmann 1979). Toxicity from sublethal long term exposures to certain metals, therefore, may inhibit shell synthesis in certain environments. Such sublethal toxicity could affect rate of shell formation through general suppression of metabolic activity. Although the sites of toxicity that would affect shell formation are not known, inhibition of critical enzymes, such as carbonic anhydrase and those involved in sclerotization such as phenol oxidase, are undoubtedly susceptible to inhibition by pollutant concentrations of metals (e.g. Albiston et al. 1940; Jackim et al. 1970; Baron 1981).

Changes in the composition of the ambient medium are known to also affect the chemical composition of the extrapallial fluid of marine bivalve mollusks (Wada and Fujinuki 1974). However, such effects are not totally passive since there is evidence for physiological regulation of sodium, magnesium, potassium and chloride in the extrapallial fluid of several species of marine bivalve mollusks (Wada and Fujinuki 1976). Crenshaw (1972b) reported that while the ionic compositions of seawater and extrapallial fluid are similar, concentrations of all major cations and total carbon dioxide were higher in extrapallial fluid. Since the chemical composition of the environment can affect the chemical composition of the extrapallial fluid, over and above physiological regulation, it is probably that the environmental effects on the chemical composition of the extrapallial fluid influence shell formation.

NUTRITIONAL, PHYSIOLOGICAL, AND METABOLIC REGULATION OF SHELL BIOSYNTHESIS

Nutritional Regulation

There have been no investigations designed specifically to elucidate cause and effect relations between nutrients ingested by bivalves and shell formation. Mixtures of unicellular algal species have been used in an attempt to alter the nutritional quality (i.e. total protein, lipid, caloric, amino acid, or fatty acid concentrations) of food available. Although significant variations in the growth rates of several species of bivalves have resulted from feeding different algal species or mixtures of algal species (e.g. Epifanio 1975; Epifanio et al. 1976; Epifanio and Ewart 1977; Flaak and Epifanio 1978), such variations may have been largely due to differences in digestibility and in the rate of passage of food through the bivalve digestive system. In addition, growth rate may be dependent upon the availability of vitamins or minerals (Walne 1970, 1973; Epifanio 1979). Berg (1981) eliminated the possible effects of digestibility and was able to examine food value per se by using one algal species but altering its protein, carbohydrate, and lipid composition. The differences in gross composition between cultures were created by varying the supply of given nutrients in the algal culture medium. He found that shell length in Crassostrea virginica larvae increased most rapidly when dietary concentrations of protein, carbohydrate, and lipid were present at 35, 24, and 39% of algal dry weight, respectively. Significantly less shell growth occurred when these proportions were changed. Although these resuls (i.e. Berg 1981) implicated assimilated nutrients in shell growth, the exact mechanisms governing the involvement of assimilated nutrients in shell synthesis may be indirect (i.e. via tissue growth). In addition, the effect of environmental conditions on shell growth are difficult to partition from effects of assimilated nutrients. For example, Palmer (1980) reported that differences in shell growth and morphology among groups of scallops (Argopecten irradians) reared on different mixtures of algal species could not be partitioned statistically from influences of light periodicity, human handling of individual animals, or temperature differences.

Although there is no strong evidence for a direct relation between diet and synthesis of shell material, several aspects of cellular metabolism in organisms other than mollusks suggest that diet would affect shell synthesis. For example, the amino acid composition of ingested protein has been observed to affect the rate of synthesis of cellular proteins in rate tissues (e.g. Cox and Mueller 1944). Using in vitro experiments with cultures of the bread mold, Neurospora sp., Beadle and Tatum (1941) demonstrated precise relations between specific nutrients and metabolic pathways for the synthesis of selected amino acids. Dagley et al. (1963) in an investigation in rats also demonstrated an association between the assimilation of specific nutrients and amino acid metabolism. Nutrient requirements for cellular metabolism in vertebrates have been observed to affect the cellular excretion of amino acids (Arnstein and Grant 1954). Relative to invertebrates, Kating et al. (1962), reported that insect tissue can synthesize several amino acids from dietary glucose. Similarly, Huggins and Munday (1968) observed that crustaceans could also synthesize free amino acids from glucose.

Since the excretion of nitrogenous material into extrapallial fluid is probably an integral component of molluscan shell biosynthesis (e.g. Kobayashi 1964; Crenshaw 1972b; Young et al. 1977), it is reasonable to speculate that nutritional factors affecting cellular nitrogen metabolism would also affect shell growth. For example, in vitro observations of specific nutrient requirements for cellular amino acid synthesis using both vertebrates and invertebrates have indicated that a series of transaminations and other metabolic transformations can result in synthesis and increased concentrations of selected amino acids (Berger 1960; Johnson and Strecker 1961; Zandee 1966; Sylvester and Krassner 1976). These processes, as observed in cells of mammalian and invertebrate tissues, could also be operative in the columnar epithelial cells of molluscan mantel tissue (Neff 1972); the cells probably responsible for secreting the nitrogenous material that presumably becomes a component of the shell matrix (Young et al. 1977).

Physiological regulation

The chemical composition of the environmental may also regulate shell biosynthesis indirectly through effects on cellular physiology. Calcium and amino acid fluxes between the mantle and the extrapallial fluid have been observed to change in response to changes in ambient salinity and concentrations of total calcium. Shumway (1977), for example, reported that osmolarity and concentrations of sodium, magnesium, and calcium in the extrapallial fluid of the oyster Crassostrea gigas were significantly different from the respective ion concentrations in ambient seawater but nonetheless were affected by seawater concentrations. Several other authors have observed ionic regulation of molluscan hemolymph and other body fluids such as the extrapallial fluid and have assumed a closed shell was necessary for such regulation (reviewed by Hayes and Pelluet 1947; Robertsen 1949). In contrast, Sick et al. (1979) observed that concentrations of total calcium in the mantle tissue of C. virginica changed curvilinearly in response to ambient calcium concentration, both in whole animals and in mantle tissue culture in vitro. The observation that changes in calcium concentration of mantle tissue were curvilinear and not proportional to ambient changes in calcium concentration, strongly suggested physiological regulation (Sick et al. 1979).

Physiological regulation of extrapallial ionic concentrations can be adequately explained by several mechanisms known to exist in molluscan mantle tissue. For example, DeWith (1977) observed that physiological mechanisms exist in mollusks for regulating concentrations of sodium, potassium, calcium, magnesium, and chloride. All cells probably possess both calcium (Baker 1972; Schatzmann 1973) and bicarbonate (Keynes 1969) pumps allowing these ions to be secreted extracellularly. However, evidence for cellular ion pumps has yet to be definitively demonstrated in marine mollusks.

Metabolic: Experimentation involving amino acid fluxes

In order to evaluate portions of previously proposed models for molluscan shell biosynthesis (e.g. Weiner and Hood 1975; Young et al. 1977), amino acid concentrations of in vitro mantle cultures and ambient perfusion fluid were examined following exposure to several concentrations of ambient calcium and salinity. While it is not known

whether it is free amino acids or peptides that react with uncomplexed calcium in the extrapallial fluid, most investigators have referred to ionic calcium as being bound by extrapallial fluid proteins (Bevelender and Nakahara 1969; Weiner and Hood 1975; Wheeler et al. 1981). However, since amino acids are actively transported across tissue membranes in vertebrates (e.g. Bergen, 1969) and invertebrates (e.g. Siebers 1976; Siebers and Rosenthal 1977) and readily bind calcium, under basic pH conditions in the molluscan extrapallial fluid (Weiner and Hood 1975), it is logical to propose that it is free amino acids or peptides and not proteins that bind calcium and thereby are prerequisite for shell biosynthesis. The hypothesis proposed for this project, therefore, was that excretion of selected free amino acids from the mantle is correlated with increases in ambient calcium concentration and salinity and serves to facilitate shell synthesis.

Experiment A: Effects of changes in ambient calcium concentration and salinity on amino acid concentrations in mantle tissue and ambient fluid.

Methods: Oysters, having an average net weight of 117.03±29.31g, were obtained from Chesapeake Bay stocks during the fall season. During an acclimation period of 7 days, oysters were maintained in filtered seawater at 25 ppt salinity. Cultures of the diatom Thalassiosira pseudonana (obtained from Dr. Charles Epifanio, University of Delaware) were batch fed daily to oysters at a concentration of 8.5×10^5 cells ml^{-1}. The temperature of the culture medium was gradually increased from 15° to 20°C during the acclimation period.

Following acclimation, mantles attached to the right valve of freshly opened oysters were exposed to various calcium-salinity treatments consisting of a factorial arrangement of salinity concentrations (15, 20, 25, 30, and 35 ppt) and total calcium concentrations (2.5, 5.0, 7.5 and 10.0 μmoles (g wet wt. culture medium $^{-1}$). The culture medium consisted of perfusion fluid (PF) (Cole 1941). Salinity and total calcium concentrations were varied independently by adjustmenting the amounts of salts added to the perfusion medium (Kester et al. 1967). Changes in osmolarity resulting from changes in Ca^{2+} salt levels were less than 0.1 m Osm. Preparations were exposed to calcium-salinity treatments for 48 hours. During the experimental period, mantle tissue was examined for vitality by observing ciliary beating and its response to tactile stimulus.

In order to examine the possible association between ambient calcium concentration and the synthesis and excretion of selected amino acids from mantle tissue, changes in both ambient and mantle tissue concentrations of total calcium were measured. An examination of the relationships between ambient calcium concentrations, tissue calcium concentrations, and changes in tissue amino acid concentrations was felt to be critical for testing our hypothesis that the concentration of ambient calcium may regulate shell synthesis via amino acid metabolism.

Tissue and perfusion fluid were each frozen, after the experimental period, and freeze dried. Amino acid concentrations in frozen tissues were compared to fresh tissue and no leakage of amino acids was detected as a result of freezing. Freeze-dried preparations were first lipid extracted with 1:1 chloroform-ether and then the free amino acids extracted three times, at 4°C with 70% ethanol. Extracts were frozen in citrate buffer until amino acid analyses could be performed. Amino acid

analyses of mantle tissue and perfusion fluid were conducted by forming N-trifluoroacetyl methyl amino acid esters (Gardner and Lee 1973) and analyzing these derivatives by gas liquid chromatography using a flame ionization detector. Calcium concentrations in mantle and perfusion fluid were determined by flame atomic absorption spectrometry.

Results: Calcium concentration in mantle tissue changed curvilinearly with increases in both ambient calcium and salinity (Figure 1). The curvilinear changes observed in excised mantle tissue mimicked changes observed when whole oysters were exposed to a range of similar ambient conditions (Sick et al. 1979).

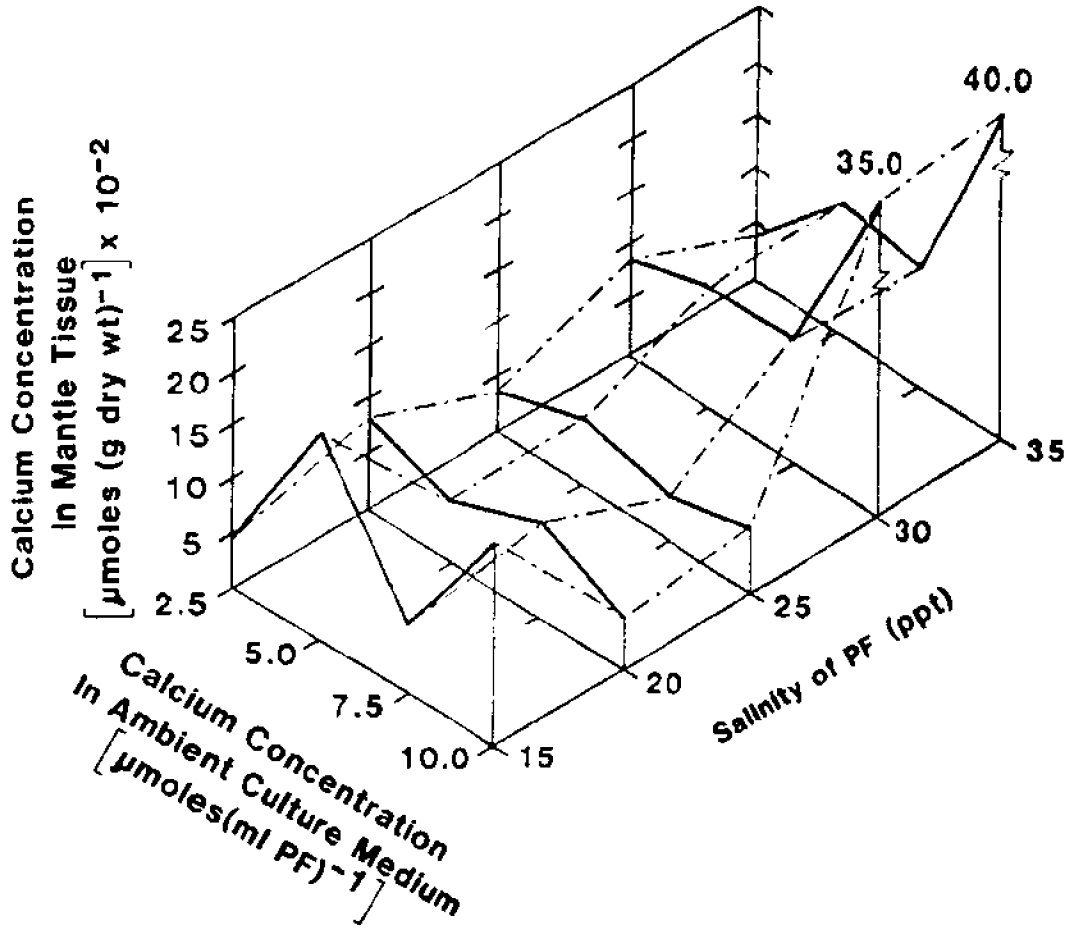

Figure 1. Effects of ambient calcium concentration and salinity on mantle tissue (Crassostrea virginica) calcium concentration. Values are based on four replicates of each treatment. Average standard errors for mantle tissue calcium concentrations over all of the ambient concentrations of calcium at salinities of 15, 20, 25, 30 and 35 ppt were 0.11, 1.1, 2.0, and 1.7, and 2.6 µmoles (g dry wt)$^{-1}$ x 10^{-2}, respectively.

Concentrations of glycine, serine, and glutamic acid measured in cultures of mantle tissue incubated at a salinity of 25 ppt tended to mirror the concentrations measured in the ambient culture medium (perfusion fluid) (Figure 2). Free serine and glutamic acid concentrations in mantle tissues initially increased with increases in ambient calcium concentration and then decreased. Concentrations of glycine, serine, and glutamic acid measured in ambient perfusion fluid were generally inversely proportional to changes in concentration observed in mantle tissue. In contrast, however, concentrations of alanine in both mantle tissue and perfusion fluid decreased with increases in ambient calcium.

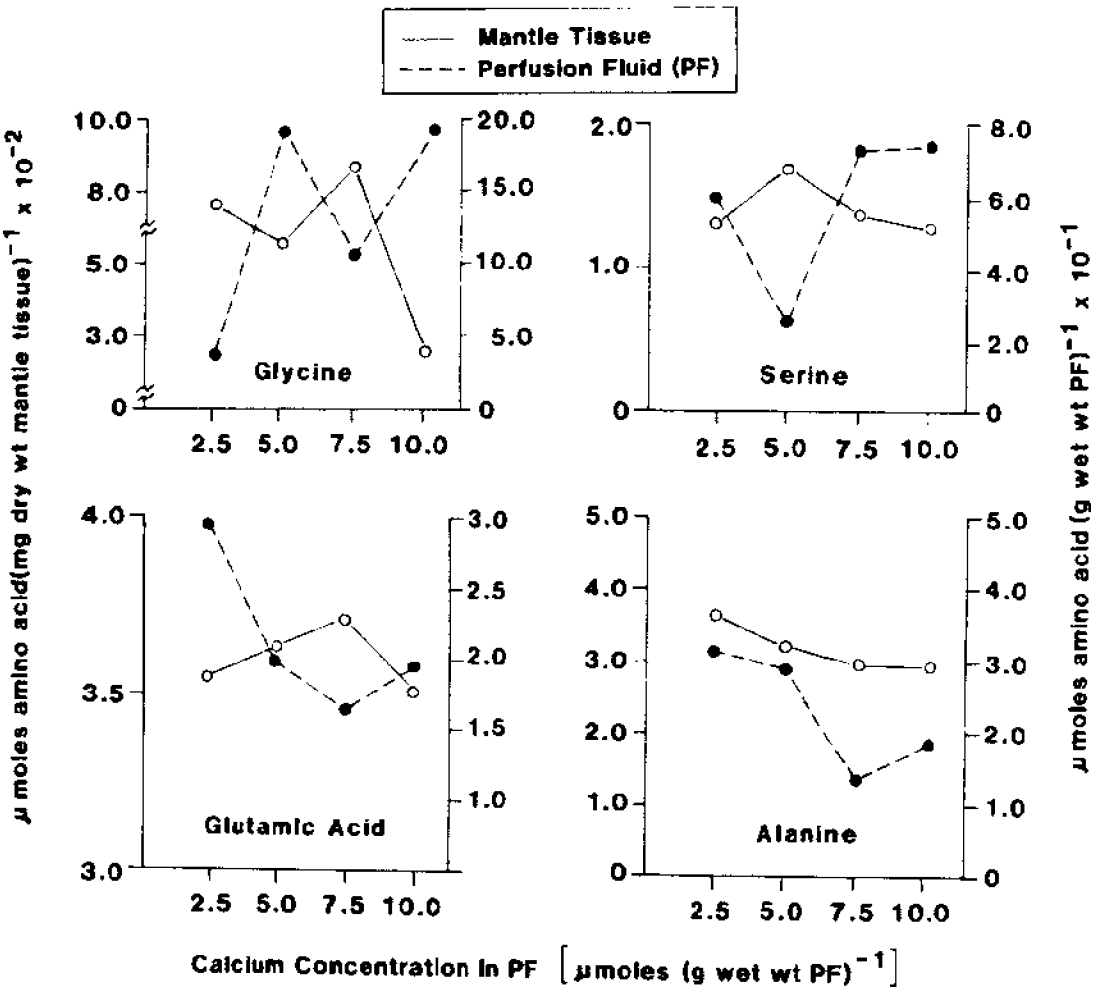

Figure 2. Fluctuations in concentrations of selected free amino acids in mantle tissue (Crassostrea virginica) and ambient culture medium in response to changes in concentration of ambient calcium. Average standard deviations (based on four replicates) were 1.8, 0.2, 1.1, and 0.9 µmoles (mg dry wt mantle tissue)$^{-1}$ x 10^{-2} for mantle tissue glycine, serine, glutamic acid, and alanine, respectively. Similarly, average standard deviations (based on four replicates) were 2.2, 1.2, 0.8, and 0.9 µmoles (g wet wt PF)$^{-1}$ x 10^{-1} for free glycine, serine, glutamic acid, and alanine, respectively, measured in perfusion fluid (PF).

Experiment B: Fluxes of free mantle tissue glycine in response to changes in ambient calcium concentration.

Methods: Oyster stocks, period of acclimation, culture conditions during acclimation, and experimental design were as described for Experiment A. However, the excised mantle tissues (attached in the right valve) were incubated in each calcium concentration and salinity treatment in the presence of ^{14}C(U)-glycine. Efflux of ^{14}C-glycine from mantle was monitored by removing the mantle preparations after the initial incubation period and placing the tissues in non - ^{14}C labelled perfusion fluid of the same calcium-salinity concentration.

Following incubation in ^{14}C(U)-glycine, a 10 mg sample of freeze-dried, lipid free, excised mantle tisue was digested in Protosol-Aquasol scintillation solution and the radioactivity determined using liquid scintillation spectrophotometry. Counting efficiency for each sample was determined using the external standard technique. Radioactivity was convereted to concentrations of ^{14}C-glycine (^{14}C was assumed to remain associated with either free or complexed glycine, Young et al. 1977) measured in respective treatments using the specific activity of the stock solution (90 mCi (mmole glycine)$^{-1}$, New England Nuclear, stock #276). In addition, incubation medium, after incubation with mantle tissue, was analyzed for ^{14}C-glycine by thin layer chromatography (TLC). Resulting spots from TLC analyses were removed from TLC plates and analyzed for radioactivity in liquid scintillation spectrophotometry.

Results: Concentrations of ^{14}C-glycine in the mantle tissue increased with increases in ambient salinity but were generally inversely proportional to increases in ambient concentration of calcium (Figure 3A). Changes in amounts of ^{14}C-glycine incorporated into excised mantle tissue were in many cases curvilinearly related to ambient salinity. The curvilinear pattern of glycine incorporation was also similar to that noted for rates of calcium incorporation in mantle tissue of whole oysters (Sick et al. 1979) and was similar to the pattern observed for the changes in glycine concentrations in mantle tissue in response to increases in ambient calcium (Figure 2).

Although not always proportional nor linearly related, the concentrations of ^{14}C-glycine in the perfusion fluid following incubation with ^{14}C-glycine labelled mantle tissue (Figure 3B), suggested increased amounts of glycine loss in response to selected changes in ambient calcium concentration. For example, concentrations of ^{14}C-glycine were higher at 10 than 2.5 µmoles calcium (g wet wt. PF)$^{-1}$ at all salinities studied, except 30 ppt. Furthermore, many of the changes in ^{14}-C-glycine loss were curvilinearly related to changes in calcium concentration.

Interpretation of Results: Results from experiments A and B concerning amino acid concentrations and fluxes in oyster mantle tissue can be summarized and interpreted as follows:

1. Based on accumulation of calcium from the ambient environment by excised mantle tissue (Figure 1), it is reasonable to implicate calcium flux as a possible "trigger" for stimulating the synthesis and efflux of selected amino acids from mantle tissue (Figures 2 and 3). Furthermore, in agreement with observations by Jodrey (1953) it is plausible that most of the calcium accumulated by mantle tissue is passed through the tissue and made available for deposition into shell.

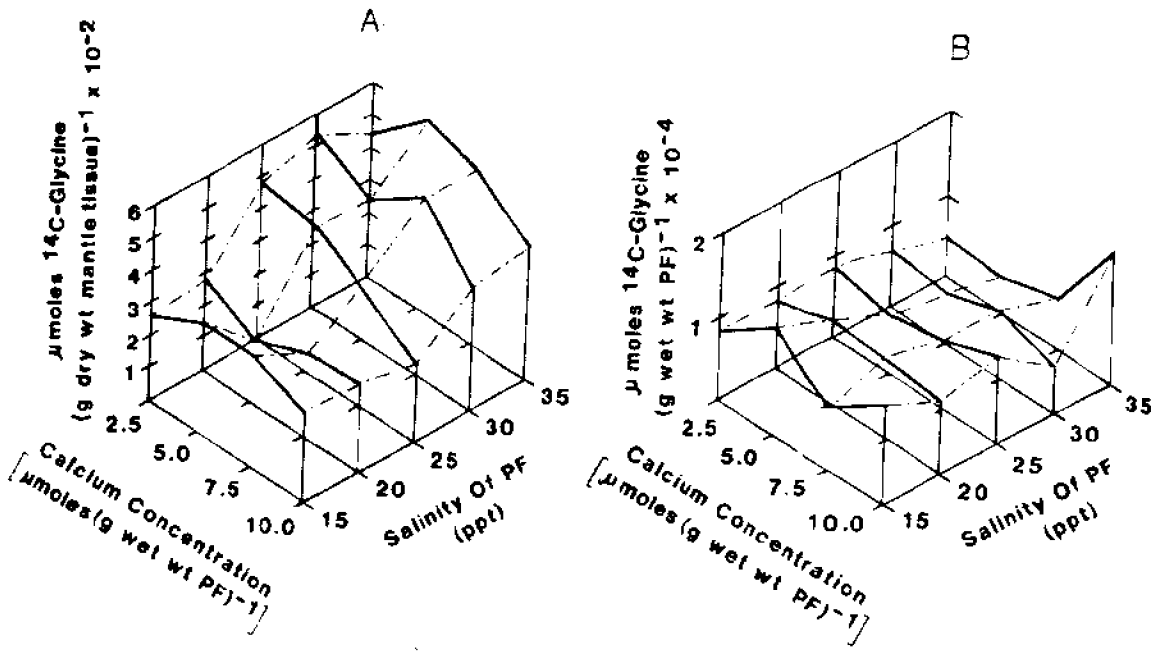

Figure 3. Fluxes of ^{14}C-glycine in mantle tissue (Crassostrea virginica) in response to changes in ambient calcium concentration and salinity. Efflux of ^{14}C-glycine from the ^{14}C-labelled mantle tissue was measured by placing ^{14}C-glycine labelled mantle tissue in non-labelled PF (perfusion fluid) following a 48-hour incubation in ^{14}C-glycine labelled medium and monitoring ^{14}C-glycine concentration in the PF.

2. Concentrations of free glycine, serine, alanine, and glutamic acid in mantle tissue changed in response to changes in ambient concentration of calcium and salinity. Such changes were assumed to occur through normal cellular processes including amino acid synthetic pathways (e.g. amino acids derived from pyruvic acid), and amino acid transamination and deamination.

3. Based on the tendency to lose ^{14}C-labelled glycine from mantle tissue to ambient fluid at some calcium concentrations and salinities, it was assumed that the mantle is capable of secreting free glycine.

4. Excretion of glycine associated with an increase in ambient calcium concentration adds further credibility to the template theory of calcification of a protein matrix as proposed by Weiner and Hood (1975) since glycine is one of the major components of their model.

5. The excretion of glycine from the mantle tissue, in an apparent association with increased concentrations of calcium (Figure 3), also suggested that the origin of shell protein matrix may be from free amino acids excreted from the mantle tissue. Although the origin of the molluscan shell soluble protein matrix has not been determined (reviewed by Wheeler et al. 1981), Young et al. (1977) interpreted an excretion of radioisotopically labelled glycine from excised mantle tissue of the clam, Mercenaria mercenaria as indicative of protein excretion by the mantle. This excreted protein presumably would somehow form the shell organic matrix. Having isolated from ^{14}C-glycine excreted from oyster mantle, our results showed that glycine can be excreted in the free form

and not necessarily as peptides. It is therefore possible that the shell protein matrix is formed from free amino acids that are excreted by the mantle and subsequently synthesized into proteins through enzyme mediation either in the extrapallial fluid or at the shell edge.

PROPOSED MODEL OF SHELL CALCIFICATION

The model of shell biosynthesis depicted in Figure 4 includes traditional concepts of mineralization of an organic matrix, (e.g. Glimcher 1960; Wilbur 1964; Simkiss 1976) but also includes effects of the environment on mantle physiology and metabolism. The model was developed using published information on the role of calcium and calcium carbonate in shell formation (e.g. Weiner and Hood 1975; Simkiss 1976) plus results from original research by the authors concerning release of amino acids from the mantle in response to changes in available calcium in the oyster, Crassostrea virginica (see above).

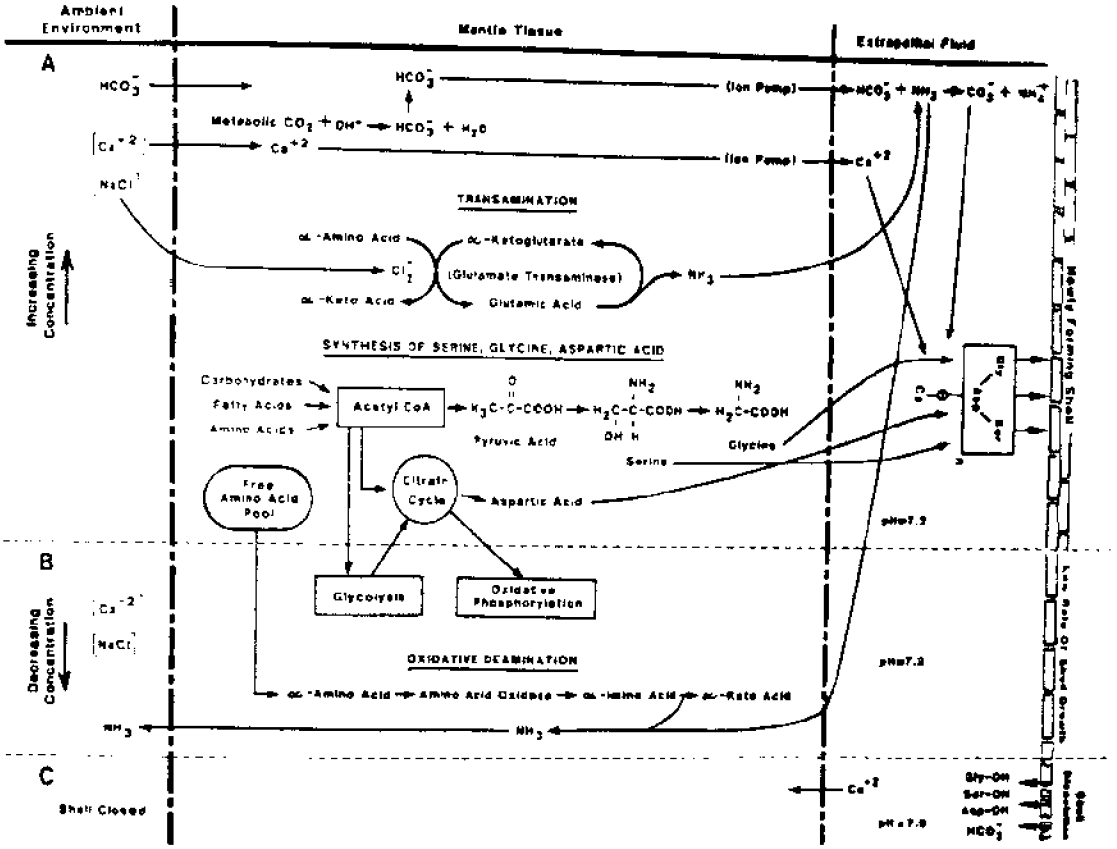

Figure 4. Proposed model describing effects of ambient calcium concentration, salinity and mantle tissue metabolism on shell biosynthesis in marine bivalves. Condition A assumes maximum rates of shell formation in response to increasing ambient salinity and calcium concentration, condition B assumes relatively slow rates of shell synthesis due to decreasing ambient salinity and calcium concentration, and condition C assumes shell dissolution due to the acid pH of the extrapallial fluid during a prolonged period of shell closure.

Calcium fluxes through the mantle tissue as proposed in this model (Figure 4) were taken from a model proposed by Wilbur (1964). In addition, recent information concerning the probable role of cellular calcium (e.g. Schatzmann 1973) and bicarbonate (Keynes 1969) ion pumps in mammalian tissue may also explain the saturated calcium and bicarbonate concentrations in the extrapallial fluid of oysters and therefore have been included in the model. It was assumed in the model that most of the calcium used by the mantle for shell synthesis is obtained from calcium dissolved in seawater rather than from ingested food.

Three environmental and physiological conditions affecting shell biosynthesis (i.e. A, B, and C, Fig. 4) were identified in the proposed model. In condition A increasing or relatively high salinity and ambient calcium concentration, and maximum rates of shell biosynthesis were assumed to be likely (e.g. Sick et al. 1979). The role of the nitrogen metabolism of mantle tissue and its relation to shell formation, as depicted in condition A, was based primarily on results of Sick et al. (1979) and others (see above) and the results of experiments described above. Under condition A, it was assumed that the induction in the mantle of glutamic acid and ammonia production occurs in response to increased ambient calcium concentration and through well studied osmoregulatory mechanisms associated with salinity change and is accomplished through transamination or oxidative deamination processes. It is proposed that the mechanism of amino acid induction is the stimulation of glutamate transaminase activity by the chloride ion. Chloride ion stimulation of glutamate transaminase has been previously observed is osmoregulatory studies in crustaceans (Gilles 1969) and if operable in bivalve mollusks would explain simultaneous induction of glutamate transaminase by increases in both calcium concentration (calcium chloride used for all experiments) and salinity (Figure 2). The proposed transamination processes would generate ammonia, which can readily pass through the mantle epithelium, become a proton acceptor for the bicarbonate radical in the extrapallial fluid, and cause further calcium carbonate formation (Campbell and Speeg 1969; Reddy and Campbell 1972). The ammonium ion formed from the acceptance of a proton by ammonia is not readily removed by diffusion through membranes. However, Carter (1972) has proposed that by carbonic anhydrase mediation, the ammonium ion and the bicarbonate radical could be coverted to ammonia, carbon dioxide, and water, thereby explaining ammonium ion removal from the extrapallial fluid. Ammonia could subsequently be removed by diffusion into mantle tissue and excretion to the ambient environment. The temporary increase in ammonia concentration in the extrapallial fluid resulting from a conversion on ammonium (as proposed by Carter 1972) may inhibit transamination and oxidative deamination reactions in mantle tissue and explain the curvilinear changes observed in mantle tissue amino acid concentrations (Figures 1-3). The "trigger" mechanism for the production of amino acids in mantle tissue in response to increases in calcium concentration and salinity is not known. However, synthesis of glycine and serine was assumed to be via citrate cycle intermediates such as pyruvate and aspartic acid (as shown in Figure 4).

It was also assumed, in the proposed model, that calcium will react with the carboxyl end of selected amino acids (either free amino acids or polypeptides) and then will complex with carbonate radicals. Reaction of calcium with the carboxyl component of amino acids is

possible in condition A (Figure 4) since at pH 7.2, typical of conditions in the extrapallial fluid (e.g. Lutz and Roads 1977; Gordon and Carriker 1978), amino acids of peptides in the extrapallial fluid would have negatively charged carboxyl groups. Furthermore, because the concentrations of mantle serine and glycine, two of the three repeating polymeric units of the Weiner and Hood (1975) template hypothesis was incorporated into the present model (Figure 4).

In condition B, (Figure 4) it was assumed that the ambient calcium concentration and salinity are either decreasing or are low relative to those necessary for normal biological activity of the mantle tissue (Sick et al. 1979). It was hypothesized that free amino acid concentrations in the mantle tissue and the rate of amino acid excretion into the extrapallial fluid decrease when bivalves are exposed to low calcium concentrations and salinity (based on net results as summarized in Figures 2 and 3). Since amino acid concentrations decreased in the mantle during conditions of low calcium concentration and salinity (Figure 2,) pathways accounting for serine, glycine, and aspartic acid synthesis from acetyl CoA in condition A are probably shunted predominately through glycolysis and oxidative phosphorylation in condition B. By virtue of a decrease in the chloride ion concentration, the activity of glutamate transaminase would probably decrease with a corresponding decrease in ammonia production. While some ammonia production may take place via oxidative deamination of cellular free amino acids, diffusion gradients (Simkiss 1976) would suggest absorption of ammonia from the extrapallial fluid into the mantle and excretion to the ambient medium from the mantle. Therefore, with the primary source of proton acceptors decreased, it is likely that the formation of carbonate in the extrapallial fluid would also decrease. The net effect of processes assumed to exist in condition B would be a slow rate of shell formation relative to condition A.

Although the proposed model is applicable to marine mollusks, metabolic and chemical phenomena proposed may also apply to freshwater mollusks. In a freshwater environment, stimulation of glutamate transaminase activity and eventual production of ammonia may still result from the chloride ion as observed in marine crustaceans (Gilles 1969). However, the primary source of chloride ion and calcium for shell formation in freshwater mollusks, is probably largely from metabolic production rather than from the ambient environment (Greenaway 1971). Shell formation in freshwater mollusks, therefore, may proceed through similar mechanisms as proposed here (Figure 4), but undoubtedly requires greater amounts of energy and may be more dependent on food than on metabolic production for organic components of the shell (Van Der Borght and Van Puymbroeck 1966; Greenaway 1971).

In condition C, the environment would not directly affect metabolic mediation of shell formation due to complete shell closure. Shell closure and eventual build up of metabolic CO_2 renders the extrapallial fluid acidic (Gordon and Carriker 1978). Under these conditions, the metabolic status of the mantle tissue is presumed to be similar to that of condition B. This condition together with an acidic extrapallial environment would result in some dissolution of shell material (Gordon and Carriker 1978). Condition C undoubtedly exists intermittently with conditions A or B.

CONCLUSIONS

Verification of chemical and biological mechanisms responsible for synthesis of molluscan shell, as proposed (Figure 4) can only be achieved by experimentally testing the many assumptions necessary for constructing the model. Specific investigations that are necessary for assessing the validity of this model should include 1) verifying the existence of calcium and bicarbonate ion pumps in molluscan mantle tissue, 2) determining the role that amino acids found in the extrapallial fluid may have in complexing calcium, 3) determining the sources (including metabolic sources) of amino acids measured in the extrapallial fluid, 4) determining the mechanism of calcium carbonate complexation in extrapallial fluid, including the possible role of metabolic ammonia as a proton acceptor and 5) examining the possibility that the chloride ion can stimulate glutamate transaminase activity in molluscan mantle tissue. Elucidation of these mechanisms would not only contribute to a general understanding of molluscan shell biosynthesis, but would further an understanding and appreciation for the interdependence between metabolic processes, chemistry of the ambient environment, and shell growth and maintenance.

Regardless of the specific mechanisms regulating shell formation, the fact that shell synthesis is integrally dependent on the chemical composition of the ambient environment and probably influenced by the nutritional quality of assimilated food, suggests that anthropogenic manipulation of shell formation is possible. Such manipulation, assuming tissue growth is commensurate with shell growth, may be advantageous for bivalve culture. Oyster spat may be seeded in natural environments selected for specific salinity, mineral-metal concentrations, and food availability suitable for rapid shell growth. In intensive mariculture systems, the ability to regulate some of the physical and biological components included in the proposed model (including quality and quantity of food), may allow control of shell formation in cultured mollusks.

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QUESTIONS AND ANSWERS

ARSENAULT (Halifax): I've been working on an energy loss, high resolution electromicroscope and I'm finding that during mineralization of the exoskeleton in lobsters, there's an intimate relationship between calcium, phosphorus and sulphur. So when you refer to calcification can you really neglect these other elements? Also, there's probably a whole gamut of other elements that haven't been looked at, but have you looked at phosphorus for example, not only in exoskeleton, but also in bone tissue culture? My co-workers and myself have looked at this and could give you some comments to add to your insights and observed relationship between calcification involving calcium and other elements.

SICK: Yes, that's an excellent question and something that is great to bring up at this time. The only experimental evidence that I can sight is the observation that concentrations of phosphorus change with observed calcium fluxes and therefore there seems to be a relation between phosphorous concentration of the extrapallial fluid and the story that I've tried to depict here this morning. Now what it actually means and how directly it's related to calcification I really can't say. My thoughts would be that in bone material, the calcium phosphate to phosphorous ratios and magnesium ratios are going to be very important as we know from classic mammalian physiology. I think these ratios are going to be less important in mineralization in a molluscan shell which is quite different in many respects from bone, bone being a tissue. You can probably use such ratios for formulating specific questions but not for generalizing. My second point is that with regard to sulphur,

sulphur binding, particularly in cross linkages of protein, is very important in exoskeleton or shell. Certainly sulphur is as important an element in calcification as phosphorus and other minerals, my point is that it may be quite different in exoskeleton synthesis and certainly it has a different roll in bone formation. I would also like to add that we really need to consider trace metals too because they are going to play a large role in mineralization and certainly are readily stored in the shell. I feel that they probably have an active role over and above storage.

ARSENAULT: Some of my findings indicate that phosphorus and sulfur precede the presence of calcium in mineralization and with our electromicroscope technique we can also quantitate how much of whatever element is present. At this point it seems that there is even more phosphorus and sulphur relative to calcium.

SICK: This is an exoskeleton now, not molluscan shell?

ARSENAULT: Right. Would that be similar again or do you think it would possibly be different than the shell in the mollusks?

LOWELL: Well, in the organic matrix of crustacean exoskeleton the glucopolysaccharides and glucosamines are cross linked at least partially by sulfhydryl cross linking within a protein matrix. We've seen these in both vertebrate and invertebrate systems and certainly they have been studied in crustacean exoskeleton. We've seen similar protein linked by sulfhydral terminal groups in molluscan shell. And so the observation at least that sulfur precedes calcium in exoskeleton formation is probably consistent with out understanding of sclerotization in the invertebrates.

ARSENAULT: How comparable is the molluscan shell to mammalian bone?

LOWELL: Well, as I said, I think if you ask specific questions concerning bone versus shell such as, kinetics of calcium flux and the role of organic matter in mineralization, maybe there are some analogues, but I think when you go beyond that there are more differences than similarities. Bone is an active specialized tissue, and, unlike the biologically inert molluscan shell, live tissue. Building an inert armorment, such as shell obviously is quite different from the continuing life support process necessary for maintenance of living tissue.

SUMMARY AND FUTURE RESEARCH NEEDS

SUMMARY AND PROSPECTS FOR THE FUTURE CRUSTACEA

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Being fortunate enough to be present at both the First and now the Second International Aquaculture Nutrition Conference, it is impossible not to feel a sense of pride in being associated with these two milestones. In reflecting on the two meetings I was also impressed by how productive the last five years have been, particularly for those working with crustaceans. Indeed, as a generalization, it can be concluded that the nutritional requirements of crustaceans are now rather well known. Based on this general understanding it is probably now possible to prepare diets which will meet the vital requirements of most arthropods. Furthermore, as emphasized by Cowey and Tacon, as well as by Dadd, these vital requirements are not strikingly different from those of vertebrates, the apparent exception being the need for cholesterol by arthropods. This would indicate the crustacean nutritionist may borrow rather freely from the greater store of knowledge relating to vertebrates in order to rapidly refine our understanding of crustacean dietary needs.

While this progress in understanding crustacean nutrition has been significant, it will have little immediate impact on commercial aquaculture outside of further reinforcing the vision of its eventual potential. This is because the commercial aquaculturist must focus on specifics rather than generalities. Consequently, while the past five years have focused on general mechanisms, it can be anticipated that future efforts will be aimed at tinkering with these mechanisms to meet production needs for specific species. This has already obviously been done to a great extent for shrimp (Penaeus japonicus) production in Japan. Indeed, as pointed out by Kanazawa, the growout diet of Penaeus japonicus has been refined to such an extent that nutritional studies on other life stages now take precedent.

In focusing on the specifics, one of the most pressing needs is in the area of feeding and digestion. Again the information available on this basic subject area, aptly reviewed by Gibson, is surprisingly narrow in scope. The lack of detailed information of feeding is particularly troublesome in connection with the overall problem of the leaching of water-soluble nutrients. The requirement for and appropriate dietary levels of individual water-soluble vitamins are very

poorly defined for crustaceans. While many of these nutrients may be supplied complexed with feedstuffs, others such as ascorbic acid which are highly labile, must be added to the diet and are subject to severe leaching tones when the diet is not consumed immediately. Certainly these losses could be reduced if feeding was optimized. Intriguingly, it appears that most commercial culturists feed at dusk while experimental culturists feed in the morning upon arriving at the laboratory. Experimental culturists also tend to overfeed their animals so as to exclude the possible impact of feed limitation. As a consequence little attention has been paid to the imput cost of increasing growth and possible wastages occurring in the processing of food by the animal.

Such techniques as microencapsulation described by Langdon or microbinding, which Kanazawa spoke of, are promising and will be particularly important in larval nutrition studies where the particle size must be reduced, greatly increasing the surface to volume ratio and consequently leaching losses. The techniques are, however, still experimental and their widespread use in aquaculture should not be assumed in the near future. Live food items such as Artemia nauplii will remain as an essential element for larval culture. Extensive information, which was summarized by Simpson and coauthors, is now available on Artemia and should be used in reducing the cost of larval culture. This could be achieved by using the live food organisms strictly as a supplement to formulated diets designed to provide the bulk nutrients.

The development of appropriate delivery techniques for water-soluble nutrients would also make the determination of optimum amino acid profiles relatively easy. Although Deshimaru has demonstrated a possible approach to this problem through the use of various protein sources differing in their amino acid composition, the ability to reliably add to the diet single amino acids would be an exceedingly useful tool for experimental aquaculture. Until the optimum balance of amino acids is known, little progress can be made in sparing expensive proteins as discussed by Capuzzo.

There is no doubt of the importance of lipids to crustaceans. While again a broad, general outline is available, provided by Castell and Teshima, the specifics are missing. It is probably that these specifics, both in terms of whether or not an animal can synthesize a particular nutrient and, of equal importance, the rate at which the nutrient is synthesized, will be important to the commercial culturist. The biosynthetic rate and needs of an animal combined will determine whether or not the dietary source of this nutrient will serve to spare the requirements and therefore stimulate growth.

I personally am looking forward to the point in the near future when various artificial fatty acid mixtures are readily available which will allow the exploration of possible interactions between fatty acids and lipid soluble vitamins. While there is no doubt that animal nutrition in general has a certain uniformity it cannot be assumed that the highly specialized regulatory systems, which have been developed around some of the fat-soluble vitamins in mammals, are present in invertebrates. This research will provide nutritionists an opportunity to focus on and devleop an appreciation for the evolution of these hormonal-like systems as well as defining the primary role of these lipid-soluble vitamins in the physiology of animals. This particular

aspect of comparative nutrition, however, may be of little interest to the commercial aquaculturists as the extensive use of marine fish oils in crustacean diets generally will provide these factors in sufficient amounts. While a host of specific answers are yet to be determined, this does not imply that the past five years have been wasted. Certainly there is a correlation with the nutritional data base of a group of animals and the success of animal production units. It is the broad outline summarized at this conference which will provide the interpretive framework for the details. It is, however, the details or the differences which are important to commercial interests as it is these specifics which will make the difference between success and failure for an aquaculture production industry.

SUMMARY AND PROSPECTS FOR THE FUTURE

MOLLUSCS

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It is clear from the foregoing papers that marine molluscan nutrition lags far behind that of crustacean nutrition. With the exception of some gastropod species, here discussed by Carefoot, there is a lack of satisfactory defined artificial diets for marine molluscs. This is especially true for bivalves, where no artificial diets have been described that will support growth that is comparable to that obtained with algal diets. The problems of food presentation and the associated difficulties of diet stability and bacterial activity are the principal reasons why marine bivalve nutrition is in such an early stage of development.

Much is known about the gross proximate biochemical composition, amino acid, carbohydrate, fatty acid, and sterol compositions of algae, as has been reported here by Epifanio and by Webb and Chu. In general, differences in the biochemical composition of algae do not explain why some algal species are better foods than others; according to Epifanio, the food quality of algal diets is best explained by the digestibility of the algal cells. An exception to this generalization is the alga Dunaliella tertiolecta which does not contain long chain polyunsaturated $\omega 3$ fatty acids (20:5ω3 and 22:6ω3) which appear to be essential for Crassostrea gigas spat. Also, Teshima has pointed out that it is probable that the limited ability of bivalves to synthesize sterols will result in some degree of dependence on a dietary supply of essential sterols for maximum growth. As yet, the sterol composition of algae has not been linked to algal food quality.

The algal cell can be viewed as a capsule containing essential dietary nutrients. Techniques in developing satisfactory artificial capsules containing all the necessary dietary constituents are being explored and Langdon has described some types that may be suitable. As an alternative method to microencapsulation, the technique of binding high molecular weight, water soluble substances, such as proteins and carbohydrates in micro-gel particles has also been described by Kanazawa for Penaeus japonicus larvae and by Langdon for Crassostrea virginica spat. Such techniques not only enable the researcher to deliver nutrients to the animal, but also improve dietary stability and reduce problems of bacterial contamination.

Ultimately, the nutritional requirements of bivalves for essential nutrients will only be determined by culturing the animal under axenic conditions on defined diets. It is now possible to readily obtain axenic oyster larvae, and this represents a major step in the right direction. As Stephens has pointed out, this technique will also be useful in the study of the role of dissolved organic material in the nutrition of marine invertebrate larvae. In combination with sensitive high pressure liquid chromatography, we can expect very significant contributions to be made in our understanding of this subject. This is potentially one of the most exciting areas of molluscan research discussed at the conference.

Another aspect of bivalve nutrition that has great potential importance in the development of non-algal diets, is the influence of particulate inorganic matter (PIM) on bivalve feeding growth. As Newell has pointed out, little is understood of the mechanism(s) whereby the addition of PIM to algal diets improves growth. Most commercial operations involved in the rearing of bivalves go to great lengths in removing PIM from seawater, and so this practice may well need to be reassessed in the light of recent findings.

Whether the bivalves' food is algae or an artificial diet, it is necessary to ensure that it is efficiently utilized for growth. Reid and Newell have discussed the value of studying the energy balance of bivalves under cultivation so as to ensure that the calorific value of the available food is sufficient to meet the requirements of the cultured animals for maximum growth. Of particular relevance to this subject are the reports that genetic heterozygosity of Crassostrea virginica is correlated with a lower basal metabolic rate, which results in more energy being available for growth. The importance of genetics in determing the growth of bivalves is only now being fully appreciated and future studies will lead to a better understanding.

Genetic expression may be modified by manipulation of the organism' endocrine system. Tombes has indicated the potential usefulness of this approach not only for improving somatic growth and development, but also in determining expression of sex of the cultured animals. Tombes notes our limited knowledge of molluscan endocrine systems and the need for more research if aquaculturists are to benefit from this method of control to the same extent as agriculturists.

This conference has underlined the importance of understanding the biochemical and physiological basis of shellfish nutrition in order to evaluate the results of present studies and plan future research. Molluscan nutrition lags far behind that of crustacea, insects and fish, and yet many helpful lessons can be learned from studies with these animals which, hopefully, will soon be applied to molluscs.

ABSTRACTS OF POSTERS

ABSTRACTS OF POSTERS

CRUSTACEANS

MINERAL DEPOSITION IN THE EXOSKELETON OF THE LOBSTER, HOMARUS AMERICANUS

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The exoskeleton of the lobster (Homarus americanus) was the subject for the detection of calcium, phosphorus, and sulphur by high-resolution microanalysis (Adamson-Sharpe and Ottensmeyer, 1981). This technique utilizing electron energy loss spectroscopy and electron spectroscopic imaging enables the visualization of these elements in their relative distribution, one to the other, and in relation to the exoskeletal matrix in which they occur. To date, the premolt endocuticle of the exoskeleton has been most extensively studied and is presented in a digitized-computerized format. In the premolt endocuticle, the relative distribution of the three principal mineral elements were as follows: 1) phosphorus occurred primarily in concentrated loci, 2) calcium was present in a less concentrated manner and was generally more randomly distributed than phosphorus, 3) sulphur, on the other hand, presented the most scattered distribution.

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EFFECT OF FEEDING A SYNTHETIC DIET ON WEIGHT GAIN AND MEAT FLAVOR IN LOBSTERS

(Homarus americanus - Milne Edwards)
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The objective of this study was to compare weight gain and me flavor of lobsters fed synthetic diet versus a diet of herring. Sal herring scrap is the conventional ration fed to lobsters held in hi density confinement in lobster pounds. The formulated synthetic di was composed of fish meal (30%), brewer's yeast (10%), alfalfa (10 kelp meal (3%) and wheat flour (47%).

Before the feeding trial began and after 48 days on the diet, t lobsters were weighed both suspended in water (SW) and in air (AW). S mean changes in wet weight were 16.0g (SW), 22.4 (AW), and 6.8 (SW 11.7g (AW), for lobsters fed the formulated diet and the herring di respectively. The mean change in weight for lobsters fed the formulat diet was significantly ($p<.01$) greater for both weight values.

For evaluation of flavor, live lobsters were cooked for 15 minut in a steam-jacketed kettle containing boiling 3% sodium chloride bri which was freshly prepared for each of four replications. Four lobste from each of the groups which had been fed on herring scraps or on t synthetic diet and eight lobsters harvested from their natural habita were cooked at a time. The meats from the tail sections were cut in pieces, coded, and presented in a randomized complete block design, wi four replications, to a sensory panel of 17 members. The panelists, w were experienced in assessing flavor qualities of foods, were asked compare the flavor of each sample with that of a labele freshly-harvested, reference standard which also was included as a cod control sample.

Scores of +3 (better than standard, large difference) to a (poorer than standard, large difference) were assigned to the 7-poi rating scale. The data were analyzed by the variance method using t treatment x judge interaction for the error term to test for significant treatment F ratio. The test for least significance was us to examine differences between the means of the two groups of f lobsters and the coded control. The flavor of lobsters which were f the synthetic diet (mean = -0.10) was rated equal to that of the cod control sample (mean = -0.06). Meat from lobsters which had received diet of herring scraps, however, was judged slightly poorer in flav (mean = -0.65) than both the freshly harvested control and the synthet diet fed lobsters. These differences were significant at the 1% lev of detection.

HISTOLOGICAL CHANGES ASSOCIATED WITH DEVELOPMENT OF THE HEPATOPANCREAS IN LARVAE OF THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*)

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This study provides details of morphological changes in the hepatopancreas of developing lobster larvae using light microscopy. Laboratory hatched larvae were raised communally and fed frozen adult *Artemia* ad lib. Hepatopancreatic tissue from embryos (obtained three days before the siblings hatched) and from intermolt larvae (molt state C) was fixed in Dietrich's fixative, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Yolk was intercalated among the hepatopancreatic tubules of the embryos and the first stage larvae but was not observed in the older stages. During development, the complexity of the hepatopancreas increased. The lumen of the tubules was small in the embryo, large in the first stage, and then gradually decreased in the older larval stages. Fourth stage larvae had the smallest diameter lumen but the thickest cells making up the tubule wall.

The hepatopancreas is composed of four cell types: embryonic (E-cells), fibrillar (F-cells), secretory (B-cells), and resorptive (R-cells). All cell types were found in each of the four larval stages but only E- and R-cells were observed in the embryos examined. F-cells may in fact be present in late embryos, perhaps in low numbers so that none were seen in this series of sections or they may develop later on. The absence of B-cells can be explained by assuming that they develop from F-cells after the stimulation of the feeding process: embryos have not yet fed. The R-cells of late embryos and of larval stages one, two, and three contained only a few vacuoles rather than the typically large number of lipid vacuoles. But these cells were morphologically distinct from F-cells which had a larger nucleus and a fibrous appearance. Only in stage four, which represents the transition from a planktonic existence to a benthic habit, were the R-cells filled with lipid reserves. The brush border was observed in the four larval stages although it was not very distinct in stage one. The presence of R-cells, generally considered to be resorptive or storage cells, in the late embryos is puzzling unless they perform some other function as well.

DO JUVENILE LOBSTERS REQUIRE DIETARY ASCORBIC ACID?

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Feeding trials were carried out with juvenile lobster, Homarus americanus, to determine their dietary ascorbic acid requirement. Lobsters were fed on semi-purified diets containing 0, 0.6, 1.2, 2.4, and 12.0g of ascorbic acid per kg dry diet for a period of 12 weeks. At termination, hepatopancreatic ascorbic acid levels were determined. (Zannoni et al. 1974)

The animals fed the ascorbic acid deficient diet showed no major differences in growth and mortality compared with those on supplemented diets, nor did they exhibit any deficiency symptoms. No significant differences were found in hepatopancreatic ascorbic acid levels of animals grown on the various diets although the level in lobsters fed the ascorbic acid deficient diet was slightly reduced. The conclusion is, therefore, that ascorbic acid is not essential in the diet of juvenile lobster.

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DIETARY COPPER REQUIREMENT AND THE POSSIBLE ROLE OF SILVER IN LOBSTER (HOMARUS AMERICANUS)

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An earlier report (Chou and Uthe 1978) showed that levels of silver (Ag) and copper (Cu) in tissues of American lobster (Homarus americanus) and rock crab (Cancer irroratus) were highly intercorrelated. Since no role for silver in nutrition has been established, we investigated the interactive effects of dietary Ag and Cu upon growth, survival and tissue levels of Cu and Ag in the juvenile American lobster.

Juvenile lobsters fed with varying semi-purified diets (Trider and Castell 1980) containing 4 levels of Cu (0, 16.2, 40.5 and 81.0 mg/kg) and three levels of Ag (0, 0.54, and 5.4 mg/kg) for 17 weeks were utilized for this study. Evaluation of the interactive effect was based on muscle tissue and hepatopancreatic metallic bioconcentrations of the supplemented metals. In addition, rate of growth, survival and hepatopancreatic somatic index (HSI) were used for the evaluation of dietary metal requirement.

The following conclusions were based on the results and observations of this study. 1) Lobsters under the present experimental conditions do not appear to have an essential requirement for a dietary Cu supplement. 2) Lobsters appear to be able to absorb sufficient copper from sea water for normal growth and survival. 3) Addition of 16.2 mg/kg Cu to the diet results in a slight improvement in growth. 4) Addition of higher than 16.2 mg/kg Cu is probably toxic as indicated by reduced growth, survival and HSI. 5) Inclusion of 0.54 mg/kg Ag in the diet reduces survival and growth in the absence of dietary Cu while improving survival, growth and HSI in the presence of 16.2 mg/kg or higher levels of dietary Cu. 6) A higher level of Ag (5.37 mg/kg) in the diets increases mortality and reduces growth, suggesting toxicity of the metal ion at this level of supplement. 7) Silver may be physiologically involved in the detoxification of excess dietary Cu as there is a direct correlation between hepatopancreatic Ag levels and excess dietary Cu levels. 8) The physiological ratio of Ag to Cu is approximately 1:30.

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BIOENERGETICS OF ELECTROLYTE METABOLISM IN LARVAL CRUSTACEANS: SALINE DEPENDENT CO FIXATION PATHWAY UTILIZED IN FORMATION OF AMINO ACIDS

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The energetic requirements for electrolyte metabolism in aquatic animals is based upon the experimental findings that every known example of net extrusion or accumulation of any substance against its concentration gradient across the cell plasma membrane involves the coupling of that movement to the electrochemical gradient of sodium created by a sodium - and potassium - dependent ATPase ($Na^+ + K^+$-ATPase). The brine shrimp (Artemia salina) is a crustacean that can adapt to salinities ranging from 10 ppt to crystallizing brine. Survival in these media is possible since in both adults and nauplii, the solutes in the hemolymph can be maintained at much lower concentrations and different compositions than those of the external environment. The hypo-osmotic state is maintained by active transepithelial ion transport and is dependent upon the cationic enzyme, $Na^+ + K^+$-ATPase. When external salinities increase there is a greater demand being placed upon the osmoregulatory mechanism, and there must be a concomitant increase in demand for chemical energy.

Noting experimental evidence suggesting that much of the needed increase in chemical energy does not come from aerobic metabolism or oxidative phosphorylation, Conte (1980) has proposed that a C-4 dicarboxylic acid pathway serves as a facultative anaerobic shunt in nauplii to help meet the energy demand created by enhanced levels of ion transport at higher salinities. The C-4 dicarboxylic acid pathway involves the fixation of carbon dioxide with phosphenolypyruvate to form oxaloacetate. The resulting oxaloacetate can be transferred into aspartate by transamination or malate by reduction.

The present study was undertaken to determine if the incorporation of carbon dioxide into free amino acids in nauplii is influenced by environmental salinity. Both the specific activity of the amino acids and the total concentration of free amino acids were measured in nauplii incubated at 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 M sterilized NaCl. In addition, the concentrations of free amino acids excreted into the acclimation media were measured. Results from nauplii acclimated in these different salines showed that incorporation of $^{14}CO_2$ into free amino acids was greatly stimulated in the higher salinities. The ^{14}C label was found highest in aspartic acid, followed by serine, glutamic acid, proline and alanine. Hypersalinity also caused an increase in concentrations of unlabeled free amino acids in the naupliar cytosol, an increase in the amount of ammonia excreted into the medium and an increase in aerobic glycolysis.

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CALCIUM-PHOSPHORUS REQUIREMENT OF PENAEID SHRIMP

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In France, a molt death syndrome has been frequently observed in intensive cultured shrimps. A high death rate at molting and the soft shell of affected shrimps indicated a possible inbalance in the mineral content of the artificial diet used. A digestibility test conducted with an artificial diet gave a negative value for calcium (-80%). This result indicated a possible lack of available calcium in the diet.

In addition to the mineral content of the constituent materials, a mineral supplement, at 4 to 5%, has been found to be necessary for the artificial diet in order to reduce shrimp mortality. The diet has a calcium-phosphorus ratio of 4:1. When this ratio falls below 2, the survival rate of the shrimp is reduced.

However, in another experiment with an artificial diet, where the calcium-phosphorus ratio was raised from 2.5 to 4.4 growth rate did not increase with the higher Ca to P ratio. Highest growth rate was obtained with a ratio of 2.5.

Using a purified diet based on casein, a Ca:P ratio of 2:1 and 6:1 were both found to be favorable for shrimp growth.

The source of calcium in the mineral supplement used in the above diets, consisted mainly of $CaCO_3$ and $CaHPO_4$. Better growth and survival rate could be obtained using calcium from organic sources, such as calcium lactate or even ground shell instead of calcium of mineral

PROTEIN COMPONENTS OF HOMARUS AMERICANUS HEMOLYMPH

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Hemolymph serum samples from newly molted (stage A) and intermolt (stage C_1-C_3) adult lobsters were examined by standard polyacrylamide gel electrophoresis methods for serum. Serum from stage C_1-C_3 lobsters was characterized by the presence of three major protein components as compared to four major components found in serum from stage A lobsters. The additional fourth protein component was found to decrease as a percentage of total protein between 0-72 hours after molt. All protein components were assumed to be hemocyanin, since the literature report 97% of serum protein in the American lobster to be hemocyanin. This assumption was confirmed by detection of copper in each of the protein bands from both stage A and stage C_1-C_3 lobsters. The presence of an additional hemocyanin band in stage A lobsters may reflect structural changes in the hemocyanin as a result of fluctuations in serum Ca^{2+} concentration during ecdysis.

AN ASSESSMENT OF CHROMIC OXIDE AS A DIGESTIBILITY MARKER IN THE AMERICAN LOBSTER

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This research investigated the effect of varying dietary levels of chromic oxide (Cr_2O_3), an inert marker for digestibility studies, on the voluntary feed intake of the adult American lobster.

Cr_2O_3 was mixed into the basal ration at levels of 0.01, 0.05, 0.10, and 1.00% and the voluntary intake of these feeds by adult lobsters was compared to that of the basal ration without Cr_2O_3. A replicated 5 X 5 Latin Square experimental design was used and the data analyzed by ANOVA.

Although the highest (1.00%) level of Cr_2O_3 in the feed slightly depressed the voluntary intake, the difference between treatments was insignificant. Cr_2O_3 does not depress voluntary feed intake at levels up to 1.00% in the diet. It was observed that with the highest levels of Cr_2O_3, where the pigment was visible to the eye, the pigment was not homogeneously mixed in the fecal strand. The Cr_2O_3 was selectively moved through the stomach before the more digestible food items. Therefore, the Cr_2O_3 was passed in the feces first, followed by the waste products left from digestion. This would lead one to falsely estimate digestibility based on the Cr_2O_3 concentration in the feces. On this basis, the user of Cr_2O_3 as in indirect digestibility marker is not adequate in the American lobster and gravimetric techniques on total fecal collections should be used.

AMPHIPODS AS A POTENTIAL DIET FOR JUVENILE LOBSTERS, *HOMARUS AMERICANUS*

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Amphipods are suggested as an alternative to brine shrimp for larval and post-larval lobster feeding. For the present feeding trial, the amphipod *Gammarus oceanicus* (Segerstrale) was chosen since it is an abundant microscopic animal found on sheltered and semi-exposed beaches between the Gulf of Maine and Newfoundland (Steele 1976). Amphipods

were collected at low tide from tidal flats at Hancock and Lamoine, Maine and fed to post-larval lobsters, obtained from the Department of Fisheries and Oceans, St. Andrews, N. B.

Post larval lobsters, stages VI to VIII were randomly assigned to one of three diets. In Experiment 1, diets of frozen brine shrimp and live amphipods were fed and in Experiment 2, a live amphipod diet and a compound diet were compared. Lobsters were fed ad libitum and uneaten feed was removed daily. The trials began in late July and ran for 96 and 117 days respectively. The lobsters were maintained at a field station at Hancock, Maine in ambient sea water at $8^{o}C$ - $13^{o}C$.

There were no significant differences in mean weight gains in Experiment I for lobsters fed frozen brine shrimp, 160 mg (n=13) and live amphipods, 210 mg (n=15) or in Experiment II for lobsters fed live amphipods, 273 mg (n = 15) and a compound diet, 206 mg (n=18). Survival was 100% for all diets, except for the compounded diet where mortality was significantly higher at 33% (P,0.01) with all mortalities occurring in the 5th week of the trial. The difference in the mean score for exoskeletal pigmentation of lobsters fed the 3 diets, as judged by a panel of 11 on a scale of 1-5, was significantly different at P,0.01, using Duncan's Multiple Range Test. Those lobsters fed the compound diet had a grey colored exoskeleton, and scored 1.54 while those fed live amphipods had a deep red, wild-type pigmentation, scoring 4.51. The Artemia fed lobsters were intermediate in color and scored 2.64.

There are a number of advantages in feeding a live, algal detritus feeder, such as Gammarus oceanicus, to lobster. There is no leaching of water soluble nutrients, and particulate organic material and fouling organisms, which may normally accumulate in a holding container and impede water flow, are consumed by the amphipods. The amphipod's exoskeleton may form an important source of calcium and phosphorus for freshly molted lobster and the amphipods provide a rich supply of carotenoids, including B-carotene in the form of undigested algal material in their intestine. As the feeding experiments indicate amphipods are comparatively nutritious, they would appear to represent an abundant untapped alternative feed source for the culture of juvenile lobsters.

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BIOCHEMICAL CHANGES ASSOCIATED WITH LARVAL DEVELOPMENT IN THE LOBSTER, *HOMARUS AMERICANUS*

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The aim of this study was to correlate changes in major biochemical constituents with larval development. Lobsters, hatched in the lab from captured egg-bearing females, were held at 21 $\pm 1^{\circ}C$ and fed frozen adult *Artemia*. The larvae were molt-staged by microscopic examinations of various spines of the tail region. Classification was based, with modifications, on the criteria of Aiken (1980); with the following stages being defined: A, B, C, D_0, D^{I}_{1}, D^{II}_{1} D^{III}_{1}, D_2, D_3

Dry weight was measured after rinsing with distilled water and lyophilization. Ash was weighed after a $475^{\circ}C$, 21 hour exposure. Protein and carbohydrate were colorimetrically determined with the BioRad and phenol-sulfuric acid assays respectively. The BioRad assay was modified by prior sample treatment with 0.1 N NaOH at $80^{\circ}C$ for 30 minutes followed by neutralization with HCl. Chitin was determined from the lipid extraction residue after successive 2% HCl and 1% NaOH treatments. Lipid analyses are currently underway.

The dry weight, ash, and carbohydrate values were all correlated with the molting cycle. Minimum values for percent dry weight (13-16%) were observed immediately after hatching and molting, rising to a maximum value (19-21%) prior to each molt.

Sudden increases were noted in percent ash after hatching and at each molt. Percent ash was maximum (26-34%) during intermolt, declining to a minimum (15-20%) prior to each molt. Gross ash values also reflected this pattern, indicating an actual loss of ash prior to each molt. Such a pattern may be homologous to calcium resorption prior to molting that occurs in juveniles and adults.

Carbohydrate levels, which were minimal (4% ashfree) during intermolt, rose to a maximum (6-7% ashfree) just prior to the molt and then declined after the molt. This may indicate the use of carbohydrate as an energy substrate during the molt recovery period.

Gross protein values showed a general rise through development which did not correlate with the molt cycle, though the greatest rates of increase appeared to occur during intermolt. On a percent basis the protein levels dropped with development from a high of 72-77% (ashfree) to a low of 40% (ashfree). This latter value appears low and may indicate an interference with the assay method.

Gross chitin levels also increased with development but did not show strong trends with the molting cycle. Percent values showed a minimum immediately after hatching which quickly rose and then remained somewhat level (8-11% ashfree) until the fourth stage when an increase became evident (15% ashfree).

These preliminary results indicate that changes in biochemical constituents of the larval stages of the American lobster are related to

both molting and developmental sequences.

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INSULIN-LIKE PEPTIDES IN THE LOBSTER HOMARUS AMERICANUS

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ABSTRACT

Peptides in Homarus americanus bind specifically to bovine insulin antibodies. Hepatopancreas, gut, and hemolymph extracts contained insulin immunoreactivity concentrations of 67.5, 14.0 and 11.0 ng, respectively in one 700 g lobster. No insulin immunoreactivity (IRI) was detected in whole eyestalk homogenates. The highest immunoreactivity was present in hepatopancreas eluate in chromatographic fractions with the same elution volume as the bovine insulin standard.

Hepatopancreas extract significantly increased the incorporation of ^{14}C-glucose into lobster muscle glycogen in vitro. Glycogen deposition was significantly increased in the same chromatographic fractions of hepatopancreas extract that had the greatest insulin immunoreactivity. Hemolymph extract also increased the rate of glycogenesis in muscle tissue but no increase was observed from the addition of gut extracts to the bioassay.

Starved lobsters contained insulin immunoreactivity ranging from 3.4 ± 0.6 to 7.2 ± 0.5 μU ml^{-1} hemolymph. Experimental results indicate that the hemolymph insulin immunoreactive peptides have no glucostatic function. An increase in exogenous glucose in the hemolymph did not stimulate secretion of IRI into the hemolymph and injection of hepatopancreas extract did not increase the rate of glucose removal from the hemolymph.

SURVIVAL AND CHANGES IN THE FINE STRUCTURE OF SELECTED TISSUES OF PENAEUS MONODON FRABRICIUS JUVENILES FED VARIOUS CARBOHYDRATES

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This study was carried out to determine the qualitative carbohydrate needs of P. monodon juveniles with a semi-purified diet and to analyze the effects of these various carbohydrates on selected tissues of the prawn: hepatopancreas, gill and cuticle. P. monodon juveniles (initial mean weight of 1.76 g) were reared individually in one-liter containers provided with a continuous flow of seawater. The juveniles were fed semi-purified diets containing 10% or 40% maltose, sucrose, dextrin, molasses, cassava starch, cornstarch, or sago palm starch. The basal diet was composed of vitamin-free casein (40%), corn oil (2.5%), fish liver oil (2.5%), cholesterol (1.0%), a vitamin (2%) and mineral (2%) mix (Deshimaru and Kuroki 1974), sodium carboxymethyl cellulose (3%), and cellufil either at 7% or at 37% depending on the amount of added carbohydrate.

Significant differences were observed between the type, as well as the level, of carbohydrate in the diet on the survival of juvenile prawns. Highest survival (52%) was obtained in juveniles fed with a diet containing 10% sucrose. Within 10 days of beginning feeding, complete mortality was observed in prawns fed with diets containing the higher level of maltose and molasses. Among the starches, 10% sago palm starch provided for best survival. Growth of prawns was generally poor on all dietary treatments. Histopathological changes in hepatopancreas, gills and exoskeleton of juveniles fed with the various diets were observed in all treatments.

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THE RELATIONSHIP BETWEEN NUTRITION, MOLTING AND MORPHOGENESIS IN DECAPOD LARVAE

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There is a complex relationship between larval nutrition, molting, and morphogenesis in the larvae of Rhithropanopais harrisii. If starvation occurred during the first 15-18 hours postmolt, larvae failed to complete the molt cycle until food became available. However, if food was available during the first 24 hours postmolt, the larvae completed ecdysis even in the absence of additional food sources. If these larvae were fed immediately after ecdysis, they progressed through metamorphosis normally. If starvation was continued for an additional 3 days, 36% of the larvae underwent a supernumerary larval stage which was intermediate between a larval stage and a post-larval stage. This suggests that when energy levels are low, molting has priority over morphogenesis. If penultimate stage larvae were allowed to feed for 48 hours, the larvae completed a larval-larval ecdysis and metamorphic ecdysis despite starvation for 72 hours during the last larval stage. This suggests that sufficient energy reserves were accumulated to sustain both molting and morphogenesis.

Larvae starved within 15 hours postmolt, later suspended the molt cycle at Stage D_0. The data suggests this is an adaptation to withstand brief (1-2 day) periods of starvation, since food at any time prior to suspension of the molt cycle at D_0 allowed the animal to proceed through ecdysis. Larvae that had suspended the molt cycle underwent a time dependent morphogenesis. Some individual animals, which had suspended the molt cycle for 144 hours, completed metamorphosis thus eliminating a larval stage. Regardless of the duration that the larvae were starved (24-144 hours), the subsequent molt cycle was significantly reduced. These results suggest that decapod larvae have evolved several mechanisms to compensate for periods of starvation.

THE USE OF MICROENCAPSULATED DIETS IN THE STUDY OF THE NUTRITIONAL REQUIREMENTS OF LARVAE OF THE MUD CRAB, *EURYPANOPEUS DEPRESSUS* (SMITH)

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We have developed the use of calcium alginate microcapsules as a tool for the investigation of critical dietary requirements of *Eurypanopeus depressus* larvae and have begun to identify the biochemical constituents necessary in their diets. We began working with nylon-protein microcapsules, and found them to be inappropriate for encapsulating certain diets. Calcium alginate microcapsules are more versatile because they can be used to encapsulate a wider range of dietary materials. These microcapsules are made by dispersing dietary material in a sodium alginate-gelatin mixture, which is then sprayed into a calcium chloride hardening bath. The resulting microcapsules are solidified pieces of a calcium alginate-gelatin matrix that contain the dietary material. We have used these microcapsules to demonstrate that specific dietary components can be added to a natural diet and that their effect on development rate and survivorship can be determined (Levine, Sulkin and Van Heukelem in press). Our experiments have shown that when the total lipid fraction of the brine shrimp is added, via a microcapsule, to a diet of rotifers (*Brachinous plicatilis*), development rate and survivorship are significantly better than on the rotifer diet alone and approximate conditions for larvae fed live brine shrimp nauplii which were previously demonstrated to be an excellent diet. Further experiments will determine the effects of fatty acids belonging to the 3 linolenic pathway on development rate and survivorship.

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POSSIBLE CHEMOAUTOTROPHIC NUTRITION IN BAHAMIAN BIVALVES

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Three species of bivalve molluscs inhabiting sulfide-rich intertidal habitats in the Bahamas are thought to derive nutritional benefit from chemoautotrophic sulfide-oxidizing bacteria found within their gill tissue. All three species (Codakia orbicularis, Codakia orbiculata, Linga pensylvanica) have thick fleshy gills characteristic of the family Lucinidae. Transmission electron microscopic examination indicates the presence of concentrations of membrane-bound procaryotic cells within the gills of C. orbicularis and L. pensylvanica. Activities of sulfide-oxidizing enzymes (ATP-sulfurylase and rhodanese) were high in C. orbicularis and activities of the Calvin-Benson cycle enzyme ribulose-1, 5-bisphosphate carboxylase were measured in C orbicularis, C. orbiculata, and L. pensylvanica. Specimens of C. orbicularis remain alive and active for four weeks in seawater-sulfide solutions prepared daily at initial concentrations of 1.5 mM sodium sulfide. Non-lucine bivalves (Asaphis deflorata (from the same locations) did not show RuBPcase activities. The sum of these preliminary findings suggests chemoautotrophy in these three Bahamian bivalves. Other lucininds from the same habitat (Divaricella dentata and Codakia costata) are also suspected of being chemoautotrophic.

The association of the bacteria with the bivalves, the nature of reduced compounds supplied by the bacteria, and the relative contribution of chemoautotrophic metabolic activities to the nutrition of these bivalves still need to be established. However, chemoautotrophy might have importance in the commercial mariculture of the large edible C. orbicularis.

GROWTH AND FATTY ACID COMPOSITION OF OYSTER LARVAE

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ABSTRACT

Previous investigators have suggested that lipids play a significant role in the metamorphosis and development of the oyster larvae (Holland 1978). Lipid rather than carbohydrate is thought to be the major metabolic energy reserve in most larvae of benthic marine invertebrates (Holland 1978). In feeding experiments, gelatin-acacia microcapsules (see Langdon, these proceedings) filled with cod liver oil were fed to Crassostrea virginica larvae. Cod liver oil is rich in long chain polyunsaturated fatty acids; the fatty acid composition is quite similar to that found in an algal diet (a combination of Chlorella sp., Pyramimonas virginica and Pseudoisochrysis paradoxa) which has been used in this laborabory as a standard food source for larvae of C. virginica. Our results demonstrated that larvae fed with gelatin-acacia microcapsules containing cod liver oil grew (increase in size from 69 µm to 152 µm) but not as much as larvae fed algae (increase in size from 69 µm to 173 µm). Results also indicated that microcapsule concentration affected growth rate.

In order to observe the changes of lipid and fatty acid composition in larvae during development, the lipid and fatty acid compositions of straight hinge, 3 day, 6 day, 8 day, and "eyed" larvae were determined by thin layer chromatography and gas liquid chromatography.

Preliminary results from the analyses of lipid composition indicated that no change in lipid class composition occurred in larvae during development. However, there were changes of fatty acid composition; the percentage of polyethylenic fatty acids was lower in younger larvae than in older larvae and the amount of total saturated fatty acids decreased in both the neutral and the polar fractions. Changes in the proportion of C-12, C-14, C-16, and C-18 saturated fatty acids were also observed. The content of monoethylenic fatty acids was rather variable. One possible explanation is that larvae do not synthesize highly unsaturated fatty acids until they are in the later stages of development. A supplement of highly unsaturated fatty acids (e.g. 20:5ω3 and 22:6ω3) may be desirable for larvae cultured under hatchery conditions.

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THE GROWTH OF JUVENILE OYSTERS *CRASSOSTREA VIRGINICA* (GMELIN) FED ALGAE SUPPLEMENTED WITH SILT OR KAOLIN

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ABSTRACT

An algal diet consisting of equal amounts of *Thalassiosira pseudonana*, clone 3H and *Isochrysis* aff. *galbana*, clone T-ISO was fed to nine groups of 20 juvenile oysters (initial $\bar{X}$ live whole weight 0.50 g) using batch or continuous feeding for periods of 6 to 7 weeks. The quantity of algal cells fed/gram oyster live weight/day was determined from the feeding ration equation developed by Pruder et al. 1978. Algal controls were tested against treatments supplemented with either natural silt (50 mg dry weight) or kaolin (10, 50, 150 mg dry weight).

The most marked improvement in growth resulted from the treatment in which the algal diet was supplemented with natural silt (50 mg/ℓ) which was 62.5% greater than the growth of the algal control. Batch vs. continuous feeding methods were compared and no significant effect on oyster growth rates was found. Supplemental kaolin did not enhance oyster growth.

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A PRELIMINARY STUDY OF GLUCOSE AND PROTEIN IN OYSTER HEMOLYMPH

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ABSTRACT

Oysters, *Crassostrea virginica* (Gmelin), obtained commercially were maintained in the laboratory under controlled conditions of temperature and salinity. Several constituents of the hemolymph serum were examined in these oysters. Overall serum glucose concentrations averaged 88.3 ± 19.8 µg/ml (± SEM) and ranged from 19 to 234 µg/ml. Serum protein

levels (total Lowry positive substances) averaged 11.0 ± 1.89 mg/ml and ranged from 3.17 to 26.5 mg/ml. No free fatty acids could be detected and serum triacylglycerol values were quite low averaging 43.2 µg/ml.

Oysters starved for up to twenty-seven days tended to maintain their serum glucose and protein levels. However, extremes in external conditions appear to affect the concentrations of these metabolites. Groups of unfed oysters maintained in 24 ppt artificial sea water at temperatures of 4° and 20°C had significantly different (P = 0.05) levels of serum glucose and protein. Oysters held at 4°C had serum glucose values of 193 ± 35 µg/ml while those kept at 20°C had serum glucose values of 84.1 ± 14 µg/ml. Similarly the average protein values were 17.56 ± 1.42 mg/ml and 9.76 ± 0.85 mg/ml for the animals at 4° and 20°C respectively.

At a low ambient salinity of 12 ppt (artificial seawater) serum glucose and protein concentrations were significantly (P = 0.05) decreased when compared to those found in oysters kept in water of 18 ppt and 24 ppt. As oysters are known to be osmo-conformers this result might be anticipated as water enters the hemolymph.

During fasting, hemolymph glucose values have been examined in several other molluscan species. As in the present report they varied over a wide range. Thus it may be inferred that these animals, including the oyster, C. virginica, are more tolerant to larger variations of hemolymph glucose concentrations than mammals. In the current report no deleterious effects of variations in serum glucose levels could be detected. Groups of animals with initial serum glucose levels of 231 to 250 ug/ml survived as well as those with initial serum glucose values of 53 to 84 ug/ml.

Oysters held at constant temperatures and in seawater of constant salinity tended to maintain their serum glucose and protein concentrations over a twenty-seven day period of starvation. This was observed in oysters kept a temperatures of 4°, 10°, 15°, and 20°C and in 12 ppt, 18 ppt, and 24 ppt saline. Also oysters stored in air at 4°C tended to maintain their hemolymph composition. Hence, there is some type of homeostatic mechanism involved in controlling the levels of these metabolites in oyster hemolymph.

YEAST AND VITAMINS AS SUPPLEMENTS IN THE DIET OF CRASSOSTREA VIRGINICA (GMELIN)

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It is desirable to develop non-algal food supplements for bivalve molluscs in order to reduce hatchery feeding costs. The following experiment was designed to determine the suitability of microencapsulated vitamins and various levels of yeast as supplements in

the diets of juvenile Crassostrea virginica.

Oysters were individually weighed before and after receiving experimental diets over a period of three weeks. At the end of each week of the experiment, the treatment groups were weighed, and the ration was increased in proportion to the weight gain during the preceding week (i.e., new ration = initial ration x $\frac{\text{new group weight}}{\text{initial group weight}}$). Algal rations were presented alone or in combination with vitamins and/or yeast, at levels of 100%, 75%, 50%, 25%, and 0%, where a 100% ration equalled 5.2 mg dry weight of food per treatment per day. The algal diet was composed of Thalassiosira pseudonana (3H) and Tahitian Isochrysis aff. galbana present in gravimetrically equal amounts. The yeast product Torutein-50 ® (Pure Culture Products, Inc.) was added at levels between 0% and 300%. A tissue culture medium vitamin mixture (TCM 199), with 50% w/w ascorbic acid added, was microencapsulated in lipid-walled capsules using menhaden oil as the lipid source (see Langdon, these proceedings). The capsules were added at either 5,000 (1X) or 25,000 (5X) capsules/ml.

The following ranking of growth was observed for animals fed at the 75% algal ration level:

75% Algae+ 25% Yeast+ 1X Vitamins	75% Algae+ 25% Yeast+ 5X Vitamins	75% Algae+ 25% Yeast	75 Algae+ 5X Vitamins	75 Algae Alone
625%*	520%	510%	390%	340%

*Percent increase in wet weight in 3 weeks.

The oysters fed the 75% algae/25% yeast/1X vitamins grew better than animals fed on 100% algae alone, which increased in weight by 585% in three weeks. In comparing groups without added vitamins, intermediate yeast supplementation (75% to 150%) generally produced the best growth at each algal level, while animals fed higher levels of yeast (150% to 300%) did not grow as well. The addition of yeast generally improved growth compared with the equivalent algae-alone treatment.

Since the microcapsules used are presently expensive to produce, it would probably not be economically feasible to use these as supplements to bivalve diets, but the observed increases in growth indicate the potential of adding vitamins and lipids to bivalve diets in a more economical manner. The increase in growth produced by yeast is significant because of the low cost of yeast compared to mass-cultured algae. However, the advantage gained by using less expensive supplements must not be neutralized by decreases in the growth rates of animals fed these supplements, if these supplements are to be useful for commercial mariculture.

INDUCTION OF SETTLEMENT AND METAMORPHOSIS IN *CRASSOSTREA VIRGINICA* BY A MELANIN-SYNTHESIZING BACTERIA

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We have repeatedly isolated a marine bacterium (designated LST) that is readily and continuously found in close association with oysters. We believe this organism has a specific beneficial role in the setting and metamorphosis of *Crassostrea virginica* larvae. LST is an aerobic, gram-negative rod, highly motile with a percent ratio guanosine to cytosine of 45.6%. It grows optimally in a 3.5% (w/v) marine salt mixture at 25°C and does not produce a sheath or spores. It has been observed to attach to a variety of surfaces, preferentially on glass and oyster shell, but also on plastic and aluminum. Biochemical characteristics have been determined and it is increasingly apparent that LST, which becomes tightly coiled in one phase of growth, is a new species.

Under optimum conditions (viz. 25°C; pH 7.3) in Marine Broth (2216), LST has a generation time of 70 min, whereas in a synthetic medium, consisting of 3.5% marine salts plus 125 mM aspartic acid and glutamic acid, it has a maximum generation time of 400 min. In the stationary and decline phases of growth, which correlates with a decrease in energy charge (ATP + 1/2 ADP/ATP + ADP + AMP) from 0.86 to 0.72, LST synthesizes and releases a brown pigment. This pigment is identified as melanin on the basis of its chemical properties and UV absorbance maxima. Pigment, purified in a column containing Sephadex 150, is comprised of polymers of various molecular weights ranging from 12,000 to 120,000 Daltons, which is typical of melanin pigments. Assays for LST tyrosinases are weakly positive.

To preliminarily verify the hypothesis that LST, and a product/precursor of melanin synthesis, attracts oyster larvae, microscope slides coated variously with either a) bacteriological growth media (control), b) *in situ* microorganisms (including LST), c) a non-pigmented marine bacterium (*Hyphomicrobium neptunium*), d) LST, e) LST pigment, f) killed LST, g) pigmentless LST mutants, or h) LST hyperpigment producers, have been immersed in tanks containing 2-5 eyed larvae/ml in sand filtered estuarine water from the Indian River inlet, Delaware.

Prefouled, LST-coated, and pigment-coated slides attracted more larvae over a 24 hour interval than control slides, as measured by attached and metamorphosing oysters. Furthermore, the pigment hyperproducing variant attracted more larvae than the prefouled or LST-coated samples. Non-pigmented *H. neptunium* and, significantly, the LST-pigmentless variant attracted neither more nor less larvae than media-coated control slides. UV-killed LST often repelled larvae, possibly due to alterations in moities of melanins and/or precursors. It is possible that such altered compounds may be competitive inhibitors

of larval settlement inducers.

One hypothesis concerning the relationship between LST and Crassostrea virginica larvae holds that LST, which adheres very strongly to cultch and some other hard surfaces, forms micro-colonies on cultch. In sufficient numbers, during the decline phase of its growth, the bacterial colonies may produce a high concentration of pigment, sufficient to attract oyster larvae. The larvae could "ingest" long LST forms (>5 μm) and induce reproduction much as a lectin produced by Halachondrea panicea stimulates the bacterium Pseudomonas insolita. The larvae would also disseminate the bacterium. In turn, a bacterial product could have a hormone-like stimulatory effect on larval development and metamorphosis.

MOLLUSCS

EFFECT OF NATURAL SILT ON OYSTER GROWTH

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Seven silt concentrations in combination with three algal rations were fed to oysters (Crassostrea virginica, Gmelin) in a twenty-one treatment matrix. Silt was added to achieve initial concentrations of 0, 12.5, 25, 50, 75, 100, and 150 $mg l^{-1}$. The silt was collected from the Broadkill River, Delaware, using continuous flow-through sedimentation tanks, and then oxidized with 30% H_2O_2. Oxidation lowered the organic content of silt from 11% to 0.28%. The algal rations were based on the weight-specific formula developed by Pruder et al. (1977). The rations offered were either a) x2, b) x1, or c) x1/2 the calculated ration. All rations were offered at the same initial concentrations of 3×10^5 cells ml^{-1} in a 1:1 mixture (by cell number) of Thalassiosira pseudonana and Isochrysis galbana, clone T-ISO. The feeding experiment lasted four weeks at 23°C. Water salinity during the experiment averaged 31.9 ppt and the pH averaged 8.

The greatest oyster growth was obtained in treatments where silt was added to the highest algal ration. With this ration, oyster growth increased with increases in silt concentrations of up to 25 $mg l^{-1}$. Further increase in silt concentration did not result in a further increase in growth. At the medium ration, silt appeared to increase growth to some extent, but the effect was not statistically significant. At the lowest ration silt had no effect on growth. In all treatments it appeared that silt at initial concentrations of up to 150 $mg l^{-1}$ had no adverse effect on oyster growth. When silt was not added, the three food rations did not result in any statistically significant differences in oyster growth rates.

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LIPID SPECIFIC STAINING: A VISUAL TECHNIQUE FOR MONITORING THE CONDITION OF BIVALVE LARVAE

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ABSTRACT

Lipid is the major biochemical energy substrate in developing and metamorphosing bivalve larvae. Adverse culture conditions and disease disrupt normal patterns of lipid accumulation and utilization. Lipid specific staining can be used to visually monitor lipid content and localization in newly spawned and developing bivalve larvae in large scale cultures.

Whole larvae are stained with Oil Red O (Gallager and Mann 1981), examined microscopically and compared with reference photographs of both healthy and selectively stressed animals.

Newly formed straight hinge larvae of Crassostrea virginica Gmelin and newly spawned larvae of Ostrea edulis L. both contain numerous small lipid droplets widely dispersed throughout their tissues. During the first three days of active feeding these droplets are lost as the digestive gland and intestine became heavily laden with lipid. In conditions of good food (Tahitian strain Isochysis glabana) and optimum temperature (25°C for C. virginica and 20°C for O. edulis) the digestive gland and intestine continue to accumulate lipid until metamorphosis. At elevated temperature (30°C for C. virginica and 25°C for O. edulis) larval shell growth rate is increased; however the percentage of larvae completing metamorphosis decreases markedly (5-8% versus 70-80% in both species) and lipid accumulation occurs only in the intestinal wall, not in the digestive gland.

Similar observations of intestinal lipid localization and low spat yield are evident when larval cultures are fed on the diatom Phaeodactylum tricornutum, a diatom of mediocre food value, irrespective of temperature. Large decreases in lipid content are observed when three day starvation periods are imposed on representative larval stages irrespective of culture conditions.

Successive spawns of single brood stocks of both oyster species produce both larvae of decreasing viability and lower spat yield. The

newly formed (C. virginica) or newly spawned (O. edulis) straight hinge larvae of these cultures exhibit a general decrease in lipid content upon staining and an increase in individual larval variability with each successive spawn.

Lipid specific staining can be used as a routine and powerful diagnostic tool in bivalve hatcheries to both monitor fluctuating larval lipid content and detect the effect of environmental stress prior to the onset of mass mortality.

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ENVIRONMENTAL AND GENETIC INFLUENCES ON OXYGEN CONSUMPTION IN CRASSOSTREA VIRGINICA (GMELIN)

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ABSTRACT

Growth is the conversion of energy to somatic tissue, and growth rate has been positively correlated with genetic heterozygosity in C. virginica (Zouros et al. 1980). Since growth rate has been positively correlated with heterozygosity of enzymes, it follows that some relationship can be expected between the energy required for growth and heterozygosity, i.e. a greater efficiency in the conversion of consumed energy to somatic tissue in more heterozygous individuals.

We have shown that the basal or standard rate of oxygen consumption is temperature dependent at any given salinity in animals acclimated to different temperature-salinity regimes for 3 weeks. In addition, oxygen consumption was found to increase with decreasing salinity from 28‰ to 7 ppt at 10 and 20°C. The acute effects of exposure to various temperature-salinity combinations on oxygen consumption were determined in pre-acclimated oysters and multiple regression equations were calculated to relate oxygen consumption rate to acclimation and experimental temperatures and salinities. These equations indicated that 1) as acclimation salinity decreases, the effect of exposure temperature becomes more pronounced, 2) as acclimation salinity decreases, the effect of exposure salinity decreases, 3) as acclimation

temperature increases, the effect of exposure salinity decreases and 4) as acclimation temperature increases, the effect of exposure temperature increases.

The oxygen uptake by C virginica in declining oxygen tensions was determined after acclimation to various temperature salinity combinations. C. virginica shows remarkable capabilities for regulation of its rate of oxygen uptake over a wide range of oxygen tensions (approximately 40-90% saturation) in animals acclimated to either 10 or 28 ppt; however, the capacity for regulation decreases considerably at all temperatures in animals acclimated to 7 ppt, the lowest capabilities being in animals at 7 ppt and 20°C.

The combined effects of temperature, salinity and oxygen tension on oxygen consumption rate indicate that animals acclimated at 28 ppt show the most suppressed response at all temperature-salinity-oxygen combinations tested, the magnitude of the response increasing with decreasing salinity.

There is a significant decline in the rate of oxygen consumption with increasing numbers of heterozygous loci per individual, the oxygen consumption rate of the most heterozygous individuals being approximately one half that of the most homozygous animals. Stressed animals showed a marked increase in oxygen uptake among all individuals; however, the relationship between the rate of oxygen consumption and the number of heterozygous loci per individual was unchanged. In addition, the line that describes the relationship between oxygen uptake and number of heterozygous loci in stressed animals is a multiple of the regression line describing the same relationship under ambient conditions. This means that the metabolic energy demand produced by the stress is more than twice as great for multiple locus homozygous individuals than for the most heterozygous individuals (see Koehn and Shumway 1982).

These findings have direct implications for the mariculture of C. virginica: a) animals should be maintained at temperature-salinity-oxygen combinations requiring the least energy expenditure and b) parental stock should be chosen so as to maximize genetic heterozygosity in offspring. Implementation of both of the above suggestions would reduce the basal metabolic demands of the individuals which in turn would reduce the necessary food rations needed to achieve specific growth rates.

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