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ABSTRACT

This document presents recent information and research data relative to the following major areas of crustacean health research: Histology-Cytology, Microbiology, Parasitology, Disease Control and Laboratory Studies. A review and summary of epizootics in crustacean populations is presented in the key-note address by Dr. C. J. Sindermann, and an overview of the workshop and crustacean health research is presented in the key-note concluding remarks by Dr. A. K. Sparks. A summary of recommendations from a planning conference on crustacean health research priorities is included as an addendum.

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Proceedings of the Second Biennial Crustacean Health Workshop

Compiled by
D. H. Lewis and J. K. Leong

TAMU-SG-79-114
July 1979

Texas A&M University Sea Grant College Program

PROCEEDINGS OF THE
SECOND BIENNIAL CRUSTACEAN HEALTH WORKSHOP

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The second Biennial Crustacean Health Workshop, held in Galveston, Texas, April 20 - 22, 1977 was a joint effort sponsored by National Marine Fisheries Services, NOAA, Galveston Laboratory and Texas A&M University. It was originally designed with an informal format in mind. However, the quality of the presentations and the potential value of the workshop material to crustacean culture led to the belief by most participants that the material should be documented. At a "Planning Conference on Crustacean Health Research Priorities" at Texas A&M University, November 18 - 19, 1977, the information presented at the workshop was recognized as best reflecting the current status of many crustacean health problems, and compilation of the workshop material was identified as a priority need. This document is an effort to meet that need and represents the efforts of many to review, revise and update materials presented at that workshop. A special note of thanks is expressed to Dr. Gisela Mahoney and staff of the Sea Grant Publication Office at Texas A&M for their efforts on the document. The patience and cooperation of all who participated is greatly appreciated.

DHL.

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KEYNOTE ADDRESS
SECOND CRUSTACEAN HEALTH WORKSHOP
Galveston, Texas
April 20, 1977

EPIZOOTICS IN CRUSTACEAN POPULATIONS

by

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ABSTRACT

Epizootic diseases have occurred in the larger commercial Crustacea, in the smaller food-chain species, and more recently in aquaculture populations. Disease outbreaks in natural populations are caused by frank virulent pathogens, whereas facultative pathogens are important in culture environments. There is some evidence that such outbreaks follow classical epidemiological principles; that the larger Crustacea are immunologically responsive; and that environmental factors -- notably aquatic pollutants and poor culture conditions -- can influence the course and severity of disease outbreaks. Recent discovery of viruses of several types in crustaceans suggests potential disease problems for the future, especially in aquaculture populations.

INTRODUCTION

Epizootics of infectious diseases and resultant mortalities have been a matter of record in natural populations of Crustacea for more than a century. This paper attempts to summarize our present understanding of such epizootics, utilizing selected examples from the world literature. To achieve some semblance of order, the subject matter has been subdivided into the following sections:

(1) The written and unwritten history of epizootics in crustacean populations;

(2) the closely related topic of internal defenses in Crustacea;

(3) the also closely related topic of environmental influences on epizootic diseases in Crustacea; and, finally

(4) recent information about viruses in Crustacea.

It might be well at the outset to discuss a few generalizations about epizootic disease and mortalities. It should be stated immediately that mass mortalities are merely an exaggerated form of natural mortality, usually distinguished by being of rapid onset, and affecting a large proportion of the population over an extensive geographic area. A "mass mortality" can be described as "an unusual and sharply defined increase in mortality rate, of sufficient proportions to significantly affect population size and to at least temporarily dislocate the ecosystem of which the population is part."

Many mass mortalities of crustaceans undoubtedly result from interactions of several environmental and biological factors; only a few have been clearly associated with a single overriding causal factor; and of these few, disease has usually been the identifiable cause.

"Disease" in its broadest sense can be defined as "any departure from normal structure or function of a living organism" Origins may be infectious or non-infectious. Included in the non-infectious category would be such phenomena as environmentally

induced anomalies in morphology, genetic abnormalities, physiological malfunctions due to chemical environmental factors, metabolic disorders resulting from nutritional deficiencies, many forms of neoplasms, and a host of others. Sparks (1973) has provided an excellent treatise on non-infectious diseases of invertebrates.

Disfunction and death due to the activity of infectious agents constitute the narrower, but often predominant concept of disease. Infectious diseases -- caused by viruses, bacteria, fungi, protozoa and others -- are usually prime suspects in searches for causes of mortalities, sometimes to the exclusion of other possible causes. In many instances of mass mortalities, it is probably the combination of an infectious agent and environmental stress that eventually kills animals on a large scale.

In considering the information about mass mortalities of crustaceans we should not overlook the continuing background mortalities that are less spectacular but not less significant in determining population size. These mortalities are represented by the slow continuous extraction of individuals from the population by deaths due to inadequate food, salinity, extremes, predation, temperature extremes, low oxygen levels, crowding effects, and many others. Actually, the dividing line between "background" mortality and "mass" mortality is hard to define and is only a matter of degree.

It should also be recognized that localized mass mortalities, involving relatively restricted geographic areas, can and do occur, and can produce significant localized effects. Predators can

increase in abundance explosively, and kill large numbers of individuals. Changes in the physical environment, such as severe flooding of rivers, with consequent reduction of salinities near river mouths, can cause and have caused localized, often complete, mortalities. Such local mass mortalities undoubtedly occur by the hundreds as compared to one of the major widespread mortalities.

So, with the qualifications mentioned above, it seems that natural mortality can be considered (somewhat artificially perhaps) in three aspects: (1) background mortality, as continuous, often unnoticed, but very significant reduction in numbers of individuals at any stage of the life history; (2) localized mass mortalities, affecting restricted geographic areas and killing up to 100% of the populations in those areas; and (3) widespread and extensive mass mortalities, affecting large geographic areas and reducing production in these areas significantly. A number of the mass mortalities in category 3 have been associated with epizootic disease in crustacean populations.

1. HISTORY OF EPIZOOTICS IN CRUSTACEA

No examination of epizootics in crustacean populations can logically start anywhere but with the great crayfish plague -- "Krebspest" in Europe, which began about 1865 and decimated much of the crayfish (*Astacus astacus*) populations of that subcontinent (Schikora, 1926; Pixell-Goodrich, 1956; Unestam, 1969). Caused by the phycomycetous fungus *Aphanomyces astaci*, mortalities in some areas reached 100%, completely eliminating many crayfish populations.

It is interesting, in reading the early and massive literature on "Krebspest", to see the sequence of events that has been repeated during modern epizootics in aquatic populations.

(1) A primary focus of disease occurs (in this case in Italy) but the disease quickly spreads to other areas (throughout Europe).

(2) There is an extended period when the etiological agent is unknown, or several pathogens are claimed separately as the etiological agent (in this case a bacillus was in the lead, and numerous other bacteria were blamed, even though the true culprit, the fungus _Aphanomyces astaci_, was named and identified by Schikora in 1906). Public arguments over favored pathogen candidates develop, often with much vituperation and scientific name-calling.

(3) As the epizootic affects new geographic areas, many new people become involved, and a few of these stay involved, to produce significant new information (in the case of the crayfish plague it seemed to be up to German workers principally -- first Leuckart and Weber, then Schikora, and then Schäperclaus -- with contributions from Swedish researchers -- Nybelin, and more recently Unestam and other contemporary scientists.

Some aspects of the epizootiology of _Aphanomyces astaci_ are very significant:

(1) The European crayfish seems to have no resistance to the fungus, so mortalities are usually 100%.

(2) In affected waters, no resistant survivors exist to form the nucleus of a resistant population.

(3) No resistant spores of the pathogen have been found, so new populations of crayfish may be introduced into previously affected areas after the original populations have disappeared.

(4) Experimentally, infectivity is high. Only a few zoospores/ml are sufficient to infect European crayfish experimentally.

(5) All U. S. species of crayfish are resistant to infection by Aphanomyces astaci, but they can be infected experimentally if many spores are added. The disease is quickly circumscribed in these species, causing localized spots. Hyphae are melanized rapidly in muscle and cuticle.

Just as we must accord the European crayfish plague a place of honor in any consideration of crustacean epizootics, so also must we pay our respects to gaffkemia of American and European lobsters, caused by the micrococcus Aerococcus viridans (var.) homari.

Gaffkemia, or "red tail disease" as it was originally called, was first recognized on the Maine coast in 1946. Two epizootics of gaffkemia occurred -- affecting lobster populations of the entire Maine coast -- in 1946-47 and 1959-60 (Goggins and Hurst, 1960). Mortalities during the epizootic years were especially apparent when attempts were made to impound lobsters -- a common practice in Maine, in Canada, and elsewhere. Losses in such impounded populations regularly reached 60% within a few weeks time.

The disease is normally enzootic in lobster populations of the North American east coast and in European lobster populations (where epizootics have been reported in England, Ireland, Norway, and The Netherlands).

Factors of significance in the epizootiology of gaffkemia include:

(1) Lack of any observable resistance of lobsters to infection;

(2) The apparently heterotrophic capabilities of the pathogen in bottom muds and sea water;

(3) Direct transmission of the pathogen;

(4) Direct temperature dependence (mortalities increase sharply if water temperature exceeds 15°C);

(5) A vanishingly small infective dose (as few as 5 organisms per lobster produced 90% mortality in experimental studies);

(6) Infectivity of the pathogen for other crustacean species (it does not cause epizootics in these other species, but they may act as reservoir hosts); and

(7) Transmission through ruptures in the integument.

The epizootics described so far have occurred in wild populations of commercial species -- but what about all the Crustacea important as food chain organisms (copepods in particular). Do we have any evidence for epizootics in such species?

We have some -- probably more than you might expect. One example of the fact that epizootics can sweep through food chain populations was called to my attention by Dr. Fleminger of the Scripps Institution of Oceanography in 1964 (Fleminger, A., personal communication). He had found in plankton tows a significant prevalence (about 15%) of systemic fungus infections in calanoid copepods of the open Pacific. The parasite was morphologically similar to *Ichthyophonus*, which had been described earlier (Jepps, 1937), from copepods in North European waters.

A better example of disease and associated mass mortalities of copepods was reported by Vallin (1951). An epizootic in populations of *Eurytemora hirundoides* on the Swedish coast of the northern Baltic in 1950 was apparently caused by a fungus pathogen, identified as a new species of *Leptolegnia*. *Eurytemora hirundoides* is the inner Baltic's most common and abundant copepod during the warmer months of the year, constituting from 50 to 90% of all copepods in plankton tows. After the 1950 mortalities the species was much less abundant in samples. Vallin's description of the mortalities is dramatic.

> "At the beginning of August 1950 it was reported that the herring fishermen in the Botten Sea, off Sundsvall, had found their nets to be clogged with a sticky substance. When the tackle was taken out of the water the substance soon began to rot and smell. It was suspected that its cause might be industrial pollution from cellulose plants. Samples of the material scraped off the nets, some treated with formalin, were however

> found to consist of dead plankton crustaceans. A single species of copepod - *Eurytemora hirundoides* - formed almost 100% of the substance ... Death of the plankton ... was first reported from fishing grounds east of the island of Alnö, off Sundsvall, at the beginning of August. Fishermen found such large quantities of dead *Eurytemora* on their nets that they could easily scrape it off by the quart. At the same time the local fishery assistant stated that the sea water in wide areas was made turbid by a lot of "small dead white eggs", which must have been the killed copepods. Within a few days the same phenomenon was reported from other fishing waters ... The area, then, stretched for some 70 km in the outer skerries off Sundsvall."

Reports were also received that the mortality was observed on the Finnish coast north of Åland.

Mortalities recurred in 1951, at a reduced level and a month later than in 1950. The severe effects of the disease on *Eurytemora* populations were indicated by reductions in percentages of the species in plankton tows at Sundsvall to only 13%, from an expected 80% characteristic of pre-epizootic years.

Eurytemora is an important food for herring, *Clupea harengus*, and other pelagic species of the area. Widespread mortalities at the trophic level of copepods could materially reduce the food supply for these commercial fishes, in addition to clogging and fouling nets, or causing their rapid decay.

Epizootic diseases have been recognized for a long time in the smaller, inconspicuous, and non-economic Crustacea. Dr. Bang of Johns Hopkins (1970) summarized much of the early literature on these diseases -- pointing out that when Metchnikoff began his classic studies on the comparative pathology of inflammation (published in 1893), his favorite experimental animal was the water flea Daphnia, in which six different types of infections had already been described. Others have been described since then.

Bacterial, fungal, and microsporidial infections, were described in the amphipod Gammarus (a sand flea) fifty years ago Pixell-Goodrich, 1928), and almost a hundred years ago a luminescent bacterial disease was described in sand hoppers from the coast of France. A similar disease was later found in sand hoppers (Talorchestia and Orchestia) from the beaches at Woods Hole.

This early literature on diseases of non-economic crustacean species is fascinating and detailed -- a legacy from a gentler, less frantic era, free from the intense preoccupation with commercial species which characterizes the present. I recommend for all of you -- particularly those of you under 35 -- a leisurely trip through this great crustacean disease literature from the past. Take two weeks and read nothing that was published after you were born. Tiptoe through some of the dim German laboratories of the late 19th century; stand on the beach at Roscoff in northern France with Cantacuzène and his students. I guarantee that it will be a learning experience that you will never forget. Dr. Bang's 1970 paper published in the American Fisheries Society symposium volume "Diseases of Fishes and Shellfishes" (edited by Dr. S. F.Snieszko) can be your entry point.

Some of you may feel that this is nonsense -- that nothing published before 1970 has any relevance to what you are doing. I suggest that many of you are treading the same paths as Metchnikoff, Cantacuzène, Cuenot, Bruntz, and other productive observers of crustacean diseases from the early part of this century -- so at least know from whence you came.

Thus far I have dealt only with epizootics in natural populations, but recently there has been increasing concern about the special case of epizootics in captive cultured crustacean populations, particularly shrimps, lobsters, and crabs. The epizootic diseases in culture populations are predominantly those of larvae -- with a whole new array of facultative pathogens and harmful epibionts which emerge under stress conditions (poor water quality, inadequate nutrition, and overcrowding primarily).

The list of facultative organisms that can kill larval and post-larval crustacean populations is growing, but is already impressive (Sindermann, 1977). Vibrio infections, filamentous bacterial infestations, and the fungi *Lagenidium* and *Sirolpidium* have been reported from penaeid shrimp larvae. Bacterial necrosis, ciliate infestations, and filamentous bacterial infestations have been identified from *Macrobrachium* larvae. The fungi *Lagenidium* and *Haliphthoros*, and filamentous bacterial infestations occur in lobster larvae.

This does not imply, of course, that there are not epizootic problems with cultured juvenile and adult Crustacea. Shell disease seems a universal problem, as do vibrio and related infections.

So, here then is just a bare handful of examples of epizootics in crustacean populations:

(1) The massive "oh-my" outbreaks in natural populations of large commercial species that command attention and immediate research response;

(2) The less obvious but probably not less significant mortalities in populations of the tiny Crustacea -- copepods, amphipods, cladocera, etc.; and

(3) The more recent outbreaks of disease in cultured populations -- outbreaks that often involve early life history stages, and that often emerge because of environmental abuses.

Before leaving this brief history, we should ask the question: "Do crustacean epizootics follow classical epidemiological principles?" By classical principles, I mean the typical epidemic wave pattern found in text books, in which prevalence of a pathogen in a susceptible host population begins to increase; numbers of acute cases and numbers of mortalities rise rapidly to a peak; then as the numbers of susceptibles decline and the likelihood of contact between pathogen and susceptible host declines, the wave recedes, in terms of numbers of acute cases and numbers of mortalities. Resistant individuals and chronic infections characterize the declining limb of the curve.

There is some evidence that crustacean disease outbreaks do follow this general pattern:

(1) An enzootic disease such as gaffkemia in lobsters periodically produces highly significant economic losses. So at least for that disease there is a strong suggestion of wave-like increases in abundance of the pathogen in wild populations.

(2) With a number of epizootics the typical epidemiological picture emerges. Increasing numbers of infected individuals lead to an epizootic peak, followed by declining levels of infection and declining mortality rates.

(3) The crayfish plague in Europe had characteristics of a pandemic of a newly introduced virulent pathogen in a highly susceptible population.

(4) Infection pressure seems to play an important role in several diseases.

There is one significant uncertainty, however, in concluding that epizootics in Crustacea follow classical epidemiological principles. That concerns the nature of the host response to infection. Several pathogens -- *Aerococcus viridans* in lobsters, and *Aphanomyces astaci* in European crayfish -- fail to produce obvious host defense response, which is disturbing (to me as well as to the animal). Reassuringly, however, several of the larger Crustacea have, in the last decade, been found to possess both heightened phagocytic and increased humoral responses to experimental infections. Since the internal defenses of Crustacea must somehow play a significant role in epizootic disease, it seems reasonable to look fleetingly at what we know and don't know about these defenses. How do crustaceans resist infection?

2. INTERNAL DEFENSES OF CRUSTACEA

As one of those sweeping and often untenable generalizations that are the prerogative of keynote speakers -- it seems that "phagocytosis, augmented by humoral factors with low specificity, constitutes the basic mechanism of internal protection in the large Crustacea" (Figure 1.).

Phagocytosis by fixed and mobile cells in gills, pericardial sinus, and sinuses at the bases of appendages seems to be a principal defense perimeter in many crustaceans. Effectiveness of phagocytes in destroying invading microorganisms varies, depending on the species of microorganism, as well as host physiology and environmental factors. Phagocytic activity is enhanced by hemolymph factors, which, in addition to immobilizing and agglutinating the invading organisms, also sensitizes them to phagocytosis (Sindermann, 1971).

Hemolymph factors, most of which seem to be of cellular origin, may also have bactericidal or lytic activity, leading to extracellular destruction of microbial invaders. A few recent studies indicate that effects of hemolymph factors may be enhanced experimentally by injection of killed or living microorganisms. Specificity of natural and experimentally enhanced humoral factors is much lower than that of vertebrate immunoglobulins, but probably such factors act synergistically with cellular protective mechanisms, as they do in vertebrates.

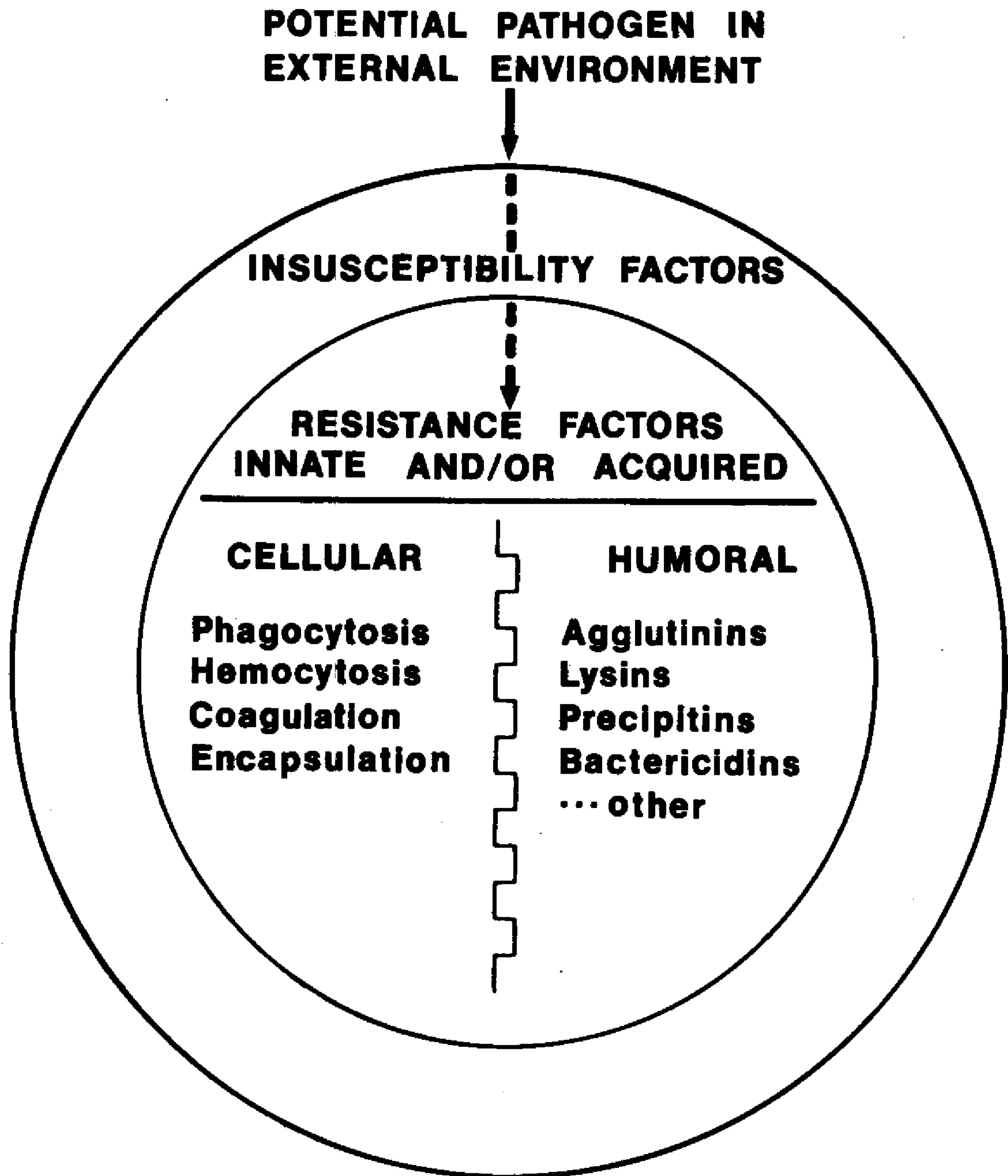

Figure 1. Mechanisms of internal defense (from Sindermann, 1971)

What examples do we have to support these generalizations? The late and much loved Les Stauber of Rutgers University wrote in 1961: "Those invertebrates with a long life span (such as the lobster) should be investigated more fully, as likely hosts capable of demonstrating acquired resistance." And indeed, since that time, it has been the larger crustaceans, the lobster and others, which have been examined for natural and acquired resistance to disease and which have been found to be immunologically competent.

A number of laboratories have been involved -- Dr. Bang and his associates at Johns Hopkins, Dr. Tripp and associates at U. Delaware, Dr. Schapiro, Dr. Rabin, and Dr. Steenbergen at San Diego State, Dr. Vago and his associates in France, and others. We could and probably should consider the accomplishments of each of these groups in invertebrate immunology -- but obviously there is not time, so instead I would like to take as a case history the studies of Dr. Stewart and associates, of Environment Canada in Halifax, on lobster internal defenses, using the gaffkemia pathogen _Aerococcus viridans_ as a model system.

I think that most of you who have read the numerous scientific papers that have reported these studies of Dr. Stewart and his associates will agree that they constitute a classical work in Crustacean biology (see, for example, Stewart and Rabin, 1970; Stewart and Zwicker, 1974).

You will recall that gaffkemia can occur as epizootics in natural as well as captive lobster populations. Stewart and associates found early that the internal defense mechanisms of the lobster, effective against a wide range of injected bacteria, and

consisting of active phagocytosis as well as agglutinating and bactericidal activity of hemolymph -- failed completely when challenged by Aerococcus viridans. Size of infective dose had little effect on mean time to death (Figure 2), whereas increasing temperature decreased the mean time to death (Figure 3). In experimental infections, hemocyte counts declined and clotting time increased concomitantly with increase in pathogen number (Figure 4). Biochemical studies indicated that death resulted from drastic reductions in hepatopancreatic glycogen and general metabolic disruption from hepatopancreatic disfunction -- that death from gaffkemia was "a result of an unsuccessful competition on the part of the lobster for its own readily available storage material."

Stewart (Stewart and Zwicker, 1974) and his associates were of course interested in keeping lobsters alive as well as understanding their internal defenses. They early prepared vaccines with killed Aerococcus but found no enhancement of bactericidal activity in vitro. Other vaccines from other bacteria did increase hemolymph bactericidal activity, but did not protect the lobster against challenge with Aerococcus.

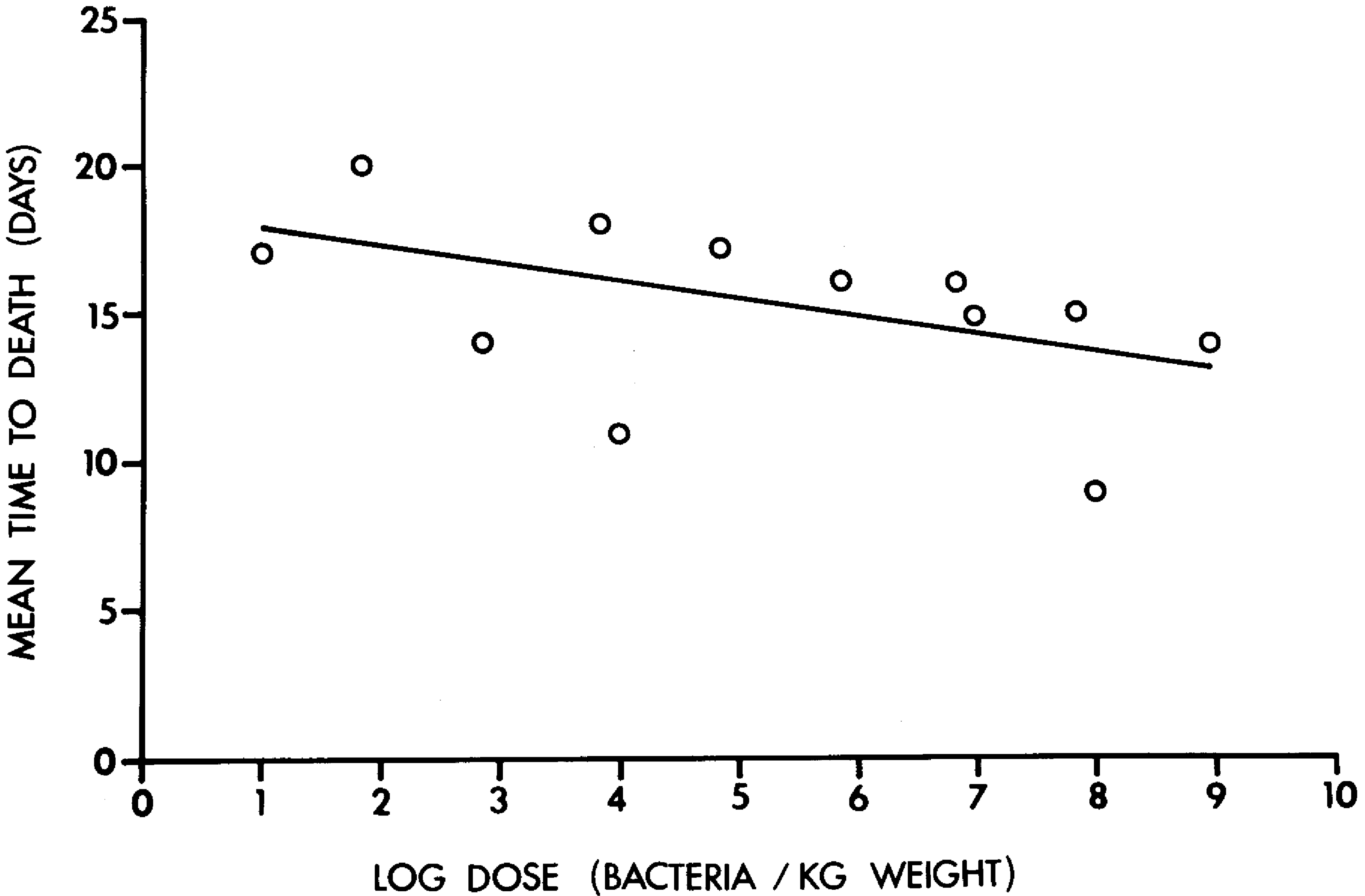

Figure 2. Relation of dosage of *Aerococcus* to mean time to death (MTD) in lobsters (calculated line of best fit for mean time to death, using experimental groups of 10 lobsters each). (redrawn from Cornick and Stewart, 1968).

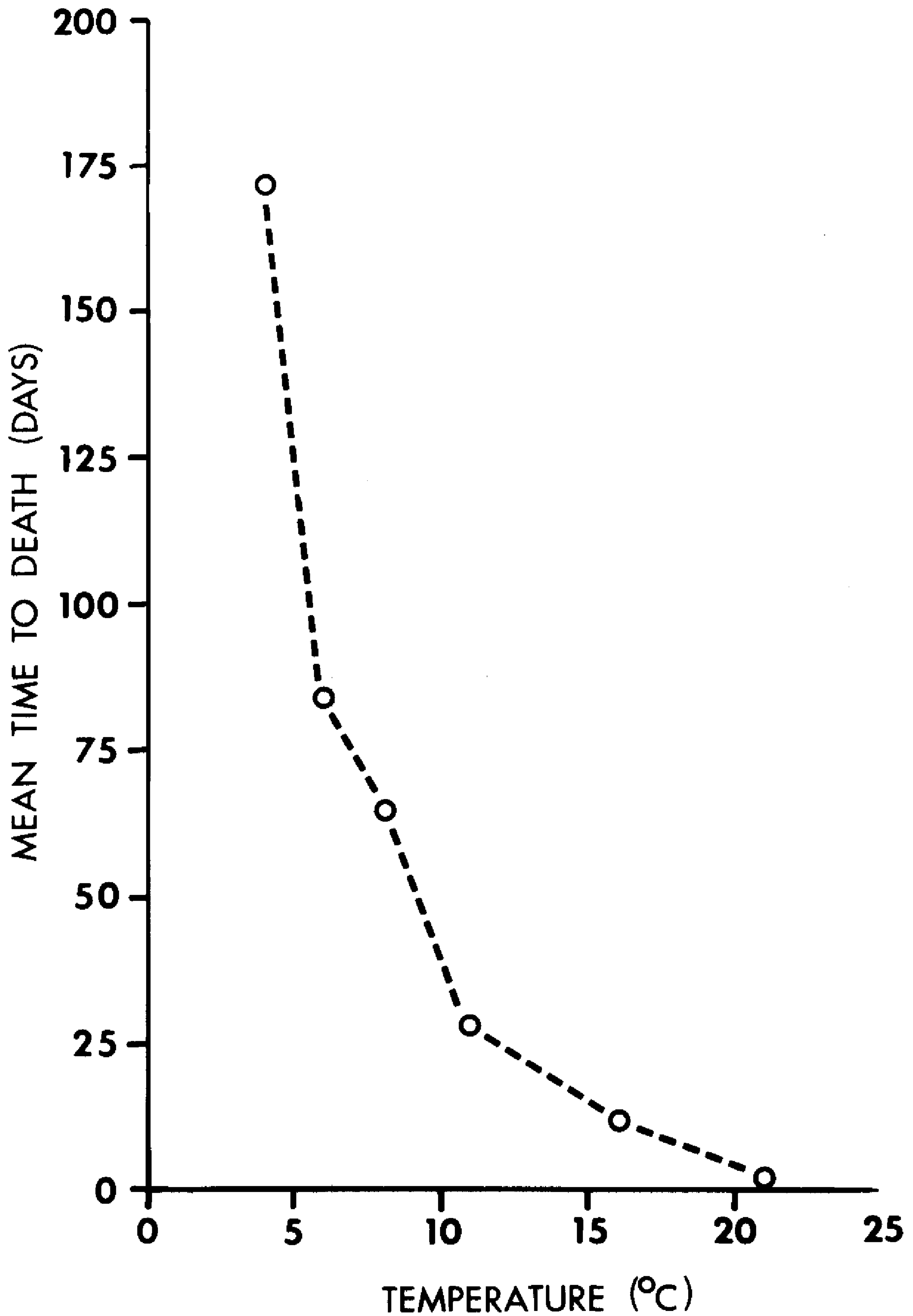

Figure 3. Relation of temperature to mean time to death in lobsters experimentally exposed to Aerococcus.

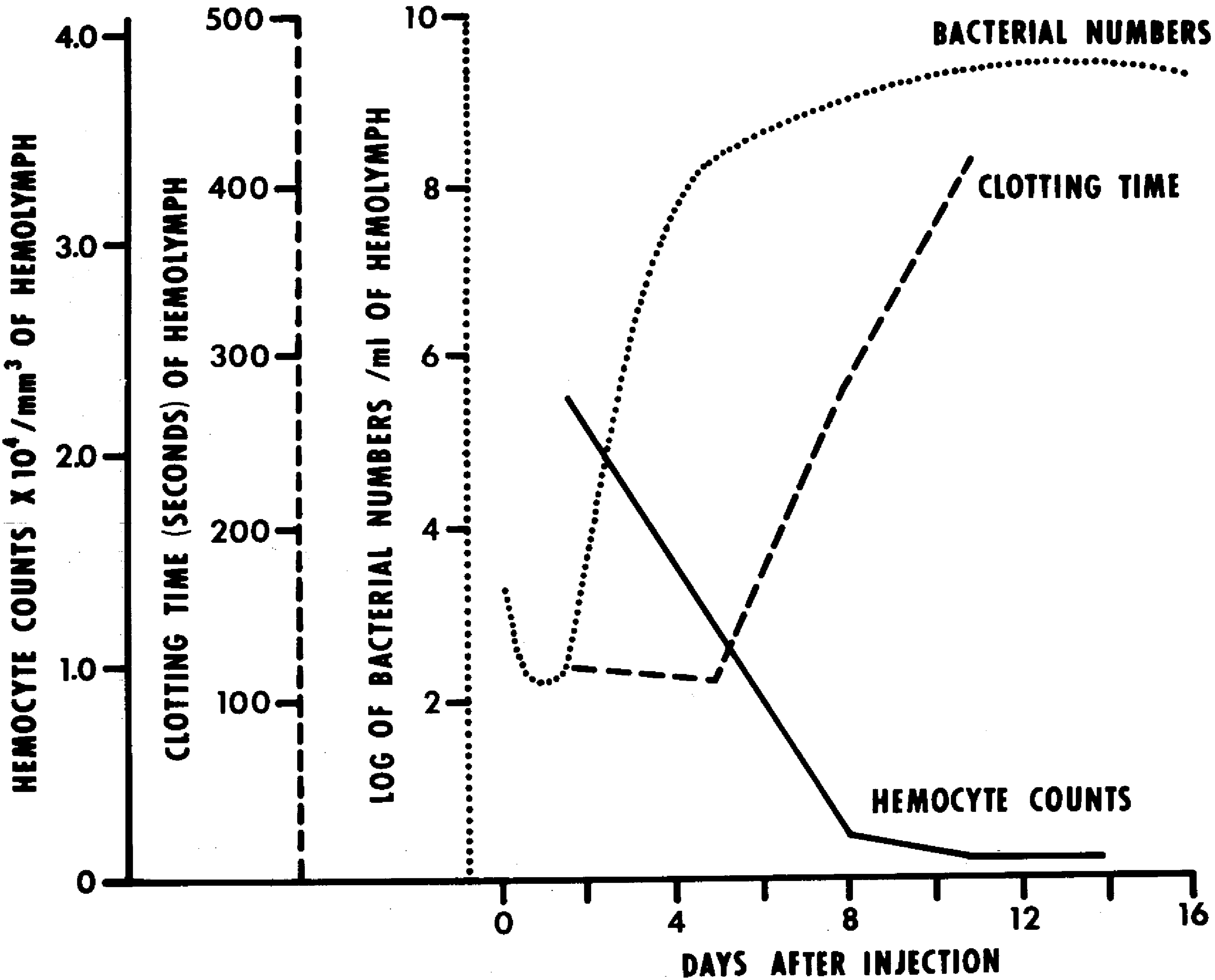

Figure 4. Relations of hemocyte counts, clotting time, and bacterial numbers to time from experimental exposure of lobsters to Aerococcus. (redrawn from Stewart et al. (1969a) and Stewart and Rabin, 1970).

Vaccines prepared from avirulent strains of the pathogen induced a degree of resistance (measured by increased time to death) to the virulent forms. Best results so far have been obtained by preliminary injection of the antibiotic Vancomycin, followed in 24 hours by injection of live pathogens -- a high degree of resistance was produced, and Stewart et al. concluded that the Vancomycin was acting as a metabolic inhibitor during the in vivo log growth phase of the pathogen. Cell wall synthesis seemed to be the target. Unfortunately, the resistance was transient. Challenge of resistant lobsters after 3 months produced 100% mortality, with a mean time to death identical to unvaccinated controls.

The Pacific spiny lobster, _Panulirus interruptus_, is also susceptible to gaffkemia. Schapiro and associates at San Diego State have increased resistance by injection of avirulent strains of the pathogen (Schapiro et al., 1974). The San Diego State group is now testing a number of avirulent strains of _Aerococcus_ of terrestrial origin as immunogens (Schapiro, personal communication).

The important conclusion that I make is that _larger crustaceans are immunologically active_ -- and we should be bright enough to understand and exploit this activity. Vaccines, I feel, will be important in future culture technology for the larger Crustacea.

But what of the _smaller_ Crustacea -- the copepods, amphipods, and cladocera -- with short generation times? Can we expect comparable immunological responsiveness to exist?

I don't know of any recent observations other than the early work on phagocytosis and the work in insect immunology, so I turn again to Dr. Stauber, who in 1961 stated:

> "That so few examples of acquired resistance are known among the smaller invertebrates may even be quite logical. Because of their relatively short generation time, their usual small size and often enormous reproductive capacities, subsequent epizootics would be much more likely to be circumvented by the appearance of resistant stocks through natural selection ....
> Even with very high mortality rates a residual stock of animals under favorable conditions later might repopulate an area ....".

This, in a sense, begs the question of acquired immunity in the miniature Crustacea -- a question which some of you may be willing to address in the future. Helpful insights may be gained from a review of present understanding of internal defenses of insects (Chadwick, 1967).

3. ENVIRONMENTAL INFLUENCES ON EPIZOOTIC DISEASES IN CRUSTACEA

The final link in our consideration of epizootics in Crustacea concerns the environment. I think most of us have probably taken it as axiomatic that environmental factors can influence the appearance, course, and severity of epizootics. But I think that in the case of the Crustacea two fairly recent phenomena have served to focus attention on this member of the triumvirate of host, pathogen, and environment.

The first is the gradual emergence of understanding of the effects of aquatic pollutants on crustaceans, and the importance of stresses caused by pollution in disease outbreaks.

The second is the experience with epizootics in crustacean aquaculture -- experience which repeatedly points to abnormal environmental factors (nutrition, water quality, temperature, salinity, and overcrowding as instigators of disease problems in cultured animals.

First the matter of pollution-associated diseases in Crustacea: Human activity has introduced or has increased environmental stresses for many aquatic animals. We have, for instance, added pesticides and other synthetic chemicals which can, even in low concentrations, drastically affect the physiology of fish and shellfish, and with which the species may have had no previous evolutionary experience. We have added heavy organic loads, in the form of sewage sludge and effluents, which can produce anaerobic or low-oxygen environments, and which are often accompanied by other contaminants such as heavy metals, that interfere with normal metabolism of aquatic animals.

During the past decade, several diseases and abnormalities of Crustacea have been described that seem associated with pollutant stresses. These can be categorized and discussed as: (1) Diseases caused by facultative pathogens related to contaminant stress, and (2) the special case of stress-provoked latent infections. A synergistic activity of chemical contaminants (or other form of pollutant stress) and an infectious agent seems to be a plausible explanation for at least some of the observed effects.

In Category 1 -- disease caused by facultative pathogens related to contaminant stress -- probably our best illustration is so-called "shell disease" or "shell erosion" or "black spot" disease or "brown spot" disease. This disease (or disease syndrome) has been associated with badly degraded estuarine and coastal waters in several parts of the world.

Lobsters and crabs from grossly polluted areas of the New York Bight were found in recent work of the Sandy Hook (N.J.) Laboratory of the National Marine Fisheries Service (Young and Pearce, 1975) to be abnormal, with appendage and gill erosion a most common sign. Skeletal erosion occurred principally on the tips of the walking legs, ventral sides of chelipeds, exoskeletal spines, on gill lamellae, and around areas of exoskeletal articulation where contaminated sediments could accumulate. Gills of crabs and lobsters sampled at the dump sites were usually clogged with detritus, possessed a dark brown coating, contained localized thickenings, and displayed areas of erosion and necrosis. Similar disease signs were produced experimentally in animals held for six weeks in aquaria containing sediments from sewage sludge dumping or dredge spoil disposal sites. Initial discrete areas of erosion became confluent, covering large areas of the exoskeleton, and often parts of appendages were lost. The chitinous covering of the gill filaments was also eroded, and often the underlying tissues became necrotic.

Dead and moribund crabs and lobsters have been reported on several occasions by divers in the New York Bight Apex, and dissolved oxygen concentrations near the bottom during the summer often approach zero. Low oxygen stress, when combined with gill fouling, erosion, and necrosis, could readily lead to mortality.

In a related study at the Sandy Hook Laboratory, Gopalan and Young (1975) examined "shell disease" in the small caridean shrimp, Crangon septemspinosa, an estuarine and coastal food chain organism on the east coast of North America -- important in the diets of bluefish, weakfish, flounders, basses, and other economic species. Examinations of samples of Crangon from the New York Bight disclosed high prevalences (up to 15%) of eroded appendages and blackened erosions of the exoskeleton. The disease condition was only rarely encountered at other collecting sites (Beaufort, N. C. and Woods Hole, Mass.). Histological examination of diseased specimens produced findings similar to those with crabs and lobsters. All layers of the exoskeleton were eroded; affected portions were brittle and easily fragmented; cracking and pitting of calcified layers occurred; and underlying tissues were often necrotic. Laboratory experiments using sea water from the highly polluted inner New York Bight resulted in appearance of the disease in 50% of individuals. Erosion was progressive; crippled individuals were cannibalized; and eroded segments of appendages did not regenerate after ecdysis. No disease signs developed in control animals held in artificial sea water. These little animals were really pitiful to behold -- with their tiny appendages often little more than blackened stubs.

A German study of the effects of industrial wastes (Schlotfeldt, 1972) on the shrimp, Crangon crangon, disclosed high prevalence of so-called "black spot disease", with signs very similar to those seen in Crangon septemspinosa from the New York Bight. Juvenile and adult shrimps from the polluted Föhr Estuary had black areas of erosion on the carapace and appendages, with necrosis of underlying tissues, and frequent loss of segments of appendages. The disease condition varied in prevalence seasonally, with a peak of 8.9% in summer. Lesions persisted and worsened after ecdysis. Experimental exposure to detergent aggravated and hastened the course of the disease.

Shell disease of Crustacea has of course been observed in many species and under many conditions, both natural and artificial (Rosen, 1970; Sindermann, 1970). Actual shell erosion seems to involve activity of chitinoclastic bacteria, with subsequent secondary infection of underlying tissue by facultative pathogens. Initial preparation of the exoskeletal substrate by mechanical, chemical, or microbial action probably is significant; thus high bacterial populations and the presence of contaminant chemicals in polluted environments -- as well as extensive detrital and epibiotic fouling of gills -- could combine to make shell disease a common phenomenon and a significant mortality factor in crustaceans inhabiting degraded environments.

Moving on to Category 2 -- stress-provoked latent infections -- there are at present published accounts of two viral diseases of marine invertebrates which suggest that latent infections may be provoked into patency by environmental stress. One, a baculovirus

infection of pink shrimp, was first recognized in stressed laboratory populations by John Couch (Couch, 1974a, 1974b). The other, a herpes-like virus infection of oysters, was discovered in a population held in a heated power effluent in Maine (Farley et al., 1972).

In Couch's work, a virus disease of pink shrimp reached patent levels and caused mortalities of 50-80% in shrimps exposed to the PCB Aroclor 1254 and to the organochlorine insecticide Mirex (Couch and Nimmo, 1974; Couch, 1974b). Other experiments in which the shrimp were crowded, but not exposed to chemicals, resulted in similar enhancement of virus infections -- suggesting that environmental stress may be an important determinant of patent infections.

Looking next at the other side of the environmental coin -- diseases associated with abnormal environmental factors in crustacean culture systems -- a clear relationship exists between frequency of disease problems and degree of departure of the culture system from some hypothetical ideal, in terms of water quality, nutrition, and population density.

While emphasis in aquaculture disease studies is on pathogens, _it should be clearly recognized that poor water quality and inadequate nutrition are often basic determinants of disease outbreaks_ and should be of primary concern in disease control.

It is important also to distinguish between what we can artificially label primary pathogens, such as _Aerococcus_ in lobsters, which can kill even when other environmental factors are adequate -- and _adventitious_ or _opportunistic pathogens_, which kill when other

physiological or environmental factors are poor or marginal. Included in the latter category would be many of the vibrios, pseudomonads and aeromonads. What we refer to as disease is often a reflection of one or more marginal environmental factors; nutrition, water quality, oxygen, temperature, salinity, and high bacterial populations.

Just as an example, we might examine some of the current disease problems in Malaysian prawn (Macrobrachium) culture. The second workshop on prawn culture was held in Charleston in 1976. At that workshop much was done to clarify the disease situation (which had been dismissed as a non-problem up to that time). Five disease entities were recognized and discussed:

(1) Shell disease;

(2) filamentous bacterial infestation;

(3) egg fungus infestation;

(4) ciliate (Epistylis) infestation;

(5) muscle opacity and necrosis.

What can be recognized about these diseases is that every one that has surfaced so far seems to be a consequence of poor water quality or some other form of stress, such as overcrowding. The disease organisms identified so far seem to be facultative rather than primary or obligate pathogens -- facultative in the sense that they exert their effects when physiological or environmental conditions become marginal for the host animal or population.

It is becoming apparent (at least to me) that too often in examining aquaculture mortality problems we focus narrowly on a search for pathogens, when the real cause may be environmental,

nutritional or physiological, with the superimposition of infection by facultative microorganisms on weakened or damaged hosts. This does not imply, however, that infectious diseases should be ignored -- simply that other causal factors should be considered, and water quality monitored constantly.

4. CRUSTACEAN VIRUSES

One final topic which should be included in this treatment of epizootics in Crustacea is that of the recently discovered viruses. The first virus disease of Crustacea was described a little over a decade ago by Vago (1966). Since then nine other viruses have been reported in the published literature (Table 1); an eleventh -- a rhabdovirus -- will be reported at this meeting by Yudin and Clarke; and others are undoubtedly waiting in the wings.

At least one virus -- baculovirus of shrimps -- has already been associated with a disease outbreak and mortalities in culture systems (Couch and Courtney, 1977), and it seems likely that this virus (or others) may emerge soon as a significant and major problem for commercial crustacean culture. Virus diseases are important in populations of the dominant group of terrestrial arthropods -- the insects -- so it is logical to expect a comparable role for these pathogens in aquatic arthropods as well. Several authors have already pointed out the similarity of the newly recognized crustacean viruses to groups found in insects (Bazin et al., 1974; Couch, 1974b; Federici and Hazard, 1975; Johnson, 1977).

One serious handicap in current studies of crustacean viruses is the absence of established serially passaged cell cultures of crustacean origin. Most of the viruses thus far recognized have

been described on the basis of electron microscopy and histopathology. Experimental studies have thus far been limited to the shrimp baculovirus, using direct host to host transmission, or viral material harvested from infected individuals. Primary explant tissue cultures or insect cell lines can be used at present to some extent, and several research groups have minor ongoing efforts in crustacean cell culture work, but there is need for a serious and substantial investment in developing the necessary methodology. Judging from the success in insect cell culture, the problem should be amenable to solution, using techniques developed for insect cells, if sufficient research effort is applied.

TABLE 1

CRUSTACEAN VIRUSES -- 1977		
Viral Description	Host	Author
Baculovirus penaei	Shrimp, *Penaeus duorarum*, from Gulf of Mexico	Couch, 1974
Herpes-like virus	Blue crab, *Callinectes sapidus*, from Chesapeake Bay	Johnson, 1976
Reovirus-like virus	Blue crab, *Callinectes sapidus*, from Chesapeake and Chincoteague Bays	Johnson and Bodammer, 1975; Johnson, 1977
Reovirus?	European crab, *Macropipus depurator*	Vago, 1966; Bonami et al., 1971; Bonami, 1973
Cytoplasmic S virus? (paramyxo and bunya viruses?)	European crab, *Macropipus depurator*	Bonami and Vago, 1971; Bonami et al., 1975
?paraspherical cytoplasmic virus in hemocytes	European shore crab, *Carcinus maenas*	Bang, 1971, 1974
Baculovirus-like virus	European shore crab, *Carcinus maenas*	Bazin et al., 1974
S virus? (in Y organ)	European shore crab, *Carcinus maenas*	Chassard-Bouchard and Hubert, 1975
Iridovirus	Freshwater cladoceran, *Simocephalus expinosus*	Federici and Hazard, 1975
Reovirus (cytoplasmic polyhedrosis virus)	Freshwater cladoceran *Simocephalus expinosus*	Federici and Hazard, 1975

CONCLUSIONS

How can we summarize all of this material on crustacean epizootics? Any generalizations can be dangerous, but some seem safer than others:

(1) A number of epizootics, with resulting mass mortalities, have occurred in crustacean populations and have been reported in the scientific literature.

(2) Frank virulent pathogens are usually associated with epizootics in natural crustacean populations, whereas facultative pathogens tend to emerge as causes of epizootics in culture populations. Such facultative organisms are often considered as benign or rare in wild populations.

(3) Because of difficulty of continuous observation, many epizootics and mass mortalities in Crustacea are probably not observed. Culture conditions do permit scrutiny of pathogens, particularly of larvae, that may be operative in wild populations as well.

(4) Epizootics involve an extremely complex interplay of host, pathogen, and environment. Resistance to infection plays a critical role, as does environmental stress. Recent experimental evidence from studies with larger Crustacea suggests that acquired immunity exists, and can be considered in epizootic disease. Other studies clearly identify a significant environmental component of disease in Crustacea.

(5) Recently discovered viruses of Crustacea raise important questions about their implications in population biology and aquaculture.

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Cytology and Cytochemistry of Hemocytes from the Freshwater Prawn *Macrobrachium rosenbergii*

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Hemolymph was withdrawn from sternal sinus, washed and suspended in van Harrevald's solution then placed on glass slides and examined with Zeiss phase contrast and Nomarski optics. Initially, hemocytes assumed a spherical shape which shortly changed to either spindle-shaped cells or to well-spread forms.

Hemocytes exhibited four types of granules: Type A (1-1.5 um, plastic, spherical), Type B (0.5-0.8 um, plastic, spherical), Type C (0.2-0.4 um, spherical), Type D (0.4-0.8 um rod). Cells were classified according to total number as well as distribution of granule types: Cytotype I - $\bar{X}$ = 169 granules (Type A 6.5%, Type B 71%, Type C 23%), Cytotype II - $\bar{X}$ = 233 granules (Type A 7.3%, Type B 36%, Type C 56%), Cytotype III - $\bar{X}$ = 70-90 granules (Type A 15%, Type B 36%, Type C 49%).

Nuclear morphology was similar in all cytotypes: spherical to ellipsoidal with prominent chromatin masses adhering to nuclear envelope.

Acid phosphatase - and NADH dehydrogenase - positive centers were identified in hemocytes and assigned to granule types. Functional role of granules was discussed.

Crustacean hemocyte types range in number from one in the brine shrimp, *Artemia* (Lochhead and Lochhead, 1941), to two and three types in the crayfish *Astacus fluviatillis* (Hardy, 1892; Tait and Gunn, 1918; Wood and Visentin, 1967) and four in the crayfish, *Cambarus boutoni*, and the blue crab *Callinectes sapidus* (George and Nichols, 1948; Toney, 1958).

Descriptions given by investigators are in some disagreement but the consensus concerning hemocyte types in Crustacea appears to be three basic types:

1) A hyaline cell sometimes referred to as a lymphoid cell containing none or relatively few granules,

2) a granulocyte containing numerous intracellular granules or "spherules," and,

3) an explosive corpuscle that was very unstable *in vitro* resulting in extensive cytoplasmic blebbing; the cell was described as relatively large containing only small granules and when blebbing was complete only the nucleus and a thin rim of cytoplasm remained.

A classification of prawn hemocytes is required before detailed physiological studies can be performed. As a consequence, specific cell functions can then be assigned to certain cell lines. This paper describes and classifies the hemocytes in *Macrobrachium rosenbergii*, as observed in *in vitro* preparation with phase - contrast microscopy.

MATERIALS AND METHODS

Prawns were obtained from the Aquaculture Laboratories, Mercer Generating Station, Trenton, N. J. Animals were maintained in 10-gallon, all-glass aquaria; temperatures were held between 23-26°C. Food consisted of Purina Marine Mix, Ration No. 25, fed daily ad libitum; diets were supplemented with fish and spinach twice weekly.

Hemocytes were collected and concentrated by a modification of the procedure of McKay and Jenkin (1970). Cysteine (40 mg/ml) was added as an anticoagulant to van Harrevald's salt solution for crayfishes (NaCl 0.205 M, $MgCl_2$ 0.0026 M, KCl 0.0054 M, $CaCl_2$ $2H_2O$ 0.0135 M, $NaHCO_3$ 0.0022 M - pH 6.2).

Hemolymph was collected from the sternal sinus with a sterile syringe and 21-gauge needle. Approximately 0.2 to 0.4ml of hemolymph was withdrawn into a 2ml glass syringe containing one milliliter of cysteine-vanHarrevald's solution. The contents of the syringe were thoroughly mixed and delivered into a graduated tube which was then centrifuged at approximately 20xg for 10 minutes. The supernatant was aspirated off and the hemocytes were gently resuspended in fresh van Harrevald's salt solution from which the cysteine was omitted. Three drops of the resuspended hemocyte solution were applied to clean glass coverslips rimmed with vaseline jelly to prevent desiccation during viewing. Coverslip preparations were allowed to sit for 5-10 minutes in a moist chamber before mounting to aid attachment of hemocytes to coverslips. Hemocytes in live preparation were observed and photographed with phase-contrast and Nomarski-interference optics of the Zeiss Photomicroscrope II.

Results

Freshly prepared cells invariably assumed spherical shapes when placed on glass coverslips (Fig. 1a); after several minutes they changed either to spindle-shaped forms (Fig. 1b) or well-spread granulocytes (Fig. 1c). Spindle-shaped cells are regarded as transitional forms since they always spread out into one of the definitive classes of cells.

Four types of granules from hemocytes were consistently observed, three of which (Types A, B and C) were used as criteria for cell classification (Figs. 2, 3).

(1) Type A granule - spherical, 1.0 to 1.5um in diameter, very dense and quite plastic;

(2) Type B granule - spherical, 0.5 to 0.8um in diameter and plastic;

(3) Type C granule - spherical, 0.2 to 0.4um in diameter;

(4) Type D granule - rod shape, 0.5 x 3um.

Cells were classified according to number and distribution of Types A, B and C granules (Table I):

(1) Cytotype I - contained an average of 76 granules (14.6% Type A, 36% Type B and 49.4% Type C) and comprised 5.5% of cell population (Fig. 2);

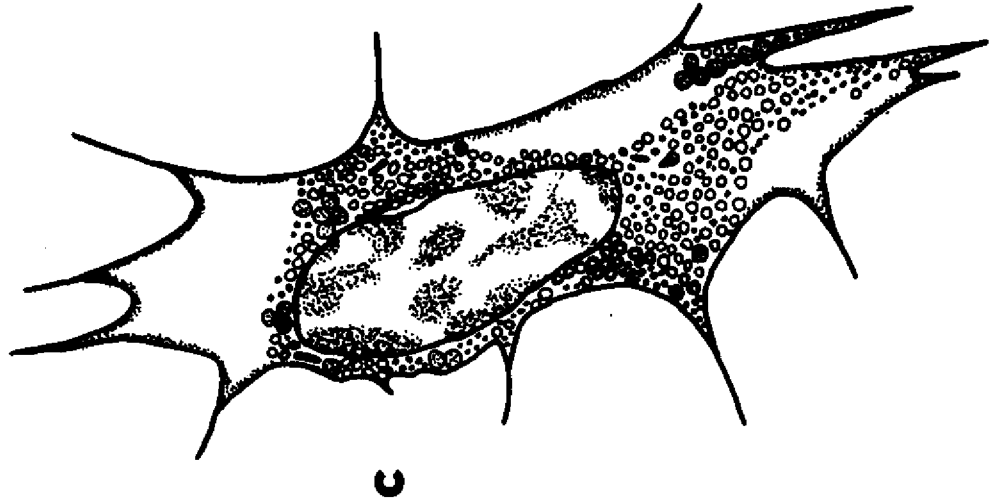
(2) Cytotype II - contained an average of 169 granules (6.5% Type A, 71% Type B and 22.5% Type C) and comprised 24.5% of cell population (Fig. 3);

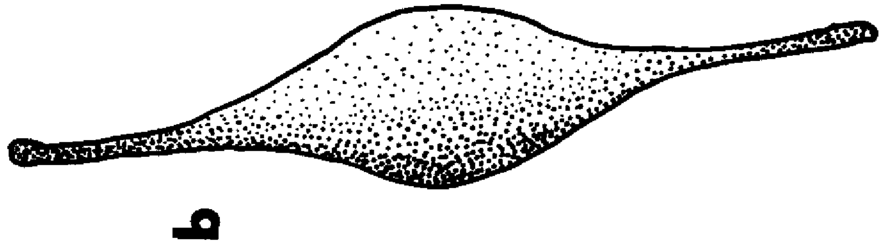
(3) Cytotype III - contained an average of 233 granules (7.3% Type A, 36.4% Type B, 56.3% Type C) and comprised 70% of cell population (Fig. 4).

Figure 1a. Spherical shape assumed by all hemocytes immediately after settling upon glass coverslips

Figure 1b. Spindle shaped cells would develop from spherical-shaped cells before spreading out into one of the definitive cytotypes

Figure 1c. Well-spread granulocyte which usually would develop from the spindle shaped forms





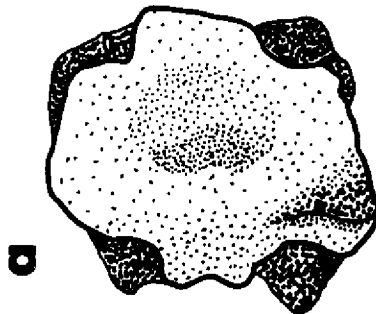


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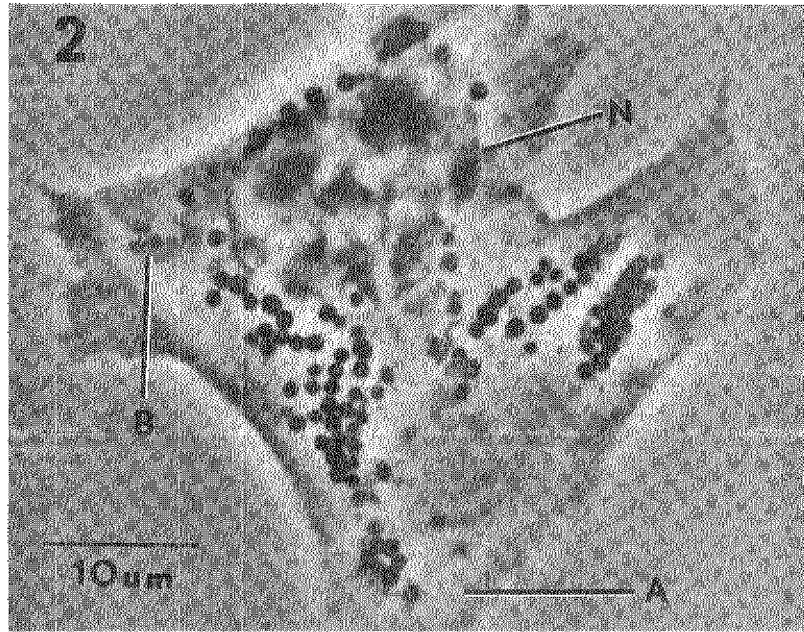
Figure 2. Cytotype I. Type A (A) and Type B (B) granules predominate in this view. Nucleus (N) shows large clumps of chromatin. Phase contrast of living cell.

Figure 3. Cytotype II. All four types of granules (A, B, C, D) may be seen. Note polarization of granules in cell process in upper left. Phase contrast of living cell.


2
N
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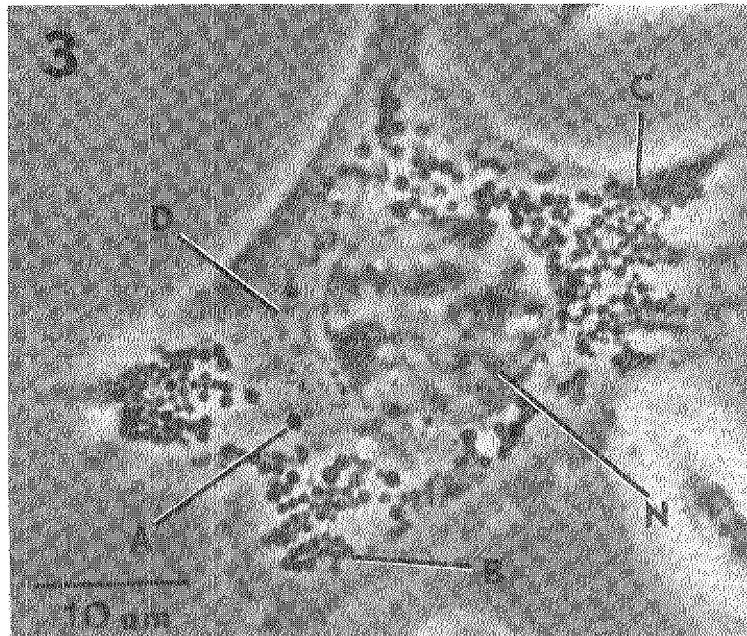

3
C
D
A
N
E
10 um

TABLE I

Cell Size, Nuclear Size and Granule Characteristics of Hemocytes of *Macrobrachium rosenbergii*

Hemocyte Type	Cell Size: Length $\bar{X}$	Cell Size: Length S.D.	Cell Size: Width $\bar{X}$	Cell Size: Width S.D.	Nuclear Size: Length $\bar{X}$	Nuclear Size: Length S.D.	Nuclear Size: Width $\bar{X}$	Nuclear Size: Width S.D.
I	31.1	5.1	21.4	5.7	15.5	2.3	9.2	2.5
II	29.2	3.7	19.6	5.7	13.6	1.5	9.6	1.7
III	30.7	5.6	20.1	4.2	13.7	1.5	10.2	1.7

Granule Composition

Hemocyte Type	Type A %	Type A $\bar{X}$No.	Type A S.D.	Type B %	Type B $\bar{X}$No.	Type B S.D.	Type C. %	Type C. $\bar{X}$NO.	Type C. S.D.
I	14.6	11	9.6	36	27	15.5	49.4	38	29.6
II	6.5	11	8.7	71	120	32	22.5	38	12.6
III	7.3	17	10.6	36.4	84.5	32	56.3	130.6	44

Cytotypes II and III were not very motile on glass coverslips. They would extend numerous filopodia from a scanty ectoplasm which served as attachment points to the glass substratum. Cytotype I was quite motile; frequently, slow undulations of the entire cell periphery were observed.

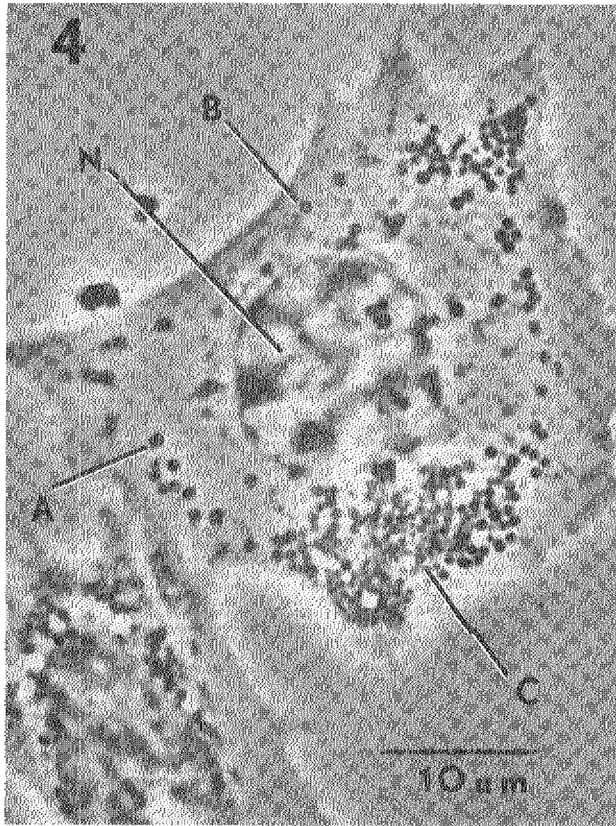
All cell types were approximately the same size, 30 x 20um (Table I); nuclear size averaged 14 x 10um (Table I) for all cytotypes. Nuclear morphology studied from living as well as from Giemsa-stained cells was constant in all cytotypes: chromatin material was gathered into 8-9 large masses scattered throughout nucleoplasm and adhering to nuclear envelope; all chromatin masses were interconnected by delicate strands of a chromatin network (Figs. 1, 4). Degranulated cells exhibited a nucleus radically changed in morphology: chromatin became fused into 3-4 large masses all associated with the nuclear envelop (Fig. 5) leaving the central portion of the nucleus filled with a clear fluid.

Reactions with acid phosphatase (Fig. 6) and NADH - dehydrogenase (Fig. 7) were always associated with granules: acid phosphatase - positive centers were usually confined to Type A granules while Type B granules were positive for the NADH - dehydrogenase reaction.

After thirty minutes on glass coverslips, many cells began to degranulate resulting in a highly vacuolated cytoplasm (Figs. 5, 7); the nucleus was filled with fluid while the chromatin was confined to several large masses associated with the nuclear envelope (Fig. 5).

Figure 4. Cytotype III. Note predominance of Type C (C) granules in lower left of cell. Type (A) and Type C (C) granules may also be seen. Phase contrast of living cell.

Figure 5. Cluster of four degranulated cells. Note characteristic clumping of chromatin (C) against nuclear envelope. Type A (A) granules may still be observed. Note Fibrillar nature of cytoplasm.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A
C
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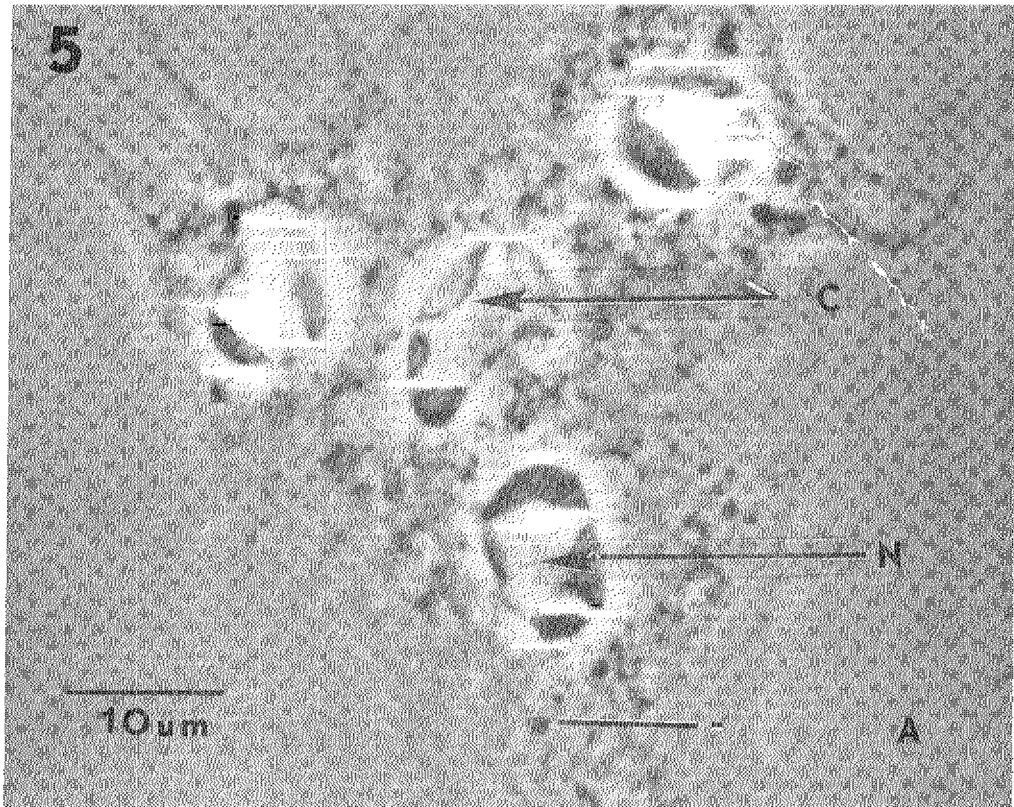
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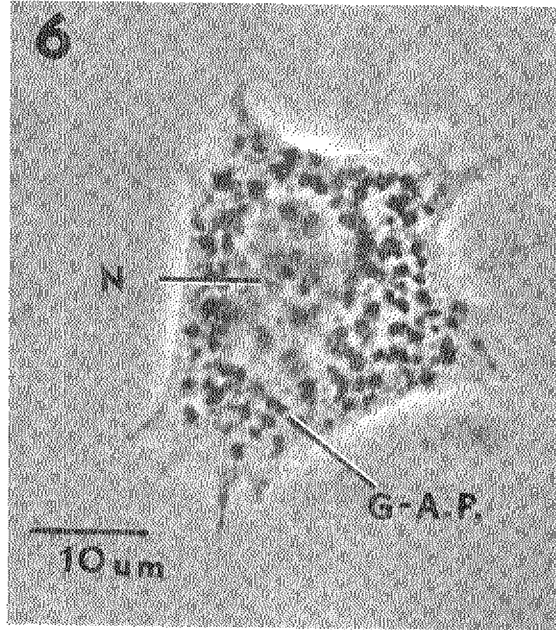
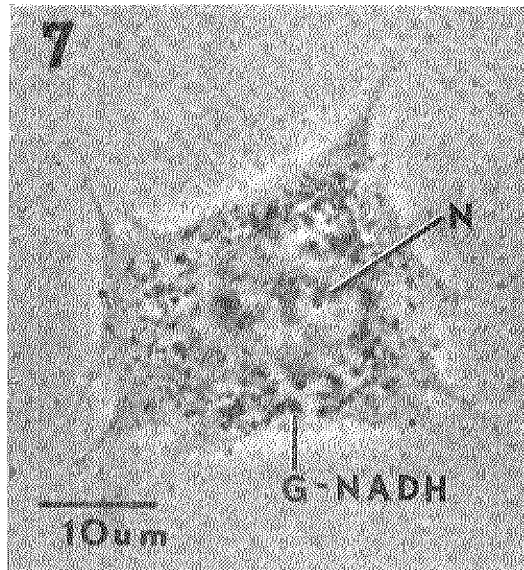
A

Figure 6. Cytotype II showing acid-phosphatase positive granules (G-A.P.) which appear to be Type Agranules.

Figure 7. Cytotype III showing granules positive for NADH - dehydrogenase (G-NADH) which appear to be Type B granules. Nucleus (N) is very prominent. Phase Contrast.


6
N
G-A.P.
10um


7
N
G-NADH
10um

DISCUSSION

Although crustacean hemocytes have been studied and classified (Hardy, 1892; Tait and Gunn, 1918; Wood and Visentin, 1967), no system described to date has used granule morphology as the major criterion for cell classification. In the present study, numbers of granules as well as their distribution were important in assigning cells to specific categories. While no true "hyaline" cells were found in *Macrobrachium*, their presence has been widely described in Crustacea (Tait and Gunn, 1918; Wood and Visentin, 1967). The present work did describe a cell, Cytotype I (Fig. 2), that had relatively few granules (less than 100) but no agranular cells were found in this study that spanned 14 consecutive months.

No significant differences in cell size or nuclear morphology could be discerned between the three cytotypes described (Table I). It is entirely possible that only one cell type exists and the three types described here are different stages of that cell line. If this is true, then Cytotype I would be the most immature stage since it contains the fewest granules and is quite motile; Cytotypes II and III (Figs. 3, 4) would represent stages in the so-called "Maturation Compartment" (Mix, 1976) and would be technically classified as granulocytes. Wood and Visentin (1967) arrived at this same conclusion working with the hemocytes of *Orconectes.* The concept that the hyaline cells are early stages of the mature granulocytes is not new: it was postulated by Cuenot (1891) and Bruntz (1905, 1907) and given a more modern interpretation by Mix (1976). We agree with Wood and Visentin (1967) that "a definitive study of the origin and differentiation of crustacean blood cells is indicated."

More work is needed before the various granules described can be assigned functional roles. Studies with acid phosphatase suggest Type-A Granules associate with this enzyme (Fig. 6); thus, these granules may be lysosomes. NADH-dehydrogenase reactions are usually found in Type-B Granules (Fig. 7) which suggest a respiratory function and indicates that such bodies may be mitochondria.

It is not known which cell type exhibits the extensive degranulation with marked nuclear changes seen in our preparations. These "explosive corpuscles" have been described and figured (Hardy, 1892; Tait, 1910; Tait and Gunn, 1917); Hardy (1892) also described the peculiar changes in the nucleus during this degranulation process. Present work with phase-contrast optics using time-lapse cinematography should shed additional light on this process.

Now that hemocytes of *Macrobrachium rosenbergii* have been described and classified, detailed physiological studies of these cells can follow.

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GILL HISTOPATHOLOGIES OF VARIOUS GULF CRUSTACEANS

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A survey of the histopathologies of various crustaceans in the Gulf of Mexico on the continental shelf of south Texas has been conducted. The most common pathological conditions observed in these crustaceans were in the gills. Almost all animals studied had various symbionts among their gill filaments most of which did not appear to be pathological. Crabs had several different metazoan symbionts which were apparently ectocommensal, while the only metazoan symbionts found in shrimp gills were parasitic trematode larvae. Most of the gill symbionts in shrimp and stomatopods were protozoans. One pathological condition, especially prevalent in the rock shrimp, *Sicyonia dorsalis*, the brown shrimp, *Penaeus aztecus*, and the spider crab, *Anasimus latus*, was caused by an apostome ciliate of the genus *Synophrya*. Not all of the pathological conditions believed to be caused by stages of infection by this protozoan could be ascertained, as the ciliate itself could not be discerned in the beginning stages of infection. Injury or other unknown diseases may have caused similar appearing histopathologies.

INTRODUCTION

The importance of marine invertebrates to the natural balance of nature in the waters of continental shelves is well known, and particularly their importance in the food chain for fish and directly as food for man is undeniable. Yet very little is known about the natural conditions in which these animals live. With man's increasing intervention in the continental shelf waters by oil and gas drilling operations as well as the increase in pollution of these waters from rivers, natural conditions must be known before it is too late.

Therefore we have undertaken a histopathological survey of benthic marine invertebrates in the south Texas region of the continental shelf. Such surveys in any specific area are rare if not nonexistent. Our collections, which were conducted from July 1976 through October 1977 excluding September of both years, and January and May of 1977, consisted mostly of crustaceans. The gills of these animals had the highest incidence of pathological conditions. These conditions will be described in this report.

MATERIALS AND METHODS

Collections were made monthly from July 1976 through October 1977 excluding September of both years, and January and May of 1977. Specimens were collected in a 35 foot otter trawl from the research vessel, the "Longhorn", of the University of Texas at 3 stations in the Gulf of Mexico. The stations were located on a transect SE from Corpus Christi Bay, Texas. Station 1 is at a depth of 22 m and is about 6 miles out from Mustang Island, station 2 is at 49 m and is about 30 miles out, and station 3 is 130 m deep and about 50 miles from shore. About 75% of the specimens collected were crustaceans, the rest being bivalves, squid and a few echinoderms. Collections were made at night, as most marine invertebrates bury in the mud during the day.

Useable specimens from each trawl were placed in a tank with running sea water. One at a time the animals were immediately dissected if large enough to dissect without the aid of a dissecting microscope, or if too small, opened sufficiently to allow penetration of the fixative, Zenker's solution. Various tissues were

taken including the gills. Further routine processing of the tissues occurred in the laboratory at Texas A&M. At least two sets of six serial sections of the gills were stained in chlorazol Black E and two sets in PAS with hematoxylin and Alcian Blue. A few sets of extra serial sections of various gill tissues were stained with a method for gram positive and gram negative bacteria in tissue sections (Brown and Brenn, 1931).

Shrimp used in this study were 114 *Penaeus aztecus*, 24 *Penaeus setiferus*, 45 *Trachypenaeus similis*, 56 *Sicyonic dorsalis* and 9 *Solenocera vioscai*. Stomatopods used were 17 *Squilla chydaea* and 10 *Squilla empusa*. Crabs used were 64 *Callinectes similis*, 52 *Portunus spinicarpus*, 14 *Portunus gibbessii*, 33 *Anasimus latus*, 5 *Ranioides louisianensis* and 6 crabs of the family Goneplacidiae.

RESULTS AND DISCUSSION

Metazoan symbionts were more commonly found associated with crab gills than with those of shrimp or stomatopods. Unidentified flatworms (Fig. 1) were observed in *Callinectes similis* and *Portunus spinicarpus*. Nemerteans (Fig. 2) were found in these crabs plus *Anasimus latus*, and coelenterates (Fig. 3) were present among the

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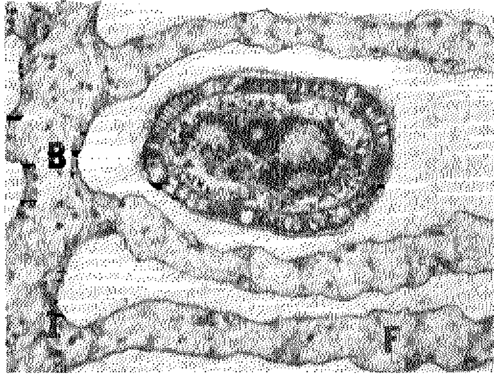
Fig. 1. Flatworm among gill filaments of *C. similis*. X200. Fig. 2. Nemertean between filaments of *A. latus*. Note small aggregates of cells in gill filaments. X75. Fig. 3. Coelentrate by filaments of *A. latus*. X100. Fig. 4. Two barnacles attached to *C. similis*. X30. Fig. 5. Unknown pathological organisms attached to gills of *A. latus*. Arrow points to necrotic part of gill. X 175. Fig. 6. Trematode metacercaria in branchial sinus of *P. aztecus*. X75. Fig. 7. Ciliate (arrow) by gill filament of *S. chydaea*. X200. Fig. 8. Ciliate attached to filament of *S. dorsalis*. X300. B = branchial sinus; D = dirt between gill filaments; F = gill filaments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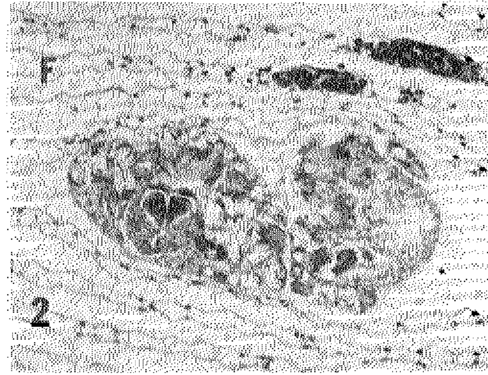
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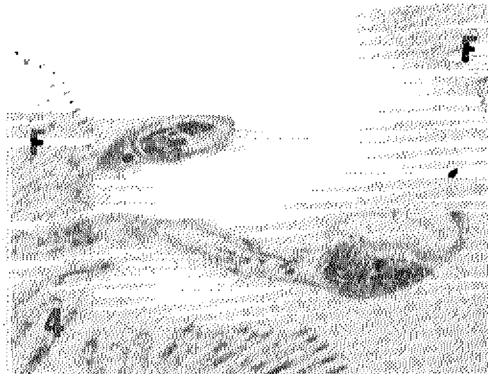
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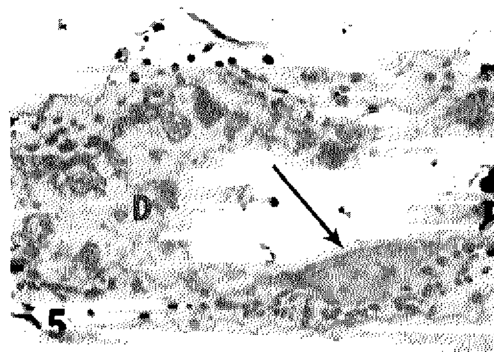
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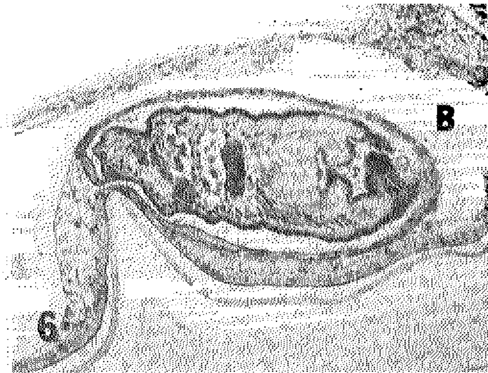
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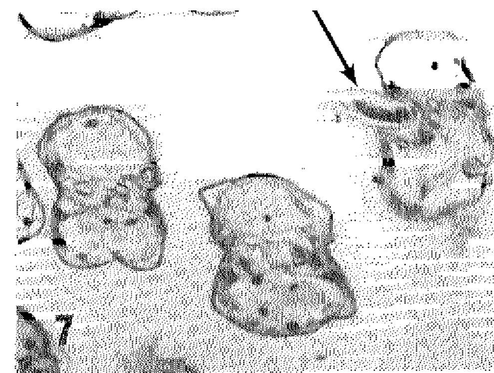
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gill filaments of C. similis and A. latus. Also, because we were not able to completely serial section the total gill tissue, we frequently saw only small portions of symbionts which were insufficient for identification. One each were found in the shrimp Penaeus aztecus and Trachypenaeus similis and a Goneplacid crab, 3 in P. spinicarpus, 8 in C. similis and 9 in A. latus. Almost all symbionts appeared to be nonpathological, but occasionally they caused thickening of the cuticle and/or swelling of the adjacent gill filaments.

In all cases of ectocommensalism in these crustacean gills, no heavy infestations were found. Such infestations could cause damage to the host merely by blocking the passage of water, and therefore respiratory exchange, to these animals.

Barnacles were found among the gills of C. similis and P. spinicarpus (Fig. 4). Their attachment sites were not observed in detail, but they caused some damage to the gills at the region of attachment. In the spider crab, A. latus, a dorvillid polychaete frequently occurred in one or both gill chambers and did not appear to be pathological. However, an unknown organism (an alga or protozoan) was found attached to the gills of three of these spider crabs which caused considerable damage. Swollen gill filaments, thickened cuticles and necrotic areas were observed among the infected gills (Fig. 5).

Shrimp rarely had metazoan symbionts among their gill filaments but they were the only crustaceans to have metazoan parasites in internal regions of the gills. Trematode metacercaria were found in the branchial sinuses of 6 Penaeus aztecus and in 2 P. setiferus (Fig. 6).

No metazoans were present among the stomatopod gills, but ciliates believed to be Synophrya sps., a suctorian, and another unidentified ciliate (Fig. 7) were found.

The most common symbionts in shrimp gills were protozoans. A stalked ciliate was attached to the gill filaments of almost half of the Sicyonia dorsalis observed and in a few specimens of P. aztecus (Fig. 8). A slight thickening of the gill cuticle at the point of attachment of the ciliate appeared to be the only damage caused by these protozoans. One case of parasitism by Nematopsis sps. was found in the gills of P. aztecus.

The shrimp studied usually had a ciliate present among their gill filaments. It is believed to be a Synophrya sps. (Fig. 9). The genus Synophrya, an apostome ciliate, has a relatively complex life cycle which was described by Chatton and Lwoff (1935). Apostome ciliates are known to parasitize marine crabs and shrimp (Chatton and Lwoff, 1935; Sprague and Couch, 1971; Johnson and Bradbury, 1976).

We have not seen these ciliates entering the gill tissues but an increase in the number of host cells in the tissue may be the host's first reaction (Fig. 10). Then a necrotic area forms (Fig. 11) which increases in extent. The distal portion of the lesion finally

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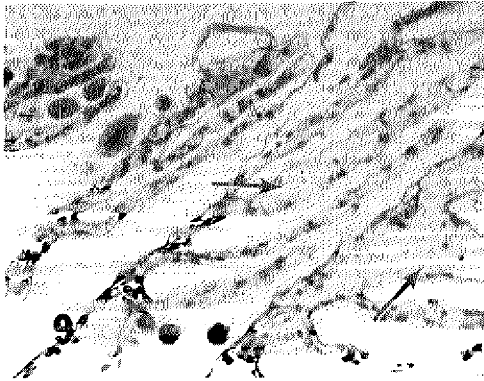
Fig. 9. Synophrya sps. Arrows point to swollen filaments of S. dorsalis. X175. Fig. 10. Possible first stage of infection by Synophrya sps. in S. dorsalis. X175. Fig. 11. Possible second stage of infection by Synophrya sps. in S. dorsalis. Arrow points to necrotic area. X200. Fig. 12. Tomont stage of Synophrya sps. in S. dorsalis. X175. Fig. 13. Trophont stage of Synophrya sps. in S. dorsalis. Note free ciliates. Atrophied gill tissue frequently chatters upon sectioning. X175. Fig. 14. Trophont stage of Synophrya sps. in the middle of a filament in S. dorsalis. X175. Fig. 15. Small aggregate of cells in P. spinicarpus. X300. Fig. 16. Large aggregate of cells with necrotic area (arrow) in C. similis. X75. A = atrophied tissue; S = Synophrya sps.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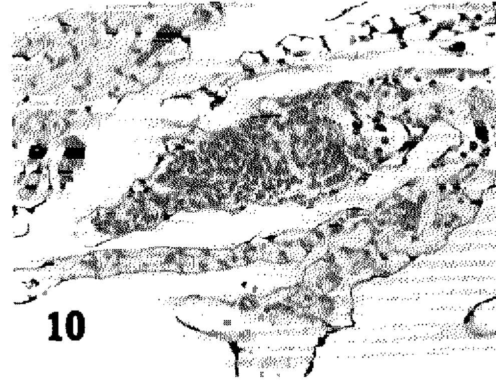
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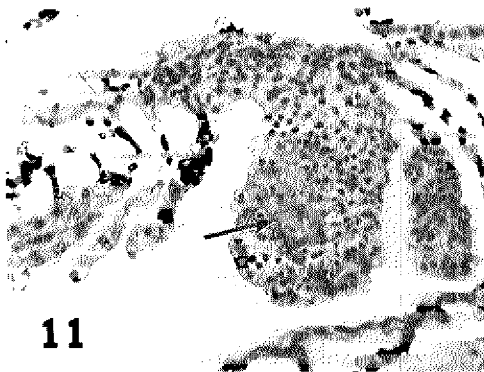
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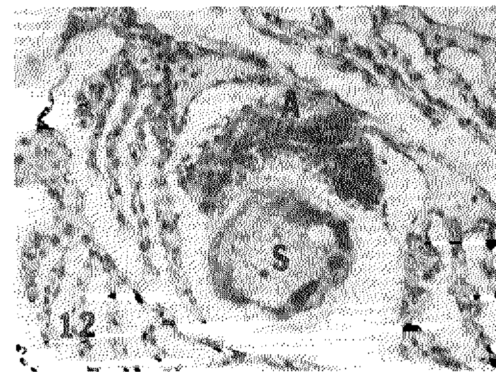
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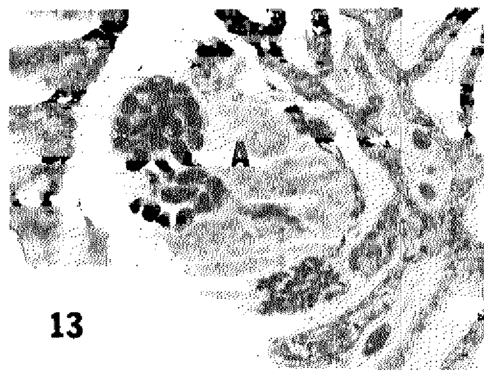
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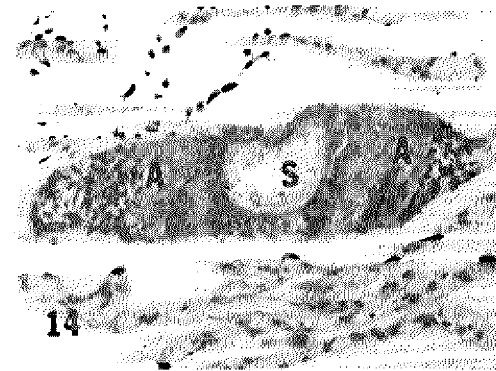
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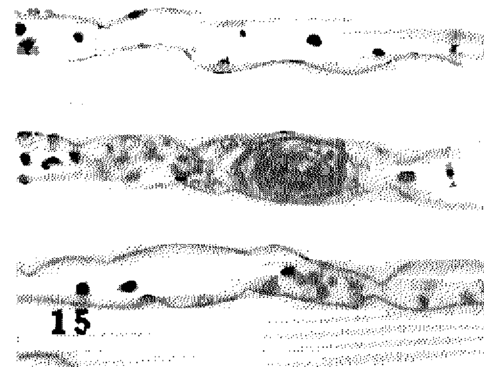
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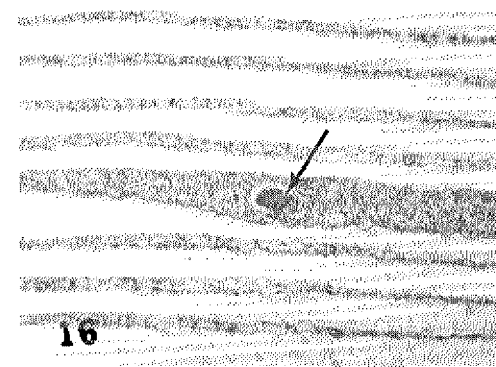
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becomes atrophied and stains PAS+, while the proximal part is swollen with excess cells grading distally to necrotic tissue. At this stage, the protozoan is visible as a large macronucleus distal to the atrophied gill tissue (Fig. 12), and is termed the tomont by Chatton and Lwoff (1935). The tomont undergoes division and forms many new ciliates (Fig. 13) which are called trophonts. These will feed on the host's exuvial fluid after excystation. Then they encyst on the substrate and divide again, finally producing many tomites, the infective stage of these ciliates. Dr. Phyllis Bradbury (personal communication) identified the ciliate in the tomont stage for us, and since the new ciliates formed in the gill tissue appear to be like those found free among the gill filaments (Figs. 9 and 13), it is presumed all of these ciliates are _Synophrya_ sps.

Lesions caused by _Synophrya_ sps. were usually found in the terminal branches of the gills but could occur anywhere, including tissue adjacent to the branchial sinuses so that the sinuses were partially blocked by excess cells and necrotic tissue. Lesions also occurred in the central part of a filament rather than at its terminal portion. In these lesions, the tomont stage was formed in the center with swelling, excess cells, necrotic regions and atrophied tissue on both sides (Fig. 14).

A longitudinal tissue section containing parts of 6 gills may contain more than 25 lesions, especially in _Sicyonia dorsalis_, or only 1 gill may have a lesion. Gross inspection of the gills of infected animals showed small dark spots sprinkled throughout the filaments.

Although none of the free ciliates were seen among crab gills, the tomont stage has been identified. Development of the lesions similar to those seen in the shrimp were found in the gills of the crabs *Callinectes similis*, *Portunus gibbesii* and *Anasimus latus*. Various small aggregates of cells, usually with the cells arranged concentrically, and possible with thickening of the cuticle and/or necrotic areas were observed (Fig. 15). Gradations of abnormal filaments from the small aggregates of cells to entire filaments swollen with excess cells were observed (Fig. 16). Since many of the latter did not contain necrotic areas, they may have been sagittal to the central lesion, as crab gill filaments are flat sheets of tissue perpendicular to the long axis, and the tissue was sectioned longitudinally. The lesions caused by *Synophrya* did not entail the entire filament. At a further stage of infection, if the lesion occurred in a distal portion of the filament, the end was atrophied, and it graded proximally to necrotic tissue followed by excess cells, the whole being swollen (Fig. 17). This atrophied region, necrotic tissue and excess cellularity is a reaction by the host to wall off the ciliate in both crabs and shrimp. Finally in the tomont stage (when the ciliate is visible as a large macronucleus), the ciliate was found distal to the atrophied part of the gill tissue (Fig. 18) as in shrimp. Occasionally a large part of the gill adjacent to the

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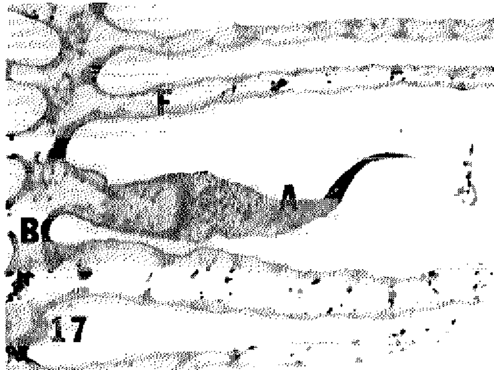
Fig. 17. Pathological condition probably caused by *Synophrya* sps. in *C. similis*. X100. Fig. 18. Tomont stage of *Synophrya* sps. in *C. similis*. X70. Fig. 19. Tomont stage of *Synophrya* sps. in *A. latus*. X75. Fig. 20 Labryinthylid in *C. similis*. X75. Fig. 21. Hyperplasia in *P. setiferus*. X90. Fig. 22. Cyst in the branchial sinus of *C. similis*. X230. Fig. 23. Cyst in the branchial sinus of *P. aztecus*. X175. Fig. 24. Deformed filaments in *C. similis*. X70. A = atrophied tissue; B = branchial sinus; F = filament; S = *Synophrya* sps.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A
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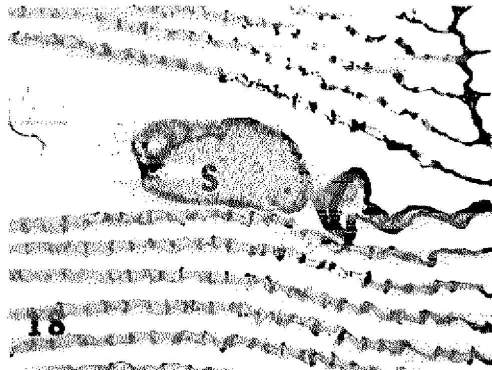
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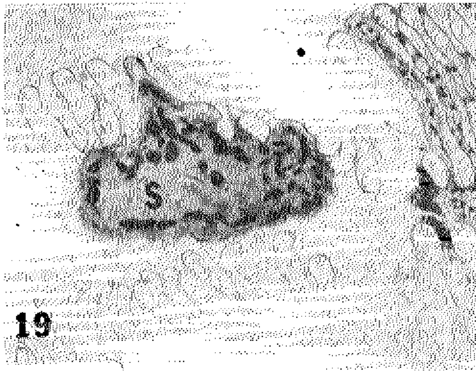
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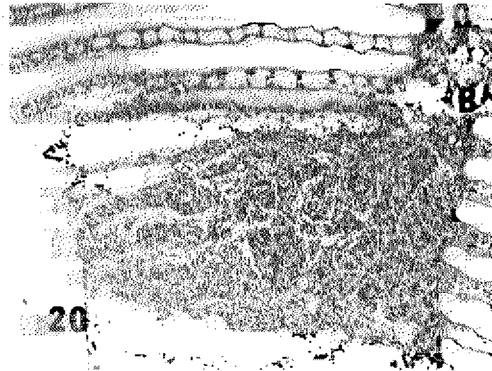
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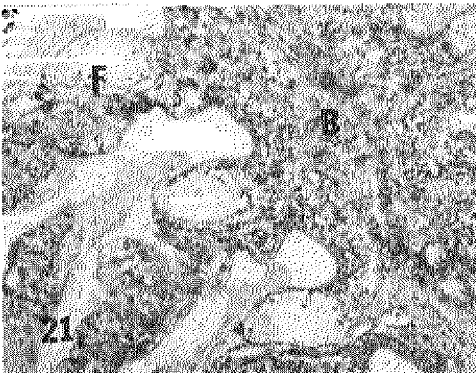
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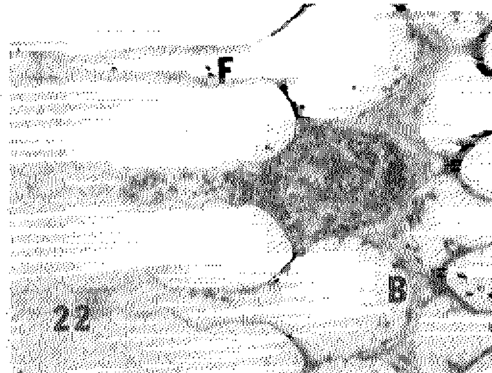
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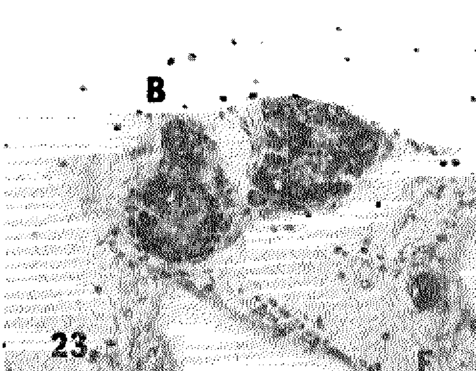
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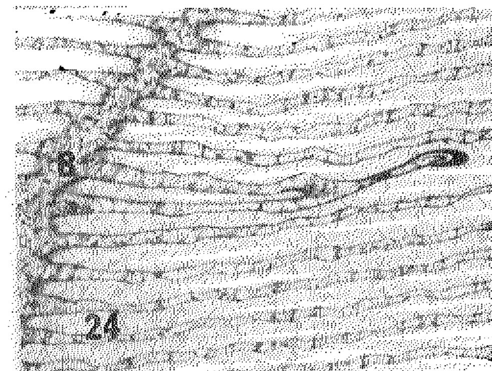
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tomont was devoid of host tissue or even debris except for the cuticle (Fig. 19). Again, as in shrimp, if the infection occurred in the center of a filament, necrotic tissue and excess cellular areas were on both sides of the ciliate. Also, as in shrimp, lesions close to the branchial sinus grew into that region and caused gross deformation of the area as well as partial blockage of the blood stream.

The tomont stage was seen only in Anasimus latus, Callinectes similis and Portunus gibbesii, and formation of the trophonts was not observed in any crab. Portunus spinicarpus had all the types of lesions which may be caused by this ciliate, but no tomont stages were observed. Since only a few specimens of Raninoides louisianensis and of the Goneplacid crab were studied, the absence of all lesions, except for a few small aggregates of cells, may not be sufficient to rule out infection by an apostome ciliate in these animals.

In both shrimp and crabs, all of the lesions described as early stages of infection (before the tomont stage) probably were not solely due to Synorphrya. Other diseases of unknown etiology or injury may have caused the same types of lesions. However, many of the large aggregates of cells in crab (Fig. 16) and shrimp (Figs. 10 and 11) gill gilaments were traced in the sets of serial sections. A few of these proved to have been sagittal to lesions with atrophied and necrotic areas and occasionally a tomont (Figs. 12, 17 and 18).

Fungi in the Order Plasmoidophorales cause internal lesions which resemble neoplasms due to their hyperplastic nature (Mackin and Schlicht, 1976). A few such lesions caused by a labryinthylid

were present in the gills of *Callinectes similis*, *Penaeus aztecus* and *Trachypenaeus similis* (Fig. 20). Hyperplasia, etiology unknown, was found in *C. similis*, *Penaeus setiferus* and *Sicyonia dorsalis* (Fig. 21).

Cysts were relatively common in the branchial sinuses of all crabs except *Raninoides louisianensis* (Fig. 22) and all shrimp except *Solenocera vioscai* (Fig. 23). Their etiology is unknown. Some tissues containing cysts were stained for bacteria with negative results.

Swollen filaments, consistently present in *Sicyonia dorsalis* (Fig. 9), and frequently observed in *Callinectes similis* and *Portunus spinicarpus* may be artifacts. However, since all filaments in the same gill or different gills in the same section were not affected, the possibility of a pathological condition exists.

C. similis, *P. spinicarpus* and *A. latus* exhibited deformed filaments, either one filament formed into two parts distally or two filaments joined into one distally (Fig. 24). These deformed filaments may be genetic or due to previous pathological conditions which healed improperly.

ACKNOWLEDGEMENTS

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GROSS AND MICROSCOPICAL OBSERVATIONS ON GILLS OF ROCK CRABS (*CANCER IRRORATUS*) AND LOBSTERS (*HOMARUS AMERICANUS*) FROM NEARSHORE WATERS OF THE EASTERN UNITED STATES

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Rock crabs, *Cancer irroratus*, were collected from Sandy Hook and Raritan Bays, New Jersey, the New York Bight Apex near Ambrose Light, and from coastal waters of the eastern United States, ranging from Maine to North Carolina. American lobsters, *Homarus americanus*, were collected only from the New Jersey-New York stations. Visual examinations of gills were made to record their condition as clean, discolored, or black, and histological studies were made to study gill-fouling microorganisms and internal parasites. Fouling organisms included bacteria, diatoms, peritrich and suctorian sessile ciliates, amoebae, and copepods. Internal parasites included amoebae, dinoflagellates, probable microsporidans, gregarines, acanthocephalans, and larval cestodes, trematodes, and nematodes. Evidence of bacterial phagocytosis and cellular nodule formation with melanization was extensive in gill, hepatopancreas, and hindgut hemolymph channels. Gross observations on over 3,000 specimens and histological findings in over 1,000 of them are summarized.

INTRODUCTION

During the past 5 years we have been making visual and histological studies on gills of the rock crab, *Cancer irroratus*, and the American lobster, *Homarus americanus*. Most of the animals were collected in waters of the New York Bight Apex near Ambrose Light, and Sandy Hook and Raritan Bays, New Jersey. Both areas are known to be affected by commercial, industrial, and residential pollution. Initially, all animals were examined for evidence of gill discoloration or blackening, and subsamples were taken for

histological examination. Microscopic studies were made to provide data on the types of microbial organisms associated with gill-fouling. Further work was designed to compare gill color as it was associated with various types of seabottom sediment (clean sand, black sludge, etc.), or with frank disease and tissue destruction.

Analyses presented here are incomplete and are presented only as a preliminary report. Observations are summarized to provide an account of our findings in the New York Bight Apex and to further document the need for additional studies in unstressed marine environments. Our literature search indicates that there are few references to comprehensive histological studies on rock crabs or lobsters native to waters of the northeastern United States. We are indebted to the MESA Project Office, National Oceanic and Atmospheric Administration, U. S. Department of Commerce, Stony Brook, New York, for supporting our studies. Continued research, based on our initial observations in perturbed study areas, should provide some of the answers to basic questions that are raised by our observations.

The following report summarizes topics presented in three separate abstracts that were presented at the Crustacean Health Workshop held in Galveston, Texas. The abstracts summarized our findings in three areas of study: (1) microbiota associated with gill-fouling, (2) histological evidence for tissue response to disease-causing agents, and (3) a summary of the parasites present in certain tissues and organs of affected crustaceans. The presence of several parasites in rock crabs and lobsters provides

new host records for some, and extends the geographic range of others.

METHODS

Two thousand and ninety-one rock crabs and 202 lobsters were collected from Sandy Hook and Raritan Bays, New Jersey, and the New York Bight Apex. Tows were made with an otter trawl for periods ranging from 15-30 minutes. All specimens were sexed, measured, and opened to permit gross evaluation of gill condition (clean, discolored, without black gills, black gill color). Subsamples of 679 of the rock crabs and all of the lobsters were processed for histological examination by placing intact gills and portions of the hepatopancreas and hindguts into vials containing Davidson's fixing solution. Fixed tissues were processed by routine methods, sectioned at 6 μm, and stained (see figure legends).

Specimens from the New Jersey-New York area were collected frequently and in large enough numbers to facilitate future statistical analyses on the influence of yearly seasons, water depth, year-class, and sex on gill-fouling microorganisms. Limited collections of rock crabs were made in areas distant from the New Jersey-New York sites to compare fouling species from less polluted environments. The numbers of animals and their source are summarized in Table 1.

Several rock crab gills which represented a clean condition and several which represented the most serious conditions of fouling and black discoloration were processed for scanning electron microscopy in order to visualize their outer surfaces (Bodammer, 1976).

RESULTS

Visual examination of intact gills from 200 lobsters taken from New Jersey-New York waters showed that virtually all of them were clean. Only four of the lobsters exceeded 21 cm in total length which suggested that most were subadults and probably were undergoing frequent molts with subsequent shedding of the gill cuticle and associated fouling organisms. In contrast, 2,091 rock crab gills were examined and, without taking molting activity and seasonal variations into account, 67 percent of them had clean gills. When seasonal differences were taken into account (Table 2), rock crabs with clean gills ranged from 23 to 72 percent with the higher value coincident to molting activity. Black gills (50 percent or more of the gill black) were noted in 4 percent of the New Jersey crabs and 7 percent of the New York crabs.

Other collections of rock crabs were made to obtain data on gill condition in coastal waters ranging from Long Island, New York to Cape Hatteras, North Carolina (Table 1). Among the 248 animals from five EPA cruises, approximately 10 percent had black gills, and, among 218 animals from the surf clam assessment cruise, 10 percent had similar blackening. Crabs examined during the scallop assessment cruise showed that 21 percent of 639 specimens had black gills. Collections in waters northward from the tip of Long Island, New York to Georges Bank provided a sample size of 158 rock crabs in which none of the specimens had black gills. Summary data from the collections showed that black gills were not uncommon in rock crabs taken from nearshore waters which range southward from New York,

but further collections are necessary to confirm their absence in specimens from northern Long Island to Maine.

Histological studies on clean, discolored, or blackened gills suggested three possible causes for the black color: (1) accumulation of black particles in specimens from areas which receive dredge spoil and sewage sludge, (2) necrosis and melanization of gill lamellae that are occluded with potentially toxic dumpsite bottom sediments, and (3) focal blackening and melanization of adjacent lamellae in response to disease or injury. Occlusion of gill lamellae by dense sludge and associated organisms was best visualized by scanning electron microscopy (Figs. 1, 2). Tissue destruction and melanization readily was observed in histological sections (Figs. 3, 4). Microscopic observations of stained tissues are summarized below under three separate headings: (1) fouling organisms on the epicuticle, (2) cellular response and nodule formation, (3) internal parasites.

Fouling Organisms on the Gill Epicuticle: Microscopic examinations were made on rock crabs and lobsters sampled from 20 field trips in the New Jersey sites and 11 trips in the New York Bight sites. Most of the New York specimens were taken from a station situated 3 miles east of Ambrose Light and used as a dumpsite for sewage sludge. The number of collecting trips was adequate for following seasonal differences in gill color as it was influenced by periods of molt. Records were kept of the following categories: microscopic debris, bacteria, diatoms, tissue nodules, ciliate protozoa, amoebae, and copepods. Seasonal variations in the incidence of each category were noted on rock crab gills from the

New Jersey-New York sites (Table 2). The number of lobsters examined was not sufficient to permit a seasonal analysis. Microscopic debris (silt, sand, debris) (Fig. 5) rarely was observed in sections of lobster gill but was found in 44 to 100 percent of the New Jersey-New York crabs. Fouling bacteria occurred either as filamentous forms or as large rounded clumps, but neither were identified (Figs. 6, 7). Bacteria were present on 17 to 80 percent of the gills examined. Unidentified naviculoid diatoms sometimes filled the interlamellar spaces (Fig. 8) where they were associated with lamellar necrosis and melanization. Diatoms were present on 11 to 38 percent of the gills. Stalked ciliate protozoa (Figs. 9, 10, 11) tentatively were identified as suctorians (*Acineta*, *Ephelota*) or as peritrichs (*Zoothamnium*, *Orbopercularia*); identifications are tentative and need to be confirmed. Crabs infested with ciliates were found more frequently in Sandy Hook Bay stations (23-63 percent) than they were in the ocean stations (11-24 percent). When the numbers of ciliates were counted in sections of gill tissue, they were more numerous in crabs from the Bay stations (Table 3). Small lobose amoebae (Fig. 12) were observed in association with clumped bacteria which probably were used for food. Probable immature parasitic copepods (Fig. 13) were found between the gill lamellae of 0-9 percent of the Bay crabs and 6-20 percent of the ocean crabs. The number of copepods never exceeded 7 per section in the New Jersey-New York rock crabs but ranged up to 40 per section in the Maine and EPA ocean collections. The remarkably small numbers of copepods in gill sections from the New Jersey-New York collections could not be attributed to any known cause.

Microscopic findings with rock crabs from the New Jersey-New York collection sites were based on 679 animals. Specimens taken from other areas were not of sufficient number to support valid comparisons on the frequency with which each condition was observed. Preliminary findings suggest that: (1) ciliate protozoa are more numerous in waters of shallow bays than they are in deeper ocean waters, (2) diatom infestations of crab gills are unique since sunlight is essentially absent beneath the dorsal carapace, and (3) unknown inhibitors may be responsible for sparse numbers of copepods and ciliates on gills from crabs in the New Jersey-New York complex.

Cellular Response and Nodule Melanization. Three different types of cellular responses were detected in histological sections of gills: (1) swollen lamellae containing large numbers of cells often associated with copepod infestations (Fig. 13), (2) large swollen hemocytes, not aggregated, and suggesting a single-cell type of response (Fig. 14), and (3) aggregations of swollen cells, often suggestive of nodule formation and melanization (Fig. 15). The occurrence of pinpoint melanized foci suggested that parasitic copepods punctured the gill cuticle, a cellular response occurred as a result of puncture, and scarring or healing of the cuticle was responsible for the thickened foci. Swollen hemocytes, often grainy and deeply stained, suggested that individual cells were responding to some type of humoral or cellular insult without involvement at the tissue level; necrotic hemocytes most often were observed in sinuses having a rich supply of hemolymph. The third type response which led to nodule formation was observed

most frequently in crustaceans taken from the New Jersey-New York sites. Aggregates of swollen cells which progressively became anucleate were present in the form of a honeycomb without evidence of encapsulation or melanization (Fig. 15). Other aggregates were compact in the form of nodules with evidence of degeneration and melanization (Fig. 16), and still others were completely melanized with virtual loss of cellular integrity (Fig. 17). Lobsters from the New Jersey-New York collections showed that upon microscopic examination nodules were present in 128 of 200 animals and that 12 of them had more than 100 nodules per 6 μm section. The nodules were observed more frequently in lobsters less than 21 cm in total length, and gill sinuses were affected more frequently than were hepatopancreas and intestine. Similar analyses of the abundance and distribution of nodules in rock crabs were not made, but nodules were found in 22 to 80 percent of the 679 animals examined. Causative organisms were not observed in the nodules, possibly because they were destroyed before frank nodule formation was detected. However, on rare occasions a parasitic amoebae, Paramoeba perniciosa, was associated with the cellular aggregates (Fig. 18).

Swollen lamellae with hemocyte infiltration were observed frequently in crabs collected outside of the New Jersey-New York area where infesting copepods were numerous. Swollen and degenerate blood cells not associated with nodule formation similarly were frequent in crabs collected outside of the New Jersey-New York area. Swollen aggregates of cells, often with evidence of melanization and progressive autolysis, were frequent in animals from the New

Jersey-New York collection sites. Conclusive cause-and-effect relationships for the three types of cellular response were not evident in histologic sections.

Internal Parasites. The parasitic amoeba, *Paramoeba perniciosa*, was recognized in 1 of 200 lobsters examined. Histologic findings were complicated by the fact that amoebae were detected only in small numbers and sometimes were degenerated within cells of developing nodules. Observations of amoebae in the lobsters suggested that invasive amoebae were destroyed by an effective host cellular response. Paired syngyns of a large gregarine, probably *Porospora gigantea* (Fig. 19), were found in sections of the digestive tract of a few of the lobsters. Cestode larvae were found in the gut lumen, and encysted nematode larvae occasionally were found in the gut wall; parasites were not found in sections of the hepatopancreas. The amount of tissue taken from each lobster was not sufficient to provide an estimate of parasite burdens.

Stained sections of rock crabs from the New Jersey-New York sites showed that *P. perniciosa* also was present in a few of them. In one specimen, large numbers of amoebae were present in hemolymph-filled sinuses of the gill and hepatopancreas without evidence of a cellular response. In several other crabs, amoebae were associated with nodule formation and they were degenerate. In tissues of animals that were effectively destroying the amoebae, residual amoeba nuclei were the only remaining evidence of infection. The hemolymph of one rock crab was heavily parasitized by the dino-flagellate, *Hematodinium perezi* (Fig. 20); it was found in one

rock crab taken from the New York Bight in October 1976. Hepatopancreas from the New Jersey-New York crabs was infected only by P. perniciosa or H. perezi, which were present in the sinuses but not the tissue. Hindguts contained unidentified gregarines and cestode larvae in the lumen, and larval trematodes and nematodes in the gut wall. The frequency of parasitism in crab tissues was not determined since relatively little tissue from each animal was processed for histological study. Gills collected from the EPA sampling sites occasionally contained parasites which resembled microsporidans. Epidermal cells of gill lamellae and large nephrocytes in the gill shaft had small round bodies resembling microsporidans while large plasmodial forms were found only once in the shaft tissue (Figs. 21, 22). Crabs with larval acanthocephalans encysted in the gut wall (Fig. 23) occurred only in the Maine collections. The acanthocephalans, Polymorphus major, are known to occur as adults in various diving ducks (Schmidt and MacLean, in preparation).

DISCUSSION

The present report summarizes our observations on gill condition and tissue pathology in wild populations of rock crabs and lobsters. Animals from heavily polluted sites in New Jersey and New York were collected in large numbers in order to estimate the influence of yearly seasons, molting activity, and year-class on the relative frequency of black discoloration and the microorganisms which made up the gill-fouling community. A total of 2,091 rock crabs were examined visually and 679 of them were processed for histological study. Lobsters were collected

coincident to studies on rock crabs, but were not taken in numbers sufficient to show seasonal difference. Additional collections of ocean rock crabs from outside of the study area provided 1,388 specimens for visual observation (258 northward from New York and 1,130 southward to North Carolina). All crabs from the northern sites were processed for histological study and 327 from the more southward collections similarly were processed.

The principal objective of observing gill condition was to determine whether or not black discoloration was due principally to the accumulation of sewage and dredge spoil particles between gill lamellae. The basic question was not answered since blackened gills also were found in crabs collected southward from New York to Cape Hatteras. It was of interest, however, that black gills were not found northward to Maine waters. We found that gill-blackening may be caused by the accumulation of noxious bottom sediments between the lamellae, melanization of diseased lamellae, and focal melanization of adjacent dead and necrotic lamellae. Our results suggest that physical and chemical tests on sediment particles trapped between the lamellae might provide conclusive data on the relation of ocean dumping to the black gill condition. The rock crab, _C. irroratus_, is ideally suited for such studies because it walks rather than swims, and is directly affected by bottom sediment characteristics. In nearshore environments, the winter season is least suited for gill-fouling studies because it is at this time that many adult males migrate shoreward and resume molting activity (Sawyer, 1976a). We observed that approximately 90 percent of the captured males may have clean gills when examined

in winter. Maximum fouling was observed during the October-December quarter because in the New York Bight Apex this season immediately precedes the adult male molting period.

The presence of suctorian ciliates on gill surfaces suggests that abundant food organisms, higher in the food web than bacteria, are present in the New Jersey-New York waters. Peritrich ciliates, often in abundance, feed upon bacteria and provide evidence that high bacterial loads are present in the water column. The same groups of ciliates were not found in waters beyond the 20-fathom line, possibly because of the greater dilution of bacterial populations in deeper waters. New studies are needed to investigate environmental influences on ciliate-crustacean relations, and the additive effects of pollution. Bacterial growth on gill epicuticle requires further study to determine differences or lack of differences in species composition between clean and polluted study sites. Microscopic studies showed that in newly molted crabs light infestations with filamentous bacteria precede the attachment of other fouling species. Definitive identifications of the species of amoebae and free-swimming ciliates which graze upon fouling bacteria are also worthy of further study. Our studies have shown that rock crabs, regardless of their source, may have excessive fouling growth on their gill surfaces. Chemical and biological analyses of agents associated with fouling may provide useful information on the effects of specific types of pollution on species diversity and abundance.

Nodules made up of clumped cells which often were anucleate and melanized were observed frequently in lobsters and crabs

collected in New Jersey and New York Bight waters. The cause or causes of nodule formation are unknown, but on several occasions the parasitic amoeba, *Paramoeba perniciosa*, was found in association with nodules. Microbial agents, if responsible for the cellular reaction, probably would be destroyed and not recognizable in nodules showing melanization. Counts of the numbers of nodules per tissue section are not complete for rock crabs, but counts that were tabulated for lobster tissues showed that they numbered up to 200 per section; 70 percent of the 200 lobsters examined had nodules. Preliminary data have shown that 22-80 percent of the rock crabs collected at the same time as the lobsters have similar nodules. We have not made specific tests to demonstrate that pigment associated with nodules is melanin. Lightner and Redman (1977) have shown, however, that pigment associated with inflammatory responses in shrimp stains specifically for melanin. New studies on the chemical and microbial characteristics of crab and lobster hemolymph are necessary before nodule formation can be explained. Hemocyte degeneration, usually involving single cells, was the usual type of response in rock crabs taken from areas away from the New Jersey-New York sites. Histological findings in crabs from the latter sites (Long Island, Maine, Georges Bank, etc.) showed that the single-cell response was present in about 50 percent of the animals, but the numbers of affected cells per tissue section usually were small when compared to the nodule counts. We are not aware of any reports on naturally occurring bacterial infections in rock crabs; however, Cornick and Stewart (1966) recovered three morphological groups of bacteria in lobster hemolymph. Data derived

from our studies are limited to histological findings from sectioned tissues. Total body burdens in terms of numbers of nodules per animal are not known, but our data suggest that they may be remarkably high. Further studies should be made to obtain qualitative data and to estimate the extent of mortality in lobsters and rock crabs.

Observations on protozoan and metazoan parasites in the crabs and lobsters provided several new host records for certain parasites, and extended the known geographical range for others. The occurrence of P. perniciosa is of interest because it is known to cause extensive mortality in blue crabs, Callinectes sapidus, at certain times of the year (Sawyer, 1969; Sprague et al., 1969). Lobsters and rock crabs now are recognized as new hosts for the amoebae (Sawyer, 1976b). The dinoflagellate, Hematodinium perezi, also was found in several new hosts, C. irroratus, C. borealis, and Ovalipes ocellatus (MacLean and Ruddell, J. Parasitol., in press). Very small parasites within tissues of the rock crab and resembling microsporidans also are worthy of further study. They were rare in the gill tissues and require considerable study before a final identification is possible. Gregarines from lobsters probably were Porospora gigantea, a well-known parasite of this host. Tissue sections of gregarines in rock crabs were not suitable for identifying the parasites. None of the larval cestodes, trematodes, or nematodes were identifiable in tissue sections, and considerably more work is required with intact specimens before their identity is known. The larval acanthocephalans were found only in crabs collected in Maine, suggesting that the definitive host is

geographically limited to more northern habitats. The acanthocephalan may be well suited as a parasite tag for studying movement patterns of the rock crab. Parasitic copepods on the outer surfaces of the gill lamellae of rock crabs were of particular interest.

Gills from 679 crabs collected in the New Jersey-New York waters showed that only 1-7 copepods were present per stained section. In contrast, counts ranging up to 30-40 copepods per section were not uncommon in crabs collected from Maine and EPA stations. The possible effects of pollution on copepod fecundity are worthy of further study. We are not aware of any published accounts of copepod infestations in gills of *C. irroratus*; however, adult female copepods have been recorded in egg masses of gravid *C. irroratus* (Connolly, 1929). New studies are planned to increase the geographical range of our studies, and to conduct comprehensive examinations of whole organ systems.

In conclusion, our studies provide an extensive data base on the frequency with which black or discolored gills are found in rock crabs collected from Cape Hatteras, North Carolina northward to Maine. Specific effects of ocean dumping on gill condition are difficult to assess because of extensive gill-fouling of crabs regardless of their source. Our findings suggest that future analyses of the nature of silt which accumulates between the gill lamellae, specific identifications for fouling bacteria, and heavy metals determinations in crab and lobster tissues may provide useful estimates of the effects of environmental pollution on crustacean health. Furthermore, the frequent occurrence of stalked ciliate protozoans on gill epicuticle, often in large numbers, merits

further consideration. Peritrich ciliates, especially, are valuable indicators of high bacterial populations since bacteria are their principal source of food. Suctorian ciliates such as Ephelota, already reported by Sawyer et al. (1977), and Acineta also are valuable indicators of the environment since they feed on microscopic organisms in the food web other than bacteria. Our studies suggest that benthic crustaceans which walk, feed, and reproduce on the seabottom are valuable indicators of the quality of the sediments with which they are intimately associated.

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Location	Number Examined	(Number) Fixed)[1]	Clean[2]	Discolored[2]	Black[2]
New Jersey	849	(310)	616 (73)	198 (23)	35 (4)
New York Bight	1242	(369)	686 (55)	468 (38)	88 (7)
Atlantic Ocean (scallop cruise)[3]	639	(39)	460 (72)	42 (7)	137 (21)
Atlantic Ocean (clam cruise)[4]	218	(15)	115 (52)	86 (40)	17 (8)
Atlantic Ocean (EPA cruises)[5]	248	(248)	109 (44)	113 (45)	26 (11)
Montauk, N. Y.	36	(36)	29 (81)	7 (19)	-
Maine	171	(71)	99 (58)	72 (42)	-
Georges Bank	51	(51)	48 (94)	3 (6)	-
North Carolina	25	(25)	25 (100)	-	-
	3479	(1164)	2187 (63)	989 (28)	303 (9)

[1] Included in number examined.

[2] Percent incidence in parentheses.

[3] Long Island, New York to Cape Hatteras, Virginia.

[4] Long Island, New York to Norfolk, Virginia.

[5] Philadelphia-Wilmington ocean-dumping sites, 35 miles offshore of Delaware and New Jersey.

Table 1. Summary of collection sites for rock crabs, *Cancer irroratus*, and number of specimens examined visually and by histological procedures.

Condition	New Jersey Winter	Spring	Summer	Fall	New York Winter	Spring	Summer	Fall
Clean[1]	72%	50%	37%	34%	56%	52%	46%	23%
Discoloration	28	50	63	66	44	48	54	77
Debris[2]	50	57	100	77	44	70	50	71
Bacteria	17	56	80	70	30	57	50	41
Diatoms	18	21	20	38	30	23	20	11
Nodules	61	61	80	72	22	60	50	23
Ciliates	23	47	63	54	22	24	13	11
Copepods	4	9	-	6	6	20	18	15

[1] Clean gills predominate in winter and spring, coincident to molt.

[2] Microscopic debris less in winter, coincident to molt.

Table 2. Summary of histological findings with rock crabs, *Cancer irroratus*, from New Jersey and New York Bight Apex. Percent incidence of each condition by season of the year. Results based on 310 crabs from New Jersey and 369 from New York.

ROCK CRABS

Location	Number Examined	Number With Peritrichs (Max.)[1]	Number With Suctoria (Max.)[1]
New Jersey	310	40 (300)	102 (573)
New York Bight	369	25 (183)	23 (60)
Atlantic Ocean (scallop cruise)	39	2 (1)	2 (1)
Atlantic Ocean (clam cruise)	15	-	-
Atlantic Ocean (EPA cruises)	248	3 (10)	5 (10)
Montauk, N. Y.	36	6 (65)	23 (200)
Maine	71	8 (9)	1 (1)
Georges Bank	51	-	6 (4)
North Carolina	25	-	-
LOBSTERS			
New Jersey	174	39 (75)	4 (9)
New York	38	15 (30)	-

[1] Maximum numbers counted on 6-µm stained sections of gill.

Table 3. Numbers of rock crabs, *Cancer irroratus*, and lobsters, *Homarus americanus*, infested with unidentified peritrich ciliate protozoa and suctorian protozoa (*Acineta* sp.).

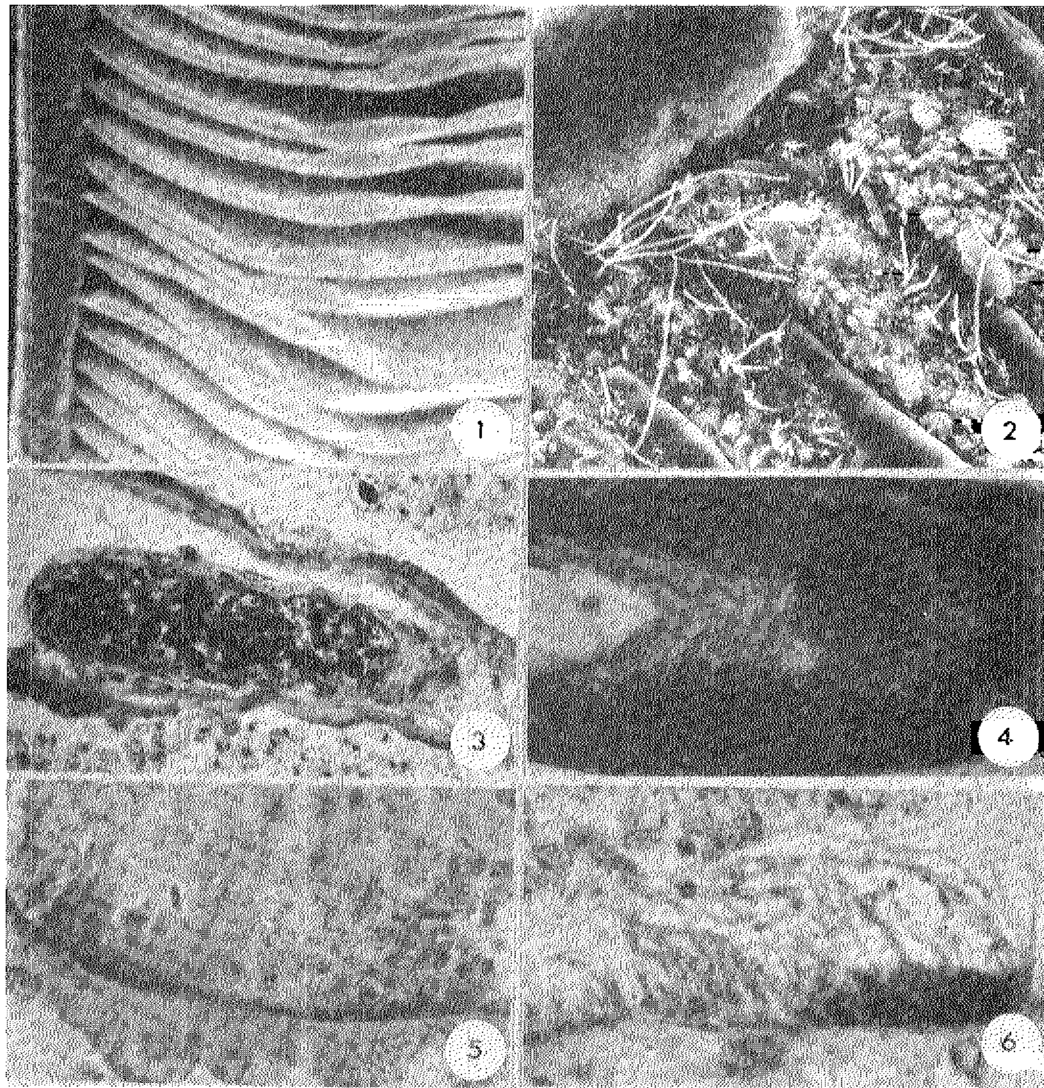

Fig.1. Scanning EM of clean rock crab gill, 60X. Fig.2. Scanning EM of fouled rock crab gill, 438X. Fig.3. Necrotic lamella of rock crab gill, Harris hematoxylin-eosin, 400X. Fig.4. Black lamella of rock crab gill, Feulgen reaction, 640X. Fig.5. Debris between lamellae of rock crab, Harris hematoxylin-eosin, 640X. Fig.6. Filamentous bacteria on gill cuticle of rock crab, Harris hematoxylin, 640X.

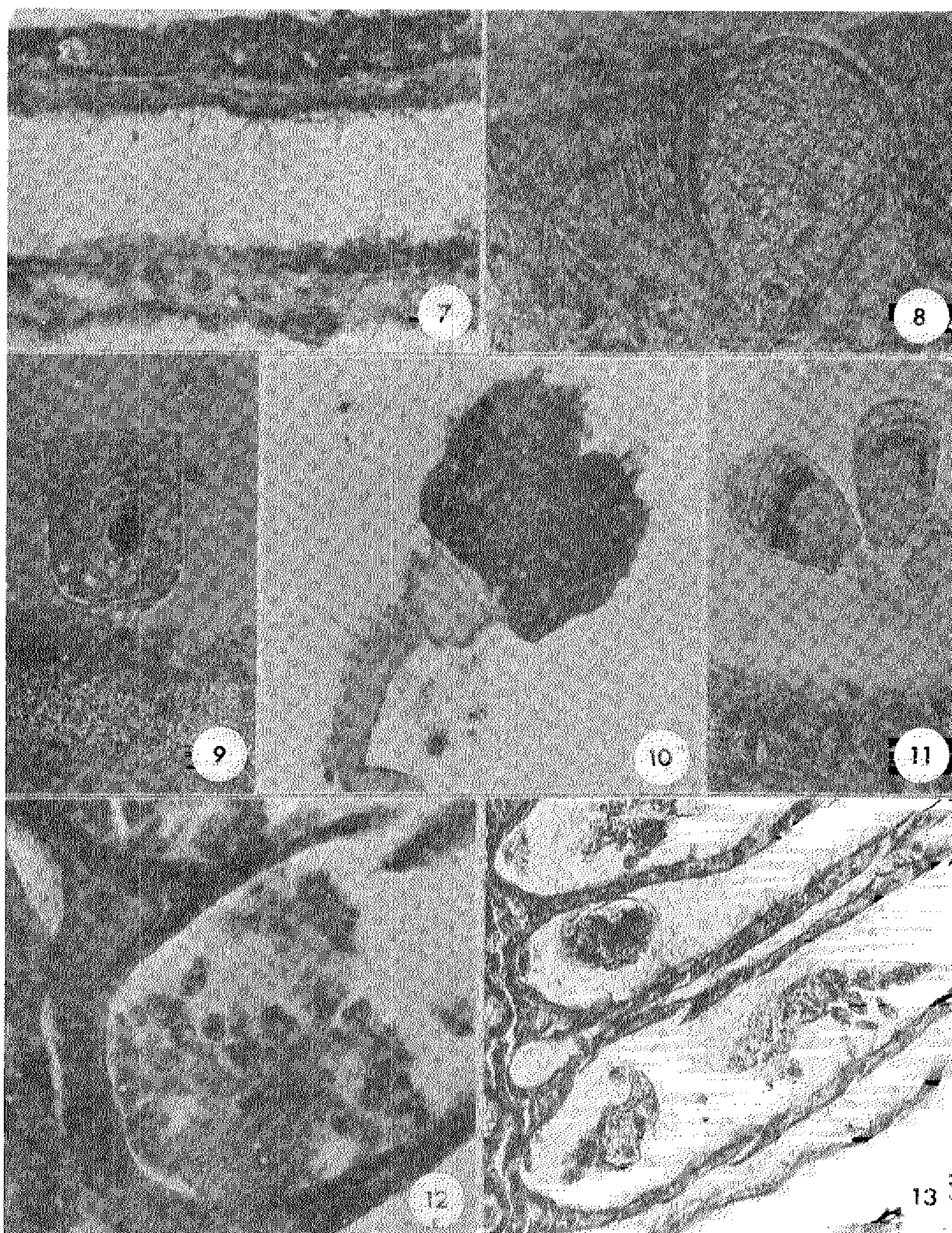

Fig.7. Clumped bacteria on gill epicuticle of rock crab, alcian blue-nuclear red stain, 640X. Fig.8. Diatoms between gill lamellae of rock crab, Feulgen reaction, 640X. Fig.9. Suctorian ciliate (*Acineta* sp.) on gill cuticle of rock crab, Feulgen reaction, 640X. Fig.10. Suctorian ciliate (*Ephelota* sp.) on gill cuticle of rock crab, Harris hematoxylin-eosin, 400X. Fig.11. Peritrich ciliate on gill epicuticle of rock crab, Feulgen reaction, 640X. Fig.12. Amoebae and debris between gill lamellae of rock crab, Harris hematoxylin-eosin, 640X. Fig.13. Copepods between gill lamellae of rock crab, Harris hematoxylin-eosin, 160X.

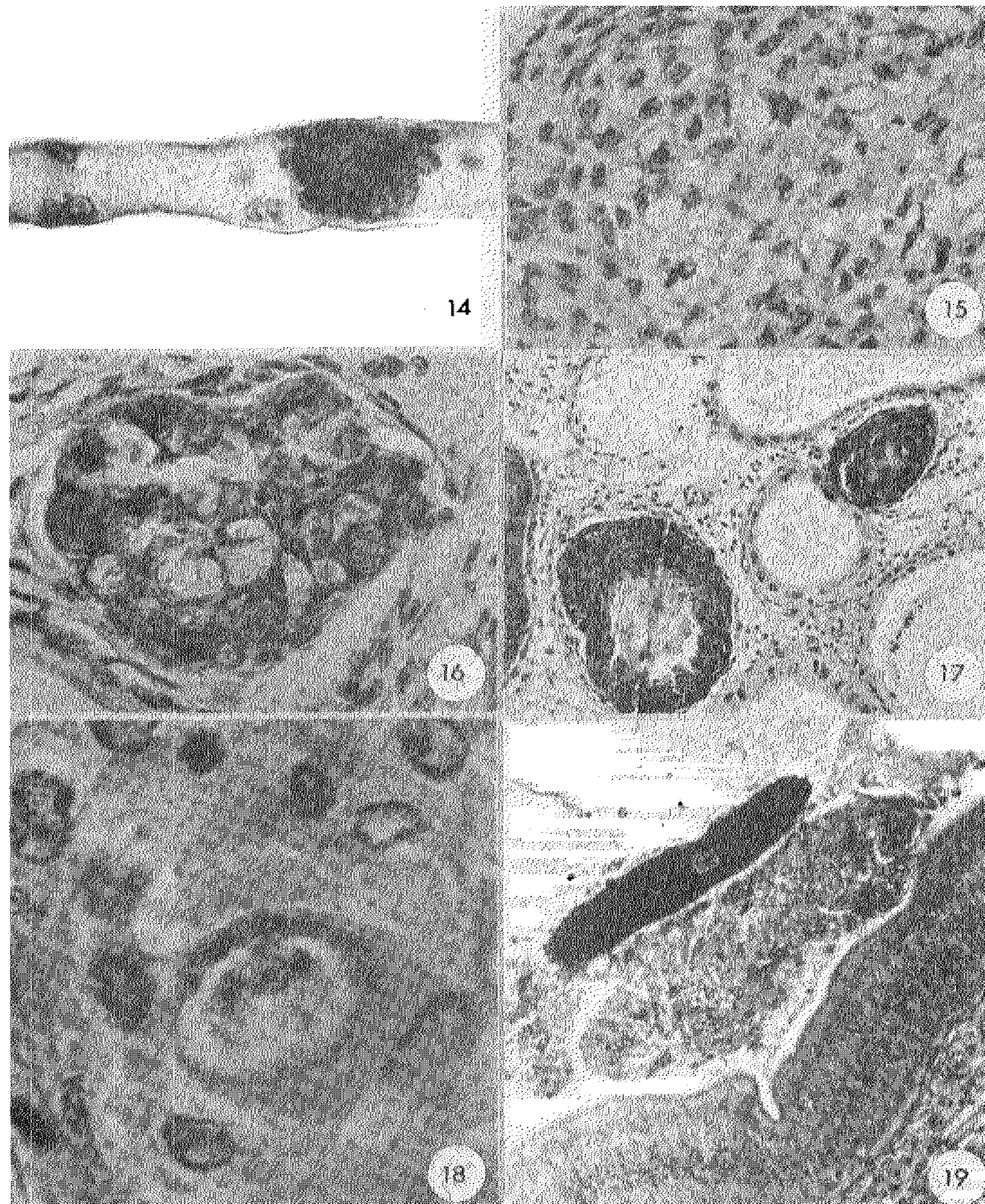

Fig.14. Localized phagocytic (?) cells in gill lamella of rock crab, Harris hematoxylin-eosin, 640X. Fig.15. Massive cellular response in gill shaft of American lobster, Harris hematoxylin-eosin, 640X. Fig.16. Possible early melanization of nodule in gill of American lobster, Feulgen reaction, 640X. Fig.17. Necrotic caseous nodule in hepatopancreas of American lobster, Harris hematoxylin-eosin, 160X. Fig.18. Paramoeba perniciosa within cell of intestine of American lobster, Harris hematoxylin-eosin, 1600X. Fig.19. Gregarine (Porospora gigantea ?) in midgut of American lobster, PAS reaction, 160X.

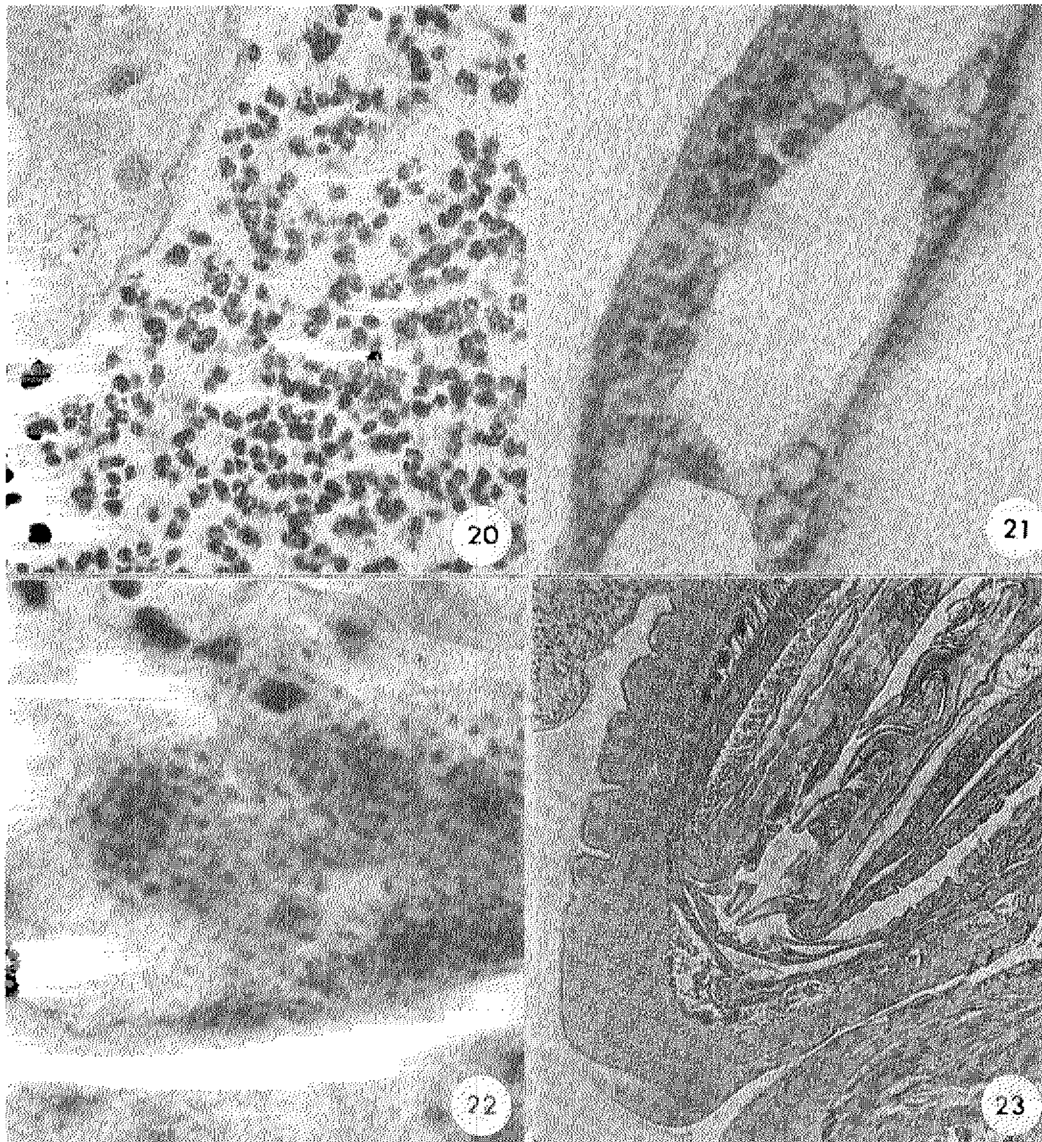

Fig.20. Dinoflagellate (Hematodinium perezi) in hepatopancreas of rock crab, Feulgen reaction, 640X. Fig.21. Possible microsporidan in gill epidermis of rock crab, Mallory trichrome stain, 1600X. Fig.22. Unknown multinucleate plasmodium in gill shaft of rock crab, Harris hematoxylin-eosin, 1600X. Fig.23. Larval acanthocephalan encysted in hindgut wall of rock crab, Feulgen reaction, 160X.

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OPPORTUNISTIC PATHOGENS AND A BACTERIAL DISEASE OF THE LOUISIANA CRAYFISH, *PROCAMBARUS CLARKII*

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A spontaneous bacterial infection was observed in the crayfish, *Procambarus clarkii*. A bacterium isolated from the hemolymph of diseased animals was tentatively identified as a Pseudomonad. The major effect of the disease was loss of equilibrium followed by death of the infected animals. Inoculation of crayfish, each with one million cells of the bacterial isolate proved to be lethal within 30 hours. Aliquots of a cell free broth culture produced no observable effects when inoculated into crayfish, but a partially purified fraction prepared by salt precipitation of the cell free broth culture caused a transient loss of equilibrium, but only 10 percent of the animals died. Our results further indicate that temperature may play a major role in either the type or quantity of toxin produced. Toxin samples prepared from the organism grown at 4C were not as effective on the crayfish or in lysing frog erythrocytes when compared with similar preparations from the same organism grown at 25C, even though the growth of the bacterium was not adversely affected.

INTRODUCTION

The fact that some diseases are caused by single biological agents has certainly contributed to our knowledge of several pathological states in aquatic animals. Thus gaffkemia of lobsters (Stewart and Arie, 1974), bacterial kidney disease of Salmonid fishes (Bullock, et al., 1974), and virus diseases of fish (Sindermann, 1974) have been extensively studied. However, as a host becomes compromised by various biological, chemical and physical stressors bacteria, which until recently were considered to be part of the normal flora of the host, now behave as active and frequent pathogens. The bacteria in question are usually members of the *Vibrio*-*Pseudomonas*-*Aeromonas*-*Acinetobacter* groups, and species of one or more of this group have been isolated from the intestinal tracts of apparently healthy fresh water fish (Collins, 1970), salt water fish (Trust, 1975), crustaceans (Sizemore, et al., 1975); and frogs (Glorioso, et al., 1974; Van der Waiij, et al., 1974).

The bacteria described above have been designated as opportunistic pathogens, As yet, the conditions necessary for disease production by these bacteria have not been defined, but high temperatures, low oxygen, inadequate diets and the presence of unidentified bacterial metabolites have been suggested to have effects on the host which tend to enhance the effects of these pathogens (Sindermann, 1974). Previous work in our laboratory

indicated a possible role for bacterial toxins in disease production in frogs and crayfish by opportunistic pathogens (Amborski, et al., 1975). This report extends these observations to a spontaneous bacterial infection in crayfish maintained under laboratory conditions.

MATERIALS AND METHODS

Animals. Crayfish, *Procambarus clarkii*, were collected from their local habitat, and maintained at room temperature in 50 gallon galvanized tanks which were coated with an epoxy paint to prevent leaching of toxic materials from the metal surface. Each tank contained an eight inch base layer of heat sterilized sand, and the water supply was aerated and filtered by means of two Dynaflow aquarium pumps. The diet of the animals consisted of carrots, live earthworms and pieces of catfish meat.

Bacteriology. Bacteriological determinations were carried out on the hemolymph and intestinal tracts of the test animals. Animals were surface sterilized with 10 percent calcium hypochlorite and washed with sterile water. Hemolymph was collected by syringe and needle from the heart, and intestinal tracts were dissected out as aseptically as possible. All sampling procedures were carried out in a laminar flow hood (Baker Co., Sanford, Maine). Isolation and identification of bacteria were performed according to previously described techniques (Glorioso, et al., 1974).

Growth curves. A preliminary growth curve was determined in a 300 ml growth curve flask (Belco Biological Glassware, Vineland, New Jersey) containing 35 ml of trypticase soy broth (Difco Laboratories, Detroit, Michigan). Each flask was inoculated with

0.2 ml of an eighteen hour culture of the bacterial sample. Flasks were shaken at 300 RPM at temperatures of either 25±1C or 4±1C. Optical densities were determined at approximately two hour intervals using a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., New York). One liter samples were prepared in a similar manner to obtain larger quantities of the test material.

Toxin preparation. After the desired bacterial growth was achieved, the bacterial cells were removed from the growth medium by centrifugation in a refrigerated centrifuge at 12,000 X G for 15 minutes. Supernatant fluids were collected, and differential ammonium sulfate fractionation was carried out. Increasing 10 percent increments between 10 and 80 percent saturation of the salt were used. The material precipitated after the addition of each increment of salt was concentrated by centrifugation, collected and solubilized in pH 7.2 phosphate buffer, and dialyzed at 4C against the same buffer for 24 hours. Samples were then frozen in two ml aliquots. Protein determinations were made on each sample by standard techniques (Lowry, et al.,1951).

Assays. Each test sample was assayed for toxicity by injecting membrane filtered (Millipore, Bedford, Mass.) aliquots of each sample into the body cavity of crayfish. Each animal received an inoculum equivalent to 20 mg of protein. Animals were held in plastic baskets and observed for effect.

Hemolysin assays were carried out on freshly collected frog erythrocytes. Blood was collected from the heart and placed in amphibian Ringer's solution (Johnson and Volpe, 1973) containing 0.1 percent sodium citrate. Blood cells were washed 2X in amphibian

Ringer's solution, and a one percent suspension was prepared. The assay consisted of mixing 0.5 ml of the one percent erythrocyte suspension, 0.1 ml of the test sample and 4.5 ml of amphibian Ringer's solution. These mixtures were incubated at either 25C or 4C for four hours. Hemolytic activity was determined both visually and by absorbance at 540 nm.

RESULTS AND DISCUSSION

The crayfish in our maintenance tanks were originally being used to study a shell disease that we have reported (Amborski, et al., 1975). Mortalities observed without signs of shell disease were originally discarded. However a recent report has indicated that other bacterial pathogens are important in crayfish mortalities (Vey, et al., 1975). Close observations of our stocks indicated that periodically, individual animals were encountering difficulty in maintaining an upright position, and were laying on their side. We had observed this behavior in the past, but it appeared to be associated with the moulting process, as we had observed the completion of the moulting process in a few of these animals.

During a one year period eight crayfish were found laying on their side, but still alive. Bacteriological studies on the hemolymph of five of the eight animals showed the presence of large numbers of gram negative rods. A single isolate was recovered from each animal, and the biochemical characteristics of the organism are shown in Table 1. With the exception of the lack of growth at temperatures above 30C, our isolate conforms to the characteriscs used to describe _Pseudomonas alkaligenes_. Both

Pseudomonas alkaligenes and Pseudomonas fluorescens have been identified as bacterial pathogens of crayfish (Vey, et al., 1975). Bacteriological studies on the intestines of healthy and diseased animals showed that the major gram negative isolates were the same organism we isolated from the hemolymph, Citrobacter freundii, Aeromonas liquefaciens, Escherichia coli and species of Achromobacter, Acinetobacter, Shigella, Flavobacterium, and Klebsiella. The major gram positive isolate was a species of Bacillus.

The growth curves for the hemolymph isolate at both 4C and 25C are presented in Figure 1. With the exception of the longer lag period at 4C, growth at the two temperatures is comparable. Samples were harvested at the times indicated by the arrows on the growth curve.

In a preliminary report on the possible relationship between bacterial toxins and disease in crayfish (Amborski, et al., 1975), we reported the possibility of toxin assay by placing crayfish into the toxic preparation. However, subsequent studies have identified a major problem in this type of assay. Bacteria excreted by the crayfish into the toxic preparation may serve as a source of infection. Thus Vey, et al. (1975) demonstrated that wounded crayfish were susceptible to exposure to suspensions of bacteria. It is therefore possible that some of our reported mortalities may have resulted from intact bacteria either present or excreted into the toxic preparation. Furthermore, the excretion and growth of any one bacterium did not appear to be uniform. For this reason, we chose to expose our test animals to the toxic preparations by injection in the present study.

Injection of individual crayfish with the hemolymph isoate at a concentration of 10^6 organisms per animal caused 90 percent mortality in 30 hours. In an attempt to demonstrate a role for toxins in the observed mortalities, various preparations of the cell free growth medium were assayed for toxicity. These results are presented in Table 2. Based on comparisons between estimated volumes of hemolymph in the crayfish and known volumes of growth medium, a dosage of 20 mg protein should have represented a massive dose of any toxin which might be produced by the bacterium. However, the injected animals demonstrated only a transient loss of equilibrium lasting from six to eight hours and only 10 percent of the injected animals died. Uniform results were observed in the 25C fractions prepared by precipitation between 50 and 80 percent salt saturation, but of even greater interest, is the fact that under the present conditions even these responses were greatly reduced when the animals were challenged with preparations from the bacterium grown at 4C.

Since the effect of our preparations could represent a nonspecific response, we determined the effect of our preparations on frog erythrocytes. These results are presented in Table 3. Lysis of erythrocytes was observed in 25C fractions prepared between 50 and 80 percent salt saturation, but similar fractions prepared from organisms grown at 4C demonstrated a substantial reduction in activity. Treatment of fractions at 60C for one hour destroyed biological activity.

It is certainly tempting to explain mortalities due to bacterial infection as being the effect of toxins. Pseudomonads and Aeromonads produce a variety of extracellular enzymes and hemolysins (Nord, et al.,

1975), but a role for these toxins in disease production in aquatic animals has not been demonstrated. One major problem is that culture conditions for optimum toxin production by these bacteria have not been determined and although substantial growth of the organism can be achieved, toxin production in culture may require specific metabolites. Thus recent studies have shown that toxin production by *Aeromonas hydrophila* (Bernheimer and Avigad, 1974), and *Pseudomonas aeruginosa* (Liu, 1973), require specific factors. Furthermore, Exotoxin A, produced by *Pseudomonas aeruginosa* is readily destroyed by proteases produced by most strains of the same organism (Liu, 1973). Thus even though toxin might be produced, the action of proteolytic enzymes might substantially reduce the final activity of a preparation. Another complicating factor is the susceptibility of the host. Whereas we assume that a host is uniformly susceptible to a potential toxin, our results might suggest that the stress of some phase of the moulting process may play an important role in the disease process. As stated previously, the behavior of the infected animals was not unlike those animals passing through the final moulting process. Work is continuing on some of these possibilities.

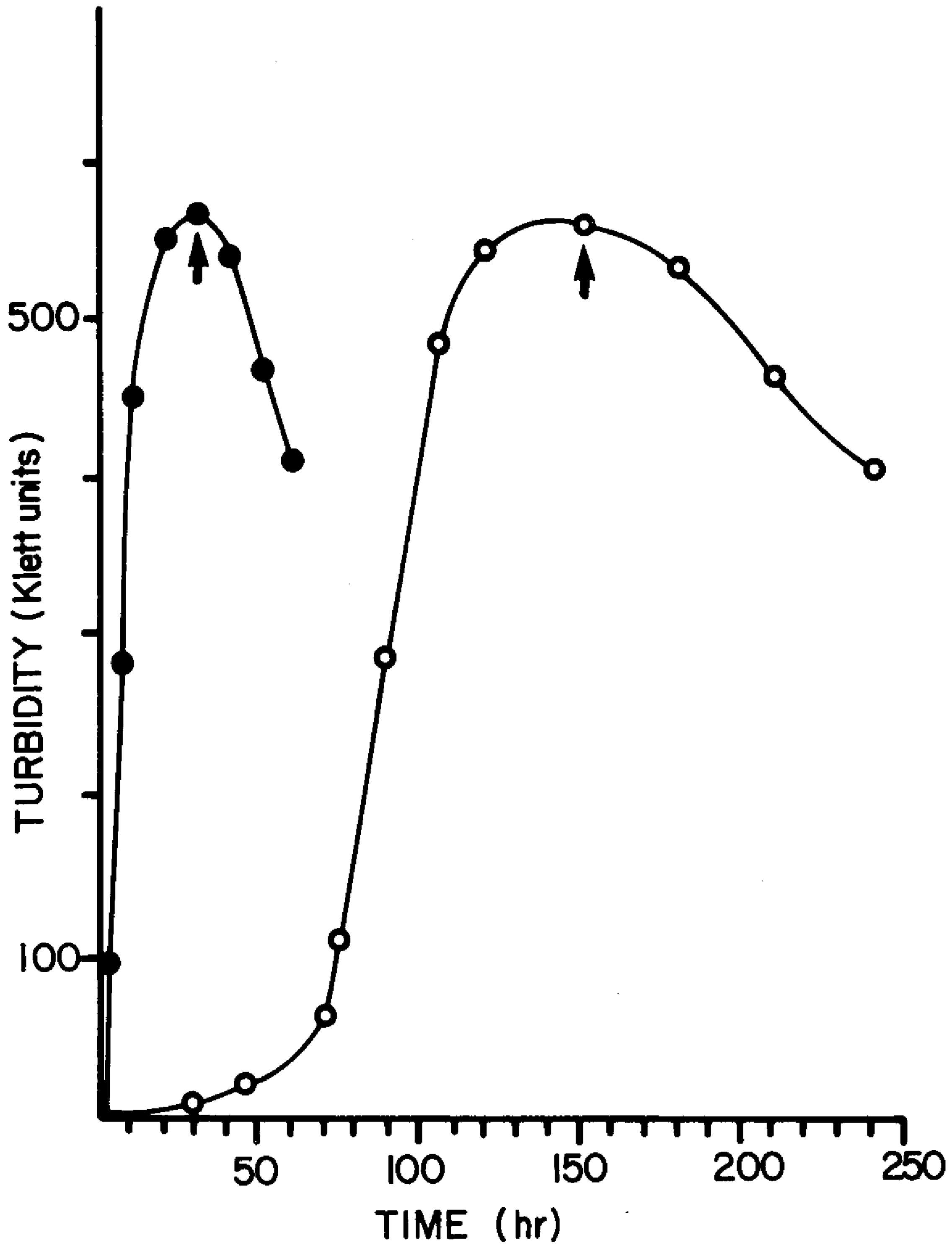

Figure 1. Effect of temperature on the growth of the Hemolymph Isolate. 25C (closed circles), 4C (open circles). Arrows designate sampling points.

Table 1. Biochemical Properties of the Hemolymph Isolate

Test	Result
Oxidase	+
Gram Reaction	-
No. of Flagella	1
Growth at 4C	+
Growth at 25C	+
Growth at 37C	-
Gelatin Hydrolysis	+
Starch Hydrolysis	-
Denitrification	+
Arginine Dihydrolase	+
Urea	+
Methyl Red	-
H_2S	-
Citrate	+
Acetamide	+
Glucose, Rhamnose, Sucrose Arabinose, Inositol, Sorbitol and Mannitol Utilization	-

Table 2. Effect of Cell Free Fractions on Crayfish

Sample Number	Number Showing Loss of Equilibrium	Number Mortalities
25-00[1]	0[2]	0[2]
25-10	0	0
25-20	0	1
25-30	2	0
25-40	2	1
25-50	5	2
25-60	8	3
25-70	3	0
25-80	3	0

Table 2. Effect of Cell Free Fractions on Crayfish (Continued)

Sample Number	Number Showing Loss of Equilibrium	Number Mortalities
04-00	0	1
04-01	0	0
04-20	0	1
04-30	0	0
04-40	0	2
04-50	1	0
04-60	2	1
04-70	0	0
04-80	0	0

1. First two digits indicate growth temperature for bacterium and second two digits indicate percent saturation of ammonium sulfate used to obtain fraction.
2. Eight crayfish were injected with each sample.

Table 3. Lytic Effect of Cell Free Fractions on Frog Erythrocytes

Sample	Absorbance at 540 nm
25-00[1]	0.261
25-10	0.000
25-20	0.000
25-30	0.060
25-40	0.263
25-50	0.282
25-60	0.304
25-70	0.032
25-80	0.000

Table 3. Lytic Effect of Cell Free Fractions on Frog Erythrocytes (Continued)

Sample	Absorbance at 540 nm
04-00	0.080
04-10	0.000
04-20	0.000
04-30	0.000
04-40	0.092
04-50	0.101
04-60	0.134
04-70	0.000
04-80	0.000

1. First two digits indicate growth temperature for bacterium and second two digits indicate percent saturation of ammonium sulfate used to obtain fraction.

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ABRASION AND BACTERIAL INFECTION AS THE PROBABLE CAUSE OF EXOSKELETAL LESIONS IN THE HAWAIIAN FRESHWATER SHRIMP, *Atya bisulcata*.

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Dark brown, shallow, crater-like lesions were found on the exoskeletons of the Hawaiian freshwater shrimp, *Atya bisulcata*. An Extensive survey of specimens captured from streams in Hawaii, Maui, and Oahu Islands showed the disease to be widespread throughout the island chain. Lesions occurred most often on the carapace and body segments, with appendages and ventral body partions infrequently infected. The highest incidences occurred in the winter months with relatively low stream temperatures and high water flow. Chitin-digesting bacteria were repeatedly isolated fron excised lesions. Specimens lightly abraded to damage the epicuticle, developed typical lesions when exposed to raw river water or cultures of chitinoclastic bacteria. Abrasions that were immediately sealed with fingernail polish did not develop inte lesions. Similarly, abraded shrimps placed in sterilized stream water with Thimerosal at 5 ppm developed a brown discoloration but not a typical lesion. In another series of experiments, chitinoclastic bacteria were confined to the abraded cuticle by means of an attached silicone rubber ring with a membrane filter seal. Under these conditions, dark, pitted lesions developed within 10 days at 25 C. When the sealed area contained either Streptomycin, triple sulfa or Thimerosal at 5 - 10 ppm, typical lesions did not develop. Naturally occurring lesions and induced lesions showed a remarkable similarity when examined by scanning electron microscopy. In both instances, bacteria are numerous in the infected area but almost entirely absent in the noninfected or undamaged area. The pitted and eroded appearance of the affected endocuticle, along with presence of numerous bacteria, strongly suggested a causal relation between chitin-digesting bacteria and the exoskeletal lesions.

EXPERIMENTAL ASSESSMENT OF THE VIRULENCE OF FOUR SPECIES OF VIBRIO BACTERIA IN PENAEID SHRIMP[a/].

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Direct intramuscular injection of *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. algosus*, and *V. anguillarum* into white shrimp (*Penaeus setiferus*) resulted in mortalities up to 100% depending on dosage ($10^4 - 10^8$ CFU/shrimp) and species of bacteria. In addition, 16% mortality occurred in brown shrimp (*P. aztecus*) when these animals were repeatedly fed white shrimp meat infected with *V. parahaemolyticus* at $10^8 - 10^9$ CFU/food piece. Based on LD_{50} and LD_{98} data within 22 hr after injection, *V. parahaemolyticus* was found to be the most virulent species to white shrimp, followed by *V. anguillarum*, *V. algosus*, and *V. alginolyticus* in that order. LT_{50} data of an injected bacterial dosage of either 1×10^7 or 2.5×10^7 CFU/shrimp also indicated the same order of virulence for those four organisms. The speed and/or pattern of dying of the shrimp in the experiments may indicate a toxin, or toxins, associated with *Vibrio* bacteria.

INTRODUCTION

Vibrosis has been implicated as a frequent mortality factor in juvenile and larval penaeid shrimp in culture (Sindermann, 1971; Lightner and Lewis, 1975). Several species of *Vibrio* bacteria, which often constitute part of the common flora in sea water, have been demonstrated in the laboratory as causative organisms of that disease (Vanderzant *et al.*, 1970; Lewis, 1973; Lightner and Lewis, 1975). Since many aquaculture ventures are considering closed-system intensive culture of penaeid shrimp, it is important to evaluate the virulence of those bacteria in penaeids. Virulence information can help elucidate, directly or indirectly, questions concerning mode of infection, pathogenesis, and necessity of treatment and prophylaxis against infection.

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a/ Contribution Number 78-47G, Southeast Fisheries Center, National Marine Fisheries Service, NOAA, Galveston, Texas 77550

In the present study, we investigated the dosage-mortality and time-mortality relationships between four species of vibrios and white shrimp (*Penaeus setiferus*) through intramuscular injection. The bacteria used were *V. alginolyticus*, *V. algosus*, *V. anguillarum*, and *V. parahaemolyticus*. Based on those mortality data, we determined and compared the virulence among the four bacteria. In addition, oral infection with *V. parahaemolyticus* was performed in brown shrimp (*P. aztecus*) to re-examine the possibility that that was one of the natural routes of infection. Other workers have reported conflicting results in their success to infect penaeids orally with *V. parahaemolyticus* (Vanderzant et al., 1970; Barkate, 1972).

Because we planned to follow and to observe the course of disease development in individual test shrimp, and at the same time to be able to establish several replicates for each dosage tested, efforts were spent to develop a bioassay system to attain those objectives.

MATERIALS AND METHODS

Test Shrimp and Aquarium Sea Water

Juvenile white shrimp (62-138 mm) and brown shrimp (54-83 mm) were purchased from a local bait camp. The clean and healthy-looking ones were held for at least one week in fiberglass laboratory tanks, equipped with under-gravel filters with air-lift pumps, before being randomly taken for use. White shrimp were used in injection experiments, while brown shrimp in oral infection tests. The shrimp were fed cooked shrimp meat.

Natural sea water was used in all the experiments. It was pumped in from the Gulf of Mexico and passed through a series of filtering and sedimenting devices to remove beach sand and particulates prior to storage in 28,000-gallon red-wood tanks lined with fiberglass. Those devices included in successive order a meshed-screened (90/in) cylindrical well-point buried under beach sand, a 30-in deep oyster-shell filter bed, and a sedimentation sump. Finally, in the Pathology Laboratory, the sea water was run through a set of diatom cartridge filters (pore sizes 5 and 1 μ) before being used in the experiments. The salinity of the water was adjusted to 20 ppt with tap water, and the water temperature was maintained at about 28^0 C.

Challenging Organisms

Four *Vibrio* species and one gram-positive sphere, *Micrococcus conglomeratus* (strain GFC-76-48), were used in the experiments.

The micrococcus and two of the vibrios, *V. algosus* (strain GFC-76-35) and *V. parahaemolyticus* (strain GFC-76-36), were isolated from postlarval white shrimp in our laboratory and identified by Dr. D. H. Lewis, Texas A&M University, College Station, Texas. The micrococcus was used as a positive control in the injection experiments assessing the pathogenecity and virulence of gram-negative vibrios. The other two *Vibrio* species were *V. alginolyticus* (strain MS-670[a/]) and *V. anguillarum* (strain MS-427[a/]), which were obtained from Texas A&M University.

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a/ Our culture code numbers were, respectively, GFC-76-5 and GFC-76-32.

All the bacteria were grown on tryptic-soy (T-S) agar plate (Difco[a/]) with 2% NaCl added. After incubation at 28° C for 24 hr, the culture was harvested by sweeping it into a sterile NaCl solution (2%) with an L-shaped glass spreader. The bacterial suspension was pipetted up and down in a sterile test tube to break up clumps and aggregates. It was then diluted by 10-fold serial dilutions for plating out on T-S agar for titration of bacterial concentration.

For the preparation of toxic extract from V. parahaemolyticus, a harvested bacterial suspension (5.1×10^{11} CFU/ml) was passed through a disposable Nalgene filter (0.2 μ pore size) immersed in an ice bath. The filtrate was collected and utilized for injection as soon as possible.

For oral experimental use, V. parahaemolyticus suspended in a 2% NaCl solution was transferred to small pieces of pre-autoclaved brown shrimp meat (5 x 5 x 5 mm), one loopful per piece. The sterile shrimp meat was held in sets of petri-dishes (15 x 100 cm) with 10 pieces of meat per set. They were laid distant from each other on a piece of filter paper soaked with a sterile NaCl solution (2%). The inoculated meat was incubated at 28° C until being used at either 24 or 48 hr. Some shrimp pieces were not inoculated to serve as control.

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a/ Trade names used in this publication do not constitute endorsement of commercial products.

Before the inoculated shrimp meat was fed to test shrimp, one piece from each of two sets of 10 pieces per set was transferred to a sterile 12-ml graduated, conical, tissue grinder (Belco Stock No. 1977-20012). A sterile NaCl solution (2%) was added to bring the total volume to 1 ml. The content was homogenized, and the homogenate was titrated for bacterial concentration on plate count agar (Difco). The average titer from the two pieces of meat was considered to represent the titer of *V. parahaemolyticus* on each piece of infected meat in two sets.

Bioassay System

Gallon-sized glass Mason jars were converted into experimental aquaria (Fig. 1). Flat-bottomed plastic food-serving bowls were used as covers. A 100-ml polypropylene Tri-Pour beaker (Sherwood #8889-206200) with a perforated bottom was seated in a 5.5-cm hole made in the bottom of the bowl. Fiberglass wool was placed inside the beaker to serve as filter.

The two vertical stems of an Eureka Baby-Saver sponge filter (sponge removed) assemblage[a/] were each passed through a hole drilled in the bottom of the bowl so that the end that had the sponge was in the jar while the other end remained outside of the bowl cover. The free end of the larger filter stem was extended by means of elbow-shaped plastic connectors and rigid plastic tubes to form an assemblage, which had a short plastic tube positioned about 2 cm above the fiberglass wool inside the beaker.

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a/ Eureka Products Co., 135 Jackson St., Newark, N. J. 07105.

When compressed air was introduced into the smaller filter stem, the sea water inside the glass jar was forced to recycle through the larger stem and the fiberglass filter. The system, with one shrimp in each jar, could maintain relatively clear aquarium water for 5 days or longer, if care was taken to remove excess food from the jar at the end of each work day.

The water temperature in our experiments was maintained at approximately 28° C by placing the aquarium jars collectively in a large water bath. The temperature of the bath water was regulated by means of submersible, thermostatically controlled, heating filaments and by circulating water pumps.

Injection Experiments

Each harvested bacterial suspension containing a test bacterial species was diluted by 10-fold dilution in 2% NaCl solution. A selected series of those diluted suspensions, usually from 10^{-2} to 10^{-5}, was separately injected intramuscularly into white shrimp by means of a 1 cc tuberculin syringe with a gauge 27 x ½ inch hypodermic needle. Each animal received a 0.05-ml inoculum of one of the diluted suspensions, the site of injection being the junction between the lateral sides of the 4th and 5th abdominal segments. Injected shrimp were maintained individually in each aquarium jar. Each treatment was replicated from 6 to 10 times. Parallel positive controls with saline (2% NaCl) injection and negative controls (no injection) were also established.

A filtrate extracted from _V. parahaemolyticus_ was also injected into test shrimp in a similar way. No dilution was made of the filtrate.

Oral Infection of Shrimp

Juvenile brown shrimp were individually isolated in aquarium jars and starved for 24 hours. Each of them was then fed with a piece of shrimp meat (5 x 5 x 5 mm) which sustained a 24-hr culture of *V. parahaemolyticus*. At three other 24-hr intervals, the shrimp were again fed with similar shrimp meats, respectively, containing 48-, 24-, and 48-hr growth of the same bacterium. After the last feeding of infected meat, the animals were maintained on non-infected meat. In control shrimp, they were fed only with non-infected meat. Thirty replicates were established for each group.

RESULTS

Injection Experiments

(a) Dosage-Mortality Response. Within 22 hours after each white shrimp was injected with 10^4 CFU or more of either of four *Vibrio* species (*alginolyticus*, *algosus*, *anguillarum*, and *parahaemolyticus*), up to 100%[a/] of the animals died, depending on the dosages employed (Fig. 2). On the contrary, a gram-positive bacterium, *Micrococcus conglomeratus*, caused no mortality in white shrimp at a dosage of 1.9×10^7 CFU per animal.

Vibrio-induced mortality was dosage-dependent. From Figure 3, the median lethal dose (LD_{50}) of the *Vibrio* species in the test shrimp were respectively determined as 1.3×10^6 CFU/shrimp for *V. parahaemolyticus*, 2.2×10^6 for *V. anguillarum*, 9.1×10^6 for *V. algosus*, and 3.2×10^7 for *V. alginolyticus*. Similarily, the

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a/ Corrected mortalities resulted from adjustments made against those in control groups (both saline-injection and non-injection) by means of Abbott's formula (Busvine, 1957).

respective LD_{98} for those bacteria in that same order were 1.1×10^7, 7.2×10^7, 1.3×10^8, and 1.8×10^8 CFU/shrimp.

(b) Time-Mortality Relationship. The time-mortality relationship between Vibrio bacteria and white shrimp is shown in Figures 3 to 6. The speed of inducing mortality by a Vibrio species, as indicated by the steepness of the slopes of the regression curves, was obviously dosage dependent. Thus, at higher dosages, such as 10^8 CFU of V. parahaemolyticus, all the inoculated shrimp were dead within 6 hr (Fig. 3). At lower dosages, such as 10^6 CFU per shrimp, it took 22 hr for V. parahaemolyticus to kill 90% of the white shrimp. A similar relationship between dosage and speed of mortality existed in V. anguillarum, V. algosus, and V. alginolyticus (Figs. 4, 5, and 6). The bacterium-free filtrate extract from a culture of V. parahaemolyticus (5.1×10^{11} CFU/ml) produced 100% mortality within 4 hr (Fig. 3).

Values of median lethal time (LT_{50}) for different dosages were determined from the time-mortality curves (Figs. 3 to 6). Those of three of the Vibrio species (parahaemolyticus, anguillarum, and alginolyticus)[a/] were plotted, and they showed that at a given dosage, the speed of causing mortality in white shrimp varied according to the bacterium species employed (Fig. 7). At a challenging dose of 1×10^7 CFU per shrimp, the LT_{50} was 7.2 hr for V. parahaemolyticus, 10 hr for V. anguillarum, and 50 hr (from extrapolation) for V. alginolyticus. The LT_{50} for V. algosus was not available at 1×10^7 CFU/shrimp, but at a slightly higher dose of 2.5×10^7 CFU, it was determined as 11 hr (Fig. 5).

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a/ V. algosus was omitted from Figure 6 due to insufficient LT_{50} points as a result of less than 50% mortality at several lower dosages.

(c) Clinical Signs and Symptoms. Clinical manifestations in shrimp injected with *V. alginolyticus* and *V. anguillarum* were monitored. Shrimp which died within a few hours after inoculation with higher doses of bacteria usually displayed no clinical signs other than general weakness, lethargy, sluggishness in swimming, and/or quiescense while lying on either the ventral or lateral side of the body at the bottom of the aquarium, with or without leg movement; occasionally such animals might also be seen swimming on their side. Shrimp which were injected with less bacteria and which lived longer exhibited other types of clinical signs such as a white patch near the site of injection, mosaic white discoloration on abdominal segments, reddening of the pleopods and less frequently of the pereiopods, and dorsal flexure usually between the 3rd and 4th abdominal segments. On two occasions, shrimp injected with *V. alginolyticus* appeared very unhealthy with pale patches all over the abdominal segments and had reddened legs. Our observations agreed with many of the clinical signs described for a natural septicemic bacterial disease of penaeid shrimp (Lightner and Lewis, 1975), although we often observed dorsal flexure between the 3rd and 4th instead of 2nd and 3rd abdominal segments, and that a white patch, which was not mentioned as a natural clinical sign, usually appeared around the site of injection. The white patch was perhaps related to injection of the *Vibrio* bacteria. In any case, we felt that the clinical signs were not specific to *Vibrio* infection. They were also frequently observed in shrimp dying from other causes. For diagnostic purposes, specific signs and symptoms of vibrosis and intoxication should be clearly defined in larval shrimp as well as in older shrimp.

(d) Molting. The frequency of molting in white shrimp was monitored in two separate experiments using *V. alginolyticus* as the challenging organisms. The results are summarized in Table 1. At dosages of 1.5 x 10^7 CFU/shrimp or more, no molting took place within 94 hr after injection. However, 5.6% and 25% of test shrimp molted either once or twice within 94 hr when they were respectively injected with 1.5 x 10^6 and 1.5 x 10^5 CFU per animal; no molting occurred in the saline-injected and non-injected groups.

TABLE 1

Molting of Juvenile White Shrimp (*Penaeus setiferus*) After Injection of *Vibrio alginolyticus*

Dose of Injected Bacteria (CFU/Shrimp)	Within 94 hr After Injection of Bacteria	
	Molting Shrimp (%) [a/]	Mortality (%) [a/]
1.5 x 10^8	0	100 (within 22 hr)
1.5 x 10^7	0	87.5 (within 70 hr)
1.5 x 10^6	5.6	25 (within 70 hr)
1.5 x 10^5	25.0	0
Saline (2% NaCl)	0	6.3 (within 22 hr)
No Injection	0	10 (within 94 hr)

a/ Average of two experiments. One experiment was replicated 6 times and the other 10 times for every dosage applied. Each replicate contained one shrimp isolated in one aquarium jar.

Oral Infection Experiment

Attempts to infect brown shrimp with *V. parahaemolyticus* through repeated oral feeding produced about 16% mortality within 96 hr. The experiment revealed that the lethal effect of the inocula wore off very rapidly. Thus, following an initial injection of the bacterium, the corrected mortality rate of test shrimp rose to and leveled off at 8.4% within 23 hr. Mortality did not increase again until a second challenging dose from a 48-hr culture was administered, and then it leveled off at 15% within a total exposure time of 47 hr (Fig. 8). A third feeding of infected shrimp meat failed to add to mortality, but a fourth feeding induced a further 1% corrected mortality to a total of about 16% within 96 hr. Thereafter, no more challenging bacteria were given, and no further death occurred in the "treatment" group.

DISCUSSION

Injection Experiments

(a) Virulence of Challenging Organisms. The dosage-mortality and time-mortality data showed that direct injection into white shrimp with the four tested vibrios could produce high mortalities depending on injected dose and time of exposure (Figs.2 to 6). Failure of parallel injections with comparable doses of gram-positive *M. conglomeratus* to induce mortality indicated that the vibrios were intrinsically pathogenic to the shrimp. Based on the LD_{50} and LD_{98} values, *V. parahaemolyticus* was apparently the most virulent species since it had the smallest LD_{50} (1.3×10^6 CFU/shrimp) and LD_{98}

(1.1×10^7 CFU/shrimp) among the four vibrios. For similar reasons, *V. anguillarum* was considered the next most virulent species to be followed by *V. algosus* and *V. alginolyticus* in that order.

With regard to the LT_{50}, *V. parahaemolyticus* also had the smallest value (7.2 hr at a dose of 1×10^7 CFU/shrimp) (Fig. 7). *Vibrio anguillarum* was the next smallest (10 hr) and *V. alginolyticus* again the next (50 hr). Since the LT_{50} indicates the speed of an organism to cause mortality, and since the length of time required to do so is inversely related to the virulence of the organism, therefore the sequential virulence of those three bacteria in injected white shrimp was in the order of *V. parahaemolyticus*, *V. anguillarum*, and *V. alginolyticus*.

The LT_{50} for *V. algosus* was not available at the dose 1×10^7 CFU/shrimp, but it was determined as 11 hr at a slightly higher dose, 2.5×10^7 CFU/shrimp (Fig. 5). Theoretically then, at 1×10^7 CFU, *V. algosus* should have a LT_{50} larger than 11 hr. That figure would place it after *V. anguillarum*, and probably before *V. alginolyticus*, in terms of virulence.

(b) Bacterial Toxin Mortality Factor. The very steep slopes of the time mortality curves at higher inoculation doses, particularly for those of *V. parahaemolyticus* and *V. anguillarum* (Figs.3 and 4), may indicate that a toxic factor, or factors, were involved in the death of the shrimp. A similar postulate was presented by Vanderzant *et al*. (1970)when these workers attempted to infect brown shrimp by adding cultures of *V. parahaemolyticus* directly to aquarium water. In our present study, the toxin hypothesis was substantiated by injecting into white shrimp a bacterium-free filtrate extracted from a

24-hr culture of V. parahaemolyticus (5.1×10^{11} CFC/ml). The resulting time-mortality curve, showing 100% mortality within 4 hr, was comparable in slope characteristics to that derived from injecting a high dose, 1.4×10^{8} CFU/shrimp, of V. parahaemolyticus (Fig. 3). Results from further experimental studies on the pathogenic relationship between Vibrio toxins and penaeid shrimp are presented elsewhere (Leong et al., 1978). The toxin mortality effect may explain why sometimes the challenging organism could not be recovered from moribund test shrimp.

(c) Molting. Table 1 shows that injection of V. alginolyticus into white shrimp apparently had a slight stimulating effect on molting. It was possible that V. alginolyticus induced molting by either stimulating the secretion of ecdysone (molting hormone) or inhibiting the production of a molt-inhibiting hormone. The absence of ecdysis at higher dosages of inoculation was perhaps due to the rapid death of the injected animals, preventing the completion of the molting processes.

Excess molting may not be favorable to the survival of the shrimp, since molting may render the animal more susceptible to environmental stresses and to invasion by opportunistic organisms. During the interim of separation of the old integument and the formation and hardening of the new one, the animal is deprived of an intimate layer of protective body covering, which has been regarded as the first line of defense against infection in arthropods. A review of the mode of invasion of Bacillus larvae, a pathogen of the honeybee, suggested that penetration of that bacterium into the gut tissues of host larvae took place when the gut peritrophic membrane sloughed off during metamorphic changes (Heimpel and Angus, 1963).

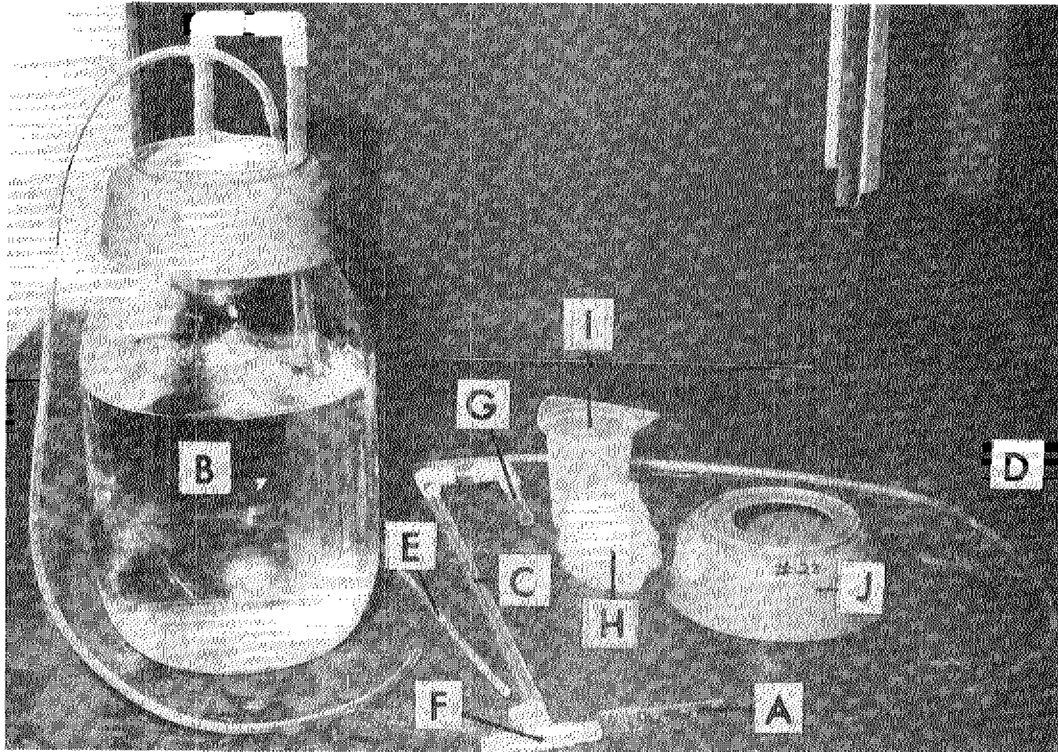

Fig. 1. Bioassay aquarium jar and components. Sponge-end "A" (sponge removed) of a Eureka sponge-filter unit is placed in aquarium jar "B", through which the aquarium water is taken in and moved by compressed air upward into plastic stem "C". (The compressed air comes in through Tygon hose "D" and the attached plastic stem "E" which is inserted into connector head "F".) The migrating aquarium water is discharged from plastic stem "G", and filtered through fiberglass wool "H" in Tri-Pour beaker "I" seated in jar cover "J" before returning to the aquarium jar. Please see text for more details.

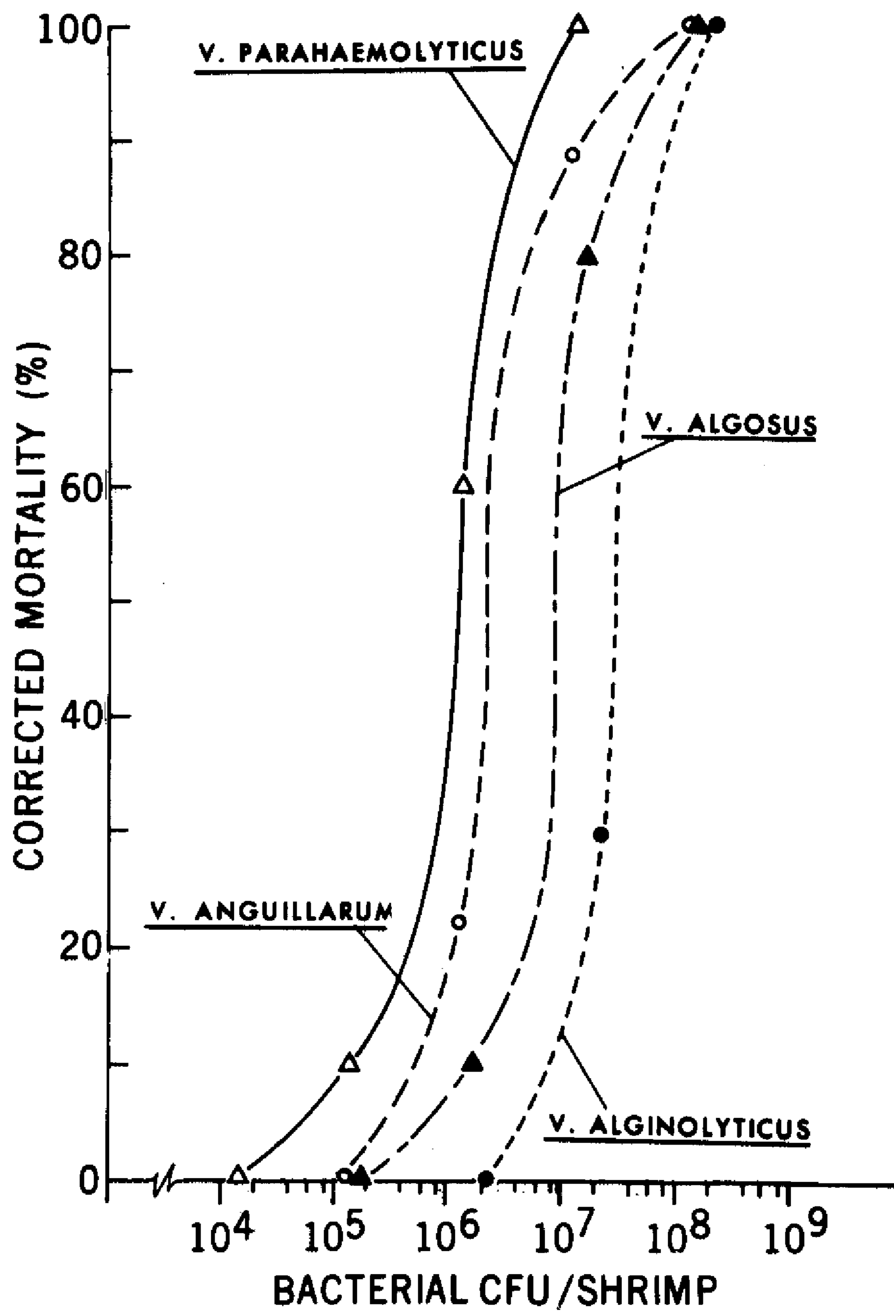

Fig. 2. Dosage-mortality response within 22 hours in white shrimp injected with four species of *Vibrio* bacteria.

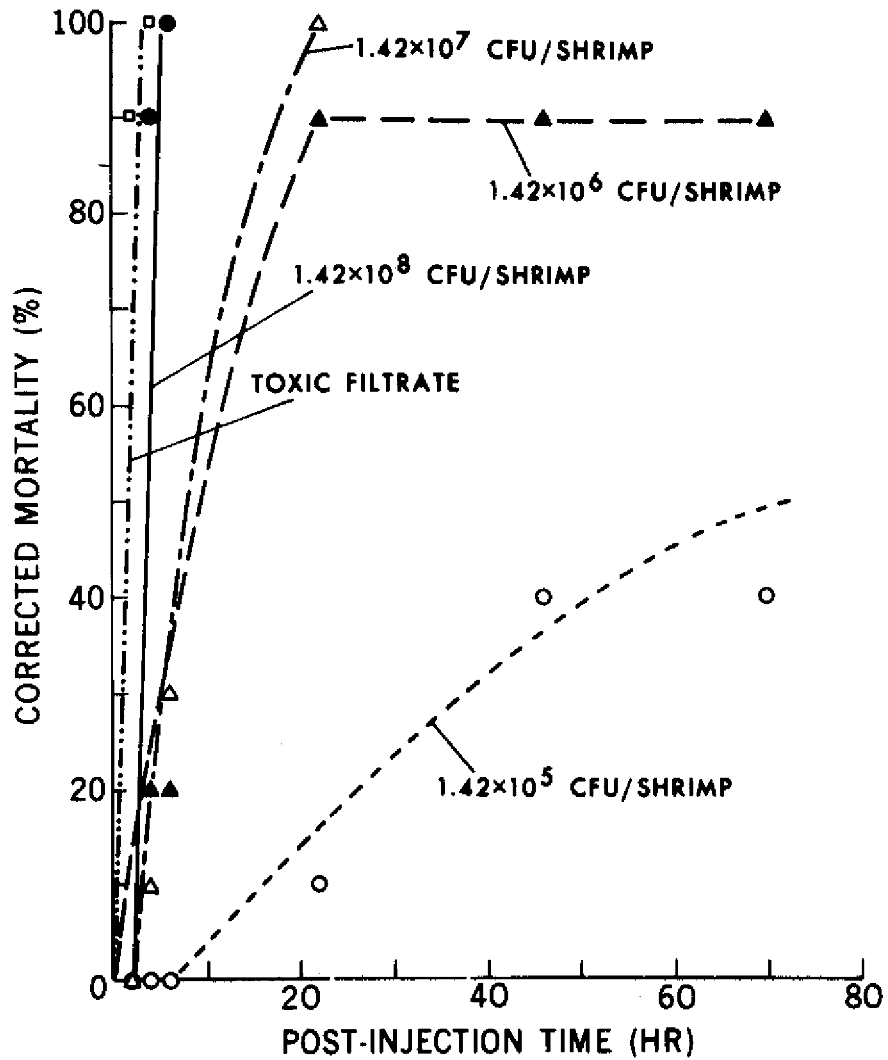

Fig. 3. Time-mortality response in white shrimp injected with <u>Vibrio</u> <u>parahaemolyticus</u> and a toxic filtrate from the same bacteria.

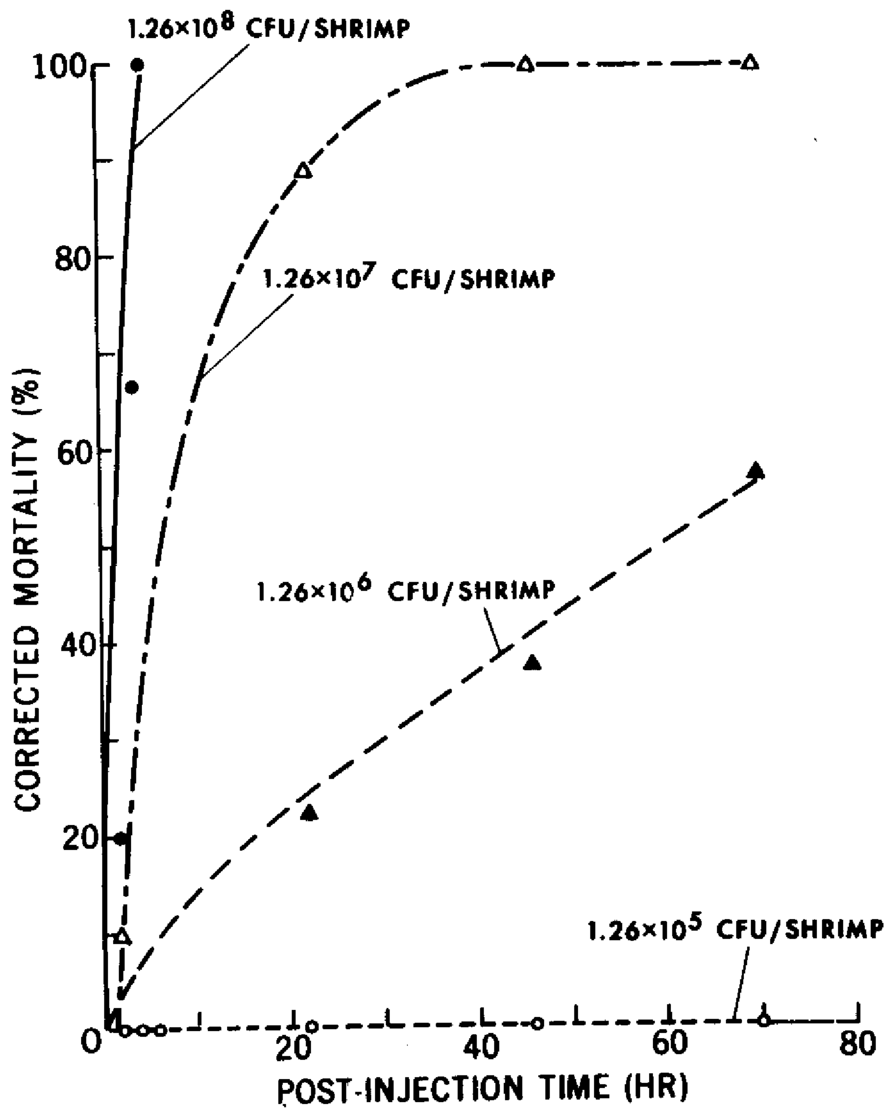

Fig. 4. Time-mortality response in white shrimp injected with *Vibrio anguillarum*.

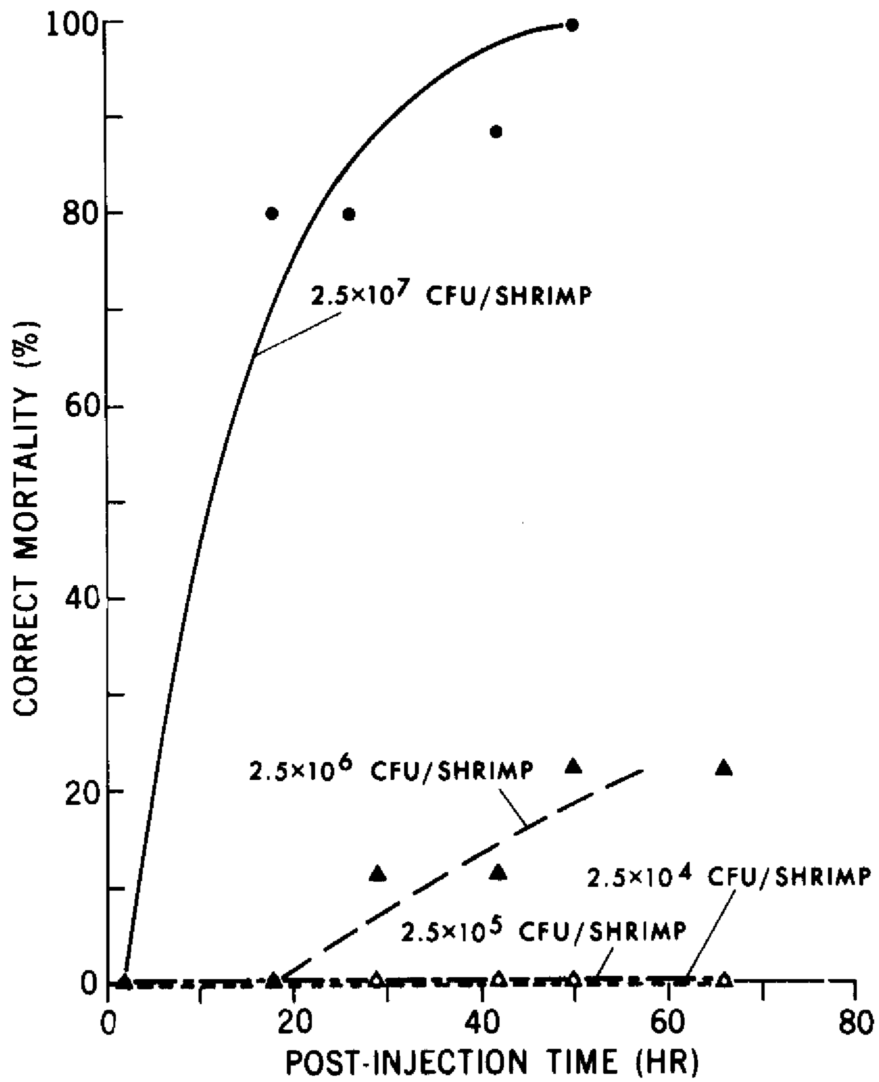

Fig. 5. Time-mortality response in white shrimp injected with *Vibrio algosus*.

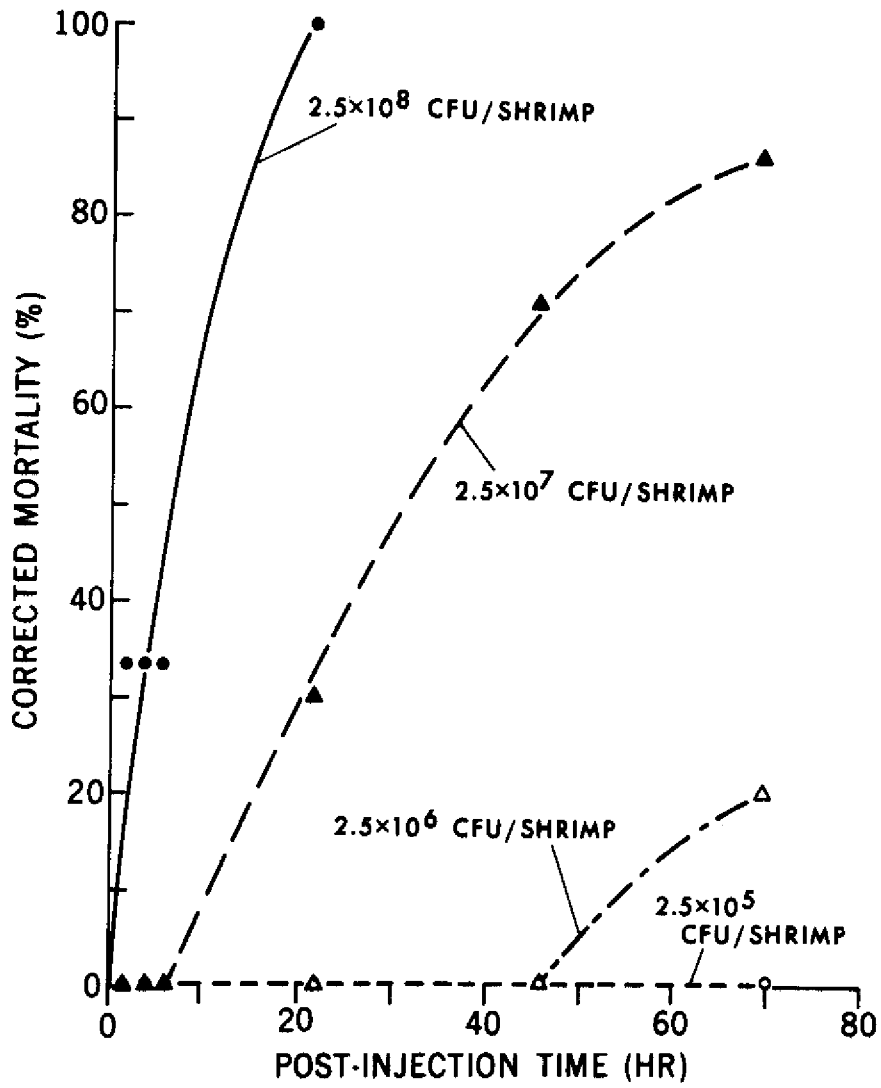

Fig. 6. Time-mortality response in white shrimp injected with *Vibrio alginolyticus*.

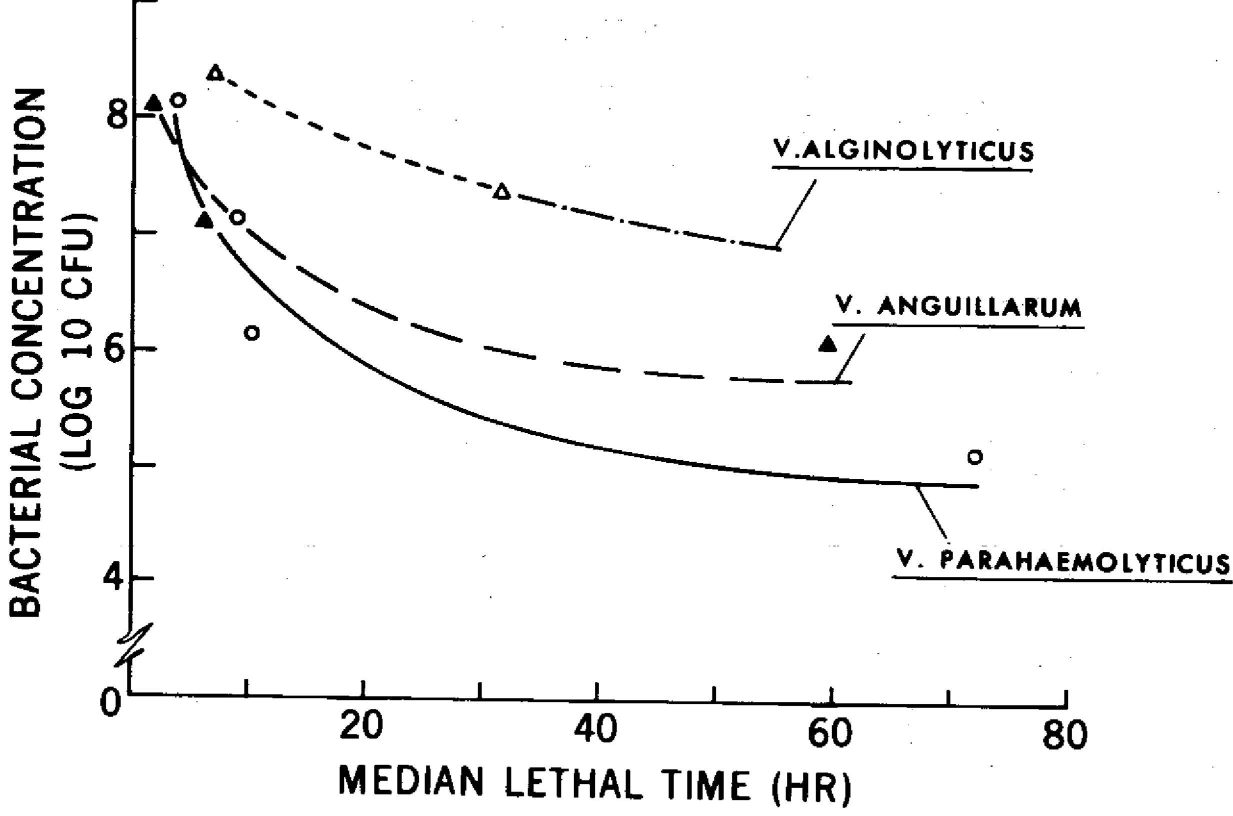

Fig. 7. Median lethal time (LT_{50}) for different doses of three species of Vibrio bacteria injected into white shrimp. Broken line (-·- -·-·-) in curve indicated extrapolated data.

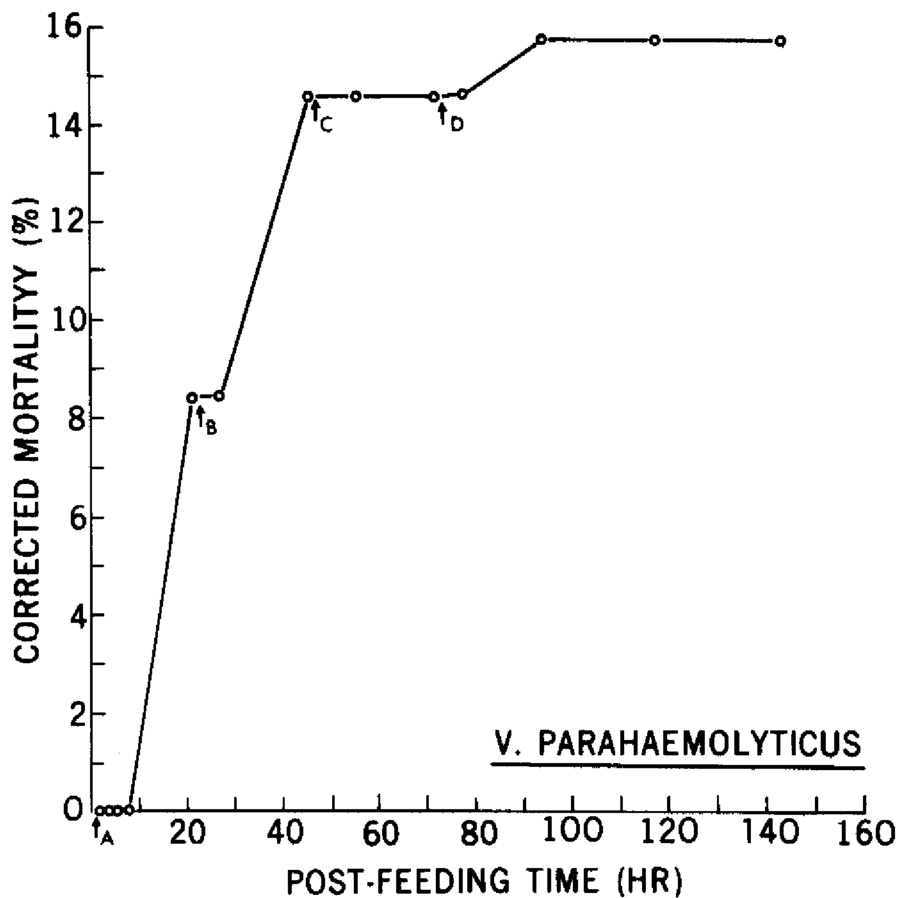

Fig. 8. Dosage-mortality response in brown shrimp fed with white shrimp meat supporting growth of <u>Vibrio parahaemolyticus</u>. Arrows indicate feeding time, and letter next to arrow indicates age of bacterial growth and dose of bacteria fed to each test shrimp: a = 1×10^9 CFU, 24-hr old; b = 2.9×10^8 CFU, 48-hr old; c = 5.4×1^{3} CFU, 24-hr old; and d = 7.3×10^9 CFU, 48-hr old. After 94 hr, the shrimp were maintained on non-infected sterile shrimp meat as in control.

Oral Infection Experiment

The results from orally feeding brown shrimp with *V. parahaemolyticus* showed that this organism was slightly pathogenic to brown shrimp through the oral route, and that oral infection by vibrios in nature was possible. Such an infection may happen when a shrimp feeds on other shrimp or fish killed by vibriosis, or ingests vibrio-contaminated materials in the water. However, since only a relatively low mortality (16% ca.) in brown shrimp was caused by repeated ingestions of relatively high dosages of *V. parahaemolyticus* (from 10^8 to 10^9 CFU/shrimp) (Fig. 8), *Vibrio* bacteria are probably not highly virulent to juvenile penaeid shrimp in nature. They are more likely opportunistic organisms, causing mortalities only when the shrimp encounter unfavorable environmental conditions. This assumption is perhaps particularly true with *V. alginolyticus*, which displayed the lowest virulence to white shrimp in our studies. *Vibrio alginolyticus* as well as *V. algosus* have been observed to be present in concentrations of from 10^2 to 10^4 CFU/ml in shrimp raceway water without causing significant loss to juvenile white shrimp (Leong *et al*., unpublished data). Experimentally, *V. parahaemolyticus*, when added directly to aquarium water to reach a final concentration of from 10^4 to 10^5 CFU/ml, was found to be either highly lethal to brown shrimp (Vanderzant et al., 1970) or harmless to juvenile pink shrimp and postlarval brown shrimp (Barkate, 1972). Piecing all the data together, it appeared that a *Vibrio* bacterium may become injurious to juvenile penaeid shrimp when its concentration reaches 10^5 CFU or more per ml. Therefore it is apparent that raceway or aquarium water should be kept from containing excess amounts of substrates, such as shrimp feed, which could sustain high concentrations of *Vibrio* bacteria.

Figure 8 showed that the lethal effect of V. parahaemolyticus subsided very rapidly when administered orally to brown shrimp. Mortality did not resume until repeated doses of bacteria were fed. Those events suggested that the death of the test shrimp probably resulted from the action of a toxin, or toxins, which were associated with V. parahaemolyticus and retained in the substrate shrimp meat, rather than the direct multiplication effect of that bacterium.

The lack of immediate mortality response in test shrimp following the feeding of the 3rd challenging dose of a 24-hr culture of V. parahaemolyticus (5.4×10^8 CFU/meat piece) indicated that the surviving shrimp either were intrinsically more resistant to the bacteria (or to their toxins) or have acquired a certain degree of immunity from the two previous injections. Penaeid shrimp have been reported to produce a hemolymph component which resembles vertebrate beta globulin within 48 hr after exposure to V. anguillarum (Lewis, 1973).

When a subsequent 4th feeding of 48-hr infected shrimp meat was made, the corrected mortality of the shrimp rose slightly again by 1%. Such increased mortality probably resulted from the use of a higher dose of challeging bacteria (7.3×10^9 CFU/meat piece) in the 4th feeding, overcoming the resistance of some of the surviving shrimp.

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Serology of Shrimp Pathogenic Vibrios

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ABSTRACT

Antigenic analysis and cross absorption studies on isolants of *V. anguillarum* recovered from diseased shrimp revealed that the pathogenic agents belonged to one of five serological groups. Serologic studies suggest that saprophytic isolants were usually not involved in the shrimp disease episodes.

INTRODUCTION

Members of the genus *Vibrio* have been recovered from most marine environments and constitute a significant portion of the indigenous microflora of marine fish and shellfish. Under conditions of compromised host defenses or adverse enviroment, certain of these organisms invade as adventitious pathogens causing widespread losses. However, a review of data on mortality patterns in aquaculture systems, cultural characteristics and apparent tissue affinity of certain isolants involved in disease processes suggests that pathogenicity probably involves a limited number of strains in most of the disease losses attributed to vibriosis. Nybelin (3) and Smith (5) recognized three biotypes of *V. anguillarum* as being associated with vibriosis in fish from the North Sea; Pacha and Kiehn categorized isolants from disease fish in the Pacific Northwest into three serotypes (4); and Tubiash et al. categorized the isolants associated with bacillary necrosis of bivalves into five serotypes (6). Several unanswered questions relative to the epidemiology of vibriosis, variability in virulence among bacteria recovered from diseased hosts and potential efficacy of select isolants as immunizing

agents could be resolved if more information on the serology of vibrios were available. The study upon which this report is based, represents an effort to serologically characterize vibrios most often recovered from diseased shrimp over a five year period from experimental mariculture ponds in Texas, Louisiana and Arizona.

MATERIALS AND METHODS

The 135 bacteria used in this study were recovered from hemolymph and lesions of moribund diseased shrimp. Primary isolation involved streaking samples on five percent bovine blood agar containing two percent marine salt (1) and TCBS agar (Baltimore Biological Laboratory, Cockeysville, Md.). Colonies typical of _V. anguillarum_ were transferred to brain heart infusion agar slants containing two percent marine salt. The isolants were characterized as described elsewhere (1). Only those organisms that reacted positively to cytochrome oxidase test, were motile by polar flagella, dissimilated glucose fermentatively without producing gas, possessed a lysine decarboxylase and were capable of utilizing citrate as a sole carbon source were used in the study. The organisms were preserved by lyophilization in sterile five percent skim milk.

The organisms were reconstituted with brain heart infusion broth and streaked onto brain heart infusion agar containing two percent marine salt. After overnight incubation at 20C, growth from two plates were harvested with 20 ml 0.85% saline and autoclaved at 121C for 15 min. The suspension was centrifuged approximately 2000xg, the supernatant (soluble antigen) collected and cell sediment (0 antigen) washed in saline five times. The cell suspension was adjusted to a turbidity

corresponding to number three McFarland turbidity standard (2).

Female white New Zealand rabbits were immunized against the soluble--and particulate antigens and antiserum processed in a manner described by Lewis and Allison (2). The resulting antisera were analyzed for agglutinins by slide agglutination and for precipitins by tube precipitation. The antigens used for serologic analysis were processed from brain heart infusion agar slants as described above.

Cross absorptivity studies were conducted on heterologous reactive antisera. Overnight growth from five brain heart infusion agar plates of hetorologous strains were suspended in 30 ml saline, autoclaved and the sediment washed five times. The sediment was resuspended in 50 ml saline, dispensed in 10 ml amounts of each of five plastic centrifuge tubes, centrifuged at 2000 xg and the supernatant discarded. Two ml antiserum were added to the cell suspension in the first tube, the preparation shaken and placed in a 37C water bath for 2 hr. This process was continued until agglutinins could no longer be detected by slide agglutination.

RESULTS AND DISCUSSION

The shrimp pathogenic isolants of *V. anguillarum* involved in this study could be classified into five serogroups based upon their soluble antigens. Two of the five soluble antigen serogroups possessed common O antigens and an O antigen was shared by the remaining three soluble antigen serogroups.

The isolants most often recovered from shrimp disease episodes belonged to two serogroups and were widespread geographically. Isolants of *V. anguillarum* recovered from marine muds and water samples usually

could not be serogrouped with the antisera developed during the course of this study. This finding suggests to us that, in most instances disease episodes are probably not precipitated by opportunistic saprophytes. It is proposed that chronically infected individuals serve as a reservoir of the pathogenic V. anguillarum. The chronically infected individuals would likely be less tolerant of adverse environmental conditions and upon succumbing would initiate epizootics such as those often observed in mariculture operations.

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A MYCOTIC DISEASE OF CULTURED PENAEID SHRIMP CAUSED BY THE FUNGUS *FUSARIUM SOLANI*

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ABSTRACT

A marine variety of *Fusarium solani* (Fungi Imperfecti) was found to be the causative agent of a chronic gangrenous-like disease of cultured penaeid shrimp. While the disease is known to occur in pond-reared penaeid shrimp, it is potentially a much more serious disease threat to intensive culture systems. Methods of drug and chemical therapy for fusarium disease are lacking, but the prevention of fusarium disease by the use of disease-resistant species of penaeid shrimp appears promising.

INTRODUCTION

Fusarium solani, a ubiquitous Fungi Imperfecti, has been known for years as pathogen of many species of plants and field crops, and for its occasional appearance in disease in man and animals. *F. solani* normally occurs as a saprophyte living on organic detritus in soil and water, and it may become a pathogen to shrimp. Strains of *Fusarium*, probably all *F. solani*, have been reported as pathogens of pond and tank-reared grooved and non-grooved penaeid shrimps in America and Japan (Egusa and Ueda, 1972: Johnson, 1974; Lightner, 1975; 1977), where it causes a chronic gangrenous-like condition in the gills, or on the general body surface of juvenile and adult shrimp. The same disease syndrome has also been described in tank-reared lobsters, *Homarus americanus* (Lightner and Fontaine, 1975), and the causative fungus was found to be a strain of *F. solani*.

All *Penaeus* spp. are probably susceptible to infection by *F. solani* under conditions favorable to the fungus. Grooved *Penaeus* spp. (the brown and the pink shrimps) appear to be more susceptible to infection by *F. solani* than do non-grooved (the white and the blue shrimps) under similar conditions.

The present paper describes fusarium disease in penaeids, reports methods for the detection of sources of *F. solani*, methods for the isolation and culture of *F. solani*, and presents new data on the sensivitity of *F. solani* "in vitro" to various forms of potential therapeutic drugs and chemicals.

MATERIALS AND METHODS

Animals

Shrimp for these studies were obtained from cultured populations reared at the University of Arizona-University of Sonora experimental shrimp culture research station at Puerto Peñasco, Sonora, Mexico. Species studied include *Penaeus californiensis*, *P. stylirostris*, and *P. vannamei*.

Histological Studies

Shrimp selected for histopathological studies were fixed live in Davidson's fixative (Humason, 1967). To enhance fixative penetration, shrimp were bisected or trisected prior to being immersed in fixative, or the *Fusarium*-affected portions were excised and fixed separately. Routine paraffin histological methods were used in section preparation and staining.

Microbiology

Fusarium solani isolates were obtained from infected shrimp tissues by culturing material from the lesions. Used in the present study were Cantino PYG broth and Sabourand's agar (Difco Laboratories), supplemented with salt or seawater to raise the salt content to 2%. Penicillin (∿200 mg/ml of media) were added to inhibit bacterial growth.

Samples of sand, soil, and feed components were cultured for *Fusarium* spp. on Sabourand's agar prepared with 2% NaCl and with added antibiotics. One gram samples of test material were homogenized in 100 ml of sterile saline. Replicate 1 ml

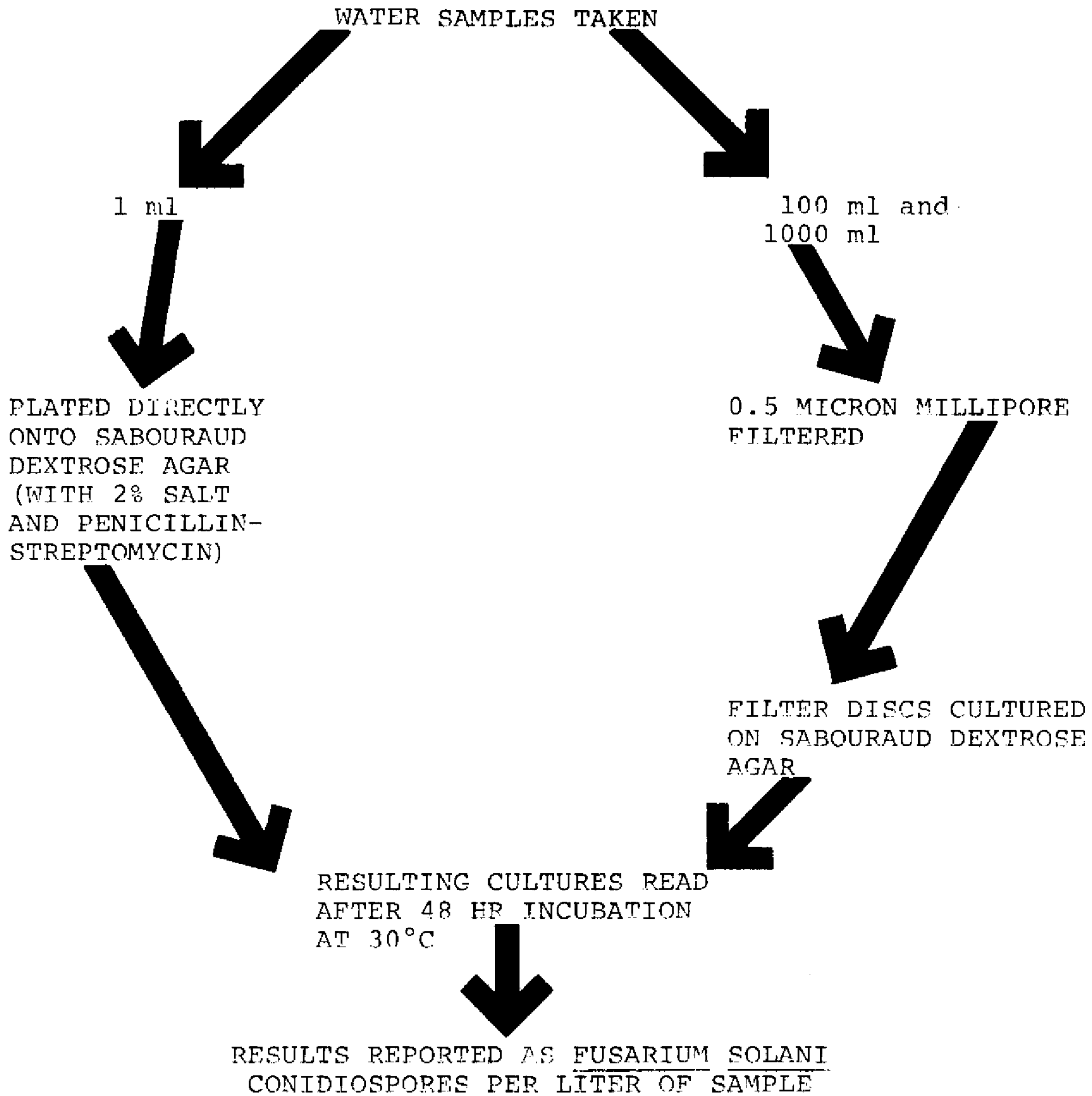

Figure 1. Schematic diagram of the method used in culturing seawater samples for *Fusarium* spp.

samples of these homogenates were plated onto Sabourand plates and incubated at 28°C for 48 hr prior to being examined for growth of *Fusarium* spp. Suspect colonies were sampled and examined for the presence of *Fusarium* spp. conidia at 100X.

Water samples taken from various tanks, raceways and from seawater wells, were cultured for *Fusarium* spp. using the method illustrated in Fig. 1.

To investigate the possibility that *F. solani* spores were airborne, Sabourand agar plates were placed uncovered for 10, 30, or 60 min at various representative sites at the research station. After incubation at 28°C for 48 hr, the resulting cultures were examined for *Fusarium* spp. colonies.

Testing of Chemotherapeutics

Potential drugs and chemicals were tested against *F. solani* using the tube dilution method in which ten-fold serial dilutions were used over the range 10^2 to 10^{-3} mg of chemical or units of drug activity per liter of media. Appropriate solvents (usually water or ethanol) were used to carry test chemicals or drugs through the dilution series. Minimum inhibitory concentrations (MIC's) were determined after 72 hr of incubation at 28°C. The MIC was considered to be the last tube in a dilution series to show no growth of the fungus.

Drugs or chemicals that gave low MIC values "in vitro" were further evaluated "in vivo" on *F. solani*-infected shrimp by addition of the material to the diet or by direct addition of the chemical to the culture water as bath treatments.

RESULTS

Pathology

Fusarium solani infects dead or damaged tissue, such as wounds resulting from crowding, gills damaged from chemical treatments, or lesions from other disease processes such as "shell disease". *F. solani* lesions are visible as expanding cuticular or subcuticular lesions that are usually blackened with melanin, occasionally with reddened necrotic centers, that occur on the general body surface, in the gills, or on the tips of the appendages (Fig. 2). Once established, the infection is chronic, usually progressive, and eventually leads to death of the infected host due to tissue destruction by toxins (Claydon, et al., 1977) produced by the fungus or to secondary bacterial infections.

Fresh mounts of *F. solani*-infected tissue typically contain hyphae and conidia of *F. solani*. Demonstration of macroconidia provides for a positive diagnosis of fusarium disease (Fig. 3).

Histopathology shows *F. solani*-caused lesions to be composed of very large numbers of infiltrating hemocytes, necrotic tissue debris, and fungal hyphae and conidia (Fig. 4). Typically, hyphae are encapsulated by multiple layers of hemocytes which frequently are melanized.

The epizootiology of fusarium disease was found to be dependent on the host species and host age. Of the 3 species of penaeid shrimp studied at Peñasco, *P. californiensis* was found to have the highest susceptibility to *F. solani*, with

P. stylirostris and *P. vannamei* being considerably less susceptible to infection by the fungus (Table 1). Populations of *P. californiensis* were found to be susceptible at a younger age to *F. solani*, and to have a higher incidence of infection and higher mortality rate (Fig. 5; Table 1) than was observed in the other two species. *P. stylirostris* was found to be intermediate in susceptibility and mortality rate between *P. californiensis* and *P. vannamei*.

Microbiology

Isolates of *Fusarium* from Puerto Peñasco shrimp were identified as a strain of *F. solani* by Dr. Paul E. Nelson (Fusarium Research Center, Penn. State U., Univ. Park, Penn. 16702) and by Dr. J. F. Grove (Univ. of Sussex, Sussex, Great Britain BNI 9QJ).

F. solani was isolated from infected shrimp of all three species; from water containing populations of shrimp in which the disease was enzootic (Fig. 6); from water containing apparently healthy populations of shrimp; from the air in the plastic covered "aquacells" that contain the raceways; and from the two seawater wells which supply the research station (Table 2). The fungus was not found in surface or near surface samples of beach sand or soil, in feed components, in near-shore seawater samples, or in wild shrimp randomly sampled from the commercial fishery.

Fusarium Therapy

MIC trials: Eighteen commercially available compounds with fungistatic or fungicidal properties were tested using the tube dilution method. The results of these trials are given

Figure 2. California brown shrimp (*Penaeus californiensis*) with a *Fusarium solani*-caused lesion on the abdomen.

Figure 3. Photomicrograph of *Fusarium solani* macroconida from a scraping of a F. solani-caused lesion. No stain x 400

Figure 2

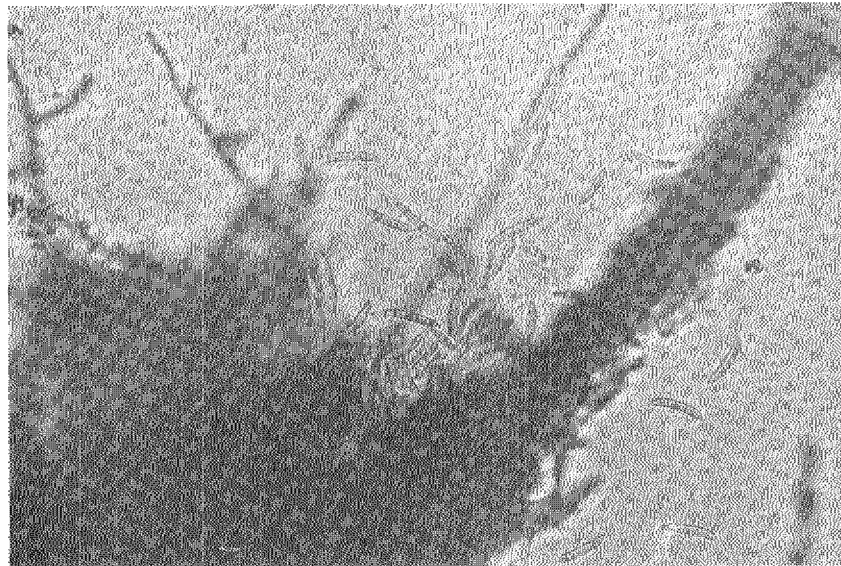

Figure 3

Figure 4. Photomicrograph of a *Fusarium solani*-caused lesion. Masses of inflammatory cells and necrotic tissue debris are present, as well as encapsulated hyphae. Masson's trichrome. x800.

Figure 5. Accumulated percent mortality by week of a population of raceway-reared *Penaeus californiensis* with an epizootic caused by *Fusarium solani*.

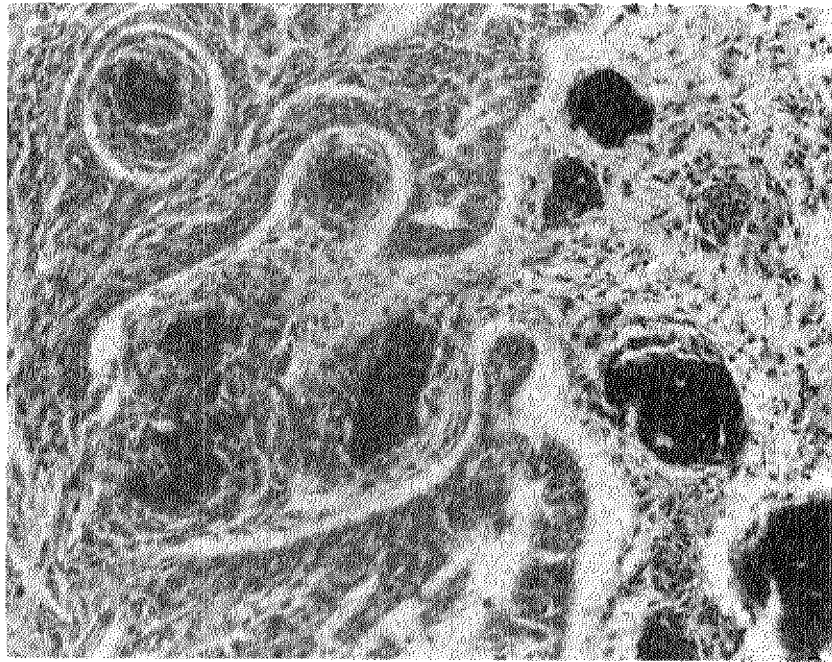

Figure 4

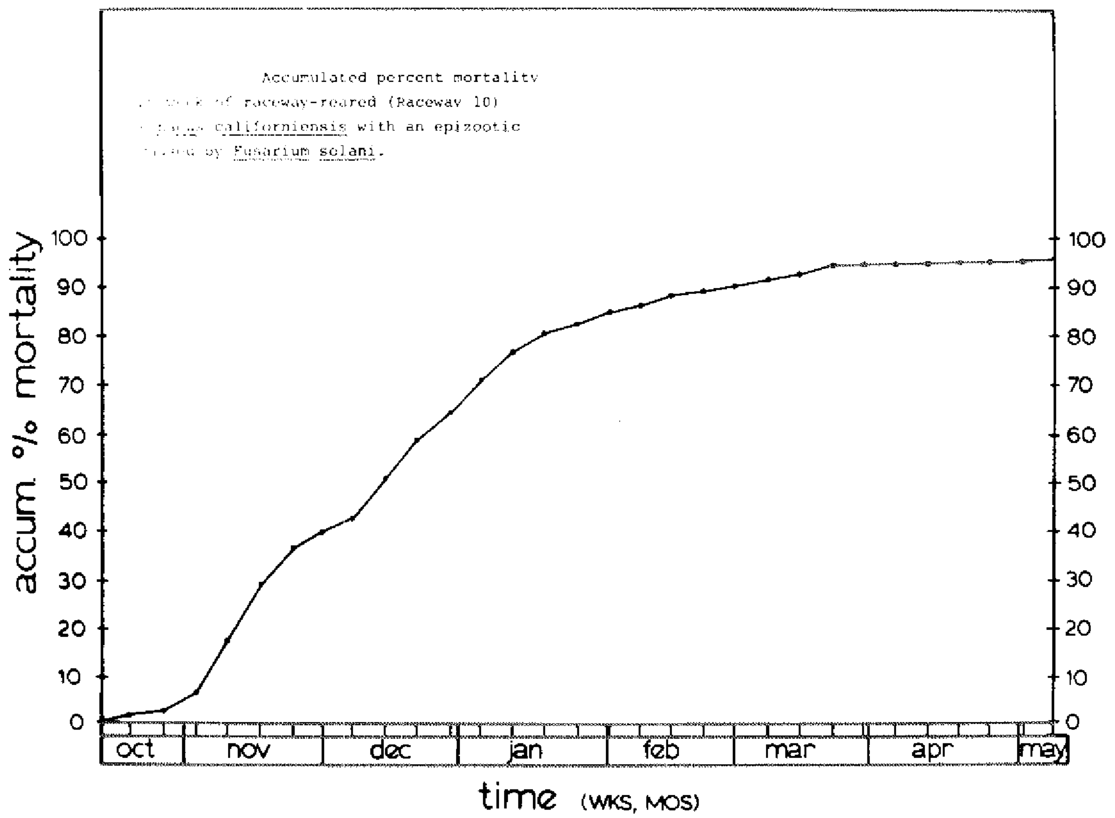

Figure 5

Figure 6. Percent incidence of _Fusarium solani_-caused mortalities in relation to spore count in a raceway-reared population of _Penaeus californiensis_ (Nov., 1976 to Feb., 1977).

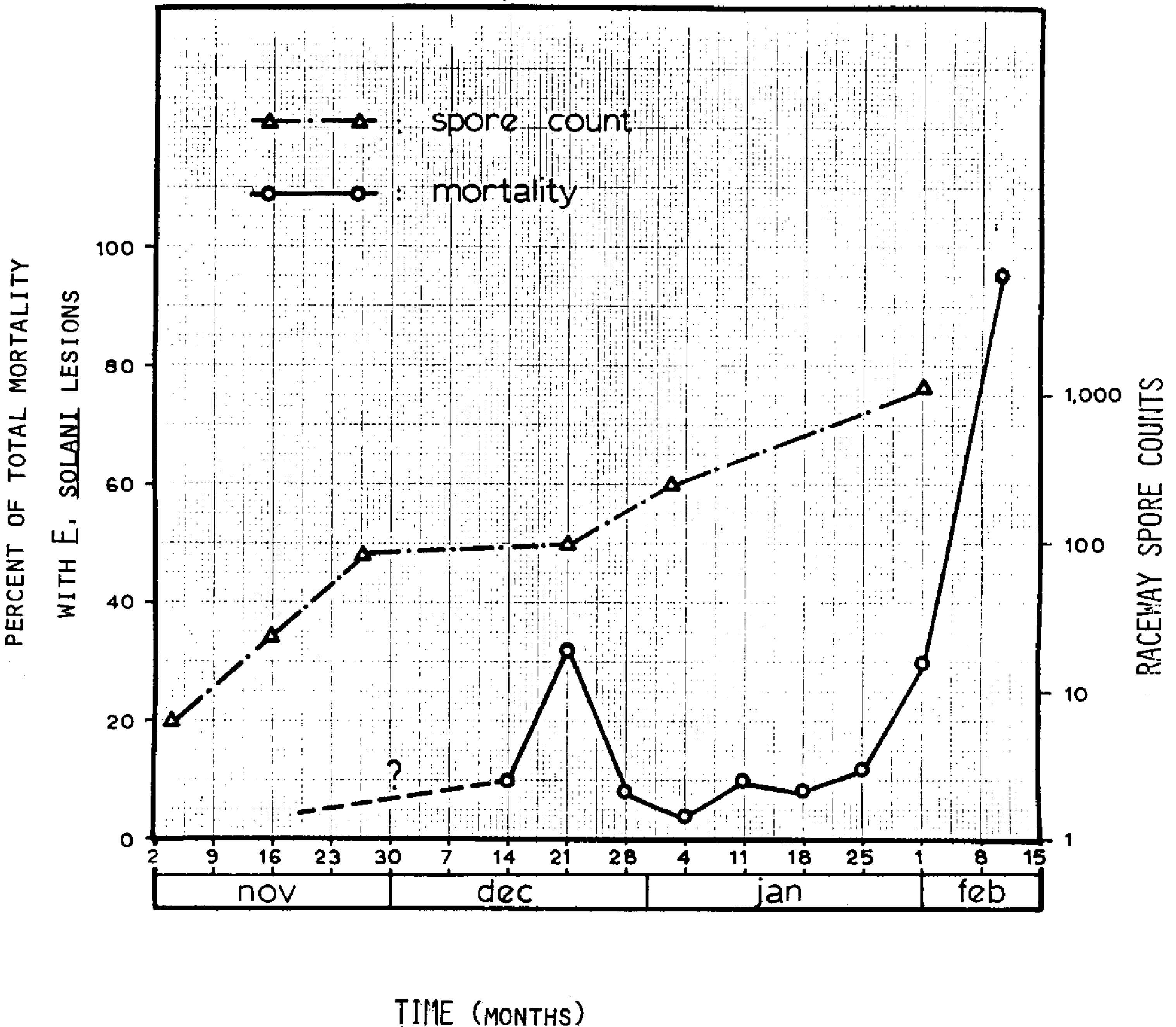

Table 1. Susceptibility and incidence of *Fusarium solani* infections in three species of *Penaeus* at Puerto Peñasco.

Species	Relative Susceptibility	Susceptible Age	Incidence*
Penaeus californiensis (Calif. Brown)	high	30-40 weeks	55[1]
P. stylirostris (Blue)	moderate	40-50 weeks	6.5[2]
P. vannamei	low	68 weeks	3.5[3]

*Incidence per thousand of population

[1]Ac.4; H37; born 4/2/76; for January to March 1977.

[2]Ac 3; H40; born 5/1/76; for January to March 1977.

[3]Ac 7; H31; born 9/4/75; for January to March 1977.

Table 2. _Fusarium solani_ spore counts per liter of seawater samples from the seawater wells and three raceways at Puerto Peñasco.

Date	Well 2	Well 5	RACEWAY 3	RACEWAY 4	RACEWAY 5
25 Feb. 76	-	-	ND	1	3500
25 Mar. 76	13	-	67	520	81
29 Apr 76	ND*	ND	180	40	10
26 May 76	ND	2	160	290	190
21 Jun 76	ND	ND	192	312	35
19 Jul 76	12	ND	366	1320	39
30 Aug 76	33	8	6	3	ND
20 Sept 76	-	37	-	-	-
8 Oct 76	ND	14	4	ND	ND
30 Nov 76	-	ND	ND	85	ND
22 Dec 76	-	ND	35	90	38
1 Jan 77	-	ND	57	293	49
9 Feb 77	ND	ND	190	1000	-

* ND = none detected

Table 3. Results of tests to determine the minimum inhibitory concentration (MIC) of various drugs and chemicals against *Fusarium solani*.

Chemical or Drug	MIC * in mg/ℓ 24 hr	48 hr	72 hr
A. Fungicides and Herbicides:			
Terraclor	1	>100	>100
Treflan	1	>100	>100
Banlate	1	10	10
Dexon	0.01	>100	> 100
Kerathane	1	10	100
Morestan	0.001	10	100
B. Drugs and anti-biotics:			
Fungizone**	0.5	5	50
Griseofulvin***	-	>1000	>1000
Thiabendazole(TBZ)	0.1	1	1
TBZ as Omnizole	0.1	10	10
Pimafucsin	-	-	>100
C. Disinfectants and Dyes:			
Formalin	0.1	1	10
Chlorine dioxide pH4	-	100	100
Chlorine dioxide pH6	-	>100	>100
Chlorine dioxide pH8	-	>100	>100
Hyamine	10	10	10
Roccal	1	10	10
Melachite green	0.01	1	1
Methylene blue	1	>100	>100
Crystal Violet	-	-	10
Copper as Cutrine Plus	0.1	>100	>100

Test concentration range:

* 0.001 to 100mg/ℓ

** 0.005 to 500mg/ℓ

*** 0.001 to 1000mg/ℓ

in Table 3. Of these 18 chemicals, Thiabendazole, Hyamine, Roccal, Benlate malachite green, formalin, and crystal violet showed activity against F. solani in the "in vitro" tests at concentrations of 10 mg/ℓ or less. Thiabendazole, malachite green, formalin, and crystal violet were tested "in vivo" tests with F. solani-infected populations of either Penaeus californiensis or Peneaus stylirostris juveniles or adults.

Malachite green at 0.1 ppm was used alone or with formalin (at 25 ppm) in 24 hr flow-through treatments administered every 3 days to a F. solani-infected population of 20 g juvenile P. californiensis. Seven consecutive treatments were given, but there was no reduction in mortality rates or in F. solani spore counts in the raceway water (Table 4). Because of the lack of favorable response to these treatments, a second series of treatments was begun. In these treatments which were 8 hr static treatments given every 7 days for 7 consecutive weeks, the concentration of malachite green was increased to 0.5 ppm. Formalin was used at 25 ppm with the malachite green. As was the case in the first set of treatments, no reduction in mortality rates or in F. solani spore counts occurred (Table 4).

Thiabendazole (TBZ) was tested two ways: a) as an externally administered bath chemotherapeutic and b) orally as a feed additive. TBZ was tested as a bath chemotherapeutic in a 4-week experiment with a population of F. solani-infected P. californiensis juveniles. TBZ (as Omnizole) was administered at 50 ppm in 8 hr static treatments given weekly for 4 weeks. The results

Table 4. Results of chemotherapy trials using malachite green-formalin mixtures to treat an ongoing epizootic in *Penaeus californiensis* due to *Fusarium solani*.

Date	Treatment Duration	Conc. (mg/ℓ) MG*	Formalin (mg/ℓ)	F. solani spore count following treatment	Accumulated mortality to date
11/1/75	24 hr	0.1	25	-	10%
11/6/75	24 hr	0.1	25	-	-
11/12/75	24 hr	0.1	25	-	20%
11/19/75	24 hr	0.1	25	80/ℓ	-
11/22/75	24 hr	0.1	25	70	35%
11/26/75	24 hr	0.1	25	270	-
12/4/75	8 hr	0.5	25	1000**	40%
12/11/75	8 hr	0.5	25	>100	-
12/18/75	8 hr	0.5	25	200	45%
12/26/75	8 hr	0.5	25	30	50%
1/2/76	8 hr	0.5	25	0	60%
1/29/76	8 hr	0.5	25	500	75%
2/5/76	8 hr	0.5	25	140	85%

* MG = malachite green.

** Pretreatment spore count 12/4/78 was 1000/ℓ.

of this experiment indicated a detrimental effect of TBZ on the treated population, as the untreated control group had better survival rates (46%) than did the group (4%) treated with TBZ (Table 5).

TBZ administered orally as a feed additive at 100 and 1000 mg TBZ/kg of feed to F. solani-infected populations of P. californiensis for 4-weeks had no effect on mortality rates, or incidence of infection, and gave results that were not different from the untreated control group.

Crystal violet was tested as a chemotherapeutic bath with F. solani-infected adult P. stylirostris. The dye at 1 ppm was administered in 6 hr static treatments given every 48 hrs for a total of 7 consecutive treatments. Following these treatments, the Fusarium-caused lesions continued to develop and mortalities continued at the same rate as in the untreated control group.

In a second experiment, a 1% solution of crystal violet was "painted" onto Fusarium-caused lesions of the carapace of adult P. stylirostris every 48 hrs for 21 days (9 treatments). No beneficial effect was noted and mortality rates were not different from the control group.

In a final experiment with crystal violet, the dye was administered in a series of three 6 hr static bath treatments given at 48 hr intervals to a population of F. solani-infected P. stylirostris. No beneficial effect of these treatments resulted.

Hyamine, Roccal and Benlate were not tested with F. solani-infected shrimp because these materials are toxic to shrimp at

Table 5. Results of experiments in which TBZ was tested in 8 hr static baths for four consecutive weeks as a chemotherapeutic for *Fusarium solani*-infected *Penaeus californiensis* juveniles (26g ave. wt.)

Treatment	Start	After 4 treatments	%Mortality
8 hr static TBZ at 50 mg/ℓ	68%	28%	46%
Untreated Control	68%	19%	4%

concentrations less than the level found to be fungistatic in the "in vitro" tests.

DISCUSSION

The results of drug and chemotherapy trials in the present paper and the results of Hatai et al. (1974) with F. solani in the Kuruma prawn in Japan, indicate that methods for the chemotherapy of Fusarium infections in crustacea remain to be developed. Furthermore, practical means of prevention of the disease have not been developed. Elimination of sources of Fusarium conidiospores by filtration or sterilization of contaminated water supplies and frequent cleaning of tanks and raceways have been suggested and may later be shown to be practical, but at present such practices are expensive in terms of labor and capital and are not completely effective. Perhaps the use and further development of Fusarium-resistant species or populations of shrimp for culture purposes may become the most practical means of preventing epizootics due to F. solani and related species.

There is some experimental evidence that indicates fusarium disease may be prevented by culturing resistant penaeid species. Species dependent resistance to infection by F. solani has been observed at Puerto Peñasco as indicated in the data given in Table 1. That data shows concurrent epizootics due to F. solani that occurred over a 6 month period in raceway-reared populations of three species of penaeid shrimp. A population of approximately 20,000 ten to 16 month-old P. californiensis was reduced by 90% by a F. solani-caused epizootic,

while a similar population of P. stylirostris was reduced by less than 10% (deaths due to F. solani-infections). Ten month-old P. vannamei were exposed to the fungus during the same period of time (exposure demonstrated by the detection of F. solani conidiospores in the seawater well water and in the raceway water), but showed no losses due to the disease until these shrimp reached approximately 16 months of age. Even then, the incidence of infection and losses due to the disease remained low (i.e., less than 10% accumulated percent mortality due to Fusarium disease to 2 years of age).

Until methods of chemotherapy and/or prevention are developed, shrimp culturists can best manage their stocks in areas where F. solani is enzootic by maintaining brood stock populations at low density and by harvesting shrimp intended for use as food before the shrimp being cultured, reach the susceptible age.

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A SYSTEM OF PHAGOCYTES IN LIMULUS POLYPHEMUS-A PRIMARY DEFENSE MECHANISM

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The hemocyte contributes a major portion of the immune processes in *Limulus*. This cell has been shown to phagocytize bacteria both *in vivo* and *in vitro*. The phagocytic process requires divalent cations and is energy dependent. Phagocytosis may be inhibited by carbohydrates which compete for receptors that are responsible for membrane binding and phagocytosis of bacteria. Hemocytes are stored in sinusoids in the gut and ventral sinuses, both of which may act as phagocytic centers. Hemocytes release clotting proteins and bactericidal factors in to the circulation after antigenic challenge. Circulating cell numbers, hemolymph protein concentration and bactericidal activity are correlated. Bacteria are rapidly cleared from the circulation by agglutination and phagocytosis. *Limulus* resembles other chelicerates in its immune capabilities and adheres to patterns evident in crustacean and insect immune conferring systems.

INTRODUCTION

The first observation of an invertebrate immune reaction was made by Metchnikoff (1884), who noted the phagocytosis of yeast by hemocytes in *Daphnia*. Although there is considerable controversey concerning the origin and function of hemocytes in arthropods, progress has been made recently regarding their role as phagocytes (Anderson et al., 1973; Gotz, 1973; McKay and Jenkin, 1970) or active contributors to immunity conferring processes.

Two cell types are found in *Limulus* hemolymph, the cyanoblast (Fahrenbach, 1970) and the hemocyte or amoebocyte (Loeb, 1921). The role of the hemocytes in blood clotting has been described by Young et al. (1972). That *Limulus* hemocytes also contribute bactericidal factors to the hemolymph has been demonstrated by Furman and Pistole (1976) and Stagner and Redmond (1975). However a phagocytic function for these cells has been unknown (Shirodkar et al., 1960; Sparks, 1972) until recently (Stagner and Redmond, 1975). Further description of these phenomena in *Limulus* and other chelicerates is important to determine the evolution of the arthropod immune system and to corroborate a model based upon insect and crustacean immune processes.

MATERIALS AND METHODS

Bacteria and bacterial cultures were prepared as described by Stagner and Redmond (1975). A culture system for *in vitro* hemocyte preparations was developed as described elsewhere

(Stagner, J. Inverteb. Pathol., in press). Briefly the method utilizes cell-free hemolymph buffered with small amounts of concentrated TRIS and maleic acid. The resulting solution is added to Leighton tubes supplied with flying cover glasses. Cell suspensions and test substances are added to the system and are allowed to incubate for six hours. Thereafter the cover glasses are removed, fixed and mounted for observation. A variety of culture conditions, including temperatures between 4-40^{0}C and pH ranging from 6.0-9.0, were employed. Pseudomonas atlantica (P.a.), latex beads, carbon particles, India ink, carmine particles and dog erythrocytes were added to test for phagocytosis. Receptors for bacterial components were determined by the addition of 100 μg of fucose, mannose or n-acetyl glucosamine to each tube prior to the addition of bacteria. Calcium-free solutions and the enzyme poisons cyanide and N-ethylmaleimide were used to test the requirements for divalent cations and energy dependent systems for phagocytosis.

In vivo phagocytosis was observed by injecting animals with heat killed bacteria (P.a.) and observing blood samples drawn at intervals for the presence of free and engulfed bacteria. Blood samples, 0.5-1.0 ml, were obtained by cardiac puncture using pre-cooled plastic syringes and 22 gauge needles. Blood smears were prepared on microscope slides for observation by phase contrast microscopy or fixed in formalin for later observation. A drop was expelled on to a chilled plastic surface and used for hematocrit determinations (Stagner and Redmond, 1975). The remainder of the sample was centrifuged at 2000g for 10 min at 4^{0}C in sterile

chilled plastic centrifuge tubes. The cell-free hemolymph was then used for bactericidal assays and aliquoted for protein determination by the method of Lowry et al. (1951). Bactericidal assays employed sterile cell free hemolymph to which 5000 P.a. per ml were added. After 90 min of incubation, an aliquot was spread on agar plates. Plates were counted after 36 hours at 22°C. In all experiments only adult female animals were used due to their larger size and blood volume. A sample drawn before injection served as a control for each animal.

Several adult animals and 10 3-4 inch juvenile animals were injected intracardially with carmine particles or heat killed bacteria which were untreated, stained with Saffranine O or labeled with tritiated mannose. After 6-24 hours, the animal was dissected. Areas of agglutinated carmine particles, bacteria or phagocytized bacteria were located visually and by use of histological sections. Smaller animals, 1-2 inch, were sectioned on a sliding microtome and examined for areas of agglutination.

In order to discern possible interconnections of the circulatory system which may provide access to phagocytic centers or hemocyte storage areas, several juvenile animals were injected with a rapidly hardening plastic solution. The monomer was colored with carmine and injected intracardially after a leg joint and gill had been cut to allow hemolymph to escape. After hardening, the animals were digested in hypochlorous acid to provide a cast of the circulatory system. Routes of blood flow were determined by the injection of concentrated Evans blue and observation using a high intensity lamp positioned beneath the animal.

RESULTS AND DISCUSSION

Phagocytosis (Fig. 1) occurred *in vitro* over the entire range of pH and temperatures tested; the optima being pH 8.0-8.5 and 30-35°C respectively (Figs. 2,3). Only erythrocytes and bacteria were phagocytized under the imposed culture conditions. The alkaline pH optimum can not be explained at this time. However the temperature effect reflects an increased metabolic rate at elevated temperatures. These data demonstrate that phagocytosis may be an efficient protective mechanism over a wide range of physiological conditions. Observations revealed that the sequence of events leading to the destruction of engulfed materials proceeded from cellular degranulation and local clumping of the antigen (P.a.), to bacterial attachment of the cell membrane, phagocytosis and the subsequent digestion of engulfed materials. As noted in molluscs by Foley and Cheng (1977), increased phagocytosis was paralled by an elevated rate of degranulation. These observations are interesting by showing at least empirically, a requirement for external cellular factors which aid phagocytosis.

Phagocytosis was abolished by cyanide and N-ethylmaleimide and was inhibited by several saccharides which competed for receptors for bacterial attachment (Table 1). The process is energy dependent and is mediated by enzymes and a complex carbohydrate sensitive receptor. Inhibition by sugars is consistent with the theory that mucopolysaccharides and similar substances common to Gram negative bacteria are targets for hemolymph agglutinins and membrane receptors (Acton et al., 1973; Cornick and Stewart, 1973;

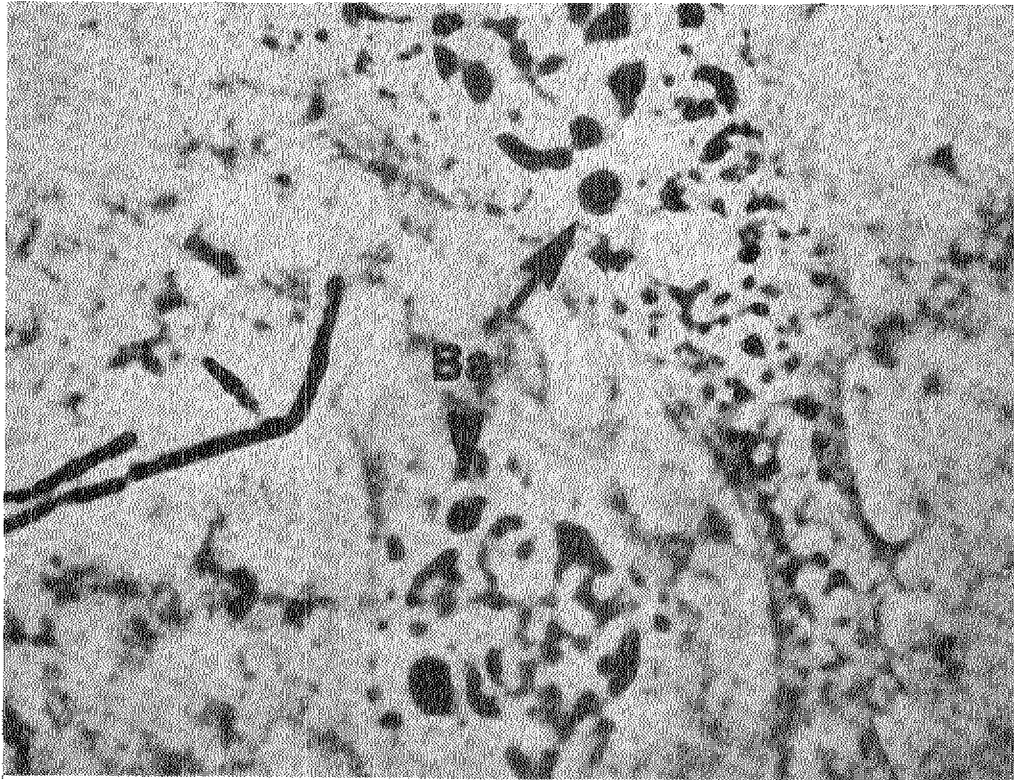

Fig. 1. Bacteria (Ba) engulfed by hemocytes. Note the swollen bacteria contained within digestive vacuoles.

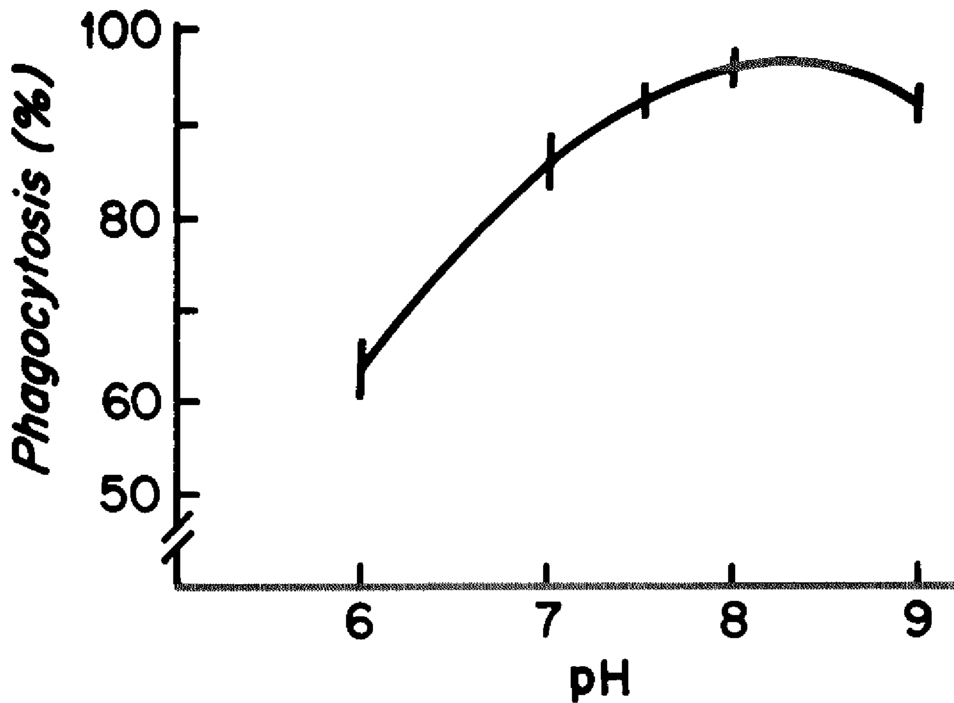

Fig. 2. Effects of pH on phagocytosis. Peak phagocytic activity is between pH 8.0-8.5.

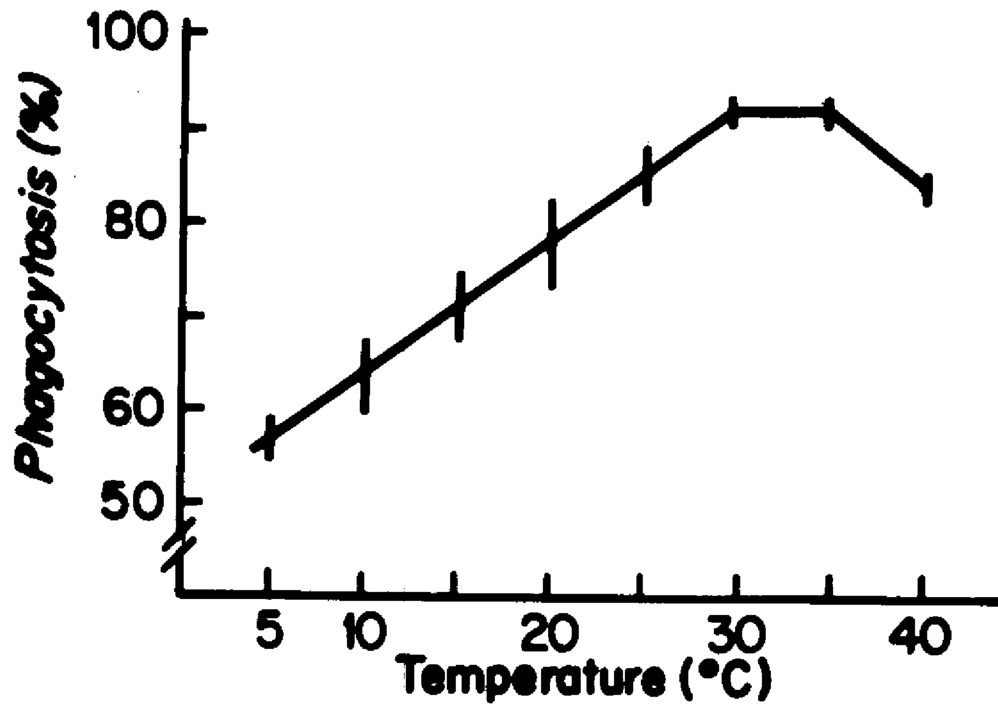

Fig. 3. Effects of temperature on phagocytosis. Peak phagocytic activity is between 30-35°C.

Table 1. Hemocyte Receptor Sensitivity.

Solution	Phagocytosis[a] %	Membrane attachment %	Bacterial agglutination
Cyanide	0	0	±
N-ethylmaleimide	0	0	±
P.a. endotoxin	82	7	+
N-acetyl glucosamine	61	13	-
Mannose	86	5	+
Fucose	72	9	+
Ca-free sea water	66	19	-
Low Mg Ca-free sea water	71	2	-
Control hemolymph	88	12	+

[a]Per cent refers to the number of cells containing engulfed P.a. after six hours of incubation.

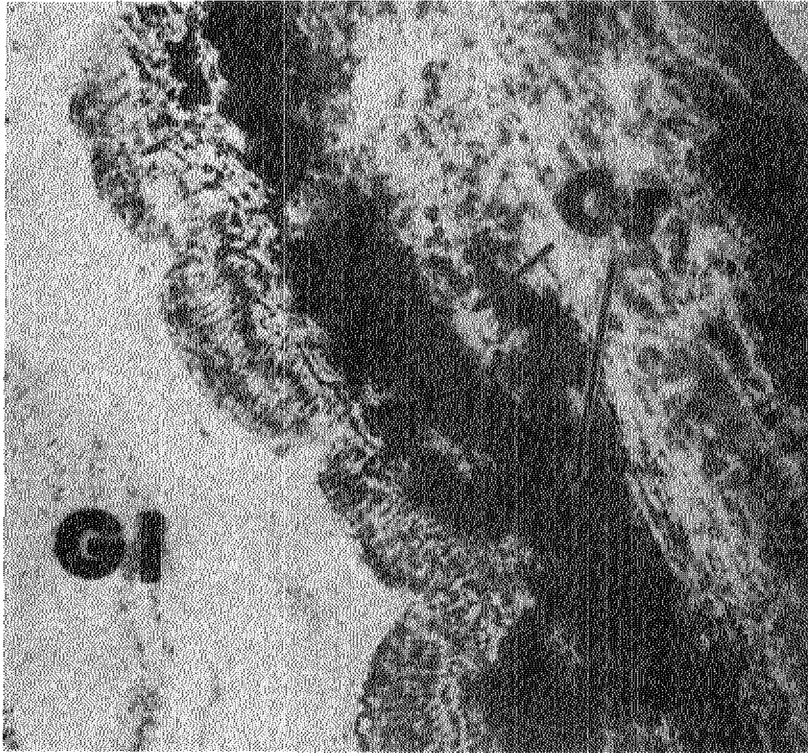

Fig. 4. Carmine particles (Cr) agglutinated in submucosal sinusoids in the digestive tract. The gut lumen (Gl) is shown to the left of the epithelium.

Lamanna et al., 1973; Renwrantz and Cheng, 1977). Divalent cations are required for phagocytosis and membrane attachment. Membrane attachment and agglutination of other materials such as carmine particles, India ink, and latex were possibly due to surface charge differences.

During *in vivo* experiments, hemocytes containing engulfed P.a. were recoveralble within 6 hours after injection. Such cells were recoverable for 24-36 hours, but in decreased numbers. During this time no free bacteria were observed in the hemolymph. Bacteria were presumably filtered out of the circulating hemolymph or otherwise removed by agglutination and phagocytosis. The presence of hemocyte storage areas and possible phagocytic centers were noted. Carmine particles and bacteria, marked and unmarked, were sequestered by agglutination in several highly vascular areas. The submucosa and muscularis mucosa of the intestine are composed of a network of connective tissues. Hemocytes fill the interstitial spaces or sinusoids and comprise a significant portion of the cells in these areas. Agglutinated materials were found in high concentrations in these areas (Fig. 4). Two ventral sinuses also were found to accumulate agglutinated materials and served as an other hemocyte storage area. Cells removed from these areas by agitation of freshly dissected organs were found to contain engulfed bacteria. It was postulated that these areas served as both phagocytic centers and storage areas for hemocytes. Plastic casts of the circulatory system and histological sections (Figs. 5,6) demonstrate these areas and perhaps explain the sudden increases in circulating cells during stress or hemorrahage (Stagner and

Explanation of legends for Figs. 5 and 6.

Fig. 5. Dorsal view of plastic cast of Limulus circulatory system.

Al	Anterior lateral artery
Be	Branchial efferent artery
H	Heart
Hm	Hepatomarginal trunk
Ma	Median aorta
Ha	Hepatic artery

Fig. 6. Ventral view of circulatory system sketched from plastic casts as shown in Fig. 5.

Am	Anterior marginal artery
Be	Branchial efferent artery
H	Heart
Pm	Posterior marginal artery
Bav	Branchial afferent vessel
Lv	Lateral ventral vessel
Mv	Median ventral vessel
Or	Oral ring
Vs	Dorsoventral sinus

Inset represents a cross section of a plastic cast demonstrating the relationship between the branchial circulation and the ventral sinuses and the gut sinusoids. The dashed lines represent the heart and aortic branches to the oral ring.

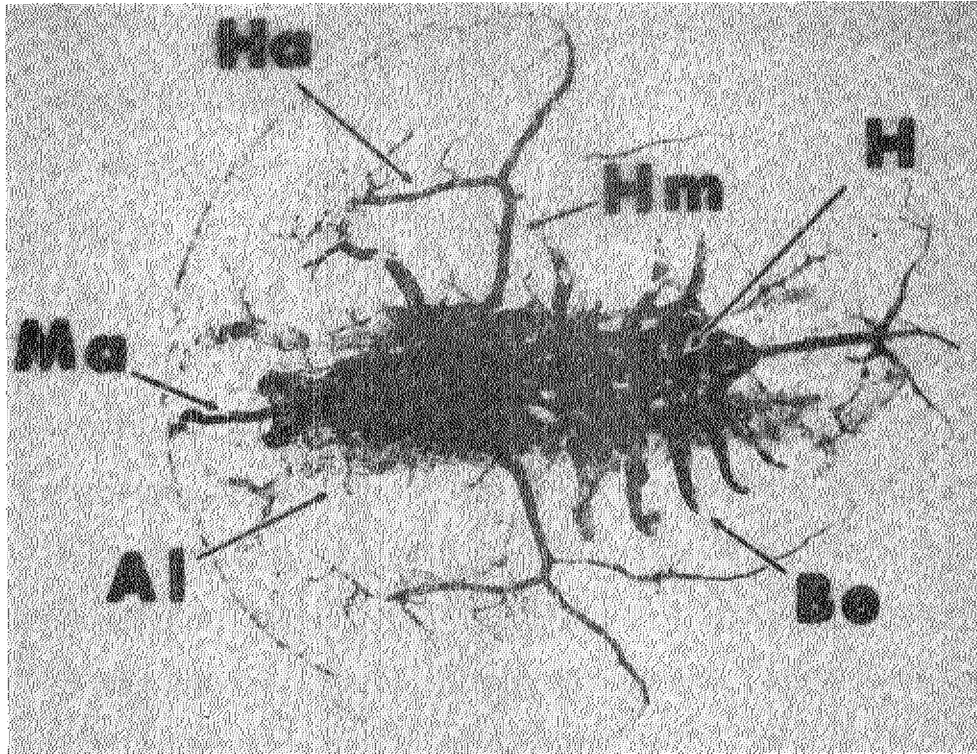

Fig. 5. Plastic cast of Limulus circulatory system. Legend explanation on next page.

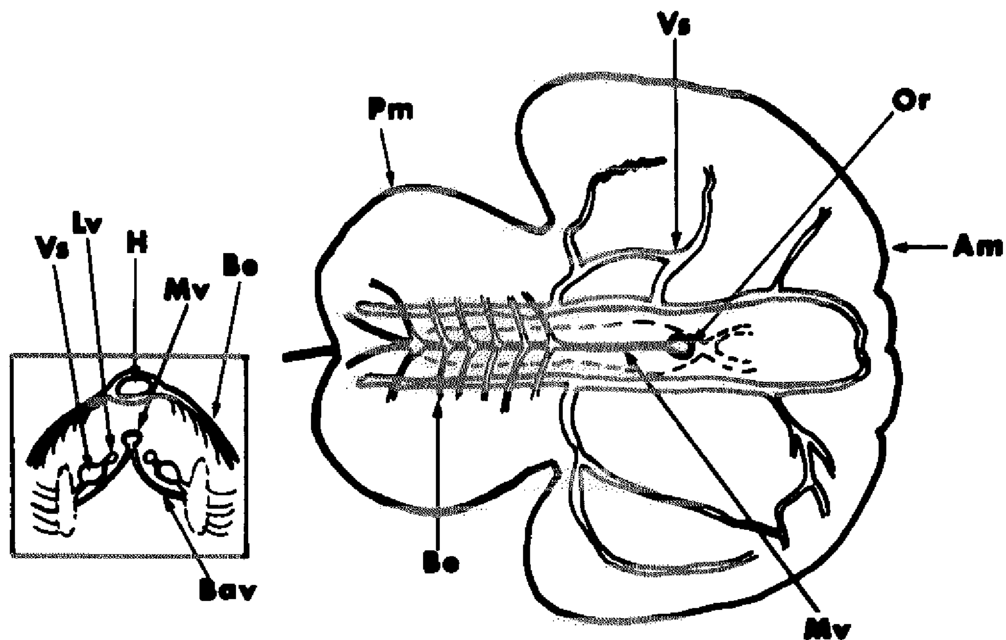

Fig. 6. Ventral view of circulatory system sketched from plastic casts. Legend explanation on next page.

Redmond,1975). Hemocytes may be released directly in to the circulation from these areas via the branchial vessels.

An additional function of hemocytes was suggested by Stagner and Redmond (1975), who noted a correlation between hemocyte numbers, protein concentration and bactericidal activity. Injection of a heat killed P.a. suspension resulted in an initial decrease of hemolymph protein and circulating hemocytes within 12-24 hours. During this time a factor with bactericidal activity was also decreased. After 24-36 hours, there was an increase in hematocrit, hemolymph protein and bactericidal activity which persisted for 84-96 hours (Tables 2,3 & 4). Maximal bactericidal activity was reached at 84 hours, whereas the hematocrit was variable and remained lower than t_0. The hemolymph protein concentration and hematocrit were elevated shortly before the maximum bactericidal activity was acheived. If animals were reinjected at 84 hours, the same sequence of events occurred with a slight enhancement of the bactericidal titer. In some cases, there was a prolonged reduction in hematocrit but an increased plasma protein concentration and bactericidal titer. It must be assumed that the hemocytes were stored and released the active principle in to the circulation. These results are similar to those reported in scorpions by Brahmi and Cooper (1974). They noted an initial decrease in hemocyte number followed by an increase which was paralled by the agglutination and clearance of sheep erythrocytes from the circulation. They postulated that this was due to an increase in hemagglutinin titer 24 hours after immunization. McKay and Jenkin (1970) also found, in crayfish, an increas in hemocyte number after exposure to an antigen which was concomitant with an increase in phagocytosis.

Table 2. Hematocrit Change Post-injection[a]

Animal number	Hematocrit Time in hours								
	0	12	24	36	48	60	72	84	96
6	2.9	8.0	2.8	2.1	2.1	-	-	2.2	-
7	4.5	3.9	2.5	7.1	3.1	-	-	2.8	-
8	4.4	4.2	4.1	4.6	4.0	-	-	3.7	-
9	4.0	3.6	4.7	4.2	4.2	-	-	4.1	-
11	2.5	1.6	1.7	2.2	0.4	-	-	1.5	-
12	0.4	0.3	0.5	0.6	0.2	-	-	0.3	-
14	1.9	0.9	1.9	1.8	1.6	-	0.8	-	0.9
16	3.6	3.2	3.4	2.6	2.6	-	-	2.7	-
18	4.0	2.7	1.5	2.3	2.1	-	-	1.5	-
19	0.7	0.5	0.9	1.1	0.4	-	-	0.5	-
20	2.4	2.3	1.7	2.5	2.3	-	-	2.1	-
6a (Reinjected at 84 hours)	2.2	2.0	2.4	3.3	2.8	1.6	-	2.2	3.0
7a (Reinjected at 84 hours)	2.8	-	4.5	3.9	3.3	7.0	-	3.9	4.9
8a (Reinjected at 84 hours)	3.7	3.4	3.3	4.0	3.8	-	-	3.5	-
9a (Reinjected at 84 hours)	4.1	3.9	4.3	4.4	4.1	-	-	3.8	-
Controls (Sea water injected)									
21	3.5	3.5	3.5	3.5	3.6	3.5	3.5	-	3.5
24	3.3	3.3	3.3	3.3	3.3	3.4	3.3	-	3.2
35	2.2	2.2	2.2	2.2	2.2	2.2	2.2	-	2.2

[a] Time 0 served as a control for each animal.

Table 3. Hemolymph Protein Change Post-injection[a]

Animal number	Protein (g/100 ml) Time in hours								
	0	12	24	36	48	60	72	84	96
6	3.0	3.5	3.0	3.2	3.2	-	-	3.1	-
7	5.3	4.8	4.7	4.9	4.8	-	-	4.9	-
8	5.3	6.2	6.6	6.4	6.5	-	-	5.9	-
9	5.0	4.8	5.2	5.5	5.5	-	-	5.1	-
11	1.0	0.9	1.1	1.0	1.0	-	-	1.1	-
12	0.7	0.6	0.6	0.6	0.8	-	-	0.8	-
14	1.8	1.7	1.5	1.7	1.7	-	1.7	-	1.8
16	3.6	4.2	3.6	3.8	3.7	-	-	3.7	-
18	0.9	0.9	0.9	1.1	1.3	-	-	1.1	-
19	1.26	1.1	1.1	1.1	1.4	-	-	1.4	-
20	2.5	2.4	2.5	2.6	2.6	-	-	2.7	-
6a (Reinjected at 84 hours)	3.1	3.1	2.9	3.0	3.1	2.8	-	3.1	2.9
7a (Reinjected at 84 hours)	4.9	4.6	3.4	4.4	4.5	5.0	-	5.2	5.2
8a (Reinjected at 84 hours)	5.9	5.7	5.6	5.3	4.5	-	-	5.1	-
9a (Reinjected at 84 hours)	5.1	5.0	4.9	4.9	5.0	-	-	-	5.0
Controls (Sea water injected)									
21	4.3	4.3	4.3	4.2	4.2	4.3	4.2	-	4.2
24	3.5	3.5	3.5	3.5	3.4	3.4	3.4	-	3.4
35	2.3	2.3	2.3	2.3	2.3	2.3	2.3	-	2.2

[a]Time 0 served as a control for each animal.

Table 4. Bactericidal Activity of Hemolymph Post-injection[a]

Animal number	Average colony count Time in hours								
	0	12	24	36	48	60	72	84	96
6	142	232	214	195	204	-	-	29	-
7	35	265	60	368	232	-	-	60	-
8	40	123	102	75	68	-	-	70	-
9	70	80	156	120	107	-	-	80	-
11	225	147	198	152	87	-	-	-	-
12	83	78	121	140	61	-	-	-	-
14	120	51	39	46	56	-	35	-	90
16	170	278	256	234	245	-	-	35	-
18	152	264	271	146	269	-	-	139	-
19	108	101	182	153	80	-	-	85	-
20	98	125	134	110	118	-	-	83	-
6a (Reinjected at 84 hours)	29	30	60	10	55	30	-	17	-
7a (Reinjected at 84 hours)	60	60	38	55	33	35	-	25	-
8a (Reinjected at 84 hours)	70	81	-	71	65	-	-	30	-
9a (Reinjected at 84 hours)	80	98	63	78	67	-	-	53	-
Controls (Sea water injected)									
21	53	55	53	57	52	-	48	-	48
24	123	125	125	160	130	130	127	-	125
35	61	65	62	61	63	58	59	-	59
Sea water control	350								

[a]Time 0 served as a control for each animal.

However Patterson et al. (1976) did not find an increase in hematocrit in lobsters while phagocytic and bactericidal activity increased after antigenic challenge. It may be that like in Limulus, hemocytes may release bactericidins while in storage. Electrophoresis and antigen-antibody tests (Stagner, 1974) demonstrated that the bactericidin and the substrate for clotting are derived from hemocytes and either are the same proteins or are similar in structure. Both are strongly bactericidal in concentrated form.

As discussed by Stagner and Redmond (1975), concentrations of hemocytes may be found in hypodermal tissues in stressed animals. These cells may entrap or phagocytize pathogens which have penetrated the carapace and may also contribute clotting and bactericidal proteins locally or in a systemic response. These authors have also demonstrated the presence of phenol oxidase in hemocytes and a melanin-like substance demonstrable by the Schmoral reaction (Bancroft,1967). The function of these proteins in Limulus is not known, but may indicate that the hemocyte may have a role in wound healing, tanning reactions or other cellular immune processes.

It must be concluded that the hemocyte in Limulus constitutes the major portion of this animal's immune capability. In its many functions, the hemocyte and these immune reactions resemble those in other arthropods. The literature concerning chelicerate reactions is sparse, however similarities between several representitives and Limulus are evident. A cell storage organ has been noted in scorpions by Brahmi and Cooper (1974) and may account for changes in circulating haemocytes and hemagglutinin titer after antigenic challenge. The lymphatic organ in scorpions was also

reported to act as a phagocytic center by Kowalevsky (1897). Deevey (1941) showed that hemocytes in tarantulas phagocytize dye particles. These observations and the data here presented, demonstrate a similarity amoung chelicerates in immune processes such as phagocytosis, cell storage and changes in hemolymph proteins after exposure to antigens. Furthermore it must be concluded that Limulus conforms to other arthropod immune patterns such as present in crustaceans (McKay and Jenkin, 1970; Patterson et al., 1976) and insects as discussed by Anderson et al. (1973); Briggs (1974); Gotz (1973) and Salt (1970). The similarities between the various arthropod classes, especially regarding receptor function or the mechanism of recognition of not-self, are striking and should contribute to the current concept of the evolution of the immune response. Precise descriptions of a putative carbohydrate based recognition system are being actively sought. It may well be that the phagocytic hemocyte will play a central role in invertebrate resistance, as such, or may contribute humoral factors to the hemolymph which augment phagocytosis.

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SEROLOGICAL AND DNA COMPARISONS OF LEUCOTHRIX ISOLATES

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ABSTRACT

Filamentous bacteria, presumed to be *Leucothrix* mucor, have been associated with mortalities in lobster and shrimp aquaculture laboratories. We have compared lobster and shrimp isolates of these bacterial with known *L. mucor* strains from the American Type Culture Collection by serological cross-reactivity and by DNA base composition. The lobster isolates were very similar to the *L. mucor* strains examined, while the shrimp isolate is distinctly different.

INTRODUCTION

The American lobster (*Homarus americanus*) and shrimp (*Penaeus* sp.) are under investigation as subjects for commercial mariculture. Under intensive culture, mortalities in juvenile stages of the lobsters and in both juvenile and adult shrimp are associated with the growth of filamentous microorganisms on the gills.

Based upon microscopic observations, the etiological agent of the gill infestations has been identified as *Leucothrix*. The genus *Leucothrix* includes colorless, gram negative, aerobic, filamentous, non-sulfur oxidizing, chemoorganothrophic marine bacteria which attach themselves to solid subtrates by means of an inconspicuous holdfast. They often occur as epiphytes on marine algae. There is a single species, *Leucothrix mucor* (Buchanan & Gibbons, 1974).

The microscopic identification of *Leucothrix* filaments *in situ* is not as straightforward as it may seem. Swollen cells often randomly occur along *Leucothrix* filaments which are difficult to distinguish from heterocysts of blue-green algae. Hence, it is necessary to isolate the disease agent in pure culture for positive identification.

Pure cultures of filamentous bacteria are difficult to isolate from crustacean surfaces because of the rapid overgrowth of the isolation media by *Vibrio* and *Pseudomonas* sp., which are part of the normal flora. We have isolated two strains from lobsters by use of an enrichment medium containing differential antibiotics. These cultures, along with one provided to us by Dr. Donald

Lightner, who isolated it from shrimp, were compared with known cultures of _L. mucor_ from the American Type Culture Collection.

MATERIALS AND METHODS

DNA was extracted and purified by modifications of the methods of Marmur (1961) and Kelly (1969). The melting curves of the purified DNA were determined on a Beckman Model DU spectrophotometer modified with a Gilford Model 2527 Thermo-programmer.

Antisera were raised in rabbits. Serological tests were run by standard techniques.

DNA Analysis

The mole percent guanine plus cytosine (% G + C), calculated from the melting curves of purified DNA, was used to compare the lobster and shrimp isolates with a number of _Leucothrix mucor_ strains from the American Type Culture Collection (ATCC). Strains HFV and HFV-II, from juvenile lobsters, and ATCC strains 25107, 25109, 25111, 25906, 25907, and 25908 all fell within a range of 48 - 51% G + C. Thus the lobster isolates are within the limits for the species listed by Buchanan and Gibbons (1974), in which 20 strains of _L. mucor_ ranged from 46 - 51% G + C. Strain PP, isolated from shrimp at the University of Arizona's laboratory at Puerto Peñasco, Sonora, had 59.5% G + C, clearly distinguishing this isolate from the _L. mucor_ group.

Serological Analysis

Antisera were raised to six ATCC strains of _L. mucor_ along with the shrimp and lobster isolates. Upon testing these sera, unique problems were again encountered. Due to the filamentous growth

Table 1. Complement Fixation Microtiters

Antigen

Antisera	HFV	25907	25111	25908	25906	25109	25107	PP
HFV	128	128	64	32	64	32	64	0
25907	128	128	64	16	32	16	16	0
25111	32	64	64	8	32	8	16	0
HFV II	32	32	32	8	64	16	16	0
25908	16	16	2	32	32	8	16	0
25906	16	16	16	16	16	8	8	0
25109	8	8	8	16	8	64	16	0
25107	8	16	32	32	8	32	128	4
PP	0	4	2	0	0	0	0	16

habit, the usual agglutination and precipitin reactions could not be used. A micro-scale complement fixation test was set up for the initial cross reactions. These data are shown in Table 1.

All strains cross-reacted to a high degree with the exception of the shrimp strain, PP. The very low cross reactivity of this strain with the known Leucothrix cultures indicates a lack of identity between these organisms. The high cross reactivity between the lobster isolates and the known cultures indicates a much closer relationship.

Removal of cross reacting antibodies for further antigenic analysis has not been possible with our system. The antigen preparations used for absorption apparently release anticomplementary activity. Improved methodology is under investigation. We are also using polyacrylamide gel electrophoresis to compare cell wall components of the various strains.

DISCUSSION

The high degree of antigenic cross reactivity between known L. mucor strains supports the conclusions of others that these organisms are a very homogeneous group (Raj, 1977). The cross reactivity of the lobster isolates, as well as the similar G + C ratio, strongly supports the presumption that these strains are also L. mucor. On the other hand, the absence of cross reacting antigens in the shrimp isolate and a markedly different percentage of G + C would lead one to suspect that it is not L. mucor. Whether this strain should be a different species of Leucothrix, or placed into a different genus remains to be established.

Filamentous marine bacteria and blue-green algae are epiphytes in nature. The attachment to living organisms apparently is advan-

tageous in keeping them close to a source of nutrients. They possess no mechanisms of pathogenesis against the organism to which they attach. The pathological effect of filamentous growth on the gills of crustaceans is an unfortunate consequence of intensive mariculture efforts, which include crowding and high rates of feeding to promote growth. It seems unlikely that the high nutrient levels in culture tanks, which greatly promote bacterial growth, would often be approached in nature. Hence, this type of problem should be rare in nature.

Due to the non-specific nature of the infestation of crustacean gills by filamentous microorganisms, it is not surprising to find two quite different organisms in two separate outbreaks involving lobsters and shrimp. Probably several types of filamentous organisms exist at any one location, and any one of these can be a potential aquaculture problem under the proper conditions.

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A DESCRIPTION OF 2 VIRUSES, EGV-1 AND EGV-2, AND THEIR ASSOCIATION IN THE ECDYSIAL GLAND OF THE BLUE CRAB, *CALLINECTES SAPIDUS*

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ABSTRACT

A rhabdovirus (EGV-1) was observed in the ecdysial gland of the blue crab, *Callinectes sapidus*, on two occasions. The EGV-1 particles were located within the calyx material, which coats the hemolymph sinuses of the ecdysial glands. The particles were bacilliform in shape and were approximately 50-70 nm in diameter and 100-170 nm in length. On one occasion EGV-1 was observed in close association with another rhabdovirus EGV-2. EGV-2 particles were frequently found proliferating in the ecdysial gland cells, 3-5 days after eyestalk ablation. Viral proliferation occurred within the nuclear envelope, in the tubular endoplasmic reticulum, and along the plasma membrane. The particles were also seen free in the hemolymph sinuses and attached to the plasma membranes of hemocytes. The cytoplasm of infected hemocytes was sometimes totally engulfed with EGV-2 particles. The particles were predominately bacilliform in shape with a diameter of 25-30 nm and a length of 100-150 nm.

INTRODUCTION

In the past decade several viruses or virus-like particles have been described in tissues of various crustaceans. Most of these viruses or virus-like particles were found in portunid crabs; however, a cytoplasmic polyhedrosis virus was reported in the freshwater cladoceran, Simocephalus expinosus (Federici and Hazard, 1975) and a nuclear polyhedrosis virus was observed in the hepatopancreatic cells of the pink shrimp, Penaeus duorarum (Couch, 1974a; 1974b). The primary sites of these viral infections in portunid crabs were the hemocytes and hemopoeitic tissue. The first reported viral infection in a crustacean was isolated from the hemolymph of the crab, Macropipus depurator (Vago, 1966). This virus, which was thought to be a reovirus, caused a slowly developing paralysis and a slight darkening of the crab (Bonami, 1973). A rhabdovirus was also found in association with the previously described reovirus in the heart cells of the same crab (Bonami and Vago, 1971). Abnormal hemolymph clotting was shown to be caused by a viral infection of the hemocytes in the European shore crab, Carcinus maenas (Bang, 1971; 1974). A reovirus was found in the hemopoeitic tissue and the hemocytes of the blue crab, Callinectes sapidus (Johnson, 1976). Johnson (1977) also described a herpes-like virus in the gills and nervous tissue of the blue crab.

Chassard-Bouchaud et al. (1976) described a rhabdovirus in the y-organ of the European shore crab. This viral particle was structurally similar to a rhabdovirus found in the ecdysial gland of the blue crab (Yudin and Clark, in press). The rhabdovirus found in the ecdysial gland was also shown to be associated with another rhabdovirus. These two rhabdoviruses were referred to as EGV-1 and EGV-2. After bilateral eyestalk ablation EGV-2 particles were observed proliferating in the ecdysial gland of the blue crab (Yudin and Clark, submitted). The purpose of this paper is to review the proliferation process

of EGV-2 in relation to eyestalk ablation, and morphologically describe the EGV-1 particles and their unique occurrence with EGV-2 in more detail.

MATERIALS AND METHODS

Blue crabs were collected from traps in the East Lagoon region of Galveston, Texas. The crabs were maintained in recirculating tanks and fed scraps of fish, shrimp and squid. Experimental animals were acclimated for a period of seven days at a temperature of 24°C and a salinity of 35 o/oo.

Eyestalk ablation was performed by hot cauterization to prevent loss of hemolymph and to deter pathogen invasion. Four separate experiments were run, each with a total of twelve animals, six experimental and six control. At 24 hour intervals, for a period of six days, ecdysial glands were excised from ablated and nonablated crabs. Excised glands were fixed in Karnovsky's (1965) solution and post fixed in phosphate buffered 1% osmium tetroxide. The tissue was subsequently dehydrated in a graded acetone series and embedded in a low viscosity epoxy resin (Spurr, 1969). Tissue for microscopic examination was sectioned with both glass and diamond knives, stained with uranyl acetate (Venable and Coggeshall, 1965) and lead citrate (Watson, 1958). Thick plastic sections (.5-1μ) for light microscopic examination were stained with toluidine blue (Dewel and Clark, 1972).

RESULTS

Light Microscopy: Each gland is composed of irregular shaped cells, approximately 20μ in diameter. The nuclei are eccentric with dense chromatin around their peripheral karyoplasm (Fig. 1). Channels of hemolymph bathe and thereby separate the gland into numerous cords. The hemolymph channels are interconnected to hemolymph sinuses which contain both granular and agranular hemocytes (Fig. 1). The EGV-1 and EGV-2 particles are normally too small to be resolved at the light microscopic level, but occasionally large aggregates

PLATE 1

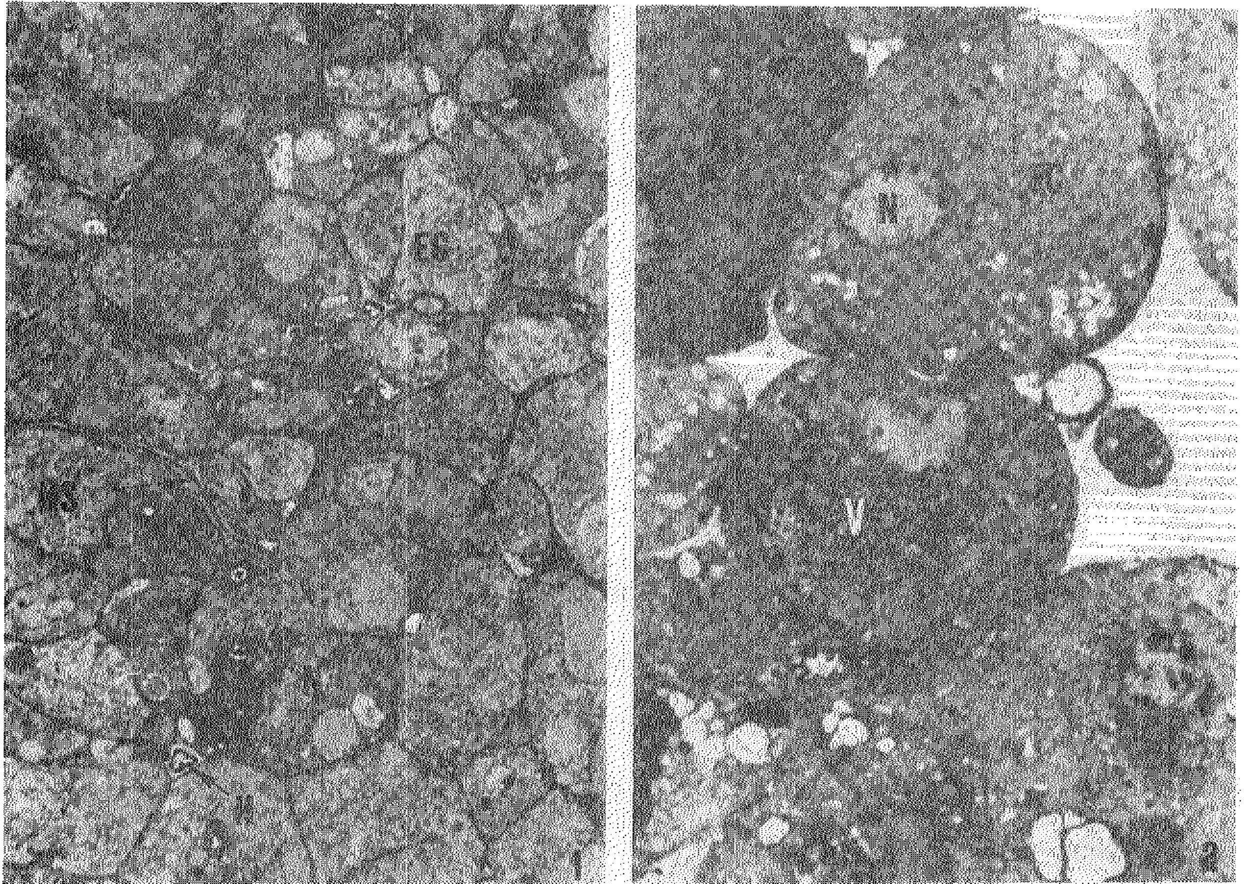
EXPLANATION OF FIGURES

Fig. 1. A light micrograph of control ecdysial gland cells (EC). Nucleus (N), hemolymph sinus (HS), hemocyte (H). x 400

Fig. 2. A light micrograph showing a large aggregate of EGV-2 particles (V) within an infected ecdysial cell (EC). Nucleus (N). x 1,050

Fig. 3. An electron micrograph of noninfected ecdysial cells revealing its typical cellular arrangement. Nucleus (N), mitochondria (M), Golgi complexes (G), smooth endoplasmic reticulum (SER), plasma membrane (PM), tubular endoplasmic reticulum (TER) and hemolymph channel (HC). x 18,000

PLATE 1



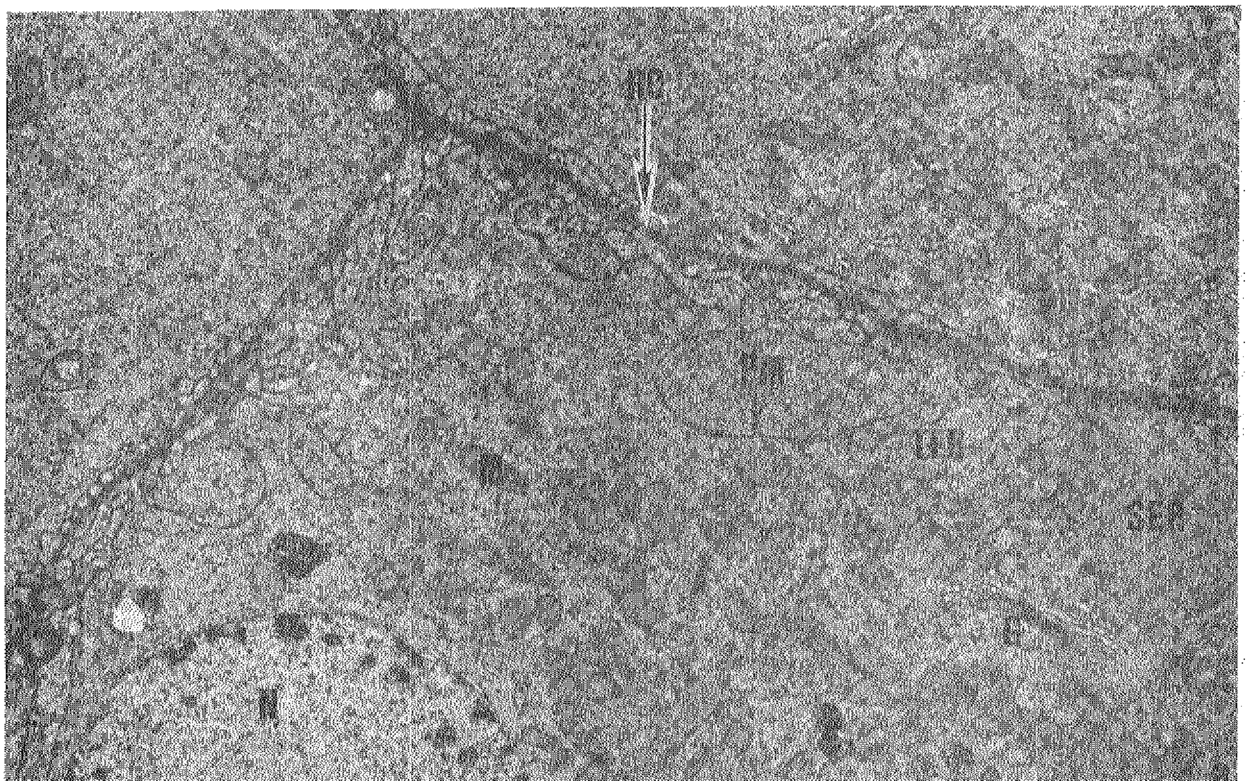


PLATE 2

EXPLANATION OF FIGURES

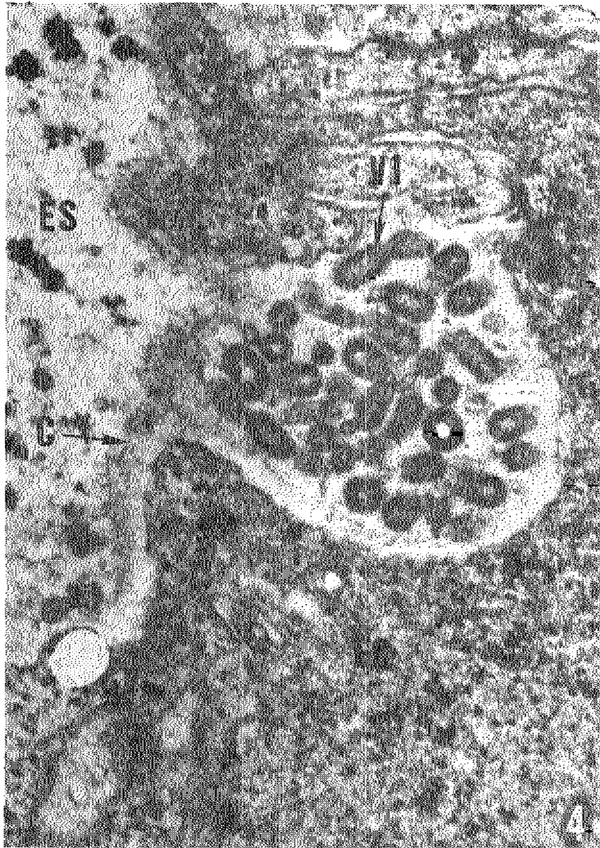
Fig. 4. A micrograph showing the location of EGV-1 particles (V1). Calyx (C), extracellular space (ES) and ecdysial cell (EC). x 62,000

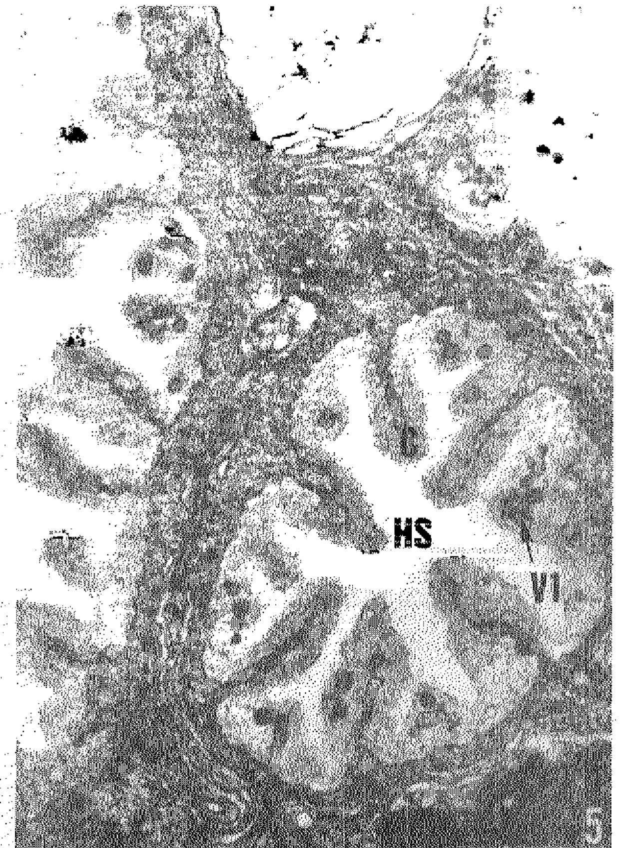
Fig. 5. EGV-1 particles (V1) within the calyx (C) material of the hemolymph sinus (HS). x 24,000

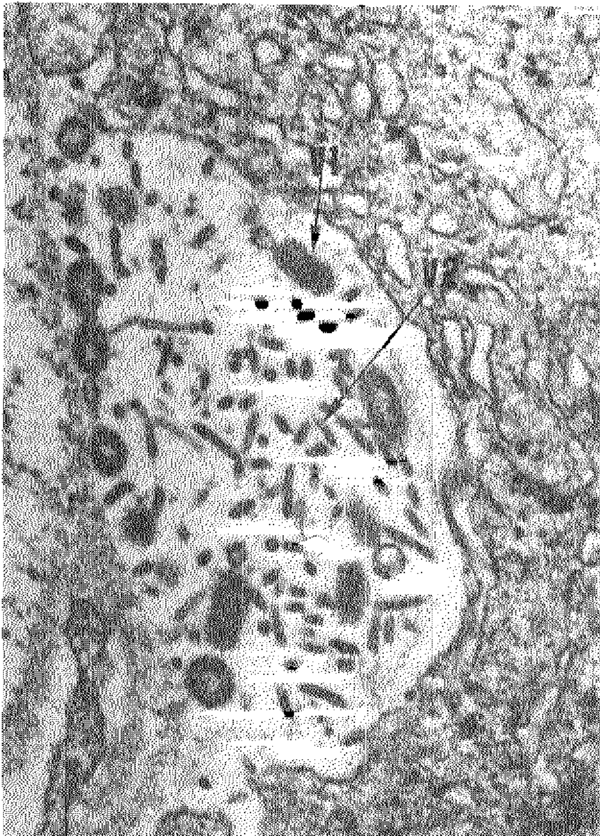
Fig. 6. An accumulation of EGV-1 (V1) and EGV-2 (V2) particles within an extracellular pocket. x 72,000

Fig. 7. Both EGV-1 (V1) and EGV-2 (V2) particles within the calyx material coating the hemolymph sinus (HS). Cluster of EGV-2 particles (CL), hemocyte (H) and nucleus (N). x 26,000

PLATE 2







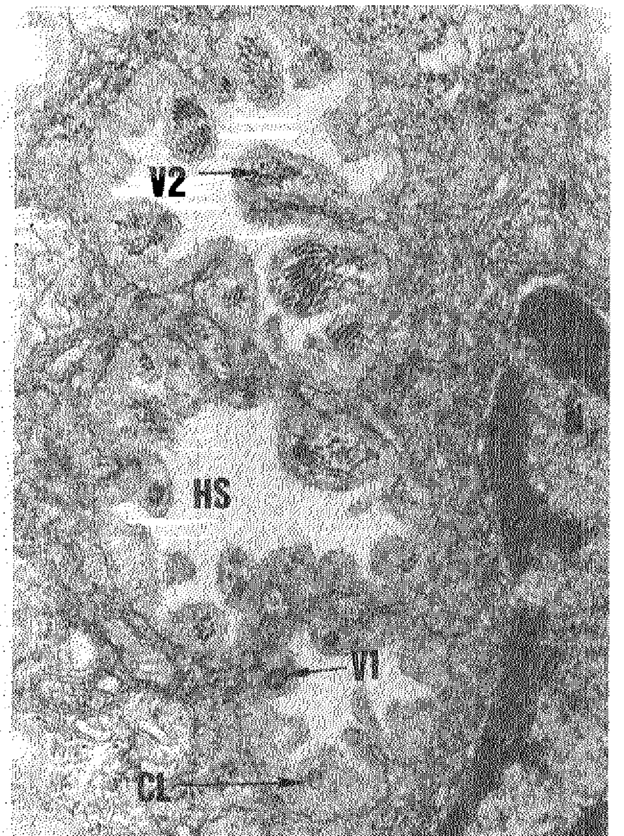


PLATE 3

EXPLANATION OF FIGURES

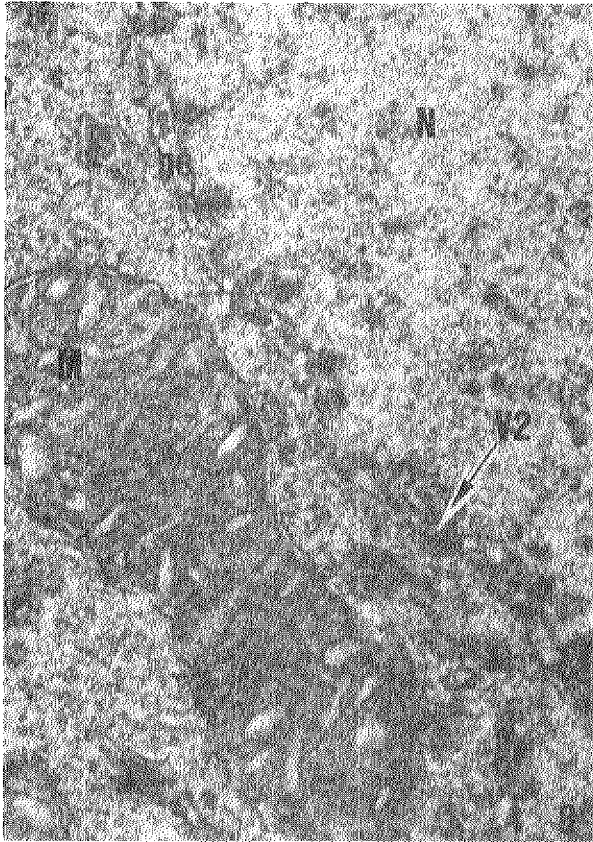
Fig. 8. An electron micrograph showing EGV-2 particles (V2) within the nuclear envelope (ne). Nucleus (N) and mitochondria (M). x 50,000

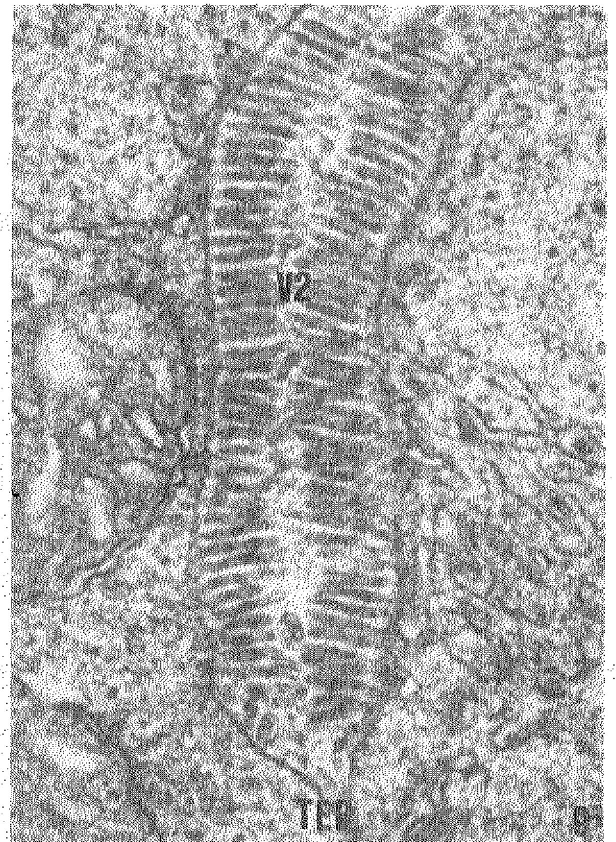
Fig. 9. EGV-2 particles (V2) proliferating along the TER. x 86,000

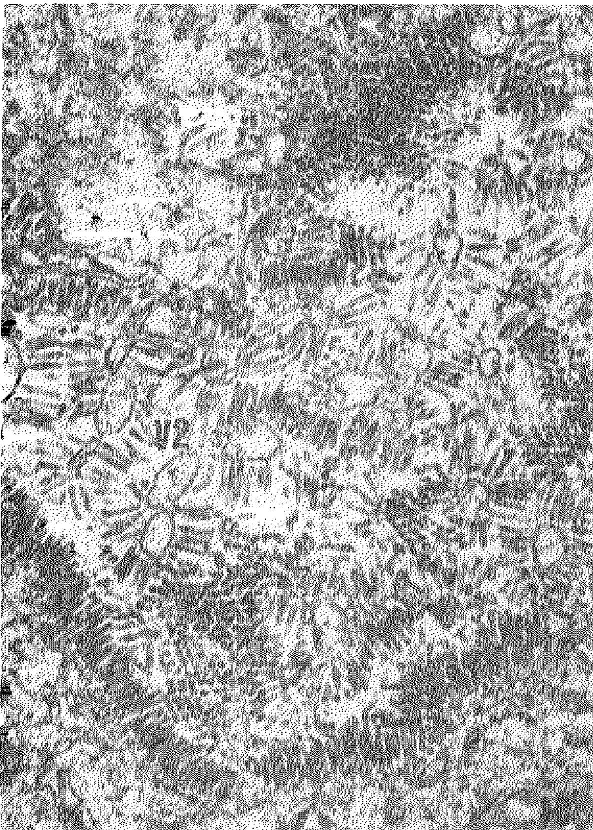
Fig. 10. EGV-2 particles (V2) being released and filling the TER. x 40,000

Fig. 11. A vacuole filled with EGV-2 particles (V2). Extracellular space (ES). x 34,000

PLATE 3







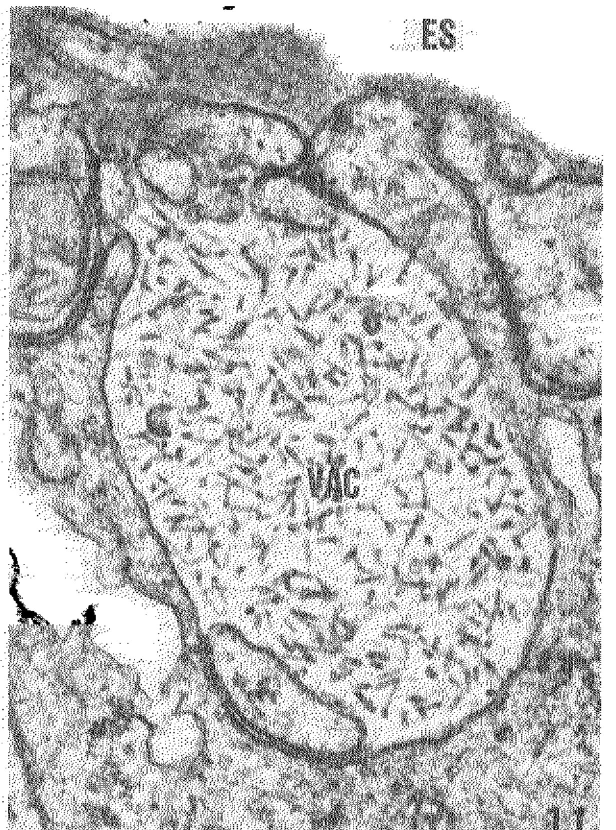


PLATE 4

EXPLANATION OF FIGURES

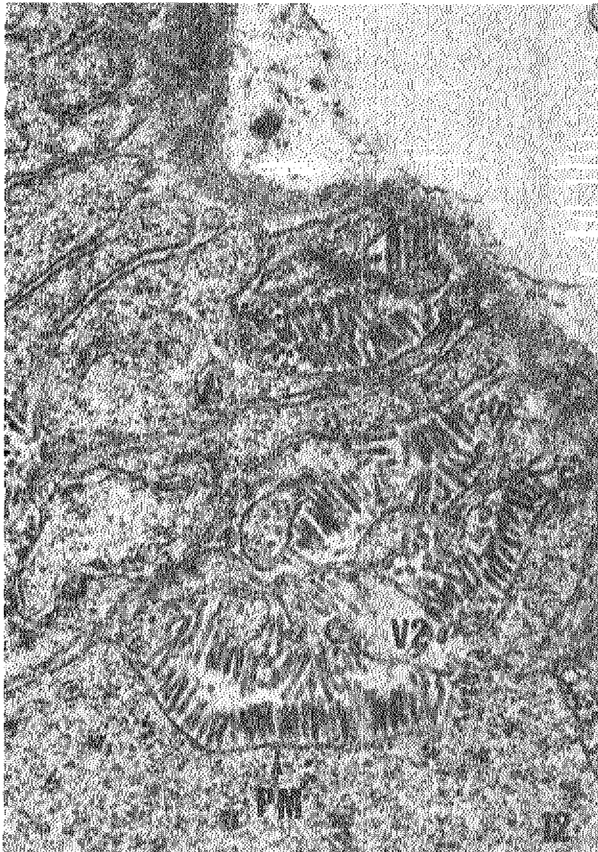
Fig. 12. EGV-2 particles (V2) lining the plasma membrane (PM) of ecdysial cells. x 39,000

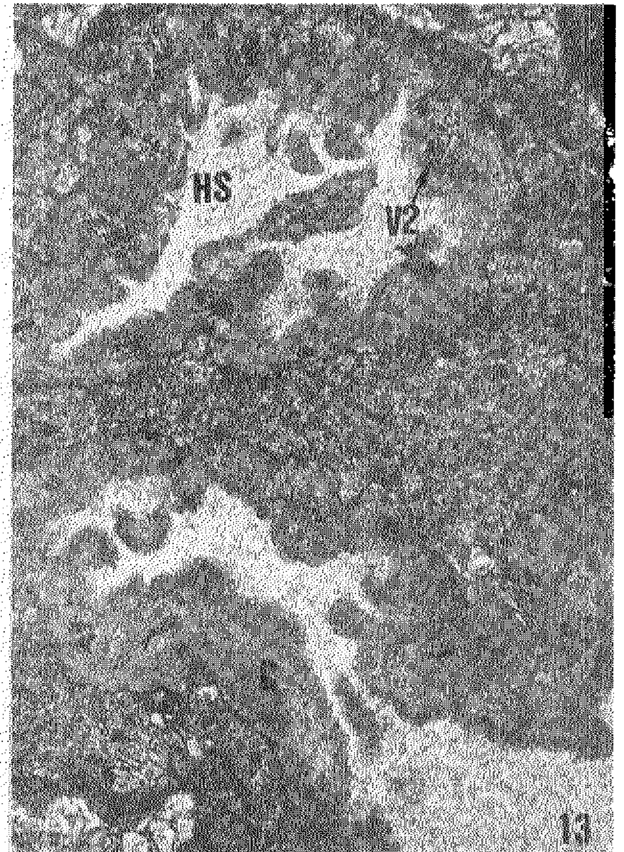
Fig. 13. Free EGV-2 particles within a hemolymph sinus (HS). Calyx (C). x 22,000

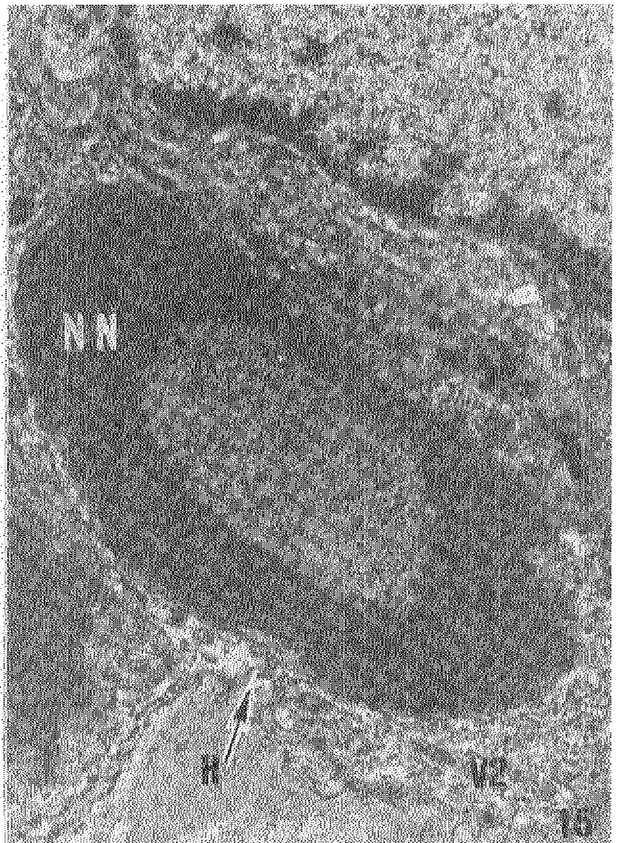
Fig. 14. A hemocyte with EGV-2 particles (V2) attached to the plasma membrane. Nucleus (N). x 28,000

Fig. 15. A hemocyte (H) engulfed with EGV-2 particles (V2). The nuclear membrane has broken down and the nucleus appears necrotic. Necrotic nucleus (NN). x 17,000

PLATE 4







of EGV-2 within the ecdysial cells cytoplasm are easily detected (Fig. 2).

Fine Structure: The ecdysial glands from nonablated, control crabs are fairly uniform in their cell morphology. The nuclei, as stated before, contain dense chromatin around the peripheral nucleoplasm. The ecdysial cells contain numerous mitochondria and Golgi complexes as well as two distinct types of agranular endoplasmic reticulum (Fig. 3). The large isolated accumulations are referred to as smooth endoplasmic reticulum (SER), while the branched, tubular form will be called tubular endoplasmic reticulum (TER). EGV-1 particles are only found in control crabs and the glands cellular arrangement is not altered by the appearance of these particles. The particles are found within pockets or extracellular spaces that line the hemolymph sinuses (Fig. 4). A calyx material coats the hemolymph sinuses and lies directly adjacent to the plasma membrane of the ecdysial cells. The EGV-1 particles are observed within the extracellular spaces and positioned between the calyx coat and the plasma membrane of the ecdysial cells. The particles are also found within the calyx material (Fig. 5). Viral maturation or budding has not been observed. The EGV-1 particles were found in the ecdysial glands of two control crabs, which showed no sign of abnormal behavior.

In another control crab the ecdysial glands contained both EGV-1 and EGV-2 particles. These particles are found closely associated with each other throughout the extracellular spaces or within the calyx material (Fig. 6-7). The EGV-2 particles are not seen proliferating within the ecdysial cells or along the plasma membrane. Occasionally clusters of EGV-2 particles are found surrounded by a membrane, which are similar in diameter to the EGV-1 particles (Fig. 7).

The EGV-2 particles have never been observed in the ecdysial gland unless in association with EGV-1 or after bilateral eyestalk ablation. A dramatic

proliferation of EGV-2 particles begins on the third, fourth and fifth day after eyestalk ablation. The site of this proliferation in the ecdysial gland cells is limited to three distinct structures; the nuclear envelope, TER and plasma membrane.

The EGV-2 particles are commonly found between the outer and inner membranes of the nuclear envelope. The particles are always longitudinally oriented and are never seen attached to the membranes (Fig. 8). The TER is also one of the principle sites for EGV-2 proliferation. Proliferation of EGV-2 particles initially begins along the inner surface of the TER (Fig. 9), and as proliferation continues the particles are released into the area between the TER membranes (Fig. 10). This continual proliferation leads to a loosely packed, viral filled vacuole (Fig. 11). The vacuoles fuse with the plasma membrane and release the particles into the hemolymph channels. The plasma membrane is another site of viral proliferation. Longitudinal arrays of EGV-2 particles are observed extending from the plasma membrane into the hemolymph channels (Fig. 12).

After release from the ecdysial cells, either by vacuole exocytosis or maturation along the plasma membrane, the EGV-2 particles are found free in the hemolymph sinuses (Fig. 13). Once the particles are free in the hemolymph sinuses, they apparently attach to the plasma membranes of hemocytes (Fig. 14). The viral infection of the hemocytes causes cytoplasmic organelle displacement, nuclear envelope breakdown and nuclear necrosis (Fig. 15).

EGV-1 and EGV-2 Morphology: The EGV-1 particles are bacilliform in shape with a diameter of 50-70 nm and a length of 100-170 nm. The particles appear to have a hollow core, which is surrounded by a nucleocapsid. A membrane envelopes the nucleocapsid and surface projections protrude from it. The EGV-2 particles are also bacilliform in shape, but are much smaller in diameter,

25-30 nm.

DISCUSSION

The apparent positioning of EGV-1 within the ecdysial gland, as well as their apparent lack of morphogenesis, suggests the ecdysial gland is not the primary site of infection. The EGV-1 particles probably mature and proliferate at another site subsequently circulating throughout the organism via the hemolymph. EGV-1 particles have only been observed in nonablated control crabs. The infection did not appear to alter the physical or behavioral nature of the animals. The structural morphology of the EGV-1 particles corresponds to other published descriptions of rhabdoviruses (Hackett et al., 1968). The association of EGV-1 and EGV-2 was only noted in one instance, therefore, the significance of this event is unknown. The appearance of EGV-2 particles was only noted in eyestalk ablated animals, except on the one occasion. The absence of EGV-2 proliferation within the ecdysial cells, during its association with EGV-1, suggests its probable maturation at another site (Jahromi, 1977). This leads us to believe that the occurrence of EGV-1 and EGV-2 together was only happenstance.

The structural morphology of EGV-2 particles, as well as their apparent site of morphogenesis, was used as preliminary evidence for EGV-2 being grouped with the rhabdoviruses (Wildy, 1971). Tubular elements similar to EGV-2 have been described in both plant and animal tissue, but their significance, to date, remains unclear (Kitajima and Costa, 1966; Zwillenberg et al., 1965). Although the EGV-2 particles exhibit a definite affinity for membranous structures, they have never been observed associated with the mitochondria, SER, or Golgi complexes of the ecdysial cells.

This paper has reviewed two rhabdoviruses, EGV-1 and EGV-2, that have been described in the ecdysial gland. The significance of these viruses are to

date unknown, though studies are now underway to complete the life cycle of EGV-2 and to determine its pathogenicity.

ACKNOWLEDGEMENTS

We thank Dr. Richard Diener and Dickie Revera for their much appreciated technical assistance.

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A SUCTOREAN PARASITE OF *PENAEUS MONODON* LARVAE[1]

by

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A pathogenic suctorean, identified as *Ephelota gemmipara* R. HERTWIG, was observed in *Penaeus monodon* larvae spawned and reared in tanks. Commonly found to inhabit hydroid colonies, *E. gemmipara* has a stalked body with two types of tentacles, the sucking and piercing types, and was observed to reproduce by multiple exogenous budding.

INTRODUCTION

The culture of *Penaeus monodon* Fabricius larvae in SEAFDEC is stymied by high mortality caused by microorganisms. Results of regular monitoring of 51 hatchery runs for 1976 showed that the most prevalent pathogens were *Lagenidium*, a phycomycetous fungus; *Licmophora abbreviata* Agardh, a pennate diatom; *Vorticella* spp. (Gacutan *et al*, 1977) and a suctorean previously unreported in the prawn species. Identification using the scheme of Kudo (1966) showed that it was *Ephelota gemmipara* R. Hertwig.

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[1] SEAFDEC Contribution # 23

This suctorean was observed for the first time on February 9, 1976 in tank-reared mysis in an indoor tank of the SEAFDEC Prawn Hatchery in Tigbauan, Iloilo. Its effects on larvae during pathogenesis were studied for 4 months until June 2 of the same year.

MATERIALS and METHODS

The occurrence and incidence of *E. gemmipara* was monitored in all hatchery runs conducted beginning from its first occurrence. For each hatchery run, 50 larvae were collected everyday until the harvest date, or in some cases due to infection until the termination of that experimental run. Each larva was mounted on a microscope slide with hatchery water provided with fresh feeds (*Chaetoceros* in zoeae, *Brachionus* in mysis). These were observed thoroughly for the presence of *Ephelota gemmipara* and other fouling organisms. Whenever possible, the number of *Ephelota* bodies in each affected specimen were recorded and the attachment site of each body determined.

RESULTS and DISCUSSION

Microscopy of affected larvae being attacked showed *Ephelota* to possess a stalked body (Fig. 1) with two types of tentacles;

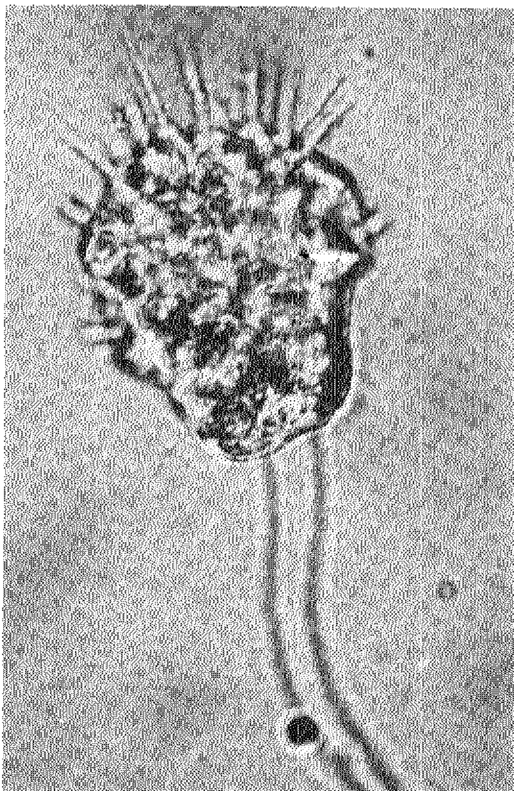

Fig. 1. <u>Ephelota gemmipara</u> showing the stalked body and tentacles (600x)

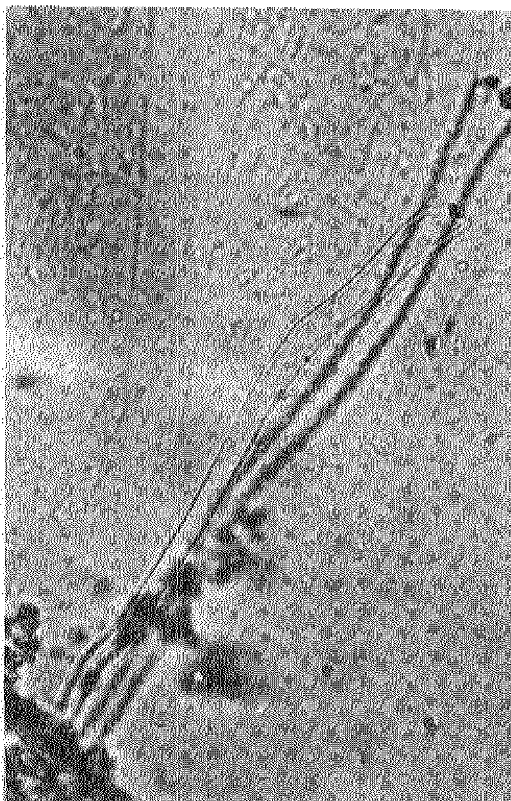

Fig. 2. Two <u>E</u>. <u>gemmipara</u> stalks to show persistence after metamorphosis of larva (400x)

the sucking and the piercing kinds. Reproduction was by multiple exogenous budding (Manwell, 1967). This involved the formation from the macronucleus of more than two dozen small buds arranged in a circle on top of the head. Division of the mother macronucleus gave rise to micronuclei which were eventually delineated into ciliated individuals (Hyman, 1940). These were released within seconds of each other at the appropriate time. Each was capable of growing into a new body. The other known mode of reproduction, anisogamous conjugation (Grell, 1953; Dogiel, 1965), was not observed to occur in any of the specimens.

A total of 15 hatchery runs involving larval populations varying from 300 to $3,000 \times 10^3$ individuals were affected Ephelota (Table 1). It was observed for the last time on June 6 of the same year. Based on the monitoring reports accumulated over the period spanning the first and last days of occurrence, the pathogens came in streaks lasting 2 to 9 days between intervals of 4 to 25 days.

The incidence of the pathogen in the monitored larval population ranged from 2 to 26 per cent. The daily levels of infection were however not consistent and could not be used therefore as a gauge of its behaviour; i.e. population dynamics, etc. It was then a routine procedure in this laboratory to change the culture water partially or completely

Table 1. Record of hatchery runs of *Penaeus monodon* larvae affected by *Ephelota gemmipara* R. Hertwig

Expt'l Run No.	Tank Used	Date	Stage	Incidence (%)	Larval Population	Remarks on Experimental Run
H_{122}	CT 11	2-9	M_1M_2	2	334×10^3	
		2-10	M_2M_3	18	301×10^3	
						10×10^3 larvae harvested February 20 at P_{10}
H_{127}	CT 3	2-25	Z_3	4	800×10^3	
		2-26	M	2	745×10^3	
						Heavy *Vorticella* load (28%) March 3; discarded March 6 at P_6
H_{128}	CT 7	2-27	Z_3	2	430×10^3	
						Discarded at P_2
H_{140}	CT 16	3-22	Z_3M_1	8	900×10^3	
		3-23	M_1	2	897×10^3	
		3-24	M_2	-	-	
		3-25	M_3	2	-	
		3-26	P_1	-	-	
		3-27	P_2	6	-	
		3-28	P_3	2	-	
		3-29	P_4	4	-	
						Fungal infection observed March 23 at M_1; no record of harvest

Expt'l Run No.	Tank Used	Date	Stage	Incidence (%)	Larval Population	Remarks on Experimental Run
H_{141}	CT 5	3-25	M_1	4	600×10^3	
		3-26	M_2	-	-	
		3-27	M_3P_1	-	-	
		3-28	P_2	-	-	
		3-29	P_3	2	-	
						No record of harvest
$H_{(?)}$	CT 14	3-31	P_1	6	-	
						No record of harvest; Fungal infection observed March 31 at P_1
H_{144}	CT 16	4-5	Z_3	26	-	
		4-6	Z_3M_1	14	860×10^3	
		4-7	M_2	8	-	
						Discarded April 8 at M_3 due to *Ephelota gemmipara*
H_{145}	CT 13	4-8	Z_2	4	$1,000 \times 10^3$	
		4-9	-	-	-	
		4-10	Z_3	2	-	
						Fungal infection detected April 10; discarded April 11 at M_1
$H_{(?)}$	CT 15	4-11	Z_3	12	-	
		4-12	Z_3M_1	4	-	
						Fungal infection April 11; no record of harvest

Expt'l Run No.	Tank Used	Date	Stage	Incidence (%)	Larval Population	Remarks on Experimental Run
$H_{(?)}$	CT 14	4-16	Z_3M_1	2	-	
		4-18	M_1	20	-	Fungal infection detected April 16; No record of harvest
H_{157}	CT 14	5-4	Z_3	4	-	
		5-5	Z_3	4	$3,195 \times 10^3$	
		5-6	M_1	6	$2,600 \times 10^3$	
		5-7	M_2	2	$2,490 \times 10^3$	
		5-8	M_3	4	$2,000 \times 10^3$	Fungal infection detected May 7; discarded May 12 at P_3
H_{159}	CT 9	5-8	Z_3M_1	2	458×10^3	Fungal infection detected May 8; discarded May 12 at P_1
$H_{(?)}$	CT 13	5-8	M_3	2	-	Fungal infection detected May 8; discarded same day
H_{171}	CT 14	6-4	Z_3	2	$2,233 \times 10^3$	
		6-5	M_1	2		
		6-7	M_3	6		Heavy *Vorticella* load detected June 4; discarded June 11 at P_2 due to *Vorticella*
$H_{(?)}$	CT 15	6-6	Z_3M_1	2	$1,407 \times 10^3$	Discarded 6-11 at P_2 due to *Vorticella*

in an effort to control the infection and to reduce mortality. These frequent changes also did not warrant observations as to which larval sub-stage was most susceptible to infection. If the earliest sub-stage when occurrence was observed could be used as basis, then the last zoeal stage (Z_3) is considered the one most prone to infection. In many cases infection surfaced and the signs became evident during the Z_2 sub-stage (H_{145}). Chances for the infection to start development during the mysis and postlarval stages were low.

Its devastating effect as a pathogen was established beyond doubt. One of the hatchery runs (H_{144}) was so severely affected that the experiment had to be halted and the larval population completely discarded. This experimental run was not affected by the usual commensals and opportunistic pathogens until the day of termination so the mortality could be ascribed entirely to the suctorean. In cases where it was not the direct cause of mortality, it nevertheless contributed to the weakening of the population. All the other runs except one (H_{122}) did not last until the harvest stage (P_{10}) owing to decimation of the populations due to Lagenidium and Vorticella.

A decreased susceptibility to infection was exhibited by the larvae with age. Out of the 100 infected specimens, 55 were in the zoeal stages (Table 2), 35 were in the mysis stages and

Table 2. Comparison of *Ephelota* infection loads among three stages of *P. monodon*

Stage	No. of Specimens Infected	No. of Ephelota attached	Infection Load/ Specimen
Zoea	55	95	1.73
Mysis	35	58	1.66
Postlarva	10	10	1.00
Total	100		

Table 3. Distribution of *Ephelota* on the various bodily parts of *P. monodon*

Body Parts	Stages		
	Zoea	Mysis	Postlarva
Body segments			
1st	2	0	0
2nd	3	5	1
3rd	4	2	0
4th	4	2	1
5th	4	4	0
6th	42	11	1
Antennae	6	1	0
Antennules	1	1	2
Rostrum	1	6	0
Eyes	2	6	0
Carapace	5	7	1
Gills	1	1	0
Telson	13	6	1
Uropods	7	5	1
Pleopods	-	1	1
Pereiopods	-	-	1
Total	95	58	10

10 were postlarvae. The mean infection loads for the three stages were 1.73, 1.66 and 1.0 Ephelota body per host. The occurrence of infection in earlier stages could be attributed to the relatively soft exoskeleton of the larvae.

There was a marked preference for the bodies to attach themselves on the broad and relatively immobile parts of the larvae such as the body segments, carapace and uropods in both zoeae and mysis stages (Table 3). Fifty-nine (59) of the 95 Ephelota that infected the zoeae were found on the body and the abdominal segments, with 42 of these concentrated on the 6th segment. The tail regions harbored 20, distributed between the telson (13) and uropods (7). In the mysis, the frequency of attachment increased in such body parts as the rostrum, eyes, and carapace; and decreased considerably in the 6th abdominal segment. This may have been due to abrupt changes in movement in the larvae. While the abdominal segments are rarely moved violently during the zoeal stages, these are now responsible for the violent, snapping dart-like movements characteristic of the mysis. These movements definitely have a thwarting role against the pathogens for they are virtually shaken off if they are not firmly attached on the exoskeleton.

This activity was observed to be the major factor affecting the physiological state of the host. Continuous and prolonged

kicking sapped the host's energy leading to marked lethargy and listlessness and minimal feeding activity. The attachment of the pathogens to the body was very persistent, so that the stalk, already devoid of tentacles and cytoplasmic contents remained attached even after repeated molts and further metamorphosis (Fig. 2).

What made Ephelota a cumbersome pathogen aside from its persistence was its remarkable ability to irritate the host by a combination of sucking and piercing actions by the tentacles. The stalk was observed to be pliant and therefore could be bent at will. Repeated brushes by the tentacles on irritable surfaces on the hosts' body weakened the latter considerably. Once lodged on the exoskeleton and on the underlying musculature, the suctorean extracts liquids and granular materials from the cytoplasm of the host. The extracted materials are formed into a food vacuole in the suctorean after a brief but rapid flow through the stalk. Another factor that favored the pathogen was its ability to reproduce fast by multiple exogenous budding.

Surprisingly, the outbreak has never recurred since June 2, 1976. Repeated attempts to find the pathogens since then failed.

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ECTOCOMMENSALS OF THE FRESHWATER SHRIMP, *MACROBRACHIUM ROSENBERGII* IN CULTURE FACILITIES AT HOMESTEAD, FLORIDA

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Abstract

During three years of observation, eight species of ectocommensals have been noted occurring on internal and external body surfaces of the larval and adult freshwater shrimp, *Macrobrachium rosenbergii*. Five species of peritrichious protozoans, *Cothurnia* sp. *Vaginicola* sp., *Vorticella* sp., and *Zoothamnium* sp., *Podophrya* sp., and *Tokophrya* sp. Direct damage or adverse effect to the host organism was not observed except on rare occasions at heavy infestations.

INTRODUCTION

Over the past three years (1973-1976), periodic ectocommensal surveys have been conducted on *M. rosenbergii* at the experimental culture facilities in Homestead, Florida. Surveys included adult and larval forms collected from the respective pond and hatchery systems. Surveys were initiated as part of a baseline biological assay of the culture system. Although ectocommensals have been reported elsewhere in Penaeid shrimp (Johnson, 1974) and *Macrobrachium* (Goodwin, 1974), they have not caused major mortality in the host organism. This paper describes identified forms from *M. rosenbergii*, respective sites of occurrence on the host, relative host and site specificity, as well as brief notes on related biological and environmental conditions.

MATERIALS AND METHODS

Observations were made at least quarterly from adult and juvenile shrimp randomly sampled from seven culture ponds. Larvae were examined from six separate hatchery larval cycles. A standard A.O. stereoscopic microscope was utilized in conjunction with camera mount for photographic record. Standard observations included fresh mounts of rostrum, antennal scales, exterior cephalothorax, interior cephalothorax, pleopods, gill lamellae, telson, uropods, and eggs. Identifications were based on Jahn (1949), Kudo (1966) and Noland (1931).

RESULTS

Of the species observed, *Cothurnia* sp., *Epistylis* sp. and *Vorticella* sp. were the most common. *Podophrya* sp. and *Vaginicola* sp. were observed on only a single occasion from the

pond environment. Developing eggs provided the surface with greatest species diversity, including Cothurnia sp., Epistylis sp., Tokophrya sp., Vorticella sp., and Zoothamnium sp. and Acineta sp. This may be indicative of the females' inability to thoroughly clean interstitial sites within the egg mass. The desirability of the egg mass as a biological substrate was further substantiated by the occurrence of rotifers and diatoms. Sites for ectocommensal attachment included egg surfaces as well as the connecting fibers.

As reported by Johnson (1974) for Penaeids, Zoothamnium sp. illustrated a preference for M. rosenbergii gill surfaces. The only other form frequently noted there was Cothurnia sp.

Larvae were host to three species, Cothurnia sp., Epistylis sp., and Vorticella sp., with the latter being the most abundant. Infections of Vorticella sp. on larvae ranged from one or two per larvae to more than 20. Occurrence was sporadic, affecting some larval tanks or cycles more than others. The telotrochs of Vorticella sp. were encountered in the larval tanks indicating their ability to reproduce there and to respond quickly to improved feeding regimes; i.e., bacterial levels. No specificity was noted for Vorticella sp. on larvae. A proportionate number occurred on floating debris and tank sidewalls. Major larval losses due to Vorticella sp. have not occurred although at the higher rates of infection reduced motility and feeding response should be assumed. A generalized site preference for Cothurnia sp., Epistylis sp., and Vorticella sp., was noted, being primarily antennal scales, rostrum, and uropods in descending order of preference.

Infections in adult animals were strongly correlated with the occurrence of extensive algal growths on the external body surfaces. The causal agent of this phenomenon has not been identified, but may be related to disrupted molt cycle or aging. The development of this periphyton community is the precursor to protozoan involvement. Adult animals without the above algal development are typically free from ectocommensals.

DISCUSSION

Ectocommensals are common on both larvae and adults of *M. rosenbergii*. With the possible exception of *Zoothamnium* sp. little specificity is shown for site location on the host. In the adult, species development of algae, rotifers and other organisms. Physiological disturbances, aging, or molt cycle disruption may result in a reduction of the normal exoskeleton cleaning activity allowing growths to develop. High nutrient levels may promote algal growth and high bacterial counts, therefore acting as principal food sources of the peritrichious protozoa. In no instances were ectocommensals found in great numbers without the accompanying algal growth. Direct mortality has not been observed from this condition.

The development of ectocommensals on eggs followed a similar pattern, but with more subtle algal involvement (usually diatoms). Development became more intense over egg development time and was most evident on nonviable and decomposing eggs within the egg mass. It did not appear that the ectocommensals themselves were a causal agent in the death of the eggs. It might, however, be surmised that extensive involvement and attachments may mechanically alter

the integrity of the egg surface, making it more susceptible to bacterial and fungal attacks.

The risk of mortality appears highest in the larval stages where motility and feeding activity may be reduced sufficiently to cause death through malnutrition. Since all the involved ectocommensal forms rely on bacteria as a food source, management strategies directed toward reducing bacterial cell counts are the best treatments.

The suctorians, _Acineta_ sp., _Podophrya_ sp. and _Tokophrya_ sp. are the only deviants from the described pattern in that they do not consume bacteria, but rather are saprotrophic on large ciliate protozoans. These forms were identified only from egg surfaces. Although not observed, the author has contemplated the possibility that they may potentially parasitize _M. rosenbergii_ eggs. Since they, unlike the peritrichs, have this potential parasitic feeding ability, careful observations should be continued in the future.

In general, ectocommensal developments appear more indicative of biological conditions within the culture system as they are affected by nutrient and bacterial levels rather than the condition of the host. No evidence has been found that they are in fact parasitic or pathogenic in habit.

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USE OF OZONE FOR CRUSTACEAN DISEASE PREVENTION: A BRIEF REVIEW

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Since natural populations of some crustacean species have become depleted, mariculture facilities have been established to supplement them. Crustacean species held in culture have a higher incidence of disease than those found in nature. Accordingly, it is of value to operators of these installations to find a reliable and effective disinfection system for culture water. Ozone has been found to be an efficient method for sterilizing seawater and some studies have demonstrated its value to shrimp and lobster culture. These reports indicate that shrimp can tolerate ozone concentrations up to 0.1 mg/l without showing distress and that lobster larvae under hatchery conditions are not affected by ozone doses up to 0.4 mg/l. By sterilizing the water used to raise commercially important crustaceans with ozone, the many diseases now affecting these species in the hatchery might be eliminated. Thus, the potential for using ozone to control disease in crustacean hatchery waters is being explored with apparent favorable initial results. This paper reviews the existing literature of ozone disinfection for crustacean disease control.

INTRODUCTION

Seawater Disinfection

Violle (1929), the first to report ozone's bactericidal effect in seawater, wrote that this chemical could be considered an excellent sterilant for that medium. His experiments concerned the disinfection of sealed tubes of seawater seeded with coliform bacteria and exposed to a stream of ozone gas. He found that ozone sterilized seawater seeded with 1×10^6 bacterial cells/ml in a maximum of 8 minutes. Violle also found that bacterially contaminated shellfish, when exposed to ozonized seawater, depurated more rapidly than normal without being altered in taste

or appearance. In a comprehensive review paper, Giese and Christensen (1954) also recognized that low concentrations of ozone killed bacteria and ciliates. In a recent study, Blogoslawski et al. (1975) found that the total marine bacterial counts decreased by three logs and no marine microorganisms could be detected when dissolved ozone dosage was increased to 0.56 mg/l. Honn and Chavin (1976), using a closed marine aquaculture system, discovered that when ozonization was suspended, bacterial populations increased from one to three logs within 24 hours. When ozonization was resumed, the counts decreased to their original levels in 24 hours. They reported ozone to be a remarkably effective control on bacterial populations in a seawater system.

Ozone Use in Aquaria

Sander and Rosenthal (1975) recommended ozone gas as an efficient disinfection method for home and public aquaria. They noted that seawater used in mariculture facilities must be sterilized to prevent disease-causing organisms from entering the water system through intake pipes. After examining several alternate disinfection systems, they selected ozone as the most likely method to achieve successful sterilization. These investigators also reviewed several different methods of ozone-water contact systems. They found that sterilization could be achieved most effectively with a secondary ozone contact chamber. First-stage ozonization removed large solids and organics by foam separation, while the second stage provided complete sterilization.

Murphy (1975) also advocated the use of ozone for closed marine aquaria systems. He listed several advantages of ozone,

which included a reduction in bacterial populations, removal of color and odor from aquaria waters, and reduction in BOD and COD.

Improve Water Quality in Aquaculture

Ozonized seawater has several beneficial applications to mariculture operations. Perhaps the most significant is that large amounts of water can be sterilized before the culture organisms are introduced into the water. This effectively prevents the introduction of a waterborne disease to the cultured stock (Blogoslawski, 1977).

Honn and Chavin (1976) indicated the need for a supplement to regular biological filtration in closed marine culture systems. Bacterial filtration normally is insufficient to cope with the large amounts of toxic wastes which accumulate in such operations. In their study, Honn and Chavin found that, after increasing the biological load of the system, the filter bed became incapable of complete nitrification and wastes reached the toxic level within 24 hours. Ozone was added to the system as a supplementary filtration method and was found to be successful in rapidly stabilizing the system. They concluded that ozone increased oxidative "flexibility" in mariculture operations. Thus, ozone not only reduces pathogenic bacteria for seawater used in a mariculture system but it provides many beneficial advantages unavailable with other disinfectants.

Diseases in Crustacea

Crustacea are susceptible to many diseases and this seems especially true for those species raised in mariculture. Due to interest and investments in shrimp and lobster culture, a considerable

literature exists concerning diseases of these organisms. Sindermann (1977) reported 14 major diseases which affect cultured shrimp: virus disease, vibrio disease, brown spot disease, filamentous bacterial disease, larval mycosis (Lagenidium), fungus disease (Fusarium), milk or cotton shrimp disease, microsporidiosis of reproductive organs, ciliate disease (Zoothamnium), black gill disease, black death disease, blisters, cramped tails, and muscle necrosis. Similarly, he noted six diseases which significantly affect cultured lobsters: gaffkaemia, shell disease, filamentous bacterial disease of larvae, fungus (Haliphthoros) disease of larvae, fungus (Fusarium) disease of juveniles, and ciliate disease.

As the natural populations of these desirable species become more and more depleted, increased mariculture efforts will become necessary to supplement them. Poor water quality has been cited as one of the primary causes of disease outbreaks in mariculture. Thus, crustacean species held in culture have a higher incidence of disease than those found in nature. Accordingly, it is of value to the operators of these installations to find a reliable and effective disinfection system for culture water.

Ozone in Disease Prevention

Inasmuch as ozone has been demonstrated to be an efficient method for sterilizing seawater, several studies have been conducted to evaluate the benefits of ozonized seawater to shrimp and lobster culture. In an early series of experiments, Crane Cochrane Environmental Systems (1973) investigators observed the reactions and health status of marine shrimp to varying concentrations of ozone. Two tanks were used for the tests; an experimental tank containing

seawater to which ozone was added, and another serving as a control, containing only raw seawater. The procedure was first to acclimate the shrimp in the control tank and then transfer them to the ozonized seawater tank. Before this transfer could be accomplished, however, many of the shrimp became diseased with a fungal infection. As soon as this was noted, one-half of the population was put into the ozonized seawater tank where they showed no signs of distress. After 3 days of exposure at an applied concentration of 0.1 mg/l and a measured oxidant residual of less than 0.1 mg/l, the shrimp had improved until no signs of the disease could be detected. When the applied ozone concentration was increased from 0.1 mg/l to 0.5 mg/l over 3 hours, the shrimp congregated as far as possible from the inlet where the oxidant residual measured 0.25 mg/l and displayed very little movement, even near the outlet where the oxidant measured 0.15 mg/l. The ozone dose was again decreased to the original concentration value of 0.1 mg/l with an oxidant residual of less than 0.1 mg/l and the shrimp resumed normal activity. Therefore, it was concluded that ozone added to seawater in concentrations below 0.1 mg/l was of benefit to the maintenance of shrimp since all of the shrimp exposed to it survived and none were lost to disease. However, the remainder of the shrimp left in the control seawater tank continued to develop the fungal infection.

Edwards (1974) advocated the use of a closed raceway system for mariculture. A series of permanent tanks was built in a closed raceway pattern, for culturing the brown shrimp, *Penaeus aztecus*. He found that an effective germicidal agent could be incorporated

into the existing design by using ozone rather than air in the air lift pump. Thus, the ozone mixed with the water reportedly removed ammonia and raised the water's D.O. level. Aside from providing an adequate safeguard for disease prevention, Edwards also observed that ozone improved the D.O. level and eliminated the odor and color that tainted the culture water. A modification of Edwards' earlier design, permitting construction of a more flexible raceway system at less expense, also employed the use of ozone in the air lift pump and suggested its use in the water purification cycle (Edwards, 1975). These measures should effectively prevent the occurrence or spread of a disease within a system. Subsequent testing on a small scale revealed that shrimp could be successfully reared in ozonized seawater (Leong, personal commun.[1]). Another investigator directly bubbled ozone three times a day for 5-minute periods into carboys containing larval shrimp (Ramsey, personal commun.[2]). He recorded 67% survival after 12 days of such treatment during which time the shrimp grew from nauplius I to postlarval stage II. While ozone dose regimes and bacterial counts were not indicated, the fact that shrimp survived direct ozonizations suggests the potential use of ozonized water for shrimp culture.

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One of the most common diseases encountered in the culture of marine shrimp is caused by the ciliate Zoothamnium. Infected shrimps may exhibit lethargy, muscle discoloration, and develop a fuzzy mat on gill surfaces, eyes, appendages, and carapace. The only preventive measure recognized is rigid control of culture water to eliminate initial introduction of the ciliates (Sindermann, 1977). As already mentioned, Giese and Christensen (1954) reported the susceptibility of marine ciliates to ozone treatment. Upon exposure to ozonized seawater, all movement displayed by these organisms ceases and, after 2 hours of exposure to extremely low concentrations of less than 0.04 mg/l, the organisms burst. This information, coupled with the existing mariculture-ozone technology, presents a strong case for incorporation of ozone into shrimp culture practice.

Similar observations have been made for ozonized seawater in lobster culture. At the University of California Bodega Marine Laboratory, lobster culture has been examined for several years (Moffett and Shleser, 1975). Using only raw filtered seawater, poor results were obtained which were traced to fouling and pathogenic organisms, such as the fungi Haliphthoros sp., Fusarium sp., and the bacterium Leucothrix mucor. Malachite green and U.V. treatment created more problems than solutions for the investigators. Seeking an alternative measure, preliminary tests were conducted using ozone to disinfect the culture water. One hundred and five first-stage larvae were exposed to a final dissolved ozone dose of 0.4 mg/l for 30 minutes. Total coliform counts were made prior to, during, and after ozonization. Survival of the lobster larvae was

comparable to that reported by successful commercial hatcheries and no disease problems were encountered. These investigators advocated the use of ozone for disease prevention in hatchery situations.

While the use of ozone in seawater does not have a lengthy history, it has proved to be a successful and reliable disinfection method. Though it has been used on only a limited basis in crustacean culture thus far, the success of these preliminary investigations, coupled with the widespread use of ozone in the culture of other marine organisms, should provide a solid basis of experimental data upon which large-scale crustacean culture operations may be established. One such study has recently been proposed in Homestead, Florida, for the culture of marine shrimp (Udey, personal commun.[3]).

While discussing the benefits gained from the study of ozone's action on marine organisms, it is also necessary to cite some of the adverse effects experienced. Early work (MacLean et al., 1973) demonstrated that high ozone doses caused abnormal mitotic division in oyster larvae. Confirmatory studies concluded that ozone must produce a residual which is detrimental to oyster larval development when present in sufficiently high quantities. Later work has suggested that this residual is not ozone but probably a hypobromite ion formed during ozonization of seawater (Mangum and McIlhenney, 1975; Blogoslawski et al., 1976; Pichet and Hurtubise, 1976).

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Considering the long half-life of these toxic residuals (in excess of 24 hours), care must be taken not to exceed the ozone dose required to produce them. These residuals, while toxic to dividing bivalve larvae, appear to have caused no problem as yet in shrimp and lobster larvae of particular ages cultured in experimental hatcheries. Perhaps the dose employed is sufficiently low so that residuals are not produced or are not present in sufficient amounts to cause larval damage.

SUMMARY

Ozonization of seawater is a proven method for seawater disinfection. Its efficacy in microbial control has been applied to many areas, including aquaria, shellfish depuration, and improvement of water quality in aquaculture.

Crustacea in culture are susceptible to several diseases which are primarily related to water quality. The potential for using ozone to control disease in crustacean hatchery waters is being explored with very favorable initial results. The technology exists for economical application of ozone to waters of mariculture operations. By sterilizing the water used to raise commercially important crustaceans with ozone, the many diseases now affecting these species in the hatchery might be avoided.

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EFFECTS OF FURANACE ON THE DEVELOPMENT OF LARVAL STAGES OF PENAEUS MONODON FABRICIUS[1/]

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ABSTRACT

Successful molts and morphological defects in *Penaeus monodon* Fabricius zoeae (Z_1, Z_2, Z_3) resulting from a 24-hr exposure to 1.0 and 2.0 mg/L furanace in baths of 1.0 and 2.0 mg/L were quantified. Molting was delayed in Z_1, but not in Z_2 and Z_3 at 1.0 mg/L; considerably delayed in all sub-stages at 2.0 mg/L. Morphological defects in the telson, carapace, uropods and pereiopods were observed in high frequency in Z_3 after the exposure. These abnormality did not result in 1.0 mg/L. In Z_2, a 6-hr exposure is deemed optimum for bath in 1.0 mg/L as gauged from higher survival of larvae after 96 hours.

INTRODUCTION

In the Philippines, larvae of the jumbo tiger prawn *Penaeus monodon* Fabricius are reared at the Mindanao State University Institute of Fisheries Research and Development (Villaluz et al.,1972) and at the Aquaculture Department of the Southeast Asian Fisheries Development

[1/] SEAFDEC Contribution No. 22

Center (SEAFDEC) in Iloilo (Santiago, 1977). In the latter laboratory, production has been and continues to be limited by the presence of various microorganisms that succeed in attaching themselves to the larvae. Mortalities have been caused by Lagenidium, Ephelota gemmipara R. Hertwig, Zoothamnium sp., Acineta sp., Vorticella sp., Licmophora abbreviata Agardh, a nematode and a filamentous blue-green alga (Gacutan et al., 1977).

Due to high frequency of diseases, the authors make use of the chemotherapeutant furanace (6-hydroxymethyl-2 2-(5-nitro-2-furyl) vinyl pyridine), reported to be effective against Macrobrachium rosenbergii and fish bacterial and fungal pathogens (Shimizu and Takase, 1967; Amend and Ross, 1970; Ross, 1972; Delves-Broughton, 1974; Holt and Conrad, 1975) during larval rearing. Toxicity studies on P. monodon larvae showed that concentrations of 1.0 and 2.0 mg/L may be tolerated by zoeae (Z_2) and mysis (M_1), respectively, when added in bath for 24 hours (Gacutan and Llobrera, 1978). The LD_{50} for each larval stage were 1.6 and 2.0 mg/L, respectively.

This paper shows and quantifies (a) the different dose-related morphological and developmental effects resulting from a 24-hour exposure to two concentrations of the chemical upon the zoeae (Z_1, Z_2 and Z_3), and (b) the optimum exposure time to 1.0 mg/L furanace for Z_2 as determined from the survival.

MATERIALS AND METHODS

Dose-related morphological and developmental effects

Source and acclimatization of larvae: Healthy zoeae (Z_1, Z_2, Z_3) were collected early in the morning from 200-ton closed system concrete hatchery tanks in SEAFDEC. Determination of the exact larval stages was based on the work of Villaluz et al. (1972). Acclimatization was effected for one hour in the culture water used throughout the experiment. Analyses showed the following physical and chemical characteristics: salinity 29$\pm$3‰; pH 7.3-8.4; temperature 25-30°C; dissolved oxygen 4.5-8.4 ppm; nitrite 0.36-0.99 ppm; and, ammonia, 0.10-0.30 ppm.

Preparation of furanace bath and stocking: The procedure previously described by Gacutan and Llobrera (1978) was followed. Three, white, circular plastic basins (6" depth, 20" diameter) were used. To leach out toxic components from the basins, these were flushed in warm freshwater for 10 days as recommended by Carmignani and Bennet (1976). After conditioning, the basins were thoroughly rinsed with a portion of the culture water.

Fifty (50) larvae were then distributed into 5L of culture water in each basin. Ample aeration was provided at all times. After the acclimatization, pre-weighed

granules of furanace (purchased from Dainippon Seiyaku, Osaka, Japan*) were dissolved in 100-m portions of the culture water and mixed thoroughly. Furanace concentrations of 1.0 and 2.0 mg/L were used. One basin not provided with the chemical served as the control.

Initial feedings of Chaetoceros and Brachionus were given right after addition of furanace, and subsequently at 5 a.m. the next day. So as not to alter the furanace concentrations, the feeds were sieved with ordinary filter paper and added to the set-ups in densities of $100\text{-}150\times10^3$ cells/mL for Chaetoceros and 5-10 individuals/mL for Brachionus.

Determination of survival and morphological defects: Survival was based on the capability of the larvae to respond to stimulation, ability to swim and feed (Gacutan and Llobrera, 1978). Borderline cases were observed under the microscope in which case the final determinant was the ability to feed from around the mounted water.

Live larvae were then transferred to a new medium in a beaker provided with adequate fresh feeds. These were then mounted on glass slides with a drop of water and observed for abnormalities, deviant allometry, necrosis, etc. Larval stages were again determined. A total of 6 runs were conducted.

*Use of trade name does not imply endorsement of product.

Optimum exposure time to furanace

On the basis of the 24-hr LD_{50} of 1.6 mg/L as worked out (Gacutan and Llobrera, 1978), the exposure duration that consistently gave the highest survival rate in potentially-weak and apparently unhealthy larvae was sought. Nine basins were set-up as done previously. After the addition of feeds and acclimatization, samples of furanace, pre-weighed to make a uniform concentration of 1 mg/L, were added to eight of the basins. The first basin was not provided with furanace and therefore served as control. The treated basins were then drained, washed thoroughly and replaced with fresh seawater containing the appropriate density of *Brachionus* and *Chaetoceros*. The whole process was done at 3-hr-interval for each basin until the eighth basin had been replaced. After 24, 48, 72 and 96 hours, the surviving population in each basin was then taken and recorded. A total of 4 trials were conducted.

RESULTS

Dose-related abnormalities; rate of development

Metamorphosis of Z_1 to Z_2, Z_2 to Z_3 and Z_3 to M_1: The results of the 3 experiments indicate a delaying effect on metamorphosis which generally increased with concentration

of furanace. Out of 180 specimens examined from a surviving population of Z_1 given no furanace, 177 or 98.3 percent progressed to Z_2 (Table 1). For those exposed to furanace baths of 1.0 mg/L, 89 out of 127, or 70.1 percent metamorphosed successfully. Comparison of these two values indicated a statistically significant difference at p=0.05. When given a dose of 2.0 mg/L, only 6 out of 47 or 13.9 percent showed successful metamorphosis.

There was a decline in the delaying effect of the chemotherapeutant with larval age. In the metamorphosis of Z_2 to Z_3, 22 out of 166 survivors or 86.7 percent molted when given a 1.0 mg/L dose; and 75 out of 159 or 52.8 at 2.0 mg/L. Statistical analysis revealed no significant difference between the control and those given 1.0 mg/L which proves that no marked effect on development is exerted. However, 2.0 mg/L definitely delays the molting function.

Experiments on the metamorphosis of Z_3 to M_1 had almost the same results as the above (Z_2 to Z_3) except for the lower percentage of successful molts in controls and in those given 1.0 mg/L. This might indicate a lower tolerance of the larvae to toxic effects of the chemotherapeutant. Out of 102 survivors from all the control populations, 89 or 87.2 percent metamorphosed to the mysis stage. This was not significantly higher than the 81.8 percent obtained from 181 individuals from those given 1.0 mg/L.

Table 1. The metamorphosis of P. monodon larvae in 2 levels of Furanace given in a 24-hr bath

Larval stages	Levels of furanace (mg/L)		
	0	1	2
Z_1 to Z_2			
Z_1	3	38	37
Z_2	177	89	6
Total	200	127	43
% Molting	98.3	70.1[1/]	13.9
Z_2 to Z_3			
Z_2	7	22	75
Z_3	94	144	84
Total	101	166	159
% Molting	93.1	86.7[3/]	52.8
Z_3 to M_1			
Z_3	13	33	57
M_1	89	148	87
Total	102	181	144
% Molting	87.2	81.8[2/]	61.4[3/]

[1/] Significantly different at all levels at p=0.05

[2/] Not significantly different from control

[3/] Significantly different from population bathed in 1.0 mg/L

Table 2. Morphological defects found in *P. monodon* larvae after a 24-hr exposure to 2 levels of furanace

Experiments	Center of Morphological defects	Number of individuals showing morphological defects in level of furanace		
		0	1	2
Z_1 to Z_2				
	Carapace	2	0	1
	Telson	5	3	2
	Individuals examined	248	178	77
Z_2 to Z_3				
	Carapace	2	3	6
	Telson	3	6	19[1/]
	Uropods	2	2	1
	Individuals examined	102	166	159
Z_3 to M_1				
	Carapace	18	3	35[1/]
	Telson-Uropods	5	2	20[1/]
	Uropods	3	3	10[2/]
	Telson	6	5	4
	Pereiopods	9	3	16[2/]
	Antennae[3/]	11	0	5
	Rostrum	2	1	0
	Individuals examined	102	168	144

[1/] Significantly different from both control and 1.0 mg/L at p=0.05

[2/] Significantly different from 1.0 mg/L not from control

[3/] Significant differences exist among 3 levels

Morphological changes: Examination of the larvae for abnormalities revealed a number of damages as shown by (a) loss of certain parts, the more notable of which are the uropodal setae; (b) deviant allometry; (c) necrosis and other conditions. Observed to result from or accompany Z_1 to Z_2 ecdysis were abnormally-shaped uropods and spread carapace (Table 2). The metamorphosis of Z_2 to Z_3 was affected most in terms of damages to the telson, the frequency of which went progressively higher with increased concentration. The very low incidence of individuals affected coupled with the absence of any statistical differences among the controls and the treated populations indicated, among other things, that these abnormalities were not dose-related at least during these sub-stages.

During the Z_3 to M_1 transition shrivelled carapaces were observed. These took the form of flat structures instead of the usual dome so characteristics of penaeid carapaces. The uropods were subjected to varying degrees and intensity of damages. This varied from erosion, necrosis, breaks, detachment and total loss of one or more of the uropodal setae to unequally-sized and shaped uropods suggestive of an altered allometry; to loss of one or more of the uropods and of the telson. The pereiopods and antennae, perhaps due to their elongated configurations, had breakages and cut-offs at points not necessarily on the joint portions. Microscopy showed high frequency of

Table 3. Survival of weak and apparently diseased *P. monodon* Z_2, exposed for varied durations in 1 mg/L 'furanace' baths[1]

Exposure (hrs)	Mean survival after ( ) hours[2]			
	24	48	72	96
0	33.0	14.3	9.5	4.0
3	42.0	32.3	30.0	8.3
6[3]	40.0	33.8	26.0	19.2
9	35.2	28.3	15.3	9.3
12	36.0	26.4	12.0	9.0
15	39.2	23.8	17.8	7.3
18	40.9	22.8	14.9	8.1
21	40.4	23.7	15.2	10.8
24	37.5	26.7	12.6	5.8

[1] Based on 4 trials

[2] Based on an initial population of approximately 50 individuals

[3] Optimum exposure based on Friedmann's rank analysis

abnormalities and damages at 2.0 mg/L compared to 1.0 mg/L where there was generally a protective effect.

Optimum exposure time

The optimum exposure duration of Z_2 to 1.0 mg/L furanace, as borne out by Friedmann's two-way analysis of variance by ranks (Table 3), appears to be 6 hours. Populations of Z_2 exposed this long had approximately 38 percent survival (19.2 out of 50.0 initial population) after 96 hours. Among other things, the data showed no significant reduction in populations in all batches after 24 hours; differences were apparent only after 48 hours.

DISCUSSION

Closed-system hatchery tanks used for the culture of *P. monodon* in SEAFDEC need a suitable chemotherapeutant which may be added to the culture water as the need arises. Aside from its potency against bacterial and fungal pathogens in low concentrations, such a chemotherapeutant must be safe to use on non-target organisms; easily absorbed by the animal in its active form, but not accumulated in tissues; and, easily degraded. It must not interfere drastically with the *Nitrosomonas-Nitrobacter* system and its residues in culture water should be easily removed after

treatment. Among the many therapeutants available at present only a few are suitable for use given these requirements.

Furanace, a nitrofuran effective against fish pathogens (Shimizu and Takase, 1967; Amend and Ross, 1970; Ross, 1972; Holt and Conrad, 1975), fulfills most if not all of these prerequisites (Delves-Broughton, 1974). Its residues in the culture water are effectively removed from the effluent after treatment by a variety of adsorptive materials especially activated carbon (Marking and Piper, 1976).

Marking *et al.*, (1977) conducted toxicity studies on 6 species of fish, 6 species of aquatic invertebrates, and on eggs and larvae of a leopard frog. The 96-hr LC 50's varied between 0.820 to 3.0 mg/L for the fish; 1.13 to 20 mg/L for the invertebrates and 0.77 mg/L for the larvae of the leopard frog. Two of the present authors (Gacutan and Llobrera, 1978) also determined the 24-hr LC_{50} for *P. monodon* zoeae and mysis to be 1.6 and 2.0 mg/L, respectively. The reported morphological defects and the inability to molt exhibited by surviving larvae used in the static bioassays earlier conducted have been quantified in the present experiments.

Unquestionably both concentrations of 2.0 mg/L of furanace administered for 24-hr is inadvisable as revealed by the results of the metamorphosis of the zoeae to the next larval stage. A 1.0 mg/L concentration is more likely

to be protective at least to Z_2 and Z_3, but certainly not to Z_1 which, as shown, are particularly sensitive to the chemical, resulting in high frequency of failure to molt. The morphological defects observed to occur on the telson, carapace, uropods, and pereiopods in many of the larvae exposed to 2.0 mg/L but not in the lower therapeutant level are believed to be dose-related.

The experiments on the varied time exposures were conducted to evolve a suitable use pattern for furanace applicable to *P. monodon* larvae. In fish, the suggested pattern is a 1-hr exposure to 1.0 mg/L for 3 consecutive days with a holding period of 10 days (Marking *et al.*, 1977). From the experiments it appears that a simple 6-hr continuous exposure is adequate for *P. monodon* Z_2. This may be gauged from the high survival after a 96-hr observation period. It is to be emphasized however that the larvae utilized in these experiments were weak and therefore were potentially susceptible to pathogens. Higher survival rates would have been obtained with healthy larvae which are actually the objects of any therapeutant treatments to be instituted.

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CANDIDATE CHEMICALS FOR CRUSTACEAN CULTURE

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ABSTRACT

A number of chemicals are used in crustacean culture to control diseases, to anesthetize organisms, or to alleviate cultural problems. Chemotherapeutants and anesthetics are listed with their potential uses, efficacy data, relative toxicity, crustacean species treated, and literature references. Data from standard toxicity tests performed at the Fish Control Laboratories with four therapeutants, on one crustacean species and three species of fish, are included for comparison. This report was compiled to evaluate the chemicals that are being used or tested in crustacean culture so that the most promising candidates can be selected for registration.

Few chemicals have been approved by the Food and Drug Administration (FDA) or the Environmental Protection Agency (EPA) for use on crustaceans. However, many of the compounds used in fish culture are in the process of being registered for fishery use. Criteria for registration of chemicals used in arthropod culture are the same as those in fish culture. Even though a compound may be registered for use on fish, data to support a label extension must be filed with FDA or EPA before the compound can legally be used to treat crustaceans intended for human consumption. Until adequate registrations become available, it is suggested that researchers in crustacean culture consider using only compounds that are approved for use on crustaceans or have a history of safe use in fish culture.

INTRODUCTION

The control of pest organisms is a problem in all types of anmal husbandry. The adverse effects of bacterial, viral, fungal, and parasitic infections increase logarithmically as population density rises. As new species are added to the list of organisms under culture, new culture systems are developed and new disease problems arise. Johnson (1975), Delves-Broughton (1974), and

Sindermann (1970, 1977) provided detailed information on the diseases of crustaceans. Solutions to new problems are generally sought by reviewing control techniques applied to similar disease agents in related cultures. Until lag time in research is overcome, crustacean culturists must look to fish culture for potential answers.

Logically, it would seem that chemicals used to treat fish pathogens should be effective against the same types of pathogens on crustaceans; similarly, if a chemical is used on fish, it presumably should be safe to use on other aquatic organisms. Unfortunately, however, only the first of these two premises applies. Effective therapeutants generally control a genus of pathogens in a variety of hosts if the delivery system assures adequate contact between the chemical and the organism; changing the host species seldom affects the activity of a chemical on pathogens. However, there are profound differences in susceptibility to treatment between families, genera, and even species of hosts. A compound readily tolerated by fish may be highly toxic to arthropods.

A long history of unchallenged use does not indicate that a therapeutant has been properly registered by the U.S. Food and Drug Administration (FDA). In fact, most of the treatments now used in fish culture lack registration. Crustacean culturists should never assume that it is permissible to use a compound merely because fishery workers are doing so. Criteria to be met in registering compounds for use in arthropod culture are the same as those in fish culture. If a compound is registered for use on fish, data to support a label extension must be filed with FDA

before it can legally be used to treat crustaceans.

Requirements for registration of a chemical include not only proof of its effectiveness but also data on toxicity to nontarget organisms, including fish, other aquatic organisms, birds, and mammals; persistence in tissues of animals and the environment; degradation rates and products; and metabolites (Lennon 1967; Meyer and Schnick in press). Some chemicals have several uses, and data developed for one use may support registration for another use. For instance, potassium permanganate has been used as a parasiticide, a detoxifier, and an oxidizing agent to relieve oxygen stress due to decaying organic matter. Lime has been used to control pH and as a pond sterilant, fertilizer, and water hardening agent.

Some of the fishery chemicals, especially therapeutants, may be useful in crustacean culture and propagation (Johnson 1977; Delves-Broughton 1974). We summarize here the registration status of chemicals currently in use, methodologies applicable to fish or crustacean studies, efficacy and toxicity to fish and crustaceans, and potential uses in crustacean culture.

Registration Status

A few chemicals used in fish culture have classifications or registrations from either FDA or the Environmental Protection Agency (EPA) which would allow them to be legally used on crustaceans. These include lime, sodium chloride, rhodamine B, copper sulfate, and 2,4-D. Unfortunately the list includes no disease control agents needed in crustacean culture.

The status of candidate and registered fishery chemicals changes continually because requirements of the regulatory agencies are frequently changed or upgraded. Essentially, fishery chemicals classified as pesticides must be registered through EPA, and therapeutants through FDA. During the past decade, Congress has enacted laws to protect the environment which require initial registration as well as periodic reviews or reregistrations (Cumming 1975).

Certain progress has been made toward registration of fishery chemicals. In 1972, about 45% of the research needed for the registration of chemicals used in fisheries had been completed, and 13 compounds had at least limited registrations. In early 1976, the percentage had risen to 75%, and 22 compounds had been registered (Meyer *et al.* 1976). Rotenone and antimycin (piscicides) and TFM and Bayer 73 (lampricides) were registered toxicants for nonfood use only. Two other fish toxicants, GD-174 and Squoxin, remain unregistered. The only anesthetic with a food fish use registration is Finquel (MS-222). Quinaldine sulfate and a mixture of it with MS-222 are still unregistered. Registered fish therapeutants include Masoten, Furanace, Terramycin, and sulfamerazine, but not all can be used on food fishes. Therapeutants that are not registered are formalin, the formalin:malachite green mixture, malachite green oxalate, furazolidone, and nitrofurazone. Povidone-iodine is used as a fish egg disinfectant and is ready for submission to FDA. Calcium hypochlorite is registered for sanitizing fish tanks, raceways, and utensils, controlling growth of algae, and killing bacteria in fish ponds. Lime has been

declared GRAS (Generally Regarded As Safe) for use in treating pond soils, and sodium chloride is classified as GRAS and used as an osmoregulatory enhancer for fish. Rhodamine B is exempted from requirements for a tolerance when used as a dye to trace water flows. Potassium permanganate is used as an oxidizing agent, but its registration is not yet complete. Aquatic herbicides registered for fishery use include copper sulfate, dichlobenil, DMA-2,4-D, diquat dibromide, endothall, Fenac, silvex, and simazine. Crustacean culturists must check each label to determine if the compound can legally be used in their situations. Diuron remains unregistered for fishery use.

Toxicity and Efficacy Data

A considerable number of chemicals have been used on crustaceans to control pests, determine chemical effectiveness in combatting disease, or assess crustacean susceptibility to chemicals used in other cultures (Kabata 1970; Muirhead-Thomson 1971; Hart and Fuller 1974; Hoffman and Meyer 1974; Sindermann 1977; Johnson 1977).

Because of the relative infancy of crustacean culture, some of the early data referenced may represent preliminary findings and may require further validation. Investigators should weigh all data carefully before using them in their work. Toxicity and efficacy data gathered from literature on laboratory studies and field uses of anesthetics and therapeutants for potential uses in crustacean culture are summarized in Tables 1 and 2. Toxicity data developed under laboratory conditions at the Fish Control Laboratories are given in Table 3.

The techniques used in toxicity tests with aquatic invertebrates are similar to those used with fish. Laboratory procedures have been standardized, and data are comparable between laboratories and geographic locations. Standardized test procedures were prepared by a committee representing private industry, contract laboratories, EPA, and the U.S. Fish and Wildlife Service (Committee on Methods for Toxicity Tests with Aquatic Organisms 1975). Since then, responsibility for the standardization of laboratory and field testing and procedures has been assumed by the American Society for Testing and Materials (ASTM).

Table 3 includes the results of standardized tests at the Fish Control Laboratories on three species of fish (rainbow trout, *Salmo gairdneri*; channel catfish, *Ictalurus punctatus*; and bluegill *Lepomis macrochirus*, and one crustacean (the glass shrimp, *Palaemonetes kadiakensis*). Exposures were for 96 hours, static, at 12°C for fish and at 16°C for glass shrimp. The fish were exposed in reconstituted water (40-44 mg/L total hardness) and the glass shrimp in limed spring water (20-24 mg/L total hardness). Survival or mortality data for 10 specimens per concentration were used to calculate toxicity in terms of LC50's and 95% confidence intervals according to Litchfield and Wilcoxon (1949). The reader must be aware that these results are based strictly on laboratory work and can be used only as guidelines in predicting levels that might be needed under other conditions.

Table 1. The relative toxicity of selected chemicals to freshwater and marine crustaceans.

Chemical	Potential Use	Relative Toxicity[a]	Crustacean Species	Reference
Acriflavin	Bacteriostat	96-h LC50 <1 mg/L	*Penaeus duorarum*	Johnson 1974a
Acriflavin	Bactericide	10-500 mg/L not toxic for 24-h exposures 45% died at 25 mg/L for 72 h 100% died at 500 mg/L for 72 h	*Procambarus clarkii* (27-35 mm)	Williams and Avault (In press)
Acriflavin	Fungicide	No mortality at single 20-h treatment with 10 mg/L One of five died at three 4-h dips at 20 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Acrinol	Fungicide	24-h MAC = 1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Aerosol OT (See Dioctyl sodium sulfosuccinate)				
2-Amino-4-nitrophenol	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Amphotericin B	Fungicide	24-h MAC = 0.1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Azalomycin F	Fungicide	24-h MAC = 5 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Benlate	Fungicide	96-h LC50 = 7.6 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Benlate	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Benzalkonium chloride	Fungicide	24-h MAC = 5 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Benzethonium chloride (See Hyamine 1622)				

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Blastin (See PCBA)				
Brilliant green	Fungicide	24-h MAC = 0.1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Brono (See Chlorathalonil)				
Butyl paraben + DMSO	Fungicide	100% mortality at 100 mg/L Butyl paraben swabbed	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Butyl paraben + vegetable oil	Fungicide	100% survival at 100 mg/L Butyl paraben swabbed	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Captan	Fungicide	49% survival at 3.2 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
Captan	Fungicide	96-h LC50 = 8.0 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Cetylpyridinium chloride	Fungicide	24-h MAC = 0.1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Chloramine T	Parasiticide	20 mg/L = not lethal at 72 h	*Penaeus stylirostris*	Johnson 1976b
Chloramine T	Parasiticide	13 of 30 died at 20 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
Chloramine T	Fungicide	24-h MAC = >100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Chloramine T		Applied LC50 2.02 mg/L for 30-60 min at 25°C Residual LC50 0.32 mg/L for 30-60 min at 25°C Applied LC50 0.56 mg/L for 30-60 min at 30°C	*Homarus americanus*	Capuzzo *et al.* 1976

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Chloramine T		Residual LC50 0.06 mg/L for 30-60 min at 30°C	*Homarus americanus*	Capuzzo *et al.* 1976
Chlorine (free)		Applied LC50 16.30 mg/L for 30-60 min at 25°C Residual LC50 2.89 mg/L for 30-60 min at 30°C Applied LC50 2.50 mg/L for 30-60 min at 30°C Residual LC50 0.41 mg/L for 30-60 min at 30°C	*Homarus americanus*	Capuzzo *et al.* 1976
5-Chlorosalicylanilide	Fungicide	24-h MAC = 1.0 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Chlorothalonil	Fungicide	96-h LC50 = 0.14 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Chloroxine + DMSO	Fungicide	One of four died when swabbed with 100 mg/L Chloroxine	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Copper sulfate (hydrated)	Herbicide, parasiticide	0.5-1.0 mg/L was not toxic	*Penaeus duorarum* *P. setiferus* *P. stylirostris*	Johnson 1974a
Copper sulfate	Parasiticide, molluscicide	1 mg/L was not toxic in ponds	*Penaeus stylirostris*	Johnson and Holcomb 1975
Copper sulfate	Algicide	1-h LC50 = 250 mg/L	*Penaeus californiensis*	Hanks 1976
Copper sulfate, tribasic	Fungicide	46% survival at 159 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Crystal violet	Fungicide	No mortality from three 4-h dips at 20 mg/L or single 20-h dip at 10 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Crystal violet	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Cu^{2+} (Bordeaux mixture)	Fungicide	96-h LC50 = 1.5 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Cutrine	Algicide	1-h LC50 = 1,000 mg/L	*Penaeus californiensis*	Hanks 1976
Cutrine Plus	Antibiotic	0.5 mg/L Cu^{++} in static tests was toxic 0.1 mg/L Cu^{++} in 24-h flow-through test was toxic	*Penaeus californiensis*	Lightner and Supplee 1976
Dehydroacetic acid	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Dichlone	Fungicide	96-h LC50 = 0.038 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Dichlone	Fungicide	35% survival at 11 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
5,7-Dichlor,8-hydroxyquinoline	Fungicide	24-h MAC = 1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Difolaton	Fungicide	6% survival at 7.2 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
Dioctyl sodium sulfosuccinate	Fungicide	24-h MAC = 5 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Dithane M-45 (See Manzate 200)				

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
DMSO (See Butyl paraben + DMSO or Chloroxine + DMSO)				
DS 9073	Fungicide	6% survival at 1.3 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
DTAS	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Dyrene	Fungicide	29% survival at 6.0 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
Ethyl paraben	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Folpet	Fungicide	96-h LC50 = <0.1 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Formalin (See also Table 3)	Parasiticide	25-500 mg/L not toxic for 24 h 5% died at 100 mg/L for 72 h 100% died at 2,500 mg/L for 72 h	*Procambarus clarkii* (33-40 mm)	Williams and Avault (In press)
Formalin	Parasiticide	10-500 mg/L not toxic for 24 h 52% died at 500 mg/L for 72 h 100% died at 1,000 mg/L at 72 h	*Procambarus clarkii* (8.5-10 mm)	Williams and Avault (In press)
Formalin	Parasiticide	Caused mortalities, molting deformities, and cessation of feeding	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Formalin	Parasiticide	96-h LC50 = 235 mg/L	*Penaeus duorarum*	Johnson 1974a

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Formalin	Parasiticide	100 µL/L for 12-72 h were not toxic	*Procambarus blandingi*	Helms 1964
Formalin:malachite green	Bacteriostat, parasiticide	1-h LC50 = 400:20 mg/L	*Penaeus californiensis*	Hanks 1976
Fradiomycin (See Neomycin sulfate)				
Fungizone (See Amphotercin B)				
Furacin (See Nitrofurazone)				
Furanace (See also Table 3)	Fungicide	No mortality from three 4-h dips at 20 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Furanace	Bacteriostat, fungicide	Tolerated 20 mg/L for 20 min Stressed 20 mg/L for 30 min	*Macrobrachium rosenbergii*	Delves-Broughton 1974
Gentian violet	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Gliseofluvin	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Glisovin FP (See Gliseofluvin)				
4-Hexylresorcinol	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Hyamine	Bacteriostat	1-h LC50 = 70 mg/L	*Penaeus californiensis*	Hanks 1976
Hyamine 1622	Fungicide	24-h MAC = 5 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Hyamine 2389	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
3-Hydroxy-5-methyl isoxazole (See Tachigaren)				
Isobutyl alcohol	Anesthetic	14.4 mL/L were nontoxic in 3-10 min exposures	*Homarus americanus*	Foley *et al.* 1966
Laurylpyridinium chloride (See Hyamine 2389)				
Malachite green (See also Table 3)	Fungicide	Long-term dietary deficiencies and malachite green treatment (40 mg/L for 10 min) made lobsters susceptible to otherwise nonpathogenic bacteria	*Homarus americanus* (juvenile)	Fisher *et al.* 1976c
Malachite green	Parasiticide, fungicide	96-h LC50 = 2.5 mg/L	*Penaeus duorarum* *P. stylirostris*	Johnson 1974a
Malachite green	Fungicide	24-h MAC = 1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Malachite green	Parasiticide	0.1 mg/L in ponds was not toxic	*Penaeus setiferus*	Johnson and Holcomb 1975
Malachite green	Antibiotic	0.1 mg/L three times a week was toxic	*Cancer magister* (larvae)	Armstrong *et al.* 1977
Malachite green	Parasiticide, fungicide	20 mg/L for 8 min and 8 mg/L for 16 min decreased survival	*Homarus americanus* (larvae)	Fisher *et al.* 1976b

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Malachite green	Antibiotic	20 mg/L for longer than 8 min were toxic	*Homarus* sp. (larvae)	Sindermann *et al.* 1977a
Malachite green	Parasiticide, fungicide	20 mg/L for 4 min decreased survival	*Homarus americanus* (larvae and postlarvae)	Fisher *et al.* 1976b
Malachite green	Fungicide	96-h LC50 = 0.04 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Malachite green	Fungicide	45% survival at 0.006 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976
		98% survival at 0.06 mg/L for 24 h	*Penaeus stylirostris* (mysis)	
		96% survival at 0.06 mg/L for 24 h	*P. californiensis* (mysis)	
		98% survival at 0.6 mg/L for 24 h	*P. californiensis* (postlarvae)	
		90% survival at 0.006 mg/L for 24 h	*P. vannamei* (mysis)	
		80% survival at 0.003 mg/L for 24 h	*P. vannamei* (postlarvae)	
Malachite green	Fungicide	2-20 mg/L = no mortality at single 1-h dip 100% mortality at 20 mg/L for single 2-h dip	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Manzate 200	Fungicide	41% survival at 2.1 mg/L 9 days after application	*Artemia salina* (eggs)	Hatai *et al.* 1974
2-Mercaptobenzothiazole sodium	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Methylene blue	Parasiticide, fungicide	96-h LC50 = <5 mg/L	*Penaeus duorarum*	Johnson 1974a
Methylene blue	Bacteriostat, fungicide	1-h LC50 = 100 mg/L	*Penaeus californiensis*	Hanks 1976
Methylene blue	Fungicide	One mortality out of five organisms at three 4-h dips at 20 mg/L or single 20-h dip at 10 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Methylene blue	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl 1,4-naphthoquinone (VK_3)	Fungicide	24-h MAC = 1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl paraben	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl pentynol	Anesthetic	Concentrations above 7.0 mg/L caused death or paralysis 100% mortality at 90 mL/L	*Homarus americanus*	Foley *et al.* 1966
Methyl violet	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Mongare (See DTAS)				
Mycostatin (See Nystatin)				
Neomycin sulfate	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Nifuroxime	Fungicide	No mortality after three 4-h dips at 20 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Nifuroxime	Fungicide	100% mortality with single 20-h dip at 10 mg/L	*Homarus gammarus* (juvenile)	Abrahams and Brown 1977
Nitrofurazone	Antibiotic	2 of 30 died at 5 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
Nitrofurazone	Antibiotic	1 mg/L was not lethal	*Penaeus setiferus*	Lightner 1977
Novobiocin	Antibiotic	15 mg/kg were lethal	*Homarus* sp.	Stewart and Arie 1974
Nystatin	Fungicide	24-h MAC = 5 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Oxytetracycline (See Terramycin)				
PCBA	Fungicide	24-h MAC = 1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
PCNB	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Penicillin G	Antibiotic	2 million IU/kg were lethal 400,000 IU/kg were not lethal	*Homarus americanus*	Stewart and Arie 1974
Pentagen (See PCNB)				
Phaltan (See Folpet)				
Polymyxin B sulfate	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Polyram	Fungicide	96-h LC50 = 5.9 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Potassium dichromate	Parasiticide	96-h LC50 = >25 mg/L	*Penaeus duorarum*	Johnson 1974a

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Potassium permanganate (See also Table 3)	Parasiticide, oxidizing agent	96-h LC50 = 6 mg/L	*Penaeus duorarum* *P. stylirostris*	Johnson 1974a
	Parasiticide, oxidizing agent	1-h LC50 = 500 mg/L	*P. californiensis*	Hanks 1976
Potassium permanganate	Parasiticide	3 mg/L in ponds were not toxic	*Penaeus setiferus*	Johnson and Holcomb 1975
Potassium permanganate	Antibiotic	5-10 mg/L - 1 h may cause severe gill damage	*Penaeus* sp.	Lightner 1977
Potassium permanganate	Fungicide, parasiticide	31% died at 25 mg/L for 24 h 43% died at 50 mg/L for 72 h 100% died at 250 mg/L for 72 h	*Procambarus clarkii* (28-35 mm)	Williams and Avault (In press)
Potassium permanganate	Antibiotic	2, 5, and 10 mg/L for 1 h and 1 mg/L for 30 min caused high mortalities	*Macrobrachium rosenbergii*	Sindermann and Le Bitoux 1977
Propyl paraben	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Quinacrine hydrochloride	Parasiticide	7 of 30 died at 5 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
		20 mg/L were not lethal at 72 h	*P. stylirostris*	Johnson 1976b
Quinaldine	Anesthetic	25 mg/L were not lethal	*Penaeus setiferus*	Johnson 1974b
Quinine bisulfate	Parasiticide	10 of 30 died at 10 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
		40 mg/L were not lethal at 72 h	*P. stylirostris*	Johnson 1976b

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Quinine sulfate	Parasiticide	6 of 30 died at 10 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
		10 mg/L were not lethal at 72 h	*P. stylirostris*	Johnson 1976b
Roccal (See Benzalkonium chloride)				
Salicylic acid Na	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Sodium benzoate	Fungicide	24-h MAC = 100 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Sorbic acid Na	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Spetol (See Benzalkonium chloride)				
Tachigaren	Fungicide	24-h MAC = 500 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Terrachlor	Fungicide	98% survival at 0.325 mg/L - 24 h	*Penaeus vanammei* (mysis)	Bland *et al.* 1976
		76% survival at 0.1625 mg/L - 24 h	*P. vanammei* (postlarvae)	
		98% survival at 1.3 mg/L - 24 h	*P. californiensis* (mysis)	
		96% survival at 1.3 mg/L - 24 h	*P. californiensis* (postlarvae)	
		100% survival at 0.65 mg/L - 24 h	*P. stylirostris* (mysis)	
		14% survival at 1.3 mg/L 9 days after application	*Artemia salina* (eggs)	

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Terramycin	Antibiotic	22 mg/kg were lethal 11 mg/kg were not lethal	*Homarus* sp.	Stewart and Aire 1974
Terramycin	Antibiotic	5,000 mg/kg + inoculated with *Vibrio* - all shrimp survived	*Penaeus aztecus*	Corliss *et al.* 1977
Terramycin	Antibiotic	2 of 30 dead at 5 mg/L for 24 h	*Penaeus setiferus*	Johnson 1976a
Terramycin	Antibiotic	360 mg/kg were not lethal to shrimp infected with *Vibrio*	*Penaeus* sp.	Lightner 1977
Treflan (See Trifluralin)				
Tri-buthyltin acetate	Fungicide	24-h MAC = 0.1 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
2,4,5-Trichlorphenol	Fungicide	24-h MAC = 10 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Trifluralin	Fungicide	90% survival at 3.0 mg/L - 24 h 94% survival at 3.0 mg/L - 24 h 100% survival at 1.5 mg/L - 24 h 90% survival at 0.1875 mg/L - 24 h 80% survival at 0.375 mg/L - 24 h 3% survival at 5 mg/L 3 days after application	*Penaeus californiensis* (mysis) *P. californiensis* (postlarvae) *P. stylirostris* (mysis) *P. vanammei* (mysis) *P. vanammei* (postlarvae) *Artemia salina* (eggs)	Bland *et al.* 1976
Trifluralin	Fungicide	96-h LC50 = 0.3 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976

(more)

Table 1. (cont'd)

Chemical	Potential Use	Relative Toxicity	Crustacean Species	Reference
Trifluralin	Fungicide	48-h LC50 = 1,200 µg/L	*Palaemonetes kadiakensis*	Sanders 1970
Tri-phenyltin acetate	Fungicide	24-h MAC = 50 mg/L	*Penaeus japonicus*	Hatai *et al.* 1974
Vancomycin	Antibiotic	20-40 mg/kg were not lethal	*Homarus* sp.	Stewart and Arie 1974
Vegetable oil + butyl paraben (See Butyl paraben + vegetable oil)				
Vitavax	Fungicide	5% survival at 38 mg/L 9 days after application	*Artemia salina* (eggs)	Bland *et al.* 1976

[a] LC50 = lethal concentration capable of killing 50% of the host organisms; MAC = minimum annihilation concentration.

Table 2. The efficacy of selected chemicals in freshwater and marine crustacean culture.

Chemical	Target Organism or Use	Efficacy[a]	Crustacean Species	Reference
Acetic acid	*Epistylis* sp.	2 ppt for 1 min were effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Acriflavin	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 1:10,000 dilution of commercial preparation	*Homarus gammarus*	Abrahams and Brown 1977
Acriflavin	*Haliphthoros* sp.	Suitable for control	*Homarus* sp. (postlarvae)	Sindermann *et al.* 1977a
Acrinol	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Aerosol OT (See Dioctyl sodium sulfosuccinate)				
Aquasol	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
2-Amino-4-nitrophenol	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Amphotericin B	*Fusarium* sp.	MIC = 1 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Amphotericin B	*Haliphthoros milfordensis*	1.5 cm growth diameter *in vitro* at 25 mg/L	*Homarus gammarus*	Abrahams and Brown 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Azalomycin F	*Fusarium* sp.	MIC = 1 mg/L *in vitro* after 2 and 7 days 1, 10, and 100γ were not effective when injected into infected prawns 1 and 5 mg/L were not effective on prawns injected with *Fusarium*	*Penaeus japonicus*	Hatai *et al.* 1974
Bay 5097	*Haliphthoros milfordensis*	1.2 cm growth diameter *in vitro* at 20 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Benlate	*Lagenidium* sp.	48-h SR = >100 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Benlate	*Lagenidium callinectes*	MLD to L-3B strain = 39 mg/L	*Penaeus setiferus*	Ruch and Bland 1974
		MLD to L-1 strain = 28 mg/L	*Callinectes sapidus*	Ruch and Bland 1973, 1974; Sindermann *et al.* 1977b
Benlate	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974
Benlate	*Lagenidium callinectes*	MLC to L-1 strain = 29 mg/L	*Callinectes sapidus*	Bland *et al.* 1976
		MLC to L-3B strain = 39 mg/L	*Penaeus setiferus*	
Benlate	*Fusarium* sp.	1, 5, 10 mg/L were not effective on prawns injected with *Fusarium*	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Benzalkonium chloride	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Benzethonium chloride (See Hyamine 1622)				
Biphenyl	*Haliphthoros milfordensis*	7.4 cm growth diameter *in vitro* in 50 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Blastin (See PCBA)				
Brilliant green	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Brilliant green	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Brono (See Chlorothalonil)				
Buquinolate	*Nosema michaelis*	Effective in preventing spores in the musculature when given in the feed	*Callinectes sapidus*	Overstreet 1975
Butyl paraben	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 10 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Captan	*Haliphthoros milfordensis*	5.4 cm growth diameter *in vitro* at 25 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Captan	*Lagenidium callinectes*	MLD to L-3B strain = 5 mg/L MLD to L-1 strain - 3.2 mg/L	*Penaeus setiferus* *Callinectes sapidus*	Ruch and Bland 1974 Ruch and Bland 1973, 1974; Sindermann *et al.* 1977b

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Captan	*Lagenidium* sp.	48-h SR = 12 mg/L	*Cancer magister* (larvae)	Armstrong *et al*. 1976
Captan	*Lagenidium callinectes*	MLC to L-1 strain = 3.2 mg/L MLC to L-3B strain = 5 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al*. 1976
Captan	*Lagenidium* sp.	0.06 mg/L retarded contagion	*Cancer magister*	Armstrong *et al*. 1977
Cetylpyridinium chloride	*Fusarium* sp.	MIC = 1 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al*. 1974
Chloramine T	*Epistylis* sp.	5 mg/L were effective after 72 h	*Penaeus stylirostris*	Johnson 1976b
		Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Chloramine T	*Fusarium* sp.	MIC = 100 or >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al*. 1974
Chloroform	Anesthetic	Not effective	*Homarus americanus*	Foley *et al*. 1966
5-Chlorosalicylanilide	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 days MIC = 100 or >100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al*. 1974
Chlorothalonil	*Lagenidium* sp.	48-h SR = 0.17 mg/L	*Cancer magister* (larvae)	Armstrong *et al*. 1976
Chloroxine	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 10 mg/L	*Homarus gammarus*	Abrahams and Brown 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Copper	Suctorian protozoans	0.4 mg/L for 6 h was effective	*Macrobrachium* sp.	Goodwin and Hanson 1975
Copper sulfate	Parasiticide, molluscicide	1 mg/L in ponds was not effective on protozoa or clams	*Penaeus stylirostris*	Johnson and Holcomb 1975
Copper sulfate	*Zoothamnium* sp.	1 mg/L was not effective in aquarium trials	*Penaeus azterus*	Johnson *et al*. 1973
Copper sulfate, Tribasic	*Lagenidium callinectes*	MLC to L-1 strain = 159 mg/L MLC to L-3B strain = 151 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al*. 1976
Copper sulfate, Tribasic	*Lagenidium callinectes*	MLD to L-1 strain = 159 mg/L	*Callinectes sapidus*	Ruch and Bland 1973, 1974
Copper sulfate, Tribasic	*Lagenidium callinectes*	MLD to L-3B strain = 151 mg/L	*Penaeus setiferus*	Ruch and Bland 1974
Crystal violet	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 10 mg/L	*Homarus americanus*	Abrahams and Brown 1977
Crystal violet	*Haliphthoros* sp.	Suitable for control	*Homarus* sp. (postlarvae)	Sindermann *et al*. 1977a
Cu^{2+} (Bordeaux mixture)	*Lagenidium* sp.	48-h SR = 4.5 mg/L	*Cancer magister* (larvae)	Armstrong *et al*. 1976
Cuprous chloride	Filamentous bacteria	1 mg/L was effective in experimental conditions	*Penaeus japonicus*	Lightner 1977
Cutrine	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Cutrine Plus	Filamentous gill disease	Effective concentration in 24-h static test = 0.5 mg/L Effective concentration in 24-h flow-through test = 0.1 mg/L	*Penaeus californiensis*	Lightner and Supplee 1976
Cutrine Plus	Filamentous bacteria	0.15 mg/L copper in 24-h flow-through treatments was effective 0.5 mg/L copper in 4- to 6-h static treatments was effective	*Penaeus* sp.	Lightner 1977
Cycloheximide	*Haliphthoros milfordensis*	5.3 cm growth diameter *in vitro* at 50 mg/L	*Homarus garmarus*	Abrahams and Brown 1977
Dichlone	*Lagenidium* sp.	48-h SR = 0.15 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Dichlone	*Lagenidium callinectes*	MLC to L-1 strain = 11 mg/L MLC to L-3B strain = 21 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al.* 1976
Difolaton	*Lagenidium callinectes*	MLD to L-3B strain = 8 mg/L	*Penaeus setiferus*	Ruch and Bland 1974
Difolaton	*Lagenidium callinectes*	MLD to L-1 strain = 7.3 mg/L	*Callinectes sapidus*	Ruch and Bland 1973, 1974
Difolaton	*Lagenidium callinectes*	MLC to L-1 strain = 7.2 mg/L MLC to L-3B strain = 8.2 mg/L	*Callinectes sapidus*	Bland *et al.* 1976
Dioctyl sodium sulfosuccinate	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Dithane M-45 (See Manzate 200)				
DS 9073	*Lagenidium callinectes*	MLC to L-1 strain = 1.3 mg/L MLC to L-3B strain = 3.2 mg/L	*Penaeus setiferus*	Bland *et al.* 1976
DTAS	*Fusarium* sp.	MIC = 10 or >100 mg/L after 2 days MIC = >100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Dyrene	*Lagenidium callinectes*	MLD to L-3B strain = 20.9 mg/L	*Penaeus setiferus*	Ruch and Bland 1974
Dyrene	*Lagenidium callinectes*	MLD to L-1 strain = 13 mg/L	*Callinectes sapidus*	Ruch and Bland 1973, 1974; Sindermann *et al.* 1977b
Dyrene	*Lagenidium callinectes*	MLC to L-1 strain = 6 mg/L MLC to L-3B strain = 21 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al.* 1974
Erythromycin	*Leucothrix* sp.	Treatments were positive, but with inconsistent results	*Macrobrachium* sp. (larvae)	Sindermann and Le Bitoux 1977
Erythromycin	Bacterial necrosis	0.65 mg/L was effective	*Macrobrachium rosenbergii* (larvae)	Sindermann and Le Bitoux 1977
Erythromycin	Bacterial necrosis	1.3 mg/L were effective	*Penaeus* sp.	Lightner 1977
Ether	Anesthetic	Not effective	*Homarus americanus*	Foley *et al.* 1966

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Ethyl paraben	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974
Folpet	*Lagenidium* sp.	48-h SR = 0.1 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Formalin	Ectocommensal protozoans	20 mg/L in pond were effective	*Penaeus setiferus*	Johnson and Holcomb 1975
Formalin	*Zoothamnium* sp.	25 mg/L were effective in aquarium trials	*Penaeus aztecus*	Johnson *et al.* 1973
Formalin	Ciliates	Weak formalin dips have been tried with some success	*Callinectes sapidus*	Sindermann *et al.* 1977b
Formalin	Cnidarians	250 mg/L for 1 h were effective	Decapod crustaceans	Sandifer *et al.* 1974
Formalin	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Formalin	Suctorian protozoans	200 mg/L for 30 min were effective	*Macrobrachium* sp.	Goodwin and Hanson 1975
Formalin	Ciliates	25 mg/L in 24-h flow-through and 75 mg/L in 6- to 9-h static successful in removing *Zoothamnium* from shrimp gills	*Penaeus californiensis*	Lightner 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Formalin:malachite green	Brown spot disease	20-75 mg/L formalin + 0.5-1.0 mg/L malachite green were effective with 0.45 g Terramycin/kg ration	*Penaeus californiensis*	Lightner 1977
Fradiomycin (See Neomycin sulfate)				
Fungistop	*Haliphthoros milfordensis*	4.5 cm growth diameter *in vitro* at 1:1,000 dilution of commercial preparation	*Homarus gammarus*	Abrahams and Brown 1977
Fungizone (See Amphotericin B)				
Fungusoff (See Sulfamethazine solution)				
Furacin (See Nitrofurazone)				
Furanace	Gaffkaemia	*Aerococcus viridans* was resistant to chemical *in vitro*	*Homarus americanus*	Sindermann *et al.* 1977a
Furanace	Bacterial necrosis	7 mg/L were effective	*Macrobrachium rosenbergii* (larvae)	Sindermann and Le Bitoux 1977
Furanace	Bacterial necrosis	1 mg/L was effective	*Penaeus* sp. (larvae)	Lightner 1977
Furanace	*Leucothrix* sp.	1 mg/L was successful	*Macrobrachium rosenbergii* (larvae)	Sindermann and Le Bitoux 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Furanace	*Leucothrix* sp.	1 mg/L for 1 h was ineffective	*Macrobrachium rosenbergii* (juvenile)	Sindermann and Le Bitoux 1977
Furanace	*Haliphthoros* sp.	Inhibited mycelial growth *in vitro* at 2.5 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Furanace	*Haliphthoros* sp.	Treatment of choice	*Homarus* sp. (postlarvae)	Sindermann *et al.* 1977a
Furanace	Bacteriostat, fungicide	20 mg/L for 20 min and a 2-mg/L constant bath should be effective to susceptible causative agents	*Macrobrachium rosenbergii*	Delves-Broughton 1974
Furanace	Black spot disease	Tested with variable results	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Furanace + Tylosin	*Vibrio* sp.	Each compound at 100 mg active drug/kg food, fed for 14 days was effective	*Penaeus stylirostris*	Lightner 1977
Gallimycin	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Garlic extract	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 1:100 dilution of commercial preparation	*Homarus gammarus*	Abrahams and Brown 1977
Gentian violet	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Gentian violet	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Gliseofluvin	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 days MIC = >100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Glisovin FP (See Gliseofluvin)				
4-Hexylresorcinol	*Fusarium* sp.	MIC = 10 or 100 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Hyamine 1622	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 and 7 days 1 and 5 mg/L were not effective on prawns injected with *Fusarium*	*Penaeus japonicus*	Hatai *et al.* 1974
Hyamine 2389	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
3-Hydroxy-5-methyl isoxazole (See Tachigaren)				
Isobutanol	Anesthetic	1.0 μL/10 g at 15°C and 2.0 μL/10 g at 6-8.5°C were effective when injected into the abdominal sinus	*Homarus americanus*	Gilgan and Burns 1976

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Isobutyl alcohol	Anesthetic	1.5-7.0 mL/L for 2-10 min were effective	*Homarus gammarus*	Foley *et al.* 1966
Laurylpyridinium chloride (See Hyamine 2389)				
Magnesium sulfate	Anesthetic	Not effective	*Homarus americanus*	Foley *et al.* 1966
Malachite green	*Haliphthoros milfordensis*	8 mg/L for 2 h every other day were effective Inhibited mycelial growth *in vitro* at 0.25 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Malachite green	Chitinolytic bacteria	20 mg/L for 8 min every other day were effective	*Homarus* sp. (larvae)	Sindermann *et al.* 1977a
Malachite green	*Lagenidium callinectes*	0.006-0.012 mg/L was fungitoxic	*Artemia salina*	Bland *et al.* 1976
Malachite green	*Lagenidium* sp.	5 mg/L for 10 min (eggs) and 5 mg/L for 2 min every other day (larvae) were effective	*Homarus* sp. (eggs and larvae)	Sindermann *et al.* 1977a
Malachite green	Filamentous epiphytes	5 mg/L for 10 min restricted infestation	*Homarus americanus*	Fisher *et al.* 1976a
Malachite green	Microbial epibionts	5 mg/L for 10 min (eggs) and 5 mg/L for 2 min every other day (larvae) were effective	*Homarus* sp. (eggs and larvae)	Sindermann *et al.* 1977a

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Malachite green	Fungus, epibiotic infections	20 mg/L for 8-16 min every other day throughout larval stage produced excellent survival as postlarvae	*Homarus americanus* (larvae)	Fisher *et al.* 1976b
Malachite green	Suctorian protozoans	0.2 mg/L (static) for 30 min daily was effective	*Macrobrachium* sp.	Goodwin and Hanson 1975
Malachite green	Larval mycosis	0.006 mg/L was effective	*Penaeus* sp.	Lightner 1977
Malachite green	Protozoans	0.1 mg/L in ponds was not effective	*Penaeus setiferus*	Johnson and Holcomb 1975
Malachite green	Microbial epibionts	0.001 mg/L increased survival	*Cancer magister* (larvae)	Armstrong *et al.* 1977
Malachite green	*Zoothamnium* sp.	1 mg/L was not effective in aquarium trials	*Penaeus aztecus*	Johnson *et al.* 1973
Malachite green	*Haliphthoros* sp.	Useful in early stages of infection	*Homarus* sp. (postlarvae)	Sindermann *et al.* 1977a
Malachite green	*Lagenidium callinectes*	MLC to L-1 strain = 0.006 mg/L MLC to L-3B strain = 0.01 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al.* 1976
Malachite green	*Lagenidium* sp.	0.002-0.010 mg/L resulted in mixed success	*Penaeus* sp.	Lightner 1977
Malachite green	Microbial epibionts	1 mg/L for 30 min three times weekly was effective	*Cancer magister* (eggs)	Armstrong *et al.* 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Malachite green	*Fusarium* sp.	MIC = 1 mg/L *in vitro* after 2 days and 10 mg/L after 7 days 1 and 0.1 mg/L were not effective on prawns injected with *Fusarium*	*Penaeus japonicus*	Hatai *et al.* 1974
Malachite green	*Lagenidium* sp.	48-h SR = 0.12 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Manzate 200	*Lagenidium callinectes*	MLD to L-3B strain = 3.0 mg/L	*Penaeus setiferus*	Ruch and Bland 1974
Manzate 200	*Lagenidium callinectes*	MLD to L-1 strain = 2.1 mg/L	*Callinectes sapidus*	Ruch and Bland 1973, 1974
Manzate 200	*Haliphthoros milfordensis*	5.4 cm growth diameter *in vitro* at 25 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Manzate 200	*Lagenidium callinectes*	MLC to L-1 strain = 2.1 mg/L MLC to L-3B strain = 2.9 mg/L	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al.* 1976
2-Mercaptobenzothiazole sodium	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Methylene blue	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 10 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Methylene blue	*Haliphthoros* sp.	Suitable for control	*Homarus* sp. (postlarvae)	Sindermann *et al.* 1977a

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Methylene blue	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl 1,4-naphthoquinone (VK_3)	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl paraben	*Fusarium* sp.	MIC = 100 or >100 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Methyl pentynol	Anesthetic	3.0-6.0 mL/L for 5-15 min were effective	*Homarus americanus*	Foley *et al.* 1966
Methyl violet	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 and 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Miconazole nitrate	*Haliphthoros milfordensis*	1.4 cm growth diameter *in vitro* at 50 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Mongare (See DTAS)				
MS-222 (See Tricaine methanesulfonate)				
Mycostatin (See Nystatin)				
Neomycin sulfate	*Fusarium* sp.	MIC = 100 mg/L *in vitro* after 2 days MIC = >100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 2. (cont'd)

Chemical	Target Organisms or Use	Efficacy	Crustacean Species	Reference
Nifuroxime	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 10 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Nitrofurazone	*Leucothrix* sp.	Treatments were positive but with inconsistent results	*Macrobrachium* sp. (larvae)	Sindermann and Le Bitoux 1977
Nitrofurazone	*Vibrio* sp.	1 mg/L was effective on *Vibrio alginolyticus*	*Penaeus setiferus*	Lightner 1977
Nitrofurazone	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Novobiocin	Gaffkaemia	7.5 mg/kg resulted in MTD of 8 days for infected lobsters	*Homarus* sp.	Stewart and Arie 1974
Nystatin	*Fusarium* sp.	MIC = 1 mg/L *in vitro* after 2 days MIC = 1 or 10 mg/L *in vitro* after 7 days 10 and 100γ were not effective when injected into infected prawns	*Penaeus japonicus*	Hatai *et al.* 1974
Nystatin	*Haliphthoros milfordensis*	5.6 cm growth diameter *in vitro* at 50 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Oleandomycin + Terramycin (See Terramycin + oleandomycin)				

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
PCBA	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al*. 1974
PCNB	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al*. 1974
Penicillin G	Gaffkaemia	80,000 IU/kg resulted in MTD of 25 days for infected lobsters	*Homarus* sp.	Stewart and Arie 1974
Penicillin G + polymyxin-B	Antibiotic	40 mg/L Penicillin G + 10 mg/L polymyxin-B into sea water were effective	*Scylla serrata*	Brick 1974
Penicillin + streptomycin	Bacterial necrosis	2 MUI, 2 g/m^3 were effective	*Macrobrachium rosenbergii* (larvae)	Sindermann and Le Bitoux 1977
Penicillin + streptomycin	Gaffkaemia	Reported successful	*Homarus americanus*	Sindermann *et al*. 1977a
Penicillin + streptomycin	Microbial epibionts	Enhanced survival of infested eggs	*Homarus* sp. (eggs)	Sindermann *et al*. 1977a
Penicillin + streptomycin	Antibiotic	Produced no significant survival of larvae	*Cancer anthonyi* (larvae)	Anderson and Ford 1976
Penicillin + streptomycin	Microbial epibionts	1 mg/L of each reduced crab egg mortalities	*Cancer magister* (eggs)	Armstrong *et al*. 1977
Pentagen (See PCNB)				

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Phaltan (See Folpet)				
Pimafucin	*Haliphthoros milfordensis*	2.8 cm growth diameter *in vitro* at 50 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Polymyxin B + Penicillin G (See Penicillin G + Polymyxin B)				
Polymyxin B sulfate	*Fusarium* sp.	MIC = 10 mg/L *in vitro* after 2 days MIC = >100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Polyram	*Lagenidium* sp.	48-h SR = 0.19 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Potassium dichromate	*Epistylis* sp.	Not effective	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Potassium permanganate	Filamentous bacteria	5-10 mg/L for 1 h were effective	*Penaeus* sp.	Lightner 1977
Potassium permanganate	*Zoothamnium* sp.	4 mg/L were not effective in aquarium trials	*Penaeus aztecus*	Johnson *et al.* 1973
Potassium permanganate	*Leucothrix* sp.	2, 5, and 10 mg/L for 1 h and 1 mg/L for 30 min were effective but caused high mortalities	*Macrobrachium rosenbergii* (juvenile)	Sindermann and Le Bitoux 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Propyl paraben	*Fusarium* sp.	MIC = 10 or 100 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Quinacrine hydrochloride	*Epistylis* sp.	0.6 mg/L was effective after 72 h	*Penaeus stylirostris*	Johnson 1976b
Quinaldine	Anesthetic	Not effective	*Homarus americanus*	Foley *et al.* 1966
Quinaldine	Anesthetic	Minimum effective dose = 25 mg/L for 1 h	*Penaeus setiferus*	Johnson 1974b
Quinine bisulfate	*Epistylis* sp.	5 mg/L were effective after 72 h	*Penaeus stylirostris*	Johnson 1976b
Quinine sulfate	*Epistylis* sp.	5 mg/L were effective after 72 h	*Penaeus stylirostris*	Johnson 1976b
Roccal (See Benzalkonium chloride)				
Salicylic acid Na	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974
Sodium benzoate	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974
Sodium bisulfite	*Haliphthoros milfordensis*	7 cm growth diameter *in vitro* at 50 mg/L	*Homarus gammarus*	Abrahams and Brown 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Sorbic acid Na	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days	*Penaeus japonicus*	Hatai *et al.* 1974
Spetol (See Benzalkonium chloride)				
Streptomycin + Penicillin (See Penicillin + Streptomycin)				
Streptomycin sulfate	Filamentous bacteria	10 μg/mL destroyed nonfungal epiphytic population which resulted in a *Lagenidium* injection	*Homarus americanus*	Nilson *et al.* 1975
Sulfa	Bacterial necrosis	3 mg/L were effective	*Penaeus* sp. (larvae)	Lightner 1977
Sulfamethazine solution	*Haliphthoros milfordensis*	2-3 cm growth diameter *in vitro* at 1:1,000 dilution of commercial preparation	*Homarus gammarus*	Abrahams and Brown 1977
Sulfaquinine	*Epistylis* sp.	Effective and consistent, but expensive	*Macrobrachium* sp.	Sindermann and Le Bitoux 1977
Tachigaren	*Fusarium* sp.	MIC = >100 mg/L *in vitro* after 2 days 1, 10, and 100 mg/L were not effective on prawns injected with *Fusarium*	*Penaeus japonicus*	Hatai *et al.* 1974

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Terrachlor	*Lagenidium callinectes*	MLC to L-1 strain = 1.3 mg/L MLC to L-3B strain = 4.3 mg/L 1.3 mg/L was fungitoxic	*Callinectes sapidus* *Penaeus setiferus* *Artemi salina*	Bland *et al.* 1976
Terramycin	*Vibrio alginolyticus*	All shrimp survived fed 5,000 mg/kg and inoculated with *Vibrio*	*Penaeus aztecus*	Corliss *et al.* 1977
Terramycin	*Vibrio* sp.	360 mg/kg/day improved survival injected with *Vibrio* 40 mg/kg biomass were effective	*Penaeus* sp.	Lightner 1977
Terramycin	Gaffkaemia	5 mg/kg injected into infested lobsters resulted in MTD of 9 days	*Homarus* sp.	Stewart and Arie 1974
Terramycin	Brown spot disease	0.45 g Terramycin/kg ration fed for 14 days + formalin:malachite green were effective	*Penaeus californiensis*	Lightner 1977
Terramycin	*Leucothrix* sp.	5, 10, 15, 20, and 30 mg/L in 1-h dips were inconsistent	*Macrobrachium rosenbergii* (juvenile)	Sindermann and Le Bitoux 1977
Terramycin + oleandomycin	*Vibrio* sp.	62.5 μg Terramycin + 25 μg oleandomycin/mL of sea water reduced bacterial populations	*Penaeus* sp. (mysis to 10-day postlarvae)	Lightner 1977
Tetracycline	Bacterial necrosis	1 mg/L was effective	*Penaeus* sp. (larvae)	Lightner 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Thymol	*Haliphthoros milfordensis*	Inhibited mycelial growth *in vitro* at 12.5 mg/L	*Homarus gammarus*	Abrahams and Brown 1977
Treflan (See Trifluralin)				
Tri-buthyltin acetate	*Fusarium* sp.	MIC = 0.1 mg/L *in vitro* after 2 days MIC = 1 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Tricaine methanesulfonate	Anesthetic	Not effective	*Homarus americanus*	Foley *et al.* 1966
2,4,5-Trichlorphenol	*Fusarium* sp.	MIC = 1 or 10 mg/L *in vitro* after 2 days MIC = 10 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Trifluralin	Larval mycosis	0.01 mg/L added continuously was effective	*Penaeus* sp.	Lightner 1977
Trifluralin	*Fusarium* sp.	96-h SR = 117.1 mg/L 48-h SR = 34 mg/L	*Cancer magister* (larvae)	Armstrong *et al.* 1976
Trifluralin	*Lagenidium* sp.	0.005 mg/L retarded contagion	*Cancer magister*	Armstrong *et al.* 1976
Trifluralin	*Lagenidium callinectes*	MLC to L-1 strain = 5 mg/L MLC to L-3B strain = 3 mg/L 3 mg/L was fungitoxic	*Callinectes sapidus* *Penaeus setiferus* *Artemia salina*	Bland *et al.* 1976
Trifluralin	*Lagenidium* sp.	1.5 mg/L yielded mixed success	*Penaeus* sp.	Lightner 1977

(more)

Table 2. (cont'd)

Chemical	Target Organism or Use	Efficacy	Crustacean Species	Reference
Tri-phenyltin acetate	*Lagenidium* sp.	MIC = 10 mg/L *in vitro* after 2 days MIC = 100 mg/L *in vitro* after 7 days	*Penaeus japonicus*	Hatai *et al.* 1974
Tylosin + Furanace (See Furance + tylosin)				
Vancomycin	Gaffkaemia	1 mg/kg + 1.6 X 10^6/kg live pathogen were effective	*Homarus americanus*	Stewart and Zwicker 1974
Vancomycin	Gaffkaemia	25 mg/kg were effective at early stage of infestation	*Homarus americanus*	Sindermann *et al.* 1977a
Vancomycin	Gaffkaemia	25 mg/kg were effective at early stage of infestation	*Homarus* sp.	Stewart and Arie 1974
Vitavax	*Lagenidium callinectes*	MLC to L-1 strain = 38 mg/L MLC to L-3B strain = 38 mg/1	*Callinectes sapidus* *Penaeus setiferus*	Bland *et al.* 1976

[a]MIC = minimum growth impediment concentration; SR = selective ratio; MLD = minimum lethal dose; MLC = minimum lethal concentration;

MTD = mean time to death.

Table 3. Toxicity of therapeutants to crustaceans at 16°C and fish at 12°C in laboratory tests with soft water.

Compound	96-h LC50 and 95% confidence interval (mg/L) for				Safety index for glass shrimp
	Crustaceans		Fish[a]		
Formalin (37% formaldehyde)	*Palaemonetes kadiakensis*	465 368-388	Rainbow trout	118 99.7-140	3.9
			Channel catfish	65.8 58.1-74.5	7.1
			Bluegill	100 80.0-125	4.7
Furanace (Technical grade)	*Palaemonetes kadiakensis*	>20	Rainbow trout	1.00 0.824-1.21	20
			Channel catfish	1.07 0.857-1.34	19
			Bluegill	3.00 2.45-3.68	6.7
Malachite green	*Palaemonetes kadiakensis*	1.90 1.76-2.06	Rainbow trout	0.248 0.193-0.319	7.7
			Channel catfish	0.112 0.0893-0.140	17

(more)

Table 3. (cont'd)

Compound	96-h LC50 and 95% confidence interval (mg/L) for				Safety index for glass shrimp
	Crustaceans		Fish		
Malachite green			Bluegill	0.0305 0.0218-0.0427	12
Potassium permanganate (Reagent grade)	*Palaemonetes kadiakensis*	0.51 0.32-0.81	Rainbow trout	1.80 1.60-2.03	0.28
			Channel catfish	0.750 0.667-0.843	0.68
			Bluegill	2.38 2.05-2.76	0.21

[a] Rainbow trout, *Salmo gairdneri*; channel catfish, *Ictalurus punctatus*; bluegill, *Lepomis macrochirus*.

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CHEMICAL CONTROL OF ZOOTHAMNIUM SP. ON LARVAL MACROBRACHIUM ACANTHURUS[1]

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ABSTRACT

Ten chemicals were tested for their ability to control an outbreak of Zoothamnium sp. on larval Macrobrachium acanthurus. Larvae were exposed for 24-h to each chemical treatment at various concentrations. Copper sulfate, Dylox®, Methylene blue, Terramycin®, Combiotic®, potassium permanganate, Roccal®, nitrofurazone, and malachite green showed little control of Zoothamnium sp. or were toxic to larvae. Formalin at 20 ppm. gave complete control of Zoothamnium sp. and had no ill effects on the larvae.

INTRODUCTION

An infestation of a stalked ciliate on Macrobrachium acanthurus larvae resulted in heavy mortalities during rearing studies at Heart of the Hills Research Station, Kerr County, Texas. The outbreak occurred in a 40-l tank with no filtration. Stocking density was 300 larvae/l. Examination of larvae revealed varying degrees of infestation. The organism was apparently shed with the exoskeleton during molting; however, reinfestation occurred in the closed system. Larval specimens were submitted to the Galveston Lab, National Marine Fisheries Service, Galveston, Texas for diagnosis and the

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[1]This project was funded by the Texas Parks and Wildlife Department and the Governor's Office through the Governor's Public Service Intern Program.

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presence of *Zoothamnium sp*. was confirmed.

Several methods have been used to control *Zoothamnium sp*. infestations. Salinities greater than 20 $^{o}/oo$ inhibit these outbreaks (C. T. Fontaine, NMFS, Galveston, Texas, Pers. Comm. 1974); however, Choudhury (1970) found these salinities toxic to *M. acanthurus* larvae. Copper sulfate (Sick and Beaty 1974) and formalin (Johnson et al. 1973) have been reported as effective controls of this epizootic on several crustaceans. These chemicals and others were tested to determine an effective control of *Zoothamnium sp*. on *M. acanthurus* larvae.

MATERIALS AND METHODS

Ten moderately infested larvae were used with each chemical treatment and ten larvae were maintained as a control. A minimum of two replicates were run for each treatment. These larvae were placed in 0.5-l containers filled with aerated artificial seawater (15 $^{o}/oo$ salinity). Malachite green, copper sulfate, potassium permanganate, Dylox®, Roccal®, methylene blue, Terramycin®, nitrofurazone, Combiotic®, and formalin were added at various concentrations (Table 1). Exposure period was for 24-h. Percent of *Zoothamnium sp*. removed from the larvae was visually estimated, and movement, body color, and percent larval mortality recorded.

RESULTS AND DISCUSSION

No mortalities in controls were observed at the end of the 24-h test period (Table 1). Nitrofurazone had no effect on either *Zoothamnium sp*. or larvae. Copper sulfate, Dylox®, methylene blue, and Combiotic®, showed little control of *Zoothamnium sp*. at the concentrations tested. Although there were

Table 1. Results of chemical treatments for the control of *Zoothamnium sp.* on larval *Macrobrachium acanthurus*

Treatment	Number of Replicates	Concentration (ppm.)	*Zoothamnium sp.* Removed (%)	Larval Mortality (%)	Larval Condition
Control	2	0	0	0	movement poor, color poor
Nitrofurazone	2	4.0	0	0	movement good, color good
Copper sulfate	2	0.5	5-10	0	movement poor, color poor
Dylox®	2	0.1	< 5	0	movement poor, color poor
Dylox®	2	0.25	< 5	0	movement poor, color poor
Methylene Blue	2	1.0	< 5	0	movement poor, color poor
Methylene Blue	2	5.0	< 5	0	movement poor, color poor
Methylene Blue	2	10.0	< 5	0	movement poor, color poor
Combiotic®	2	5.0	< 5	0	movement poor, color poor
Combiotic®	2	10.0	< 5	0	movement poor, color poor
Combiotic®	2	20.0	< 5	0	movement poor, color poor
Roccal®	2	2.0	95-100	40	movement poor
Malachite green	2	0.25	5-10	0	movement good, color good
Malachite green	10	0.5	90-100	10-80	movement and color variable
Terramycin®	2	2.0	< 5	100	precipitate on appendages
Potassium permanganate	2	5.0	< 5	80	percipitate on appendages
Formalin	2	20.0	100	0	movement good, color good
Formalin	2	25.0	100	0	movement good, color good

few larval mortalities using these chemicals, larvae were in poor condition. Sick and Beaty (1974) reported copper sulfate (0.4 ppm. at 6 to 12-h exposure) is an effective treatment of this epizootic on larval *M. rosenbergii*. Roccal® was effective in killing the *Zoothamnium sp.*, but caused 40% larval mortality. The 0.5 ppm. malachite green treatment controlled the organism, but larval mortalities were variable (10-80%). Terramycin® and potassium permanganate caused high larval mortalities. Formalin treatments at 20 and 25 ppm. gave 100% control of *Zoothamnium sp.* and caused no larval mortalities. All these larvae were in good condition at the end of the 24-h test period. Johnson et al. (1973) found 25 ppm. formalin an effective control of *Zoothamnium sp.* on adult penaeid shrimp in ponds. This agrees with our results; however, D. M. Dugger of Aquaprawns Inc., Port Isabel, Texas (Pers. Comm. 1976) noted formalin had some adverse effects (deformities and mortalities) in large scale larval production of *M. rosenbergii*.

Other *Macrobrachium sp.* culture activities at this laboratory have shown larvae reared in aquaria with biological filtration do not experience *Zoothamnium sp.* epizootics. Apparently, good water quality is important in preventing infestations of this organism. However, if *Zoothamnium sp.* outbreaks should occur in aquaria, a 20 ppm. formalin treatment for 24-h will prevent excessive larval mortalities.

ACKNOWLEDGEMENTS

We wish to express appreciation to Dr. D. V. Lightner and C. T. Fontaine of the Galveston Laboratory, National Marine Fisheries Service, for diagnosing the *Zoothamnium sp.* We also thank J. W. Wagner, Fish and Wildlife Technician III, Texas Parks and Wildlife Department, who assisted in making observations.

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CRUSTACEAN HEALTH RESEARCH AT THE GULF COAST RESEARCH LABORATORY*

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Considerable research has been or is being conducted on crustacean health at the Gulf Coast Research Laboratory. In fact, studies of various types and intensity have been carried out in the sections of Parasitology, Microbiology, Physiology, Microscopy, and Fisheries. Some of this work was done cooperatively among sections, and other aspects were conducted individually. Much of the information on diseases and symbionts of shellfishes as well as of finfishes has already been condensed in a booklet by Overstreet (in press).

The Blue Crab

Of all the diseases of the blue crab, we have put the most emphasis on a microsporidosis. That disease could greatly affect the soft-shelled crab industry if people started rearing or maintaining young crabs for the purpose of obtaining mature molting individuals (Overstreet and Cook, 1972). Not depending on an intermediate host, the organism can rapidly reproduce asexually and involve most of a crab's striated musculature. Muscle adjacent to spores becomes lysed, and infected crabs die more readily than noninfected ones when under stress. Overstreet (1975) and Overstreet and Whatley (1975) have found that the drug buquinolate as well as another drug presently under study are both fairly successful in preventing spore development. Other studies are also in progress (by Solangi and Overstreet) which should help determine

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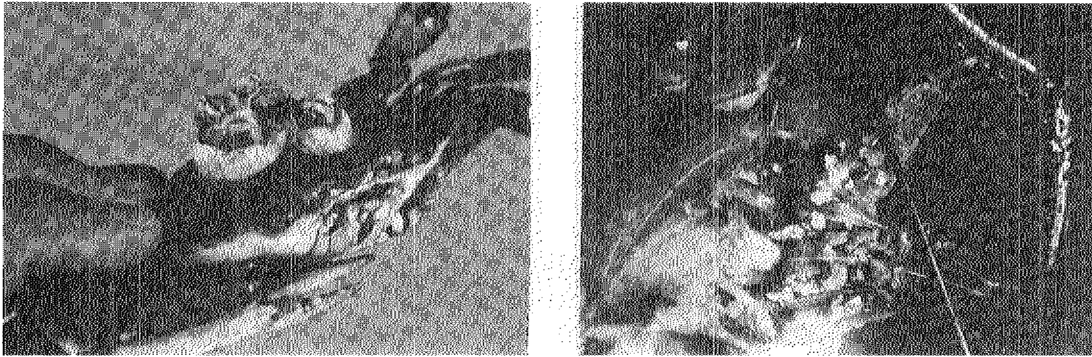
how the drugs affect the organism. Systems can be readily disinfected using either commercial bleach or a disinfectant containing iodine.

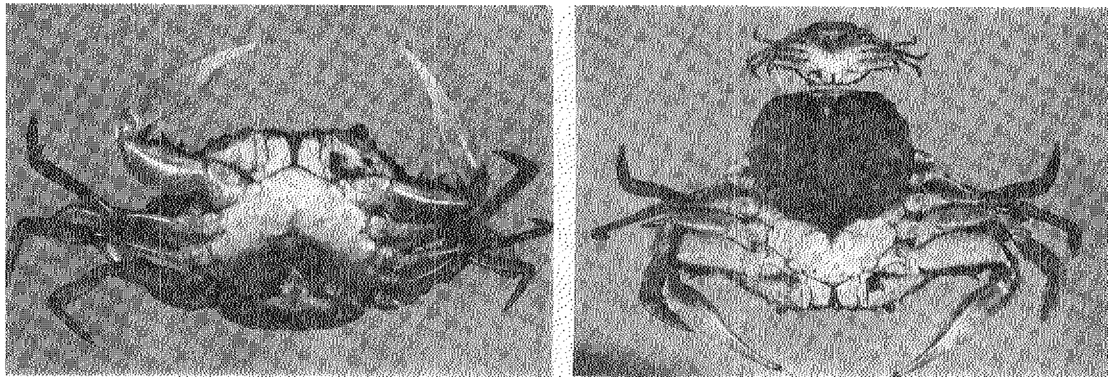
An ultrastructural study of the anatomy and development of an undescribed microsporidan from the blue crab is also in progress. Other protozoan diseases have and are being investigated, but less intensively.

Digenetic trematodes have also received our attention. Overstreet and Perry (1972) described the largest known microphallid (Levinseniella capitanea) from a local blue crab. Richard Heard and a student of his are completing its entire life cycle. Heard (1976) has already established the life cycles of most local microphallids utilizing the blue crab, fiddler crabs, and a variety of other crustaceans. This work is especially important because some of these worms have a potential for infecting man. The common species in the blue crab (Microphallus basodactylophallus) can be detected easily with naked eyes when a urosporidan hyperparasitizes the metacercarial cysts because that protozoan enlarges them and makes them turn dark brown to black. Infected crabs are called "pepper-crabs."

In conjunction with Harriet Perry of the Fisheries Section, we are beginning to examine the importance of the rhizocephalan Loxothylacus texanus in relation to the blue crab fishery. Blue crabs host a variety of barnacles, but only L. texanus truly parasitizes its host. Balanus venustus niveus and Chelonibia patula (Fig. 1) attach externally on the carapace and add considerable weight to crabs from high salinity water that molt infrequently or not at all. The gooseneck barnacle Octolasmis muelleri infests the gills and branchial chamber (Fig. 2), and several hundred individuals presumably greatly influence oxygen consumption by the host. On the other hand L. texanus does not seem to produce excessive sluggishness in its host, but it does inhibit growth and cause



Figures 1 and 2. Barnacles on the blue crab. 1. The acorn barnacle Chelonibia patula, a rather host-specific barnacle, usually infests specific crabs. 2. A moderately heavy infestation of the gooseneck barnacle Octolasmis muelleri on the underside of the gills. Note the lack of individuals on the dorsal surfaces. (Fig. 2 from Overstreet, in press.)



Figures 3 and 4. The rhizocephalan barnacle Loxothylacus texanus in the blue crab. 3. The externa protrudes from under the "mature female" abdomen. 4. The same crab shown in Fig. 3 situated next to an egg-bearing female to exemplify the stunted condition of infected crabs.

castration (Figs. 3 and 4). Lawler and Shepard (in press) compiled a bibliography on all rhizocephalans.

The blue crab hosts a variety of other protozoan and metazoan symbionts. Most of these have little bearing on the unstressed host.

One of these, the leech _Myzobdella lugubris_, has been implicated in causing fatal lesions by Hutton and Sogandares-Bernal (1959), but we have obtained no evidence corroborating their suggestion even though we have observed thousands of specimens on crabs. Sawyer, Lawler, and Overstreet (1975) discussed temperature and salinity tolerances relative to reproduction and growth of the leech. The authors also pointed out that _M. lugubris_ obtains a blood meal from a fish, usually a killifish, flounder, or mullet along the Gulf coast, but more frequently from a white catfish along the Atlantic seaboard. These then attach to but do not feed on the crab. The crab just provides its carapace as a substratum for deposition of cocoons and for dispersion to other localities. Grass shrimp (mostly _Palaemonetes pugio_) and occasionally penaeid shrimp also harbor the leech.

Like the leech, a branchiobdellid annelid (_Cambrincola mesochoreus_) infests the crab when occupying fresh or nearly fresh water. Also like the leech, it does not appear to affect its host, even though some crabbers in Florida attribute summer mortalities to these "mullet bugs."

Several different viruses might occur in the Gulf and harm confined crabs like they do along the northeastern seaboard (Johnson, 1977). Crabs have not been examined with these in mind. Cook and Lofton (1973), however, have investigated chitinoclastic bacteria. They found that on the basis of several bacterial strains from different crustaceans with black spot, or shell disease, one bacterium, _Benekia_ Type I, was common to all. Cook and Lofton have also aided the Parasitology Section by diagonosing or establishing on several occasions the presence or absence of bacteria in diseased crustaceans.

The blue crab occasionally exhibits anomalous conditions, usually deformed claws which often result from injury. On the other hand, Lawler and Van Engel (1973) reported a triple regenerated swimming leg, and Lawler and Shepard (1978) reported a partially albino crab with a white claw that remained white following a molt.

Penaeid Shrimps

Diseases of penaeids have received more of our attention than those of the blue crab. We have investigated brown and white shrimp in ponds at Grand Terre, Louisiana, and elsewhere. When shrimp were densely stocked, the commensal *Zoothamnium* sp. heavily infested the gills and body (Fig. 5). Unless the shrimp are further stressed, such as by low oxygen tension, the ciliate does not seem to cause apparent harm. With low oxygen concentrations, however, an entire infested stock can die leaving lightly infested ones in other ponds alive (Overstreet, 1973).

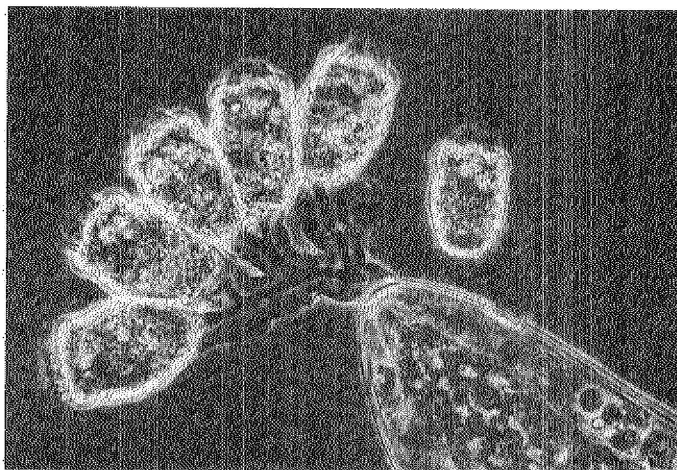


Figure 5. The peritrich ciliate *Zoothamnium* sp. on a penaeid's gill filament. Note the dark connected spasmonemes in the contractile stalk which differentiates it from members of other genera (photo supplied by Carolyn Foster - from Foster, Sarphie, and Hawkins [in press]; by permission of Blackwell Scientific Publications).

Foster and Howse (1978) provided an ultrastructural analysis of the brown shrimp gill, and Foster, Sarphie, and Hawkins (in press) showed that the ciliate's attachment provokes no appreciable pathological host-response. This is in direct contrast to the relationship between the related Epistylis sp. in the southeastern U.S. and its fish hosts (Overstreet and Howse, 1977).

Other potentially harmful protozoans include microsporidans. Measurements and characteristics of all four common species (Ameson nelsoni, Pleistophora sp., Thelohania duorara, and Agmasoma penaei) have been reported (Overstreet, 1973). All those intracellular species involve muscle cells in a different fashion, and some are more harmful than others. Agmasoma penaei can castrate the majority of a white shrimp stock, whereas the other three infect abdominal muscles and turn the shrimps' tails a chalky white color. Consequently, people call infected hosts "cotton shrimp" or "milky shrimp." Infections appear to inhibit normal migration patterns, at least for many individuals. Infected individuals have no commercial value, and infections in young shrimp are poorly understood. Another microsporidan (Inodosporus spraguei from grass shrimp) has been studied ultrastructurally (Fig. 6), and Overstreet and Weidner (1974) suggest that that species, because of its pansporoblast chamber, prevents harmful metabolites and lytic agents from damaging adjacent host tissue as occurs extensively in a blue crab with Ameson michaelis. The spore of Inodosporus spraguei is unusual in that it has external tails. These tails appear to aid in transporting essential materials for growth in developing spores and to allow the mature, free, single spores to attach to debris permitting the proper foraging hosts to have access to them.

Several helminths infect penaeids (Overstreet, 1973). All of the cestodes, digeneans, and nematodes can kill their hosts if the relative size of the worm is great enough or if the worm interferes with vital organs. A larval trypanorhynch cestode (Prochristianella hispida) occurs on, in, and surrounding the hepatopancreas of penaeids. The relatively large blastocyst housing the larva can be seen through a shrimp's carapace when embedded in the hepatopancreas' membrane. A graduate student, Tom Mattis, has not been able to infect shrimp with the infective larva; however, he has obtained infections in some copepods.

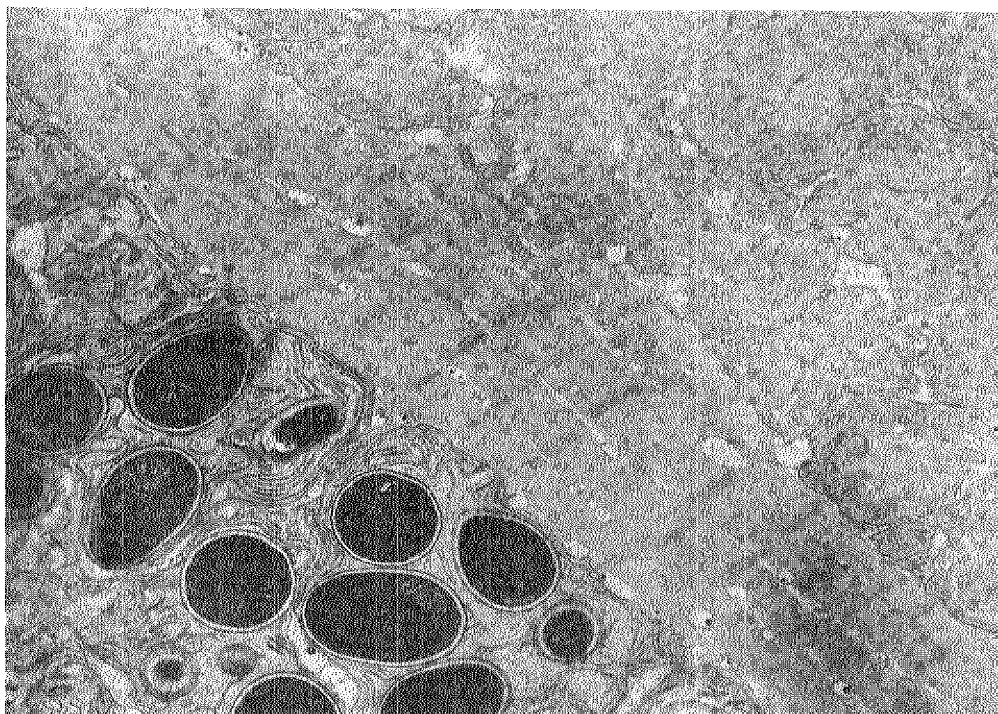


Figure 6. Electron-micrograph of Inodosporus spraguei from abdomen of grass shrimp. Mature spores with internal non-inverted polar filaments and external tubular tails occur in pansporoblasts of eight spores on the upper right hand side of the photo. An early developing stage occurs on the lower left of the photo separated from the mature spores by relatively healthy muscle tissue. (Photo from Overstreet, in press.)

The worm matures in stingrays that feed on penaeids and other infected crustaceans. Mattis' work also exemplifies differences in the ability of hosts to tolerate different cestodes. A small harpacticoid can survive infections of up to twenty procercoids of *P. hispida* for quite some time, whereas the same copepod species usually dies quickly after five coracidia of *Poecilancistrium caryophyllum* invade its hemocoel. The latter species is larger, but it also tends to locate anteriorly in the copepod. Mattis has been able to infect different crustaceans with a variety of cestodes. Heard (1976) has experimentally infected shrimp and other crustaceans with a variety of microphallid metacercariae. Members of this family infect penaeids only rarely in nature; however, most individuals of some crustaceans such as *Palaemonetes pugio* possess many cysts (*Microphallus choanophallus* in the abdominal musculature). Microphallids are probably less likely to cause human health problems than are ascaridoid nematode larvae. The human disease anisakiasis is best known for infections of ascaridoid larvae that normally mature in marine mammals. Such is not always the case. Norris and Overstreet (1976) have already shown that the smaller of two unidentified species of *Thynnascaris* in penaeids quickly penetrates a mouse's gut and survives long enough to evoke a host response. Both authors are continuing with related studies. Thomas Deardorff, a graduate student, is trying to relate larval stages of a few different species of *Thynnascaris* with their adult counterparts. All species of *Thynnascaris* mature in fish. Fusco (1978) completed the life cycle of a nonrelated nematode (*Spirocamallanus cricotus*) using penaeids and other crustaceans. Like *Thynnascaris*, its adult matures in a fish (Fusco and Overstreet, 1978). Once again in summary, members of all three discussed helminth groups

can harm shrimp, especially if capable of infecting confined shrimp in culture operations.

Shrimp as well as crabs and other crustaceans contract black spot disease from chitinoclastic organisms. Because shrimp molt more often than many crustaceans, the disease is seldom problematical. However, shrimp cultured in ponds with excess chitin casts and with optimal temperatures for the causative organisms are vulnerable. Also, shrimp fed brine shrimp seem more prone to infections (see Venkataramiah, Lakshmi, and Gunter, 1975).

A virus reported by Couch (1974) and characterized by Summers (1977) has been implicated in mortalities, especially when interacting with other organisms or specific toxins (Couch, 1978; that current paper also reviews parasites and diseases of penaeids extensively). The virus also occurs in Mississippi (Overstreet and Howse, 1977) and probably elsewhere.

Several other disease conditions occur in penaeids from the northern Gulf of Mexico and have received attention. Two that are especially important to those rearing shrimp or holding them in bait tanks have been called "spontaneous necrosis" and "cramps." The first results from a variety of stresses often involving low oxygen concentrations or rapid environmental changes (Lakshmi, Venkataramiah, and Howse, 1978). If the necrotic shrimp progresses to a threshold stage before unfavorable conditions are improved, the abdominal degeneration can not be reversed and the shrimp dies. On the other hand, the muscle tissue can repair itself if water quality improves early enough. Once the terminal portion of a shrimp turns chalky white, the shrimp usually

dies. Cramping (Fig. 7), unlike necrosis, is associated with an internal ionic imbalance, usually during periods of low temperatures and low salinity levels (Venkataramiah, Lakshmi, Biesiot, Valleau, and Gunter,

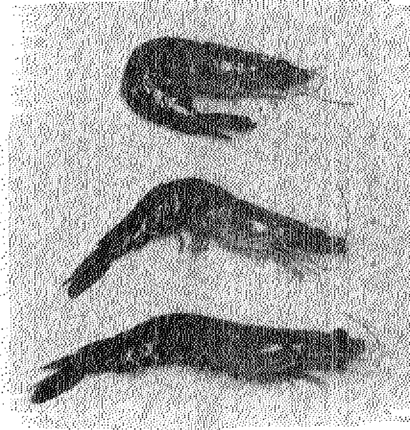


Figure 7. Abdominal cramping of brown shrimp. Top and middle shrimp illustrate completely and partially cramped condition compared with normal bottom shrimp (photo supplied by Biesiot, Lakshmi, and Venkataramiah).

1977; Venkataramiah, personal communication). Those physiologists are presently trying to determine the ion or ions involved in inducing the condition as well as other physiological and nutritional factors that harm penaeids.

Two other rarely observed conditions are "golden shrimp" and tumorous growths. Shrimp affected by the former have a golden coloration which is not lost following molting. These shrimp may become useful in tagging populations. The tumor is not neoplastic, but rather an overgrowth of the sixth abdominal segment (Fig. 8). Overstreet and Van Devender (1978) suggest that these growths, seen in postlarvae only, result from an interaction between developing shrimp and an unidentified toxicant.

Carolyn Foster (personal communication) has been studying phagocytosis in penaeids using the electron microscope. She is trying to determine the functional relationship between fixed and free phagocytes in both the gills and heart using carmine as the injected foreign particulate.

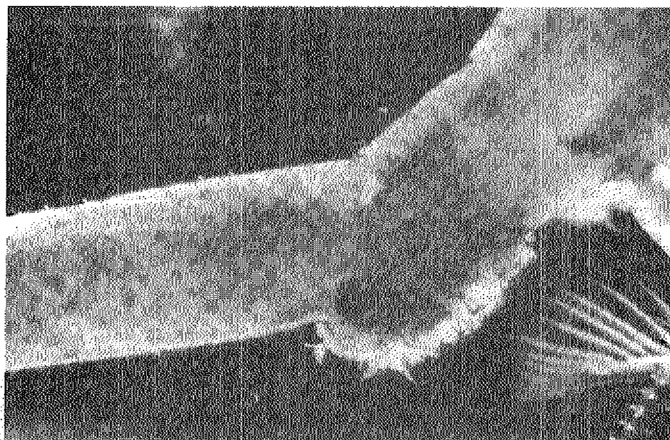


Figure 8. Relatively large, frayed hamartoma of sixth abdominal segment of postlarval brown shrimp (photo from Overstreet and Van Devender, 1978; by permission of Academic Press, Inc.).

Other Crustaceans

Crustaceans other than the blue crab and penaeids have and are being studied as experimental hosts for parasites. These include grass shrimp, mysids, fiddler crabs, amphipods, isopods, and copepods, and they all have been infected or at least an attempt was made to infect them with a variety of digeneans, cestodes, and nematodes by Heard, Mattis, Deardorff, or Overstreet. Infections in amphipods of two acanthocephalans have been described by Buckner, Overstreet, and Heard (1978), and Overstreet (1970) described a relatively large larval digenean on a copepod.

Rearing larval crustaceans may be hampered by fungal and bacterial infections. One of these, Leucothrix mucor, can be devastating (Fig. 9). Solangi, Overstreet, and Gannam (in manuscript) tested the effectiveness of several chemicals in treating brine shrimp infested with the filamentous bacterium. They also studied the bacterium's ultrastructure.

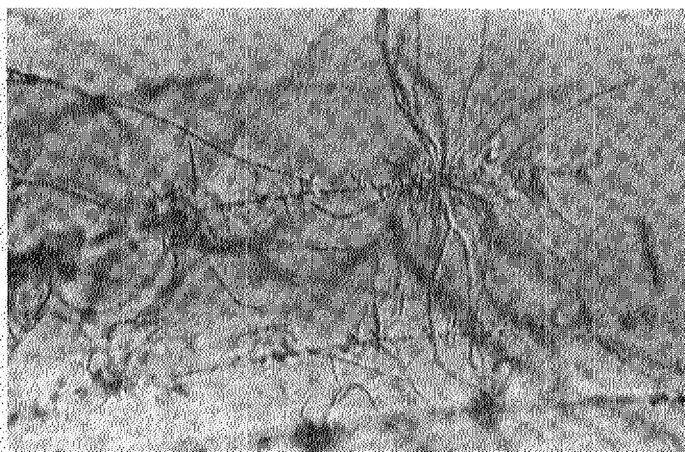


Figure 9. A light initial infestation of the bacterium Leucothrix mucor on a brine shrimp revealing a rosette of filaments.

Bioassays

Several bioassay-toxicity studies utilizing penaeids, the blue crab, and a mysid have been conducted by Walker, Lawler, and Burke. One of these (in press) showed that concentrations as low as 25 ppm Starlicide®, a toxicant used to control starlings and other specific bird pests, killed all tested penaeid shrimp within 8 hr. Crabs showed slightly more tolerance. Results of some of the other studies will also be published.

Acknowledgments

A large number of people are involved with conducting or aiding the studies discussed; many of them are cited and all are appreciated by me or others. For purposes of this overview, I would like to show special appreciation to Ronnie Palmer, Roswitha Buxton, and Ann Miller who are currently aiding me in several of the continuing studies.

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CARCINOGENIC MECHANISMS IN SELECTED ARTHROPODS
METABOLISM OF BENZO(A)PYRENE

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Several mixed-function oxidase activities, including that of benzo(a)pyrene (BP) hydroxylase (AHH) have been measured in aquatic and terrestrial arthropods. The basal AHH level in insects is inducible by environmental pollutants, such as polychlorinated biphenyls; this effect has yet to be shown in crustaceans. The specific activity of arthropod AHH is apparently lower than that of the comparable system in laboratory mice. Arthropod AHH activity, like its mammalian counterpart, is O_2 and NADPH dependent, highest in microsomal preparations, and inhibited by CO. In crustaceans, mixed-function oxidase activity is usually most easily detected in the green gland, hepatopancreas and gut. Since environmental polycyclic aromatic hydrocarbon carcinogens, such as BP, require metabolic activation, the presence of AHH in arthropods suggests that more detailed studies of chemical carcinogenesis in these animals are warranted.

INTRODUCTION

Environmental xenobiotics of many kinds are metabolized by the mixed-function oxidase (MFO) systems of vertebrate and invertebrate animals. Among these compounds is benzo(a)pyrene (BP), a widely distributed pollutant, which can give rise to mutagenic and/or carcinogenic derivatives after MFO-mediated metabolism. These BP metabolites have been shown to bind covalently to various biological macromolecules including DNA, RNA, and proteins; other metabolically produced BP derivatives are not active in this regard. The hydroxylation of polycyclic aromatic hydrocarbons (PAH), using BP as substrate, has been extensively

studied in mammalian systems; however, there have been few similar studies of this enzyme in invertebrates. The question of invertebrate response to chemical carcinogens is generally unresolved. As the initial step in an attempt to develop a model system for the study of invertebrate carcinogenesis, we decided to assay the enzymatic capacity of representative organisms to metabolize BP. The comparatively low reported incidence of neoplasia among invertebrates could be partially explained by an intrinsic inability to metabolically activate important classes of environmental carcinogens.

In this paper some of the characteristics of aryl hydrocarbon hydroxylase (AHH) of arthropods are discussed. The animals to be considered are an insect, the larval form of *Spodoptera eridania*, and aquatic arthropods including a crayfish (*Cambarus* sp.) and a lobster (*Homarus americanus*).

MATERIALS AND METHODS

Sixth instar larvae of *Spodoptera eridania* were decapitated, and the gastrointestinal tracts excised and washed with ice-cold 0.05 M Tris buffer, pH 7.8. Enzyme assays were performed using either midgut homogenates or 100,000 g microsomal preparations. One ml reaction mixtures were prepared in 10 ml Erlenmeyer flasks containing: 0.05 M Tris-HCl buffer (pH 7.8), 5 mM NADPH, 3 mM KCl, 1.6 mg/ml bovine serum albumin, midgut homogenate or microsomes (3-5 mg protein), and 80 nmoles (0.1 μCi) 7,10-[^{14}C]-benzo(a)pyrene. The reactions in control samples (T_0) were immediately stopped by the addition of an equal volume of cold

acetone; experimental samples were incubated for 10 min at $30^{o}C$ in a reciprocating water bath, before the addition of acetone.

Crayfish or lobster hepatopancreas and green glands were homogenized in ice-cold 0.1 M HEPES buffer, pH 7.8. AHH assays were done using either homogenates or microsomes. One ml reaction mixtures contained: 0.1 M HEPES buffer (pH 7.8), 2.5 mM NADPH, 5 mM $MgSO_4$, 1.6 mg/ml bovine serum albumin, homogenate or microsomes (2-4 mg protein) and 80 nmoles (0.1 μCi) ^{14}C-BP. The reactions in the control and experimental samples were stopped with acetone as described above. The reaction vials were incubated at $27^{o}C$ on a shaking water bath.

Radiolabeled BP derivatives were extracted by a method similar to that of Abramson and Hutton (1975). The reaction mixtures were extracted with 2 volumes hexane for 15 min at $30^{o}C$. An aliquot of the hexane layer was further extracted with 1 N NaOH. A portion of the alkali extract was placed in a scintillation vial. The aqueous layer was extracted again with hexane and an aliquot pipetted into a scintillation vial. The protein present in this aliquot was solubilized by overnight incubation with Protosol at room temperature. The radioactivity present in the alkali and aqueous aliquots was quantified using a liquid scintillation spectrometer. The AHH specific activity was expressed as nmoles ^{14}C-BP metabolized per mg protein per unit of time.

RESULTS AND DISCUSSION

The results from our work on insectan AHH will be presented here in condensed form; a more detailed discussion can be found in Anderson, R. S. 1978. Aryl hydrocarbon hydroxylase in an insect *Spodoptera eridania* (Cramer). *Comp. Biochem. Physiol.* C, in press. These data are included in this paper for comparative purposes, since they were obtained from a terrestrial arthropod and are representative of the typical AHH characteristics.

The preferred electron donor for the reaction is NADPH; little or no enzyme activity was observed in the absence of exogenous NADPH. Concentrations >5 mM either failed to enhance activity or were inhibitory. NADH could not substitute for NADPH in promoting insectan AHH activity.

The activity of insectan AHH was temperature dependent. Little activity was seen at 0°C; activity increased to maximal at 30°C but was not detectable at 56°C. The pH optimum was in the range pH 7.6 - 8.0. Oxygen dependency was shown by measuring N_2 inhibition of this reaction, which averaged about 40%. The similarities between the conditions required for maximal activity of insect AHH and other MFO activities were marked. Aldrin epoxidation also required NADPH and O_2, had a pH optimum of 7.8, and was most active at 30°C (Krieger and Wilkinson, 1969). *Spodoptera* AHH was effectively inhibited by the presence of carbon monoxide, indicating cytochrome involvement. Ray (1967), Gil *et al*. (1974), and other investigators have measured cytochrome P450 in insect preparations.

Studies of the time course of BP metabolism by Spodoptera showed that the rate was linear for approximately the first 15 min of incubation. The rate of reaction was linear with enzyme concentration to about 1.5 mg/ml. The experimental length of incubation and enzyme concentration were selected to insure that the data fell on the linear portion of the curve to permit comparison of the rates of reaction between individual experimental groups of animals.

The activity of AHH in Spodoptera is most concentrated in the midgut region of the gastrointestinal tract. Low levels of activity were measured in the crop, anterior intestine, rectum and Malpighian tubules; no activity was present in the head capsule or integument. In fact, the addition of aqueous homogenates of head capsules to midgut homogenates or microsomes caused partial inhibition of enzyme activity. This effect was probably mediated by the presence of natural eye pigments such as xanthommatin, which has been shown to inhibit insect MFO (Schonbrod and Terriere, 1971). Early in these studies it was shown that midgut preparations had little AHH activity if they were not thoroughly washed. We prepared a buffered gut-contents homogenate which could abolish more than 98% of normal enzyme activity. Similar inhibitory proteolytic enzymes had been previously described in the gut contents of insects by Kreiger and Wilkinson (1970), Brattsten and Wilkinson (1973), and Kreiger and Lee (1973). The presence of endogenous AHH inhibitors emphasizes the necessity to work with clean, homogeneous

tissue samples and explains why it may be difficult to measure MFO activities in homogenates of whole organisms.

When subcellular organelles were separated from Spodoptera midgut homogenate by centrifugation, it was apparent that AHH specific activity was highest in the 100,000 g microsomal fraction. This association of enzyme activity with the endoplasmic reticulum is typical of MFO activity in all animal species studied. The fine structure of insectan microsomes compared favorably with that of mammalian liver microsomes (Cassidy *et al*., 1969).

We have also measured the effect of polychlorinated biphenyls (PCBs) on Spodoptera AHH specific activity (Anderson, R. S. 1978, Aryl hydrocarbon hydroxylase induction in an insect, *Spodoptera eridania* (Cramer), by polychlorinated biphenyls (PCBs). *Comp. Biochem. Physiol.* C, in press). These ubiquitous and stable environmental pollutants are potent inducers of MFO in many mammalian and invertebrate species. Since MFO mediates many important chemical transformations including metabolism of drugs, insecticides, steroid hormones and chemical carcinogens, agents which alter their activity can have profound effects on host organisms. In these studies, AHH specific activity was measured at various times after topical administration of certain commercial PCB mixtures including several Aroclors, Capacitor, and microscope immersion oils.

Exposure of the integument to any type of PCB tested caused marked AHH induction, usually ranging from 8- to 15-fold. PCB induction did not significantly alter the relative proportion

of alkali- and water-soluble BP metabolites recovered. Maximum induction was measured 24 hr after a single skin application of PCB; however, induction was transitory in duration. This suggested that detoxifying enzymes were rapidly induced after exposure to environmental pollutants, but the effect was reversible when the animal was no longer exposed to the noxious agent. In *Spodoptera* the AHH response was dose dependent, with the maximal induction following application of 1-2 mg PCB to each sixth instar larva.

It has been suggested that the MFO inductive capacity of PCBs was directly proportional to their chlorine content. For example, Rhee and Plapp (1973) reported that aldrin epoxidase induction in houseflies was proportional to Aroclor chlorine content. Using PCBs of various chlorine contents, we were unable to measure such a consistent relationship. Commercial PCB preparations are impure mixtures of various isomers and congeners. In fact, some of their AHH inductive activities may be caused by impurities, such as chlorinated dibenzofurans (Bowes et al., 1975). Pure PCB isomers can be categorized into two types of inducers; commercial PCB mixtures commonly have both types present (Goldstein et al., 1977). Biphenyls chlorinated symmetrically in both *meta* and *para* positions are good AHH inducers, whereas those chlorinated in the *para* and *ortho* positions are weak AHH inducers. Therefore, pharmacological properties of PCB mixtures are probably determined more by their isomeric composition than solely on the basis of their degree of chlorination.

The data indicate that AHH activity can be measured in insects and that the basic properties of the insectan enzyme are similar to MFO from other species, including mammals. However, from the point of view of chemical carcinogenesis, it is important to identify the particular metabolites produced. The expression of chemically induced neoplasia probably depends on the balance between detoxification and/or activation reactions, as well as target tissue sensitivity. Recently, a technique for efficient separation of BP metabolites using high-pressure liquid chromatography (HPLC) has been developed by Selkirk et al. (1974). We have begun to use this method to separate and identify ^{14}C-BP metabolites produced by insectan AHH. The identity of certain metabolites generated by Spodoptera microsomes can be tentatively made, based on published retention times and Rfs. Several epoxides were produced and converted to dihydrodiols by epoxide hydrase; these were tentatively identified as the 9,10-diol, the 4,5- and/or 7,8-diol. The 1,6- or 3,6-quinone, 3-OH BP, and an unidentified monohydroxylated derivative were also detected. Apparently, the quantitatively major BP metabolite was 3-OH BP, as is the case for mammalian systems. Exact identification of these metabolites awaits coelution studies with known standards. The BP quinones and 3-OH BP are not particularly active mutagens. However, BP-4,5-epoxide is mutagenic for both mammalian cells (Chinese hamster V79) and bacterial cells (Salmonella typhimurium), as reported by Wislocki et al. (1976a). The 7,8-dihydrodiol is the precursor of the corresponding BP-7,8-diol-

9,10-epoxide, which is the putative ultimate carcinogenic BP derivative (Wislocki et al., 1976b). Certain monohydroxylated BP metabolites (6-OH and 12-OH BP) have been shown to be weak mutagens; as mentioned previously, an as yet unidentified monohydroxylated BP derivative is produced by Spodoptera. The probable generation of mutagenic carcinogen metabolites by insectan AHH indicates the need for further studies of the interaction of PAH carcinogens with invertebrate species.

On the basis of the preliminary data reported below, it would appear that crustacean AHH share many of the characteristics already described for the analogous insectan enzymes. Unless otherwise noted, the data presented below were obtained from the crayfish Cambarus.

In crayfish hepatopancreas, AHH activity was consistently measured. Approximately equal quantities of alkali- and water-soluble BP metabolites were produced. The enzyme was shown to be heat labile; no activity could be measured in homogenate after incubation for 30 min at 56°C. The optimal incubation temperature for the reaction was about 30°C; little activity was seen at 0°C. Requirements for O_2 and NADPH were demonstrated for crayfish AHH. Treatment of homogenates or microsomes with CO produced an average inhibition of 54.7%, suggesting cytochrome involvement. Enzyme specific activity of microsomes was 7- to 10-fold higher than that of the corresponding homogenates. AHH activity was also measured in Cambarus green gland homogenates. On the other hand, we could not detect AHH in Homarus hepatopancreas homogenates; green gland homogenates also had little

activity. Only minimal activity was measured when microsomes were used as an *in vitro* MFO source. The difficulties experienced in detecting MFO activity in lobster preparations might be due to contamination with digestive juice. Other workers report that lobster digestive juice is a potent *in vitro* inhibitor of MFO activity in preparations from other species (Pohl et al., 1974).

The AHH specific activities in crayfish hepatopancreas, insect midgut, and mouse (♂ C57BL/6) liver homogenates are 0.300±0.105 (n = 10), 0.398±0.088 (n = 15), and 2.370±0.333 nmoles BP metabolized/mg protein/10 min. In the case of each species, approximately equal amounts of alkali- and water-soluble products are formed. The above data were all obtained in this laboratory using the same extraction procedure; the reactions were carried out under conditions appropriate for each species. It is apparent that baseline AHH levels were consistently found in these arthropods. We have shown that the enzyme was readily inducible in insects by PCBs, but have not as yet studied the effect in crustaceans. Payne (1977) reported that AHH activity is not induced in various crustaceans exposed to emulsified petroleum.

Recently, other investigators have studied AHH, and other MFO activities, in certain crustacean species. Crayfish MFO has been shown to require NADPH and O_2, to be inhibited by CO, and to be associated with microsomes (Khan et al., 1972). The authors report good activity in the hepatopancreas, green gland, and gut. Green gland MFO was active in the epoxidation

of cyclodienes, O-demethylation of methoxychlor and hydroxylation of photoisodrin and BP. Several authors have reported the absence of various MFO activities in lobster hepatopancrea, such as analine hydroxylase, d-benzphetamine demethylase, 7-ethoxycoumarin deethylase and BP dehydroxylase (Pohl et al., 1974), and the failure of lobster microsomes to convert parathion to paraoxon or p-nitrophenol (Carlson, 1973; Elmamlouk and Gessner, 1976). However, Payne (1977) has reported basal level AHH in the lobster. Even though lobsters are reported to be deficient in many MFO activities, significant levels of cytochrome P450 have been measured (Elmamlouk et al., 1974; Bend et al., 1977). In addition, both lobsters and spiny lobsters have high epoxide hydrase activity (Bend et al., 1977). Epoxide hydrase usually functions in the further metabolism of epoxides produced by cytochrome P450-dependent MFO-mediated reactions. The rock crab Cancer borealis was reported to lack MFO activity (Pohl et al., 1974); Cancer irroratus lacks natural or hydrocarbon-inducible nitroanisole demethylase and aniline hydroxylase but has basal levels of AHH and imipramine demethylase (Payne, 1977). Burns (1976) measured aldrin epoxidase in the green gland of the fiddler crab Uca pugnax. The blue crab Callinectes sapidus had highest AHH activity in the green gland (particularly in mature females) and strong activity in the pyloric stomach of both sexes; the hepatopancreas was not a particularly good AHH source (Singer and Lee, 1977).

The evidence suggests that at least some MFO activities can be measured in both aquatic and terrestrial arthropods. Probably

this important detoxification pathway has persisted throughout much of metazoan evolution. There is good reason to think that the system (at least in insects) is inducible by xenobiotic substances, permitting a protective response to noxious environmental materials. The ability of many arthropods to metabolize BP suggests that further studies are required to determine if these invertebrates are subject to the tumorigenic effects of vertebrate carcinogens.

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NITROGEN TOXICITY TO CRUSTACEA AND ASPECTS OF ITS DYNAMICS IN CULTURE SYSTEMS

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ABSTRACT

Nitrogen enters culture systems primarily as organic compounds that are metabolized to ammonia, nitrite and nitrate by resident culture species and/or bacteria. The toxicity of these inorganic nitrogen compounds to crustacea decreases in the order nitrite $>$ ammonia $>$ nitrate. Toxicity is highly pH-dependent and since literature on pH-toxicity relationships in nitrogen poisoning is very scarce for crustacea and other animal groups, conclusions on whether ammonia or nitrite is of greater toxicity must be considered provisional at this time.

Toxicity is presented in several sets of units, e.g., mg NO_2^- N/ℓ or mg NO_2/ℓ, but molarity seems best for comparing ammonia to nitrite to nitrate within and between animal groups. This is because concentrations are expressed on the number of molecules per unit volume and not as some fraction of their formula weight or weight normalized to a particular element.

Nitrite is the most toxic of the three compounds to crustacea with effective concentrations of about 0.5 mM NO_2/ℓ. Ammonia adversely affects shrimp, lobsters and caldocerans at about 1.1 mM ammonia/ℓ and nitrate, the least toxic, as low as 12.5 mM NO_3/ℓ. Comparing the toxicity of ammonia among several aquatic animal groups, sensitivity increases from molluscs to crustaceans to fish. There is too little data to formulate such comparisons for nitrite and nitrate.

INTRODUCTION

Culture of crustaceans on a small scale for a variety of laboratory research purposes has been done for several decades, and now the financial lure of commercial-size operations is attracting extensive interest world-wide in crustacean culture. While measurable success has been achieved in the aquatic husbandry of fish and molluscan species, sustained and profitable cultivation of crustaceans has not been realized, but to this end a vast research effort in academic and private organizations is currently underway.

The literature is replete with articles on important topics to be considered in the culture of any aquatic species including crustaceans. Criteria in selecting a species amenable to culture include reproductive biology, sensitivity and duration of larval stages, tolerance to crowding, water chemistry requirements, food preferences, and marketability (Bardach et al., 1972; Weber and Riordan, 1976; Kinne, 1977). Designs of systems for rearing larval and adult stages of crustaceans on large and small scales are numerous and have been discussed by Ebert et al. (1974), Mock (1974), Rice and Williamson (1970), and Schuur et al. (1976). Disease has a profound effect on the success of culture programs and often the resident crustacean species seem to be no more than a nutrient media for a myriad of lethal pathogens (see Sindermann et al., 1977, for reviews). As crustacean culture has expanded from laboratory husbandry of a few hundred animals to enormous commercial operations of many acres, the need for cheap, artificial food of proper nutritive composition has become a foremost research

topic as discussed by New (1976), Colvin and Brand (1977), Kinne (1977), and Conklin et al. (1978).

Water quality is often given consideration in senarios of crustacean culture, particularly in terms of nitrogen compounds, but is a topic about which little is known for this class of animals. Most literature on the toxicity of inorganic nitrogen, specifically ammonia and nitrite, to aquatic species comes from work with fish (for reviews read Spotte, 1970; Colt et al., 1975; Hampson, 1976; Kinne, 1976), and generalization on "safe" concentrations for all aquatic species stem from this work (Spotte, 1970).

The purposes of this paper are to familiarize readers with several topics relevant to nitrogen toxicity in culture systems and direct them to useful literature, to summarize existing information on ammonia, nitrite and nitrate toxicity to crustacea for both lethal and sub-lethal concentrations, compare crustacean sensitivity to these compounds with that of other animal groups, and draw conclusions on the potential importance of these compounds in culture situations.

SOURCES OF NITROGEN IN CULTURE SYSTEMS

Water of most aquaculture facilities receives nitrogen primarily from organic sources that are processed in two ways within the system. First, the proteins of foods such as frozen adult *Artemia*, fish and artificial chows (all high in protein) are converted to amino acids and then to ammonia and keto acids by heterotrophic bacteria in the oxidative process of *ammonification* (Fig. 1) (Stanier et al., 1970). Of course resident culture species themselves add to this organic pool as lethal factors such as cannabalism and

disease cause the mortality of a portion of the population. Second, organic nitrogen ingested and assimilated by cultured animals is catabolized as the basis of energy production, with ammonia released from amino acids which are transaminated and deaminated to furnish carbon units to the tricarboxylic acid cycle. Ammonotelism describes a mode of nitrogen excretion common among marine and freshwater animals wherein the ammonia of deamination is the principle form of nitrogen excreted into the medium (Fig. 1).

Thus, the most important form of inorganic nitrogen entering a culture system is ammonia. This toxic molecule is utilized as an energy source in the process of nitrification by autotrophic, aerobic bacteria, primarily of the genus *Nitrosomonas*, (Stanier et al., 1970; Wetzel, 1975) which oxidize NH_3 to NO_2^- nitrite (Fig. 1). Further oxidation of NO_2^- by *Nitrobacter* sp. results in NO_3^-, nitrate, with respective ΔG values for these exothermic reactions being -65 and -18 Kcal/mole (Fig. 1). Nitrate is the most oxidized form of nitrogen in the culture system and is readily assimilated by associated algae, diatoms, and higher plants.

Although not a common occurrence, culture systems using biological filtration with poor aeration might have denitrifying processes operative. In this situation (e.g., in an anerobic layer of a deep biological filter) anerobic bacteria use NO_3^- and NO_2^- for terminal H^+ acceptor as organic compounds are oxidized. Nitrate and nitrite may be reduced to nitrous oxide and nitrogen gas and thereby lost from the system.

Bacterial conversion of inorganic nitrogen from ammonia to nitrate in oxidative reactions is the essence of biological filtration widely used in crustacean aquaculture systems (Hirayama, 1974;

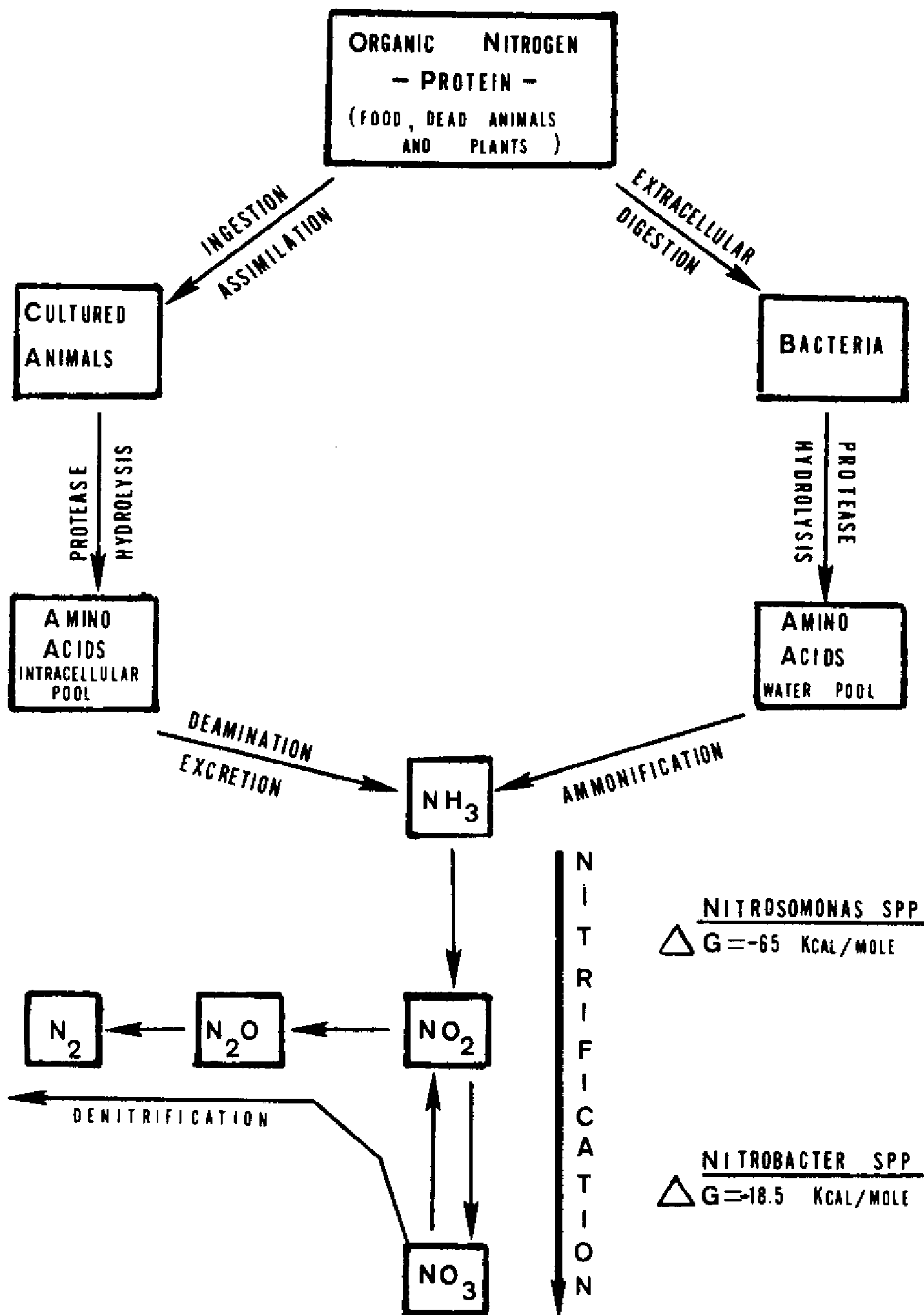

Figure 1. Principal routes of nitrogen metabolism in a closed-circulation culture system. Nitrification is represented as the main process involving inorganic nitrogen, and denitrification as a very minor pathway in well oxygenated systems.

Schuur et al., 1976). In an interesting paper Collins et al. (1975) studied the change in bacterial populations and water chemistry in catfish culture systems using new, unconditioned biological filters. After an initial increase due to excretion, the ammonia concentrations declined as NO_2^- levels rose, followed then by increases in NO_3^-; they concluded that establishment of nitrification required 33 days, although Hirayama (1974) warns this process may take 50 days.

pH AND NITROGEN SPECIATION

An important relationship exists between the various forms of inorganic nitrogen and chemical-physical parameters of the water that influence the speciation of the compound. Ammonia may exist as NH_3 or NH_4^+ ammonium ion, while nitrate might be NO_2^- or HNO_2, nitrous acid. Whether a molecule is ionized or not can be the most important factor in determining its toxicity to an organism. Generally, the un-ionized, non-polar form of ammonia is considered most toxic as it should most easily traverse biological membranes (Forester and Goldstein, 1969). Indeed, experiments with fish (Wuhrmann and Woker, 1949; Downing and Merkens, 1955) and shrimp (Armstrong et al., 1978) indicate that NH_3 is toxic in much lower proportions than NH_4^+. It has been suggested by Colt (1974) and Colt and Tchobanoglous (1976) that NHO_2 rather than NO_2^- may be the most toxic form of nitrite, but no research has been done to test this hypothesis.

The most crucial factor determining the proportion of a molecule in its ionized or un-ionized form is pH (Warren, 1962). The chemistry of ammonia and nitrite equilibria relative to pH has been discussed by Whitfield (1974), Colt and Tchobanoglous (1976), and Armstrong

et al. (1978). Tables for determining NH_3:NH_4^+ proportions have been prepared by Trussel (1972), Skarheim (1973), and Emerson et al. (1975). As pH rises, increasing proportions of ammonia exist as NH_3 simply because less hydrogen ion is available to protonate $NH_3 \rightarrow NH_4^+$. An increase of 1 pH unit (e.g., pH 7-8) raises the NH_3 proportion 10-fold (Emerson et al., 1975). Consequently, the toxicity of ammonia should increase at higher pH values since the easily diffusable NH_3 concentration increases. The pKa of ammonia (dependent on salinity and temperature; see Whitfield, 1974; Armstrong et al., 1978) is about 9.3 and, therefore, at a pH of 7.6 almost 98% of the total ammonia present is NH_4^+.

The relationship of un-ionized nitrous acid and hydrogen ion concentration lies in the opposite direction on the pH scale. As pH lowers, more H^+ is available to protonate $NO_2^- + H^+ \rightarrow HNO_2$ which, like NH_3, may be the more toxic form of nitrite (Colt and Tchobanoglous, 1976). The pKa of nitrite is about 3.3 which means that at pH = 7.6 only about 0.004% exists as HNO2 (Colt, 1974).

MODES OF NITROGEN TOXICITY

The aspects of toxicity to be listed in this section include actual bio-chemical modes of toxicity, generalized symptoms, and pathology whose underlying chemical causes are unknown. Many of the aspects of ammonia and nitrite toxicity are documented from studies with mammals and fish, and whether parallel processes are operative in crustaceans is unknown.

AMMONIA

Several reviews of the mechanisms of ammonia toxicity have been written which are pertinent to aquaculture (Spotte, 1970; Maetz, 1972; Campbell, 1973).

Blood pH: Un-ionized ammonia (NH_3) entering an organism's blood via diffusion from ambient waters or metabolic production, encounters a fluid pH of about 7.6 which causes its rapid protonation to NH_4^+. The subsequent elevation of blood (and perhaps intracellular) pH can have a pronounced effect on enzyme functions such as rates of reactions (Hochachka and Somero, 1973).

Additionally, pH seems to affect membrane stability through structural alterations in lipid components. Bell et al. (1971), Nakamaru and Schwartz (1970), reported an abnormal release of Ca^{+2} by sarcoplasmic reticulum of muscle at high pH. Indirect evidence of ammonia effect on membrane stability, perhaps via a significant alteration in pH, was provided by Lloyd and Orr (1969). They found that trout exposed to various levels of ambient ammonia had greatly increased rates of urine production, and this diuretic response was attributed to increased permeability of the fish, probably by a structural change in membrane components.

Na Transport: A great deal of literature has discussed the physiological relationship between Na^+ uptake and utilization of blood NH_4^+ instead of K^+ as a counter-ion in an exchange process for fish (Maetz and Garcia-Romeu, 1964; Maetz, 1974; Carrier and Evans, 1976; Maetz et al., 1976), crustacea (Shaw, 1960; Mangum et al., 1976; Mangum and Towle, 1977; Armstrong et al., 1978), and amphibia (Guggenheim et al., 1971). Carrier and Evans (1976) found that exposure of fish to 10 mM NH_4^+/ℓ reduced Na^+ influx by 38 to 80%, while Maetz and Garcia-Romeu (1964) reported that injection of NH_4^+ into goldfish caused a marked rise in the rate of Na influx. The crayfish Astacus pallipes experiences a 70-95% reduction in Na^+ influx when 1 mM NH_4^+/ℓ is added to water (Shaw, 1960).

Armstrong et al. (1978) postulated that one mode of ammonia toxicity responsible for death of shrimp larvae at lower pH (6.8) was inhibition of normal Na^+ influx rates due to high ambient NH_4^+ concentrations.

The presence of ammonia, especially as an ion, can, therefore, have toxic effects on aquatic animals by impairing normal osmoregulatory processes as Na uptake is disturbed.

Effects of Ambient Ammonia on Ammonia Excretion: Loss of metabolic ammonia from aquatic animals occurs by three principal routes: 1) diffusion of NH_3 from blood to water since water levels are usually much lower, 2) exchange transport with Na^+ ion as previously discussed, and 3) conversion to a non-toxic compound like urea which is subsequently excreted. Several studies have shown that increasing concentrations of ambient ammonia may change the rate of normal ammonia excretion, increase blood levels of this compound, and cause some shift from a typical ammonotelic excretory pattern to a ureotilic one.

Trout exposed to increasing ammonia concentrations of 0 to 8 mg total ammonia/ℓ water had blood levels rise from 40-70 mg/ℓ, with a concomitant decrease in ammonia excretion rate of about 43% (Fromm and Gillette, 1968); however, total nitrogen output did not decrease proportionally. Olson and Fromm (1971) followed this lead to learn if urea excretion increased with ambient ammonia concentrations as a possible detoxification pathway. Urea production in trout did not increase but goldfish excretion of urea increased up to four-fold as water ammonia rose. Interestingly, trout show symptoms of ammonia stress at concentrations eight times less than those affecting goldfish.

In high concentrations of ambient ammonia, the crab, Callinectes sapidus, has about half the normal excretory rate of metabolic ammonia as controls over a wide salinity range (Mangum et al., 1976). Armstrong (unpublished data) has found that ammonia excretion by the shrimp Macrobrachium rosenbergii, is greatly reduced over an ambient concentration range of 0.09 to 2.5 mg ammonia/ℓ.

Thus, the normal rates at which ammonia is flushed from the bodies of aquatic animals can be decreased as levels outside the body increase. If tolerable body levels are exceeded, stress and death may ensue if alternate detoxification pathways are not available. Even so, conversion of ammonia to urea or, more so, uric acid is an energy-dependent reaction and thus diverts quantities of energy from other normal functions.

Pathology: There is almost no information on the histopathological consequences of ammonia exposure and those that exist deal with fish. Both Burrows (1964) and Reichenbach-Klinke (1967) report cell damamge to gill epithelia of salmonid and other species of fish when exposed to ammonia. Gill lamellae were described as being "clubbed" (Burrows, 1964) with hyperplasic and hypertrophic aspects to the damage which were thought to impede gas diffusion across the gill membranes. Such thickening of diffusion routes may partially explain increased respiratory rates of trout exposed to ammonia (Fromm in Fromm and Gillette, 1968).

Generalized Effects of Ammonia Toxicity: A variety of behavioral, metabolic and physiological anomalies in animals exposed to ammonia have been reported and reviewed by Kinne (1976). Such symptoms include reduced growth rates, greater susceptibility to diseases, decrease in the number of blood cells and hyperexcitability.

NITRITE AND NITRATE

Alterations of blood chemistry and circulatory systems are the major consequences of nitrite poisoning and, again, almost all research on this compound with aquatic species has used fish (see Russo et al., 1974).

Nitrite can cause relaxation of smooth muscles of small blood vessels which, in this dialated state, results in a pooling of blood (Gleason et al., 1969). The most notorious result of excessive nitrite uptake is methemoglobinemia. This condition is caused by the oxidation of iron within the hemoglobin molecule from its ferrous to ferric state (i.e., $Fe^{+2} \rightarrow Fe^{+3}$). Such a transformation renders the molecule incapable of binding oxygen which causes hypoxia and death (Bodansky, 1951). It is likely that the same reaction can occur with the iron of crustacean hemocyanin but such a conversion has never been reported for this animal class.

Nitrate can also cause methemoglobinemia, but is not nearly so strong an oxidizing agent as nitrite. In accord with this fact, nitrate has consistently been found less toxic than either ammonia or nitrite, and is the desired final oxidation product of ammonia excretion. Nitrate can be reduced to nitrite by certain gut bacteria and to this extent may be dangerous (Gleason et al., 1969), but such a phenomenon has not been shown for crustacea.

CONCENTRATIONS OF INORGANIC NITROGEN TOXIC TO CRUSTACEA

Literature on inorganic nitrogen toxicity to crustaceans is scarce and much research on the toxicity of ammonia and nitrite, particularly, to this class and other invertebrate groups is still required to understand how factors such as pH and life stage may

alter susceptibility to these toxicants. For instance, no studies have investigated nitrite-pH interactions on survival of aquatic animals or the possible synergistic effects of ammonia, nitrite, and pH in affecting survival, growth or various physiological parameters.

AMMONIA

Concentrations of this compound causing death or adversely affecting crustaceans on a sub-lethal basis are given in Tables 1 and 2. Data are given mainly as total ammonia ($NH_3 + NH_4^+$) since all conventional methods of measuring this compound quantify in these units (Solórzano, 1969, ion-specific electrode, Orion Co.). However, to the extent that ammonia toxicity is primarily caused by the un-ionized NH_3 molecule, some comparative data as NH_3 is also given. The NH_3 proportion of the total ammonia measured can be calculated from the equation:

$$[NH_3] = \frac{\text{total ammonia}}{1 \times 10^{pKa-pH}}$$

The pKa is dependent on salinity and temperature, and its calculation has been discussed by Whitfield (1974), Emerson et al. (1975), and Armstrong et al. (1978); the pKa ≈ 9.30 in freshwater at 23°C and ≈ 9.31 in 12°/₀₀ at 28°C. Because ammonia and nitrite speciation is so dependent on physical-chemical factors, comparisons of data between experiments of different authors can be misleading unless a reader notes variations in the experimental conditions between tests. Even presentation of data as units of NH_3, i.e., based on un-ionized ammonia, may be inadequate as discussed by Armstrong et al. (1978). Sometimes the total ammonia levels used in tests are so great due to lower pH values that the NH_4^+ concentration

is undoubtedly contributing to toxicity (Shaw, 1960; Mangum et al., 1976; Armstrong et al., 1978).

The total ammonia levels reported as toxic in Table 1 are generally high and notably influenced by pH. The larvae of M. rosenbergii are killed by 5.7 times less total ammonia at pH 8.34 than at pH 6.83 (Fig. 2), primarily due to the higher proportion of NH_3 at the higher pH. Delistraty et al. (1977) reported that for larval lobsters, Homarus americanus, equal concentrations of about 76 mg ammonia/ℓ resulted in LT_{50} values (time by which 50% of test animals die) of 8.2 days and 45 minutes at respective pH values of 7.4 and 8.4, at which the NH_3 levels were 0.56 and 5.4 mg/ℓ. Armstrong et al. (1978) estimated an incipient lethal leval (level of an environmental factor beyond which 50% of a population cannot live for an extended period; Sprague, 1969) of 14 mg ammonia/ℓ for larval M. rosenbergii (pH = 8.34), and Delistraty et al. (1977) calculated that 37 mg ammonia/ℓ represented an incipient lethal level for lobster larvae at pH 8.1. Over short-term exposures of about 48 hr, extremely high concentrations of ammonia may be needed to kill crustacea. For instance, 48 hr LC_{50} values were 162 mg ammonia/ℓ for M. rosenbergii (Armstrong et al., 1978, pH = 6.8) 227 mg/ℓ for Penaeus sp. (Wickins, 1976, pH = 7.0), and 70 mg/ℓ for H. americanus (Delistraty et al., 1977, pH = 8.1).

The earliest study of ammonia toxicity to a crustacean was that of Anderson (1944) using the cladoceran Daphnia magna (Table 1). Little information on water conditions is given along with this report, notably missing are pH values. Of the three ammonia compounds tested, NH_4OH had the lowest 16 hr threshold (i.e., a

Table 1. Concentrations of ammonia, nitrite, and nitrate lethal to various crustacea plus pertinent conditions of the experiments.

Species	Life stage	Temp (°C)	Salinity (°/°°)	pH	Toxic Concentrations mg/ℓ NH_3-N[1/] NO_2-N NO_3-N	Toxic Concentrations mg/ℓ TA[2/] NO_2 NO_3	Toxic Concentrations mM/ℓ TA NO_2 NO_3	Toxicity units	Reference
					Ammonia				
Macrobrachium rosenbergii	larvae	27	12	6.8	0.27	80.0	4.7	144 hr LC_{50}	Armstrong et al., 1978
				7.6	0.83	44.0	2.6		
				8.4	1.19	14.0	0.8		
Macrobrachium rosenbergii	juvenile	29.2	3	7.0	2.81	378[3/]	22.2	9.3 hr LT_{50}	Wickins, 1976
					1.43	192	11.3	28.3 hr LT_{50}	
Penaeus sp.	juvenile	28	33	7.0	1.29	24.0[3/]	1.41	48 hr LC_{50}	Wickins, 1976
				8.0	1.29	227.0	13.4		
Daphnia magna	8 hr after release	25	0	--[4/]	--[5/]	23.3	1.37	16 hr[6/] threshold	Anderson, 1944
					--	2.6	0.15		
					--	14.8	0.87		
Homerus americanus	4th stage larvae	22	33.4	8.1	1.40	42.9	2.5	144 hr LC_{50}	Delestraty et al., 1977
					Nitrite				
Macrobrachium rosenbergii	larvae	28	12	8.1	5.0	16.5	0.36	144 hr LC_{50}	Armstrong et al., 1976a
Macrobrachium rosenbergii	juvenile	22.2	3	7.4	204	673	14.6	15 hr LC_{50}	Wickins, 1976
					15.4	51.0	1.1	4 wk LC_{50}	
Penaeus sp.	juvenile	28	30	8.0	170	561	12.2	48 hr LC_{50}	Wickins, 1976
					62	205	4.5	120 hr LC_{50}	
Palaemonetes pugio	larvae	25	25	--	14.5	48	1.04	no survival to metamorphosis	Hinsman, 1977
					7.2	24	0.52	significant decrease in survival	
					Nitrate				
Macrobrachium rosenbergii	juvenile	23	1	7.1	1840	8149	131	35 hr LC_{50}	Wickins, 1976
					160	709	11.4	21 day LC_{50}	Wickins, 1976

Table 1. (con't)

Species	Life stage	Temp (°C)	Salinity (°/₀₀)	pH	Toxic Concentrations mg/ℓ NH_3-N NO_2-N NO_3-N	TA[1/] NO_2 NO_3	mM/ℓ TA NO_2 NO_3	Toxicity units	Reference
Penaeus sp.	juvenile	28	30	8.0	3400	15057	243	48 hr LC_{50}	Wickins, 1976
Palaemonetes pugio	larvae	25	25	--	452	2001	32.3	no affect during development	Hinsman, 1977

1/ NH_3-N = unionized ammonia only.

2/ TA = total ammonia (NH_3 + NH_4^+)

3/ Total ammonia calculated from unionized concentrations

4/ --, data not available

5/ Data in these three rows is for ammonia added as NH_4Cl, NH_4OH, and $(NH_4)_2SO_4$, respectively

6/ Threshold concentration just less than that required to immobilize animals on the bottom of aquaria (Anderson, 1944)

concentration just less than one causing immobilization of animals) of 2.6 mg total ammonia/ℓ or 0.15 mM/ℓ. Such a low level is likely due to a higher pH caused by this basic compound, but still these toxicity values are some of the lowest reported.

Experiments designed to document sub-lethal effects can be used to study growth responses, physiological changes, morphological anomalies, etc. Sub-lethal effects of ammonia to crustacea are given in Table 2. The lowest total ammonia concentration adversely affecting crustacea was 9.4 mg/ℓ, which reduced the growth of Penaeus sp. by 50% during three week tests (Wickins, 1976). Both Wickins (1976) and Armstrong et al. (1978) found that as little as 0.10 mg NH_3/ℓ reduced the growth of juvenile and larval M. rosenbergii (Fig. 3, Table 2). Generally, concentrations of un-ionized ammonia in the range of 0.1 to 0.6 mg NH_3/ℓ have produced various forms of sub-lethal stress in crustacea, while comparable total ammonia levels range from about 9.5 to 180 mg/ℓ depending on pH (Table 2).

NITRITE

The toxicities of this compound to three species of crustacea are listed in Tables 1 and 2. Larvae of M. rosenbergii are killed at a water concentration of 5 mg NO_2^- N/ℓ during a 144 hr exposure, and a time-course change in the sensitivity to nitrite is depicted in Fig. 4 (Armstrong et al., 1976a). A decline in LC_{50} values is most abrupt in the first 72 hr, and such values reach an asymptotic minimum, indicative of incipient lethal conditions, at about 96 hr (Fig. 4). Other toxic concentrations of this compound range from a 5 day LT_{50} of 62 mg NO_2^- N/ℓ for juvenile penaeids (Wickins, 1976) to 7.2 mg/ℓ (significant reduction in survival metamorphosis) for larvae of Palaemonetes pugio (Hinsman, 1977).

Although admittedly difficult to compare toxicity information from different studies, it is interesting to note the differences in susceptibility of various life stages of the same species to a toxicant. Wickins (1976) calculated a four week LC_{50} value of 15.4 mg NO_2^- N/ℓ for juvenile M. rosenbergii (water pH = 7.4), while Armstrong et al. (1976a) found that 5 mg/ℓ killed half of larval test populations in six days (ph = 8.1). The greater sensitivity of the larvae is even more dramatic if the hypothesis that nitrite toxicity increases at lower pH (as $NO_2^- + H^+ \rightarrow HNO_2$) is true (Colt and Tchobanoglous, 1976), since the pH of water in Wickins' test was 0.7 units lower than that of Armstrong et al. (1976a).

Increased sensitivy of larval stages to toxicants is a common finding. Armstrong et al. (1976b) found that larvae of the Dungeness crab, Cancer magister, were about 8 and 80 times more sensitive to the insecticide methoxychlor than juvenile and adult stages, respectively, during chronic exposures. Larvae of M. rosenbergii are killed by three times less nitrite in one-fourth the time than are juveniles. This disparity between larval and juvenile sensitivity to nitrite is greater when sub-lethal criteria are used for comparison (Table 2). Growth of larvae over eight days was reduced 35% in 1.8 mg NO_2^- N/ℓ (Armstrong et al., 1976a); a concentration 8.3 times less than the 15 mg/ℓ level that Wickins (1976) reported to affect juveniles during 28 day growth studies.

NITRATE

This compound is the least toxic of the three inorganic nitrogen forms found in culture systems (Tables 1 and 2). Wickins (1976) reported a 35 hr and 21 day LC_{50} of 1840 and 160 mg NO_3^- N/ℓ, respectively,

Table 2. Concentrations of ammonia, nitrite and nitrate producing sub-lethal effects in crustacea.

Species	Life stage	Temp (°C)	Salinity (°/₀₀)	pH	Toxic concentrations mg/ℓ NH_3-N / NO_2^--N / NO_3^--N	mg/ℓ TA / NO_2 / NO_3^-	mM/ℓ TA / NO_2^- / NO_3^-	Toxicity units	Length of test	Reference
					Ammonia					
Macrobrachium rosenbergii	larvae	27	12	6.8	0.11	32	1.9	31% less growth	7 day	Armstrong *et al.*, 1978
				7.6	0.63	32	1.9	21% less growth	7 day	
				8.4	0.98	32	0.6	no effect	7 day	
Macrobrachium rosenbergii	juveniles	29.2	3	7.2	0.10	12.5[1]	0.74	30 % less growth over this range	6 wk	Wickins, 1976
					0.32	40.1	2.36			
Penaeus sp.	juvenile	28	33	8.0	0.45	9.4[1]	0.55	50% less growth	3 wk	Wickins, 1976
Astacus pallipes	adult	12	0	--	--	18.0	1.06	80% reduction in Na influx		Shaw, 1960
Callinectes sapidus	adult	22	5	6.8[2]	0.57	180.0	10.6	50% reduction in ammonia excretion rate	30 min	Mangum, *et al.*, 1976
					Nitrite					
Macrobrachium rosenbergii	larvae	28	12	8.1	1.8	5.9	0.13	35% less growth	8 day	Armstrong *et al.*, 1976a
Macrobrachium rosenbergii	juvenile	22.2	3	7.4	15	49	1.10	no growth inhibition significant mortality	28 day	Wickins, 1976
Penaeus sp.	juvenile	28	30	8.0	6.4	21.0	0.46	50% less growth	34 day	Wickins, 1976
Palaemonetes pugio	larvae	25	25	--	3.1	10.2	0.22	extended development time, more larval stages	--	Hinsman, 1977
					Nitrate					
Macrobrachium rosenbergii	juvenile	as above			175	775	12.5	50% less growth	3-5 wk	Wickins, 1976
Penaeus sp.	juvenile	as above			>200	885	14.3	no growth effect	3-5 wk	Wickins, 1976
Palaemonetes pugio	larvae	as above			450	1993	32.0	extended larvae development	--	Hinsman, 1977

[1] Total ammonia calculated from un-ionized concentrations given, pKa = 9.3

[2] pH value from Mangum and Towle, 1977

for juvenile M. rosenbergii; in the same paper a 48 hr LC_{50} of 3400 mg/ℓ was calculated for penaeids. Interestingly, he found a 50% reduction in Macrobrachium growth over a five week period at 175 mg/ℓ indicating some potential for toxicity in culture systems. Hinsman (1977) found that nitrate concentrations up to 450 mg NO_2^- N/ℓ did not kill larval P. pugio but did extend development times.

MOLARITY AS A COMPARATIVE BASIS FOR NITROGEN TOXICITY

Nearly all toxicity information is reported on a weight per volume basis; (often normalized to a particular element) i.e., milligrams of a compound per liter water or, roughly, parts per million (ppm). Accordingly, information presented in this paper has been given in these units although they are not the best with which to compare ammonia to nitrite to nitrate. For such comparisons the measure of moles or millimoles is more useful because these units base data on the number of toxicant molecules per volume of water needed to cause an effect (death, growth reduction) within the experimental parameters listed. It is the molecule NH_3 or NO_2^- that is changing blood pH or oxidizing hemaglobin iron, not the nitrogen atom alone or some fraction of the compound's weight (e.g., 3 mg NH_3/ℓ). Therefore, the millimole (mM) concentrations of ammonia, nitrite and nitrate are also listed in Tables 1 and 2. Again, the problems of variation in experimental conditions must be considered in comparisons of data, but levels of general toxicity are evident for ammonia, nitrite and nitrate. Armstrong et al. (1978) reported levels of ammonia toxic to larval M. rosenbergii ranging from 11.80 mM total ammonia/ℓ (pH = 6.8, 24 hr) to 0.80

mM/ℓ (pH = 8.4, 144 hr). Nitrite toxicity to this species was even greater as 0.13 mM NO_2^-/ℓ reduced growth by 35% during eight day exposures (Armstrong et al., 1976a). Based on the data compiled in Tables 1 and 2, nitrite is somewhat more toxic to crustaceans than ammonia. The lowest nitrite concentrations causing lethal or sub-lethal effects have an average molarity of about 0.47 mM NO_2^-/ℓ, and such ammonia concentrations average 1.03 mM NH_3/ℓ (from data of Tables 1 and 2).

Nitrate is still comparatively innocuous even in units of molarity, although toxicity values more closely approximate those of ammonia and nitrite than when compared on a weight basis. This is because nitrate (NO_3^-) has a formula weight of 62 while ammonia (NH_3) is 17, a difference of 3.6 fold in comparisons by weight, whereas molarity values tend to equate these compounds in terms of their numbers.

Values of nitrate toxic to crustacea range from highs of 243 mM NO_3^-/ℓ (48 hr LC_{50} for penaeids, Wickins, 1976) and 131 mM/ℓ (35 hr LC_{50} for *Macrobrachium*, ibid) to lows of 12.5 mM/ℓ (50% growth reduction of *Macrobrachium*, ibid) and 11.4 mM/ℓ (21 day LC_{50} for *Macrobrachium*, ibid). The low values are only about 12 times greater than those for ammonia and 24 times those of nitrite (Tables 1 and 2).

COMPARATIVE NITROGEN TOXICITY: FISH, CRUSTACEA, MOLLUSCS

The extremes in toxicity values recorded for a single class of animals are exaggerated even more when comparing the response of widely divergent groups to a chemical. It must be noted again that investigations on the tolerances of aquatic animals, particularly crustaceans and molluscs, to inorganic nitrogen are too few for basing

definitive conclusions. Still, comparative data given in Table 3 show trends in greater or diminished sensitivity to nitrogen compounds that may be used to gauge or predict the relative hardiness of an animal in a culture system.

Exposed to ammonia, fish seen most sensitive followed by crustacea then molluscs with approximately two-fold differences in toxic concentrations between groups (Table 3). Species of snails and bivalves are killed at concentrations of about 7.0 mM total ammonia/ℓ, whereas fish succumb in levels near 1.2 mM/ℓ. The response to nitrite can be highly variable within a group of related animals as evidenced by the 96 hr LC_{50} values for trout and catfish of 0.19 and 0.93 mM NO_2/ℓ, respectively, (Table 3); a 48-fold difference. Here again, molluscs are the least sensitive and fish and crustaceans comparably susceptible. Finally, the little data available on nitrate indicates that catfish are more resistant than oysters, although the methods for determining "death" could easily account for part of the difference between these and other toxic values between phyla.

CONCLUSIONS

It is axiomatic to state that maintenance of animals in culture water of low inorganic nitrogen levels will enhance their general health and contribute to good survival and growth. The problem in crustacean culture has been lack of information on many aspects of water chemistry that affect health, either by direct and massive chemical alterations that cause death, or more subtly by increasing susceptibility to diseases or reducing growth. Crustacean mortalities that cannot be blamed on more obvious causes such as aeration failures, malfunctioning of heating or cooling units, or pathogens may be attributable to ammonia, nitrite, pH or combinations thereof (Wickins, 1976).

Table 3. Comparative toxicity of ammonia, nitrite and nitrate to fish, crustacea and molluscs.

Species	Life stage	Temp (°C)	Salinity (‰)	pH	Toxic concentrations mg/ℓ NH_3-N NO_2^--N NO_3^--N	Toxic concentrations mg/ℓ TA[1/] NO_2 NO_3	Toxic concentrations mM/ℓ TA NO_2 NO_3	Toxicity units	Reference
					Ammonia				
Fish									
catfish	juvenile	26	0	8.7	3.20	14.5	0.85	96 hr LC_{50}	Colt and Tchobanoglous, 1976
trout	older juvenile	10.8	0	8.0	0.40	24.4	1.44	incipient LC_{50}	Ball, 1967
trout	juvenile	20.1	0	8.1	1.75	30.5	1.79	168 hr LC_{50}	Merkens and Downing, 1957
rudd	adult	12.6	0	8.2	0.36	13.5	0.79	96 hr LC_{50}	Ball, 1967
Crustacea									
shrimp	larvae	28	12	8.34	1.40	15	0.88	96 hr LC_{50}	Armstrong *et al.*, 1978
shrimp	juvenile	28	33	7.0	1.29	275.6	16.2	48 hr LC_{50}	Wickins, 1976
				8.0	1.29	29.0	1.7		
lobster	larvae	22	33.4	8.1	2.31	49.0	2.88	96 hr LC_{50}	Delestraty *et al.*, 1977
Molluscs									
oyster	juvenile	20	27	7.9	6.05	158.0	9.3	96 hr TL_m	Epifanio and Srna, 1975
clam	juvenile	20	27	7.9	3.33	87.0	5.1	96 hr TL_m	Epifanio and Srna, 1975
aquatic snail	adult	20	0	--[2/]	--	109.0	6.4	96 hr TL_m	Patrick *et al.*, 1968
aquatic snail	adult	20	0	5.5	0.006	25.0	1.5	20% growth reduction	Thomas *et al.*, 1976
				5.5	0.017	75.0	4.4	40% growth reduction	
				8.3	3.10	25.0	1.5	40% growth reduction	
					Nitrite				
Fish									
trout	juvenile	26	0	7.9	0.27	0.88	0.19	96 hr LC_{50}	Russo *et al.*, 1974
catfish	juvenile	26	0	8.7	13.1	43.0	0.93	96 hr LC_{50}	Colt and Tchobanoglous, 1976
Crustacea									
shrimp	larvae	28	12	8.1	8.5	28.1	0.61	96 hr LC_{50}	Armstrong *et al.*, 1976a
shrimp	juvenile	22.2	3	7.4	15.4	50.8	1.10	28 day LC_{50}	Wickins, 1976
Molluscs									
oyster	juvenile	20	27	7.9	532	1757	38.2	96 hr TL_m	Epifanio and Srna, 1975
clam	juvenile	20	27	7.9	756	2496	54.3	96 hr TL_m	Epifanio and Srna, 1975
					Nitrate				
Fish									
catfish	juvenile	26	0	8.7	1400	6200	100	96 hr LC_{50}	Colt and Tchobanoglous, 1976
Crustacea									
shrimp	juvenile	28	30	8.0	3400	15057	243	48 hr LC_{50}	Wickins, 1976
shrimp	juvenile	23	1	7.1	160	709	11.4	21 day LC_{50}	Wickins, 1976
Mollusc									
oyster	juvenile	20	27	7.9	275	1222	19.71	96 hr TL_m	Epifanio and Srna, 1975

[1/] TA = total ammonia (NH_3 + NH_4^+)

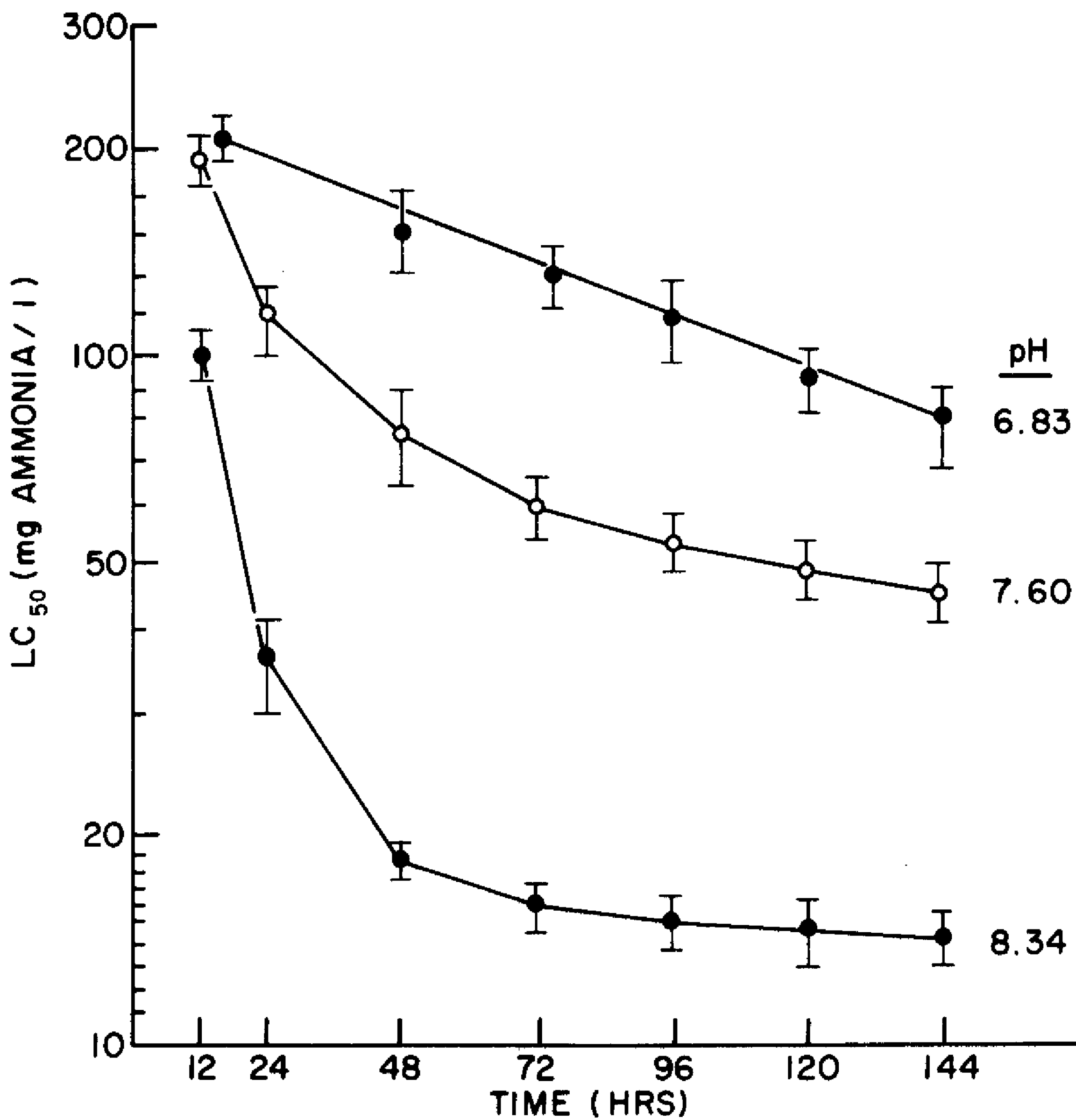

Figure 2. Ammonia toxicity to larvae of *M. rosenbergii* in waters of three different pH. Units are as total ammonia, $NH_3 + NH_4^+$ (after Armstrong *et al.*, 1978).

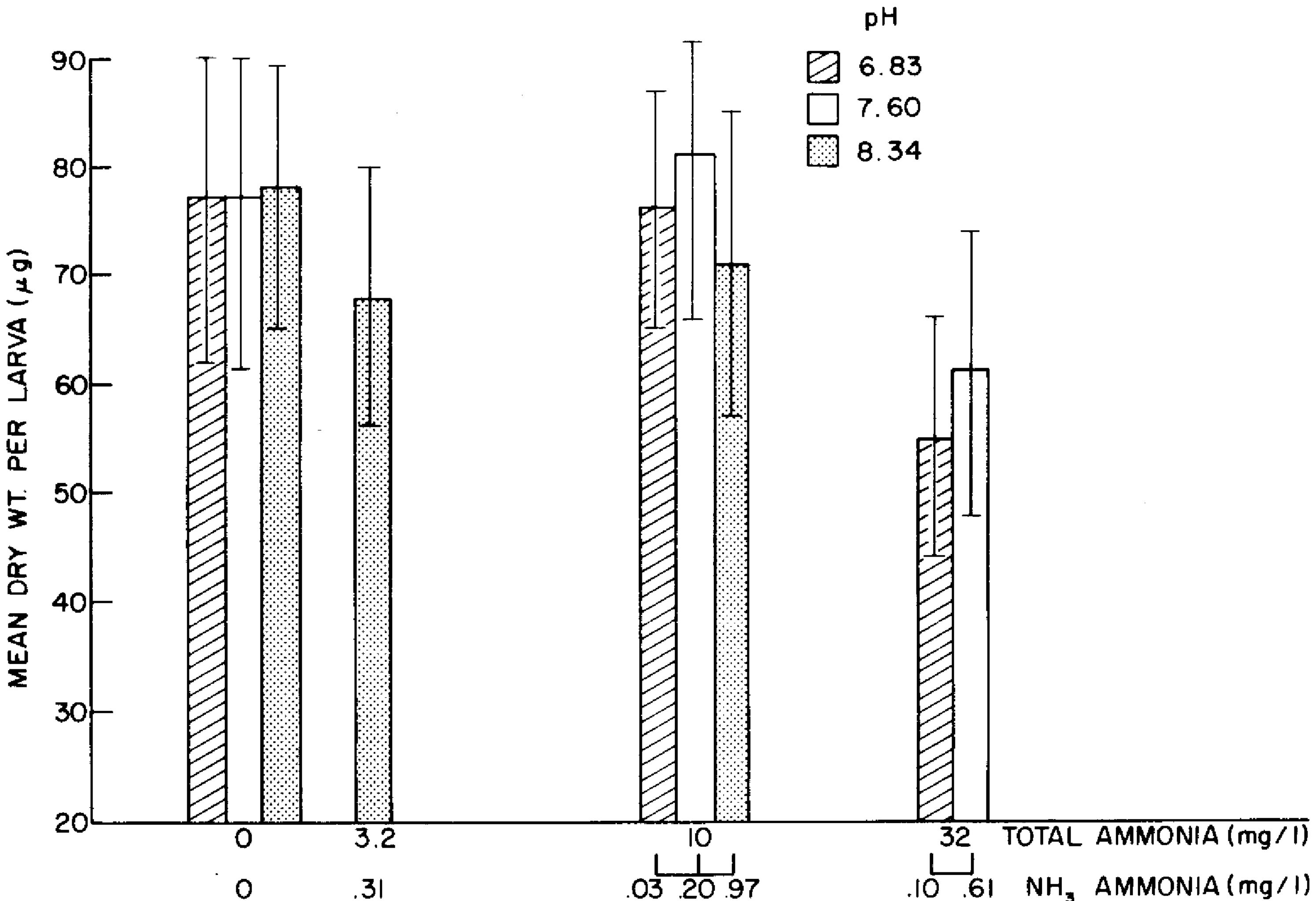

Figure 3. Growth of *M. rosenbergii* larvae in various treatments of ammonia and pH. Larvae had an initial weight of 35 ± 6 mg/animal and were exposed 7 days. Growth of animals in 32 mg/ℓ at both pH 6.83 and 7.6 was reduced from controls ($P < 0.05$), see Armstrong *et al*., 1978).

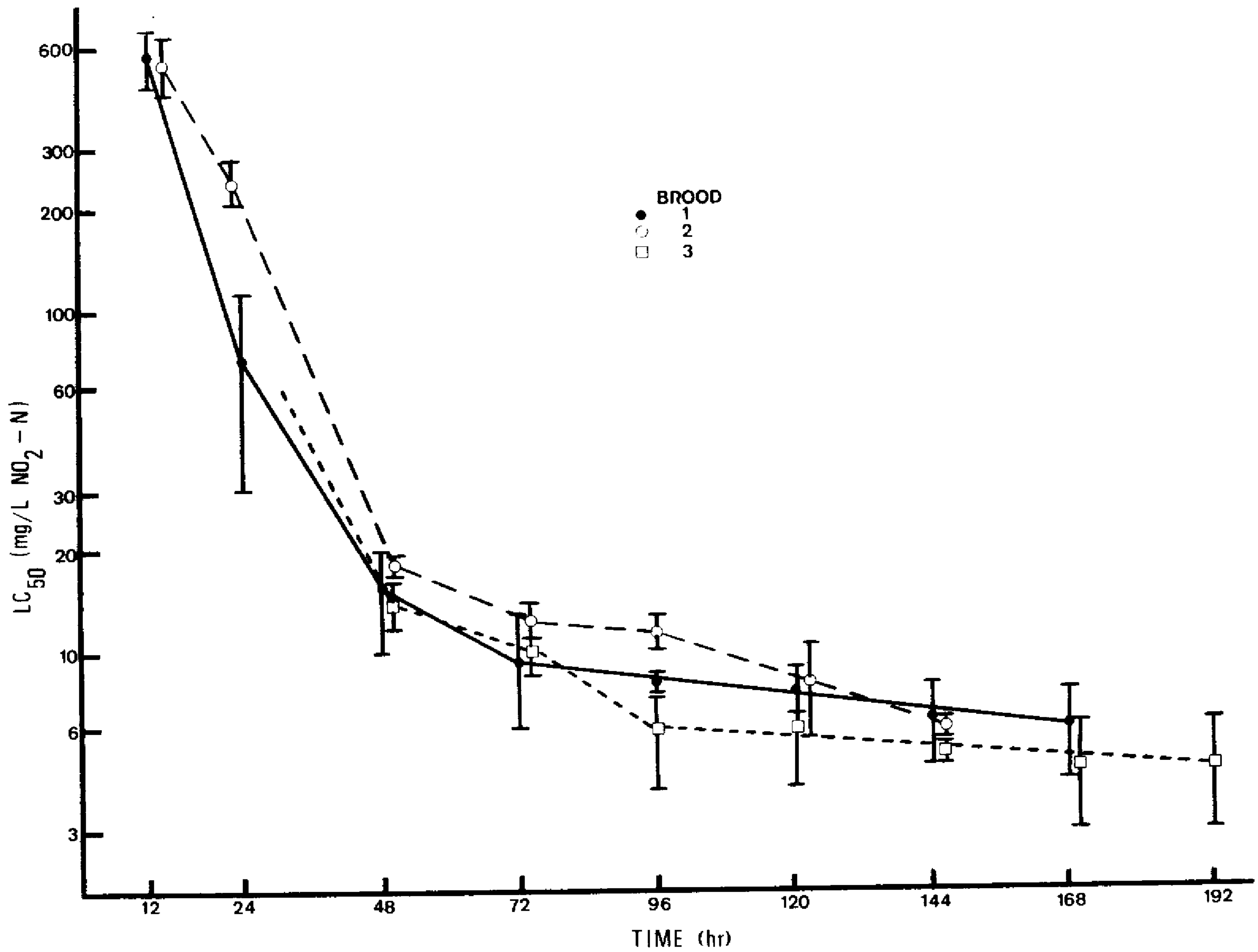

Figure 4. Nitrite toxicity to *M. rosenbergii* larvae over eight days. The pH of exposure water was 8.1 (after Armstrong *et al*., 1976a).

The primary means to ensure proper water quality in a great many closed and semi-closed culture systems, whether near-stagnant ponds or re-circulating raceways, is biological filtration (discussed previously in Sources of Nitrogen in Culture Systems). While the need to metabolize ammonia seems the principle function of these filters, the intermediary product, nitrite, is lethal (Russo et al., 1974; Collins et al., 1975) and must be converted to nitrate. Proper aging of filters to ensure a complete bacterial fauna for total oxidation of ammonia is imperative.

Given a properly functioning biological filter, its use is still often guess-work f r many culturists. Certain numbers of an aquatic species are housed in biologically filtered water, inorganic nitrogen measured is low, animals survive and grow; therefore, the system works. However, the need to maximize animal density, feed new food-types, and assure more probability of success than provided by trial and error requires greater sophistication in the application of filtration technology to culture systems. Scientific approaches to this end have been reviewed by Kinne (1976) and formulated by Speece (1973), Hirayama (1974), and Delistraty et al. (1977). For fish hatcheries, Speece (1973) provided equations to calculate the needed nitrification capacity based on fish size and temperature (as determinants of food consumption and ammonia production rates) and the water concentration of ammonia. These factors and the number of fish defined the specific surface area required for nitrification and, depending on the substrate size, the total volume of the nitrification facility could be calculated. Delistraty et al.

(1977) sought to equate ammonia tolerance and excretion rates of larval lobsters in various culture conditions to the maximum stocking density amenable to high survival. They provided examples of the theoretical stocking number of larvae at various combinations of flow rate, fractions of water reused in a system, and fractions of ammonia removed during passage of water through biological filters.

Consideration of these factors in conjunction with the toxicity data provided, and a certain amount of trial-and-error practical experience in crustacean culture will greatly minimize the potential danger of nitrogen toxicity.

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PRELIMINARY STUDIES TO EVALUATE THE POTENTIAL OF USING EMBRYO AND LARVAL STAGES OF THE GOOSE BARNACLE, *POLLICIPES POLYMERUS* FOR MARINE BIOASSAYS[1]

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ABSTRACT

The general purpose of these studies was to determine if the developmental and larval stages of the goose barnacle, *Pollicipes polymerus* would be useful organisms to use as a marine bioassay system. Different culture systems and culturing conditions were developed and evaluated. We designed a simple, inexpensive system that can be used to culture fertile eggs under normal laboratory conditions (room temperature and ambient light-dark cycle). The culture medium consists of Instant Ocean sea salts mixed in tap water to a salinity of 28-30 parts per thousand. Antibiotic concentrations of 10 mg/l seawater of streptomycin sulfate and 10 mg/l seawater of penicillin G are then added to the artificial seawater. As a result of these and other studies, it seems likely that *P. polymerus* may be a valuable bioassay organism. More complete studies, necessary to gather large amounts of data for developing and statistically evaluating different criteria to determine deleterious effects, are required before a final judgment can be made about the future use of *P. polymerus* for bioassays.

INTRODUCTION

It has been suggested that pollution is essentially a biological phenomenon in that its primary impact is on living organisms. However, most measurements of water quality are based on chemical observations which measure conditions rather than on

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biological criteria that evaluate effects. That situation continues to persist, despite growing recognition of the need for biological yardsticks and the recent development of a small number of suitable bioassay systems (Woelke, 1972).

Existing marine bioassay systems utilize developmental stages or embryos of oysters (*Crassostrea* species) or echinoderms (sea urchins, sea stars) and juvenile or adult stages of selected mollusks, echinoderms or crustaceans. Such organisms are subjected to different concentrations of a chemical for varying time periods and, depending on the results and criteria utilized for evaluation, certain conclusions can be made about the toxicity of the compound being studied. Developing embryos and larvae are most effective for determining the effects of chronic, low-level releases of a particular contaminant because they are more sensitive to many physical and chemical variables. Adult or juvenile invertebrates are frequently utilized to assess the effects of acute, high-level releases of environmental contaminants. However, such levels are rarely encountered in nature and thus, the use of those systems has little practical value for use in setting environmental standards.

To date, virtually no reliable bioassay systems have been developed that utilize early developmental and embryonic stages of a crustacean species. Crustaceans are a critical component of marine food webs and are extensively utilized as a food source by man. Thus, it seems desirable to develop a number of crustacean assay systems because of their direct and indirect importance to man and their occupation of every niche in the marine environment.

Recent studies (Abbott, 1975; Lewis, 1975a; Lewis, 1975b) using a goose barnacle (Pollicipes polymerus) have provided information about the development and maturation of a crustacean that may prove to be an extremely valuable marine bioassay system. This species is found world-wide in rocky, exposed, intertidal areas (Cornwall, 1970) and is an ecological dominant in the Mytilus-Pollicipes-Pisaster (mussel-barnacle-starfish) community (Rickets et al., 1973). The clustered adults play important roles as predators, scavengers and omnivorous general feeders (Howard and Scott, 1959). Hermaphroditic adults are believed to begin breeding when the water temperature reaches 12.3°C, and their breeding season lasts from May to December in California (Hilgard, 1960) and from May or June to October or November in Oregon. The eggs are fertilized internally and then extruded into a pair of sacs, the ovigerous lamellae, located on both sides of the mantle cavity. There are several thousand eggs per brood; a mature adult goose barnacle probably raises four to six broods a year, and about 50 to 60% of the mature adults can be found to contain developing embryos during the peak of the breeding season. Embryos contained in the ovigerous lamellae of an individual start development synchronously (Barnes and Barnes, 1959a), but the adults in a cluster are not synchronized breeders. The protostomous, yolky zygote undergoes unequal holoblastic, spiral, determinate cleavage (Anderson, 1973; Barnes, 1974). The development rate of the zygote and embryo is temperature dependent, and hatching and release of nauplii larvae normally occurs about one month after extrusion (Barnes and

Barnes, 1959b). Six naupliar larval stages are followed by a cypris larva which about one month after hatching is chemically attracted to existing adults and settles nearby. After settling, the cypris larva metamorphoses into an adult. Methods for culturing the larvae, which are probably an important food source in the littoral zone, have been previously developed (Barnes and Barnes, 1959b; Lewis, 1975a; Abbott and Mix, Unpublished Research).

To recapitulate, _P. polymerus_ possesses many unique characteristics which make it attractive to consider for use in marine bioassays; these include: developing eggs are available during 6-9 months of the year; the egg masses are fertilized and develop inside the adult barnacle and thus, there is a high degree of genetic homogeneity; the eggs develop synchronously; the two egg masses present in an adult barnacle each contain several thousand zygotes, thus providing large numbers required for statistical analyses; they are easily reared in the laboratory and they are easily collected.

The purpose of this report is to describe the results of preliminary studies to determine if developmental and larval stages of _P. polymerus_ would be suitable organisms for marine bioassays.

METHODS AND MATERIALS

Egg Availability and Collection of Eggs

To determine when fertile eggs are available, barnacles were examined weekly beginning on 15 April 75 through 30 November 75 and 15 April 76 through 30 September 76. Eggs contained in

the ovigerous lamellae of ripe P. polymerus were collected at Yaquina Head, Oregon. An abundant barnacle population exists at this location which is readily accessible at low tides. Additional populations may be found at Seal Rocks, Yachats, and other exposed rocky locations, provided there is not too much sand present. To obtain eggs, barnacles were opened in situ by severing the adductor muscles with a small pair of scissors and exposing the mantle cavity. Ovigerous lamellae were then removed with plastic forceps and placed in 25 ml glass vials filled with synthetic seawater containing antibiotics. The vials were then placed in a styrofoam container filled with cold (ambient) natural seawater and transported to our laboratory in Corvallis.

Preparation of Seawater

Seawater was prepared by mixing Instant Ocean synthetic sea salts with tap water to salinities of 28-31 parts per thousand (ppt). In earlier studies in our lab (Abbott, 1975), antibiotic concentrations of 100 mg/l seawater of streptomycin sulfate, 100 mg/l of penicillin G and 20 mg/l of Nystatin were used. Because of the possibility that such high concentrations could affect hatching rates and cause abnormalities and/or mortality of developing embryos, studies using antibiotic concentrations of 10 mg/l seawater of streptomycin sulfate, and 10 mg/l seawater of penicillin G were also conducted.

Culturing Apparatus

Two different systems were developed to culture P. polymerus. The major components of the culturing apparatus used in early studies in our lab (Abbott, 1975) and reevaluated during

this study are shown in Figure 1. This system consists of a double water bath; a cooling unit; culture chambers; an aeration system; and an evacuation hood. The inner water bath (d) is a small, two-gallon glass aquarium which is supported in the outer water bath (b), a 15-gallon aquarium, by rubber-coated test tube racks (c). This double water bath was used to minimize decontamination problems in the event of leakage from the culture chambers (The culture chambers are clinical cytology monitors, Millipore MSMP 037 HO.) (a). The experimental and control solutions were stored in Erlenmeyer flasks (e). A Hush III pump (f) connected to surgical tubing provided the air supply. A Westinghouse model WNC10KG1 cooling unit (not shown) coupled with a submersible pump (g) was used to maintain a temperature of 13 ± 1°C throughout the experiment.

The egg masses were placed in the culture chambers and then maintained in the inner water bath for the duration of the experiments (typically, 10-30 days). Water, with drugs, was changed daily and, although the chambers have a volume of 10 ml, only 5 ml of solution was used to prevent the water from bubbling out. The culture chambers (Fig. 2) have plugs (f) which could be removed for changing water and inserting a bored-out one ml disposable syringe barrel (b) which was part of the aeration system designed to prevent escape of the solution being analyzed. To move air from the surgical tubing to the culture chamber, a removable device (c, d, and e) was constructed. A disposable hypodermic needle (d) was glued onto a capillary tube (c), a short piece of rubber tubing (e) was glued to this and another

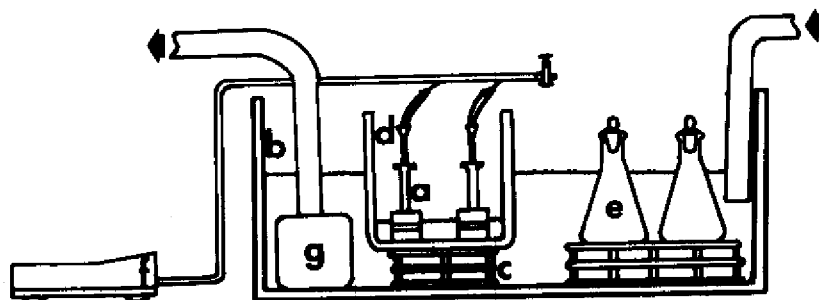


Figure 1. The first system used to culture *P. polymerus*. See the text for details.

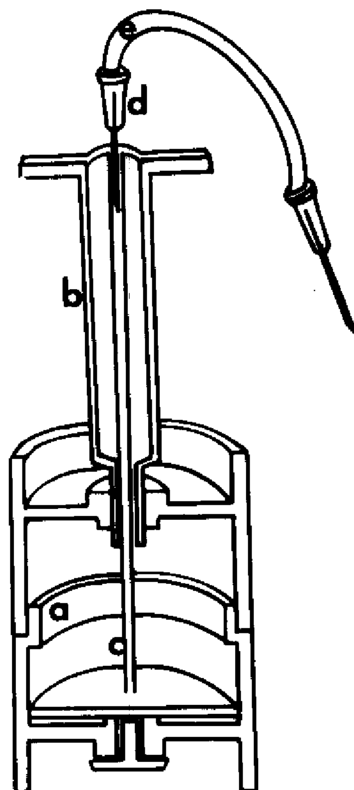


Figure 2. The culture chamber used in the first studies. See the text for details.

needle (d) was attached to the free end of the tubing. This last needle could be plugged into the air supply (the surgical tubing) or disconnected. The capillary tube (c) was then inserted down the syringe barrel (b) into the culture chamber (a) to provide gentle aeration. Except during the daily water changes, aeration was continuous in the chambers.

Figure 3 shows a second culturing system we developed during later studies. The primary difference between the systems involves the culture chamber (Fig. 4). The air hose consisted of 9 mm of latex tubing (1/8 x 1/32 in) (a) with 1-2 mm of a 0.1 cc B.D. Yale tuberclin syringe (b) attached at each end. B.D. Yale disposable hypodermic needles (c) (22G) were used to connect the air supply from the base and leading into the culture chamber. Twleve mm of a 50 μl Corning glass disposable micropipet (d) was used to introduce air into the chamber. The micropipet was held in place by silicon aquarium seal (e) which also prevented evaporation through the connection. The most suitable culture chamber (f) was a 30 ml Buchner funnel containing a coarse glass filter (Pyrex No. 36060). The bottom funnel (g) was closed by size 00 cork stoppers covered with Parafilm. The glass top (h) was constructed by a glass blower and served to introduce air into the chamber and to prevent evaporation. Subsequently, a second type of cover (Fig. 5) was developed that consisted of a 5 mm watchglass with a hole drilled in it to accommodate 6 mm of a micropipette which was sealed and held by silicon aquarium seal.

Experimental Design

Two experiments were designed to examine developmental

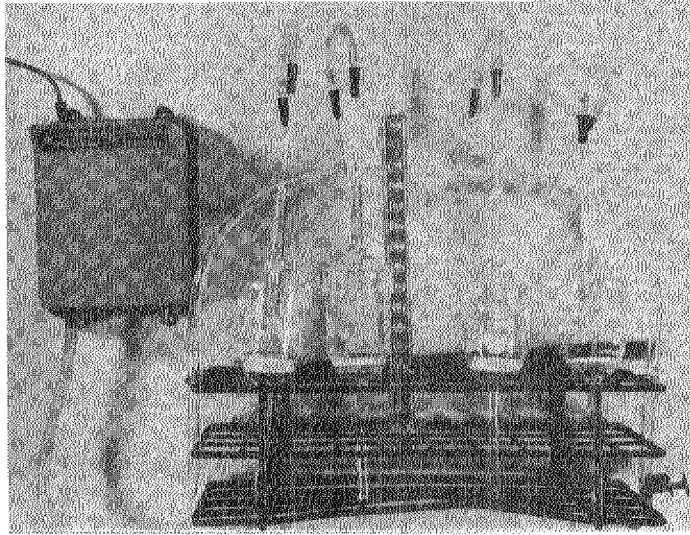


Figure 3. A second system used to culture *P. polymerus*. See the text for details.

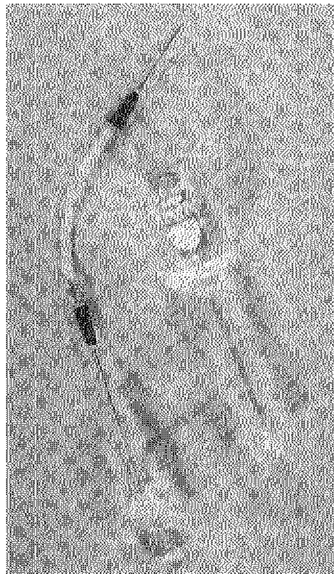


Figure 4. The culture chamber used in the second culturing system. See the text for details.

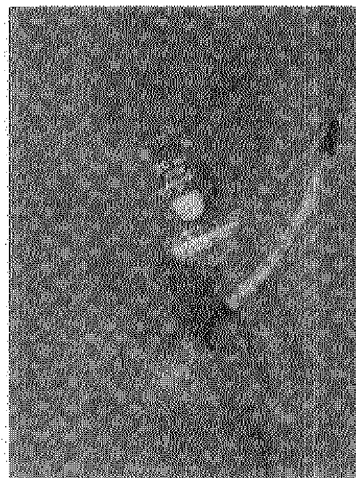


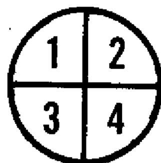
Figure 5. A second type of culture chamber used in the second culturing system. See the text for details.

rates of P. polymerus egg masses at different temperatures and light regimes. For both experiments, Instant Ocean was mixed with tap water to salinities of 28 to 31 ppt. The culture water, with antibiotics, was changed each day. Attempts were made to collect egg masses of the same orange color, indicating that the ovigerous lamellae contained the same developmental stage.

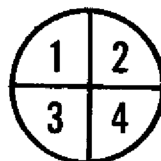
The purpose of the first study was to compare developmental rates of embryos maintained at 10-11°C and at room temperature, usually 21-25°C under ambient light-dark conditions. The egg mass was quartered, and a diagonal pair of sections was maintained at each temperature. Antibiotic concentrations were 10 mg/l seawater of streptomycin sulfate and 10 mg/l of penicillin G. Each culture chamber was checked daily and hatching dates were kept for each egg mass.

The purpose of the second experiment was to compare hatching rates at room temperature under light/dark laboratory conditions and under constant dark conditions. Two egg masses from one adult Pollicipes were placed in culture chambers according to the following scheme:

Egg Mass "A"



Egg Mass "B"

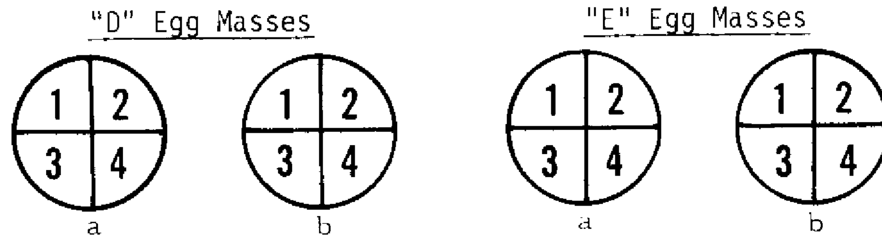


A1, A4, B1 and B4 were maintained at room temperature and ambient light-dark conditions.

A2, A3, B2 and B3 were maintained at room temperature in constant darkness.

Culture conditions were the same as described for the first study and mean hatching dates were noted for each egg mass.

In a final experiment, both egg masses were obtained from two barnacles and cultured as described below:



Da1, Da4, Db1, Db4, Ea1, Ea4, Eb1 and Eb4 were maintained at room temperature and ambient light-dark conditions.

Da2, Da3, Db2, Db3, Ea2, Ea3, Eb2 and Eb3 were maintained at room temperature and constant dark conditions.

Culture conditions were the same as described previously. Prior to placing the quartered egg masses into the culture chambers a small sliver of the lamellae were removed from each quarter and the stage of embryonic development was determined. It was found that all quarters contained embryos at the same stage of development. At days 10, 13 and 15, the egg masses were removed and the entire contents of the culture chambers—loose eggs, stage I and stage II larvae—were fixed in buffered neutral formalin. Subsequently, the number of each type of larvae was determined by placing the fixed organisms in a counting chamber with a grid consisting of 3 mm x 3 mm squares. Counts were then made using a Wild dissecting scope with an incandescent light source and an American Optical Co. Register QR-1 counter.

Statistical Analyses

The statistical analyses were very complex and only a simplified explanation will be given. For each of the three time periods when the contents of the chamber were fixed, counts were made of the egg mass (EM), loose eggs (LE), stage I nauplii larvae (NI), stage II nauplii larvae (NII) and detached egg mass (DEM). The counts were then converted into proportions as follows:

$$EM(J) = EM(J + 1) + DEM(J + 1) + LE(J + 1) + NI(J + 1) + NII(J + 1)$$

where J = 0,1,2 (first, second and third time period)

then

$$P_U, J + 1 = \frac{EM(J + 1) + DEM(J + 1) + LE(J + 1)}{EMJ}$$

= the proportion of eggs that were unhatched at the beginning of time period J + 1

$$P_I, J + 1 = \frac{NI(J + 1) + NII(J + 1)}{EM(J)}$$

= the proportion of eggs unhatched at the beginning of time period J + 1 which reached stage I larval development during period J + 1

$$P_{II}, J + 1 = \frac{NII(J + 1)}{EM(J)}$$

= the proportion of eggs unhatched at the beginning of period J + 1 which reached stage II larval development during period J + 1

By converting the counts to proportions, an F statistic could be used to determine the effects of light-dark and dark conditions on hatching, the differences between egg masses, the differences between barnacles and fixed responses due to light, random effects due to barnacles, random light-barnacle interaction and random effect due to egg mass within a particular light-barnacle

combination. A CDC 3300 computer was used to analyze the data.

RESULTS AND DISCUSSION

Egg Availability and Collection of Eggs

Ovigerous lamellae containing fertile eggs are available beginning in May or June depending on when the water temperature increases to 13-14°C. Fertile egg masses are then readily available throughout the summer and fall until October or November. We would not recommend using eggs obtained after September for bioassay studies because fewer embryos tend to develop normally from the last egg masses of the spawning period. This phenomenon has been described for a number of marine invertebrates that spawn repeatedly during the spring and summer.

Preparation of Seawater

We found that Instant Ocean synthetic sea salts mixed in tap water to a salinity of 28-31 ppt works as well for culturing *P. polymerus* as natural seawater. That is advantageous because it eliminates the inconvenience associated with obtaining and transporting seawater and there is no storage requirement. After testing various combinations and concentrations of antibiotics for use in inhibiting microbial blooming in the culture chambers, we have concluded that 10 mg/l seawater of streptomycin sulfate added with 10 mg/l of penicillin G is most adequate for routine culturing. There was an occasional loss of a developing egg mass because of microbial contamination, but in our opinion, fewer developmental anomalies, caused by antibiotics, are likely to occur.

Culturing Apparatus

During this study, we evaluated two different culturing systems (see Figs. 1 and 3). The system developed during the first study was adequate for culturing *P. polymerus* and the use of equipment to maintain constant temperature conditions would be an advantage if room temperature were high and uncontrolled. The primary disadvantage is that it is cumbersome and requires a great deal of space. Thus, we developed a simplified system that lends itself to convenient mass culturing. The advantages of the second system include: the culture chambers are larger; the total system requires little space; except for the custom-made glass tops, it is cheaper (the use of modified watch glasses—Fig. 5—greatly reduces the cost); it is simpler to change the water and developing barnacles can be easily monitored without disturbing the system. We found that barnacle embryos develop normally at room temperatures of 21-25°C (water temperatures in the chambers will fluctuate between 16-19°C) and thus, there is no need to attach a cooling system to the culturing apparatus.

Experiments

The purpose of the first experiment was to determine if there were significant differences in the rates of development between barnacle embryos maintained at 10-12°C and in water at room temperature. A second objective was to determine if barnacles developed normally if they were maintained in water with temperatures of 16-19°C.

The mean hatching time at 10°C was 25 days (range of 20-32 days) compared to a mean hatching time of 15 days (range of 12-20

days) for barnacles maintained at room temperature. It should be noted that 8 cultures were maintained at 10°C and 6 cultures at room temperature. Barnacles maintained at room temperature developed normally. The significance of finding that barnacles can be cultured at room temperature is twofold: first, bioassays could be completed more quickly and second, there is no need for cooling equipment under ordinary conditions.

The purpose of the second experiment was to compare hatching rates of barnacles maintained at room temperature under either ambient light-dark conditions or constant darkness. We found that mean hatching times did not appear to differ significantly between different light regimes or between the two egg masses.

Thus, our first data suggests that *P. polymerus* can be cultured under normal laboratory conditions and that contrary to reports in the literature, embryos may not require constant darkness while they are developing.

The final experiment was designed to provide data for statistically analyzing our system of culturing *P. polymerus* and to provide numerical information that would be useful in developing criteria if *P. polymerus* were to be used in bioassays. Tables 1-4 are summaries of the count data and the proportions derived from these data. All possible interactions were subjected to F tests (0.05 level of significance) and none were found to be significant. Briefly, this shows that there were no significant differences between: eggs obtained from the two barnacles (E and F) used in the study; the quartered egg masses of each lamellae; the quartered egg masses of all lamellae;

Day fixed	Egg mass	Number Eggs	N I larvae	N II larvae
10	Da1	26	53	33
	Da4	19	93	60
	Db1	24	55	25
	Db4	65	99	50
	Ea1	172	182	174
	Ea4	35	106	39
	Eb1	10	19	10
	Eb4	143	135	83
13	Da1	44	77	101
	Da4	195	150	134
	Db1	153	170	90
	Db4	225	241	124
	Ea1	20	35	27
	Ea4	9	14	12
	Eb1	5	28	12
	Eb4	226	189	146
15	Da1	346	208	178
	Da4	413	187	138
	Db1	207	168	151
	Db4	---	---	---
	Ea1	374	199	121
	Ea4	224	258	85
	Eb1	345	136	47
	Eb4	443	238	153

TABLE 1

Count Data for Barnacles Maintained at Room
Temperatures and Ambient Light-Dark Conditions

Day fixed	Egg mass	Number		
		Eggs	N I larvae	N II larvae
10	Da2	234	153	151
	Da3	29	152	87
	Db2	468	175	85
	Db3	24	146	46
	Ea2	75	116	56
	Ea3	25	84	96
	Eb2	64	148	90
	Eb3	51	172	107
13	Da2	83	53	36
	Da3	18	93	61
	Db2	112	42	16
	Db3	200	88	129
	Ea2	363	63	47
	Ea3	35	140	121
	Eb2	75	88	79
	Eb3	13	78	44
15	Da2	35	20	11
	Da3	91	70	92
	Db2	---	---	---
	Db3	98	54	43
	Ea2	264	108	88
	Ea3	306	511	420
	Eb2	346	415	235
	Eb3	---	---	---

TABLE 2

Count Data for Barnacles Maintained at Room Temperature and Constant Dark Conditions

Egg mass	Time 1 (10 days)	Time 2 (13 days)	Time 3 (15 days)
D,a,1	U = .925±2 (.007)	U = .828±2 (.010)	U = .525±2 (.014)
	NI = .075±2 (.007)	NI = .172±2 (.010)	NI = .475±2 (.014)
	NII = .029±2 (.004)	NII = .097±2 (.008)	NII = .219±2 (.012)
D,a,4	U = .905±2 (.006)	U = .802±2 (.009)	U = .653±2 (.013)
	NI = .095±2 (.006)	NI = .198±2 (.009)	NI = .347±2 (.013)
	NII = .037±2 (.004)	NII = .093±2 (.007)	NII = .147±2 (.010)
D,b,1	U = .932±2 (.007)	U = .758±2 (.011)	U = .518±2 (.015)
	NI = .068±2 (.007)	NI = .242±2 (.011)	NI = .482±2 (.015)
	NII = .021±2 (.004)	NII = .084±2 (.007)	NII = .228±2 (.014)
D,b,4	U = .844±2 (.010)	U = .507±2 (.009)	U = θ
	NI = .156±2 (.010)	NI = .493±2 (.009)	NI = θ
	NII = .052±2 (.006)	NII = .168±2 (.012)	NII = θ
E,a,1	U = .785±2 (.008)	U = .945±2 (.006)	U = .694±2 (.011)
	NI = .215±2 (.008)	NI = .055±2 (.006)	NI = .306±2 (.011)
	NII = .105±2 (.006)	NII = .024±2 (.004)	NII = .116±2 (.008)
E,a,4	U = .902±2 (.006)	U = .980±2 (.003)	U = .729±2 (.010)
	NI = .098±2 (.006)	NI = .020±2 (.003)	NI = .271±2 (.010)
	NII = .026±2 (.004)	NII = .009±2 (.002)	NII = .067±2 (.006)
E,b,1	U = .970±2 (.005)	U = .972±2 (.005)	U = .798±2 (.011)
	NI = .030±2 (.005)	NI = .028±2 (.005)	NI = .202±2 (.011)
	NII = .010±2 (.003)	NII = .013±2 (.003)	NII = .013±2 (.006)
E,b,4	U = .887±2 (.006)	U = .786±2 (.009	U = .661±2 (.013)
	NI = .113±2 (.005)	NI = .214±2 (.009)	NI = .389±2 (.013)
	NII = .043±2 (.004)	NII = .093±2 (.006)	NII = .152±2 (.010)

U - unhatched eggs; NI - stage I larvae; NII - stage II larvae.

TABLE 3

Proportions ± 2 Standard Deviations Determined From the Count Data for Barnacles Maintained at Room Temperature and Ambient Light-Dark Conditions

Egg mass	Time 1	Time 2	Time 3
D,a,2	U = .641±2 (.012)	U = .711±2 (.021)	U = .705±2 (.035)
	NI = .359±2 (.012)	NI = .289±2 (.021)	NI = .295±2 (.035)
	NII = .179±2 (.011)	NII = .117±2 (.016)	NII = .105±2 (.025)
D,a,3	U = .738±2 (.012)	U = .760±2 (.014)	U = .656±2 (.016)
	NI = .262±2 (.012)	NI = .240±2 (.014)	NI = .344±2 (.016)
	NII = .095±2 (.008)	NII = .095±2 (.010)	NII = .195±2 (.015)
D,b,2	U = .761±2 (.011)	U = .840±2 (.004)	U = θ
	NI = .239±2 (.011)	NI = .160±2 (.004)	NI = θ
	NII = .078±2 (.007)	NII = .044±2 (.009)	NII = θ
D,b,3	U = .782±2 (.012)	U = .674±2 (.015)	U = .610±2 (.025)
	NI = .218±2 (.012)	NI = .326±2 (.015)	NI = .390±2 (.025)
	NII = .052±2 (.006)	NII = .194±2 (.013)	NII = .173±2 (.020)
E,a,2	U = .880±2 (.007)	U = .907±2 (.007)	U = .725±2 (.013)
	NI = .120±2 (.007)	NI = .093±2 (.007)	NI = .275±2 (.013)
	NII = .039±2 (.004)	NII = .040±2 (.005)	NII = .124±2 (.010)
E,a,3	U = .904±2 (.006)	U = .844±2 (.008)	U = .322±2 (.010)
	NI = .096±2 (.006)	NI = .156±2 (.008)	NI = .678±2 (.010)
	NII = .051±2 (.004)	NII = .072±2 (.005)	NII = .306±2 (.011)
E,b,2	U = .881±2 (.006)	U = .901±2 (.006)	U = .552±2 (.010)
	NI = .119±2 (.006)	NI = .099±2 (.006)	NI = .448±2 (.010)
	NII = .045±2 (.004)	NII = .047±2 (.004)	NII = .162±2 (.008)
E,b,3	U = .437±2 (.019)	U = .262±2 (.014)	U = θ
	NI = .563±2 (.019)	NI = .738±2 (.014)	NI = θ
	NII = .216±2 (.016)	NII = .266±2 (0.29)	NII = θ

U - unhatched eggs; NI - stage I larvae; NII - stage II larvae.

TABLE 4

Proportions ± 2 Standard Deviations Determined From the Count Data for Barnacles Maintained at Room Temperature and Constant Dark Conditions

barnacle development under ambient light-dark conditions or constant dark conditions. The results indicate that our culture techniques are sound and that P. polymerus may be a dependable organism for use in bioassays.

In order to develop definitive criteria to be applied to a bioassay system, it is necessary to obtain substantial additional data. It was not the intent of this preliminary research to conduct studies that would yield such data. Nevertheless, from our studies it seems that the following criteria may be useful in utilizing P. polymerus for marine bioassays (it is assumed that barnacles will be cultured at room temperatures and ambient light-dark conditions and that controls are being compared to experimentals maintained in different concentrations of the compound being tested):

1. the percentage of eggs that hatch under each condition after an appropriate time period;
2. the proportion of eggs to first stage larvae after an appropriate time period;
3. the proportion of eggs to second stage larvae after an appropriate time period;
4. the proportion of stage I to stage II larvae after an appropriate time period;
5. the percentage of abnormal stage I larvae;
6. the percentage of abnormal stage II larvae.

Additional experiments utilizing different experimental designs and statistical analyses will be necessary to further evaluate the barnacle system for use in marine bioassays.

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CIRCADIAN RHYTHMS AND CRUSTACEAN HEALTH*

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Literally thousands of biological rhythms with periods reflecting the environmental cycles of the day, tide, month and year have now been described in animals and plants. From the earliest phases of these investigations and continuing up to the present, studies on the Crustacea have contributed significantly to our basic understanding of the phenomenon. Beginning in the late 1940's, F. A. Brown and his students at the Marine Biological Laboratory undertook an investigation of the rhythms of pigment dispersion in crabs and prawns that called attention to the extraordinary rhythm properties of persistance, temperature-independence of frequency, and response to such synchronizers as light and temperature. What seemed to many at the time to be very remarkable but esoteric characteristics of crustacean color change rhythms are now recognized as nearly universal properties of a phenomenon that is itself ubiquitous. Within the Crustacea the knowledge of biological rhythms has been extended to an ever-increasing list of species for many processes (biochemical, endocrine, nervous, metabolic, locomotor) occurring

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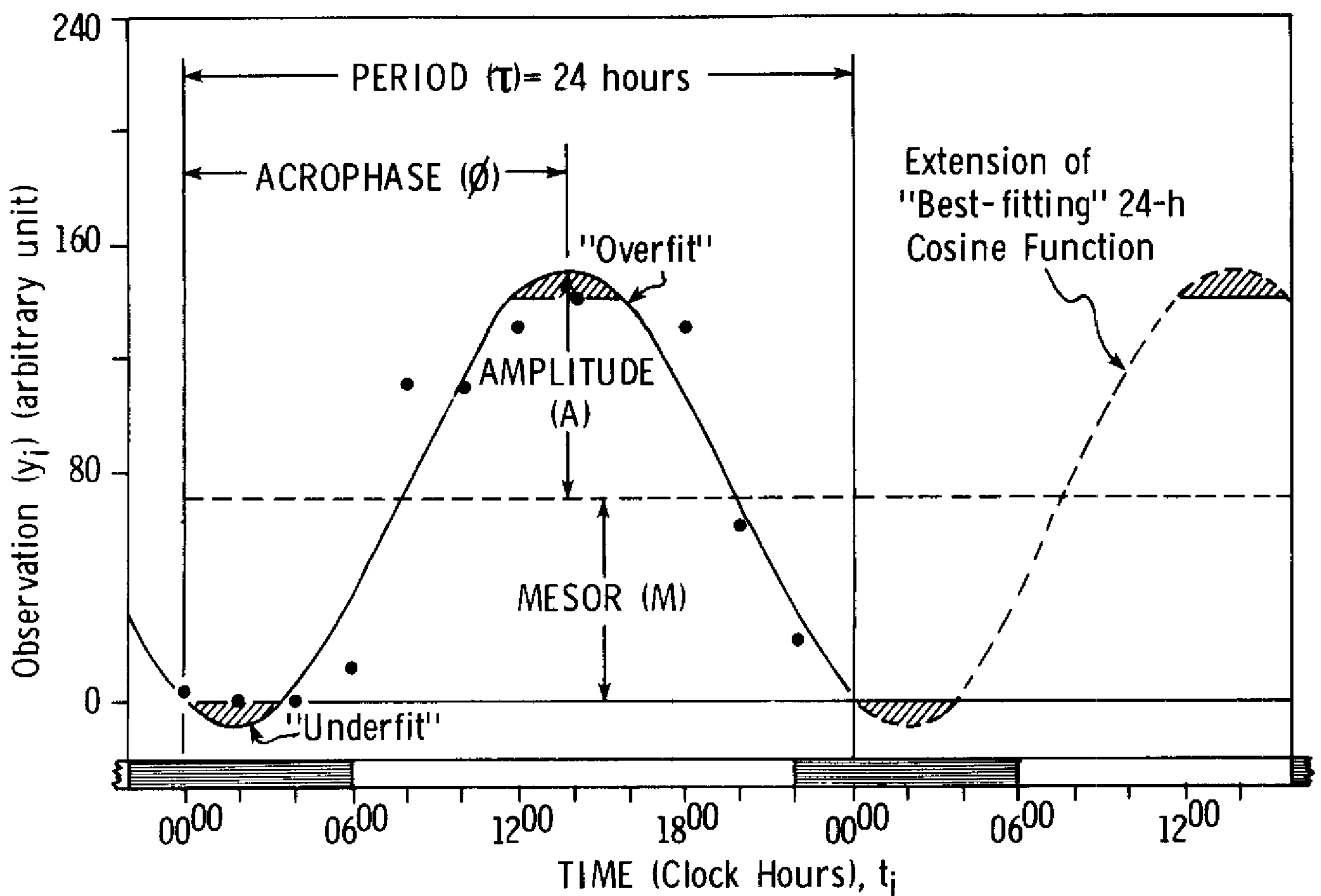
at all levels of biological organization (molecular, organ system, organism, population). With this growing awareness an increasing number of workers in widely different areas of crustacean biology have taken cognizance of the potential significance of biological rhythms for their own studies. From the standpoint of this workshop on crustacean health, the application of rhythms might profitably take its lead from the voluminous work on human and other mammalian clinical studies. As one example we turn to a discussion of the concept and application of the chronobioassay.

Tests of physiologic, pharmacologic or environmental polluting agents can be carried out to assess concomitantly two interdependent, yet distinguishable rhythm-related phenomena: first, any effect of the agent tested upon circadian (Figure 1) or other rhythm characteristics and second, any dependence of effect upon the stage of rhythm(s) at the time of agent administration.

Let us consider a basic practicable scheme designed to assess the behavior of circadian rhythm characteristics by the study of responses to drugs or environmental changes, e.g., high or low concentrations of environmental pollutants. Data collection may be automatic, e.g., by recorders of motor activity or by time lapse photography in a serially dependent (longitudinal) fashion, each animal being sampled repeatedly, as is indicated at the bottom of Figure 2. Any other determinations (whatever may be pertinent for a given problem, such as chemical ones that might require killing the animal) can then be carried out in a serially independent (transverse) fashion, to cover, for instance, a full circadian cycle, with data from separate groups of animals. The

ESTIMATION OF PARAMETERS M, A AND Ø BY LEAST SQUARES FIT OF COSINE MODEL WITH FIXED PERIOD*



*$y_i = M + A \cos(\phi + \omega t_i) + e_i$; $\omega = 2\pi/\tau$

t_i=time

y_i=observation at t_i

e_i=error at t_i, assumed to have the same independent normal distribution with mean zero and unknown variance σ^2, regardless of time.

Figure 1

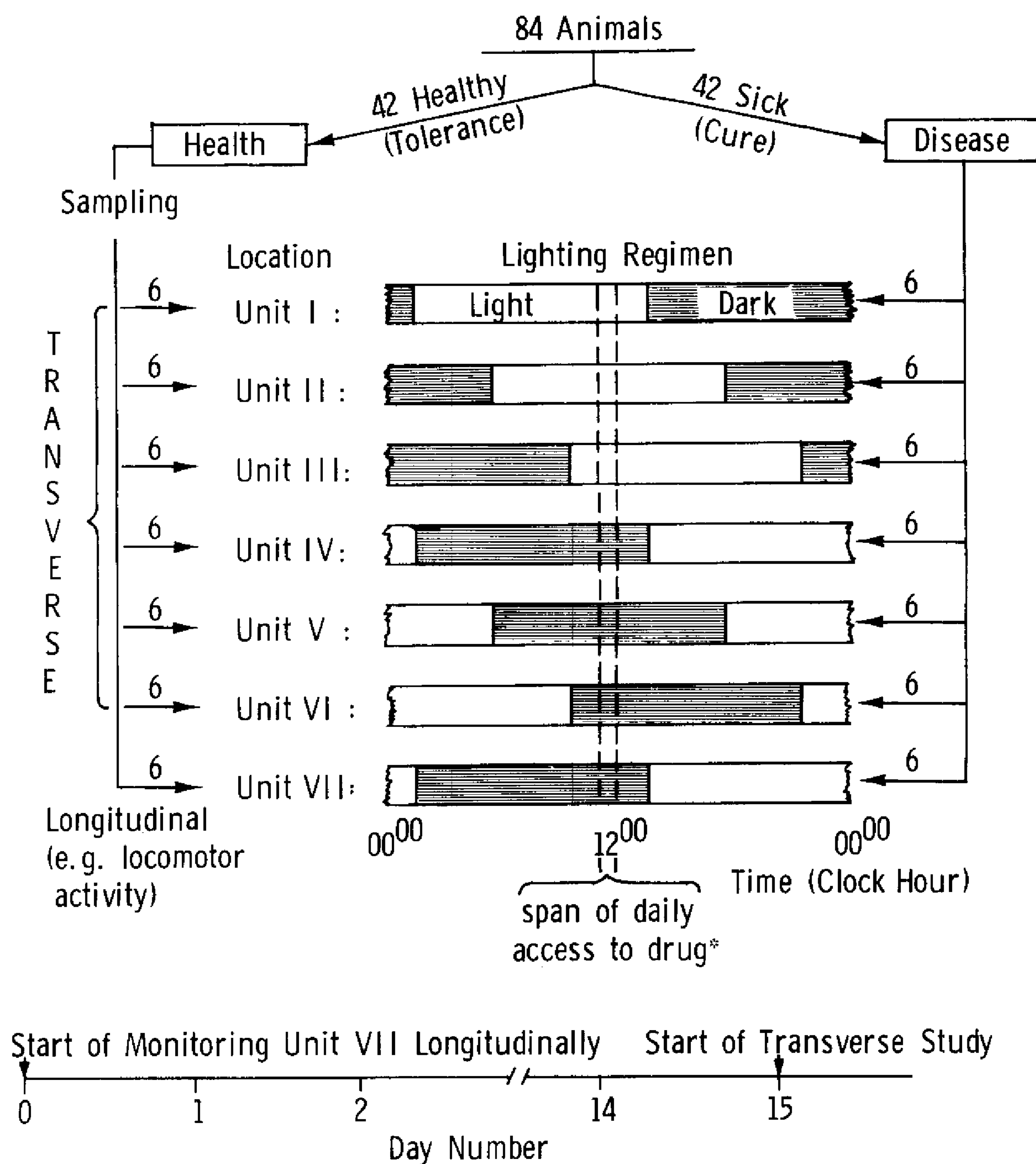


Figure 2

single (or a few such) cycle(s) covered may be picked on the basis of the longitudinally monitored (locomotive or other) marker rhythms.

Chronobioassays carried out at critical times serve to explore rhythm characteristics under one, several, or all of the following conditions, among others:

a) exposure to one or several (24-hour) synchronizers such as the alternation of (1) light and darkness and of (2) the availability and unavailability of food (a design of interest in itself, in view of the demonstration that feeding schedules can be optimized themselves for a better utilization of calories).

b) behavior during and following one or several changes in the temporal placement along the 24-hour scale of one or several synchronizers, such as the schedules of lighting, meals, pollutants, or drugs. Such metarhythmometry (involving the manipulation of one or more synchronizers) dissociates physiologic mechanisms in time just as chromatography dissociates compounds by travel in space.

c) isolation of the organism in a presumably constant environment, compatible with a "self-selected" schedule (and thus with a "free-running" period of a rhythm).

d) completion of study by resynchronization with a synchronizer for sampling, transversely, once on each organism from separate groups for a series of special, e.g., biochemical, determinations on blood and/or tissues (that may require killing). This may be done after one has adjusted the timing of the animals to that of the investigator, rather than vice versa (as also shown in the top 6 rows of Figure 2).

Survival of Leukemic* and Non-leukemic Mice
Treated with Arabinosyl Cytosine on Different Schedules:

CHRONOTHERAPY (CR_X) versus HOMEOSTATIC THERAPY (HR_X)

Leukemia* No. of mice	CRx	CRx	CRx	CRx	CRx	CRx	CRx	CRx	HRx	Subtotal
No ▨	174	175	175	175	175	175	174	175	139	1537
Yes ■	119	103	120	120	174	120	120	103	161	1140
									Total:	2677

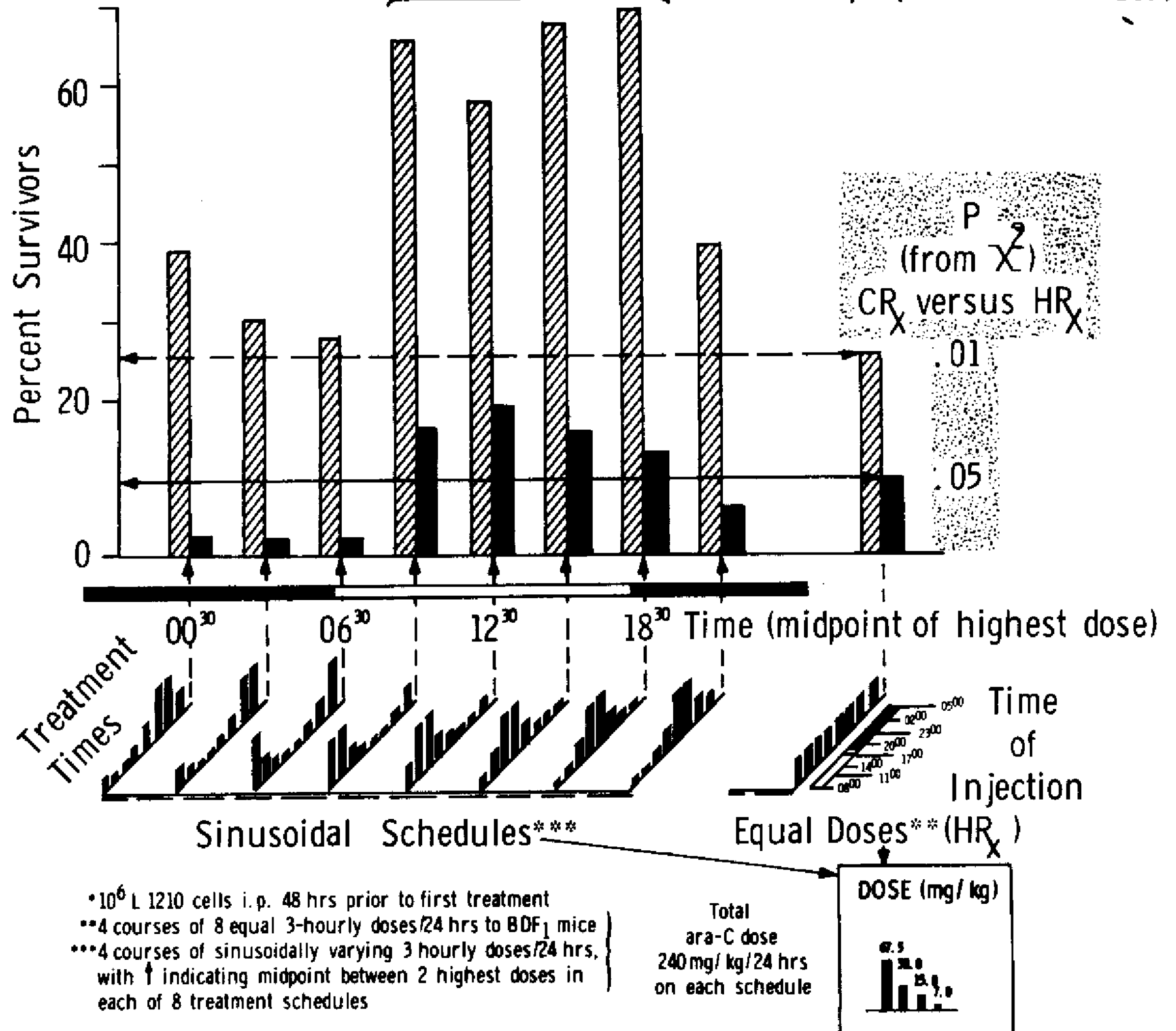


Figure 3

The 7 rows in Figure 2 can represent 14 groups of individuals - 7 control groups and 7 experimental ones. Each group in the top 6 rows could be tested at a different circadian stage yet at the same convenient clock hour. Different rhythm stages can be made to be available at the same clock hour -- whenever one can manipulate environmental synchronizers by placing separate tanks or other housing units on different schedules as, for instance, in Figure 2.

The two groups at the bottom (labeled longitudinal control) could be monitored continuously, as discussed, e.g., for oxygen consumption or locomotor activity. Actually, during transverse sampling one of these two groups may again by monitored as the control and the other as the test group of an agent or condition - disease, drug, or pollutant.

The longitudinal data may be analyzed on a daily basis for rhythm characteristics, shown in Figure 1, such as (I) a measure of timing - the acrophase, (II) a measure of extent of change - the amplitude, (III) the overall mean - the mesor and (IV) the wave from (which also is testable but not shown in Figure 1). Once the mesor, amplitude, acrophase or waveform changes [and if the rhythm in different individuals remains (group-)synchronized], one can examine the possobility that these rhythms can be shifted in both the test and control groups. If such shifts also are possible, one can then institute a set of lighting regimens such as those shown in the 6 top rows of Figure 2. These would allow one to carry out certain tests at a fixed clock hour on one day or on consecutive days if some groups are being picked from a larger group to study what happens in consecutive cycles as the effect of a given condition tested.

Survival of Leukemic* and Non-leukemic Mice Treated with Arabinosyl Cytosine on Different Schedules:

CHRONOTHERAPY (CR_x) versus HOMEOSTATIC THERAPY (HR_x)

Leukemia* No. of mice				CR_x					HR_x	Subtotal
No ▨	174	175	175	175	175	175	174	175	139	1537
Yes ■	119	103	120	120	174	120	120	103	161	1140

Total: 2677

Percent Survivors

60

40

20

0

P (from χ^2) CR_x versus HR_x

.01

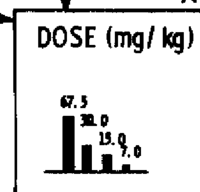
.05

00^{30} 06^{30} 12^{30} 18^{30} Time (midpoint of highest dose)

Treatment Times

Time of Injection

Sinusoidal Schedules*** Equal Doses** (HR_x)



Total ara-C dose 240 mg/ kg/ 24 hrs on each schedule

*10^6 L 1210 cells i.p. 48 hrs prior to first treatment

**4 courses of 8 equal 3-hourly doses/24 hrs to BDF_1 mice

***4 courses of sinusoidally varying 3 hourly doses/24 hrs, with ↑ indicating midpoint between 2 highest doses in each of 8 treatment schedules

Similar tests in human beings have yielded, already, chronotherapeutic leukemia in mice where a comparison was made of equal doses of the antimetabolite arabinosyl cytosine (briefly ara-C) in a so-called homeostatic treatment with a sinusoidal treatment. The sinusoid consists of the same total dose per day, but with the doses rearranged so that more is given when the organism is resistant (in terms of toxic side effects) and less drug is given at the time when the organism is more susceptible. It can be seen in Figure 3, a study of 2,667 animals, that the treatment of an experimental cancer can be optimized in the mouse. Perhaps similar consideration of chronotherapy more broadly may apply to crustacean disease.

It seem possible that not only certain drug treatments but also the effect of food can be optimized in crustacenas. For this consideration we can turn to data on the mouse, human beings, and most recently on an aquatic form, the Yamuna River catfish. In cooperation with Dr. Sundararaj of Delhi, it was possible to show that a colorie is not the same calorie for the equivalents of a catfish "breakfast" and "dinner". It matters whether a calorie is offered at the beginning or end of a ligh or dark span to fish kept on a regimen involving 12 hours of light alternating with 12 hours of darkness. The body weight gain is quite different according to the timing of feeding schedules. In human beings of usual weight as well there is a consistent relative body weight loss on a regimen of breakfast only as compared to one of dinner only.

In this energy poor world, wherein we search for food, it will be important for human beings to know when to consume their food. For those who produce the food, e.g., in the form of crustaceans, it will be important to know when, at this point in the food chain, one gets "most

mileage" out of the calories offered to the animal. Thus, the questions as to <u>when</u> to <u>eat</u>, <u>when</u> to <u>treat</u> and <u>when</u> to <u>test</u> for a pollutant (among many other applications of chronobiology to the health of crustaceans) can be approached by chronobioassay in the laboratory.

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CONCLUDING REMARKS

BY
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This Second Crustacean Health Workshop has been a veritable kaleidoscope of where we've been, where we are, and where we're going in crustacean health sciences. The "where we've been" was admirably documented in the scholarly keynote address by Dr. Sindermann. The rather amazing, to me at least, "where we are" was amply demonstrated by the scope and sophistication of many of the papers presented. "Where we are going" is, of course, speculative, but can be predicted with some assurance by examining the present state of the art along with present and anticipated future problems.

I shall briefly address these three topics in the perspective of this workshop.

"Where We've Been"

Dr. Sindermann prefaced his discussion of epizootics in crustacean populations with a few, but cogent, concepts that we should keep constantly in mind when studying the mortality pattern of any population. The concept that "mass mortality" is merely an exaggeration of natural mortality is obvious but often forgotten. Every organism in a population will die from one

cause or another; it is the rapid acceleration of the mortality rate that causes concern, usually because it decimates the population before we have the opportunity to do so.

Another concept frequently overlooked is that invertebrates, like man, can suffer from non-infectious diseases. We speak of heart disease, dietary deficiency diseases, the numerous neoplastic diseases, etc., but are inclined to think only in terms of infectious agents in relation to diseases of invertebrates. Equally germane is the discussion of the effect of sudden environmental changes and the combination of an infectious agent, often an innocuous one, and environmental stress resulting in mass mortalities.

The historical review of crustacean epizootics in the keynote address is a concise documentation of the subject, including not only the familiar crayfish plague and lobster gaffkemia but also the less well known epizootics of non-economically important crustaceans. I concur with Dr. Sindermann's recommendation for a trip through the older literature on crustacean diseases.

The speaker then discussed the epizootiology of crustacean diseases and summarized the present knowledge of immune responses in the group. He completed his address with a masterful discussion of the interaction of host, pathogen and environment. As Dr. Sindermann points out, it is becoming increasingly apparent that environmental factors play <u>a</u> major, if not <u>the</u> major, role in crustacean diseases. This is particularly obvious in culture situations, but recent investigations indicate that synergistic interactions between degraded environment and facultative pathogens are equally important in feral populations.

"Where We Are"

The research papers presented at this workshop are impressive from two aspects: their breadth and the sophistication of much of the research. They fall into several broad categories: infectious disease organisms, cytology and immunology, disease control and surveys of disease in feral populations. Rather than discuss each paper, I would like to make some comments on each of these categories.

(1) Disease organisms

At one of the first scientific meetings I attended, I heard a famous herpetologist state, in a discussion of the zoogeography of snakes, that man's knowledge of the geographical distribution of snakes was as dependent on the distribution of herpetologists as on the snakes themselves. This is, of course, equally true of diseases of crustaceans. Just a few years ago, for example, the known virus diseases of crustaceans, indeed all marine invertebrates, could easily be counted on the fingers of one hand. Now, it is almost impossible to keep up with this rapidly expanding field to which Yudin and Clark have added at this workshop a new rhabdo-like virus in the blue crab. Like the shrimp baculovirus, many insect viruses and mammalian viruses, it is enhanced by stress, in this instance by eye-stalk ablation.

These viruses have unquestionably been around for a long time; they are new to science because we have only recently had the competent investigators, sophisticated equipment such as the electron microscope and adequate facilities to maintain populations of crustaceans while they are being studied.

Although less spectacular, the discovery of other organisms causing disease in crustaceans is also the result of the fortuitous combination of, usually, cultured populations, outbreaks of diseases, and invertebrate pathologists. In this category, I would include the report of Lightner, et al. on _Fusarium solandi_ in cultured penaeids, the suctorean parasite of _Penaeus monodon_ larva by Gucatan, et al. and Chan's report on bacterial infections as the cause of exoskeletal lesion in feral population of the Hawaiian fresh water shrimp _Atya bisulcata_.

The reports on a bacterial disease of the Louisiana crayfish by Amborski and Amborski and on ectocommensals of the freshwater prawn by Hall add emphasis to Dr. Sindermann's remarks on the opportunistic pathogen effect of commensals under conditions of stress.

(2) Cytology and immunology

Several reports fit in the important category of cytology and immunology. It is patently obvious that the next step in crustacean health following the discovery of a pathogen is investigation of the host's response to it. Such studies typically begin with the cytology of the hemocytes as in Eble and Blewetts report on the hemocytes of _Macrobrachium rosenbergii_, followed by investigation of phagocytosis and other hemocytic defense mechanism exemplified by Stagner's study with _Limulus_.

Another aspect of immunology is the determination of the virulence of a particular pathogen. Information on the virulence of vibriosis to shrimp was provided by the two reports of Leong and Fontaine and Leong and Hanrahan. Lewis presented data on the

serology of Vibrios pathogenic to shrimp and Steenbergen described his studies on the serology of *Leucothrix*.

(3) Disease control

The final step in the maintenance of health in a population is the prevention and treatment of disease. In crustacean health we are just beginning to address this facet of the problem of growing the maximum number of animals in the minimum amount of space. Blogoslawski and Brown made a strong case for the use of ozonized water as a means of preventing bacterial diseases. Gacutan, et al. have done much of the testing of toxicity levels of furanace to shrimp that is essential for any candidate chemical prior to its use for prophylaxis or disease treatment and Roegge, et al. reported on their experimental use of ten chemicals at various concentrations to control *Zoothamnium* infestations in larval cultures of *Macrobrachium*.

Fred Meyer, in the paper by Schnick, et al. quite correctly called to our attention the requirement that all chemicals used in crustacean culture must be approved by FDA or EPA and pointed out that only a few have been approved to date. Their compilation, in tabular form, of the known data on the efficacy and toxicity of all the chemicals used in crustacean culture is a worthwhile contribution that will be widely used by crustacean culturists.

(4) Surveys

The fourth category, surveys of disease in feral populations, is a relatively unexplored area in crustacean health. Such studies

are now being supported to provide baseline information in areas to be developed in the offshore petroleum industry and to measure the effects of pollution. Sawyer, et al. and Ernst and Neff elected to study the gills of large numbers of crustaceans rather than more complete investigation of a smaller number of animals. Since the gill is more readily exposed to pollutants and is vulnerable to injury, such emphasis is probably the best means of determining the effects of declining water quality. I can't help regretting however, the lost opportunity to ascertain the overall state of health in a wild population over a wide geographic area. I'm sure the authors of these papers share my regrets and I'm aware a complete histopathological workup on the 200 lobsters and 2,100 rock crabs in Sawyer, et al.'s study would have been a physical impossibility.

Two papers presented at the workshop do not fit into any of my arbitrarily selected categories. They are the sophisticated study of the metabolism of Benzo(A)pyrene in certain arthropods by Anderson and Mix, et al.'s report on the potential use of embryo and larval stages of the goose barnacle for bioassays.

"Where We Are Going"

In my concluding remarks at the first crustacean workshop I commented on the rather amazing acceleration of research on crustacean health that had occurred in the last several years. This workshop has shown that the pace has increased rather than slowed. I am confident that the trend will continue, at least for the foreseeable future.

There are a number of reasons for this optimistic view. First, as crustacean aquaculture approaches commercial viability, more and more disease problems will arise with the necessity for researchers to address their diagnosis, prevention and treatment. Second, the recent realization by funding agencies that pollutants can have significant effects on wild populations over wide geographic areas will lead to increased field investigations, both baseline studies and studies of the effects of environmental degradation. Finally, laboratory studies of the specific effects of pollutants will lead to accelerated effort in crustacean histopathology and physiopathology.

ADDENDUM

SUMMARY OF PLANNING CONFERENCE ON CRUSTACEAN HEALTH RESEARCH PRIORITIES HELD AT TEXAS A&M UNIVERSITY NOVEMBER 17, 18, 1977

The conference was designed to identify priority areas of health-related problems of crustaceans. During the first day, case histories exemplifying some of the more significant health-related problems of wild and cultured crustacean populations in the United States were presented to provide the basis for analyzing problems related to various species and management practices. The remainder of the conference concerned summarizing the available information, clarifying areas of need and formulating recommendations. The following recommendations were made:

1. Industry representatives concerned with disease and disease control should prepare projections on cost of disease prevention and control as a proportion of total production cost. These projections would provide justification for inclusion of disease research in planning total production systems. There is a need to quantify effects on population abundance of known crustacean diseases.

2. Greater attention must be paid to the status of legislation, regulations, and registration of drugs used in disease control. An

annual update of these matters should be prepared and distributed at the World Mariculture Society Meeting.

3. Development of vaccines for disease control is a highly important area. An annual update on developments in this field should be prepared and distributed to World Mariculture Society members.

4. Virus diseases of marine animals are being recognized, and can be of significance to marine aquaculture. Research in this area must be intensified.

5. An attempt must be made to develop standard diagnostic methodology for important diseases in marine aquaculture.

6. The role of introductions of non-indigenous species in disease transmission is poorly understood, and research on the subject should be a part of any planned introductions. The development of brood stock that will eliminate importing disease problems should be emphasized.

7. Water quality and water chemistry are intimately associated with disease outbreaks, and measures to describe diseases must take into account the often dominant role of water quality.

8. Information on histology is lacking for many aquaculture species. Because histopathology is dependent upon knowledge of normal histology, greater efforts in normal histology should be supported.