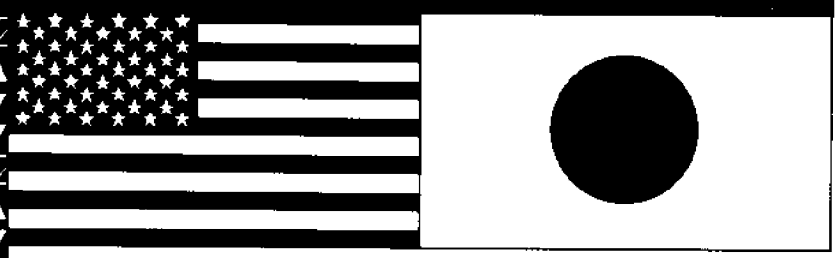
Spawning and Maturation of Aquaculture Species



PROCEEDINGS OF THE TWENTY-EIGHTH US-JAPAN NATURAL RESOURCES AQUACULTURE PANEL

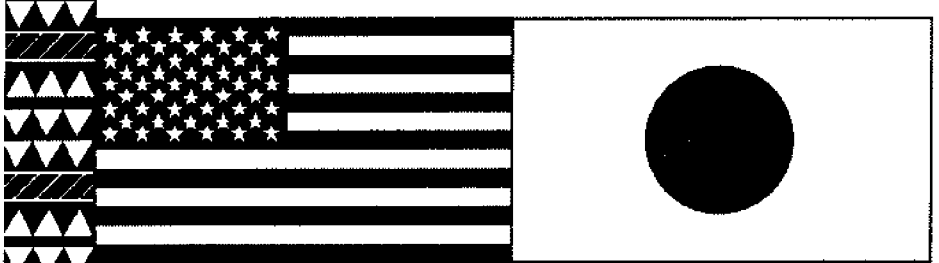
HAWAII

Edited by C.C.-T. Tamaru, C.S. Tamaru, J.P. McVey, and K. Ikuta

UJNR Technical Report No. 28

Spawning and Maturation of Aquaculture Species

Kihei, Hawai'i U.S.A.
10-12 November 1999



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PREFACE

The United States and Japanese counterpart panels on Aquaculture were formed in 1969 under the United States-Japan Cooperative Program in Natural Resources (UJNR). The UJNR-Aquaculture panels currently include specialists drawn from the government and academic departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture that could be of benefit to both countries.

The UJNR was begun during the Third Cabinet Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, current subjects in the program include toxic microorganisms, air pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resource research, development, and utilization.

Accomplishments of the Aquaculture panels include: increased communication and cooperation among technical specialists; exchanges of scientists and students; focusing of efforts to issues of major international concerns such as disease transmission and stock enhancement; exchanges of information, data, and research findings; annual meetings of the panel, a policy-coordinating body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects of international relations.

The 28th U.S.-Japan Aquaculture Panel Business Meeting, two Symposia, and several field trips were held from 7-16 November 1999 on the islands of O'ahu, Maui, and Hawai'i. The primary Symposium was held in Kihei, Maui, from 10-12 November and was organized by program chair Dr. Charles E. Helsley, Sea Grant Director and Dr. Clyde S. Tamaru, Sea Grant Extension Aquaculture Specialist at the University of Hawai'i at Manoa. The mini-symposium was held on O'ahu covering current aquaculture research activity in the State of Hawai'i.

Participants in the 28th UJNR Aquaculture Panel Symposium, held in Kihei, Hawai'i, U.S.A., November 10-12, 1999.

Dr. Clyde Tamaru calling the meeting to order.
Photo courtesy of Dr. H. Ohta.

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ACHIEVING NATURAL COLORATION IN FISH UNDER CULTURE

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ABSTRACT

Fish that are colored in nature often acquire faded coloration under intensive culture conditions. Experiments adding top-coated algae to the diets of ornamental fish have resulted in color enhancement. Freshwater red velvet swordtails *Xiphophorus helleri*, rainbowfish *Pseudomugil furcatus*, and topaz cichlids *Cichlasoma myrnae* became significantly more intensely colored when fed a diet containing 1.5-2.0% of a carotenoid-rich strain of *Spirulina platensis* and 1.0% of a specially grown *Haematococcus pluvialis* for 3 wk. Though color enhancement was apparent after only a wk, when the fish consumed these doses of algae, lower doses (0.5% and 0.4%, respectively) were not significantly different for kissing gouramis *Helostoma temmincki*, 24 K mollies *Pachouli latipinna*, and rosy barbs *Barbus chunkiness*, were examined after the 3-wk feeding period. Both treatments were significantly more effective than control treatments with no added carotenoid, and better than treatments with traditional carotenoid sources. Color enhancement appeared to occur via natural carotenoid receptors. Thus, color intensity diminished when fish were stressed, coloration appeared only in males in species where only the males are normally colored, and between rosy barbs and topaz cichlids color enhancement was environment-sensitive. Topaz cichlid color developed only after the aquaria were divided into territories and rosy barb color intensified when floating substrate was present. It is concluded that ornamental fishes are good models for color enhancement through diet and that this enhancement may be achieved using products made by marine biotechnology companies.

INTRODUCTION

The fledgling freshwater ornamental fish industry in Hawai'i has experienced the problem of faded coloration in fish, especially those grown in clear water. These fish are traditionally rejected by ornamental fish wholesalers, leaving the growers with fish that can't be sold or fish sold at a very reduced price. For this reason it was decided to investigate fish coloration. The current work was based on prior studies using cultured clownfish *Amphiprion ocellaris* and *Premnas biaculeatus* which only achieved color patterns and intensities seen in fish in the wild when treated for 2 wk by incorporating astaxanthin into the diet (Ako and Tamaru 1999). Several species of fish at the Waikiki Aquarium and Sea Life Park on O'ahu, Hawai'i, USA, responded similarly, and maintenance of the bright coloration appears achievable with incorporation of 25 mg/kg

astaxanthin into the various diets on a continual basis. However, chemically synthesized astaxanthin is a different stereoisomer from natural astaxanthin (Ako and Tamaru 1999) and is difficult for the small grower to incorporate into fish diets. Fortunately, carotenoid sources were available from a marine biotechnology company in Hawai'i in the form of carotenoid-rich strains of *Spirulina platensis* and specially grown *Haematococcus pluvialis*. Incorporation of these algae into the diets of ornamental fish is the subject of this report.

MATERIALS AND METHODS

All fish were treated (fed enhanced diets) in clear water aquaria equipped with biofilters. They were fed three times/d with meal sizes adjusted so that very few feed particles (<5% of the applied dose) remained in the aquarium after 10 min. All treatment periods were a duration of 3 wk.

Treatment algae containing carotenoids were obtained from Cyanotech Corporation (Keahole, Hawai'i, USA) and were added to the high palatability salmon fry feeds we are recommending to ornamental fish farmers (Tamaru and Ako 1997; Ako et al. 1997; Tamaru et al. 1998; Ako et al. 1999). The algae easily adhered to the 14% lipid feed particles.

Swordtail fish of the red velvet variety were obtained from grazed down (clear water) ponds. They were treated with the feed described above ± 1.5% *Spirulina* and 1% *Haematococcus*. Rainbowfish were obtained from clear water, indoor aquaria and treated with the feed containing 2% *Spirulina* and 1% *Haematococcus*. Control fish of each species were fed a flake feed which reported no source of carotenoids in the listed ingredients. Topaz cichlids were obtained as juveniles and were reared in the same way as the rainbowfish. Treated cichlids were fed as described above but control fish animals were fed a commercial cichlid feed containing alfalfa meal as the carotenoid source. Among the cichlids, no color changes were observed after 3 wk and the experiment was continued for an additional 3 wk after bottles were put into the tanks to provide territories.

The rosy barbs used in the experiment were obtained from a grower who had reared the fish in green water. Because the fish has bright coloration when obtained, they were held for 1 mo. in shaded, algae-free aquaria before the trials began. The rosy barbs were treated with one of four diets: a flake with no added carotenoid source, a flake with traditional carotenoids, a flake incorporating 0.5% *Spirulina* and 0.4% *Haematococcus*, and a flake with 1.5% *Spirulina* and 1.0% *Haematococcus*. Kissing gouramis and 24K mollies were also obtained from growers but were from shaded aquaria containing no algae and they received the same diets as the rosy barbs.

Color was judged by test panels of persons randomly recruited from around the biochemistry laboratory at the University of Hawai'i. The treatments were not revealed to the individuals who were asked to rank the fish according to intensity of color. Color ranking was by a score of 1-4 (one being the lowest) for fish with four treatment groups. Ties were allowed but did not often occur. Color rankings were either one or zero for fish of two treatment groups with the score of one relating to more intensely colored fish. Scores were subjected to ANOVA or *t*-test. In all cases, pairs of control and treatment fishes were photographed and can be seen at http://www.sandersbshrimp.com.

RESULTS

Panel members clearly identified the most intensely colored swordtails, rainbowfish and topaz cichlids. Those treated with *Spirulina* and *Haematococcus* (biotech algae) received significantly higher scores than control fish fed no carotenoid (Table 1).

Table 1. Mean scores of treated and control fish. Different alphabetical designations indicate significantly ($P<0.05$) different scores.

Fish	Number of panelists	SCORES Control	SCORES Experimental
Swordtails	8	0.0b	1.0a
Rainbowfish	15	0.1b	0.9a
Topaz cichlids	13	0.0b	1.0a

While the panelists convened after the 3-wk treatments, differences were already noticeable between treated and control swordtails after only 1 wk. It should be emphasized that, unlike some of the other fishes tested, only male rainbowfish and topaz cichlids acquired color.

Rosy barbs fed the *Spirulina* and *Haematococcus* were judged to be significantly ($P<0.05$) more intensely colored than rosy barbs given feed containing no carotenoids (control) or those given control feeds containing traditional carotenoid sources (traditional) (Table 2). There was a dose effect of low (low biotech) or medium (med biotech) levels of *Spirulina* and *Haematococcus* in the feeds but the effect was opposite of expectations. The 24K mollies fed *Spirulina* and *Haematococcus* were significantly ($P<0.05$) more intensely colored than those fed no carotenoids or traditional sources of carotenoid though there was no detectable dose effect among fish fed *Spirulina* and *Haematococcus*. Kissing gouramis fed carotenoids were significantly ($P<0.05$) more intensely colored than those fed no carotenoids.

Table 2. Mean scores of treated and control fish. Different alphabetical designations indicate significant ($P<0.05$) differences.

Fish	Number of panelists	SCORES Control	Traditional	Low biotech	Med biotech
Rosy barbs	16	1.7d	1.9d	3.9a	3.0b
24K mollies	16	1.9c	1.4c	2.8a	3.6a
Kissing gouramis	16	1.0cd	3.0a	2.6a	2.8a

DISCUSSION

The treatments reported here are efficacious. Since they use natural algae that mimic the absorption of carotenoid that occurs in the wild, they should be more acceptable to consumers who may have concerns about the use of chemicals or hormones to enhance color.

A "cocktail" of algae supplying natural stereoisomers of β-carotene, zeaxanthin, lutein, canthaxanthin, and astaxanthin was used in the current work. The cocktail approach was taken because fish sometimes seem to metabolize carotenoids before depositing them onto natural receptors in the skin in a species dependent way (Miki et al. 1985; Matsuno et al. 1985; Katsuyama et al. 1987; Katsuyama and Matsuno 1988). This was observed in preliminary results whereby the blue-green fluorescent colors in discus fish *Symphysodon* var. seemed to be enhanced by feeding sources of β-carotene and red colors seemed to be enhanced by feeding sources of canthaxanthin and astaxanthin. However, the red color of red swordtails and tinfoil barbs *Barbus schwanenfeldi* seemed to be enhanced by β-carotene sources.

The test panels used to judge color intensity in the current work were modeled after panels used to judge taste and texture of tropical fruits. Panelists were blinded as to treatments and did not interact with those who recorded results, as the latter stood physically behind the panel members. Chemical extraction (Miki et al. 1985; Matsuno et al. 1985; Katsuyama et al. 1987; Katsuyama and Matsuno 1988) and fiber optic reflectance spectrophotometry (Wallat and Lazur 1999) are other methods of quantifying color intensity. The test panel method, however, offers the advantages of low cost and convenience but color photographs (which are expensive to produce) have an immediate and long-lasting impact. Photographs of control and experimental swordtails and rainbowfish may be viewed on the Internet at http://www.sandersbshrimp.com.

The current work should be of immediate interest to pet feed manufacturers who service customers who keep their fish in clear water aquaria. The coloration of pet fish will fade if the fish are not provided with carotenoids. Traditionally, manufacturers have used complex formulations which may result in reduced palatability when introducing carotenoids into their feeds. The current work may also be of interest to ornamental fish feeds manufacturers who service growers with grazed down or otherwise clearwater systems, but it should be noted that ornamental fish growers do not use large amounts of feed because their fish are small. The largest market for the enhancement formulas may be food fish growers who wish to color the flesh of their fish or who grow fish such as the

red sea bream that lose their coloration under culture. The algae described here work well to provide color and also offer advantages in application. They may be used to topcoat other feeds, which is appealing in simplicity, reducing losses during extrusion. Because the algae used in this study are natural products, their use would negate possible hesitation of the public to consume food fish grown on chemically produced algae and satisfy those who want to feed more "organic" feeds to their pets.

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ARTIFICIAL INSEMINATION AND SPAWNING OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI*: IMPLICATIONS FOR A SELECTIVE BREEDING PROGRAM

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ABSTRACT

Through the U.S. Marine Shrimp Farming Program, the Oceanic Institute has established a breeding program where Pacific white shrimp *Litopenaeus vannamei* are selected for rapid growth and resistance to Taura Syndrome Virus. Until recently, OI produced maternal half-sib families by mating one female with two different males within a 2-wk spawning period. The artificial insemination (AI) technique used to produce these families relied on the removal of both spermatophores from a single male and the application of the spermatophores over the thelycum of a ripe female. The female was then placed in a spawning tank where fertilized eggs were liberated. If a previously inseminated female developed ripe ovaries before the 2-wk period elapsed, she was inseminated again with spermatophores from a different male. In an effort to maximize the number of half-sib families and reduce the time that families are produced, a different AI technique was used to produce paternal half-sib families. With this technique, each of the two spermatophores from a single male was manually extruded and placed on the thelycum of two different females. This later technique resulted in a significantly higher ($P < 0.001$) spawning success (84% *vs* 58%) and females produced significantly more ($P < 0.001$) viable nauplii per spawn (24,400 nauplii *vs* 8,500 nauplii). Importantly, the time to produce selected families was reduced from 14 d to 9 d, and the number of half-sib families increased. These improvements have significant implications for a selective breeding program.

INTRODUCTION

Most shrimp cultured worldwide are either collected from the wild or offspring from wild-caught broodstock. This practice is risky because wild-caught shrimp may be carriers of pathogens, including viruses. Several of these viruses have devastated the global shrimp farming industry in recent years, resulting in the emergence of novel production systems that rely on pathogen exclusion (McIntosh 1999; Moss 1999). Additionally, there are concerns about the ecological effects of harvesting wild shrimp for aquaculture, and this activity has been implicated in changing the dominant species composition of wild shrimp caught by fishermen in coastal Ecuador (Landesman 1994).

A significant disadvantage in culturing wild-caught shrimp is the inability of the farmer to benefit from domestication and genetic improvement of stocks. Many penaeid shrimp possess characteristics that are amenable to selective breeding, including the ability to close the life cycle in captivity, a short generation time, and high fecundity. Recently, there has been an emergence of shrimp breeding programs in Asia and the Americas, and the Oceanic Institute (OI) has played a significant role in establishing some of the fundamental principles of operating such a program, including techniques of artificial insemination (AI).

Most commercial shrimp hatcheries rely on natural matings to produce larvae (described by Yano et al. 1988). Advantages of natural mates over artificial insemination are a greater number of nauplii produced per spawn and decreased labor costs. However, the male is unknown and this may be important information for selective breeding programs. The male may be identified by DNA fingerprinting if it is undesirable to use AI (Moore et al. 1999; Hetzel et al. 2000), but this approach requires sophisticated procedures and equipment that typically are unavailable to shrimp farmers. In the OI breeding program, specific males are mated with specific females by AI to produce half/full-sib families for estimation of genetic parameters, including heritability estimates, phenotypic and genetic variation, and phenotypic and genetic correlations. However, spawning success and the number of nauplii produced per

spawn are lower than with natural mates. Originally, OI operated its breeding program by producing maternal half-sib families. Production of half-sib families depended on multiple spawns of a single female within a 2-wk period. However, this approach may confound genetic analysis by introducing maternal and environmental effects. In addition, the ratio of full-sib families to number of dams was 1.2, whereas the goal of the breeding program was to obtain a ratio of 2.0. A reduction in the number of half-sib families in the breeding program results in less accurate estimates of genetic parameters. In light of the drawbacks associated with producing maternal half-sib families by AI, an experiment was conducted to compare two AI techniques in order to determine the most efficient method to produce offspring for the breeding program at OI.

MATERIALS AND METHODS

Broodstock shrimp were obtained from the shrimp production facility at OI and were negative for specifically listed pathogens (Lotz 1997). Three 4.3-m diameter maturation tanks were stocked with 70 female and 40 male Pacific white shrimp *Litopenaeus vannamei* at an initial mean weight of 54 g (SD = 9.0 g) for females and 42 g (SD = 7.0 g) for males. Prior to stocking, all broodstock were tagged with colored, numerically coded, plastic eyestalk tags (National Band and Tag Co., Newport, KY, USA) to facilitate individual identification of the shrimp. Unilateral eyestalk ablation was performed on all females within 1 wk after the first molt in the maturation system (Wyban and Sweeney 1991). Broodstock shrimp received a maturation diet consisting of enriched *Artemia*, bloodworms, and squid and were fed between 24-28% of their biomass per day. The maturation diet was provided four times daily at 0830, 1100, 1330, and 1600. Environmental conditions were standardized among the three maturation tanks; flow rates were 13-15 L/min (200% daily exchange), water temperature was 28-29 C, salinity was 33-35 ppt, and dissolved oxygen was 4-5 mg/L. Photoperiod was set for gradual sunrise at 0300 and sunset at 1600 (13 h light and 11 h dark). Sourcing for ripe females (stage IV and V, described by Yano et al. 1988) began 2 wk after feeding was initiated and was conducted daily at 1300 h. Ripe females were randomly inseminated by one of two different techniques detailed below.

Double Spermatophore Technique: (used to produce maternal half-sib families)

1) Capture a female with full ovarian development (stage IV-V), visually identifiable by well-developed ovaries which are thick from the posterior edge of the carapace through the posterior end of the abdomen. The ovarian lobes at the base of the carapace should also be fully developed and olive-green in color.

2) Identify a male with fully developed spermatophores and manually eject both spermatophores by applying gentle pressure to the base of the outer corner of the spermatophore until it slips out of the genital pore. Healthy spermatophores show no signs of melanization, are white in color, slightly swelled and are hard to the touch.

3) Carefully hold the ripe female so that her thelycum is exposed. The fourth and fifth sets of pereopods should be directed posteriorly and held against the ventral surface of her abdomen. Dry the exposed thelycum by blotting it with a paper towel.

4) Place the first spermatophore anterior to the thelycum between the base of the third and fourth pereopods perpendicular to the long axis of the body. Return the fourth set of pereopods to their normal position, securing the first spermatophore in place. Place the second spermatophore posterior to the thelycum between the base of the fourth and fifth pereopods perpendicular to the long axis of the body. Return the fifth set of pereopods to their normal position, securing the second spermatophore in place. Using an index finger, spread the glutinous material surrounding the spermatophore structure to cover the thelycum. Place the female in a spawning tank overnight. The insemination process should be completed in less than 1 min to reduce stress to the female.

Single Spermatophore Technique: (used to produce paternal half-sib families)

The procedure for the single spermatophore technique is identical to that of the

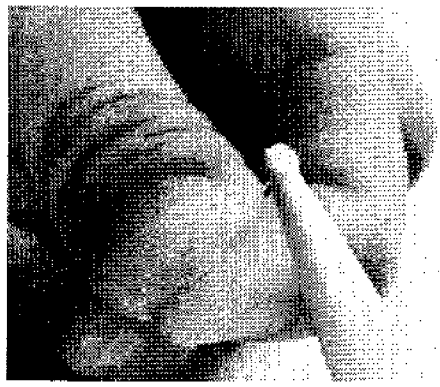

Figure 1. Liberating sperm mass from the spermatophore of a male *L. vannamei* broodstock.

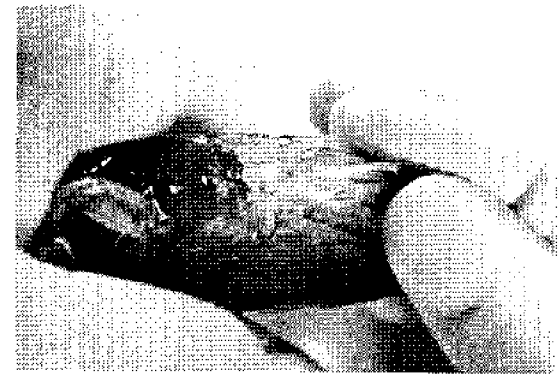

Figure 3. Completed artificial insemination via single spermatophore technique.

double spermatophore technique through step 3.

4) Place a single spermatophore between the thumb and index finger with firm constant pressure being applied from the bottom (closed end) toward the top (open end) of the spermatophore. This pressure ruptures the sperm sac and liberates a sperm mass that forms a droplet between the thumb and index finger. It also separates the sperm mass from a sheath of glutinous material and the spermatophore. Using angled forceps, remove the sperm mass so that the droplet sits on top of the closed tip of the forceps (Fig. 1).

5) Hold the female securely in the position described in step 3 and carefully place the sperm mass inside the thelycum by inverting the forceps (Fig. 2). The thelycum serves as the seminal receptacle and is enclosed by the coxae of the third and fourth set of pereopods and also partially by the ventral setae of these structures (Dall et al. 1990). After the sperm mass is correctly positioned, return the pereopods to their normal position, which helps to "lock in" the sperm mass,

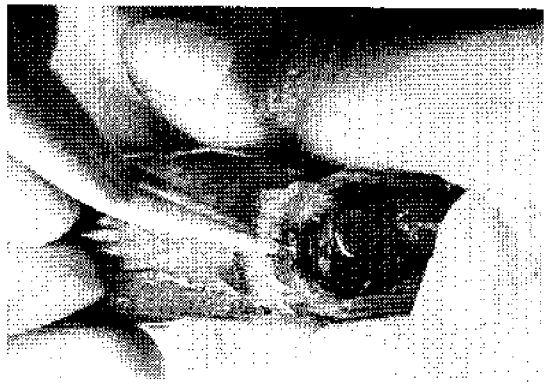

Figure 2. Placement of sperm mass into the thylecum of a female *L. vannamei* broodstock.

and place the female in a spawning tank overnight (Fig. 3). This process should be completed in less than 1 min to reduce stress to the female.

Prior to insemination, all spawning tanks were filled with 300 L of filtered sea water at 29 C and aerated with a 2-cm air stone. A solution of 10 ppm EDTA (disodium salt, Sigma Chemical, St. Louis, MO, USA) at a volume 150 ml was added to each spawning tank. Tanks were checked for eggs the following morning at 0800 h and females were returned to the maturation tanks. In tanks containing successful spawns, aeration was increased to promote gentle mixing of the water column and eggs were allowed to hatch for 2-3 h. Between 1000 and 1100, air stones were removed and viable nauplii were collected using a light attached to the side of the spawning tank. Positively phototactic nauplii were harvested by siphoning into a 10-L bucket and rinsed with fresh sea water. Nauplii were sampled using a 10-ml Hensen-Stemple pipette (Wildlife Supply Company, Saginaw, MI, USA) and counted volumetrically. A successful spawning event was defined by the production of at least 3,000 viable nauplii by a single female. Spawning success and number of viable nauplii produced per spawn were compared between the two AI techniques with ANOVA using SAS version 6.12.

RESULTS AND DISCUSSION

The single spermatophore technique resulted in a significantly higher ($P < 0.001$) spawning success (84%; n = 120) than the double spermatophore technique (58%; n = 133), and it produced significantly more ($P < 0.001$) viable

nauplii per spawn (24,400 nauplii *vs* 8,500 nauplii). The number of half-sib families produced with the single spermatophore technique also increased. The ratio of full-sib families to number of sires was 1.7 for the single spermatophore technique, whereas the ratio of full-sib families to number of dams was 1.2 for the double spermatophore technique. In addition, the time it took to produce 80 families decreased from 14 d to 9 d. In a recent breeding run using only the single spermatophore technique, the mean number of viable nauplii per spawn was 36,500, spawning success was 87%, and the half-sib ratio was 1.7.

An important distinction between the two AI techniques described above is that, in the single spermatophore technique, the sperm sac is ruptured thereby liberating the sperm mass from the chitinous spermatophore. Also, in this procedure, the sperm is separated from a sheath of glutinous material surrounding the spermatophore that may interfere with fertilization. Using forceps to apply the sperm mass inside the thelycum allows for more effective fertilization of eggs and increases the number of viable nauplii. In addition, using a single spermatophore per female is a more efficient use of male gametes and increases the number of half-sib families because it precludes the need for a specific female to spawn twice in a 2-wk period.

One advantage of producing an increased number of viable nauplii in a shorter time is that potential confounding environmental effects are minimized. Increasing the number of half-sib families is advantageous to a breeding program because it allows for more accurate estimates of genetic parameters, such as heritability and genetic correlations. Heritability describes the percentage of phenotypic variance that is inherited in a predictable manner and is used to determine the potential response to selection. Heritability estimates may be used to estimate progress in a breeding program, whereas phenotypic and genotypic correlations may reveal an indirect response, either positive or negative, to a breeding plan. Use of the single spermatophore AI technique and production of paternal half-sib families has improved the efficiency of the breeding program at OI and should be considered in other shrimp breeding programs.

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PROBLEMS IN SPECIES IDENTIFICATION OF THE MUD CRAB GENUS *SCYLLA* (BRACHYURA: PORTUNIDAE)

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ABSTRACT

Mud crabs in the genus *Scylla* inhabit brackish waters such as mangrove areas and estuaries and are widely distributed throughout the Pacific and Indian Oceans, from Tahiti, Hawai'i, New Zealand, Australia and Japan to southern Africa. This species is an important fisheries and aquaculture resource in Australia, Japan, Taiwan, Indonesia, and the Philippines.

The Japan Sea-Farming Association (JASFA) has developed technologies for stock enhancement activities, such as broodstock management, seed production, and experimental release of hatchery-raised juvenile mud crab in Japan. Seed production is performed using three morphologically distinguishable species, *S. serrata*, *S. tranquebarica*, and *S. oceanica*. If these three morphologically distinguishable species are, in fact, genetically different, it is recommended that resource management activities be tailored to each species, individually.

Stephenson and Campbell (1960) regarded four varieties of mud crab as one species using samples collected from Queensland and New South Wales, Australia, and suggested that the morphological differences were produced by environmental differences. Fuseya and Watanabe (1996), however, studied the genetic variability at three loci in the mud crab and reported that the species *S. serrata*, *S. tranquebarica*, and *S. oceanica* are clearly distinguishable from one another.

Distribution and Commercial Value

Mud crabs in the genus *Scylla* inhabit brackish waters, such as mangrove areas and estuaries, throughout the Pacific and Indian Oceans, from Tahiti, Australia, and Japan to southern Africa (Chahpgar 1947; Hill 1975; Sakai 1976; Dai and Yang 1991). This crab is an important fishery resource in Australia, Japan, Taiwan, Indonesia, and the Philippines where it is also targeted for aquaculture (Fukunaga and Fukumoto 1960; Fushimi 1983a; Cowan 1984; Oshiro 1988; Chin and Amandakoon 1992; Cholik and Hanafi 1992; Jamari 1992; Larda and Lin 1992; Rattanachote and Dangwatanakul 1992; Watanabe and Sulistiono 1993; Watanabe et al. 1996). In recent years, this mud crab has been selected as one of the target species for stock enhancement programs in Japan (Fukunaga and Fukumoto 1960; Fushimi 1983b; Oshiro 1988). Stock enhancement activities, such as broodstock management, induced spawning, seed production, and acclimatization for release, have been carried out by the Japan Sea-Farming Association and some Prefectural Fisheries Experiment Stations. Artificial seed production has been successfully achieved for three morphologically distinguishable species, *Scylla serrata*, *Scylla tranquebarica*, and *Scylla oceanica* (Cholik and Hanafi 1992; Larda and Lin 1992). Seed production and release data for *Scylla tranquebarica* from 1984 to 1997 are shown in Fig.1. Seed production of the mud crab has steadily increased year after year and over 4 million individuals were produced in 1996. Annual release is also increasing steadily from an average of 500,000 during the early years to 1 million individuals in 1996. The annual fluctuation in seed production and release of *Scylla oceanica* is summarized in Fig. 2. In contrast to *Scylla tranquebarica*, seed production of *Scylla oceanica* is more difficult. Controlling the environmental conditions, such as salinity, has

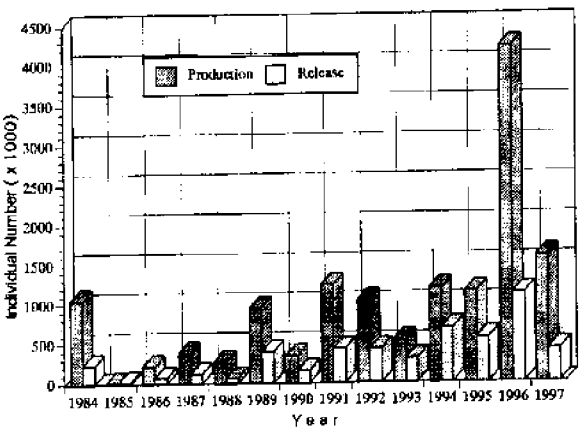

Figure 1. Annual fluctuation of individual number of seed production and release of *Scylla tranquebarica* (Data from JFA and JASFA).

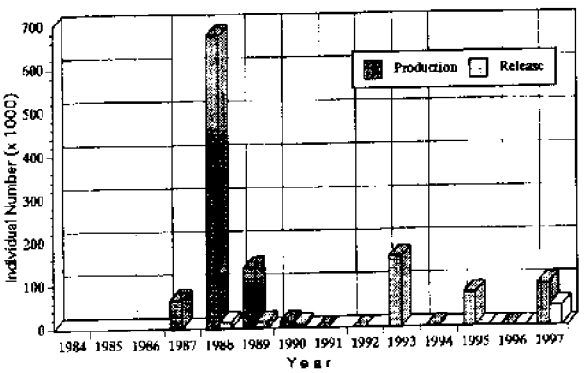

Figure 2. Annual fluctualtion of individual number seed production and release of *Scylla oceanica* (Data from JFA and JASFA).

hampered successful larval rearing past the zoeal stage. Although seed production has at times reached 700,000 individuals, the overall production and release of *Scylla oceanica* remain comparatively low.

Species Identification of the Mud Crab

The species identification of mud crab has been controversial, and for many years, only one species was recognized in the genus *Scylla* (Fuseya 1998). Recently, however, researchers have reported that the genus *Scylla* includes several species (Estampador 1949; Serene 1952; Stephenson and Campbell 1960; Ong 1964; Fushimi 1983a; Joel and Raj 1983; Oshiro 1988; Kathirval and Srinivasagam 1992; Fuseya and Watanabe 1995, 1996; Watanabe and Fuseya 1997; Fuseya 1998). As summarized in Fig.1 and 2, seed production of mud crabs in Japan is performed using three morphologically distinguishable species. Estampador (1949) classified the mud crab into three species and one variety, *S. serrata*, *S. oceanica*, *S. tranquebarica* and *S. serrata* var. *paramamosain* using specimens collected in the Philippines, based on their external morphology (e.g., color of carapace and legs, anterolateral teeth of carapace, and outer spines of cheliped carpus) and gametogenesis. Serene (1952) also recognized the existence of four forms in Vietnam, in accordance with the finding of Estampador (1949). However, Stephenson and Campbell (1960) regarded the four forms as only one species and based their conclusions on samples collected from Queensland and New South Wales, Australia. They suggested that the morphological differences were produced by environmental differences and it is their recommendation that: "Therefore, and tentatively, the four forms of Estampador and of Serene are fused into synonymy." Subsequently, Ong (1964) in Malaysia and Joel and Raj (1983) from India also noted differences in forms between specimens.

Fushimi (1983b) pointed out the presence of three forms of mud crabs in Hamana Lake, and Oshiro (1988) recognized at least three species based on specimens from Japan. Fuseya and Watanabe (1996) carried out a study on genetic variability at three loci in the mud crab and determined that three species, *S. serrata*, *S. tranquebarica*, and *S. oceanica*, could be clearly distinguished. Overton et al. (1997) carried out a multi-variate analysis of mud crabs from four locations in Southeast Asia. Although they could distinguish three distinct morphological forms, their conclusion was the same as that of Stephenson and Campbell (1960), that the morphological differences were produced by environmental variations. They did not, however, specify what environmental condition(s) would produce the three morphological forms. Keenan et al. (1998) made a revision of the genus *Scylla* using specimens collected from the Red Sea and from locations throughout the Indo-Pacific region. Two independent genetic methods, allozyme electrophoresis and sequencing of two mitochondrial DNA genes (cytochrome oxidase I and 16s RNA) were employed in an attempt to differentiate species. They recognized up to four

species using morphological criteria but there were differences in nomenclature.

Fuseya (1998) carried out morphometric analyses of specimens within the genus *Scylla* collected from throughout the geographic distribution of the mud crab. She also examined morphological characteristics of the first and second pleopod of the male sex. Based on her analysis, the species *S. serrata*, *S. tranquebarica*, and *S. oceanica* were clearly distinguishable. Morphological characteristics of these three species were found to correspond to those described by Estampador(1949). The dorsal and ventral views of the carapace are compared in Fig. 3. The morphological characteristics of the rostrum and antero-lateral teeth and cheliped are summarized as follows:

S. serrata: Front cut into four lobes or blunt teeth of about equal size and prominence. Antero-lateral border cut into nine sharply acuminate teeth of about equal size.

S. tranquebarica: Front cut into four sharply acuminate teeth with spine. Antero-lateral border cut into nine sharply acuminate teeth with spine.

S. oceanica: Front cut into four acuminate teeth of about equal size. Antero-lateral border cut into nine sharply acuminate teeth.

There are also differences in the numbers of spines of the cheliped carpus and behind the finger joint The first pleopod tip of each of the three species was examined using scanning electron microscopy and found to be different from each other (Fuseya 1998).

Genetic information is essential for the identification of the three species of the mud crabs in the genus *Scylla*. From June 1994 to May 1995, Fuseya and Watanabe (1996) collected and identified 342 mud crabs from seven locations (Lake Hamana and Okinawa in Japan, Bali and Cilacap in Indonesia, Chantaburi and Surat Thani in Thailand, and Madagascar). They initially classified the crabs into three species, *S. serrata*, *S. tranquebarica* and *S. oceanica*, according to Estampador (1949). Horizontal starch gel electrophoresis was used to analyze muscle tissue

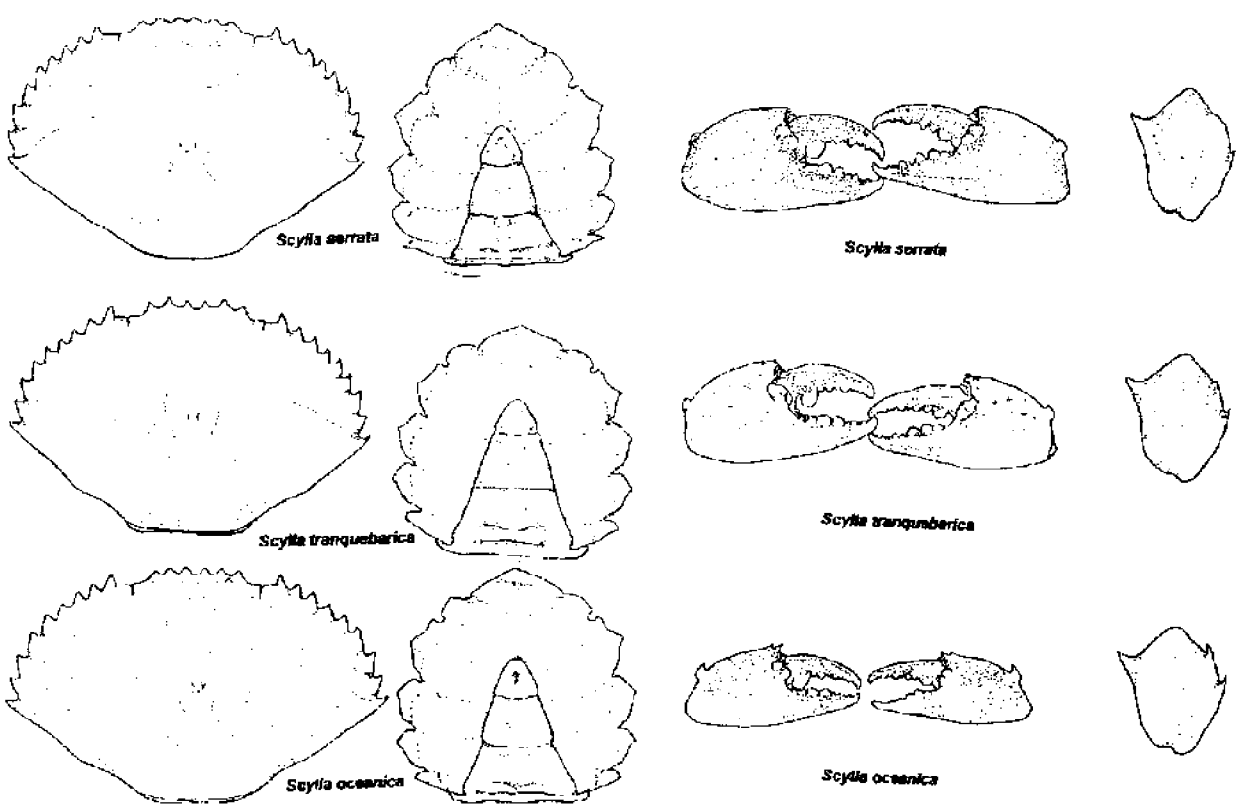

Figure 3. Comparison of carapace (dorsal and ventral view) and cheliped in three species of genus *Scylla* (male). (After Fuseya 1998).

for variation in 11 enzymes at 17 allozymic loci in the three proposed species. Seven of the 17 loci were found to be polymorphic, and fixed differences were detected at three loci (EST, LAP-2, and SOD). The fixed differences found at 3 of the 17 loci sampled and the relatively large genetic distances calculated between the species verify the existence of at least three species of mud crabs in the genus *Scylla*. The three species classified by Estampador (1949) based on morphological traits coincide with the genetic results obtained in this study, however, the genetic analysis shows that *S. serrata* and *S. tranquebarica* are more closely related than *S. oceanica*. The mean heterozygosity of the genus *Scylla* (the whole population) is 0.108. This value is high compared with those reported for other crustacean species. For example, values of mean heterozygosity in other crustacean species are 0.007-0.014 in the swimming crab *Portunus trituberculatus*, 0.0004-0.02 in the snow crab *Chinonoecetes opilio*, 0.072-0.077 in the spider crab *C. japonicus*, and 0.023-0.032 in the hair crab *Erimarcus isenbeckii*. The mean heterozygosity found in this study for each of the mud crab species within the genus *Scylla* is 0.049 for *S. serrata*, 0.014 for *S. tranquebarica*, and 0.004 for *S. oceanica*. The genetic variability of *S. serrata* is higher than that of *S. oceanica*. The information obtained in the current investigation establishes that the three species of mud crabs are clearly distinct from each other and implies that the stocks of each species should be managed separately.

We would recommend that the morphological description of Estampador (1949) is sufficient for species identification of the genus *Scylla*, and it is necessary to confirm the identification of the three species for further study on the crabs in the genus *Scylla*.

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HAWAI'I OPEN OCEAN AQUACULTURE DEMONSTRATION PROGRAM

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ABSTRACT

Although Hawai'i has been a leader in aquaculture research and development in the Pacific for several decades, no offshore aquaculture activity has been undertaken primarily because our offshore waters tend to be quite rough. The Hawaii Open Ocean Aquaculture Project (HOARP), a joint project between University of Hawai'i (UH) Sea Grant, Safety Boats Hawai'i, and the Oceanic Institute, began in 1998. An OceanSpar SeaStation 3000 sea cage was ordered in the fall of 1998 and growout operations commenced in April 1999.

Pacific threadfin *Polydactylus sexfilis*, locally known as *moi*, was chosen as the test species. The brood stock spawned in early February 1999 and 70,000 70-d-post-hatch juveniles (5-7 cm BL) were transferred to the cage in mid April. A special nursery cage was placed inside the main cage to contain the small juvenile fish and was used for approximately 4 wk, until the fish had reached sufficient size that they could no longer escape from the outer net.

Pacific threadfin are surf zone fish and one goal of the demonstration was to determine how these shallow water fish would grow at deeper depths. The top of the cage was at approximately 12 m depth during the growout of the fish. Although the fish are naturally adapted to very shallow water, they performed well at the greater depth.

Feeding was accomplished by means of a tube (10 cm in diameter) connected to the surface. A slurry of water and pelletized feed was introduced for several hr each d. Feed was supplied to the growing fish at a rate of several percent of total fish mass/d. Divers monitored the feeding which was terminated when a slight rain of pellets began to reach the bottom of the cage. Harvesting began in late August of 1999, about 4.5 mo after the fish were introduced into the cage, and continued on an incremental basis until October when the harvest of fish was completed.

The experiment successfully demonstrated the feasibility of growing *moi* at depths of 15 to 30 m and showed that this could be done in an economically viable way with no adverse environmental impact to water column, the sea floor, or the nearby coral reefs.

INTRODUCTION

Hawai'i, an island state with limited land area but extensive offshore waters, has long viewed aquaculture as a viable means of adding to the economy. Beginning more than 800 yr ago, the ancient Hawaiian culture built fish ponds on the reef flats surrounding most of the islands for the culture of both brackishwater and saltwater fish (Kikuchi, 1976; Tamaru et al. 1998). The species cultivated in these ancient facilities included the grey mullet (*Mugil cephalus*) and the pacific threadfin or *moi* (*Polydactylus sexfilis*) as well as a broad mixture of other wild species.

With the growth of an industrialized society over the past century, most of these fishpond facilities have been allowed to deteriorate or have been filled with rock and soil, given the value of land. Few ponds remain today, and there is question as to whether the low-density culture technique practiced in the fishponds is economically viable given the cost of labor and the regulatory regime under which the operation must function (Tamaru et al. 1998).

However, the past practices can also be looked upon from another perspective. These rock structures constructed on the reef flats were a 'state-of-the-art' means of cultivating the ocean many centuries ago. But in today's culture, modern high-strength materials such as steel and Spectra netting replace the rocks and sticks of yesteryear and thus the modern fishpond becomes a net-enclosed structure supported by a steel frame. And these modern materials permit the utilization of waters further offshore and thus further from the environmentally sensitive coastal region.

RATIONALE AND OPPORTUNITIES

It is well recognized worldwide that capture harvest of wild fisheries has reached critical levels and will not be sustainable in the near future. Aquaculture production of target species has been viewed as a means to supplement increasing demands for fish and fishery products. Seafood demand is projected to nearly double in the next quarter century based upon population growth alone and, as New (1997) has pointed out, aquaculture production must rise to meet this increase in demand.

Great strides have been made in marine aquaculture technologies in the past decade and it is now possible to produce many species of fish in land-based intensive culture systems at costs that are comparable to or below those of harvesting wild stocks. Expanding the use of the ocean to include aquaculture will provide economic opportunities for fish farmers, engineers, commercial fishermen, and seafood processors by enabling the production of aquaculture products at commercially significant levels.

The commercial development of offshore aquaculture in the United States has been impeded by the lack of demonstrated feasibility in critical areas, such as engineering of containment structures to withstand open-ocean conditions, adequate information on rate of growth and survivorship in containment structures for interesting species, and efficient offshore production management and harvesting methods. These broad issues, as well as more regionally specific issues such as regulations regarding marine deployment and environmental protection, were addressed by the Hawaii Open Ocean Aquaculture Project (HOARP). This project was the first integrated demonstration of the potential for offshore production in the State of Hawai'i, and was designed to demonstrate the feasibility and practicality of offshore culture of a high-value, ethnically desirable, locally endemic fish.

The project was funded by the National Sea Grant Office of the National Oceanic and Atmospheric Administration (NOAA) and called for the demonstration of the feasibility of offshore aquaculture in Hawaiian waters as the primary goal. This project made maximum utilization of existing information including the marine finfish culture technologies for *moi* developed by the UH Sea Grant College Program (May 1976) and the Oceanic Institute (Ostrowski 1998) with a commercial offshore containment system supplied by Net Systems (Bainbridge Island, WA, USA). Throughout the project considerable effort was made to assure that the project was conducted in an environmentally acceptable way, for environmental acceptability is a critical factor in the long-term viability of an offshore mariculture industry in the State of Hawai'i.

Site Location

The primary constraints to conducting offshore aquaculture in Hawai'i are twofold: environmental compatibility issues, and the physical constraints of weather, oceanographic conditions, and port access. To minimize these constraints, we considered and evaluated several sites along the south and west shores of O'ahu. In the end we chose to do the initial experiments about 13 km west of Honolulu Harbor at a site about 3 km offshore in water about 30 m deep. A site with a sandy bottom was chosen as ideal for the type of anchors being used and also to minimize the impact on the coral reef ecosystem (an ecosystem perceived to be environmentally sensitive and easily damaged). The nearest reef is about 800 m away. The site for the research project is located to the west of the entrance to Pearl Harbor, approximately 3 km SSE of the coastal community of Ewa Beach and is adjacent to a military testing area.

Project Objectives

Feasibility of offshore culture of finfish is dependent upon several real issues that deal with the biologic performance of the fish and the culture system, several perceived issues that deal with the environmental issues of cage culture in an area that prides itself on scenic beauty and the high water quality in offshore areas, and the general need for the determination of economic costs and benefits of this type of activity. Within these general issues in mind, several specific project objectives were defined to:

- Demonstrate that environmentally compatible, offshore aquaculture can function in Hawai'i
- Develop a cooperative, effective, and coordinated partnership among the key Hawai'i contributors to the project, and serve as a test model for offshore aquaculture research and demonstration projects throughout the United States
- Conduct test marketing of aquaculture products derived from project activities both locally and worldwide
- Demonstrate feasibility of submerged growout of *moi*
- Develop means of underwater feeding and harvesting
- Identify and examine issues that might constrain future industrial development

ACCOMPLISHMENTS

A site was identified and research activity was permitted in approximately 30 m of water at a location with a sandy bottom with no nearby coral formation. All offshore waters in Hawai'i are classified as a conservation district and thus special permits are required for any activity within 5 km of shore (State of Hawai'i waters). Rather than go through the lengthy full permitting process required by the regulations governing an offshore lease, the project chose to avail itself of the simpler procedure of applying for a permit to do research within the conservation zone. But even this process is cumbersome, for consultations were required with many agencies or divisions of those agencies. These included five divisions of the Department of Land and Natural Resource (DLNR) - (Aquatic Resources, Land, Boating and Recreation, and the Aquaculture Development Program) the Department of Health, the Department of Economic Development and Tourism, the Office of State Planning (particularly its Office of Environmental Quality Control), the United States Coast Guard, the United States Army Corps of Engineers, and the United States Navy. In the end three written permits were required (DLNR Conservation District Use Permit, Army Corps of Engineers, DLNR-Boating and Recreation Mooring Permit) and more than 9 mo elapsed between the start of the process and the issuance of the final permit.

Once we were fairly confident that the necessary permits would eventually be forthcoming, anchors were placed on the sandy sea floor in the four corners of an imaginary rectangle about 100 by 300 m in extent. Four anchors, with anchor lines five times as long as the water depth, were used and, since bi-directional strong currents were known to exist in the area, safety considerations required that two anchors, each capable of holding the cage in place, be deployed to each side of the cage. Each anchor is 3 t or more in weight (more than needed but the price was right - free) and each line has a subsurface float near the anchor end to hold the line in a constant taut configuration. All anchors and cage deployment activities were constrained to be within 300 m of the permitted location.

Cage System

A totally enclosed, semi-submersible cage produced by Net Systems was chosen for the project. Several types of cages were considered but the perceived need for a cage that could be maintained in a fully-submerged condition was thought to be an important, and perhaps even critical, parameter. Thus an OceanSpar Sea Station 3000 was purchased.

The cage is bi-conical in shape, approximately 25 m in diameter and 15 m in depth with a working volume of about 2,600 cubic m (see Fig. 1). The cage was moored on a four-point anchor system with a working anchor scope in excess of 5:1. Each anchor was larger than required since they were from a stock of surplus anchors and the smallest had a mass of greater than 3 t. Each anchor was attached to the cage with Spectra mooring line and a short piece of heavy anchor chain. Subsurface floats were attached to the Spectra line approximately 20 m from the chain to provide a compensation system that assures that the anchor lines remain taut, yet shock absorbing, at all times.

The skin of the cage consists of a hexagonal mesh of Spectra netting with an opening of 2 cm. The netting is stretched taut between 12 guy lines that attach the ring to the spar. For purposes of regulation, the Coast Guard

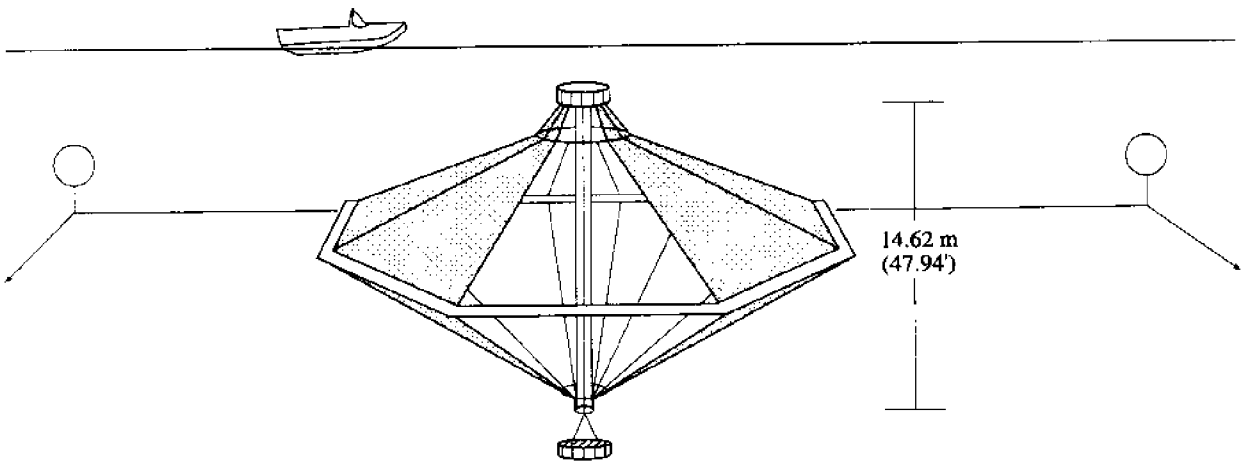

Figure 1. Schematic presentation of the sea cage used in this project.

has defined the cage structure as a moored vessel, a barge, when it is at the surface.

The cage was constructed at the surface of the sea. Once construction was completed, half of the spar was flooded and the cages settled until its ballast weight contacted the sea floor. The cage remained fully submerged, with the very top of the cage about 12 m below the surface, for the duration of the experiment. All stock introduction, feeding, and harvesting was done from this fully-submerged location.

Species of Culture

The species of fish chosen for culture is the indigenous Pacific threadfin (*Polydactylus sexfilis*), known locally as *moi*. This fish has been highly regarded in Hawai'i since ancient times when it was known as the food of the *ali'i*, only to be consumed by members of Hawaiian royalty. *Moi* was commercially fished in the past but over-fishing and over-regulation effectively removed it as a commercially exploitable stock (see Fig. 2). Today, annual commercial catch from the wild averages less than 1,000 kg, although considerably more is thought to be caught for individual consumption.

In the 1970s the UH Sea Grant College Program supported research into the life cycle of *moi* and over the last 5 yr the Oceanic Institute has developed culture technologies appropriate for the threadfin making it the newest commercially grown species in Hawai'i. The species grows well in captivity, reaches a market size (350 g) in 6 mo and sells in the round for about US$10/kg at the farm gate.

Moi is the only marine species currently being cultured in Hawai'i in numbers sufficient for the demonstration project. Methods for the hatchery production of fry have been described by Ostrowski and Molnar (1998). The parents of the cultured *moi* are captured from the indigenous wild stock of Hawai'i and genetic mapping has indicated that fish are of one genetic stock. *Moi* from the same parents are currently being grown for release for stock enhancement purposes. Thus an accidental release of fish from the cage would have no adverse genetic impacts on wild populations. A cooperative export marketing plan was devised to ensure that local farm producers would not be adversely affected by our test offshore production efforts.

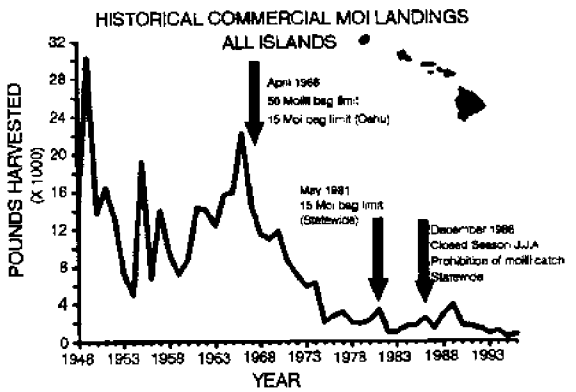

Figure 2. Commercial landings of the *moi* in Hawai'i between 1948 and 1996. Arrows indicate implementation of fishing restrictions. Source: Division of Aquatic Resources, State of Hawai'i.

Operations

The cage was deployed in March 1999 and is expected to remain in the water 18-24 mo. It was constructed and deployed approximately 1 mo prior to fish delivery to assure it was sea worthy. A few days prior to the introduction of the juvenile fish to the cage, an inner nursery net (0.5 cm mesh) was deployed around the central spar inside the main net. This nursery net has about one-tenth the volume of the main net. The nursery net within the main cage was stocked in mid-April 1999 with approximately 70,000 fingerlings of two cohort classes: 70-d-old Pacific threadfin (approximately 10 cm long and 12 g in weight) and 50-d-old fish weighing 4 g. The fish were fed to satiation each morning with a commercially pelleted feed (initially 2 mm pellets and later 5 mm pellets) for the duration of the project (6 mo). After about 1 mo, the inner nursery net was removed giving the young fish access to the entire main cage.

Harvest commenced in early September when fish reached 180 d of age (350 g in weight) and continued for approximately 2 mo until all the fish were harvested. At the end of the experiment, the cage and its associated mooring system was secured and left at the site to await a second experiment that was planned for the following year.

Fish were sampled once every month for weight and length and compared with growth studies conducted in tanks on shore. Growth was comparable in the tanks and in the cage although those in the cage grew slightly less rapidly and they appear to have had a wider size spread. Samples of fish were provided to the State Fish Pathologist (Dr. James Brock) on a more or less monthly basis and no disease was noted. The health of the fish was examined daily and any dead fish were removed and examined for parasites, bacteria, and other indicators of disease. None were noted. Mortality was generally low once the pulse of stress-induced mortality associated with the transfer operations ended. At harvest, 52,000 fish were taken from the cage with a total harvest weight of slightly more than 18,000 kg.

Feed conversion efficiency was less than expected and the feed conversion ratio (FCR) was 1.8 kg dry food/kg fish. Based upon previous tank experiments we had expected an FCR of 1.3 to 1.5. The fish fed in tanks on land, however, had a similar low FCR of 1.7 when fed under the same one-feeding-per-day regime. Thus, the relatively poor FCR performance for the cage at sea was probably due to the once-a-day feeding strategy. This strategy has been modified for the second experiment currently underway.

Environmental Analysis

Quantitative and qualitative assessments of pelagic and benthic animal and plant communities around the cage was conducted prior to cage deployment, during growout, and after fish harvest. Bottom samples taken from points around the cage showed little systematic change except during a period of overfeeding. During this period, feed was allowed to fall through the cage and this abundant source of food produced a change in the ratio of indicator organisms under the cage (Dave Bybee, personal communication). Once the extra feed was removed, the bottom biota rapidly returned to its pre-cage ratios.

Fish and algal communities on the outside of the cage were also monitored. Initially, algae rapidly accumulated on the mesh of the net and the divers were barely able to remove it by scrubbing as rapidly as it grew. But during the summer, an equilibrium was established between the growth rate of the algae and the foraging rate of the herbivores external to the cage. This community of fish, our 'worker fish', took about 3 mo to accumulate to an extent that they grazed off the algae as rapidly as it grew. The primary components of this fish community were broomtail filefish or *loulu* (*Alutera scripta*) and various surgeonfish or *palani* (*Acanthurus dussumieri*), *pualo* (*Acanthurus blochii* and *Acanthurus xanthopterus*). *Kāhala* (*Seriola dumerilii*), a carangid, also took up residence beneath the cage. Other fish that were observed from time to time include, Sandbar sharks (*Carcharinus milberti*), *'opelu* (*Decapterus macarellus*), *akule* (*Selar crumenophthalmus*), *kawakawa* (*Euthynus affinis*) and *ono* (*Acanthocybium solandri*).

Hydrological data also was collected three times during the experiment: once at the start, once near the time of maximum biomass,

and again at the end of the experiment shortly after harvest. Observations made included temperature, velocity and direction of the current, wave height, and turbidity. All parameters were within the range expected for Hawai'i waters although the current was generally less than that anticipated (it was rarely above one knot). Water samples also were taken down current and up current from the cage to determine water chemistry and total suspended solids. Unfortunately, the sampling frequency was too infrequent to establish a clear correlation with feeding rate or biomass. In fact, the data on water chemistry showed essentially no signal from the presence of the fish except for a slight elevation in the NH_4 level just downstream of the cage. The site is located in Class A waters between two point source high nutrient discharges (sewage discharge diffusers) and this, and the natural but variable outflow of nutrients from Pearl Harbor, produced high variability in the background water quality readings in the same range as those produced by the fish in the cage.

Prior to the experiment a number of UH scientists at both the Mānoa and Hilo campuses participated in several offshore aquaculture planning and advisory sessions. In general, the assessments and projections this group made prior to the experiment were born out by the observations made during the experiment. Assessment of the feeding levels proposed indicated that the nutrients from the cage would have no detrimental impact on the water column or the benthic communities surrounding the cage due to the large volumes of water involved. This was verified by observations during and after the experiment, for only temporary transitory effects were noted or no effect could be found. The advisory group also stated that given the currents present at the site and the rapid exchange of offshore waters and the immense expanse of the seas surrounding the islands, it is unlikely the small addition of organic carbon and nutrients to nearshore waters would have a measurable effect on water quality. Again, no effects were noted.

Retrospective Analysis

Looking back on the experiment, we can see that we learned may things. We established that a locally desirable fish, *moi*, can be grown in a totally submerged offshore cage. We also established a baseline protocol for the feeding and harvesting of the fish in the submerged cage and we established that no lasting negative environmental impact would result from the deployment of an offshore fish farm stocked with a limited number of fish.

But we also learned that the experiment had many shortcomings. We did not succeed in stocking the cage at a commercial density and our number of fish at harvest was far too low for the cost and effort it took to raise them. The cost of feeding the fish was between US$100,000 and $160,000 for labor and operations and US$60,000 in feed and supplies. This suggests our cost, without amortization of the cage or the consideration of maintenance and management costs, was about US$9/kg. In other words, the experiment was sub-economic and the number of fish would have to be increased by at least a factor of three to begin to have an economically viable growout effort. Moreover, many of the environmental effects were too small to be noticed due to the relatively small number of fish. Thus, in order to examine the economic viability and the potential environmental impacts on the ocean immediately outside the cage, the experiment should be repeated using between 100,000 and 150,000 fish.

Several technical issues must be addressed as well. First, a more efficient feeding methodology must be developed to include multiple feeding stations as well as a means for more continuous feeding. This may mean more time on station by those overseeing the feeding or it may require the development of an underwater feeding system that can provide one or more supplemental feedings each day.

A second technical issue is the method of harvesting. The OceanSpar SeaStation 3000 is designed to be bulk-harvested all at one time. A means to undertake a progressive harvest is needed for the market has yet to be developed for large quantities of *moi* at any given time. The project developed a rudimentary progressive harvest technique using a fish pump methodology. This worked, but it required a large expenditure of manpower, three or four divers, on harvest days.

Thus, a more efficient progressive harvest system is necessary.

The size of fish in any one harvest was quite variable. Initially this ranged from 250 g to 500 g and by the last harvest the range had increased to 300 g to 750 g. Since the market prefers fish of more uniform size, a means of grading the fish prior to harvest is desirable. This issue was not addressed during this experiment.

Marketing was not addressed in a systematic way. Naively, we thought we only had to call a wholesaler when the fish would be ready to harvest. It was a surprise to find that there was no market for these highly-prized fish outside of Hawai'i. Thus, if this fish is going to be used for a culture industry, much effort has to be spent on the development of markets outside the state.

Transfer of the fish to the cage proved to be more difficult than envisaged. This was in part due to the size of the fish. The large 10 to 12 g fish proved to be much more susceptible to stress from handling than anticipated. We deliberately chose to raise the fish to 70-d-post-hatch on land rather than transferring them at the usual 30 to 50 d so as to reduce the stress of placing the fish in a high current environment when they were still small. But this almost proved to be our undoing for nearly 30% of the fish died during the transfer operation. Lack of oxygen, poor water quality, possible elevated temperatures, and surface abrasions and the resulting secondary infections proved to be the primary causes of mortality. It is felt that a more rapid transfer, and a transfer at a younger age, could have avoided most of these problems. (This surmise was proven in the second experiment currently underway were younger fish (50-d-old) were transferred successfully with very low mortality).

Local sales were conducted at the UH Mānoa campus where we found good market demand when fish were sold at US$5-$6/lb (US$12-$14/kg.). Clearly there is a fairly large and untapped local market for this fish. International sales were primarily to Mexico where this unknown fish was readily accepted at a rate of about 3 mt/wk. The price was low for this introductory effort but it is thought that future international sales could be substantial if a market is properly promoted.

CONCLUSIONS

The Hawaii Open Ocean Demonstration Program successfully grew and marketed more than 50,000 fish (*moi* or *Polydactylus sexfilis*) in a subsurface offshore cage in an effort to define the constraints and impediments an offshore aquaculture industry in Hawai'i might face. For the most part, the operation went very smoothly and no insurmountable problems were found in the culture, operation, or marketing of the fish. The most difficult part of the project was acquiring the required permits. The project used a research permit approach, which required a minimum of permits, but even so, the permitting problem is probably the most significant issue to be overcome. Public acceptance of the project was high and the results suggest that offshore aquaculture, conducted with an appreciation for minimizing the real and perceived impacts on the environment, can be a viable business opportunity in Hawai'i.

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REPRODUCTION OF DOMESTICATED STRIPED BASS: COMMERCIAL MASS PRODUCTION OF FINGERLINGS

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ABSTRACT

Most commercial hybrid striped bass (HSB) farms rear sunshine bass produced by mating wild-caught female white bass *Morone chrysops* with male striped bass *Morone saxatilis*. The opposite hybrid (palmetto bass; *M. saxatilis* X *M. chrysops*) has been used less often because wild or domestic female striped bass are viewed as too difficult to reproduce when needed. Beginning in the early 1990s, our investigations at North Carolina State University, focused on developing detailed understanding and control of the reproductive biology of female striped bass while domesticating the species in captivity. These studies established the basic methodology required to reliably reproduce the domestic females to generate striped bass and palmetto HSB. Results of recent fingerling production trials using palmetto and sunshine HSB fry from domesticated (F_2-generation) parents demonstrated unequivocally the value of female striped bass as broodstock in the HSB farming industry. We repeatedly used domesticated striped bass females to generate palmetto HSB fingerlings using extensive (pond) culture methods on private farms with egg fertility and hatching rates, larval survival, and fingerling yields comparable to those obtained using fully mature, wild female striped bass captured on or near their spawning grounds. Reproductive performance of the female striped bass and survival of their progeny through the fingerling production cycle were also equal to or better than corresponding values for domesticated female white bass and their sunshine HSB progeny. This is the first report, of which we are aware, on the use of domesticated striped bass for commercial mass production of fingerlings.

INTRODUCTION

Farming of hybrid striped bass (HSB; genus *Morone*) is one of the fastest growing segments of United States aquaculture, with production of foodfish approaching five million kg/yr. Although efforts at domesticating *Morone* species are underway (Harrell and Webster 1997), most commercial HSB hatcheries still generate sunshine bass produced from wild-caught female white bass *Morone chrysops* and male striped bass *Morone saxatilis*. The opposite hybrid (palmetto bass; *M. saxatilis* X *M. chrysops*) has been used less often because wild or domestic striped bass females are viewed as too difficult to reproduce when needed. Beginning in the early 1990s, our investigations at North Carolina State University (NCSU) focused on developing detailed understanding and control of the reproductive biology of female striped bass (Sullivan et al. 1997). Parallel investigations were simultaneously conducted on white bass and white perch *M. americana*. We exploit the latter species as a laboratory model for *Morone* reproductive biology (Jackson et al. 1995).

Captive broodstocks were established and a detailed picture of their gametogenic cycle was acquired with respect to circulating levels and actions of important sex steroids (estradiol 17β; [E_2], testosterone [T] and 11-ketotestosterone) relative to specific stages of gonad maturation (Woods et al. 1992; Woods and Sullivan 1993; Tao et al. 1993; Blythe et al. 1994ab; Berlinsky et al. 1995; Jackson and Sullivan 1995; Sullivan et al. 1997). Considerable knowledge of the

physiology of oocyte growth was developed with regard to the main yolk precursor protein, vitellogenin (Vg), including development and use of immunoassays for circulating Vg as a marker of female maturity (Tao et al. 1993, 1996; Blythe et al. 1994; Berlinsky et al. 1995; Folmar et al. 1995; Sullivan et al. 1997; Heppell et al. 1999).

We also made many discoveries about the process of final oocyte maturation (FOM), including calibration of blood levels of maturation-inducing steroid (MIS) hormones (17α,20β-dihydroxy-4-pregnen-3-one, DHP; 17α,20β-trihydroxy-4-pregnen-3-one, 20β-S) to specific stages of FOM, detailed characterization of their actions and mechanism(s) of action, functional description of the ovarian MIS receptor, and identification of 20β-S as the main *Morone* MIS (King et al. 1994ab, 1995ab, 1997). We recently found that insulin-like growth factor I (IgF-I) can induce *in vitro* FOM of striped bass oocytes at stages much earlier than either gonadotropin (hCG) or MIS. Other new findings suggest that acquisition of follicular responsiveness to IgF-I may be a critical early step enabling FOM in *Morone* species (Weber et al., 2000; Weber and Sullivan, In Press).

The new knowledge of *Morone* reproductive physiology was exploited to develop improved techniques for tracking the onset and course of maturation while maturing broodstock out-of-season via photothermal conditioning (Blythe et al. 1994ab; Sullivan et al. 1997). Our most recent experiments, involving exposing striped bass to various combinations of static or annually-cycling day length and water temperature, revealed a striking dependency of vitellogenesis and oocyte growth on low temperature (Clark 1998). Based on these findings, we utilize mixed well and surface water sources to expose broodfish held in outdoor tanks to a thermal cycle appropriate for normal maturation (Hodson and Sullivan 1993; C.V. Sullivan, unpublished).

Our fish are induced to mature and spawn using implanted pellets that chronically release a potent, synthetic analog of gonadotropin-releasing hormone (GnRHa), a technique we pioneered for *Morone* species (Woods and Sullivan 1993; Hodson and Sullivan 1993; Sullivan et al. 1997). However, problems remained with identifying when females become responsive to GnRHa. We recently discovered that female striped bass whose (biopsied) follicles are capable of undergoing FOM in response to IgF-I *in vitro* respond to GnRHa implants *in vivo* to complete FOM and fully ovulate. This finding forms the basis for a new bioassay to select females competent to respond to the GnRHa implants (Weber et al., 2000).

The basic and applied research on striped bass maturation was accompanied by empirical development of improved methods for broodstock husbandry and efforts to fully domesticate the species. Aside from proper photothermal conditioning, improved husbandry involves minimizing handling and associated stress and feeding the fish an adequate ration of an appropriately formulated broodstock diet (Hodson 1995; Sullivan et al. 1997). We have now taken several different stocks of striped bass through a number of filial generations of domestication. For the purposes of this paper, "domesticated" refers to fish reared for two or more generations in captivity.

The present study was undertaken to test the value of our domesticated striped bass, broodstock husbandry methods, and spawning techniques for mass production of HSB fingerlings at commercial scale. In collaborative research with North Carolina HSB growers, we repeatedly spawned domesticated females to generate palmetto HSB fry which were used to produce fingerlings through application of extensive (pond) culture methods on private farms. For these females, egg fertility and hatching rates, larval survival, and fingerling yields were comparable to those obtained using fully mature wild fish captured on or near their spawning grounds. Our results also showed that reproductive performance of the female striped bass and survival of their progeny through the fingerling production cycle were equal to or better than corresponding values for domesticated female white bass and their sunshine HSB progeny.

MATERIALS AND METHODS

Striped Bass Broodstock

The 4-yr-old domestic (F_2-generation) female striped bass broodstock (avg. BW 4.32 kg) used in this study were produced at the NCSU Pamlico Aquaculture Field Laboratory (PAFL) in Spring of 1994. They were generated as a cross between domestic (F_1-generation) female striped bass from PAFL with domestic (F_1- and F_2-generation) male striped bass of Chesapeake Bay origin that were obtained from the Crane Aquaculture Facility (CAF; University of Maryland) in Baltimore. The F_1-generation domestic females were obtained in 1990 from the South Carolina Department of Natural Resources striped bass hatchery in Bonneau, where they were produced from wild parents captured from the Santee-Cooper drainage. The F_2-generation broodfish were reared to maturity out of doors in 0.1-ha ponds and then 6-m-diameter circular pools at PAFL as described previously (Hodson and Sullivan 1993) with the exception that they were fed at a rate of 1.5% BW, three times/wk with a special diet (CAF-Striped Bass Broodstock Diet 'B': Ziegler Brothers; Gardners, Pennsylvania, USA). Male striped bass used to produce sunshine HSB were from the same stock as the domesticated striped bass females.

The wild striped bass used in this study to produce sunshine HSB were obtained from commercial pound nets near the mouth of the Roanoke River in North Carolina and transported to a local fish farm (North State Fisheries[NSF]; Pinetown, North Carolina, USA) for spawning.

White Bass Broodstock

The domestic (F_2-generation) white bass broodfish used in this study (BW range 0.45-0.52 kg) were 2 yr of age and generated as a cross between 2-yr-old, F_1-generation domestic parents performed at PAFL in 1996 (Hopper 1999). The parents originated as a cross of wild adults obtained from tributaries of the Ohio River with adults captured from the Lake Erie basin that was performed in an attempt to enhance genetic variability. Various (sibling) groups of fry arising from this cross were pooled and used to generate fingerlings under extensive culture conditions at PAFL, which were then reared to maturity in 0.1-ha ponds using standard HSB culture practices (Harrell and Webster, 1997). Separate sibling groups were pooled, transported to a local fish farm (Carolina Fisheries [CF]; Aurora, North Carolina, USA), and stocked into ponds there for fingerling production and growout to maturity. To avoid inbreeding, mature CF males were crossed with the mature PAFL females to generate the F_2-generation domesticated white bass, which were then reared to maturity in ponds at PAFL (Hopper 1999). The white bass were fed a standard HSB growout diet (Southern States, Inc.; Farmville, North Carolina, USA) according to the feed manufacturer's recommendations based on the average weight of fish subsampled from the ponds at irregular intervals.

Wild white bass used at one commercial hatchery (CF) to produce palmetto HSB were obtained from commercial pound nets in Lake Erie and those used for this purpose at the other hatchery (NSF) were captured by hook and line from impoundments of various tributaries to the Catawba River in North Carolina.

Spawning of Female Striped Bass

The female striped bass were seined from their home pools in mid-April (1998) and subjected to ovarian biopsy under anesthesia to obtain oocytes for staging maturity as described by King et al. (1994ab). All of the fish used in spawning trials had naturally initiated at least the early stages of final oocyte maturation (oocyte stage $\leq$ 14 h; Rees and Harrell 1990) as made evident by coalescence of the lipid droplets in the ooplasm or migration of the oocyte germinal vesicle.

The females were treated with a synthetic analogue of human gonadotropin-releasing hormone (GnRHa) at doses of 50 to 150 µg administered in implanted (i.m.) cholesterol/cellulose pellets (Hodson and Sullivan 1993; Sullivan et al., 1997), tagged for rapid identification with strips of colored yarn tied to their dorsal fins through small punctures, and held individually or in small groups (2-3 fish) in 2-m-diameter circular tanks until ovulation and spawning. Eight of the 27 females required a subsequent injection of human chorionic

gonadotropin (hCG; 330 IU/kg BW) to hasten ovulation (Hodson and Sullivan 1993). All of the females ovulated and were spawned within 48 h after GnRHa implantation.

At spawning, females were stripped of their eggs for *in vitro* fertilization with semen from the F_2-generation domestic male white bass. Aside from the use of GnRHa implants, spawning operations and incubation of fertilized eggs and fry were conducted using standard HSB hatchery procedures (Rees and Harrell 1990).

Spawning of the wild female striped bass was conducted similarly at a private farm (NSF) where the resulting palmetto bass fry were stocked into a fertilized pond for growout.

Spawning of Female White Bass

Approximately 50 of the domesticated (F_2-generation) female white bass were seined from their home ponds in early April and taken into the hatchery where they were held for approximately 1 wk in 2-m-diameter circular tanks supplied with excess fresh well water at 18 C. To induce final maturation and ovulation the fish were injected with hCG at a dosage of 150 IU/kg BW. Approximately 32 h later, the females were stripped of their eggs for *in vitro* fertilization with semen from the F_2-generation striped bass males. The fecundity (range), egg fertility (average) and yield of fry per female (range) were approximately 100-150,000, 50%, and 50-60,000, respectively. Spawning operations and incubation of fertilized eggs and fry were conducted using standard sunshine HSB hatchery procedures (Kohler 1997).

Fingerling Production Trials

Once they had fully developed mouth parts and could commence feeding (4-6 d after hatching), the palmetto HSB fry were transported to the local fish farms (CF, NSF) for stocking into fertilized, outdoor, fingerling production ponds. In general, the ponds were fertilized and zooplankton blooms therein were managed according to the standard procedures (Geiger and Turner 1990). Production of HSB fingerlings in North Carolina is a highly competitive business and the exact details of pond management on the private farms are proprietary and cannot be reported here.

When they were old enough to begin feeding (3-4 d after hatching), the sunshine HSB fry were transported to the local fish farms (CF, NSF) for stocking into fingerling production ponds as described above for palmetto HSB.

Approximately 30-40 d after stocking, the fingerling production ponds were harvested by repeated seining, the fingerlings were transported to rectangular vats for subsampling and enumeration, and the number and percentage return of palmetto or sunshine HSB were recorded (Brewer and Rees 1990; Hodson 1995).

RESULTS

Representative results of spawning trials utilizing the domesticated (F_2-generation) female striped bass and male white bass to produce palmetto HSB fry are shown in Table 1. In several successive spawning trials involving implantation of 27 female striped bass with GnRHa, 96% of the females spawned successfully with an average

Table 1. Production of original-cross hybrid striped bass fry at the NCSU Pamlico Aquaculture Field Laboratory (see Table 1) using domestic (F_2-generation) female striped bass and male white bass parents. GnRH-a was administered in cholesterol/cellulose implants (Hodson and Sullivan 1993).

Trial	Fish (N)	GnRH-a Dose	Oocyte Diameter Mean (SEM)	Number Spawned	Fry Produced Mean (SEM; N[1])
1	4	100 µg	1,082 (38.6) µm	4	163,547 (31,774; 3)
2	8	100 µg	1,036 (14.1) µm	7	96,199 (43,600; 5)
3	6	100 µg	1,050 (22.3) µm	6	243,898 (37,667; 6)
4	9	50 - 150 µg	1,101 (29.3) µm	9	247,699 (40,215; 8)
					Average 187,836

[1]Fry production not quantified for all females spawned

Table 2. Pond production of original-cross hybrid striped bass (palmetto HSB) fingerlings (Phase-I; 30-40-d-old) on commercial farms using fry produced at the NCSU Pamlico Aquaculture Field Laboratory (see Table 1) from domestic (F_2-generation) female striped bass and male white bass parents. Results for fry produced from wild broodstock at a private farm (NSF) are shown for comparison. CF=Carolina Fisheries. NSF=North State Fisheries.

Farm Pond	Size (ha)	Stocked (N)	Rate (N/ha)	Harvested (N)	Harvested (%)
Domestic Broodstock					
CF-3	1.21	500,000	413,233	350,000	70
CF-B3	1.62	1,000,000	617,284	500,000	50
CF-B4	1.62	900,000	555,555	400,000	44
NSF-1	1.72	900,000	523,256	600,000	67
					Average 58%
Wild Broodstock					
NSF-3	1.72	1,200,000	697,674	400,000	33

yield of 187,836 4-6-d-old fry/female or 43,480 fry/kg female BW, based on results recorded for 22 of the fish of 4.32 kg average BW.

Table 2 shows results of fingerling production trials utilizing these F_3-generation domesticated palmetto HSB fry on commercial farms to generate Phase I (30-40-d-old) fingerlings with conventional pond culture techniques. Various farm ponds ranging from 1.21 to 1.72 ha in size were stocked with the fry at rates from 413,223 to 617,284 fry/ha, yielding survival rates to fingerling harvest ranging from 44 to 70% with average survival of 58%. The overall yield of Phase I fingerlings was 1,850,000 from the four ponds or 299,838 fingerlings/ha. Comparable results for a commercial 1.72 ha pond stocked with fry produced from a wild female striped bass at a rate of 697,674 fr/ha were 33% survival and 232,558 fingerlings/ha, respectively.

Results of the fingerling production trials utilizing palmetto HSB fry produced from domesticated female striped bass can be compared to those of trials conducted simultaneously on the same farms that utilized sunshine HSB produced from domesticated female white bass and male striped bass (Table 3). Various ponds (0.81-1.72 ha) were stocked with domesticated (F_3-generation), sunshine HSB at rates ranging from 174,419 to 962,963 fry/ha. Those stocked with fry produced from domestic parents yielded

Table 3. Pond production of reciprocal-cross hybrid striped bass (sunshine HSB) fingerlings (Phase-I; 30-40-d-old) on commercial farms using fry produced at the NCSU Pamlico Aquaculture Field from domestic (F_2-generation) female white bass and male striped bass parents. Results for fry produced from wild broodstock at private farms (NSF, CF) are shown for comparison. CF=Carolina Fisheries. NSF=North State Fisheries.

Farm Pond	Size (ha)	Stocked (N)	Rate (N/ha)	Harvested (N)	Harvested (%)
Domestic Broodstock					
CF-N	1.21	700,000	578,512	150,000	21
CF-N1	1.01	600,000	594,059	220,000	37
CF-M	1.21	700,000	578,512	250,000	36
NSF-2	1.72	1,200,000	697,674	450,000	37
					Average 33%
Wild Broodstock					
CF-M1	0.81	780,000	962,963	200,000	25
CF-N2	0.81	400,000	493,827	200,000	50
NSF-4	1.72	300,000	174,419	100,000	33
NSF-5	1.72	600,000	348,837	330,000	55
					Average 41%

207,767 fingerlings/ha or an average survival rate of 33%. Corresponding values for ponds stocked with fry produced from wild parents were 164,032 fingerlings/ha and 41% survival, respectively. In either case, fingerling yields were less than achieved using palmetto HSB produced from domesticated female striped bass.

DISCUSSION

The results of this study are remarkable in several respects. They represent the first report, of which we are aware, of repeated mass production of palmetto HSB fingerlings from domesticated striped bass on commercial farms. It is clear that yields of fry from the domesticated female striped bass are equivalent to those from fully mature, wild female striped bass captured on or near their spawning grounds (Table 2, bottom). The average yield of 43,480 4-6-d-old fry/kg female BW from the domesticated female striped bass can be compared with average values of 43,260 (range 20,000-60 143) 1-d-old fry/kg (Experiment 4, Hodson and Sullivan 1993) or 67,402 (range 30,000-121 927) 5-d-old fry/kg (Experiment 5, Hodson and Sullivan 1993) obtained for wild fish spawned by the same investigators using similar methods as in the present study.

The equivalency between domesticated and wild striped bass in reproductive performance appears to hold as well in comparisons with wild fish volitionally spawning together to produce striped bass fry, especially if we take into account the expected decrease in fertility due to hybridization and the difficulty in predicting ovulation time of striped bass as required to strip eggs for *in vitro* fertilization to produce HSB (Rees and Harrell 1990). As an example, the average yield of striped bass fry from several wild Roanoke River (North Carolina, USA) females that we induced with GnRHa implants to spawn in tanks in an experiment at Edenton National Fish Hatchery was 57,485 fry/kg female BW (Table 2.6; Sullivan et al. 1997).

Rates of recovery of Phase-I fingerlings from commercial farm ponds stocked with the F_3-generation domesticated HSB fry were outstanding, among the best ever recorded on the two farms (L. Brothers - Carolina Fisheries and H. Griffin III – North State Fisheries, personal communication). Average production of domesticated palmetto HSB for the trials shown in Table 2 was 29,8412 fingerlings/ha. The corresponding value for domesticated sunshine HSB produced in the trials summarized in Table 3 is 202,507 fish/ha. For comparison, a 1986 survey of 12 successful striped bass hatcheries located in the southeastern United States revealed that, during 1984-85, average production of Phase-I fingerlings at these hatcheries was 135,905 fish/ha (Brewer and Rees 1990), although yields of up to 197,680 Phase-I fingerlings/ha have been reported (Fitzmayer et al. 1986).

Review of the data for ponds stocked with progeny produced from wild fish in this study (Tables 2 and 3, bottom) suggest that the extraordinary yield of fingerlings from ponds stocked with domesticated HSB were likely due, in part, to the skill of the farmers involved at creating and maintaining suitable zooplankton blooms as well as the prevalence of favorable weather patterns during Spring of 1998. Nonetheless, results of the fingerling production trials demonstrate unequivocally that domesticated striped bass can be used to produce palmetto HSB fry with survival rates in outdoor ponds equivalent to progeny of wild striped bass and domesticated or wild white bass. Although data could not be obtained from the farmers on growth rates or size-frequency distributions for the Phase-I fingerlings at harvest, they did not report any unusual incidence of stunting and described the fingerlings as 'normal' by comparison to those produced from wild broodfish in prior years.

We attribute the excellent reproductive performance of our domesticated striped bass to several factors. First, the fish have been in captivity for two generations and we have likely benefited from passive domestication (Hallerman 1994). Second, our fish are fed a special diet (CAF-Striped Bass Broodstock Diet "B"; Ziegler Brothers) based on the U.S. Fish & Wildlife Service open formula salmon diet, with the exceptions that squid meal is substituted for fish (herring/menhaden) meal and squid oil replaces up to 25% of the fish oil in order to increase levels

of ω-3 and ω-6 fatty acids that promote larval development and survival (Watanabe 1982). Third, we use multiple well- and surface-water sources to provide the fish with proper thermal conditioning during gonadal maturation (Sullivan et al. 1997). Specifically, we alter flow rates of CastleHayne aquifer well water (~18 C) versus brackish surface (South Creek) water to provide seasonally cycling temperature similar to what the fish would experience in nature (Blythe et al. 1994ab; Clark 1998). Large or abrupt fluctuations in water temperature just prior to and during the spawning season are strictly avoided, as they impair final maturation and ovulation (Sullivan 1997). Fourth, the fish are held at low density in large (6- m diameter) circular pools and, other than activities associated with hatchery spawning, handling is kept to an absolute minimum, being limited to semi-annual census as needed to update inventory records and calculate growth and feeding rates. Handling stress is especially damaging to the health of adult striped bass (Harms et al. 1996) and it is known to disrupt the reproductive neuroendocrine system at multiple levels (Sumpter et al. 1994). Before introduction of these advances in broodstock husbandry the reproductive performance of our striped bass was highly unreliable.

In contrast, our domesticated white bass broodstock were produced and reared in outdoor ponds using the very same methods used for production of HSB foodfish without any special diet, photothermal conditioning, or handling practices (Hopper 1999). The fish were reproduced shortly after being harvested from the ponds. After being held for only 1 wk in hatchery tanks at 18 C, they were injected with hCG and then spawned to produce sunshine HSB for use in the fingerling production trials. Other investigators have made considerable progress on domesticating white bass and reproducing the domestic fish both in- and out-of-season using rearing tanks supplied by recirculating water systems and intensive culture methods (Kohler et al. 1994; Smith et al. 1996; Kohler 1997). However, this is the first report we know of that indicates white bass can be domesticated and reproduced using simple pond rearing techniques, established HSB hatchery procedures, and extensive fingerling production methods. The North Carolina HSB producers have been quick to adopt this approach and at least two commercial hatcheries now maintain domestic white bass and lesser numbers of male striped bass as broodstock in outdoor ponds.

Rearing white bass broodstock outdoors precludes reproducing the fish year-round after maturing them when needed under artificial photothermal cycles (reviewed by Sullivan et al. 1997). However, we have extended the natural spawning season of our domestic white bass by moving selected fish indoors in early spring for coldbanking and delayed spawning up to 3 mo after the normal season, allowing us to triple crop some outdoor, commercial fingerling production ponds (Hopper 1999). Coldbanking refers to the practice of holding spermiating males or females with nearly fully grown oocytes at low temperature (10-12 C) for extended periods before they are warmed to normal spawning temperature for reproduction after the natural season. Because HSB fry stocked into fertilized outdoor ponds, where they feed on natural zooplankton, can be harvested as (Phase-I) fingerlings after only 30 d, those produced from coldbanked parents can be used to restock the ponds two or more times over a single extended spawning season.

Full development of HSB farming to a level comparable to the 'broiler' chicken industry in the United States will require complete control of the reproductive biology of both striped bass and white bass, year-round reproduction of the fish to produce HSB for growout, and selective breeding of both parental lines to improve production efficiency (Harrell et al. 1990; Harrell and Webster 1997). The results of the present study clearly demonstrate that captive breeding and domestication of striped bass are achievable goals and that the domesticated fish can exhibit reproductive performance equal to or better than their wild counterparts. Future research on striped bass reproduction should involve out-of-season spawning of the fish to produce commercially significant quantities of fingerlings, and eventually deliver proven methods for year-round mass production of palmetto HSB.

Our results also show that white bass can be domesticated and utilized as broodstock for

sunshine HSB production using simple pond culture methods. This finding should be especially significant to producers growing HSB in outdoor ponds, a farming practice which presently encompasses about half of the total production of HSB in the United States. Coupled with the coldbanking methods described above, pond production of white bass broodstock will lessen the dependence of growers on wild fish for spawning, decrease fingerling costs, and increase yields of HSB for growout.

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LABORATORY SPAWNING OF CORAL REEF FISHES: EFFECTS OF TEMPERATURE AND PHOTOPERIOD

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ABSTRACT

The culture of marine tropical fish would help safeguard coral reefs and develop a new source of organisms for the aquarium trade. Although many of the freshwater tropical species sold to the public are now cultured, more than 90% of all ornamental marine organisms are still collected from the wild. Increasing pressures on natural populations of coral reef animals due to their expanding popularity in the aquarium trade has stimulated interest in the culture of marine tropical fish. Over the past few years, we have conducted experimental studies with several species of coral reef fishes including pygmy angelfish *Centropyge argi*, spotfin hogfish *Bodianus puchellus*, bluehead wrasse *Thalassoma bifasciatum*, and clown wrasse *Halichores maculipinna*. All are protogynous hermaphrodites, have sexual size dimorphism, and some show sexual dichromatism. Spawning was induced by manipulating temperature and photoperiod. Environmental conditions of at least 22 C, 11 h light (13 h dark) and 15 min dawn and dusk induced regular spawning in paired adult *C. argi*, *T. bifasciatum*, and *B. puchellus*. *H. maculipinna* spawned primarily during simulated summer conditions. All species produced small, spherical, pelagic eggs containing a single oil globule that hatched within 18-26 h. Fecundity was strongly affected by day-length and temperature in all species, and by length of time in captivity. Newly-hatched larvae were small with rudimentary development. Though offered a wide variety of food beginning on d 3 after hatching, no larvae survived to metamorphosis.

INTRODUCTION

The culture of marine tropical fish conserves natural reef resources by offering alternatives to wild capture and develops a new source of organisms for the aquarium trade. The aquarium hobby draws 10-20 million enthusiasts who keep more than 90 million tropical fish (Andrews 1990). Two-thirds of the aquarium hobbyists worldwide live in the United States. With this popularity, it is not surprising that there is concern for the impact of the aquarium hobby on natural populations of marine organisms and their habitats. Although many of the freshwater tropical species sold to the public are now cultured, more than 90% of all marine ornamental organisms continue to be collected from the wild.

Increasing pressures on natural populations of coral reef animals due to their expanding popularity in the aquarium trade has stimulated interest in developing culture techniques for marine tropicals. Controlled spawning through temperature and photoperiod manipulations to simulate seasonal changes and bring about gonadal maturation has been successful with several temperate species (Arnold et al. 1976; Arnold 1978). Slight changes in day-length in the tropics may act as seasonal cues for tropical organisms (Wolda 1989). Coral reefs experience seasonal changes in day-length of 0.5-2.0 h and temperature differences of 10-12 C at their northern and southern limits (Rezak et al. 1985). Lobel (1978) reported that *Centropyge potteri* reproduction was influenced by both seasonal and lunar changes. Likewise, many Caribbean reef fishes in Jamaica show a high level of spawning activity between January and May (Munro et al. 1973) and seasonal spawning peaks have been reported for other tropical marine teleosts (Johannes 1978; Foster 1987). Thus, egg

production in tropical reef fishes may be tied to seasonal changes.

This study was initiated to determine if coral reef fishes could be induced to spawn in captivity using photoperiod and temperature cues. Over the past few years we have conducted experimental studies with several species of coral reef fishes including pygmy angelfish *Centropyge argi*, spotfin hogfish *Bodianus puchellus*, bluehead wrasse *Thalassoma bifasciatum*, and clown wrasse *Halichores maculipinna*. All are protogynous hermaphrodites, have sexual size dimorphism, and the clown wrasse and bluehead wrasse show sexual dichromatism. There are scattered reports of these popular ornamental fish species spawning in captivity but not in any controlled or predictable manner.

Pygmy angelfish are pair spawners with relatively small territories (Moyer et al. 1983). Several species of *Centropygi* have spawned in captivity (Bauer and Bauer 1981; Hioki and Suzuki 1987; Karanikas 1989; Hioki et al. 1990). The spotfin hogfish spawns in harems or in pairs and both sexes are similar (Thresher 1984). The dichromatic bluehead wrasse and clown wrasse spawn in harems or large multi-male groups or occasionally in pairs (Warner and Hoffman 1980; Thresher 1984). There are no reports of aquarium spawning in the spotfin hogfish or clown wrasse but two *Thallasoma* spp. have spawned in captivity (Thresher 1984).

The overall goal of this research was to develop a protocol for predictable spawning of coral reef fishes. This paper reports spawning and fecundity results and data on egg size and hatch rates for the four species of Caribbean reef fishes.

MATERIALS AND METHODS

Tropical reef fish were hand-collected from the Florida Keys or from reefs near Veracruz, Mexico. Paired adults were maintained in separate 300- or 900-L, fiberglass tanks and fed twice daily with commercial flake food and raw shrimp or fish. Rocks covered with algae and invertebrates (live rock) were stacked in the tanks to simulate the reef habitat (Fig.1A). Effluent water from spawning tanks was airlifted into an external filter box fitted with four vertical plates covered with polyester filter material as substrate for nitrifying bacteria (Fig.1B). Crushed oyster shell was added to buffer pH. Water returned from the filter box at a rate of 2.1 L/min resulting in a total tank water exchange every 2.4 h. Using light timers and heat pumps, photoperiod and water temperature were manipulated to simulate seasonal changes. Fish were subjected to environmental manipulations that ranged from 10 h light and 17-20 C for the simulated winter season to 13 h light and 25-30 C for summer. Spring and fall conditions were 11 h light and 21-24 C. Spawning tanks were equipped with both overhead and underwater lights (Fig.1A) on separate timers to produce a 0.25 h dawn and a 0.25 h dusk. Salinity was maintained at 32 to 36 ppt, pH=8.2, NO_2 and NH_3 < 0.03 ppm

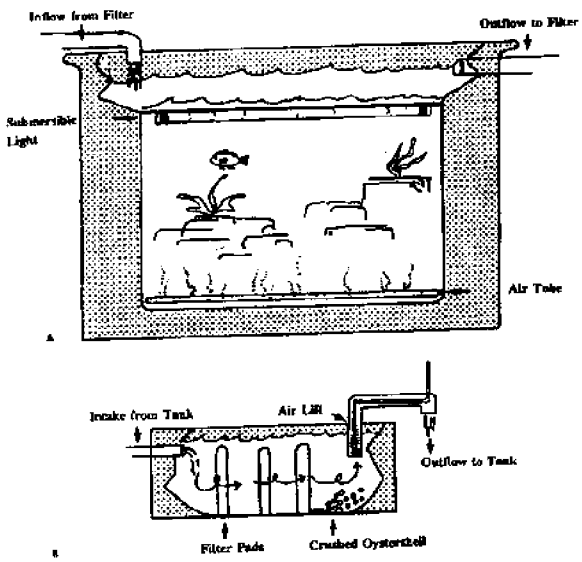

Figure 1. A) 300-L (60 x 90 x 60 h cm) fiberglass spawning tank with live rock, internal underwater light, and surface level water outflow for filtration. **B)** External biological filter box of fiberglass resin coated plywood fitted with vertical filter plates.

Eggs were collected in 150-µm mesh bags (10 x 20 cm) attached to the intake pipe inside the filter box. A subsample of eggs was measured and the remaining embryos were placed in 5 µm filtered sea water in small (1-6 L) glass aquaria, at 25 C, pH = 8.2, salinity = 32-36 ppt (to match the spawning salinity). Only floating eggs were considered fertilized and percent hatching was calculated as the number of viable larvae on the morning after hatching divided by the number of fertilized eggs.

During the study three pairs of a pygmy angelfish and bluehead wrasse and two pairs each of spotfin hogfish and clown wrasse were exposed to laboratory controlled annual cycles. The mean sizes of the captive fish are given in Table 1. Fecundity was examined for individual females exposed to one or more annual cycles. Fecundity was measured as the total number of eggs produced/d per fish, regardless of fertility.

RESULTS

During their first 6 mo in captivity, adult fish were subjected to environmental changes that mimic typical seasonal changes in northern tropical latitudes (Fig.2). After an abbreviated summer, fall, and winter, spawning began when conditions were changed to spring. Spring conditions of 22 C, 11 h light, 13 h dark, and 0.25 h dawn and dusk, induced continuous daily spawning in pygmy angelfish, bluehead wrasses, and spotfin hogfish. Clown wrasses began to spawn during simulated summer conditions (25 C; 13 h light). All species produced small spherical, pelagic eggs containing a single oil globule that hatched within 18-26 h.

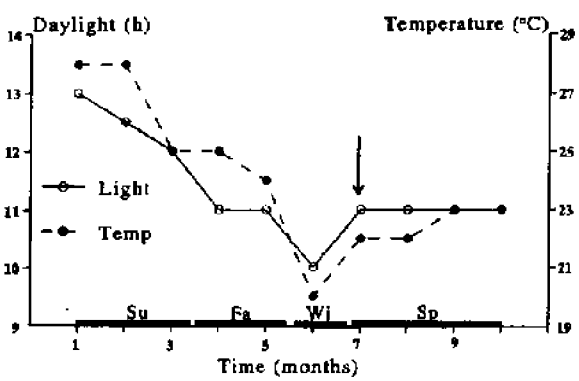

Figure 2. Photoperiod and temperature regime used to induce laboratory spawning of adult *Centropyge argi*, *Thalassoma bifasciatum*, *Halichores maculipinna* and *Bodianus puchellus*. Adults collected in summer were subjected to an abbreviated annual seasonal cycle in the laboratory. Lengths of each simulated season are represented by bars at the bottom of the graph, where Su=summer, Fa=fall, etc. Arrow indicates date when spawning began.

Pygmy angelfish spawned 15-35 min before total darkness after an elaborate courtship lasting 30-45 min. Duration of the actual spawning event was less than 1 min. The large rocks and rubble in the tank were used during courtship. During the final spawning burst, the male pushed the female toward the surface into the open water and spawning occurred approximately 50 cm above the tank floor. Pygmy angelfish spawned continuously over a broad range of day-length and temperature conditions (10-13 h light and 21-27 C). Daily fecundity was strongly influenced by photoperiod and temperature with highest fecundity occurring at 11-13 h light and 24-25 C and lowest fecundity at 10 h light and 21 C. Spawning ceased when water temperature was increased to 28 C or decreased below 20 C. The number of eggs produced averaged 100 eggs/female (N=478) (Table 2) with a mean percent fertilization of 87% (Table 3). The small spherical eggs (0.73 mm in diameter) contained a single oil globule and eggs hatched after approximately 18 h at 24 C (Table 3).

Bluehead wrasses spawned at midday following a courtship during which the male displayed by swimming up and down in the water column (looping). The final spawning event involved the pair rushing up toward the surface and releasing a gamete cloud. Daily egg production by individual female bluehead wrasse varied from 3-7221 and was influenced by

Table 1. Sizes (mm SL) of males and females of four species of marine tropical fish spawned in captivity at UTMSI under temperature photoperiod regulation.

	Males		Females	
	Mean	Range	Mean	Range
Pygmy angelfish (*Centropyge argi*)	5.43	4.8-6.0	4.06	3.6-4.6
Bluehead wrasse (*Thalassoma bifasciatum*)	12.04	10.5-14.0	6.87	5.4-9.2
Clown wrasse (*Halichoeres maculipinna*)	11.10	7.8-14.4	7.25	7.4-8.0
Spotfin hogfish (*Bodianus puchellus*)	13.00	10.0-16.0	8.42	6.8-10.0

Table 2. Summary of egg production data for four species of marine tropical fish spawned in captivity at UTMSI under temperature photoperiod regulation. N= the number of spawns, Mean = mean daily number of eggs produced by an individual female when spawning occurred, Max= maximum number of eggs produced in any one day, Temp/light= temperature (°C) and day length of maximum egg production, Range= range of temperatures (°C) for good egg production.

	N	Mean eggs	Max eggs	Temp/light optimum	Range
Pygmy angelfish *(Centropyge argi)*	478	100	648	24.5/11h	23-25.5
Bluehead wrasse *(Thalassoma bifasciatum)*	529	425	7221	27.0/13h	27-30
Clown wrasse *(Halichoeres maculipinna)*	114	144	662	27.5/13h	24-29
Spotfin hogfish *(Bodianus puchellus)*	494	366	1980	25.5/11h	23.5-27.5

Table 3. Egg size (mm), percent fertilization, percent hatching, and longest larval survival for four species of marine tropical fish spawned in captivity at UTMSI under temperature photoperiod regulation.

	Egg Size	Fertilization	Hatch	Larval Survival
Pygmy angelfish (*Centropyge argi*)	0.73	87%	97%	7 days
Bluehead wrasse (*Thalassoma bifasciatum*)	0.56	61%	88%	7 days
Clown wrasse (*Halichoeres maculipinna*)	0.59	78%	78%	5 days
Spotfin hogfish (*Bodianus puchellus*)	0.85	56%	98%	21 days

photoperiod, temperature and fish size. A small female (5.6 cm) averaged 257 eggs/spawn during the first spawning cycle but during a second spawning period when the female had grown to 6.5 cm SL, she produced 504 eggs/spawn. Spawning occurred between 10-13 h light and 21-30 C, but fecundity increased with rising temperature and was highest at 25-29 C, and lowest at 10 h light. Spawning ceased when temperatures reached 20 C or lower. The 0.56 mm diameter eggs hatched after incubating 26 h.

Clown wrasses spawned under summer conditions in the early afternoon after a simple courtship display by the brightly colored male. The male and the female rushed upwards toward the surface in the final spawning ascent. Highest fecundity (Table 2) occurred at 13 h light and 27.5 C. There was no spawning below 22 C or at 11 h light. Percent fertilization of the 0.59 mm eggs was 78% (Table 3).

The spotfin hogfish spawned in the late afternoon (1630- 1730). Courtship consisted of the larger of the pair (presumably the male) displaying, with dorsal and anal fins erect, in front of the smaller fish by bobbing up and down horizontally. The final spawning rush by the pair was very short in duration. Maximum fecundity occurred at 25.5 C and 11 h light. In one pair, an individual female spawned every day for 1 mo averaging 703.1 eggs/spawn with a range of 25 to 1691 eggs/spawn. This was followed by a week of little or no spawning and then another month of spawning at a reduced rate (average of 423.6 eggs/spawn). During the first year of spawning, when conditions were favorable, this female spawned continuously missing only a week or so at any one time. The eggs were relatively large (Table 3) and hatched in 18-24 h.

DISCUSSION

Courtship of pygmy angelfishes in this study was identical to that described by Bauer and Bauer (1981) for six species of *Centropyge* based on reef and tank observations and by Moyer et al. (1983) for pygmy angelfish in the Netherlands Antilles. Bauer and Bauer (1981) reported spawning every month of the year averaging 119 eggs/female with a percent fertility of 80%, similar to our data of 100 eggs/spawn with 87% fertilized, but we found spawning to be limited by temperature extremes.

There are no previous reports of the captive spawning behavior of bluehead wrasses but spawning observed in this study is similar to that reported for natural pair spawning (Feddern 1965; Reinboth 1973; Thresher 1984). An increase in fecundity but not in frequency of spawning with increased size of the female (Feddern 1965; Schultz and Warner 1991) was observed in our captive fish. Our mean fecundity data was lower than that reported by Schultz and Warner (1991) for natural populations of bluehead wrasses but the maximum fecundity we recorded (1350 eggs for 45-50 mm and 7221 eggs for > 60 mm SL female) was greater. We were unable to find any published reports of captive spawning for spotfin hogfish, or clown wrasses, but in our laboratory each of these species spawn during specific daylight hours as suggested in Thresher (1984).

Spawning of these tropical fish species conform to the "continuous spawning strategy" proposed by Bauer and Bauer (1981) in that fish generally spawn regularly every day when temperature and day-length are constant at appropriate levels. Our laboratory data suggest a variant of this strategy may occur in subtropical latitudes where temperature and day-length are reduced during winter, causing cessation or reduction of spawning during short-day and low-temperature periods. Extremely high temperatures (29 and 30 C) resulted in reduced fecundity in the pygmy angelfish.

Temperature and photoperiod have not typically been described as important determinants of fecundity in tropical fish. Moyer and Nakazono (1978) found that in the more temperate waters of Japan, *C. interruptus* spawned from May to October when temperatures increased to 21 or 22 C, while Munro et al. (1973) established that in Jamaica many shallow reef species spawn during the season when water temperatures are minimal (below 28 C). As we found in this study, water temperatures between 22 and 28 C appear to be optimal for spawning in many coral reef fishes.

Conditions for controlled spawning of several marine tropical fishes have been identified. These species, as well as most coral reef fishes, produce tiny eggs and larvae that require special rearing conditions. The small planktonic larvae develop quickly and need food at 36-48 h after hatching. The development of an adequate rearing protocol is at present a major problem in raising these and many other reef fishes in captivity. To help resolve this problem the diets of wild planktonic larvae captured in light traps over coral reefs were examined (Riley and Holt 1993). Micro-zooplankton, found to be important larval prey, are under culture in the lab and tank design and water quality parameters are being evaluated to identify optimal growing conditions.

The long-term goal of this research is to develop aquaculture techniques for raising reef fish in captivity to increase our understanding of their ecological requirements, to preserve rare and endangered species, and to reduce harvesting pressure on natural populations.

ACKNOWLEDGMENTS

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EFFECTS OF ACIDIFICATION ON FISH REPRODUCTION

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ABSTRACT

Acid rain induces the acidification of inland waters which results in damage to aquatic ecosystems that contain fish. In northern Europe and America, where damage by acid rain has been manifested, many populations of fishes have vanished. At present, rapidly expanding industrial activities in Asia have led to a continuous increase in emissions of acidic pollutants, and rain at acidic levels of pH 4 has often precipitated throughout Japan. In order to investigate the effects of acid rain on fish ecosystems and forecast the damages that will be sustained in the future, it is necessary to clarify the biological responses of fish to an acidic environment. Therefore, we have investigated the effects of acid exposure on physiological changes in fishes. Levels of pH 4 constitute acidic conditions lethal to most species of fish. When fish were exposed to low pH, loss of sodium (Na^+) and chloride (Cl^-) ions from the body fluid occurred, resulting in a decrease in plasma osmotic pressure. Dace *Tribolodon hakonensis* inhabiting Lake Osorezan in Japan, which is highly acidic (pH 3.6), had profoundly differentiated gill chloride cells which showed high V-ATPase activity that stimulates the proton pump of the cell membrane to excrete H^+ ions. Moreover, it was found that somatolactin, a pituitary hormone, is possibly involved in the acid-base regulation. Even if pH is not low enough to be lethal, the stress of acidification induces various physiological and ecological problems in fish. When juvenile carp were exposed to pH 4.5, plasma cortisol levels peaked in response to acid stress, and immuno-globulin (IgM) levels subsequently decreased. This result suggests that acid stress depresses the immune system of fish. When mature salmonid fishes are exposed to pH 4.5-5.0, inhibition of development and increases in malformation are observed in the embryos of their offspring. Plasma levels of sex steroids and gonadotropin exhibited abnormally high levels and there was a possibility that acid stress disrupted the endocrine control over reproduction. Additionally, the acidic condition of pH 5.8 completely inhibited the homing migratory behavior of land-locked sockeye salmon, and extremely slight acidification (near pH 6) inhibited their spawning behavior. These results suggest that salmonid fish are highly sensitive to slight changes in acidity and they attempt to avoid an environment perceived to be deleterious to their offspring.

INTRODUCTION

The phenomenon called acid rain results from industrial activities where sulfuric and nitric acid are produced by the release of sulfuric oxides (SOx) and nitrogen oxides (NOx) into the atmosphere. Acid rain induces the acidification of inland waters which results in damage to aquatic ecosystems, including fish. In northern Europe and America, as damage by acid rain has been manifested since the 1960s, many populations of salmonid fishes constituting important fisheries resources have vanished (Schofield 1976). In particular, great numbers of Atlantic salmon *Salmo salar* and brown trout *S. trutta* were destroyed by the acidification induced

by the rapid inflow of acid pollutants into rivers during spring snow-melts (snow-melt acid shock) in Scandinavian countries. This phenomenon is called "fish kill" (Leivested and Muniz 1976). Countries in these areas dedicate an enormous percentage of their national budgets to the neutralization of acidification by means of liming (Appelberg et al. 1995).

At present, rapidly expanding industrial activities in Asia have led to a continuous increase in emission of acidic pollutants resulting in rain at acidic levels of pH 4 precipitating throughout Japan (Japan Environment Agency 1997). In order to investigate the effects of acid rain on fish ecosystems and forecast the probable damage that could be sustained in the future, it is necessary to clarify detailed biological responses of fish to an acidic environment in Japan. Therefore, we have investigated the effects of acid exposure on physiological and ecological processes in fishes, especially regarding reproduction.

ACUTE EFFECTS AND ACID TOLERANCE

Levels of pH 4 constitute acidic conditions lethal to fish (Ikuta et al.1992). Fish have the ability to regulate their acid-base balance in order to maintain normal pH of their body fluids under acidic ambiance. When fish are exposed to a low pH, chloride cells in the gill tissue take up bicarbonate (HCO_3^-) ion from the outside to neutralize the hydrogen (H^+) ion flowing in the body. At this time, the loss of sodium (Na^+) and chloride (Cl^-) ions from the body fluids occurs, and plasma osmotic pressure decreases (Fig.1) (Iwata et al. 1990). This process is considered to be one of the major reasons why freshwater fish die under acidic conditions. In tilapia *Orechromis niloticus*, *O. mossambicus*, and medaka *Oryzias latipes*, Na^+,K^+-ATPase activity in chloride cells increases in association with Na^+ loss when exposed to low pH; this suggests that Na^+,K^+-ATPase may act to affect Na^+ uptake under an acidic hypotonic environment (Yada and Ito 1997, 1998).

From these results, it was considered that plasma Na levels could be used as an indicator to estimate the acute effects of acidification on fish. When rainbow trout *Oncorhynchus mykiss* were exposed to various acidic conditions, the fish showed lower plasma Na^+ levels and the Na^+ levels and pH were found to be significantly correlated (Fig.2) (Yada et al. 2000). Thus, we began checking the health of fish in the natural environment by means of a medical blood ion and gas meter (i-STAT Corp. USA).

Dace *Tribolodon hakonensis* inhabiting Lake Osorezan in Aomori Prefecture, Japan, which is highly acidic (pH 3.6), are well-known to be acid tolerant (Satake et al. 1995), whereas the same species inhabiting areas of neutral pH exhibit a low tolerance to low pH. A recent study revealed that dace in Lake Osorezan have especially differentiated chloride cells which enlarge and gather to form a follicle-shaped structure. Since these cells show high V-ATPase activity that stimulates the proton pump of the cell membrane, their function is possibly to excrete H^+ ions which flow into the body from the outside (Fig. 3) (Kaneko 1997). Moreover, it was found

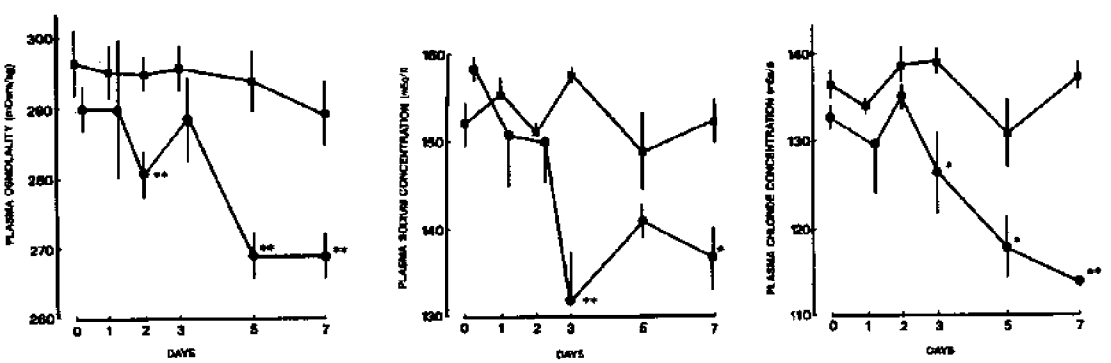

Figure 1. Changes in the plasma osmolality (left), sodium concentration (middle), and chloride concentration (right), in the pH 3.9-exposed (circle) and the control (square) yearling Japanese char. Data are shown as means ± SE (n=6). *; $P<0.05$, **; $P<0.01$.

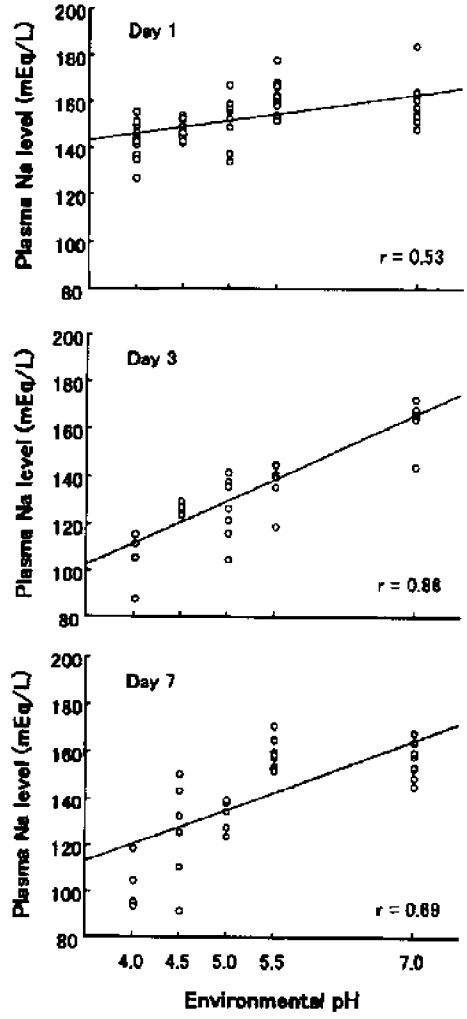

Figure 2. Correlation between the plasma Na levels and environmental pH in rainbow trout on d 1, d 3 and d 7 after acid-exposure.

that somatolactin, a pituitary hormone of the growth hormone and prolactin family, is possibly involved in the acid-base regulation of chloride cells (Kakizawa et al. 1996, 1997).

EFFECTS OF SUB-LETHAL ACIDIFICATION

Even if pH is not at a lethal level, the stress of acidification induces various physiological and ecological problems in fish. As observed under laboratory conditions, chum salmon *O. keta* juveniles could significantly perceive low acidity such as pH 5.8 and displayed avoidance behavior (Ikuta et al. 1996). At this time, juvenile salmon showed a high peak of plasma cortisol which is known as the "stress

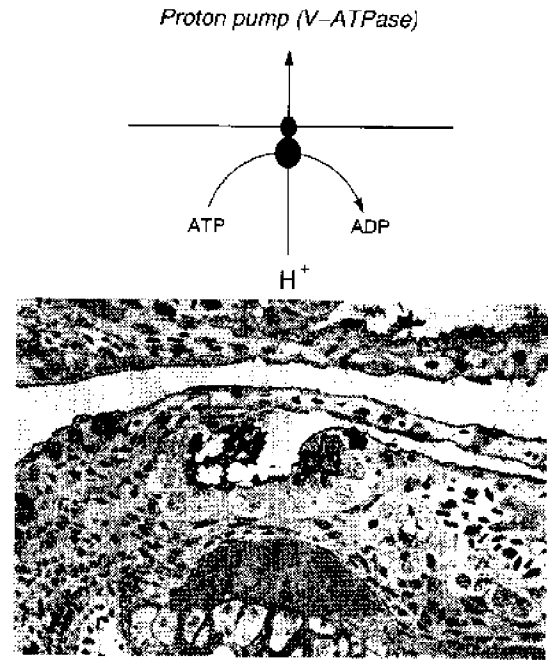

Figure 3. Schematic of proton pump in cell membrane (upper) and cross section of chloride cells positively stained with antibody to V-ATPase in gill membrane of dace in Lake Osorezan (lower).

hormone" secreted from the adrenal cortex. This indicates the possibility that salmon will disappear from the areas where acidification is beginning, due to their instinctive avoidance behavior.

It is well-known that cortisol secreted due to stress reduces immune and reproductive functions in fish (Carragher et al. 1989). When juvenile carp *Cyprinus carpio* were exposed to water of pH 4.5, plasma cortisol levels peaked rapidly in response to acid stress, and immuno-globulin (IgM) levels subsequently decreased (Fig. 4) (Ikuta et al. 1997). IgM is a protein constituting antibody that specifically binds to alien substances such as viruses and bacteria, and prevents infection. This result suggests that acid stress depresses the immune system in fish. Therefore, it is also very important to consider the indirect effects of acidification on physiological mechanisms of fish, even if the acidification itself is not lethal.

EFFECTS ON FISH REPRODUCTION

Recent research has also revealed that sub-lethal acid stress affects reproduction of fish. If mature salmonid fishes are exposed to sulfuric acid water of pH 4.5-5.0, inhibition of

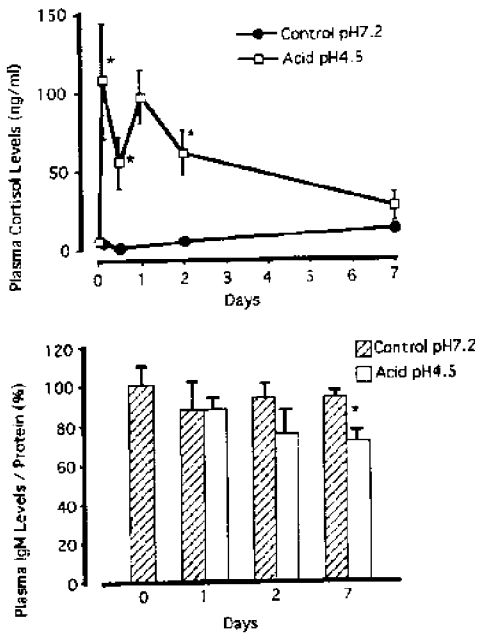

Figure 4. Changes in the plasma cortisol (upper) and immunoglobulin (lower) levels during acid exposure in the juvenile carp. Data are shown as means ± SE. *; $P<0.05$.

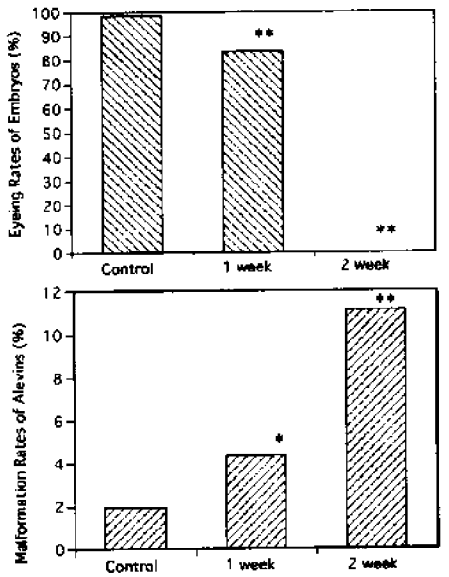

Figure 5. Eyeing rates of embryos produced between the acid-exposed (0, 1 and 2 wk) females and the control males (upper), and malformation rates of hatched alevins produced between the acid-exposed (0, 1 and 2 wk) males and the control females (lower) in rainbow trout. *; $P<0.05$, **; $P<0.01$, compared with the control.

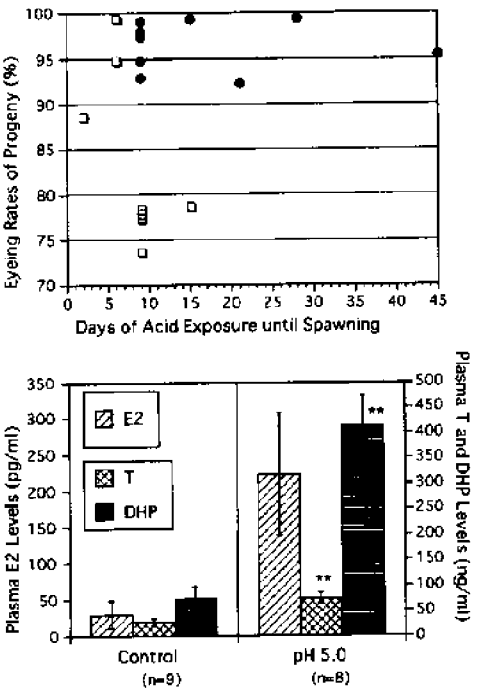

Figure 6. Correlation between days on which pre-ovulatory female sockeye salmon were exposed to pH 5.0 (square) and pH 7.2 (circle) until spawning and eyeing rates of progeny of each individual (upper), and their plasma levels of sex steroids, i.e. estradiol-17β (E2), testosterone (T) and 17α20β-dihydroxy-4-pregnen-3-on (DHP) at spawning (lower). Data of hormone levels are shown as means ± SE. **; $P<0.01$, compared with the control.

development and increases in malformation are observed in the embryos of their offspring (Ikuta and Kitamura 1995). When mature rainbow trout were reared in pH 4.5 just prior to spawning, the eyeing rate (index indicating normal development) of embryos from females exposed to acid decreased drastically, and the malformation rate of embryos produced with sperm from males exposed to acid increased in a time-dependent manner, even if the embryos were cultured in neutral water after fertilization (Fig. 5). As plasma levels of sex steroids and gonadotropin showed abnormally high levels in both male and female fish exposed to acid, there was a possibility that acid stress disrupted the endocrine system of reproduction in fish. In land-locked sockeye salmon *O. nerka*, mature female fish reared at pH 5.0 showed ovulation earlier than did the controls, and the eyeing rates in embryos produced from them rapidly decreased after one wk (Fig. 6). Since the pH 5.0 group showed extremely high plasma levels of 17α20β-dihydroxy-4-pregnen-3-on (DHP), which is a steroid hormone inducing final maturation in oocytes, abnormal oocyte maturation and ovulation might be induced by acid stress (Ikuta et al. 1999). Because similar depression of embryonic development was

reported from female land-locked sockeye salmon, called kokanee in North America (Parker & Mckeown 1987), exposed to pH 5.6, this species is considered very sensitive to acidity especially with regard to reproduction.

Additionally, it has been clarified that slight acidity (in the range of pH 6) is sufficient to depress reproductive behavior of salmonid fish. It is common knowledge that salmonid fishes exhibit homing or migration behavior in which they return to their native rivers for spawning. In observational experiments using artificial two-way channels in which water of neutral and various pH was allowed to flow, acidic conditions such as pH 5.8 completely inhibited upstream migratory behavior of land-locked sockeye salmon, and all of the salmon selected the neutral channel to begin migration. Female adult salmon also show digging behavior by forming a nest on the gravel bottom of the river; this is followed by mating and spawning behavior (Fig. 7). In tank observations, very slight pH changes from 6.8 to 6.4 and less, significantly inhibited their spawning behavior (Fig. 8) (Kitamura and Ikuta 2000). These results suggest that salmonid fish are very sensitive to slight acidity and avoid an environment perceived to be deleterious to their offspring.

CONCLUSION

In the series of studies presented, it has been clarified that acidification of inland waters induced by acid rain or other acidic pollutants

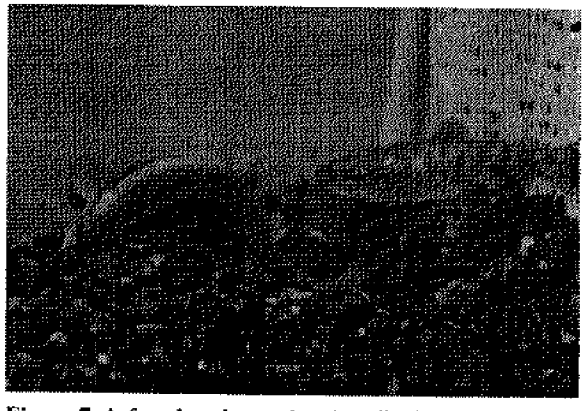

Figure 7. A female salmon showing digging behavior by forming a spawning nest on the gravel bottom (left) and a male fish attending the female in an observation tank.

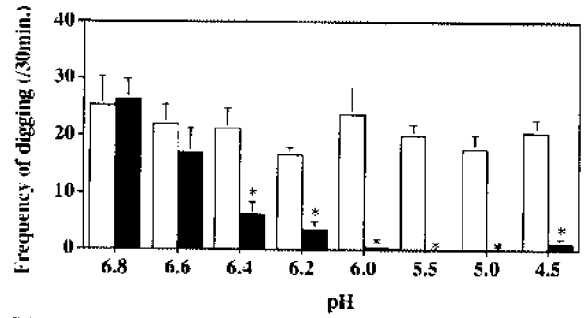

Figure 8. Changes in the frequency of digging behavior of female sockeye salmon. White bars indicate the mean frequency in neutral water (pH 6.8) and dark bars indicate that after transferred to each pH (6.8-4.5). Vertical lines indicate SE. *; $P<0.05$.

causes various physiological and behavioral impediments in fish, even if the acidity is not at a lethal level. Summarizing the results obtained in various experiments using salmonids, such as sockeye salmon, the following scenario in association with inland-water acidification can be proposed in fish (Fig. 9):

1. Extremely slight acidification at pH 6 and lower ranges inhibits homing migration and/or spawning behavior.
2. Sub-lethal acid stress at pH 5 and lower ranges stimulates avoidance of acidic areas or induces failure of immune and reproductive functions via the alteration of physiological mechanisms, including the actions of endocrine factors.
3. Acidification in the range of pH 4 rapidly affects the acid-base regulatory function of gill chloride cells, resulting into mortality due to the efflux of NaCl from body fluid.

At present, the chronic acidification of inland waters by acid precipitation, as seen in North America and Europe, has not yet been observed in Japan, with the exception of volcanic or mineral acid rivers and lakes. However, evidence of potent acid rain has often been observed. Even if the acidity of the water is very slight or at sub-lethal levels, it may adversely affect the physiological functions of immunity and reproduction. Additionally, it is a surprising result that even extremely slight changes in acidity (pH 6) is enough to inhibit instinctively programmed behavior such as homing and spawning in the salmon. Therefore, there is the possibility that the

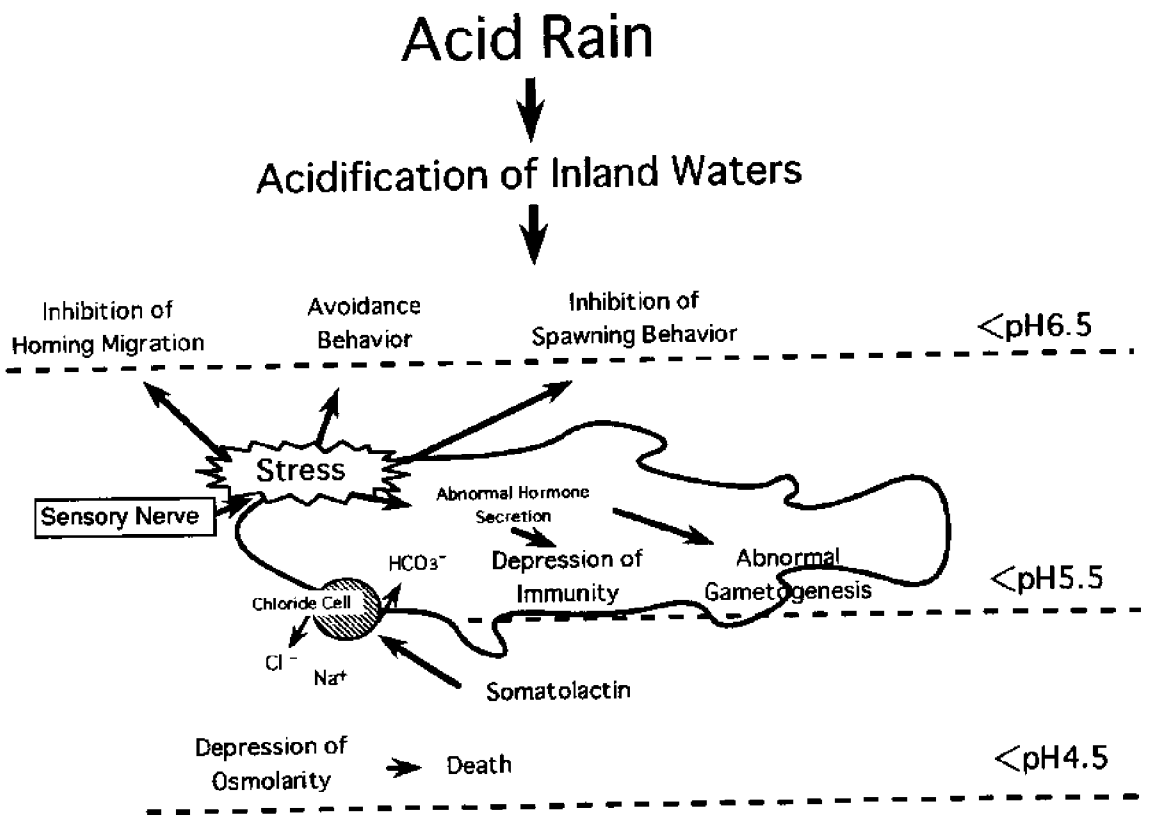

Figure 9. Summary of the effects of water acidification on physiology and behavior of fish.

effects of acid rain will be strongly manifested in the near future in Japan as well. Although we formally used toxicity tests or physiological examinations to evaluate the effects of pollution on organisms, these results suggest that behavioral-specific responses to environment changes are also very important in this kind of research. This is because the inability of fish to reproduce is equivalent to the avoidance of reproductive behavior, even if fish are physiologically capable of doing so.

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HORMONAL MANIPULATION OF DIGESTIVE ENZYME ONTOGENY IN MARINE LARVAL FISHES - EFFECTS ON DIGESTIVE ENZYMES

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ABSTRACT

It has been demonstrated previously that the ontogeny of digestive enzymes in the Pacific threadfin *Polydactylus sexfilis* (locally known as *moi*) follows a pattern in which amylase is the first to become activated, followed by lipase and protease later in development. The timing of digestive enzyme development suggests a greater importance of carbohydrates during the early, critical, period of first feeding than might be expected for carnivorous larval fishes.

Treatment of larval threadfin with a combination of hormones including triiodothyronine and cortisol has been shown to improve survival in this species, at least in part by advancing the timing of initial intestinal absorptive function. A single, brief (1-h) exposure to the same hormones at hatching also advances the pattern of expression of intestinal enzyme patterns at the onset of feeding. Although the advance is only by a matter of a few hrs, it does appear to cause the increase in specific activities of amylase and serine protease throughout the experimental period. This suggests that these enzymes are inducible, characteristically appearing around the time of first feeding. The hormone-dependent increase in digestive capacity coincident with first feeding may improve nutrient utilization, and may therefore be one mechanism by which exposure to these development-promoting hormones increases larval survival.

Starved larvae undergo a decrease in the production of amylase at approximately the latest time that feeding can begin (termed the *point of no return*; Blaxter 1969). These results suggest that amylases and their products may be of importance in early larval differentiation, and indicate a key role of hormones in the regulation of their expression.

INTRODUCTION

Many regulatory compounds such as hormones, neurohormones, neurotransmitters, and mRNAs which encode for growth factors and other compounds are maternally derived and deposited into the yolk of vertebrate eggs (reviewed by Brown and Núñez 1994). These regulatory compounds are particularly important in ontogenetic development and initiation of function in larval organ systems. It is possible that the rates of deposition of these regulatory compounds can vary, and that they may be affected by artificial diets and other environmental parameters to which broodstock female fish are subjected. Though hatchery techniques have improved steadily over recent years, early mortality at first feeding remains a primary bottleneck in mass production of marine juvenile fishes.

Thyroid hormones are important regulatory hormones that increase epidermal mitotic rate by controlling the synthesis of specialized proteins during cell differentiation within the digestive system, in the formation and inflation of the swim bladder, and in the development of muscle tissue (Hourdry 1993). They are also reported to frequently have the net effect of improving larval health and survival. Thyroid hormone treatments increased larval survival in tilapia, rabbitfish, striped bass, walleye, goldstriped amberjack, and carp (Lam 1980; Lam and Sharma 1985; Brown et al. 1988, 1989; Miwa et al. 1992; Ayson and Lam 1993; Hey and Farrar 1996; Tachihara et al. 1997). The results from the experimental thyroid hormone treatment of fish

eggs (embryos) and larvae are, however, not conclusive mainly due to the sensitivity to species-specific timing and dosage applied. It is not uncommon that thyroid hormone treatments resulted in altered body morphometrics and overstimulated growth of specific tissues including bones, muscles, and scales (reviewed by Eales 1979).

Thyroid hormones are deposited into eggs against the concentration gradient during vitellogenesis (Norberg et al. 1989; Babin 1992; Bjornsson et al. 1998). Maternally-introduced radioiodine was found in embryonic larvae of coho salmon *Oncorhynchus kisutch* as protein-bound forms (Kobuke et al. 1987), and maternal triiodothyronine (T_3) injection elevated T_3 level of striped bass *Morone saxitilis* (Brown et al. 1989). Secretion of thyroid hormones varies seasonally (Brown and Stetson 1985), and the uptake of T_3 is favored during oogenesis of marine fish eggs. Tagawa (1996), however, demonstrated the possibility of passive diffusion into eggs by estimating the T_4 level with which vitellogenin can bind in chum salmon. T_3 levels detected in unfertilized eggs of Japanese flounder, barfin flounder, striped jack, and yellowtail were variable between batches, and those levels were not correlated with survival of starved larvae (M. Tagawa, unpublished). Despite unresolved issues in the depository mechanism of thyroid hormones into eggs, those hormones decrease to nearly undetectable levels during the yolk absorption period, suggesting the utilization of thyroid hormones during embryonic development (Tanaka et al. 1995; Tagawa 1996). It has long been established that the period of yolk absorption is a time of sensitivity to thyroid hormones (Brown and Bern 1989). These results imply a role of maternally deposited thyroid hormones in rapidly developing marine embryos and larvae, which may be one determinant of "egg quality." Variation of hormonal levels could contribute to variation in egg quality, which in turn might compromise the consistency of hatchery production. Since hatchery success is largely affected by the survival of larvae through the critical, first-feeding period, egg quality as a consequence of variable maternal deposit may influence such survival.

Another important regulatory hormone in teleosts is cortisol, which is involved in the maintenance of hydromineral balance, osmoregulation, and glucose metabolism (Idler and Truscott 1972; McCormick 1995). Cortisol also acts as a regulator of development throughout the vertebrates. Maternal deposition of cortisol into eggs was evident in Japanese flounder, chum salmon, and tilapia, but cortisol declined to undetectable levels prior to hatching (de Jesus et al. 1991; de Jesus and Hirano 1992; Hwang et al. 1992). Cortisol influenced the timing of hatching in steelhead trout (Yeoh 1993; Mathiyalagan 1996). Cortisol also plays an important role in the regulation of carbohydrate utilization. Cortisol-injected rats had increased amylase activity (Kumegawa et al. 1980) and similar results have been reported in goats and pigs (Sanglid et al. 1994; Lopez et al. 1997). Since amylase activity is elevated during the first half of larval development (Kim 1999; Kim et al. submitted), cortisol may be a particularly important regulatory compound in larval threadfin.

Corticoid and thyroid hormones are known to interact in the regulation of a range of processes of within the target tissues. Cortisol decreased plasma level of T_3 in the European eel *Anguilla anguilla* (Redding et al. 1986) and increased hepatic conversion of T_4 to T_3 in brook char *Salvelinus fontinalis* (Vijayan et al. 1988). During metamorphosis of the Japanese flounder *Paralychthys olivaceus*, resorption of the dorsal-fin rays occurred under the influence of direct peripheral interactions between T_3 and cortisol (de Jesus et al. 1990) as found in amphibian metamorphosis (Norris and Dent 1989; Hourdry 1993; Denver 1997). In human premature infants, glucocorticoid and thyroid hormones are commonly used in the treatment of respiratory distress syndrome (Warburton et al. 1988).

In the current study, the effects of treatment with exogenous T_3 and cortisol were tested during larval threadfin development under hatchery conditions. Development of the digestive system and particularly the timing of patterns in the digestive enzymes was studied after exposing newly hatched larvae to these hormones by 1-h immersion. A combination of both hormones was administered under identical conditions to the

applications used in previous studies (Brown and Kim 1995; Kim and Brown 1997). A control group was subjected to similar handling, but without hormone exposure.

MATERIALS AND METHODS

The Pacific threadfin larvae were reared as described in an earlier series of experiments (Brown and Kim 1995; Kim and Brown 1997). All batches of eggs were from the same broodfish which were fed a mixture diet of frozen squid, krill, and artificial pellets while kept in an earthen pond at The Oceanic Institute. Embryonic development indicated that eggs were the products of multiple females, and spawns occurred within 2-4 h.

Newly-hatched larvae were immersed for 1-h in sea water containing a combination of hormone(s); 2.6 ppm of T_3 and 0.1 ppm of cortisol (TF group), or untreated sea water serving as a control (C). All larvae for each group were immersed in a bucket containing 12 L of water for each treatment. Following hormone treatment, larvae were stocked into separate rearing tanks (1500-L, fiberglass). The initial stocking density was determined as 40 larvae/L. Additional larvae were kept in a separate tank without live feed supply (starvation) to compare the digestive enzyme activities. The experiment was terminated at the onset of cannibalism (metamorphosis) on d 29 (Kim 1999).

Serial samples of larvae were taken from d 1 after hatching until d 14. More than 1,000 larvae were sampled and specific activities of digestive enzyme activities of whole body tissue were tested as described previously (Kim et al. submitted). In summary, chilled, pooled larval tissues were homogenized in a solution of glycerol saline (Maugle et al. 1982). Following centrifugation, supernatents containing soluble enzymes were divided into aliquots and stored at -70 C until the standard assays for digestive enzymes were performed. The technical aspects of the assays for serine protease, aspartic protease, collagenase, lipase, amylase, chitinase, cellulase, and phosphatase are described in detail by Kim et al. (submitted).

Larvae with induced levels of specific activity (units/mg protein) of digestive enzymes by the combination of T_3 and cortisol treatment (TF) were compared with those of untreated larvae (C). The changes in enzyme activities related to hormonal treatment at the time of first feeding were calculated as percentage change occurring between d 0 and d 3 relative to the range of values detected throughout the larval phase for each particular enzyme.

RESULTS

Among the hormone-treated larvae (TF), specific activities of amylase (Fig.1a), aspartic protease, collagenase, and phosphatase (Fig.1b) were elevated relative to those of untreated larvae by the time of first feeding. After the initiation of feeding, the activities of most digestive enzymes were similar in TF-treated and untreated larvae (Fig.1; d 3.3 or 8 h into d 3). The TF treatment

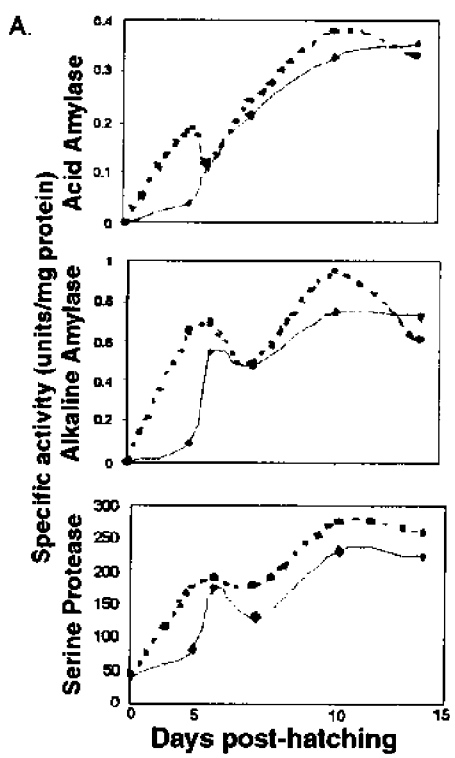
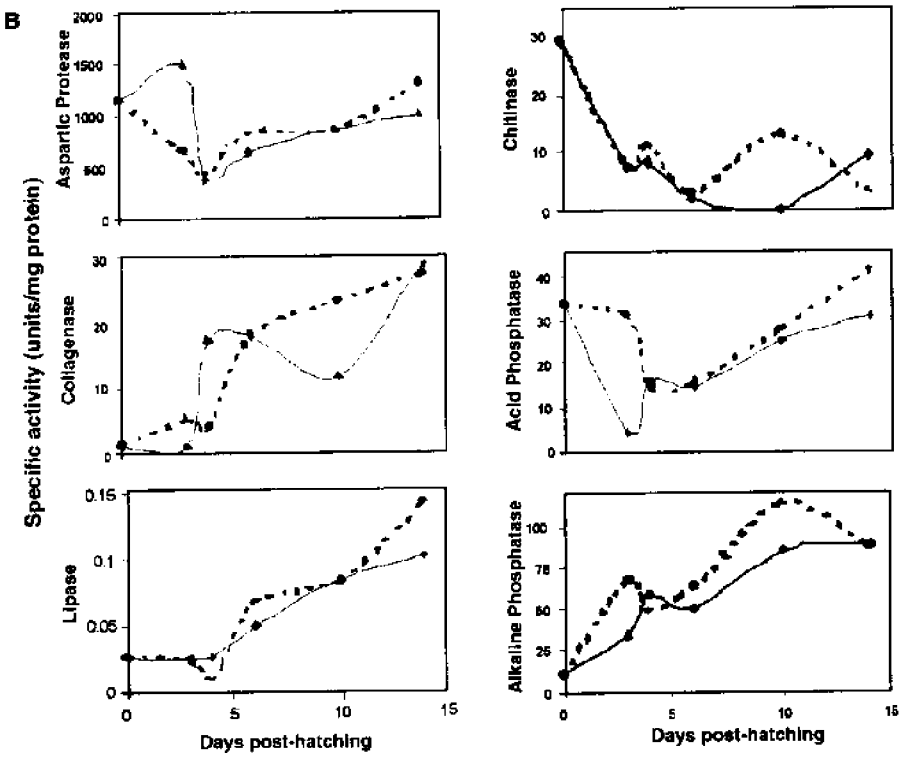


Figure 1. Specific activities of digestive enzymes of larval threadfin after treatment with a combination of T_3 and cortisol (- - - -) as compared with untreated larvae (——). A. Enzymes that responded to treatment with increased specific activity and B (following page) enzymes that were not affected.



elevated amylase and serine protease activities consistently during the first 2 wk of age (Fig.1 A), while patterns suggesting possible increases in the specific activities of other enzymes were not as clear (Fig.1 B).

The changes in digestive enzyme levels during early development are summarized in Fig. 2. This figure graphically presents the difference in each of the enzymes measured between hatching (d 0) and first feeding (d 3.3 or 8 h into d 3) corresponding to the critical period for larval survival in this species. The differences between specific activity between d 0 and d 3.3 varied considerably among the enzymes assayed, and appeared to be hormone dependent. Larvae sampled from the TF-treatment group had a sharp increase in amylase activity by the time of first feeding, relative to the control group (Fig. 2). Aspartic protease activity was reduced when larvae were TF-treated, while activity increased slightly in the control group of larvae. Reduction of acid phosphatase activity was less in larvae with

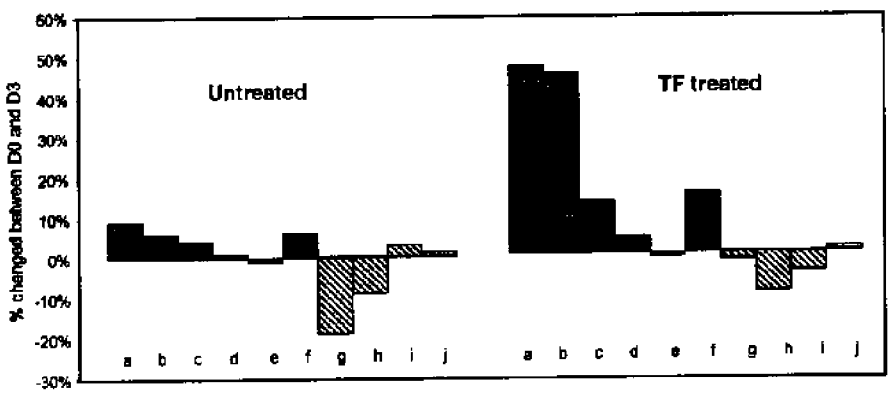


Figure 2. Inductive effect of the hormone treatment on changes in specific activity of digestive enzymes measured in larval threadfins, occurring between d 0 and d 3 after hatching. Percentage of change was the difference between the two values expressed as a percentage of the range of specific activities detected (see Methods and Materials). a: acid amylase, b: alkaline amylase, c: serine protease, d: collagenase, e: lipase, f: alkaline phosphatase, g: acid phosphatase, h: chitinase, i: aspartic protease, and j: cellulase.

the hormone treatment relative to larvae in the control group. Hormone treatment, however, did not affect specific activity of lipase.

DISCUSSION

We have reported previously that a single hr of immersion in sea water containing a combination of T_3 and cortisol (TF) conveys survival benefits to Pacific threadfin during the larval period (Brown and Kim 1995). This effect has been attributed at least partially to the hormonal stimulation of the onset of gastrointestinal function (Kim and Brown 1997). We interpret these results as an indication that some hormone effects or interactions are sufficiently potent to override other variables responsible for differences in cohort survival (i.e., egg quality). An episodic increase in mortality was observed in three stages in the course of the experiment. Mortality occurred from the time of hatching (d 0) through the first feeding (d 5), immediately prior to notochord flexion (d 12 to d 14), and post-flexion prior to metamorphosis (d 20 to d 22).

These results are consistent with patterns experienced routinely in marine hatcheries involving a dietary shift. Survival was not quantified in this experiment, although we have seen a consistent and positive survival effect among the TF-treated groups over several years of experimentation (Kim and Brown 1997).

Specker (1988) has proposed a dietary supplement of thyroid hormones for preadaptation to prepare larval intestinal tissues for feeding, and Tanaka et al. (1995) demonstrated that T_4 treatment enhanced the thickness of epithelial cells of the alimentary tract, suggesting improved absorptive function. When larval *moi* were treated with a combination of T_3 and cortisol, the specific activities of most digestive enzymes increased prior to first feeding (Fig.1) suggesting that these regulatory hormones preconditioned the digestive tract for increased digestion (this study) and nutrient absorption (Kim and Brown 1997).

Amylases were relatively most elevated and certainly the most changeable digestive enzymes for larval *moi* during the first 2 wk of age. The TF treatment enhanced the specific activity levels of amylase at both pH 5.4 and 7.4 as well as serine protease activity (Fig. 1). These data suggest that a combination of T_3 and cortisol advanced the capacity of larval threadfin for prey digestion, which may have physiological benefits both in the first-feeding stage and in subsequent dietary shifts (e.g., the transition from the consumption of rotifers to *Artemia sp.*, during d 12 and d 13). The present results suggest that the net effects of hormone treatment consisted of both an advance in the timing of the ontogenetic pattern of amylases and serine proteases in the threadfin, and an increase in the specific activities of these enzymes particularly during the first few d after hatching.

Digestive enzyme activities increased at the time of mouth opening, even among starved larvae (Fig. 3), indicating that enzymes were produced prior to the presence of live feed in the gastrointestinal tract. During the first-feeding period (initiation of feeding until the point-of-no-return), amylase activity increased by more than threefold for the control group but decreased among starved larvae. This suggests some reliance during this stage on carbohydrate metabolism, possibly for energy, during the critical period. Specific activities of most digestive enzymes other than amylase were low even when prey (substrate) was abundant in the lumen. This indicates that protein and fat digestion are relatively unimportant during the first half of the larval period.

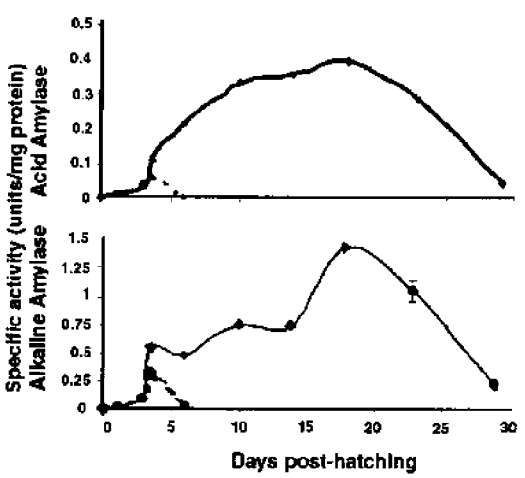


Figure 3. Effect of starvation on the ontogenetic patterns of digestive enzymes. Starved larvae are indicated by dashed lines (- - - -) and fed (controls) are shown as solid lines (__).

Nonetheless, when larvae were TF-treated at hatching, specific activities of lipase, protease, and alkaline phosphatase increased. In other words, digestive enzyme activities are inducible by hormones.

Improved protein and fat uptake by Japanese flounder treated with T_4 (Tanaka et al. 1995) and high absorptive function by cortisol-treated tilapia (Ayson et al. 1995) also support the maturation-promoting function on alimentary tissues of these hormones applied individually. Multiple hormonal regulators are known to interact in the control of gastrointestinal tract changes during amphibian metamorphosis (Dent 1988); cortisol and T_3 are among the most prominent, working in concert against the antimetamorphic actions of prolactin. Despite the lack of evidence to discriminate between possible direct and indirect actions of T_3 and cortisol on gut development and function, these hormones have been shown to have some direct peripheral interactions on developing target tissues (Redding et al. 1986; Vijayan et al. 1988; de Jesus et al. 1990).

Maternal deposit of cortisol into eggs and its importance in the regulation of embryonic development are still questioned (Brown and Bern 1989; Hwang et al. 1992; Tanaka et al. 1995; Tagawa 1996), but cortisol has been detected prior to larval corticosteroidogenesis (Perez et al. 1999). When both hormones are applied exogenously, as in this study, they appear to interact to promote one or more vital developmental processes, which can convey survival advantages even under compromised conditions.

The conclusion that the enzymes measured here reflect early digestive physiology appears consistent with other results, particularly when considered with evidence of development-promoting effects of these hormones at the same dosage, on the larval intestine in the same species (Kim and Brown 1997; Kim 1999). Nevertheless, because whole larvae were pooled for the extraction of enzymes, their specific tissue source can only be considered speculatively. It is possible, and in fact likely, that the early appearance of amylases and their promotion by thyroid hormone and cortisol is not restricted to the gastrointestinal system. Certainly these and other enzymes reported in this study could be involved in carbohydrate metabolism and tissue reorganization elsewhere in the developing larvae. More tissue-specific technical approaches such as histochemistry or *in-situ* hybridization may allow finer-resolution and more definitive examination of some of the ontogenetic questions raised in this study.

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ASSESSMENT OF THE GROWTH POTENTIAL OF THE ROTIFER *BRACHIONUS PLICATILIS* BY EVALUATING BIOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

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ABSTRACT

To anticipate the changes that occur during continuous mass culture of the rotifer *Brachionus plicatilis*, biological characteristics such as extent of digestive organs in the body, frequency distribution of lorica length, and physiological tolerance to a hyper-saline environment were examined. The extent of digestive organs in the body was measured as the proportion of digestive-organ area to the body (PDA) of non-egg-bearing females fed on freshwater *Chlorella*. Tolerance to hyper-saline environment was evaluated as the percentage of individual rotifers still swimming after exposure to 70 ppt saline water for 3 h. The PDA and size frequency distribution of lorica length of feeding individuals changed only after culture conditions deteriorated sharply. The non-feeding individuals in the size range of 210-240 μm increased from d 7 during culture under unfavorable conditions. Hyper-saline tolerance suddenly dropped when rotifer growth was close to peak level and this was more evident in egg-bearing than non-egg-bearing females. From those results, the size frequency distribution (210-240 μm) of non-feeding individuals seemed to be useful in forecasting changes during mass culture. Hyper-saline tolerance (70 ppt for 3 h) was also evaluated and found to be a suitable index of rotifer activity and both criteria can be used to assess the growth potential of rotifers under mass culture conditions.

INTRODUCTION

Marine rotifers are widely used as a live food in the early stages of marine fish larval rearing. Mass rotifer production incurs problems, however, such as a sudden decrease of rotifer density and decline of population growth. If these problems can be anticipated, countermeasures can be taken to prevent or curtail a total collapse of the culture. Swimming speed (Snell et al. 1987; Korstad et al. 1995), egg ratio (Korstad et al. 1995), and tolerance to chemical toxicity (Juchelka and Snell 1994; Janssen et al. 1994) have been good indices to monitor the population growth of rotifers. These indices require intensive effort, however, and accordingly they are not practical at the rotifer production site. Therefore, it is important to determine simple yet accurate indices for monitoring the changing conditions when rotifers are under mass culture. To understand these changing conditions that occur under high-density rotifer culture conditions, biological characteristics, such as the extent of the digestive organs in the body, size frequency distribution of lorica length, and physiological tolerance to a hyper-saline environment were examined in individual *Brachionus plicatilis*.

MATERIALS AND METHODS

The Kinki L-type strain of *B. plicatilis* was used in all experiments. This strain has been cultured and used for larval rearing at the Amami Station of the Japan Sea-Farming Association since 1994. Three experimental batch cultures were performed (culture I, II and III) using 500-L polycarbonate (PC) tanks for 12 d. Filtered sea water was used as the culture medium, and temperature was regulated at 26 C. Commercially available concentrated freshwater *Chlorella*, (Nisshin Science Co. Ltd; average cell density, 150 x 10^8 cell ml^{-1}) was used as food for the rotifers. This algae suspension (0.4 -1.0 L) was added twice/d to each culture tank. The number of rotifers in 0.5 ml of culture medium was counted three times in order to estimate the rotifer density.

Proportion of Digestive-organ Area to the Body (PDA)

A green-colored area, corresponding to the digestive organs, appeared in the rotifer body when fed *Chlorella*. The proportion of this green-colored area to the body (PDA) was used as a index of feeding activity. The PDA was measured on d 1, 4, 7 and 10 in cultures I and II using the following procedure. Rotifers were transferred from the experimental culture to a 1-L flask containing algae suspension with a density of 10 x 10^5 cells ml^{-1}. PDAs of 30 non-egg-bearing individuals were then measured at 1, 5, 10, and 15 min using a microscope with a picture-analysis system (Olympus Co. Ltd; RS-3100). Data were statistically analyzed using the *t*-test.

Size Frequency Distribution of Lorica Length

The size frequency distribution of lorica length of both feeding and non-feeding rotifers was evaluated on d 1, 3, 5, 7, 9 and 11 in culture III. One sample containing 100 feeding and 50 non-feeding individual rotifers was measured under the microscope described previously.

Tolerance to Hyper-saline Environment

The hyper-saline challenge test was performed by calculating the percentage of swimming rotifers when exposed to high salinity. At first, to determine the adequate experimental conditions, the changes in the percentage of swimming rotifers were evaluated at various salinity and exposure times. Salinity was adjusted from 34 to 80 ppt (at 6 ppt increments) by adding sodium chloride (NaCl) to sea water. Exposure time was 0.5, 1, 2, 3, 5, 10 and 20 h. From the results of those preliminary experiments, the hyper-saline challenge test was designed as follows: rotifers were transferred into wells of a Multiwell Plate (Iwaki Glass Co. Ltd; 6-well type) containing 70 ppt saline water. After 3 h, the percentages of swimming rotifers were calculated. The hyper-saline challenge test was performed every day during culture I.

RESULTS AND DISCUSSION

Proportion of Digestive-organ Area to the Body (PDA)

Culture I resulted in good growth during which rotifer density attained 825 ind/ml^{-1} on d 8. Conversely, the rotifers in culture II did not obtain desirable growth and the peak was not clear (Fig.1). The digestive organs of egg-bearing rotifers could not be clearly distinguished from the developing ovary area. Therefore, only the change in PDA of non-egg-bearing rotifers is

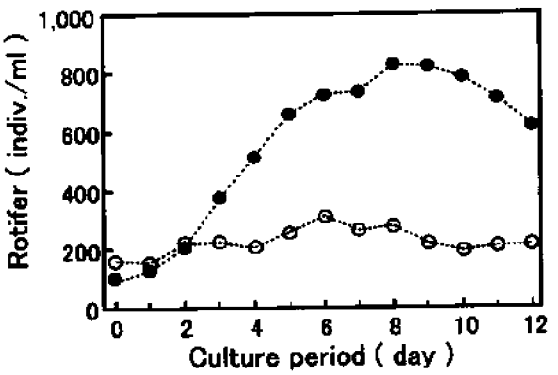

Figure 1. Growth of rotifer in cultures I and II. ●, culture I; ○, culture II.

shown in Fig. 2. PDAs in both culture I and II were not different from d 1 to d 7. On d 10, however, when the growth peak passed, the PDA of rotifers in culture II was significantly (P <0.01) less than that of rotifers in culture I. These results mean that PDA did not fully reflect the changing

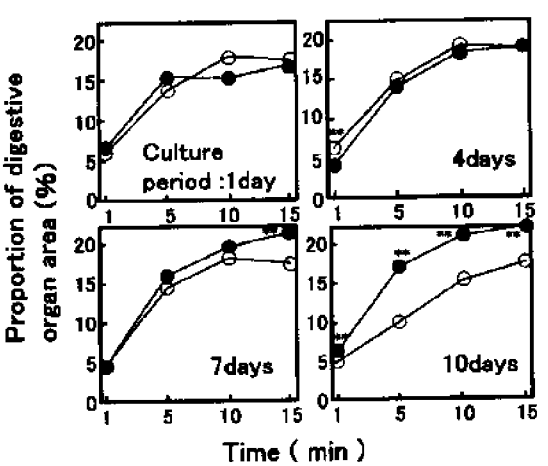

Figure 2. Changes in proportion of digestive-organ area of rotifer in cultures I and II. ●, culture I; ○, culture II; Significantly different between culture I and culture II (**:P<0.01).

conditions of the culture. One explanation is that reproductive activity may be affected by declining environmental conditions prior to feeding activity. Therefore, the PDA index is apparently not useful as a index to readily monitor rotifer population growth.

Size Frequency Distribution of Lorica Length

Culture III showed good growth, with 839 ind/ml^{-1} at the peak of rotifer density on d 7 (Fig. 3). The size frequency distribution of feeding rotifers did not change from d 1 to d 9. But on d 11, when the growth peak passed, production of offspring decreased and the proportion of rotifers over 250 μm increased (Fig. 4). The size frequency distribution of lorica length of feeding rotifers was not suitable as an index to monitor population growth, since it changed only when culture conditions deteriorated sharply. On the other hand, in the non-feeding rotifers (Fig.5), the small size group of 160-200 μm and the large size group of 250-300 μm were observed mainly from d 1 to d 5. The former are offspring, while the latter may be old and weak adults which can not feed. The intermediate group between 210 and 240 μm appeared from d 7, and increased over the course of culture. As first spawning size of this rotifer is about 240 μm, this intermediate group appeared young and active. It was concluded that the appearance of these young and non-feeding rotifers give an early warning of an impending collapse of the culture. Therefore, this result indicates that the size frequency distribution of non-feeding rotifer seems to be useful as a index to monitor rotifer population growth.

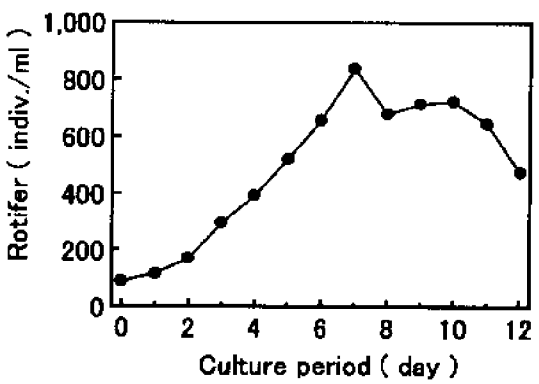

Figure 3. Growth of rotifer in culture III.

Tolerance to Hyper-saline Environment

The percentage of swimming rotifers at various salinity and exposure times is shown in Fig. 6 where changes are reflected when salinity ranged between 60 and 80 ppt. The midpoint (70 ppt) was therefore chosen for the hyper-saline challenge test. The percentage of swimming rotifers in 70 ppt was observed to be stable from 3 to 5 h, which indicated a suitable incubation time. Therefore the challenge test of 70 ppt salinity

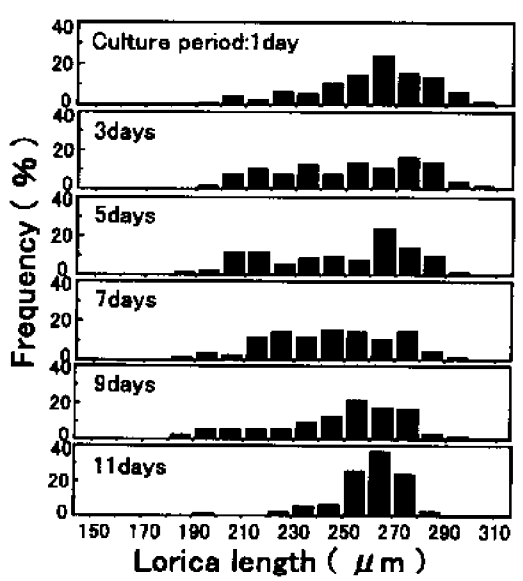

Figure 4. Changes in frequency distribution of lorica length of feeding rotifer in culture III.

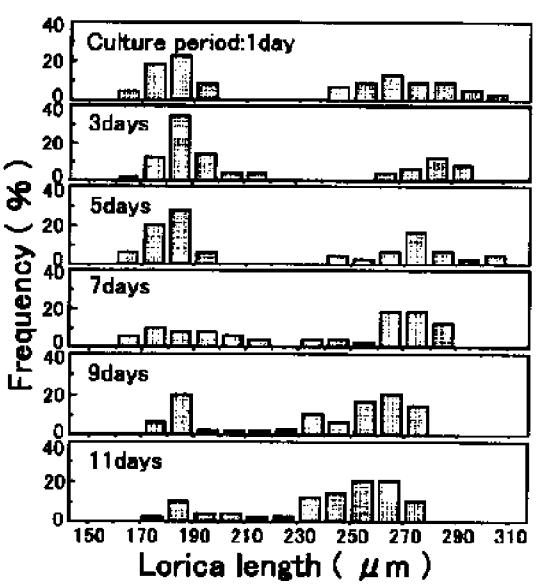

Figure 5. Changes in frequency distribution of lorica length of non-feeding rotifer in culture III.

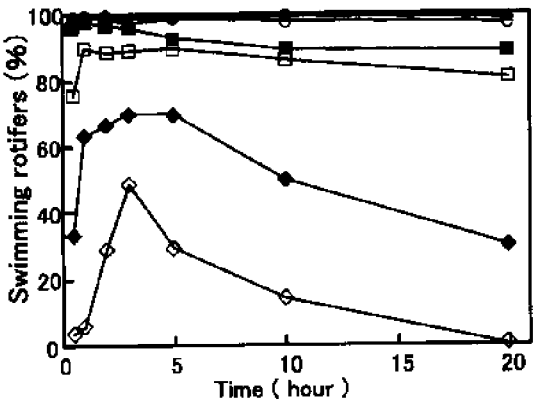

Figure 6. Changes in percentage of swimming rotifers exposed to the hyper-saline water. ●, 34 ppt; ○, 40 ppt; ■, 50 ppt; □, 60 ppt; ◆, 70ppt; ◇, 80 ppt.

for 3 h was adopted. The relationship between the percentage of swimming rotifers and population growth in culture I is shown in Fig.7. The percentages were nearly constant at 90% from d 1 to d 6. But in egg-bearing rotifers, a sudden drop to 68% was observed on d 7 when growth was near peak level. Therefore, tolerance of the egg-bearing rotifers to a hyper-saline environment was concluded to be a suitable index to measure rotifer activity, since this index changes prior to the peak of population growth.

If we can use this index during the mass culture of rotifers, it may be possible to forecast the impending collapse of a culture in a relatively short amount of time and also select vigorous rotifers that are in a rapid growth phase. Consequently, mass rotifer production can be designed to be more stable and efficient.

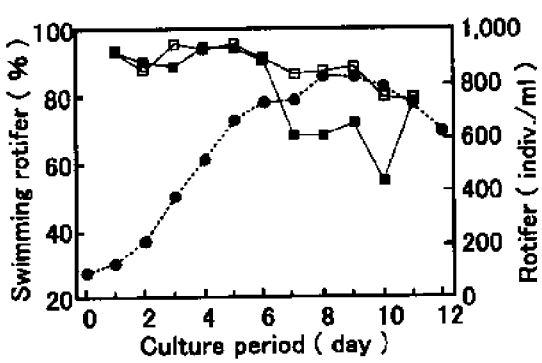

Figure 7. Population growth (●, rotifer density) of rotifer in culture-1 and changes in the percentage of swimming rotifer (■, egg-bearing; □, non-egg-bearing rotifer) in hyper-saline challenge test (70 ppt).

ACKNOWLEDGMENTS

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ACHIEVING ADVANCED MATURATION AND SPAWNING IN YELLOWTAIL *SERIOLA QUINQUERADIATA* BY THE MANIPULATION OF PHOTOPERIOD AND WATER TEMPERATURE

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ABSTRACT

Yellowtail *Seriola quinqueradiata* held in captivity begin maturing and spawning in late April to early May, while yellowtail in the wild spawn about 2 mo earlier. The 2 mo difference in spawning periods is significant, as the cultivated fry are smaller than those in the wild when they are released for stock enhancement. The purpose of this study was to develop techniques for obtaining eggs of yellowtail in captivity earlier than usual to coincide with the natural spawning season of yellowtail in the wild. The first experiments examined the effects of extended daylength (1800-2400; EDL) on ovarian maturation and the use of human chorionic gonadatropin (HCG) to induce final maturation and spawning. In 1991, 1992, and 1993, female broodstock were induced to mature more rapidly than those under natural photoperiod by extending daylength by 6 h for 28 ds (1991, 1992) or 20 ds (1993). Each year, the mean number of eggs from fish under EDL was higher than that of the control group. The results demonstrate that manipulation of daylength is an effective method for accelerating maturation in female yellowtail broodstock.

Further experiments in 1994-1995 and 1995-1996 examined the combined effects of photoperiod control on the maturation of yellowtail, namely, a short-day (SD) treatment of 1 mo followed by a long-day treatment and water temperature control (≥19 C) for one mo. In both years, the daylength was set to 8 h (8 L: 16 D) for 1 mo followed by a 10 h extension (18 L: 6 D) for the next mo under controlled water temperature. Female broodstock kept under controlled photoperiod and water temperature were induced to mature more rapidly than those maintained under natural conditions. However, neither controlled photoperiod nor water temperature alone was sufficient to induce maturation in yellowtail. After HCG injection, during both years, fish kept under EDL or warm temperature spawned earlier than usual in captivity. Consequently, photoperiod and water temperature manipulations are effective in accelerating maturation of female yellowtail broodstock to the point where fertilized egg production can be achieved by the induction of spawning using HCG.

INTRODUCTION

The wild population of yellowtail *Seriola qiunqueradiata* is one of the most valued fishery resources in Japan. However, the natural stock has steadily declined over the years. In 1978, the first program for broodstock management and production of the juveniles of yellowtail for stock enhancement was initiated by the Japan Sea-Farming Association (JASFA) to offset the decline. Due to the advancements in techniques for the induced spawning of broodstock and the rearing of larvae and juveniles, as many as 1 million juveniles/yr have been produced.

The spawning of wild yellowtail in the waters around Shikoku and Kyushu has been observed from late February to April (Umeda 1991). However, the spawning season of yellowtail reared in net cages under natural conditions at the JASFA Komame Station in Kochi Prefecture occurs about 2 mo later. Due to this delay in spawning in captivity, artificially-produced juveniles are much smaller than wild juveniles at the time of release, resulting in poorer survival. The project initiated by JASFA was designed to obtain eggs at an earlier period in order to release tagged juveniles close to the same size and same age as juveniles found in the wild.

There have been many investigations of controlling the natural spawning season in fish by manipulating environmental factors such as photoperiod and water temperature (Breton and Billard 1977; MacQuarrie et al. 1978; Whitehead et al. 1978; Beacham and Murray 1993). The present study focused on determining appropriate photoperiod and temperature regimens to result

in the production of spawned eggs at an earlier time than usual from captive yellowtail broodstock.

MATERIALS AND METHODS

Yellowtail Broodstock

Experiment 1 was conducted during 1991 and 1993. Yellowtail used as broodstock in 1991 (Table 1) were captured by set-nets in Komame inlet (Kochi Pref.) and reared on moist pellets (MP) (Mushiake et al. 1993) for about 2 yr in a floating rectangular net cage (10 x 5 x 6 m) at the Komame Station of JASFA. All females used for the experiments were marked individually by a personal identification tag (PIT) (Identification Devices Inc., USA), implanted into the dorsal muscle when they were transferred from the net cage into indoor spawning tanks (110 m^3). Fish used in the 1992 and 1993 experiments were transferred from a private farm in Ehime Prefecture to the Komame Station and fed on moist pellets for 1.5 yr as captive broodstock.

In experiment 2 , during 1994-1995 and 1995-1996, the adult yellowtail were captured by set-nets in the Komame inlet and fed moist pellets (MP) or commercial soft-dry pellets (SDP: Sakamoto Fish Feed Co. Ltd., Chiba, Japan) (Mushiake et al. 1995) during 1994 and 1995, respectively, at Komame Station (Table 1). The fish were reared in net cages (10 x 10 x 6 m) for a period of 8 mo and transferred to indoor spawning tanks (65 m^3) for spawning on 14 November in 1994 and 1995. All females were marked individually by PIT.

Experimental Rearing Conditions

Table 1 shows a summary of the two different groups in experiment 1 and the four groups in experiment 2. Each group in experiment 1, either the EDL-treated or control, consisted of 20 (1991) or about 10 (1992, 1993) individuals. The experiments were begun by placing the fish in the spawning tanks (110 m^3). All fish were kept under natural lighting conditions from sunrise to 1800. The group exposed to EDL received

Table 1. Details of the yellowtail broodsotock used for the experiments

Year	Group No.	Origin	No. of fish (M:F)*2	Diet*3	Fork length ±SD (cm)	Body weight ±SD (kg)	Condition factor ±SD	Rearing condition Photoperiod*4	Water temperature*5
Experiment 1									
1991		Wild-1	20 (12:8)	MP	82.5±3.3	11.38±1.53	20.22±1.21	controlled-1	natural
		Wild-1	20 (12:8)	MP	82.4±2.9	11.42±1.60	20.24±1.34	natural	natural
1992		Wild-2	10 (6:4)	MP	77.8±2.1	9.31±0.83	19.79±1.36	controlled-1	natural
		Wild-2	9 (6:3)	MP	76.6±1.3	8.70±0.54	19.38±1.46	natural	natural
1993		Wild-2	12 (6:6)	MP	76.3±1.9	8.51±0.67	19.17±0.70	controlled-1	natural
		Wild-2	12 (6:6)	MP	73.8±1.9	7.78±0.66	19.36±1.05	natural	natural
Experiment 2									
1995	1	Wild-1	6 (3:3)	MP	81.9±1.7	10.36±1.24	18.65±1.06	controlled-2	controlled
	2	Wild-1	6 (3:3)	MP	81.6±1.4	10.14±1.86	18.67±1.15	controlled-2	natural
	3	Wild-1	6 (3:3)	MP	81.9±1.6	10.28±1.26	18.71±1.04	natural	controlled
	4	Wild-1	6 (3:3)	MP	81.8±1.8	10.31±1.45	18.83±1.11	natural	natural
1996	1	Wild-1	12 (6:6)	SDP	80.7±2.1	9.86±1.22	18.79±1.27	controlled-2	controlled
	2	Wild-1	12 (6:6)	SDP	81.1±1.9	9.95±1.71	18.66±1.34	controlled-2	natural
	3	Wild-1	12 (6:6)	SDP	81.0±1.9	9.77±1.68	18.39±1.08	natural	controlled
	4	Wild-1	12 (6:6)	SDP	80.9±1.7	9.91±1.49	18.71±1.42	natural	natural

*1 Wild-1: Captured by set-net fishery and reared for 2 years, Wild-2: Captured at juvenile stage and reared for 4 years in all.

*2 M: male, F: female.

*3 MP: Moist pellets (formula feed prescribed by National Research Institute of Aquaculture, Japan + raw fish (1:1)), SDP: Commercial soft dry pellets (Sakamoto Fish Feed Co. Ltd., Chiba, Japan).

*4 Controlled-1: Extended daylength treatment (EDL) from 18:00 to 24:00, controlled-2: short-day treatment for one month followed by EDL treatment for the next one month.

*5 Controlled: Water temperature was kept at a minimum of 19 C.

additional lighting provided by two tungsten flood lights (200 W/l) supported above each tank from1800 to 2400. The EDL treatment was initiated on the day the fish were stocked into each experimental aquarium, and continued for 28 (1991, 1992) or 20 (1993) days at which time they were injected with human chorionic gonadotropin (HCG) to induce final maturation and spawning. The water temperature in experiment 1 was maintained around 19 C for the duration of the experiment.

In experiment 2, the broodstock in group 1 were maintained under both controlled photoperiod and water temperature. In group 2 the photoperiod and in group 3 the water temperature was controlled, respectively. No environmental manipulation was provided for fish in group 4. Photoperiod manipulation consisted of a short-day (SD) treatment followed by EDL treatments. The SD treatment was performed by spreading a matted black sheet (light transmittency 0%) over the surface of each tank from 1700 in the evening until 0900 the next morning (8 L-16 D). The SD treatment was initiated on d 3 (17 November: d 0 in Fig. 2) after transferring the fish into the indoor spawning tank, and continued for either 31 d (until 18 December 1994) or 32 d (until 19 December 1995). The EDL treatment commenced on the day following the termination of the SD treatment and continued for either 32 d (until 19 January 1995) or 34 d (until 22 January 1996). The EDL treatment was attained by extending the daylength 1 h every 3 or 4 d until daylength reached 18 h. The long photoperiod (18 L) was maintained from 19 January 1995 and 22 January 1996, throughout the spawning period.

The water temperature of the temperature-controlled tanks (1 and 3) in 1994-1995 and also 1995-1996 was kept at a minimum of 19 C by inflow of sea water heated with thermostatic devices. In groups 2 and 4, the water temperature was allowed to fluctuate naturally.

Examination of Ovarian Maturation

Ovarian tissue was sampled by inserting a cannula into the genital pore of fish which had been individually identified by their PIT tag. For each fish, the diameters of 100 sampled oocytes were examined under a stereoscopic microscope (Nikon) and the mean oocyte diameter was calculated. Statistical analysis comparing the mean oocyte diameter between experimental groups was performed by *t*-test.

In experiment 1, the state of maturation in the females was assessed by cannulation three times in 1991 and 1992, just prior to distributing the fish into the spawning tanks (d 0), and on d 14 and d 28 after the start of experiments in both the EDL and control groups. In 1993, maturation examinations were carried out on d 0, d 10 and d 20.

In experiment 2, the stage of ovarian maturation was examined four times: just prior to distributing the fish into the spawning tanks on d 31 and d 32 (when the SD treatment was completed), on d 63 and d 66 (when the EDL treatment was completed), and on d 76 and d 97 (when fish were injected with HCG) in 1995 and 1996.

Induced Spawning by Hormone Injection and Evaluation of Egg Quality

In experiment 1, in order to induce spontaneous spawning in the indoor spawning tanks, HCG was injected at a dosage of 600 IU/kg BW into the dorsal muscle of both sexes, on d 28 (31 March 1991; 4 April 1992), or d 20 (25 March 1993) of EDL treatment.

In experiment 2, HCG was injected on d 76 in 1995 (1 February) and d 97 in 1996 (19 February). About 2 d after the administration of HCG in both experiments, fish began to spawn. Eggs were collected each day from 1700 to 0900 for as long as the fish continued to spawn. The number of eggs produced per fish/d was estimated by counting the number of eggs in a volume of 1 ml.

The diameters of 50 buoyant eggs, percent fertilization and the number of eggs having more than one oil droplet (abnormal eggs) were examined using a profile projector (Nikon). Percent hatching was also determined by estimating the numbers of larvae in each net. The percentage of normal larvae was estimated by counting the deformed and abnormal larvae (larvae having more than one oil droplet or having unusual oil deposition).

RESULTS

Ovarian Maturation by the EDL Treatment

The changes in oocyte diameters with time in all test groups in experiment 1 (1991-1993) and 2 (1994-1995, 1995-1996), are summarized in Fig. 1 and 2, respectively. Mean oocyte diameters at the start of experiment 1 ranged between 562 and 584 μm as shown in Fig. 1. In 1991 and 1992, mean oocyte diameters in the EDL group on d 28 were significantly ($P<0.01$) larger than those in the control groups. In 1993, the oocyte diameters in the EDL group on d 20 were also significantly ($P<0.01$) larger than those obtained from the control group.

In experiment 2, conducted in 1994-1995 and 1995-1996 (Fig. 2) the mean oocyte diameters at the beginning of experiments ranged from 309 to 314 μm and 339 to 347 μm, respectively. In 1994-1995, the mean oocyte diameters in the group 4 (that did not receive any environmental control) were 343 μm on d 31, 438 μm on d 63, and 504 μm on d 76 of the experiment. Mean

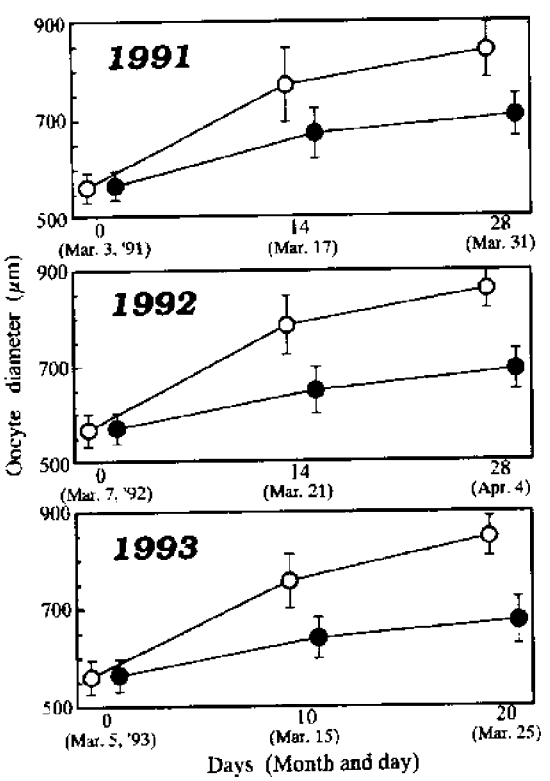

Figure 1. Changes in mean oocyte diameter of yellowtail in experiment 1. Vertical lines represent the standard error. ○, EDL treatment; ●, control.

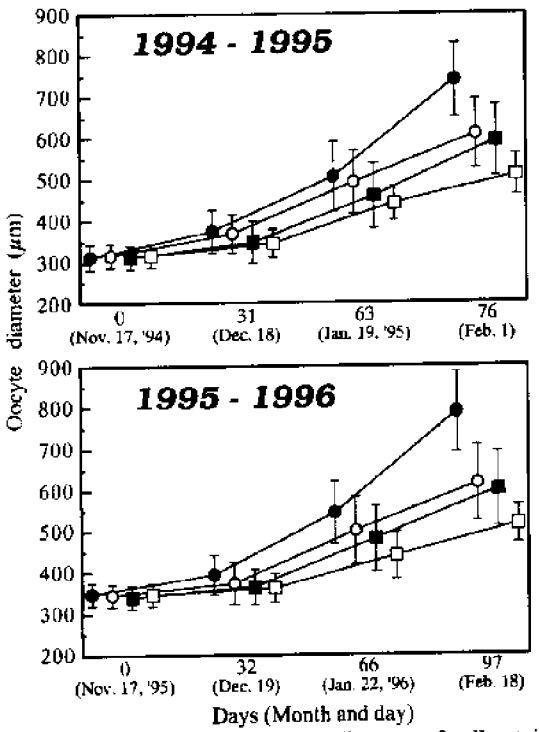

Figure 2. Changes in mean oocyte diameter of yellowtail in experiment 2. Vertical lines represent the standard error. ●, group No. 1; ○, No. 2; ■, No. 3; and □, No. 4.

oocyte diameters in fish from the photoperiod and water temperature-controlled group (1) were 373, 503, and 735 μm on d 31, d 63, and d 76, respectively. The mean oocyte diameters of fish from group 1 on d 76 was significantly larger ($P<0.01$) than those obtained from group 4. The mean oocyte diameters of females from either the photoperiod (2) or water temperature (3) controlled groups were intermediate to those of groups 1 and 4. In 1995-1996, the mean oocyte diameters were 359, 436, and 511 μm in group 4 and 393, 542, and 784 μm in group 1 on d 32, d 66, and d 97, respectively, indicating a significant difference ($P<0.01$) in the state of maturation between the two groups. The trend of increasing mean oocyte diameters in the other two groups (2 and 3) was similar in both years.

Induced Spawning

The results of induced spawning trials of yellowtail broodstock injected with HCG in experiment 1 and 2, are summarized in Tables 2 and 3, respectively. In experiment 1, the numbers of eggs produced in the EDL treatment during 1991 and 1993 were significantly ($P<0.01$) higher

Table 2. Induced spawning results of yellowtail injected with HCG in experiment 1

Year		1991		1992		1993	
Test group		EDL*1	Control	EDL	Control	EDL	Control
Spawning period		Apr.3-Apr.15	Apr.3-Apr.17	Apr.7-Apr.16	Apr.7-Apr.16	Mar.28-Apr.9	Mar.28-Apr.9
Spawning days		13	15	10	9	13	13
Eggs							
Eggs produced/fish	($X10^3$)	2029.8*2	958.2	2139.1*2	1058.9	2246.3*2	987.1
Buoyant eggs/fish	($X10^3$)	1657.1*2	607.6	1418.7*2	628.1	1680.1*2	605.8
Rate of buoyant eggs	(%)	81.6*3	63.4	66.3	59.3	74.8	61.4
Rate of fertilized eggs	(%)	77.0*3	54.9	57.1	49.3	58.4	49.1
Hatched larvae							
Total larvae obtained from total eggs	(%)	42.3*2	18.2	42.2*2	21.3	43.6*2	25.6
Normal larvae obtained from total eggs	(%)	28.4*2	10.7	31.5*2	11.7	32.4*2	15.2

*1 EDL: extended daylength treatment.
*2 Significantly different ($p<0.01$) as compared with the result of the control in the same year (t-test).
*3 Significantly different ($p<0.05$) as compared with the result of the control in the same year (t-test).

Table 3. Induced spawning results of yellowtail injected with HCG in experiment 2

Year		1994 - 1995				1995 - 1996			
Test group		No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4
Spawning period		Feb.3-Feb.9	-	-	-	Feb.21-Mar.3	-	-	-
Spawning days		6	0	0	0	9	0	0	0
Eggs									
Eggs produced/fish	($X10^3$)	418.0	0	0	0	1541.7	0	0	0
Buoyant eggs/fish	($X10^3$)	155.7	-	-	-	759.2	-	-	-
Rate of buoyant eggs	(%)	37.2	-	-	-	49.2	-	-	-
Rate of fertilized eggs	(%)	14.8	-	-	-	66.6	-	-	-
Hatched larvae									
Total larvae obtained from total eggs	(%)	4.2	-	-	-	24.7	-	-	-
Normal larvae obtained from total eggs	(%)	1.8	-	-	-	23.6	-	-	-

than those from the control groups. There was also a significant difference ($P<0.01$) in the number of normal larvae obtained between the two groups.

In experiment 2, only the fish in test group 1 spawned on d 2 after administration of HCG in 1994-1995 and in 1995-1996. In 1994-1995, after the injection of HCG, the broodstock began to spawn on 3 February and spawned daily until 9 February. They produced 418.0 x 10^3 eggs per fish, 37.2% of which were buoyant. In 1995-1996 the fish injected with HCG began to spawn on 21 February, and spawned daily from 24 February to 3 March. They produced 1541.7 x 10^3 eggs/fish, 49.2% of which were buoyant.

DISCUSSION

Changes in the mean oocyte diameter with time in experiment 1 (Fig.1) clearly indicate that ovarian maturation was accelerated by the EDL treatment. All data concerning egg quality indicated that the broodstock exposed to EDL treatment produced eggs that were superior to those in the control groups. This result demonstrates that the maturity of yellowtail broodstock can be manipulated by EDL to result in large numbers of eggs of suitable quality at a time that should result in the production of seedlings of appropriate size for use in stock enhancement activities.

As shown in Fig. 2, changes in mean oocyte diameters of broodstock in experiment 2 indicate that ovarian maturation in yellowtail was accelerated by manipulating both photoperiod and water temperature. The broodstock of all groups were injected with HCG on 1 February (on d 76) and 19 February (on d 97) in 1994-1995 and 1995-1996, respectively. The fish having mean oocyte diameters around 700 µm could be spawned by HCG injection, although the quantity and quality (percent buoyancy, fertilization, and hatching) were low. HCG-treated fish that possess mean oocyte diameters around 800 µm (1995-1996) responded by spawning a larger number of eggs that were decidedly of better quality. Therefore, it was concluded that the success of accelerated egg production from yellowtail broodstock would also depend on administering HCG at the appropriate state (mean oocyte diameter of 800 µm) of maturity.

Spawning results (number of eggs and egg quality) of group 1 in 1995-1996 were superior to those in 1994-1995, but not as good as those reported previously from other culture activities (Mushiake et al. 1995). However, for the stock enhancement of yellowtail, in order to release juveniles of similar size to those in the wild, egg production earlier than the normal captive spawning period is required. Further research is necessary to improve the egg quality of from the advanced spawning yellowtail when maintained under controlled photoperiod and water temperature conditions.

For pink salmon *Oncorhynchus gorbuscha* (Beacham and Murray 1988, 1990), it has been suggested that acceleration of maturation is more likely to be achieved through manipulation of photoperiod rather than water temperature. The distinction between which environmental parameter is more important could not be discerned for the yellowtail broodstock, as the mean oocyte diameters from (photoperiod-controlled) and (water temperature-controlled) did not differ significantly from each other.

Although the mechanism by which ovarian maturation is accelerated remains to be clarified, it was found that yellowtail kept under controlled photoperiod and water temperature were able to spawn in February, 2 mo earlier than those held under ambient conditions. These results indicate that yellowtail juveniles can be produced earlier than usual at the JASFA Yashima Station, Kagawa Prefecture. In 1996, seed production was conducted and tagged juveniles were released into the sea 2 mo earlier than previously reported. The released fish, which were similar in size to their counterparts in the wild, showed a high percent recovery (12.9%) compared with the usual 0.2–3.1% of fish produced and released during the usual time near the eastern part of the Seto Inland Sea (unpublished data).

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THE APPLICATION OF DEEP SEA WATER IN JAPAN

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ABSTRACT

Deep sea water (from a depth of more than 200 m) has cold temperature, abundant nutrients, and good water quality that is pathogen-free and stable. Basic research on the utilization of this water for fisheries in Japan began in 1976 and at present, deep-seawater pumping systems are established in Toyama and Kochi Prefectures and under construction in Shizuoka and Okinawa Prefectures. The research emphasis of many national organizations, prefectures, universities, and private companies is shifting from basic research to feasibility studies or practical applications of deep sea water. For example, in Kochi Prefecture, located in southern Japan, it was found that deep sea water is advantageous in the aquaculture of cold-water species. Current fisheries-related projects include:

- aquaculture (sea vegetables, fishes, shellfish, etc.)
- basic research on deep sea organisms
- restoration of sea grass habitats

A wide range of projects unrelated to fisheries that are utilizing deep sea water to develop new industries and contribute to local economies include:

- the food industry
- medical treatment facilities
- cooling water for power stations
- agriculture of cold climate vegetables

Future investigations should focus on further explorations of deep sea water attributes, a cascade system for using deep sea water, reduction of costs, and potential environmental impacts.

INTRODUCTION

Currently, the ultilization of deep sea water (DSW) is receiving much attention due to its high productivity, large quantity, and potential for recycling energy. Deep sea water, accounting for 95% of all sea water, generally refers to sea water from a depth of more than 200 m. DSW circles the globe over a period of about 2000 yr, and the up-welling of DSW occurs regularly in the oceans and seas throughout the world. Although it constitutes no more than 0.5% of all water in the seas, up-welled deep sea water is highly productive, supporting nearly 50% of all sea products. With the worldwide population explosion contributing to an ever-increasing consumption of animal protein, the focus on aquaculture will undoubtedly also intensify. The high productivity of DSW as a renewable energy source may increase the role of aquaculture to cultivate food for the expanding human population.

History of Research on the Uses of Deep Sea Water

Research on the applications of DSW is occurring worldwide, particularly in Hawai'i, USA, at the Natural Energy Laboratory of Hawai'i Authority (NELHA), and in Norway. The focus in Japan on the research and developmental studies for further applications of deep sea water forms the basis of this paper.

The history of research efforts in Japan on the applications of DSW is depicted in Table 1 (Nakajima 1998). In 1976, basic research efforts were begun by the Agency for Science and Technology. Between 1976 and 1986, we conceptualized deep sea water, developed an understanding of the characteristics of deep sea water, and developed the technology for the pumping of DSW. From 1986, the agency funded a 5-yr research program which resulted in the establishment of deep-seawater pumping systems in Kochi and Toyama Prefectures.

Table 1. History of research.

1976	Basic research
1986	Granted by the science and technology agency
1989	Water pumping systems in Kochi and Toyama Pref.
1997	Exploration of practical applications

Water Pumping Systems in Japan

In 1989, a buoyant catenary system of pumping water was installed in Toyama Bay, Toyama Prefecture, to examine whether on-site productivity could be increased by mixing deep sea water with surface water. That system was replaced by an above-ground system in 1994. In Kochi Prefecture, the first above-ground system of water pumping was installed in 1989 and the second in 1995. Some of the intake water has been shared with private companies, which have designed many products around the use of deep sea water. In Okinawa Prefecture, private companies jointly installed a buoyant catenary system called "Umi-Yakara 1-gou" (Fujii 1998), bringing the number of locations with DSW pumping systems in Japan to three (Fig. 1).

In 1997, egg production of cold-water organisms such as Japanese flounder was accomplished in Kochi Prefecture using DSW (Okamura and Doi 1998). This led to the shift from basic research to feasibility studies and further exploration of practical applications.

The quantity of DSW intake is 920 t/d in Kochi Prefecture and 3000 t/d in Toyama Prefecture (Table 2). The depth of water intake is about 300 m and water temperature is 9.5 C in Kochi and 2 C in Toyama Prefecture. As of yet, there are no large systems in Japan with the capacity or economic efficiency of the system at NELHA in Hawai'i, which pumps water from the deep sea at 88000 t/d (Hachmuth 1991).

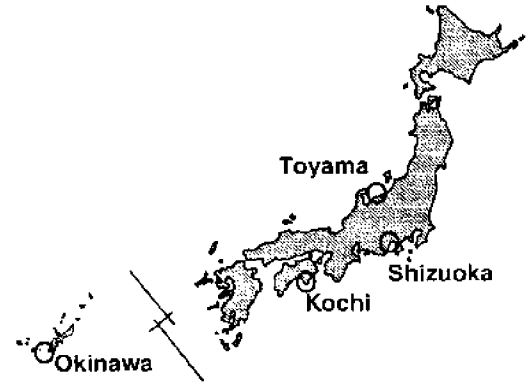

Figure 1. Water pumping places in Japan.

Research in Toyama and Kochi Prefectures indicates many practical possibilities for future applications of DSW and currently many requests for the water are from private companies which have many projects in the planning stages along the coasts of Japan (Table 2). In Kochi Prefecture, a third DSW intake pipe was installed early this year. This system will provide 4000 t/d, 2000 t of which will be utilized for fisheries, with the remainder of sea water provided to companies such as cosmetic firms and chemical companies. In Okinawa Prefecture, a large system is planned which will provide 15 000 t/d of deep sea water (Shimoji and Tominaga 1997). This system will service research facilities as well as a resort. In Shizuoka, plans are being made for the intake of deep sea water from two different sources, one originating from the Kuroshio Current and the other originating from

Table 2. Water pumping systems in Japan.

System	Capacity (m^3/day)	Intake depth (m)	Length from shore (m)	Temp. (degrees C)	Date Installed
Kochi 1	460	320	2,650	9.5	1989
Kochi 2	460	344	2,650	9.5	1994
Toyama	3,000	321	3,060	2	1995
Okinawa 1	-	600,1400	30,000	9,2.6	1997
Okinawa 2	15,000	600	-	-	2000
Kochi 3	4,000	300	2,074	-	2000
Shizuoka	3,000	350,700	-	-	2001

the Oyashio Current.

MATERIALS AND METHODS

Fisheries Related Applications: Feasibility Studies

New ways of using deep sea water for fisheries, agriculture, energy, medical treatment, and environmental purposes are under constant investigation in Japan. Currently, the major use by fisheries is in the aquaculture of fishes, shellfish, sea vegetables, and phytoplankton. The fisheries' sector is also looking into the practical application of handling of captured fish with deep sea water to maintain freshness. Salinity and environmental restoration using the abundant nutrients of deep sea water is another avenue of research.

Aquaculture

A major advantage of using deep sea water for aquaculture is the ability to culture cold-water organisms and deep-ocean organisms in tropical areas. Another is the ease at which water temperature can be controlled by mixing surface water with deep sea water. A third advantage is disease control, as there are few viruses and pathogenic bacteria in deep sea water. A disadvantage of using surface sea water is the maintenance required to keep the water intake pipes free of organisms that cling to the pipes and foul the water. However, when DSW is used for aquaculture purposes, maintenance of the pipes to remove harmful bacteria and other organisms is not necessary. In the Kochi Prefectural Deep Seawater Laboratory, the intake pipes haven't required cleaning for the past 10 yr (Miyamoto 1999).

The species targeted for aquaculture in Japan are mainly those requiring cold, deep, ocean water (Table 3). Most of the aquaculture projects that rely on that type of water are carried out in Kochi and Toyama Prefectures (Fujita 1997; Taniguchi 1997). Almost all the projects are at experimental level, but the egg production of Japanese flounder has been demonstrated to be practical since 1997 (Okamura and Doi 1998).

The abundant nutrients in deep sea water have instigated many projects for the production of sea vegetables and micro algae. In Kochi Prefecture, they have succeeded in producing edible *konbu*, and cold water sea vegetables. The growth of *konbu* in pumped deep sea water is reportedly the same as in its natural habitat in Hokkaido Prefecture, in the northernmost section of Japan, which is known as the production center of *konbu* (Yamaguchi et al. 1994).

Table 3. Target organisms of aquaculture using deep seawater in Japan.

Target organisms	Organizations
Fishes	
Japanese flounder	Kochi Pref., Toyama Pref., Kinki Univ.
Flatfish	Kochi Pref.
Globe fish	Kochi Pref., Kinki Univ.
Butterfish	Kochi Pref.
Trout	Toyama Pref.
Sea bream	Kochi Pref.
Anglerfish	Toyama Pref.
Sandfish	Toyama Pref.
Pacific cod	Toyama Pref.
Shellfishes	
Abalone	Kochi Pref., Kochi Univ.
Snow crab	Toyama Pref.
Firefly squid	Toyama Pref.
Shrimp	Japan Sea-Farming Association, Okinawa Pref.
Whelk	Toyama Pref.
Oyster	Japan Marine Sci. & Tech. Center
Vegetables	
Japanese tangle	Kochi Pref., Toyama Pref.
Wakame	Kochi Pref.
Laver	Kochi Univ.
Sea trumpet	Kochi Pref.
Microalgae	Kochi Pref., Toyama Pref., etc.
Precious coral	Kochi Pref.
Plankton	Kochi Pref., Toyama Pref.

Handling of Captured Fishes

In Japan, there are many instances when captured fish are not taken directly to market. One such instance is when fishermen hold them in port until prices increase. Therefore, it is necessary to keep the captured fishes sanitary and fresh until sold. Research for the application of DSW in the

handling of captured fishes is focused on the purity of the water. Studies include the use of DSW to wash captured fish in the fishing ports in order to keep them fresh and also to transport the fish. Frozen deep sea water, for example, has already been shown to be effective in the transporting of fish (Kawasaki and Kuyou 1998).

Environmental Restoration

Trials are underway to examine whether the abundant nutrients of deep sea water can be applicable to environmental restoration efforts. The loss of the sea grass habitat is an important topic in Japan. Many efforts have been made to restore sea grass habitats, but most have not been very successful thus far.

In Kochi Prefecture, however, the DSW is discharged into the near-shore ocean waters after it is used. As a result, sea grass was discovered growing along the coastal areas, even where it had not grown before. Accordingly, a new avenue of research developed recently in Kochi Prefecture (Taniguchi et al. 1998) using DSW to initiate restoration of the coastal habitat. In Toyama Prefecture, mixing DSW with surface water to raise on-site productivity was attempted, but it met with limited success as the quantity of DSW was low and the vertical mixing was difficult due to the heavier density of deep sea water (Iseki et al. 1994).

Applications Unrelated to Fisheries

Businesses unrelated to fisheries, such as the food industry, medical treatment facilities, utility companies, and agriculture have found the usage of DSW advantageous. Applications in the food and medical industries are now practical. The use of DSW to cool water for power stations and utilization for agriculture are being explored as future applications.

Food Industry

Various foods and beverages are being produced using desalinized or concentrated deep sea water. Products such as jelly, mineral water, soy sauce, Japanese sake, confectioneries, and salt are made in Kochi Prefecture, contributing to the local economy (Hisatake 1997). These products are very popular due to the "mellow" flavor

Photo 1. Products using deep seawater in Kochi Prefecture.

associated with the water. The exact role of DSW in changing the flavor or taste, however, is not fully understood.

Medical Treatment

It is empirically known that sea water is effective for the treatment of atopic dermatitis. However, because of the many bacteria and viruses in surface sea water, it is not recommended for daily application. As an alternative, pure, deep sea water was tested for daily use with promising results (Nomura 1995). Other medical researchers are trying to extract valuable chemicals from micro algae grown in deep sea water (Matsunaga et al. 1997; Komai et al. 1997).

Agriculture

In the subtropical environment of Okinawa Prefecture, it is not possible to grow cold climate vegetables, such as spinach, during the summer months. However, when cold, DSW was pumped through fields of spinach in underground pipes, the spinach grew very well. Such applications of deep sea water for agriculture are performed at NELHA in Hawai'i, USA, where they have succeeded in producing various cold-season vegetables and crops in the tropics (Daniel 1994).

The abundant nutrients of deep sea water are also favorable for agriculture. When watered with diluted deep sea water, spinach actually grew faster than when tap or surface water was used (http://www.lizard.co.jp/deep-sea/hourensou.html).

DISCUSSION

Future Applications

A variety of possible applications of deep sea water are topics of future research. One possible use is to cool the water emitted by power stations. Preliminary studies demonstrate that the cold temperature of DSW increases the efficiency of heat exchange. The small variation in temperature between discharged deep sea water and coastal waters reduces any potential damage to the near-shore environment. However, if deep sea water is to be used for cooling water at power stations, quantities as large as million t/d would be needed. The intake of such a large quantity of DSW could affect the global environment, for example, by changing the balance of carbon dioxide or altering ocean currents. Therefore, environmental impact studies have been initiated.

Thalassotherapy, or medical treatment using sea water, is being examined. Currently, some resort facilities in Toyama Prefecture perform thalassotherapy using DSW (http://www.micnet.ne.jp/hotaru-n/museum/english/index.html).

Future uses of DSW in agriculture might be for hydroponic plant culture or maintenance of seed at cool temperatures.

Future Problems

Problems that might result from increased use of DSW are also under consideration. Although it is recognized that deep sea water is effective in various applications, the exact functions and ramifications are not clearly understood. Methods must be developed to reduce the high costs of constructing DSW water pumping systems. Further studies should be done to understand and assess the environmental impacts of intake and discharge of deep sea water on the coastal environment.

To alleviate some of the anticipated problems, a cascade system of using deep sea water has been proposed (Fig. 2, Ikeda 1997). In this system, intake water is used for air-conditioning and then used for aquaculture. After it is used for aquaculture, the DSW is discharged into the ocean for environmental restoration. This system can decrease the negative environmental impact of discharging cold water while maintaining the positive attributes of deep sea water. In Kochi Prefecture, DSW that has been used to culture sea vegetables then used in the aquaculture of abalone, is one method of using a cascade system whereby the deep sea water pumped from deep depths is utilized to the fullest and the environmental impacts are minimized.

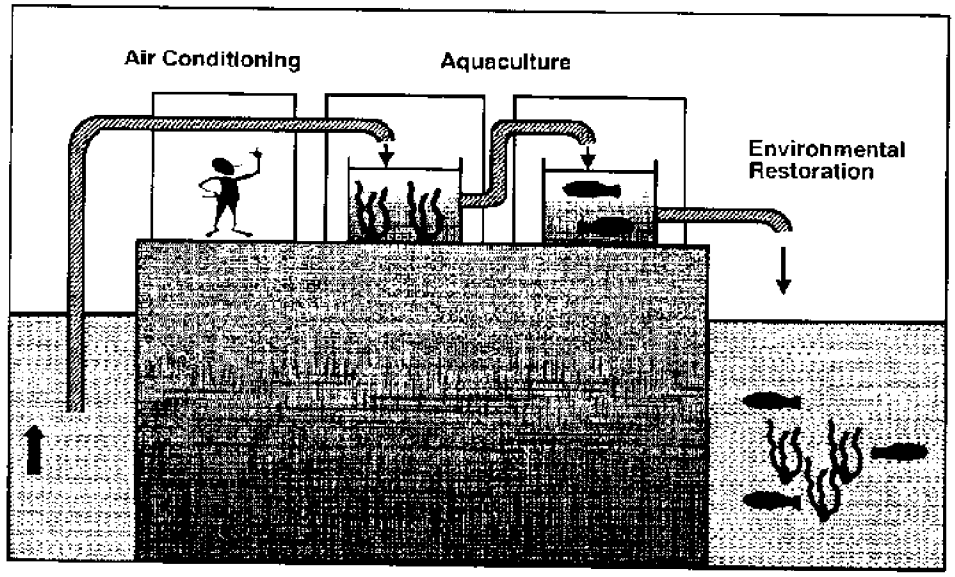

Figure 2. Cascade system of using deep seawater.

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ACQUISITION AND LOSS OF POTENTIAL FOR MOTILITY OF SPERMATOZOA OF THE JAPANESE EEL *ANGUILLA JAPONICA*

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ABSTRACT

To date, cultivated male Japanese eels have not matured sexually in captivity under normal conditions. However, spermatogenesis and spermiation can be induced by the injection of gonadotropins. During this study, most males spermiated after the fifth or sixth weekly injection of human chorionic gonadotropin (HCG; 1 IU/g BW/wk) and the milt weight gradually increased as the number of injections increased. Motility of spermatozoa (percent motility after dilution with 450 mM NaCl) from HCG injected males showed significant individual differences and periodical changes after each weekly injection of HCG. Motility of spermatozoa in Japanese eel milt was found to have an intimate relationship between the pH of the milt and concentration of potassium in the seminal plasma. Motility of spermatozoa could be regulated by changes in the ionic constituents of the isotonic incubation media before dilution with a hyperosmotic solution. The percent motility of spermatozoa in eel milt increased significantly after incubating for 60 min in isotonic artificial seminal plasma (ASP), which consists of NaCl + KCl + $CaCl_2$ + $MgCl_2$ + $NaHCO_3$ buffered with TAPS-NaOH at pH 8.1, and with Ca^{2+}, Mg^{2+} free-ASP. Motility, however, decreased rapidly in K^+ free-ASP and in HCO_3^- free-ASP. These results indicate that acquisition and loss of the potential for motility of eel spermatozoa can be altered by changing the potassium and bicarbonate ion concentrations of the incubating medium irrespective of the initial potential for motility. The methods to obtain good quality spermatozoa described in this experiment should compensate for low volume of milt at the time of artificial fertilization.

INTRODUCTION

The freshwater Japanese eel *(unagi)* is one of the most widely cultivated species in Japan due to its popularity as a food fish. This popularity has caused a decrease in wild fry over the last 25 yr, as the eel fry under cultivation are wild-caught elver which have been captured in estuaries. The shortage of fry for cultivation has recently become a serious problem, leading to the intensive study of techniques for artificial breeding of the eel. In the present study, we investigated the factors which affect the milt quality in males artificially induced to mature, and examined the techniques for controlling the motility of eel spermatozoa.

INDUCTION OF TESTICULAR MATURATION BY HCG INJECTION

Cultivated male Japanese eels at 200-300 g BW are sexually immature and do not mature under normal culture conditions (Yamamoto et al. 1972). However, injection of gonadotropins can easily induce spermatogenesis and spermiation in the male for purposes of artificially propagating this species (Yamamoto et al. 1972). Chiba et al. (1997) reported that injections of human chorionic gonadotropin (HCG) administered to male eels less than 29 cm BL had no effect on the serum 11-ketotestosterone (11KT) levels, a major androgen in the eel (Miura et al. 1991), or on spermatogenesis. However, in fish more than 32 cm BL, testicular maturation was induced, accompanied by increases in plasma 11-KT levels. HCG has been used exclusively for the induction of sexual maturation in male Japanese eels, and a single injection of HCG at a relatively high dose (5 IU/g BW, Miura et al. 1991; 8 IU/g BW, Ohta and Tanaka 1997) is reportedly effective in inducing spermatogenesis.

To develop techniques for the artificial maturation of male eels, we investigated the number of weekly HCG injections required to obtain an adequate volume of high quality milt (Ohta et al. 1996a). Ten sexually immature males (252 g average BW) received 14 weekly injections of HCG (250 IU/eel/wk). Two out of 10 males spermiated after the fifth injection, and most had

spermiated after the sixth. The expressible milt weight gradually increased as the number of injections increased and became stable after injections 11-13. Percent motility of the spermatozoa measured after dilution with 450 mM NaCl increased after 7-9 injections and reached about 60-70% after the tenth injection. These results indicated that repeated weekly injections of HCG at 1 IU/g BW over 10 wk artificially induced sexual maturation in immature male eels.

PROBLEMS WITH INDUCTION OF TESTICULAR MATURATION

Although artificial induction of maturation in the immature male eel is not difficult, the milt obtained from an artificially matured male has not been sufficient in quantity or quality for high fertilization of eggs. First, the amount of obtainable milt from one male is small compared to the egg volume from one female. As already described, milt can be obtained from most of the males which received 10 or more weekly injections of HCG. The milt volume averages about 1 g (Ohta et al. 1996a), while the egg weight from one ovulated female often exceeds 300 g. Therefore, techniques must be developed to enable successful fertilization using the small quantity of milt.

Another problem is that individual differences in sperm motility are significant among the males induced to mature by artificial means. For example, Fig. 1 shows the percent motility of the milt from 10 randomly selected males which received 14 injections of HCG. Although the mean motility value was 55.3±8.2%, the lowest value was less than 10% and the highest more than 90%. It is clear that these differences in milt quality among males will lead to varying degrees of success in the fertilization of eggs. Furthermore, sperm motility changes with time after the administration of HCG. The changes in percent motility of spermatozoa in milt during the weekly injections of HCG are presented in Fig.2. The motility increased sharply 6 h after the injection and peaked after 24 h. Then, motility decreased by d 3 after injection, and remained at low level when tested on d 7. Milt obtained just prior to injection13 also showed low sperm

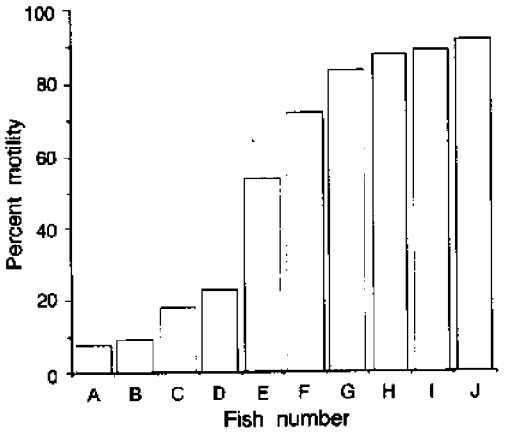

Figure 1. Percentage of motile spermatozoa of the milt from ten randomly selected males when the milt was diluted at 1,000 times with 450 mM NaCl buffered with 20 mM HEPES-NaOH at pH 7.5. Spermatozoa were classed as motile when the sperm head showed forward movement at 15 sec after dilution when analyzed under the VTR-light microscope.

motility. These periodic changes in percent motility will certainly affect the fertilization of eggs if the qualitative changes in milt quality at the time of artificial fertilization are disregarded. One possible solution to this problem is to give an additional injection of HCG 1 d prior to artificial fertilization (Ohta et al. 1997c).

RELATIONSHIP BETWEEN IONIC CHARACTERISTICS OF SEMINAL PLASMA AND SPERM MOTILITY

Changes in sperm motility observed during the weekly administration of HCG suggests

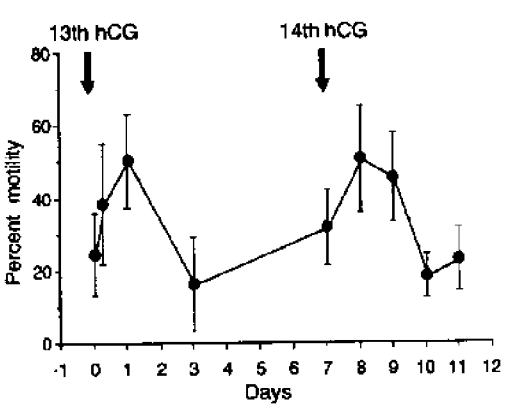

Figure 2. Changes in sperm motility during weekly injections of HCG (1 IU/g BW/wk). A small amount of milt was obtained from each male (n=5) just prior to injection 13, and 6 h, 24 h, and 72 h after, and just prior to injection 14, and 24 h, 48 h, 72 h, and 96 h after.

that the increase or decrease of HCG concentration affects the aqueous environment surrounding the spermatozoa in the sperm duct. Therefore, the potential for changes in the motility of spermatozoa appear to be related to the time elapsed after administration of HCG. It is possible that the composition of the seminal plasma can also affect the motility of the eel spermatozoa.

The biochemical characteristics of seminal plasma in 109 male Japanese eels were investigated with reference to sperm motility. After measuring the pH of the milt, the percent motility of spermatozoa and the ionic concentration of the seminal plasma in the milt were measured, respectively. An intimate relationship was found between sperm motility, milt pH and potassium concentration in the seminal plasma. The mean milt pH was 8.05 ± 0.02 and the potassium concentration of the seminal plasma was 20.85 ± 0.52 mM (n=109). We classified the milt from the 109 males into four groups: 1) milt which showed both milt pH and potassium concentration of seminal plasma more than the mean values (n=29), 2) milt which showed milt pH more than the mean value and potassium concentration less than the mean value (n=12), 3) milt which showed milt pH less than the mean value and potassium concentration more than mean value (n=24), and 4) milt which had both pH and potassium concentration less than the mean values (n=44). The mean percent motility of spermatozoa in the milt of these four groups is shown in Fig. 3. Percent motility of spermatozoa in the milt, of which pH and potassium concentration of seminal plasma were above the means, were significantly ($P<0.05$) higher than samples with both values less than the means. Similar relationships could not be found for other cations (Na^+, Ca^{2+}, or Mg^{2+}). These results indicate that the increase of potassium ions and decrease of protons in the seminal plasma stimulate the acquisition of sperm motility in the Japanese eel.

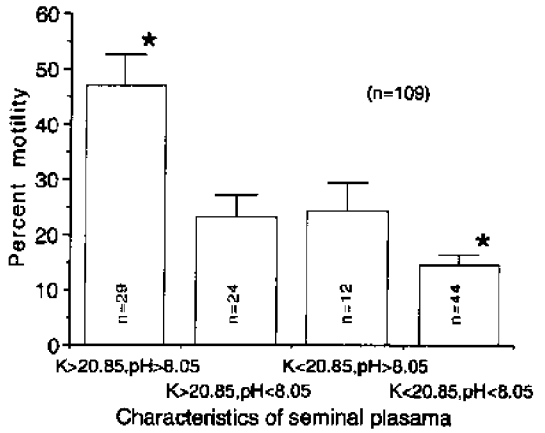

Figure 3. Relationship between percent motility of the spermatozoa when diluted with 450 mM NaCl and pH of the milt, and concentration of potassium ions in the seminal plasma. Asterisks mean significant difference ($P<0.05$) between them.

ACQUISITION AND LOSS OF POTENTIAL FOR MOTILITY IN THE MILT SPERMATOZOA *IN VITRO*

An artificial seminal plasma consisting of 149.3 mM NaCl + 15.2 mM KCl + 1.3 mM $CaCl_2$ + 1.6 mM $MgCl_2$ + 20 mM $NaHCO_3$ (buffered with 20 mM TAPS-NaOH at pH 8.1) was produced during the current study based on previous measurements of seminal plasma (Ohta et al. 1997a). Spermatozoa in milt was incubated in the ASP and changes in the potential for motility following dilution with 450 mM NaCl were recorded (Fig. 4). Before incubation, spermatozoa in the milt was observed at 53.4 ± 11.8% motility (initial control). The motility increased significantly when the milt was incubated with the ASP or Ca^{2+} and Mg^{2+} free ASP for 60 min (83.4 ± 2.5% and 86.1 ± 2.1%, respectively). In contrast, spermatozoa incubated with K^+ free ASP or HCO_3^- free ASP showed a sharp decrease in motility within 30 min, and reached 1.8 ± 0.7 %

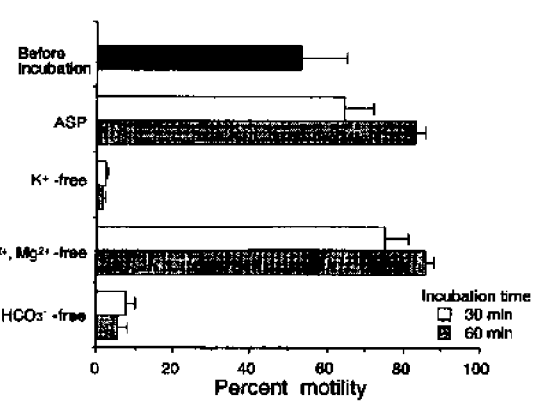

Figure 4. Effects of cations or anions in the ASP on the acquisition and loss of sperm motility. Horizontal bars indicate means ± SEM of results from 10 eels.

or 5.7 ± 2.2%, respectively. These results indicate that potassium and bicarbonate ions are essential for the acquisition and maintenance of motility of eel spermatozoa.

SOLUTIONS TO TECHNICAL PROBLEMS

The main problems with artificial induction of testicular maturation in the Japanese eel are: 1) the scarcity of milt compared with egg volume for successful fertilization, 2) the extent of individual differences in sperm motility among males artificially induced to mature, and 3) the change in motility with time after injections of HCG.

Low milt volume can be resolved by dilution of milt with appropriate diluent(s). Milt dilution has been reported to improve percent fertilization compared to undiluted milt which is low in volume (Poon and Johnson 1970; Billard et al. 1974; Rieniets and Millard 1987). Billard (1992) stressed the advantages of using diluents for artificial insemination compared to the dry method, and indicated the optimum sperm dilution of milt in salmonids is around 1000 times. In earlier studies of the Japanese eel, we reported the validity using artificial seminal plasma to dilute the milt up to 30 times (Ohta et al. 1997b) and 100 times (Ohta et al. 1996b; Kagawa et al. 1998) to improve percent fertilization.

Results from the present study suggests that the cause of the other two problems (individual differences and changes over time in sperm motility) is the lack of an effective regulatory mechanism of potassium and bicarbonate ions and/or proton concentrations in the seminal plasma of males treated with HCG. Although spermatogenesis can be readily induced by multiple injections of HCG, further improvement in hormonal treatment appears to be necessary for obtaining high quality milt.

A relationship between motility and the capacity for fertilization of teleost spermatozoa has been confirmed by several authors (Billard and Cosson 1992; Harvey and Kelley 1984; Ohta et al. 1995). The present study indicates that the potential for sperm motility in the Japanese eel can be regulated by incubating milt in an isotonic incubation media with a high concentration of potassium and bicarbonate ions. Techniques for obtaining good quality spermatozoa of the Japanese eel by the incubation method used in the current investigation should compensate for the shortage of milt at the time of artificial fertilization and improve percent fertilization.

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ENHANCED NUTRIENT SUPPLY TO NORWEGIAN COASTAL WATERS: EFFECTS ON GROWTH OF SCALLOPS AND BLUE MUSSELS

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ABSTRACT

An experiment including an enhanced nutrient supply to coastal waters was performed during 1996-1999 in a landlocked bay, Hopavågen, located in Central Norway. The aim of the study was to quantify effects of the nutrient enrichment on the food web structure and growth of blue mussels *Mytilus edulis* and scallops *Pecten maximus*.

INTRODUCTION

Hopavågen has a surface area of 27 ha, a total volume of 5.4 mill m^3 and a mean depth of 20 m. The volume of euphotic waters is estimated to be 3.7 million m^3, which corresponds to 68% of the total volume. Due to a narrow inlet, the tidal range in Hopavågen is limited to 0.3–1.0 m, compared to 0.8 – 2.3 m in the coastal waters outsideof the bay. Daily water exchange in the bay averages 0.61±0.22 mill m^3 or 0.11±0.04 % of total volume d^{-1}, corresponding to 19% of the productive waters d^{-1}.

MATERIALS AND METHODS

In the first 2 yr of the investigation, 1996 and 1997, physical, chemical and biological data were provided for an undisturbed situation. From early May to mid October 1998 and 1999 nutrients (phosphorus, silicate, and nitrogen) were added during the early tide period with inflowing water to the bay, corresponding to approximately two additions of nutrients daily. The addition of nutrients in 1998 corresponded to 0.4 µg phosphorus L^{-1} d^{-1}or an estimated 100% increase in the phosphorus supply to the bay. The molar ratio for N:Si:P was 15:5.4:1. In 1999 the addition of phosphorus was increased to 0.8 µg P L^{-1} d^{-1}, and the molar ratio of N:Si:P was close to 16:8:1.

Data on growth of scallops and blue mussels are available for 1997 and for the first year nutrients were added, 1998. In the former year, scallops (40 mm) and blue mussels (45 mm) were placed in polyethylene baskets at three different depths and two different depths, respectively, in the central area of the bay. The growth of the shells was followed for 9 months (May 1997-February1998). During the period June 1998–April 1999, the growth of scallops (25 mm) and blue mussels (40 mm) was followed at 2 and 10 m depth at four different sites in the bay and at a control station (Værnes) in a fjord about 1 km from the inlet of Hopavågen. Growth was measured as increase in shell height (SH). In 1998 the content of shell tissue (wet weight, dry weight and ash free weight) of blue mussels and scallops was measured at the end of the growth period.

RESULTS

Physical measurements revealed a 4-5 C higher temperature at 10 m depth in the late summer period (August and September) in 1997 (18 C) compared to 1998 (12 C). The temperature of the surface layer reached 20 C in early September 1997, compared to about 15 C in the following year. The salinity was in the range of 31 – 33‰ in both years, and the water current at 10 m at the four stations in Hopavågen varied from 1.1 – 1.8 cm s^{-1}.

The mean chlorophyll *a* content (June-September) was in 1996 and 1997 estimated to be 2.1 μg l^{-1} and 1.8 μg l^{-1}, respectively. About 80% of the mean chlorophyll *a* content was in the fraction less than 20 μm in both years, and particles less than 2μm contributed 20% of the total chlorophyll *a* on average. The mean daily production for the period May-October in 1996 and 1997 was estimated to be 410 and 420 mg carbon m^{-2} d^{-1}, respectively.

In the first year nutrients were added to the bay, the mean daily primary production increased to 580 mg carbon m^{-2} d^{-1}. However, the mean chlorophyll *a* content, 2.0 μg l^{-1}, was at the same level as the previous year, and the chlorophyll *a* content, in different size fractions, did not reveal any change in size distribution of the phytoplankton following the nutrient additions. The bacteria biomass and production remained at the same level during the investigation.

The growth rate of blue mussels in the period July-September 1997 and 1998 was in the range of 0.14–0.29 % d^{-1} at the different depths and locations. The highest daily increase in SH was recorded in Hopavågen in 1998, but it was not significantly higher than in 1997 or at the control station at Værnes. In the late autumn period (September-October), the growth rates varied from 0 to 0.07% d^{-1} at the sampling stations. The tissue content (wet and dry weight) at the end of the season was significantly higher in blue mussels in Hopavågen compared to mussels from the control station at Værnes.

The growth rate of scallops in Hopavågen increased from 0.16% d^{-1} in the period July-September in 1997 to 0.53% d^{-1} in 1998. During the latter year the recorded growth of SH of scallops in Hopavågen was significantly higher than the mean value for scallops grown in the fjord outside Hopavågen (0.44% d^{-1}). Also, between September and late October, the growth rate in the bay (about 0.20% d^{-1}) was much higher than in the previous year (0.04% d^{-1}). The tissue content (dry weight, ash free dry weight) in the scallops grown in Hopavågen was 2-4 times higher than in shells farmed at Værnes.

DISCUSSION

The addition of nutrients corresponding to an estimated annual supply of phosphorus and a molar ratio for N:Si:P of 15:5.4:1 caused a 46% increase in primary production in Hopavågen, compared to the pre-fertilization year. As the mean chlorophyll *a* content remained at the same level through the investigated period, the results indicate an increased turnover rate or increased grazing rate of the phytoplankton following the nutrient additions. The increased primary production in 1998 did not affect the SH growth of blue mussels *Mytilus edulis*. However, the shell content in mussels grown in Hopavågen was significantly higher than in mussels from the control station, which indicate that the increased production affected the somatic growth of the mussels. The SH of scallops *Pecten maximus* in the bay increased from 0.16% d^{-1} in 1997 to 0.53 % d^{-1} in 1998. The recorded growth of scallops in Hopavågen in 1998 was also significantly higher than for scallops outside the bay (0.44% d^{-1}). Even more pronounced was a 2-4 times higher content of shell tissue in the scallops grown in Hopavågen in 1998, compared to individuals from the control station at Værnes.

The results reveal that nutrients may be considered an important resource also in management of aquatic systems. However, dose-response experiments in marine waters with different water qualities are needed to establish a general knowledge of effects of nutrient supply on the productivity of different aquatic organisms and the environmental impact in a long-term perspective.

SEED PRODUCTION TRIAL OF THE DEEP-SEA WHELK *BUCCINUM BAYANI* USING DEEP SEA WATER

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ABSTRACT

The edible deep-sea whelk *Buccinum bayani* is distributed at depths from 200-800 m in the Sea of Japan, and especially Toyama Bay. It is one of the most important commercial shellfish in Toyama Prefecture. In order to establish seed production and to obtain ecological information necessary for the management and stock enhancement of this species, indoor tank culture of the whelk was carried out at the Toyama Prefectural Fisheries Research Institute using running, deep sea water at a temperature of about 3 C that was pumped from a depth of 321 m. For preparation as broodstock, the whelks were cultured on a diet of fish such as sardine or mackerel and after 1 yr of culture, as many as 80% of the whelks survived and some of the females laid eggs. Most of the younger whelks that were initially < 60 mm in shell height (SH) grew, while the presumed older ones (up to 120 mm SH) showed very little growth. Histological examination, low variation in the gonad index (GI), and the recorded mo during which eggs were layed all strongly suggest that *B. bayani* has no definite reproductive season. Spawned eggs were deposited as a mass of 80 to 250 egg capsules; each capsule containing1500-3500 eggs. Whelks with larger SH produced many more egg capsules with about 10-30 individuals hatching from one egg capsule 6 mo after being spawned. By feeding whelks the fish as mentioned previously, juvenile whelks grew well and 50% of them survived after 1 yr in culture.

INTRODUCTION

Toyama Prefectural Fisheries Research Institute has the capability to pump deep sea water (DSW) from Toyama Bay in the Sea of Japan at a depth of 321 m. The temperature of this DSW remains around 3 C year-round. The cold, clean, DSW is mainly used for stock enhancement or ecological studies on cold- and deepwater species such as Masu salmon *Onchorhyncus masou*, Pacific cod *Gadus macrocephalus*, red tanner crab *Chionocetes opilio elongatus* and Toyama shrimp *Pandalus hypsinotus*, as well as edible deep-sea whelks inhabiting the continental slope in the Sea of Japan. The whelks are among the most important commercial shellfish in Toyama Prefecture and are caught using chained basket traps. The catch of whelks in Toyama Bay (greater than 300 t) accounts for around 85% of the total catch of marine shells (Fig. 1). Because little biological information is available on the whelks, indoor culture trials of *Buccinum bayani* were carried out to establish mass-culture techniques and to promote ecological studies for the management and stock-enhancement of this important shellfish species.

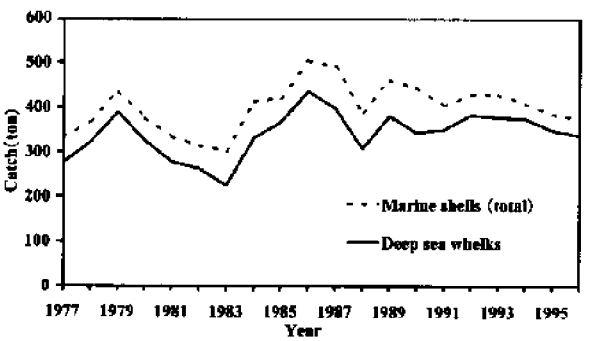

Figure 1. The total catches of marine shells and the catch of deep-sea whelks in Toyama Prefecture from 1977 to 1997. solid line indicates the catch of deep-sea whelks and broken line indicates the total catches of marine shells.

MATERIALS AND METHODS

Broodstock Culture

B. bayani of various sizes were collected for broodstock from Toyama Bay and cultured in 1-t tanks using running DSW under atmospheric pressure. Fish, such as sardine or mackerel, were given as food. Survival and growth, as determined by changes in shell height, were recorded over the course of 1 yr. When egg laying was observed, the mo of egg laying, SH of the egg-laying female and number of egg capsules were recorded.

Culture of Juveniles

The culture of juveniles was carried out in two 5-L tanks stocked with 100 juveniles/tank. The tank bottoms were covered with mud collected from the sea bottom at a depth of 200 m. To validate the hypothesis that juvenile *B. bayani* feeds on organic matter in the mud, fish meat such as sardines were given to the juveniles in one tank, while no food was given to those in the other tank. After 1 yr of culture, the survival and growth of juveniles were assessed.

Reproductive Cycle

B. bayani, more than 90 mm SH and presumed to be sexually mature, was sampled from Toyama Bay at 3 mo intervals. Fifteen to 20 shells were examined during each sampling and the gonad index (GI) was determined in the manner described by Takamaru and Fuji (1981); where GI is equal to gonad weight x 100/BW without the shell. The gonads were fixed in a formalin solution, dehydrated in alcohol, and embedded in paraffin wax in preparation for histological examination. Sections were cut at 8-12 μm, stained with Mayer's hematoxylin and counterstained with eosin.

RESULTS AND DISCUSSION

Broodstock Culture

After 1 yr in culture, as many as 80% of the whelks survived. Most of the younger cultured whelks that were less than 60 mm SH grew, while the older ones (up to 120 mm SH) rarely grew (Fig. 2). The reason for this difference is unknown, but perhaps a better food source must be investigated for older whelks to grow and mature.

During the course of culture, some of the females laid eggs (Fig. 3). Since egg laying was observed throughout the year (Fig. 4), it is suggested that *B. bayani* have no definite egg-laying season. During the egg-laying process, female *B. bayani* formed an 'egg mass' by producing 80-250 egg capsules (each capsule containing 1500-3500 eggs). A female, 73.5 mm

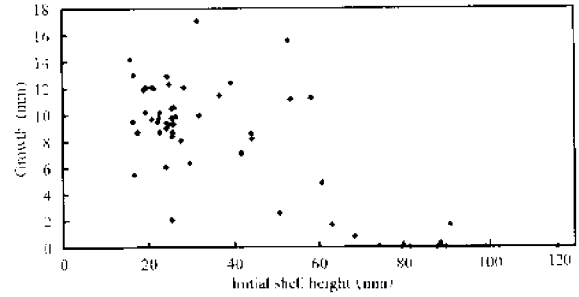

Figure 2. Growth in shell height of *Buccinum bayani* after a year of culture. Growth = Shell height after a year of culture — Initial shell height.

Figure 3. Laying eggs of *Buccinum bayani*. Dome-shaped egg mass was comprised of egg capsules.

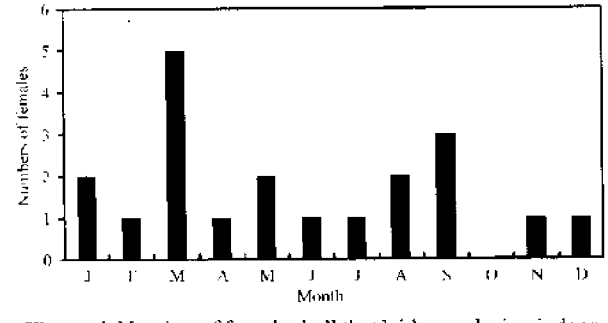

Figure 4. Number of female shell that laid eggs during indoor culture by months.

SH, was the smallest whelk to lay eggs, and it was noted that the larger whelks laid more egg capsules. At the given temperature (3 C), it took 6 mo before the eggs hatched. About 10-30 individuals hatched from one egg capsule, while the other eggs in the capsule appeared to serve as nurse eggs. The shell height just after hatching was about 2 mm. As the females did not lay eggs again for at least 2 yr after previously laying eggs, further investigation is necessary to determine the life span and egg-laying cycles of whelks. Tag-and-release experiments may bring further information on the growth and survival rates of *B. bayani*, particularly in their natural habitats.

Culture of Juveniles

Juvenile *B. bayani* survived and grew on the mud substrate under both conditions; given fish meat and without any solid food (Table 1). Surviving and growing without any solid food for 1 yr strongly suggests that the juveniles could survive only on the organic matter in the mud. However, the growth of the whelks in the 'mud group' was inferior to that of the 'mud + fish group' where the weight of the 'mud group' was only one-sixth of the 'mud + fish group.' When given fish meat, the SH increased from 2.5 to 7.1 mm after 1 yr of culture and the survival was 51%. It appears that fish meat is a suitable food for juvenile as well as adult whelks.

Reproductive Cycle

The maximum gonad index value of the female whelks was about 12, which is approximately twice of that observed for males (Fig. 5). In both the males and the females examined, however, whelks that revealed a GI greater than half of each maximum GI value were reported in each mo throughout the yr. This was coincident with the above results that egg laying was observed year-round. Histological examination of testes and ovaries revealed the presence of mature spermatozoa and mature oocytes filled with large yolk granules (about 20 μm in diameter) in more than 80% of the males and more than 50% of the females, respectively, every representative mo of the four sampling periods (Fig.6, 7). In addition, the year-round maturity in the males (>80%) suggests that there is no resting period in spermatogenesis. We are speculating that sperm is constantly produced in the testis and transported to the seminal vesicle.

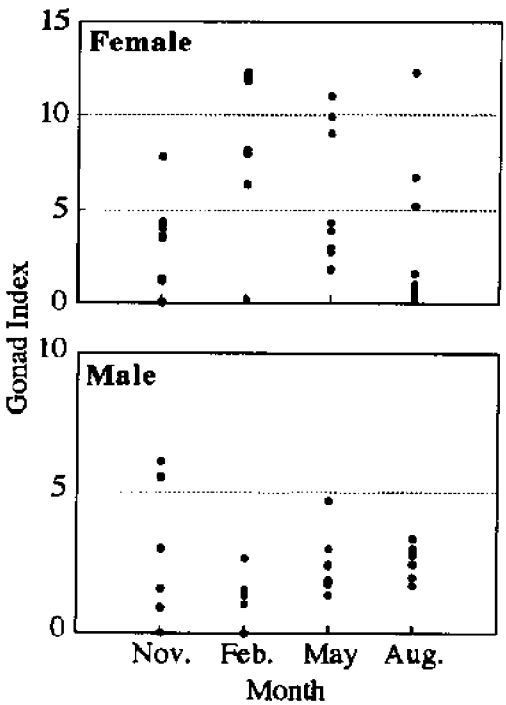

Figure 5. Gonad index of Buccinum bayani. Gonad Index = gonad weight X 100/body weight without shell

Table 1. Growth of juveniles of *Buccinum bayani* after a year of culture.

	Average survival rate (%)	Average shell height (mm)	Average weight (mg)
Mud+fish meat group	51.0	7.1	58.4
Mud group	31.0	3.5	10.0
*Initial juveniles			
Average shell height (mm) = 2.5			
Average weight (mg) = 3.2			

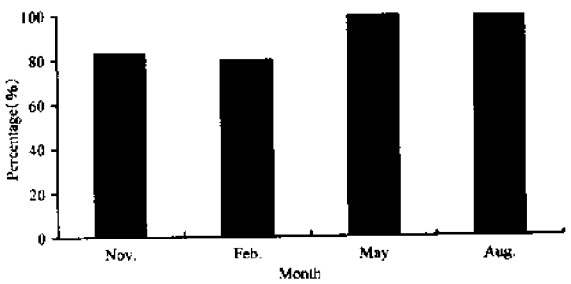

Figure 6. Percentage of males with mature testis.

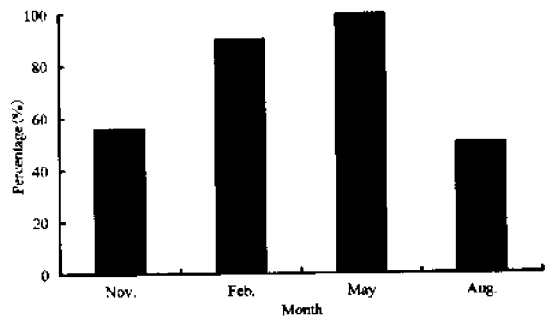

Figure 7. Percentage of females with mature oocytes.

In other studies on the reproductive cycle of subtidal neogastropods of the family Buccinidae, *Buccinum undatum* (Martel et al. 1986), *Nucella freycineti* (Kawai and Nakao 1993) and *Neptunea arthritica* (Takamaru and Fuji 1981; Fujinaga 1985), a clearly defined reproductive season was exhibited and seasonal changes of water temperature was considered to be an important factor in gonad development. The water temperature in the zone (200 to 800 m) where *B. bayani* lives was below 3 C throughout the year (Nakura and Nagata 1989). It is quite likely that there is no environmental cue to induce or arrest maturation of *B. bayani* in their deep-sea habitat.

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MANAGING THE CULTURE OF A COMPLETELY PISCIVOROUS AND VORACIOUS LARVAE, JAPANESE SPANISH MACKEREL *SCOMBEROMORUS NIPHONIUS*: EXPERIMENTAL ESTIMATION OF DAILY FOOD CONSUMPTION

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ABSTRACT

To establish an appropriate feeding schedule in captivity, feeding rhythm and daily ration of larval Japanese Spanish mackerel *Scomberomorus niphonius* were experimentally estimated and compared to those of wild-caught larvae. Using the model of Elliott and Persson (1978), instantaneous gastric evacuation rates (*R*) were estimated for reared fish (d 8, d 10, and d 15 after hatching) from starvation experiments, and for wild fish (3.0-10.3 mm SL) from the depletion of stomach contents (percent body weight) over time when collected during the night. Japanese Spanish mackerel exhibited piscivorous habits from first feeding and a remarkable peak of feeding activity during the evening, under both laboratory and wild conditions, although primarily a daylight feeder. The estimated value of daily consumption for reared larvae ranged between 90.6 and 111.7% and that of wild larvae was 111.1% of their body weight.

INTRODUCTION

Japanese Spanish mackerel *Scomberomorus niphonius* is an important fisheries resource distributed throughout southwestern Japan, particularly in the Seto Inland Sea. Total catch exceeded 6000 t in the mid-1980s but it has recently decreased to less than one-twentieth of that amount due primarily to overfishing (Nagai et al. 1996; Kono et al. 1997). It is hoped that the depleted stock can be restored by fishing regulation and seedling release.

To stabilize the catch and establish more effective fisheries management practices, it is necessary to accumulate biological information and understand the recruitment process. Previous biological studies have focused only on the biology of adult fish (Kishida 1986, 1989; Kishida and Aida 1989; Kishida et al. 1985). Recently, information on the early life history of this species has been accumulated and several peculiar ecological features have been clarified: precocious development in the digestive system (Tanaka et al. 1996), piscivorous habits from the first-feeding stage (Shoji et al. 1997), short-term larval occurrence synchronized with peak abundance of prey fish, rapid growth in early life stages (Shoji et al. 1999a), and diel changes in vertical distribution and feeding rhythm (Shoji et al. 1999b).

Japanese Spanish mackerel is anticipated to be an important target for sea-farming because of a high growth potential (reaching 10 mm TL in one month and 600 mm TL in the first growing season: May to November). However, intensive cannibalism during the larval stages has prevented their mass production (Higuchi and Oshima 1974; Fukunaga et al. 1982). Before culture and mass

production can be managed effectively, an appropriate feeding schedule must be established.

In this study, using the model of Elliott and Persson (1978), the daily consumption of Japanese Spanish mackerel larvae was estimated based on data obtained from successive 24-h samplings under both laboratory and wild conditions.

MATERIALS AND METHODS

Rearing of Fish

Artificial fertilization was carried out with a pair of adult Japanese Spanish mackerel captured by drift gill-net in Harima-nada Sea (the eastern Seto Inland Sea, Japan) in May 1999. Artificially fertilized eggs were transported to Yashima Station, Japan Sea-farming Association (JASFA: Fig. 1), Takamatsu, Kagawa, and maintained in two 0.5 m^3 tanks under natural light conditions. Water temperature ranged from 18.2 to 19.5 C during the experiments. Newly-hatched larvae of red sea bream *Pagrus major* were used as the larval feed.

Instantaneous gastric evacuation rates (R) for reared fish were estimated from starvation experiments at d 8, d 10, and d 15 after hatching. Because Japanese Spanish mackerel larvae are piscivorous and begin to cannibalize without piscine prey, each 70 or 80 fish were isolated using

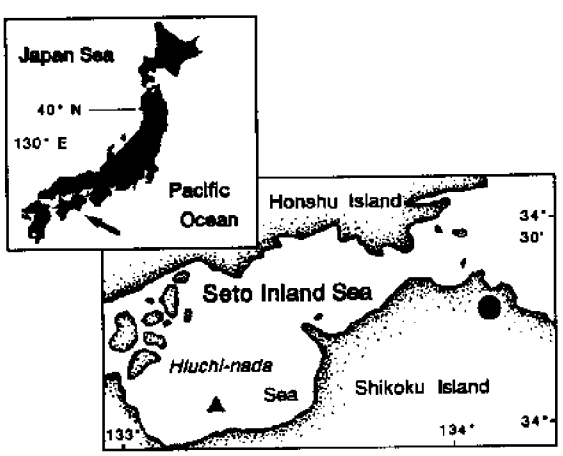

Figure 1. Map of the central waters of the Seto Inland Sea showing the JASFA Yashima Station (closed circle) where rearing experiments were conducted May to June 1999 and a sampling station (triangle) where Japanese Spanish mackerel larvae were collected over a 24-h period on 3-4 June 1997.

2-L plastic cups on the day of the starvation experiments. Ten fish were sampled from each cup at intervals of 30 min to 3 h over a period of 10 h from the beginning of fasting. The stomach content weight index (SCWI) of the sampled fish was calculated as follows: SCWI = 100*dry stomach content weight (SCW) / dry body weight (DBW)

To understand the diel changes in SCWI, a total of 31 samplings were conducted throughout a 24-h period from 0300 on the same day the starvation experiments were conducted. Each 15 to 20 fish used in this determination were removed from the rearing tank every 30 min or 1 h and SCWI was calculated as described previously.

Field Sampling

A 24-h survey was carried out during a cruise on the R/V *Hiuchi* (Ehime Prefecture, Chuyo Fisheries Experimental Station) in the Hiuchi-nada Sea, central Seto Inland Sea (Fig. 1). A total of 10 sets of larva-net tows were conducted at intervals of about 2 h from 1033 on 3 June 1997. Details of the sampling method are described in Shoji et al. (1999b). The stomach contents of 209 larvae, ranging between 3.0 and 10.3 mm SL, were examined and SCWI was calculated.

Estimation of Gastric Evacuation Rate (R) and Daily Ration

The daily ration of Japanese Spanish mackerel was estimated in terms of percent body weight using the model from Elliott and Persson (1978):

$$C_t=(S_t-S_0e^{-Rt})Rt/(1-e^{-Rt})$$

where the (C_t) is the consumption of food during the time interval from t_0 to t_t observed from the average amount of food in the stomach expressed as stomach, content weight index (SCWI) at time t_0 (S_0), the average stomach content index at time t_t (S_t), and the instantaneous evacuation rate (R). The estimates of C_t calculated for each time interval are then summed to give the total daily ration.

The value R for reared fish was estimated from the reduction of SCWI during the starvation experiments using the following equation:

$$R=(1/t)\ \ln(S_0/S_t)$$

The SCWI of wild-caught Japanese Spanish mackerel larvae was high during the daytime and declined during the night while the percentage of those with empty stomachs increased after sunset (see Results: Fig. 6). Therefore, assuming no feeding between sunset and sunrise, R for the larvae was estimated from the reduction of SCWI during the night. Evacuation rate is therefore given by

$$S_{sr}=S_{ss}e^{-Rt'}$$

which, in its logarithmic form, is

$$\ln(S_{sr})=\ln(S_{ss})-Rt'$$

therefore,

$$R=(1/t')\ln(S_{ss}/S_{sr})$$

where the instantaneous evacuation rate (R) is calculated from the average SCWI of the sample collected at sunset t_{ss} (S_{ss}), the average SCWI at sunrise t_{sr} (S_{sr}), and the time interval between t_{ss} and t_{sr} (t').

RESULTS

Estimation of Gastric Evacuation Rate (R) and Daily Ration for Japanese Spanish Mackerel Larvae Under Rearing Conditions

Japanese Spanish mackerel larvae initiated feeding on d 5 after hatching. Mean sizes of fish sampled for the experiments at d 8, d 10, and d 15 after hatching were 6.84, 8.72, and 12.11 mm SL, respectively (Fig. 2). In the starvation experiments, R was derived from the set of SCWI values during 10 h since the onset of fasting, which were plotted in the exponential equation (Fig. 3). The values of R obtained from these data of fish

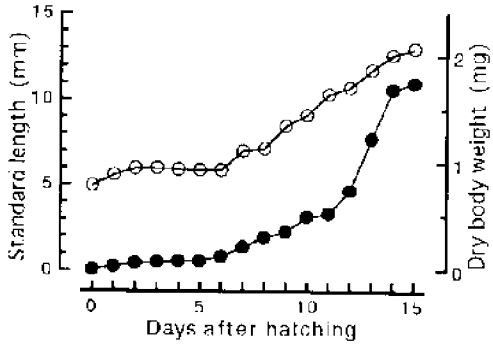

Figure 2. Mean standard length (open circle) and dry body weight (closed circle) of Japanese Spanish mackerel larvae reared at JASFA Yashima Station in 1999. The larvae initiated feeding on d 5 after hatching.

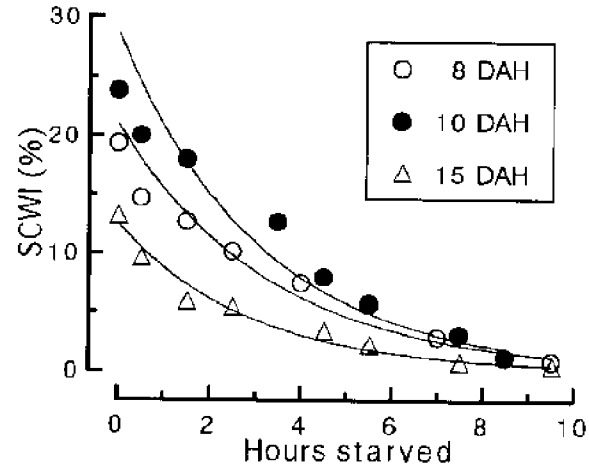

Figure 3. Changes in stomach contents weight indexes (SCWI) during the starvation experiments of Japanese Spanish mackerel larvae at d 8, d 10, and d 15 after hatching. Each reduction of SCWI was used for estimating the gastric evacuation rate (see text). Estimated Rs for fish at d 8, d 10, and d 15 after hatching were 0.282, 0.324, and 0.311, respectively.

at d 8, d 10, and d 15 after hatching were 0.282, 0.324, and 0.311, respectively.

Diel changes in SCWI of Japanese Spanish mackerel larvae increased after dawn until evening and decreased throughout the night (Fig. 4). The SCWI and the R were used to estimate the food consumption for each time interval (C_i). A few of the estimated values of food consumption per hour (C/t) for fish at d 8 and d 15 after hatching appeared to be negative in captivity. Durbin et al. (1983) considered that the negative values were caused when the decline in

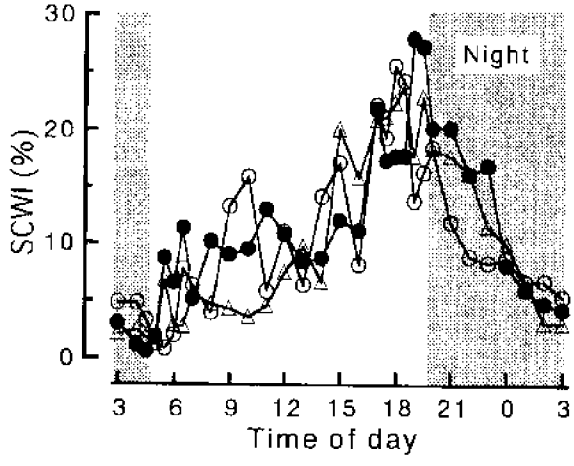

Figure 4. Diel changes in stomach contents weight index (SCWI) of the reared Japanese Spanish mackerel larvae at d 8, 10, and 15 after hatching. Legends same as in Fig. 3.

the amount of food in the stomach from one period to the next was greater than predicted from the evacuation rate used in the calculation, and they summed the amount of food ingested during each period, including the negative values, to obtain the daily ration. In this study, daily ration was determined by summing both positive and negative values. Daily ration of fish at d 8, d 10, and d 15 after hatching reached 104.9, 111.7, and 90.6% of body weight, respectively (Fig. 5).

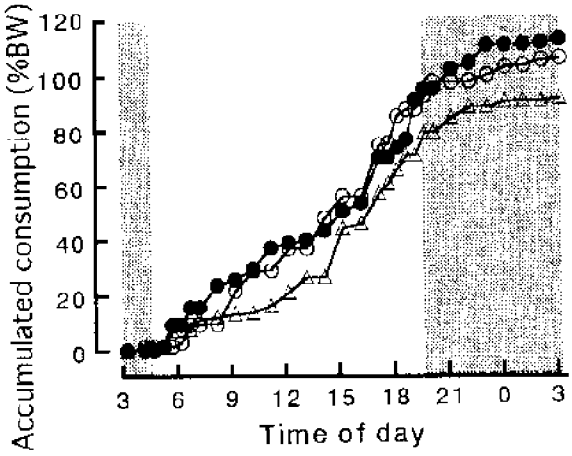

Figure 5. Diel changes in accumulated values of food consumption (% BW) for reared Japanese Spanish mackerel larvae at d 8, d 10, and d 15 after hatching. Elliott and Persson's method was applied to estimate the values of food consumption. Using the gastric evacuation rate of 0.282 (8 DAH), 0.324 (10 DAH), and 0.311 (15 DAH), accumulated values of food consumption were calculated as 104.9, 111.7, and 90.6% of body weight, respectively. Legends same as in Fig. 3.

Estimation of Gastric Evacuation Rate (R) and Daily Ration for Japanese Spanish Mackerel Larvae Under Wild Conditions

Stomach contents of the wild-caught Japanese Spanish mackerel larvae consisted exclusively of other fish larvae (Shoji et al. 1999b). Diel changes in the percentage of fish with empty stomachs and SCWI were observed (Fig. 6) and the percentage of fish with empty stomachs increased during the night and reached a maximum at dawn. SCWI reached a maximum at dusk, then consistently decreased during the night to a minimum at dawn. This reduction of SCWI during the night was plotted in the exponential equation and the gastric evacuation rate (R) for

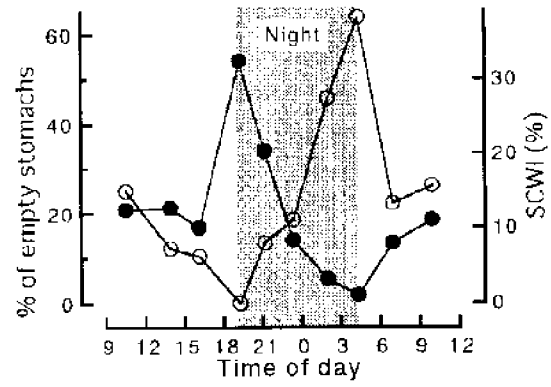

Figure 6. Diel changes in percentage of empty stomachs (open circle) and stomach content weight index (SCWI; closed circle) of Japanese Spanish mackerel larvae collected during the 24-h sampling in the central Seto Inland Sea on 3-4 June 1997. Using the reduction of SCWI during the night when the larvae were considered not to feed, gastric evacuation rate of wild larvae (0.338; see text) was estimated.

the wild-caught larvae was estimated as 0.338. The SCWI and R were used to estimate the food consumption for each time interval. Daily ration for wild-caught larvae reached 127.2% of body weight (Fig. 7).

DISCUSSION

Comparison of Daily Rations Among Scombrid Larvae

Daily ration of Japanese Spanish mackerel larvae was estimated as 127.2 % of BW

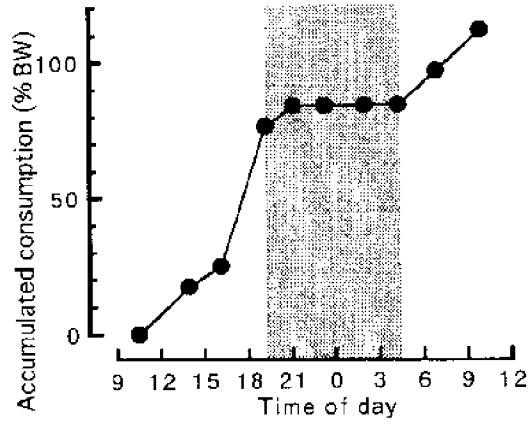

Figure 7. Diel changes in accumulated food consumption for Japanese Spanish mackerel larvae collected during the 24-h sampling in the central Seto Inland Sea on 3-4 June 1997. Elliott and Persson's (1978) method was applied to estimate the values of food consumption. Using the gastric evacuation rate of 0.338, daily ration was calculated as 127.21% of body weight.

for fish in the wild and between 90.6 and 111.7 % for fish in captivity. From these values it is reasonable to characterize the feeding behavior of Japanese Spanish mackerel larvae as voracious. Lower values of daily ration for other scombrid larvae, Atlantic mackerel *Scomber scombrus* (Peterson and Ausubel 1984), southern bluefin tuna *Thunnus maccoyii*, and albacore tuna *Thunnus alalunga* (Young and Davis 1990) have reportedly been 25 and 50% of BW. Hunter and Kimbrell (1980) reported a higher value (87%) for Pacific mackerel *Scomber japonicus* larvae under culture conditions.

Voracious habits exhibited in this study might account for the high growth potential of Japanese Spanish mackerel larvae. Mean gross growth efficiency (percentage of increase in BW to weight of accumulated food consumption) of Japanese Spanish mackerel from first-feeding stage until d 15 after hatching under rearing and wild conditions were calculated as 33 and 26%, respectively. Hunter and Kimbrell (1980) reported mean growth efficiency of 33% for Pacific mackerel larvae under culture conditions. Compared with other scombrids such as Atlantic mackerel (Kendall and Gordon 1981), southern bluefin tuna (Jenkins and Davis 1990), bluefin tuna (Scott et al. 1993), and yellowfin tuna *Thunnus albacares* (Lang et al. 1994), which are planktivorous during their early larval stages, Japanese Spanish mackerel exhibits a higher growth rate (approximately 1.0 mm/d) (Shoji et al. 1999a) during the larval and early juvenile stages. The faster growth can be attributed to the unique feeding habits of Japanese Spanish mackerel larvae: piscivorous from first-feeding larval stage (Shoji et al. 1997). In addition, the histological observations of Tanaka et al. (1996) described a functional digestive system of reared Japanese Spanish mackerel larvae at the first-feeding larval stage: a large expanded blind-sac with consumed fish larvae. The precocious development of the digestive system could account for the voracious and piscivorous habits and high growth potential from the first-feeding larval stage.

Implication for Stock-enhancement of Japanese Spanish Mackerel

Japanese Spanish mackerel are a commercially important fisheries resource and recovery of the depleted stock in the Seto Inland Sea is critical. Because of the unique and fierce feeding habits of Japanese Spanish mackerel, however, particular attention should be given to possible influences which might be caused by the mass release of seed or sudden increase in biomass of the target species upon other related species. Japanese anchovy *Engraulis japonica*, an important prey for larval and adult Japanese Spanish mackerel (Kishida 1986; Shoji et al. 1997), would suffer from more intensive predation by an increase in biomass of Japanese Spanish mackerel. In addition, several species of young and adult piscivorous fish, such as chub mackerel and ribbon fish *Trichiurus leptulus* (Hashimoto et al. 1989), might face interspecific competition with Japanese Spanish mackerel. Recently, techniques for the mass culture of Japanese Spanish mackerel have been established in Japan. To reduce any potential negative impacts upon other species by the stock-enhancement activities of Japanese Spanish mackerel, monitoring the availability of prey species, their distribution and abundance in the sea is imperative.

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CONTROLLED SPAWNING OF SOUTHERN FLOUNDER *PARALICHTHYS LETHOSTIGMA*: ISSUES AND PROGRESS

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ABSTRACT

The southern flounder *Paralichthys lethostigma* is a popular recreational and commercial species along the coasts of the southern United States. In recent years it has become one of the flatfishes of interest for aquaculture development. Ecological data and research results indicate that this species is both eurythermal and euryhaline, making it attractive for coastal and, possibly, inland culture. During the past several years, research has focused on developing controlled reproduction techniques for captive wild southern flounder. In South Carolina, post-metamorphosed juveniles are recruited to inshore waters from December-February with spawning believed to occur along the continental shelf. Collection of non-spawning adults can be accomplished during spring and summer and in fall prior to the offshore migration. Adult fish caught by trammel net sustain little damage during capture and can be readily adapted to tank conditions. Feeds consisting of live fishes and crustaceans (e.g., *Fundulus* spp., penaeid shrimps) are readily eaten as are chopped fish (e.g., mullet, *Mugil* spp.; mackerel, *Scombrus* spp.). Time in captivity affects reproductive results. The percent of GnRH-a spawnable females increased from 29% for fish in captivity only 1.5-3.5 mo to 70% for those held under captive conditions for 5.5-6.3 mo. All females held for more than 24 mo could be spawned, and due to their increased size, these fish produced about three times the number of eggs as the recently captured fish. However, there appeared to be a decrease in percent fertilization among fish held in captivity longer. Fertile eggs could be stripped from naturally ovulating females but timing of ovulation and frequency of success were substantially lower than that obtained from GnRH-a-treated females. Some previously hormone spawned females could be re-implanted with GnRH-a and re-spawned. However, number of eggs produced and apparent percent fertilization decreased. Availability of milt was a concern during strip spawning research in 1997. Recent work indicated males could be repeatedly stripped and produce high volumes of milt, if not stressed by handling and captive conditions. A series of studies was conducted to improve tank spawning techniques. When fish are subjected to spawning conditions (10-11 h light; 17-18 C) and left undisturbed in a 3.7 m diameter x 1 m deep tank, females often produce a large number of eggs but typically there is low or no fertilization in spite of the presence of ripe (spermatogenic) males. GnRH-a treatment of larger females (2 kg) often results in the production of fertilized eggs during some tank spawning events. Treatment of males with testosterone or methytestosterone and GnRH-a did not induce male participation. GnRH-a treatment of both males and females in a tank offered no benefit over treating only the females.

In summary, southern flounder readily acclimate to captivity and will mature under photothermal conditioning. Use of GnRH-a implants in females improves the timing of strip spawning and currently appears necessary for predictable production of fertilized eggs from volitional tank spawning. Methods to improve male participation in tank spawning events need to be identified.

INTRODUCTION

The southern flounder *Paralichthys lethostigma* supports commercial and recreational fisheries along the Atlantic coast and Gulf of Mexico. Commercial landings during 1992-1996 of summer *P. dentatus* and southern flounder combined (species not distinguished) averaged 8,726 mt. However, in 1997 landings were less than half of this value (NMFS 1998). Southern flounder is also a target of recreational fisheries that occur along the south Atlantic coast and in the Gulf of Mexico (NMFS 1998).

Based on studies to date, this species appears to have desirable aquaculture characteristics. Reported growth rates of wild fish in South Carolina indicate that females attain a mean size of about 0.9 kg at 24-28 mo of age (Wenner et al. 1990). Recent work supported by South Carolina and North Carolina Sea Grant Programs showed that southern flounder is euryhaline and displays increased tolerance to low salinity and fresh water with increasing age (Daniels and Borski 1998; Smith et al. 1998, 1999a). Thus, there may be opportunity to grow this species in inland as well as coastal sites as is

done with the euryhaline hybrid striped bass (Smith et al. 1995). Although a detailed economic analysis of southern flounder aquaculture has not been conducted, market prices appear supportive for commercial development of this species. In 1997, ex-vessel price of summer and southern flounders was US$4.10/kg (NMFS 1998). However, live east coast flounders are regularly sold to upper-scale Japanese restaurants in the northeastern United States, where they command a premium price (~US$15/kg). They are also shipped live to Tokyo, Japan, where they are priced at US$45-60/kg (Ackerman 1997).

A number of studies have focused on the spawning of southern flounder. Arnold et al. (1977) reported the first successful spawning of this species. They used photothermal conditioning to tank spawn 3 of 6 females and produced 120,000 eggs from 13 separate spawns with 30-50% fertility. The following year, Lasswell et al. (1978) reported the strip spawning of 25,000 eggs (average 5,000 eggs spawn^{-1}) from 14 females induced to ovulate using carp pituitary extract. Later, Henderson-Arzapalo et al. (1988) examined photothermal conditioning alone and in combination with LHRH-a implants. However, the females only spawned during simulated natural winter spawning conditions in December - February, and there was no fertilization, apparently due to lack of male participation (Henderson-Arzapalo et al. 1988).

Over the past several years, a number of collaborative spawning trials have been conducted with Dr. Craig Sullivan (North Carolina Sea Grant Program) and Dr. David Berlinsky (University of Rhode Island) at the South Carolina Department of Natural Resources (SCDNR) Waddell Mariculture Center (WMC)in Bluffton, and the Marine Resources Research Institute (MRRI) in Charleston. All studies utilized captive wild fish which were held under controlled photothermal conditions. Results indicated that females containing oocytes ≥500 μm in diameter could be induced to ovulate using GnRH-a implants inserted intramuscularly. Eleven of 12 fish were strip spawned in our initial tests, and they produced a total of 1.6 million eggs with batch fertility ranging from 7 to 95% (Berlinsky et al. 1996). In an effort to reduce broodstock handling stress, increase egg production, and extend the spawning period, GnRH-a implants were also given to broodstock which were placed in spawning tanks. Initial results with three females were very impressive with a mean of 277,800 eggs/d being produced on 64 d during a 99-d spawning period. Total egg production was 17,782,000 and mean fertility was 32.8% (Smith et al. 1999b).

Other studies focused on aquaculture development have been conducted in South Carolina. Larval rearing trials during 1995-1997 indicated that small juveniles can be grown in tanks to a size of 25-30 mm TL using live foods but that pigmentation abnormalities occur (Denson and Smith 1997). This problem with pigmentation is similar to findings in North Carolina (Daniels et al. 1996). A recently completed study indicated that photoperiod may influence larval survival and that the presence of a sand substrate improved ocular side pigmentation (Carter and Smith unpub.). In addition, we have demonstrated production of small juveniles (25-125 mm TL) in fertilized earthen ponds (Jenkins et al. 1997; Jenkins and Smith 1999). This suggests that perhaps phase I juveniles (50+ mm TL) could be mass-produced using extensive pond systems similar to those used for red drum and hybrid striped bass culture. However, survival during these first attempts was poor (~5-6%) suggesting that pond management techniques need to be improved. Weaning of young pond-reared juveniles (2.5 mo old) to artificial diets was rapid (2 wk) and incidence of pigmentation abnormalities was low (Jenkins and Smith 1999). In North Carolina, it was shown that tank-reared juveniles could be rapidly converted to commercially produced rations (Daniels and Hodson 1999).

In summary, research results to date indicate that southern flounder have characteristics which make them attractive for culture. However, additional work is required to develop cultured broodstock, improve predictability of spawning, assure high quality gamete production, and to improve nursery and grow-out conditions.

This manuscript presents results from recent studies focused on initiation and control of

spawning. In particular, a variety of studies on strip spawning and tank spawning were conducted to improve the predictability of successful spawning of captive broodstock and to elucidate mechanisms which control tank spawning.

METHODS

General

For the various studies, wild adults were captured using trammel nets set in coastal waters (Smith et al. 1999b). For 1-4 wk after capture, fish were held in tanks under ambient estuarine conditions. During this time the fish were acclimated to captive conditions, treated for external parasites, and converted to chopped natural feeds. After this period, fish were typically moved to indoor tanks and maintained under controlled conditions.

Results reported herein are based on these wild fish held in indoor tanks located in separate rooms each of which had independent temperature, lighting, and water quality control. Each environmental room housed a holding/conditioning/spawning tank (3.7 m x 1.1 m deep), a biological bead filter (model PBF-6, Armant Aquaculture, Vacherie, Louisiana, USA); a UV filter (80W, Aqua Ultraviolet, Temecula, California, USA); a heat exchanger tank (1.2 x 0.6 x 1.2 m deep); and an external egg collector tank (110 cm high x 70 cm in diameter). The egg collector which contained a mesh bag, was connected to the spawning tank at the surface during the tank spawning trials. During the conditioning phase, fish were typically held at densities of 50-75 fish/tank (4.8-7.1 fish/m^2 bottom area; ~1.7-2.5 kg/m^3). The flounder were fed to satiation (usually three times/wk) a diet consisting primarily of squid and mackerel. Sampling data indicated that most fish grew substantially while in captivity. Males used in the various studies ranged from ~330-400 mm TL and weighed ~500-700 g. Due to sexual dimorphism, mature females were much larger and ranged in size from ~410-670 mm TL and weighed ~900-4000 g.

Photothermal conditions were strictly controlled. Lighting (297 lux at the surface) was provided by overhead fluorescent lights (two double 43 W T8 electronic bulbs). The lights were automatically controlled as either on or off without phasing (no dusk or dawn simulation). Temperature was controlled to ± 1 C using heat exchangers connected directly to the main heated and chill water systems for the building. During the strip and tank spawning studies, photothermal conditions were maintained at 10-11 h light and 17-18 C. Recirculated water was provided at a rate of 150 L/min to cause a circular rotation. During the various studies, typical water quality conditions were: 6-9 mg/L dissolved oxygen; salinity 32-34 g/L; pH 7-8; total ammonia nitrogen <1 mg/L; nitrite <0.1 mg/L; and nitrate1-35 mg/L.

The number of eggs reported are based on counts of sub-samples of water hardened fertilized eggs. On six occasions, a 1 ml sample of eggs was obtained and counted under a microscope. Egg counts ranged from 836 to 1,104, and overall mean was 956 eggs/ml. For purpose of convenience, eggs are presented as 1,000 eggs/ml.

Strip Spawning Research

Fish were placed in the environmental control rooms and subjected to simulated natural photothermal conditions. However, the natural spawning period was extended for several months by maintaining the photothermal conditions during which flounder are believed to spawn. Females used in these studies were selected from the tank populations based on gonadal biopsy. Selected fish had oocytes ≥500 µm in diameter. These fish received a 95% cholesterol, 5% cellulose pellet (Sherwood et al. 1988) containing 100 µg of GnRH-a (Peninsula Lbs, Belmont California, USA) as described in Smith et al. 1999b. Males received no hormone treatment during any of the studies. Females and males were held in 2-m diameter tanks (33-34 ppt salinity) during the strip spawning studies. Females were visually inspected several times during 48 h post hormone treatment until ovulation occurred. They were then checked at successive 24 h intervals until egg production ceased. Typically, ovulation occured in 48 h and eggs were stripped 3-5 times during a 5-7-d period. To reduce handling stress, females were examined only when their

abdominal area was sufficiently swollen to cause a protrusion around the vent and when scales in the area were slightly raised. Females were anesthetized in a solution of sea water and tricaine methanesulfonate (MS-222) before stripping. Males were selected based on the expression of milt with slight abdominal compression. Motility of sperm was confirmed by activation with sea water and observation under a compound microscope. Milt was routinely collected from the males using a 5-ml syringe and then stored in an ice bath until use (within1h). Eggs were manually expelled by providing slight abdominal pressure and collected in a ceramic bowl. Eggs were covered with milt from at least two males and the mixture stirred for 2 min. Next, sea water was added and the mixing continued for an additional 2 min. After fertilization, eggs were placed in a 20-L bucket and slightly aerated for 2 h. After this period, eggs were drained into a graduated cylinder and volume of floating and sinking measured. During the strip spawning studies in 1998, the volume of floating eggs relative to total egg production was used as an index of fertilization. However, in 1999, ~200 eggs were sub-sampled from the floating eggs and examined under a dissecting microscope for evidence of development. In 1999, percent fertilization was based on this sub-sample and calculated based on the total number of eggs taken (floating + sinking). Based on previous work (Smith et al. 1999a) it was determined that sinking eggs were dead (unfertilized, broken, deformed).

Study - SS1: Natural vs Hormonal Induction

Captive females will naturally mature and ovulate in tanks having photothermal control (Arnold et al. 1977). However, we sought to compare the efficacy of stripping naturally ovulated eggs to hormone induced ovulation. In 1998 two studies replicated two treatments: 1) controlled environmental conditioning coupled with strip spawning; and 2) controlled environmental conditioning coupled with GnRH-a implants and strip spawning. The studies were run sequentially using females (and males) from the same broodstock holding tank. Study duration was 26-30 d.

Study - SS2: Re-induction of Ovulation of Recently Spawned Fish

At times it may be beneficial to re-use recently spawned fish. A study was conducted to determine whether southern flounder could be re-induced to produce additional clutches of eggs after completion of a spawning event (multiple days of stripping or tank spawning). After initial spawning, females were placed in 2-m diameter recovery tanks for a period of 1-3 wk. In this study 12 females were selected based on ovarian biopsy results that showed that oocyte diameters remained >500 μm after spawning trials were completed and recovery period was over. All fish were implanted with a hormone pellet. All techniques were as described above.

Study SS-3: Relationship of Captivity Time to Spawning Success

Over the years there appeared to be improved predictability in maturation, ovulation and fertilization of eggs from fish that had been in captivity for more than several mo. During 1998, adult flounder were collected on 30 June and 27 October. Spawning of these animals was attempted from December - February. The results of these strip spawning trials were compared to those from fish which had been held in captivity for > 2 yr. Males collected on 27 October were used in all spawning trials to minimize possible affects which could occur if different groups of males were used with the various time in captivity groups of females. Results are presented only for females which could be discriminated as mature females based on their larger size and ovarian biopsy samples. Smaller fish could be either males or immature females. Thus, the actual number of males was not known at time of the spawning attempts.

Study - SS4: Milt Production

Previous work had suggested that milt production could be a limiting factor in fertilization of strip spawned eggs (Berlinsky et al. 1996). In 1998, a study was conducted to measure milt volume and quality from a group of males that had been in captivity for at least 24 mo. Near the end of a strip spawning study, a group of 8 males was isolated in a 2-m holding

tank and feeding was continued three times a wk. During this 10-d study, males were sampled every other d by applying abdominal pressure and collecting all available milt.

TANK SPAWNING RESEARCH

To reduce stress associated with strip spawning and to increase egg production, a tank spawning study was conducted in 1997 which was highly successful (Smith et al. 1999b). However, attempts to duplicate this success have indicated that results can be variable. Thus, a series of studies were conducted to identify possible controlling factors in volitional spawning to improve the predictability of tank spawning techniques. All studies utilized fish which had been in captivity for 24-36 mo. Males and females used in the studies were selected using the same criteria as those in the strip spawning studies (e.g. ≥500 µm oocytes, running milt). Females were implanted with 100 µg of GnRH-a in studies indicating hormone treatment of females. Unless otherwise noted, males were not treated with hormones. In the 1998 studies, 3 females were placed in a tank with 6 males (1:3 sex ratio) while in the 1999 studies 3 females were placed in a tank with 4-6 males. Water salinity was maintained at ≥32 ppt so that the eggs would float (Smith et al. 1999a). Eggs were skimmed off the water surface and collected in the external egg collector tank which contained a 250-µm mesh bag, 58 cm diameter x 66 cm deep. The egg collectors were inspected at least daily and eggs were removed and volumetrically measured in a 1-L graduated cylinder containing a known volume of sea water. Percent fertility was based on observation of embryonic development in a sample of 200 eggs randomly taken from the floating eggs and expressed based on total egg production (floating *vs.* sinking eggs). The total number of floating eggs collected in 1 d may be from one or more spawns within a tank as well as from remnant groups of eggs left over from the spawn the previous day.

Study TS1: Size of Females and Hormone Treatment

Published information indicated that only larger females could be induced to spawn (Arnold et al. 1977). Thus, a study was conducted to examine the effect of size of females on spawning as well as the need for hormone inducement of ovulation. Three treatments were examined consisting of: 1) GnRH-a implanted females ≤1.5 kg (actual mean size 1.2 kg, range 1.0 - 1.5 kg); 2) GnRH-a implanted females ≥2.0 kg (actual mean size 2.9 kg, range 2.0 - 5.5 kg); and 3) no hormone treatment, females ≥2.0 kg (actual mean size 2.5, range 2.0 - 3.3 kg)(control). Due to limitations in the number of tanks, only treatment 2 could be replicated in two tanks. Due to their smaller size, 4 females (and 8 males) were used in treatment 1 while 3 females (and 6 males) were utilized in treatments 2 and 3.

Study TS2: Approaches to Improve Male Participation with Naturally Ovulating Females

Results of study TS1 suggested that lack of male participation was an issue which should be examined. Study TS2, was a non-replicated experiment with several components which attempted to address this issue. The fish used in this study were those from study TS1, treatment 3 (control, no hormone treatment females or males). After completion of study TS1, males were inspected and found to be running ripe. These males were returned to the tank with the non-hormone treated females and removed 1 wk later as no spawning had occurred. Six new spermiating males which were being used in a strip spawning study were placed in the tank with the 3 females. During the following week no spawning occurred. The males were removed and implanted with testosterone silastic elastomers and then returned to the tank for an additional wk. As no appreciable spawning had occurred, the females were removed and given 100 µg GnRH-a implants and the study continued for an additional 11 d.

Study TS3: Approaches to Improve Control of Volitional Spawning

Results of study TS2, conducted in 1998, suggested hormone-treated females stimulated male participation. In 1999, study TS3 was conducted to further examine the use of hormones to improve male participation in volitional spawning. In this study, three hormone treatments (and a control) were examined: 1) GnRH-a treatment of females, no hormone treatment of males; 2) no hormone treatment of females, 50 µg GnRH-a + 1 mg/cm fish TL methyltestosterone treatment of males; 3) GnRH-a treatment of females, 50 µg GnRH-a treatment of males; 4) control - no hormone treatment of females or males. Due to tank limitations, the treatments were replicated during sequential studies. However, treatment 2 was not replicated. Duration of study 1 was 40 d while study 2 was concluded after 21 d, as all spawning had ceased.

RESULTS AND DISCUSSION

Due to the number of studies conducted and their different objectives, results from each study will be presented and discussed separately.

Study SS1: Natural vs Hormonal Induction

Although fish would naturally mature and ovulate under controlled photothermal conditioning, timing of ovulation was difficult to predict. Consequentially, repeated handling was necessary to attempt to identify the appropriate time to strip eggs. In study 1, no eggs were obtained from the three environmentally conditioned eligible females over a 26-d period. In contrast, the three eligible females which had been treated with GnRH-a implants completed spawning within 5 d and produced a mean of 453,000 eggs/fish (Table 1). In study 2, the two environmentally conditioned fish both ovulated and could be repeatedly strip-spawned. One fish was stripped 9 times over 30 d and produced a total of 1 million eggs of which 74% floated (Table 1). The other fish was stripped 3 times and produced 230,000 eggs of which 54% floated. The GnRH-a treated females all ovulated and produced a mean of 488,000 eggs of which 86% were floating. These fish were stripped 3-4 times and stripping was completed in 6 d.

Table 1. Data from strip spawning of natural and hormone induced ovulating southern flounder.

Treatment	Eggs ($x10^3$)		Spawnings (No.)	Duration (Days)
	Total	Floating (%)		
	STUDY 1			
Natural	0	-	-	26
Natural	0	-	-	26
Natural	0	-	-	26
GnRh-a ♀	660	18	3	5
GnRh-a ♀	360	56	4	5
GnRh-a ♀	340	65	4	5
	STUDY 2			
Natural	230	54	3	30
Natural	1000	74	9	30
GnRh-a ♀	450	91	3	6
GnRh-a ♀	595	72	4	6
GnRh-a ♀	420	95	3	6

These results indicate that naturally ovulating females can be strip-spawned, however, timing of ovulation is difficult to predict. In contrast, hormone treatment of eligible females resulted in greater predictability in timing of ovulation, and spawning was completed in a shorter time period (within several d). Unfortunately, percent fertilization was not determined for the floating eggs so no conclusion was possible concerning relative fertility of natural vs. hormone induced production of eggs.

Study SS2: Re-induction of Ovulation of Recently Spawned Fish

Results of implantation work showed that 11 of 12 females responded to the hormone treatment. Five of the 12 spawned only once. Egg production ranged from 50 to 270,000 eggs. Egg quality was also highly variable ranging from 0-100% floating eggs. Six of the fish were from a tank spawning trial and individual egg production from the first implantation was not known. However, previously strip-spawned individuals were used to compare first and second

Table 2. Data from previously spawned fish re-implanted with GnRh-a. Fish were strip-spawned.

Fish No.	Initial Implantation			Re-Implantation		
	Spawnings (No.)	Total eggs ($x10^3$)	Floating (%)	Spawnings (No.)	Total eggs ($x10^3$)	Floating (%)
1	4	440	91	3	336	33
2	4	472	92	2	270	33
3	3	426	63	2	330	39
4	3	660	18	1	150	60
5	4	360	56	2	200	45
6	4	340	65	0	-	-

implantation egg production. One fish from this group produced no eggs after the second implantation, and egg production and percentage of floating eggs was generally lower amongst the other females (Table 2).

Study SS3: Relationship of Captivity Time to Spawning Success

There was a clear relationship between occurrence of females that could spawn and egg production with time in captivity (Table 3). The percent of females that could spawn increased from 29% for fish in captivity only 1.5-3.5 mo to 70% for those held under captive conditions for 5.5-6.3 mo. Females held for more than 24 mo were all eligible and could be spawned. Egg production per female was similar for those in captivity ≤6.3 mo and over three times greater for fish held in captivity >24 mo (Table 3). Production of fertilized eggs per female was almost double for fish in captivity >24 mo but on a weight basis production of fertilized eggs was similar among all groups (mean 71,000/kg). Mean percent egg fertilization per fish was almost double for the newly acquired groups of fish as compared to those in captivity for > 24 mo and all groups of fish showed, substantial within group, variation in percent fertilization (Table 4).

The results showed that the fish in captivity grew larger with time. As a result, more fertilized eggs per female were produced and the predictability of spawning improved with captivity time. However, the number of fertilized eggs decreased, with the oldest fish in captivity

Table 3. Data on captivity time and spawning success of female southern flounder.

Captivity Time (mo)	Eligible/ Total	Mean Wt. (g)	Spawned (No.)	Total Eggs/ (%)	Total Fertility[1] female ($x10^3$)	(%)
1.5 - 3.5	5/14	944	4	29	232	30.8
5.5 - 6.3	7/10	1048	6	70	203	33.3
>24	7/7	1776	7	100	754	17.3

[1]Total fertility = all fertilized eggs divided by all eggs produced.

Table 4. Data on captivity time, egg production, and fertilization for southern flounder.

Captivity time (mo)	Fertilized eggs ($x10^3$) per female	kg	Fertility per fish (%)[1] Mean	Range
1.5 - 3.5	72	76	37.7	8.0 - 79.9
5.5 - 6.3	68	65	32.9	10.4 - 64.6
>24	130	73	17.2	4.0 - 48.2

[1]Based on batches of eggs which were fertilized.

providing the lowest fertility. The reason for this is not clear and may be related to a number of factors including physiological changes with age or perhaps nutritional factors associated with captivity, both of which can affect egg quality.

Although the number of potential males could not be determined due to the possible presence of immature females, running ripe males did readily occur with the fish in captivity for only 1.5-3.5 mo. In tanks where the >24 mo captive females were held, all smaller fish were running ripe males during spawning conditions.

Study SS4: Milt Production

Although milt volume was often measured in µl during the work of Berlinsky et al. (1996), this was not the case in the present study. Males were prolific in milt production and most could be stripped every other d over a 10-d period (Table 5). Milt was viable in all cases and total volume per fish ranged 3.6 to 20.0 ml. Greatest production came from the two largest males (18.5, 20.0 ml). Maximum period for milt stripping was not determined as these fish had been previously stripped and were still running at the time the study was terminated. It appears that reduced stress associated with gentle stripping (only 1.0 to 1.5 ml per stripping event is routinely taken) and adequate holding conditions were responsible for the improved performance of the captive males. During this study, the fish actively fed indicating that stress levels were minimal.

Study TS1: Size of Females and Hormone Treatment

Size of females and hormone treatment influenced results (Table 6). The larger females which relied on natural photothermal conditioning alone, spawned 36 times over the 90-d study and produced a total of 5.3 million eggs. However,

Table 5. Milt production of male southern flounder stripped every other day during a 10-day period.

Fish Size		Stripping No. (Milt ml)					Total
Wt (g)	TL(mm)	1	2	3	4	5	(ml)
493	339	1.3	1.0	2.0	1.8	0.3	6.4
534	338	2.4	3.4	0	1.0	0.5	7.3
547	387	2.7	2.1	2.5	1.2	0.5	9.0
554	344	1.1	1.5	0	0	1.0	3.6
631	366	3.3	1.5	0.8	2.5	2.0	8.3
634	362	1.3	0.8	2.0	1.3	0.3	5.7
726	356	6.1	3.8	4.0	2.8	3.3	20.0
826	393	7.2	3.8	3.5	3.0	1.0	18.5

Table 6. Effect of size of females and hormone treatment on tank spawning success of environmentally conditioned (EC) fish.

Treatment	Duration (Days)	Spawns (No.)	Total Eggs (x10⁶)	Overall Fertility (%)	Fertility (Days)	Batch Fertility (%) Mean	Range
EC + GnRh-a <1.5kg ♀	60[1]	13	1.5	0		-	-
EC >2.0 kg ♀	90	36	5.3	0		-	-
EC + GnRh-a >2.0 kg ♀							
Rep 1	90	40	6.3	12	23	32	5-69
Rep 2	90[2]	42	4.6	18	18	33	8-59

[1]Spawning ceased on day 30

[2]Spawning continued for 150 days, data not presented.

there was no fertilization, apparently due to lack of male participation. In contrast, the hormone treated larger females produced an average of 41 spawns and an average of 5.5 million eggs (Table 6). Average overall fertility was 10%. Males on average participated 21 d and average fertility was 32.5%. The smaller females which also received the hormone treatment spawned 13 times during the initial 30 d and then ceased to spawn. Total production was 1.5 million eggs, but none were fertilized. These results suggest that hormone treatment and use of larger females are required to produce successful tank spawning events. However, even under these conditions, this study showed that males don't participate in all spawning events and that daily fertility levels vary considerably.

Study TS2: Approaches to Improve Male Participation

Although this study was not replicated, results strongly suggested that females control male participation. Replacement of nonperforming males with other ripe males did not stimulate spawning nor did use of testosterone-treated males. However, treatment of females with GnRH-a resulted in ovulation and spawning on 10 or 11 d and production of 2.3 million eggs (Table7). Further, males participated on 5 of the 10 d during which 87% of the eggs were spawned. Mean fertilization was 7.1% and ranged from 11-34%.

Study TS3: Approaches to Improve Control of Volitional Spawning

Results of this study clearly suggest female motivation causes male participation in the spawning event. In study 1, multiple spawning occurred in the tank containing the males which were hormone treated with GnRH-a + methyl testosterone. However, there was no fertilization. In the tank containing the GnRH-a treated females, spawning occurred on 29 days and 3.9 million eggs were produced (Table 8).

Table 7. Results of study to induce male participation in tank spawning.

Treatment	Time (Days)	Spawns (No.)	Egg Production ($x10^3$)	Fertilization (%)
No hormones ♀ + ♂	7	0	-	-
No hormones ♀ + new ♂	7	0	-	-
No hormones ♀ + ♂ with testosterone	7	1	10	0
GnRh-a ♀ +♂ with testosterone[1]	11	10	2,265	6.2

[1]Fertilized eggs were produced during 5 spawns. Mean fertility was 7.1%, range 11-34%.

Table 8. Effect of hormonal treatment on tank spawning of southern flounder.

Treatment	Duration (Days)	Spawnings (No)	Total Eggs ($x10^6$)	Total	Fertility (%) Daily Max
		STUDY 1			
♀ GnRh-a	40	29	3.9	4.8	59.2
♀ GnRh-a/MT[1]	40	11	0.5	0	0
Control[2]	40	11	1.5	0.3	25.9
		STUDY 2			
♀+♂ GnRh-a	21	14	1.6	6.3	6.0
♀+♂ GnRh-a	21	13	2.3	0	0
♀ GnRh-a	21	16	1.5	4.8	13.8
Control[2]	21	7	0.4	0	0

[1]MT = methyl testosterone

[2]Control = no hormone treatment of % and &.

Fertilization occurred on 11 d and percent fertilization ranged from 0.8 to 59.2%. Fish in the control group (no hormone treatment) spawned on 11 days. On one of these days natural fertilization occurred (25.9%).

In study 2, the tank with the GnRH-a treated females produced fertilized eggs while the control tank (no hormone treatment) produced only unfertilized eggs (Table 8). GnRH-a treatment of both males and females was not an improvement over treating just the females and in one replicate there was no fertilization.

The controlling mechanism for motivation of males is not clear but GnRH-a treatment of females alone does result in production of fertilized eggs. However, hormone treated males were not stimulated to participate in tank spawning.

CONCLUSIONS

Information obtained from these various studies will help improve the predictability of spawning southern flounder. As was shown with a number of other species including sea bass *Lates calcarifer* (Almendras et al. 1988), winter flounder *Pseudopleuronectes americanus* (Harmin and Crim 1992), and striped bass *Morone saxatilis* (Hodson and Sullivan 1993), GnRH-a was effective in inducing final maturation and ovulation in southern flounder (Berlinsky et al. 1996; Smith et al. 1999b). The use of GnRH-a implants to induce ovulation substantially narrowed the time frame for egg taking and improved the predictability of spawning success relative to strip spawning based on natural ovulation. Re-implantation of GnRH-a was also useful for production of additional batches of eggs from some previously spawned females. As observed during these and previous strip spawning studies, there was variability in fertilization success and this may be related to egg quality. Current assessment techniques used in selection of eligible females may not be adequate for evaluation of egg quality (C. Sullivan NCSU, personal communication). Work focused on this issue is underway in North Carolina and South Carolina. Additionally, correct timing of ovulation to maximize egg viability is very difficult to achieve using strip spawning techniques and this no doubt accounts for variability in spawning success as well (Smith et al. 1995).

Volitional tank spawning has a number of advantages over strip-spawning. First, spawning can be controlled to occur over an extended period and tank spawning typically results in very high egg production. Second, labor requirements are minimized as the eggs are easily collected external to the spawning tank. Third, handling stress on broodstock is essentially eliminated and at termination of the spawning activity, fish are normally in good health and fitness. However, hormone induction coupled with strip spawning does result in more concise initiation and conclusion of the spawning event but overall production of eggs is lower.

There appear to be physiological and behavior issues associated with captive wild males which need to be addressed. Males naturally mature in captivity and can produce copious volumes of milt if not severely stressed. This was reflected in the study where the males were repeatedly stripped five times over 10 d and produced up to 20 ml of milt. However, there appears to be a controlling mechanism(s) which influences the involvement of the males in volitional tank spawning events that are not well known at present. Although running ripe, males did not normally participate in spawning with naturally ovulating females during our tank studies. Treatment of the females with GnRH-a had a mitigating influence and often resulted in male participation in tank spawning events. However, timing and predictability of spawning was not well controlled. Additional tank spawning research is needed to better simulate natural conditions including the use of larger and perhaps deeper spawning tanks.

In summary, a basic spawning technology is available to produce southern flounder. However, refinements are needed to improve efficiency and predictability. Besides the issues identified above, additional research is needed to identify suitable broodstock diets, and to develop and evaluate cultured broodstocks.

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USING COMMERCIAL FEEDS FOR THE CULTURE OF FRESHWATER ORNAMENTAL FISHES IN HAWAI'I

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ABSTRACT

Since 1993, various public and private institutions have supported the development of a freshwater ornamental industry in Hawai'i which, based on current success, must be intensive and utilize the latest in culture technologies to remain competitive. Feeding and palatability trials using commercial feeds suggests that very little is understood about the nutritional requirements of the 1500 species that characterize the ornamental trade. Rather than focusing on a particular food for any one species, we examined feeds already developed for food fish to find whether they are suitable as feed for ornamental fish. Investigations on various aspects (price, palatability, color enhancement, growth supporting characteristics, maturation and spawning) of commercially available diets were conducted using a variety of species of freshwater ornamental fishes and form the basis for this report.

INTRODUCTION

Developing a freshwater ornamental fish industry in Hawai'i is believed to be one means to diversify the agricultural output and stimulate the stagnant local economy. Approximately 75% of the freshwater aquarium fish imported into the United States originate from Southeast Asia (Chapman et al. 1997), reflecting a trade deficit of approximately US $34 x 10^6 (Fig. 1). Of the nearly 1500 different species of ornamental fish that are imported yearly, the quantities are dominated by only a few species. The top 10 species of imported ornamental fish are listed in Table 1. Three of the top 10 species are live-bearing tooth carps belonging to the Poecilid family. The guppy *Poecilia reticulata* is considered by many to be the most popular aquarium fish (Whitern 1979) and in 1992, it alone accounted for nearly 26% of the total number of freshwater ornamental fishes imported into the United States.

In Hawai'i, researchers and extension agents are often stumped when asked by growers of ornamental fish, "What is the best feed for my fish?" A closer examination of this question has revealed that, from an aquaculture standpoint, very little information is actually available about the nutritional requirements of the various ornamental fishes being cultured. In this report, some of the preliminary findings and experiences regarding fish food marketed for the freshwater ornamental trade and feed marketed for the culture of food fish are presented.

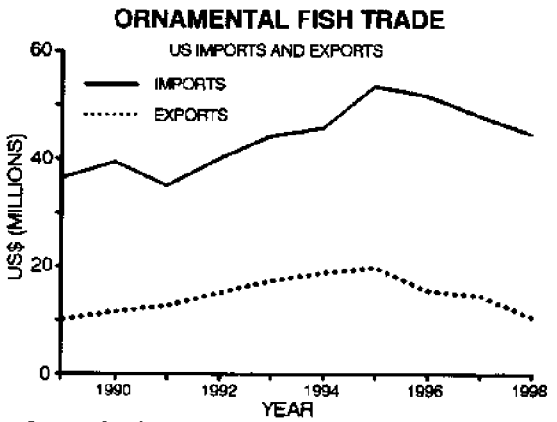

Figure 1. Summary of USA imports and exports of ornamental fish from 1990 to 1998.

Table 1. Summary of top 10 freshwater ornamental fishes imported into the USA in 1992 (from Chapman et al. 1997).

Common Name	Scientific Name	Percentage of Total Fish Imported (1992)	Number of Individuals Imported (1992) (x 10^6)
Guppy	*Poecilia reticulata*	25.8	51.9
Neon Tetra	*Paracheirodon innesi*	11.3	22.7
Platy	*Xiphophorus maculatus*	5.4	10.9
Siamese Fighting Fish	*Betta splendens*	2.7	5.4
Goldfish	*Carrasius auratus*	2.4	4.8
Chinese Algae-eater	*Gyrinocheilus aymonieri*	2.4	4.8
Shortfinned Molly	*Poecilia sphenops*	2.0	4.0
Cardinal Tetra	*Paracheirodon axelrodi*	1.5	3.0
Glassfish	*Chanda lala*	1.5	3.0
Tiger Barb	*Barbus terazona*	1.3	2.6
Total	10	56.3	113.1

Prices of Various Feeds

Not being conversant with the feeds currently marketed in the aquarium industry we asked basic questions such as "What about price?" Retail prices of nine feeds available for koi *Cyprinus carpio* were compared to prices of the few aquaculture feeds available in Hawai'i. The prices were standardized in US dollars/kg (Table 2). A USA flake (staple) feed is also included as we used it in a feeding trial described later. Two conclusions are obvious in this initial examination of the feed groups. The first is that all the ornamental fish feeds are higher in price by 10-60 times the price of aquaculture feeds. Second, the prices of the feed targeted for a single ornamental species vary dramatically compared to prices of the food fish feeds, each of which is targeted for a specific species. Another major difference is that feeds for ornamental fish are marketed in much smaller packages, the largest being just over 0.5 kg. In contrast, the smallest commercial package of aquaculture feed we know of is 22 kg.

Since the prices of the various feeds designed for one species vary significantly, one would logically presume differences in their efficacy. Our presumption was viewed from an aquaculture standpoint where promoting growth and survival of the target species are primary concerns. This framework presented an opportunity to test whether observations gathered over several years of working with the marine food fish mahimahi *Coryphaena hippurus* and *moi* or *Polydactylus sexfilis* would stand as objective and quantitative. One finding was that fish do have definite preferences among feeds and attack the preferred feeds with greater gusto. They also feed vigorously for a longer period of time if they like the feed. It follows then that, nutrition being equal, more palatable feeds should yield faster growth and to test this hypothesis, a method to assess

Table 2. Comparison of retail prices for ornamental fish feeds and aquaculture feeds.

Type of Feed	US $ per Kg
Japanese Staple	15.18
Japanese Gold	24.09
Japanese Spirulina	41.56
"German" Pond Regular	24.16
"German" Pond Pigmented	28.60
"German" Pond Spirulina	74.34
USA Koi Staple	26.01
USA Koi Color	27.96
USA Koi Growth	29.77
USA Flake (Staple)	14.00
Mahimahi Feed	1.25
Salmon Fry	1.85
Catfish Chow	1.10

Retail prices in Honolulu, Hawai'i, in 1997.

palatability was designed utilizing the various koi feeds.

Method of Assessing Palatability

Eight different koi feeds were investigated for palatability and each was assigned a code (e.g., Feed 1) to simplify presentation of the results. The various feeds in this study were provided by Rolf A. Hagen, Inc., USA. Some of the feeds are still undergoing testing and many aspects of the data (e.g., proximate analysis) are not available at this time. The proximate analysis provided by the manufacturers of the various feeds used in the current study is summarized in Table 3. The mix of ingredients include: minimum crude protein of 25-40%, minimum crude fat of 2-7%, maximum fiber of 2-5%, maximum moisture 7-12% and maximum ash of 10-12%. With the exception of protein content, no outstanding differences in the major components of the various feeds were found.

Testing for Differences in Palatability

Juvenile koi were purchased from a commercial breeder and kept in four 120-L tubs (7 fish/tub) equipped with aerators. Water was pumped through all tubs and changed 50% each wk. The water temperatures ranged from 19 to 25 C. The koi were fed twice/d (morning and evening) and the feeding schedules were maintained rigorously. Fish were offered a weighed amount of feed and if the feed particles were too large for the fish, the pellets were crumbled by hand. If the koi ate all of the feed (about 5 g in our tests) within 10 min, they were provided an additional 0.3 g of feed and the next meal was also increased by this amount. (If this was a morning feeding, the following morning 5.3 g would be offered). If 5-10 particles of feed remained in a tub at the end of the feeding period, the amount of feed in the next meal was the same. If more than 10 particles remained, the next meal was decreased by 0.3 g. Using this method, the quantity of feed fed morning and evening generally stabilized in 1 d, although there were occasional small variations (usually due to water temperature). Each wk, the feeds provided to a group of fish were rotated among the tubs. For example, to test four different feeds, group 1 was fed Feed 1, group 2 was fed Feed 2, group 3 was fed Feed 3, and group 4 was fed Feed 4 during the first wk. Then during the second wk, group 1 was fed Feed 2, group 2 was fed Feed 3, group 3 was fed Feed 4, and group 4 was fed Feed 1. Eventually, all fish in each tub sampled each feed.

The first 2 d of feeding were ignored in the calculations. Otherwise, daily feed consumption was averaged for the wk and daily feedings for each of the feeds were compared to the average on a weekly basis. These normalized data were subjected to statistical analysis to see whether a particular feed was eaten in greater or lesser quantity than the other feeds. Normalization was necessary because the fish grew to twice their size during the course of the test.

More than 400 meals were fed to the koi during the 8-wk experiment. The feed preferences are summarized in Fig. 2. It can be seen that feed preference surfaced in three statistically significant ($P<0.05$) groupings. A growth test was then performed using three of the feeds, two with the highest relative palatability and one on the

Table 3. Reported proximate analysis of koi feeds used in the current study.

Feed Type	% Protein Min.	% Lipid Min.	% Fiber Max.	% Moisture Max.	% Ash Max.
Feed 1	na	na	na	na	na
Feed 2	40	4	4	10	12
Feed 3	na	na	na	na	na
Feed 4	25	2	2	7	na
Feed 5	32	3	2	7	na
Feed 6	37	7	5	12	10
Feed 7	36	4	4	7	12
Feed 8	35	3	5	10	12

na = data not available

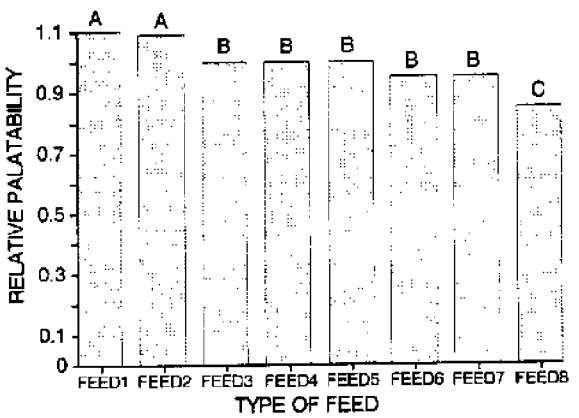

Figure 2. Relative palatability of various feed for koi. Bars with a different alphabet are significantly ($P<0.05$) different.

basis of its high market position. The koi were fed to satiation using the previously described method for 1 mo. At the beginning of the trial, the fish were about 15 cm in length and 80 g in weight. After the 1-mo trial, Feed 1 and Feed 2 supported an increase of about three times in fish length and about three times in weight gain (Table 4). This is consistent with the hypothesis that more palatable feeds should yield faster growth with nutrition being equal. Most interesting of the observations in this particular experiment was that the performance (e.g., palatability, growth) of a particular feed was not correlated to the retail price.

Comparing the Performance of Different Feeds

In the previous investigation, a method to compare the palatability of different feeds one would normally find in any pet shop was determined. Further investigation focused on feeds for one particular species of fish. However, the majority of feeds developed for use by aquarium hobbyists are in the form of flake food.

Table 4. Summary of body length and weight increases during the growth trial. Values that have different letters are significantly different ($P<0.05$).

Feed Type	Body Length (cm)	Body Weight (g)
Feed 1	1.5a	27.7a
Feed 2	1.1a	29.9a
Feed 5	0.4b	9.8b

Most brands of commercially available flake feeds (basic or staple) formulated for maintaining freshwater ornamental fishes meet the nutritional requirements of multiple species. It becomes obvious upon examination of the package label of these feeds that the long list of ingredients are combined to suit the diet for almost every species of ornamental fish. In this way, the manufacturer can satisfy a large consumer base. A problem with this approach is that this "all purpose" feed may not be suitable for fish that require a high-protein or high-vegetable diet. Similarly, these "basic" or "staple" diets appear to be designed to provide sustenance and not to optimize growth or reproduction, which is important in the food fish industry.

In contrast, formulated feeds designed for the aquaculture of food fishes are manufactured to result in optimal growth at minimal costs. They are subjected to extensive testing to insure maximum performance and also include a feed conversion ratio. To meet this criteria, aquaculture feed manufacturers in the USA have focused on feed for "popular fishes" such as channel catfish, salmon, and trout. The obvious drawback to the aquaculture farmers is that they are limited to feeds formulated for a limited number of fish species. In the next series of experiments we compared aquaculture feeds designed for mahimahi and salmon against a generic USA flake (staple) feed designed for maintaining many kinds of fish in the aquarium industry (Table 2). For our initial investigation we chose the angelfish *Pterophyllum scalare*. A comparison of the composition of the feeds used in this experiment is presented in Table 5. While there is a slightly higher crude protein content in the aquaculture feeds, the major difference appears in the fat (lipid) content.

All feeds used in the trial were subjected to amino acid analysis as described in Tamaru et al. (1992) and are summarized in Table 6. The mahimahi feed contained significantly ($P<0.05$) higher amounts of total amino acids than the other feeds used in the current study. Although the flake and salmon fry feeds were found to contain similar total amino acids, the salmon fry feed was found to contain significantly ($P<0.05$) higher levels of several essential amino acids (methionine,

Table 5. Proximate composition of various feeds used in the investigation of angelfish.

Treatment Feed	% Minimum Crude Protein	% Minimum Crude Fat	Moisture (%)	Ash (%)	Price US$/kg
Flake Feed	45	4	8	19	14.00[a]
Mahimahi Feed	56	14	-	-	1.25[b]
Salmon Fry Feed	50	23	6	10	1.85[b]

Superscript a relates to prices in Honolulu, Hawai'i and superscript b relates to FOB factory prices.

histidine, and lysine). The mahimahi feed contained significantly ($P<0.05$) higher amounts of all of the essential amino acids, except methionine, when compared with the flake feed and has higher levels of several essential amino acids (i.e., threonine, isoleucine, and arginine) when compared with the salmon fry feed. The salmon fry feed contained the highest level of histidine of all the feeds investigated.

Fatty acid profiles of the various feeds used in the study of angelfish were determined as described in Tamaru et al. (1992) and are

Table 6. Essential amino acid profiles of flake feed, mahimahi feed, and fry feed (mg/100 mg dry weight). Numbers with different alphabetical suffixes are significantly different ($P<0.05$).

Amino acid	Flake feed	Mahimahi feed	Salmon fry feed
Thr	1.36 ± 0.24a	2.43 ± 0.13b	1.77 ± 0.16a
Val	1.11 ± 0.13a	1.58 ± 0.20b	1.19 ± 0.10ab
Met	0.73 ± 0.06a	0.91 ± 0.17ab	1.07 ± 0.08b
Ile	0.70 ± 0.08a	2.12 ± 0.15b	0.69 ± 0.06a
Leu	2.35 ± 0.14a	3.69 ± 0.25b	2.92 ± 0.58ab
Phe	1.14 ± 0.09a	1.89 ± 0.32b	1.38 ± 0.17ab
His	0.67 ± 0.04a	0.88 ± 0.09b	1.53 ± 0.10c
Lys	2.04 ± 0.11a	3.70 ± 0.37b	2.81 ± 0.27b
Arg	2.07 ± 0.31a	2.65 ± 0.18b	2.14 ± 0.15a
Total amino acids	30.5 ± 1.41a	45.5 ± 3.35b	33.0 ± 2.00a

Table 7. Fatty acid profiles of flake feed, mahimahi feed, and fry feed (mg/100 mg dry weight). Numbers with different alphabetical suffixes are significantly different ($P<0.05$).

Fatty acid	Flake feed	Mahimahi feed	Fry feed
14:0	0.86 ± 0.24a	1.36 ± 0.06a	1.83 ± 0.05b
16:0	1.70 ± 0.08a	2.71 ± 0.14b	4.48 ± 0.13c
16:1n-7	0.58 ± 0.02a	1.21 ± 0.05b	1.77 ± 0.02c
18:0	0.52 ± 0.05a	0.20 ± 0.00b	0.82 ± 0.01c
18:1n-9	1.45 ± 0.06a	0.52 ± 0.03b	1.76 ± 0.04c
18:2n-6	0.76 ± 0.04a	0.75 ± 0.45ab	0.90 ± 0.00b
18:3n-3	0.17 ± 0.01a	0.33 ± 0.01b	0.27 ± 0.01b
18:4n-3	0.19 ± 0.02a	0.34 ± 0.01b	0.42 ± 0.01c
20:1n-9	0.05 ± 0.00a	0.12 ± 0.00b	0.29 ± 0.01c
20:4n-6	0.05 ± 0.00a	0.21 ± 0.01b	0.23 ± 0.01b
20:5n-3	0.83 ± 0.03a	2.51 ± 0.08b	2.40 ± 0.19b
22:1n-11	0.32 ± 0.02a	0.08 ± 0.00b	0.08 ± 0.00b
22:6n-3	0.93 ± 0.03a	1.68 ± 0.10b	2.05 ± 0.21b
Total fatty acids	8.7 ± 0.61a	12.0 ± 0.05b	17.3 ± 0.19c

summarized in Table 7. In summary, the flake feed contained the lowest levels of essential (18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3) and total fatty acids. Mahimahi feed was found to consist of significantly ($P<0.05$) higher amounts of total fatty acids and all of the essential fatty acids, except 18:2n-6, in comparison to the flake feed. The salmon fry feed contained similar amounts of all of the essential fatty acids in comparison to mahimahi feed, but it is also significantly ($P<0.05$) higher in total fatty acids.

Palatability Trial Using Angelfish

The same experimental design described previously was used to test the palatability of the USA flake (staple), mahimahi, and salmon fry feeds using angelfish (marble variety). The angelfish were from multiple broods at the age of 8 or 9 wk after hatching, reared collectively in one tank. Three 10-gal glass aquaria equipped with a single sponge bio-filter and aeration were stocked at 50 individuals/ aquarium. Because only three feeds were investigated, the entire experiment was completed in 3 wk. For purposes of illustration, the actual levels of feed consumed during each rotation is summarized in Fig. 3. During all three rotations (i.e., three separate wk), the flake feed was consumed at significantly ($P<0.05$) lower levels than the other two feeds tested. In general, the mahimahi and salmon fry feeds were consumed at equal rates indicating similar preferences by the angelfish for both feeds.

Growth Trial Using Angelfish

For the growth experiments, the three 10-gal treatment aquaria were stocked with 50 angelfish each. Throughout the feed trials, the fish were fed three times/d during the weekdays and twice/d on weekends. Each treatment (i.e., feed) was replicated. Feeding to satiation was conducted by first offering a predetermined amount of food. If the food had been consumed entirely in 5-10 min, additional feed was then provided. If residual feed remained, feeding was terminated for that particular feeding and the amount of food provided during the next feeding was decreased. The goal was to provide just enough food and no more. The entire experiment was carried out for one mo and then repeated with a new set of fish.

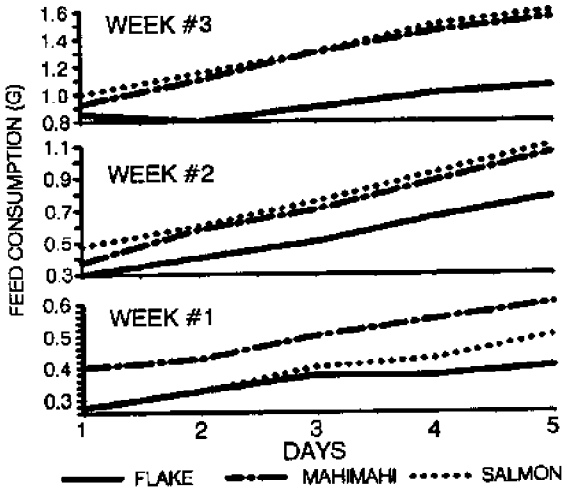

Figure 3. Summary of palatability trials of flake, mahimahi, and salmon fry feeds using the angelfish *Pterophyllum scalare*.

An artificial light source was on for 10 h and off for 14 h/d during the course of the experiment. Complete water changes were done approximately every 2 wk, and water chemistry measurements were taken weekly.

A summary of initial and ending body weights of angelfishes fed the various test feeds is presented in Table 8. Results of this investigation demonstrate that the mahimahi feed and salmon fry feeds are superior for growing marble angelfishes. Similar results were achieved with the golden angelfish variety. The average feed conversion ratios for USA flake (staple), mahimahi, and salmon feeds were 1.3, 0.8, and 0.8, respectively. The results show that although the mahimahi and salmon fry feeds result in superior growth of angelfishes, they are approximately 10 times more inexpensive than the flake food used in the current study. Once again, feed performance (in terms of growth, survival, and feed conversion) does not necessarily correlate with the price of the feed.

Maturation Diets

A continuous area of research interest for investigators and hobbyist alike is the maturation and spawning of freshwater ornamental broodstock. As reported in Tamaru and Ako (1997), investigations in this area rely heavily on the input of members of the Honolulu Aquarium Society who share their expertise in this domain as they have, through trial and error, found ways

Table 8. Feed trials with marble angelfish. Initial weights were 284 ± 69 mg and 300 ± 82 mg for Trial 1 and Trial 2, respectively. Numbers with alphabetical suffixes of a and b are significantly different (P<0.05). Numbers with alphabetical suffixes of c and d are significantly different (*P*<0.01). N

Treatment	# of Replicates	# of Individuals	Final Body Weight (mg)	Final Body Length (cm)	Survival (%)
Trial #1					
Flake Feed	2	25	525 ± 138a	3.1 ± 0.3a	100
Mahimahi Feed	2	25	994 ± 269b	3.5 ± 0.3b	100
Salmon Fry Feed	2	25	858 ± 331b	3.5 ± 0.4b	100
Trial #2					
Flake Feed	2	18	504 ± 176c	3.2 ± 0.4c	94
Mahimahi Feed	2	18	781 ± 229d	3.7 ± 0.4d	100
Salmon Fry Feed	2	18	840 ± 229d	3.7 ± 0.3d	100

to spawn their respective fish. In that report, the fatty acid profiles of both prepared and live feeds that are commonly used by Hawai'i breeders in conditioning freshwater ornamental fish broodstock for spawning were investigated.

Live earthworms and mosquito larvae were obtained from a compost bin and bucket of rainwater, respectively, located in Maunawili, O'ahu, Hawai'i, USA. *Moina* was obtained from Lance Pang of Wainani Kai, Honolulu, Hawai'i, USA. The beef heart preparation and black tubifex worms were obtained from Fred Lum of Pacific Discus Hatchery, Honolulu, Hawai'i, USA. Red tubifex worms were obtained from Patrick Vahey of Hanohano Enterprises, Punaluu, Hawai'i, USA. All live and prepared feeds were collected, rinsed in fresh water and stored frozen at -20 C until analyzed. Results for beef liver are from a previous investigation (Iwai et al. 1992). Fatty acid analysis was conducted as summarized in Tamaru et al. (1992). The values presented are the averages from triplicate determinations and, unless specified otherwise, are expressed in terms of mg/100 mg dry weight.

Essential Fatty Acid Profiles of Maturation Feeds

A summary of the total fatty acids and essential fatty acids found in the various feeds used for the maturation and spawning of freshwater ornamental fishes is presented in Table 9. Total fatty acids ranged between a low of 0.81 found in earthworms to a high of 8.95 found in beef liver. With the exception of the beef heart preparation, all other feeds were found to be deficient in 22:6n3. All feeds contained 20:5n3 ranging from a low of 0.07 found in *Moina* to a high of 0.61 found in black tubifex worms. Relatively high levels of 20:4n6 were observed in all of the feeds tested ranging from a low of 0.16 found in *Moina* to a high of 0.90 found in black tubifex worms. Both 18:3n3 and 18:2n6 ranged from 0.00 to 0.51 (beef liver, black tubifex

Table 9. Essential fatty acids of maturation feeds used for freshwater ornamental fishes. Values are reported in mg/100 mg dry weight.

Fatty Acid	Beef Heart Diet	Beef Liver	Black Tubifex Worms	Red Tubifex Worms	*Moina*	Earthworms	Mosquito Larvae
18:2n6	1.71	1.56	1.68	1.43	0.11	0.11	0.48
18:3n3	0.20	0.00	0.51	0.19	0.04	0.10	0.31
20:4n6	0.51	0.22	0.90	0.64	0.16	0.22	0.33
20:5n3	0.11	0.00	0.61	0.33	0.07	0.09	0.23
22:6n3	0.33	0.00	0.00	0.00	0.00	0.00	0.00
Total Fatty Acids	4.86	8.96	6.22	4.68	4.22	0.81	8.27

worms) and 0.11 to 1.71 (earthworms, *Moina*, beef heart), respectively.

The essential fatty acid data were summarized in a non-conventional fashion (i.e., percent composition) where each of the essential fatty acids within a particular feed was divided by the observed total fatty acids in the respective feed. The value was then multiplied by 100. Each of the essential fatty acids were then ranked on the basis of the percent composition of total fatty acids as summarized in Table 10. When the data are presented in this fashion, all feeds exhibit a high percent composition of either 18:2n6 or 20:4n6. On a percent composition basis, 18:2n6 ranged from a low of 2.61% found in *Moina* to a high of 35.19% found in the beef heart diet. On a percent composition basis, 20:4n6 was ranged from a low of 3.42% found in *Moina* to a high of 27.16% found in earthworms.

The various live- and fresh-feed preparations examined during the current study are invariably used by freshwater ornamental fish breeders who have found them to be effective in bringing about maturation and spawning of particular species. It is interesting to note the inclusion of skipjack tuna roe in the beef heart diet prepared for growth and maturation of discus *Symphysodon discus*. Discussions with numerous freshwater ornamental fish breeders reveal similar inclusions of marine products (e.g., blood worms, fish roe, squid, crustacean meat) with a preparation of beef heart or beef liver. While inclusion of marine products in freshly prepared diets is being practiced, many hobbyists rely solely on the live feeds under investigation. The results indicate that these feed items do not have detectable levels of 22:6n3. In addition, the live-feeds examined possess relatively low levels of 20:5n3. This would lead one to hypothesize that a large number of freshwater ornamental fishes do not require the long chain polyunsaturated fatty acids as reported to be essential for marine fish species (Watanabe et al. 1983, 1984). Both 18:2n6 and 20:4n6 were found to be the major essential fatty acids present in the live-feeds investigated in the current study, implying that they play a critical role in reproduction, as these feeds are used religiously by the hobbyists/breeders of ornamental freshwater fishes. It has been reported that juvenile chinook salmon do not exhibit the ability to convert dietary 18:2n6 to 20:4n6 (Dosanjh et al. 1988) and it remains to be demonstrated to what extent the conversion of 18:2n6 to 20:4n6 takes place in teleosts. If this situation is pervasive in freshwater teleosts, then it is not surprising that 20:4n6 is present in large quantities in the prey that the fish consume.

The genus *Corydoras* is one of two important genera of catfishes as far as freshwater aquarium enthusiasts are concerned. Members of the genus originally came from regions of South America, however, many other species have now been domesticated. Most of the 'Corys', as they are affectionately called, are hardy and highly adaptable to most aquarium conditions and it is often said that a community aquarium is not complete without a few armored catfishes. Their reputation as a bottom "cleaner" makes them one of the more sought after fishes in the aquarium trade. That this species is being cultured in Hawai'i served as further justification to carry out this investigation.

Three 15-gal aquaria were individually stocked with two male and one female *C. aeneus*. The aquaria were part of a single recirculating system so that water quality parameters in each aquarium would be equivalent. Fish in each aquarium were fed one of the following treatment

Table 10. Summary of essential fatty acids found in maturation feeds ranked by percent composition.

Ranking	Beef Heart Diet	Beef Liver	Black Tubifex Worms	Red Tubifex Worms	*Moina*	Earthworms	Mosquito Larvae
1	18:2n6	18:2n6	18:2n6	18:2n6	20:4n6	20:4n6	18:2n6
2	20:4n6	20:4n6	20:4n6	20:4n6	18:2n6	18:2n6	20:4n6
3	22:6n3	18:3n3	20:5n3	20:5n3	20:5n3	18:3n3	18:3n3
4	18:3n3	20:5n3	18:3n3	18:3n3	18:3n3	20:5n3	20:5n3
5	20:5n3	22:6n3	22:6n3	22:6n3	22:6n3	22:6n3	22:6n3

diets (beef heart + seafood, beef heart only, or Nutrafry) for approximately one mo. For example, groups 1, 2, and 3 received beef heart + seafood, beef heart, and Nutrafry, respectively. In the second trial fish in groups 1, 2, and 3 received beef heart, Nutrafry and beef heart + seafood, respectively. Feed for each tank was switched again after one mo so that all trios were fed a particular diet. Fish in each treatment group were fed equal weights of each of the diets. This meant that they were fed equal amounts of Nutrafry and wet weights of the beef heart and beef heart + seafood diets. The fatty acid profiles of the three treatment diets are presented in Table 11. The commercial pellet, Nutrafry, was found to contain the highest amount of total fatty acids, EPA (C20:5n3), DHA (C22:6n3) and ADA (C20:4n6).

During the entire experiment, the d on which a spawn occurred and the number of eggs spawned were recorded. During the latter part of the experiment, eggs from each spawn were measured using a compound microscope equipped with an ocular micrometer. A summary of the spawning activities of the fish fed the various treatment feeds is presented in Table 12. From the data presented, some consistent patterns

Table 11. Fatty acid profiles of three maturation diets used for *C. aeneus*. Values are reported in mg/100 mg as fed.

Fatty Acid	Beef Heart + Seafood	Beef Heart	NutraFry
14:0	0.01	0.03	1.83
16:0	0.20	0.33	4.48
16:1n7	0.01	0.05	1.77
18:0	0.20	0.29	0.82
18:1n9	0.17	0.62	1.76
18:2n6	0.51	0.30	0.90
18:3n3	0.06	0.03	0.27
18:4n3	N.D.	0.00	0.42
20:1n9	0.00	0.02	0.29
20:4n6	0.15	0.11	0.23
20:5n3	0.03	0.03	2.41
22:1n11	N.D.	0.01	0.08
22:6n3	0.10	0.00	2.05
Total Fatty Acids	1.46	1.82	17.30

Table 12. Summary of spawns for *C. aeneus* fed three different maturation diets.

Treatments	Total Number of Eggs Spawned	Number of Spawns	Average Number of Eggs/Spawn
Trial #1			
Nutrafry	331	9	37
Beef Heart	457	6	76
Beef Heart + Seafood	1127	7	161
Trial #2			
Nutrafry	56	1	56
Beef Heart	996	4	249
Beef Heart + Seafood	1750	6	292
Trial #3			
Nutrafy	57	1	57
Beef Heart	244	1	244
Beef Heart + Seafood	666	2	333

emerge. First, the fish fed beef heart + seafood produced a significantly larger number of eggs during the time they were fed the diet. The differences are approximately twice that of the nearest treatment (beef heart). The number of spawns, however, did not differ statistically. Although it appears that there may be a trend in the number of eggs/spawn, no statistical difference could be detected between fish fed beef heart + seafood and beef heart alone. The only statistical difference is with the lower number of eggs/spawn produced by individuals fed Nutrafry.

Spawned eggs were collected only from the latter spawns due to an oversight. However, a summary of the average (n=20) egg diameters from some spawns that were measured is presented in Table 13. Fish fed beef heart + seafood were found to have significantly larger eggs than fish fed any of the other diets.

Table 13. Average spawned egg diameters from *C. aeneus* fed three different maturation diets.

Treatment Diet	Number of Spawns	Egg Diameter (mm)
Beef Heart + Seafood	3	1.69 ± 0.04
Beef Heart	3	1.61 ± 0.01
NutraFry	1	1.63 ± 0.00

It is clear from the data that the beef heart + seafood diet does result in the production of a larger quantity of eggs from *C. aeneus* broodstock. It would appear that this is due to increasing the number of eggs/spawn and not by increasing the frequency of spawns. It should be noted however, that reasonably similar results can be obtained with the use of beef heart alone. Subjectively, the addition of seafood seemed to make the beef heart diet more palatable, i.e., the fish did not like the beef heart diet compared to beef heart + seafood. Although percent hatching for each spawn was not measured, hatching did occur in all nests that were put aside for observation. The egg size of the armored catfish fed beef heart + seafood also produced larger eggs, the biological significance of which remains to be determined.

At present there is no explanation for the increase in egg production and egg size observed when the armored catfish were fed the beef heart + seafood diet. Research activities always produce more questions than answers, at times, and this is definitely true for the current investigation. It is rather surprising that the reproductive performance of Nutrafry was so poor compared with the other diets tested. Nutrafry, however, does well against other commercial pellets. It is also clear that the essential fatty acids EPA (C20:5n3), DHA (C22:6n3) and the total fatty acids are not correlated with the observed reproductive performance. Arachidonic acid (C20:4n6), however, is still correlated with the observed reproductive performance (i.e., increase in egg production and egg size) again suggesting it has an important role in the reproduction of fishes.

The relatively high levels of both 18:2n6 and 20:4n6 in the feeds investigated are consistent with their reported biological roles. In higher vertebrates, metabolic conversion of 18:2n6 is the source of 20:4n6 (Galli 1980; Kinsella 1987) and 20:4n6 has been identified as the precursor for prostaglandins (Kinsella 1987). Prostaglandins have been demonstrated to play a critical role during the ovulatory process in teleosts or serving as pheromones that stimulate mating behavior in fishes, synchronizing both the physiological and physical components of the spawning event between both sexes (Goetz 1983; Kobayashi et al. 1986; Stacey et al. 1986). Investigations into the fatty acid profiles of spawned eggs from striped mullet *Mugil cephalus* maturing under natural and artificial conditions suggest a link to the observed low levels of 20:4n6 detected in spawned eggs and the reproductive process (i.e., lower percent fertilization) observed in striped mullet (Tamaru et al. 1992). The data on the essential fatty acids observed in the various live feeds and prepared diets presented in the current investigation strongly suggest that 20:4n6 plays an integral part in the reproductive mechanism of a large number of freshwater ornamental fishes. A concerted effort should be initiated to determine the function of 20:4n6 in the reproductive processes of teleosts.

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SHRIMP MATURATION AND SPAWNING

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ABSTRACT

The lack of a reliable supply of disease-resistant postlarvae (PL) continues to contribute to the uncertainty, inefficiency, and economic loss facing shrimp farmers worldwide. Many of the world's estimated 375 913 shrimp farms rely heavily on wild stocks (for brood and seed to stock ponds) and many of the 5777 hatcheries (Rosenberry 1999) rely on ready-to-spawn adult females from the oceans as a source of nauplii. As the shrimp aquaculture industry has matured, the number of farms relying on hatcheries for seed has increased and hatcheries are adopting technology to control the reproductive process and to produce generation after generation of shrimp without totally relying on the wild populations. This technology offers independence from the unpredictable fluctuations in wild populations, accessibility to the superior non-indigenous species, improvement in performance through artificial selection, and some control over the diseases found in feral stocks through development of disease-resistant strains.

The technology for control of shrimp reproduction has not changed much since the most important breakthroughs in this area occurred more than 20 yr ago. The United States Department of Agriculture (USDA) Shrimp Farming Consortium has made progress toward domesticating the western white shrimp *Penaeus vannamei*, (the newly proposed genus by Perez and Kensley (1998) is *Litopenaeus*), and has worked toward developing High Health, Genetically Improved (HHGI) animals for the industry. More progress is needed to stay ahead of the shrimp virus problems plaguing the industry. Problems with male shrimp quality have been overcome to some extent by using artificial insemination techniques but, again, more research is needed.

The technology for controlling shrimp reproduction is under constant refinement by commercial and academic groups. Initial breakthroughs occurred more than 65 yr ago when a Japanese researcher published the first written account of shrimp culture (Hudinaga 1935). The same researcher spawned the kuruma shrimp and described the techniques in detail (Hudinaga 1942). Panouse (1943) described shrimp eyestalk ablation, but it was not used in commercial shrimp maturation until the early 1970s. A few advanced farms were familiar with the techniques, but there was reluctance to share information. For many years, the industry generally preferred wild-caught PL over hatchery-reared PL, but with the advent of shrimp viruses like the White Spot Syndrome Virus (WSSV) and evidence of measured growth from captive stocks, the industry is gradually becoming more dependent on captive stocks. Disease-resistant, hatchery-reared animals are becoming more popular for pond stocking. Practicing the HHGI concept involves strict biosecurity measures at the hatchery and farm to control disease which generally involve limiting access and maintaining strict quarantine procedures. Pond growout comparisons have been made with PL from different sources, and it is documented that domestication and the HHGI concept (with added biosecurity measures practiced) have benefits over wild-caught stocks. The USDA funded US Marine Shrimp Farming Program, The Oceanic Institute, and a number of other organizations have made progress in domesticating and selecting faster-growing, disease-resistant families of shrimp and have brought them forth in their breeding programs. Several private companies are now utilizing the offspring from this work to produce future generations of shrimp for the aquaculture industry (Dr. James Wyban, Hawaii, personal communication, http://www.hihealthshrimp.com). Several research groups have selected animals through numerous generations that do not require ablation and the resulting animals selected spawn without ablation (Dr. Robert Shleser, Hawaii, personal communication). Through selective breeding, virus-resistant strains of *P. stylirostris* called the Super Shrimp were developed in Venezuela without eyestalk ablation (Chris Howell, Venezuela, personal communication) and this has assisted Mexico to reestablish itself as one of the top producing shrimp mariculture countries in Latin America.

INTRODUCTION

Maturation - Research History

Dr. Motosaku Fujinaga (Hudinaga 1935) made some of the most important contributions to the development of shrimp culture when he first accomplished captive spawning of mature *P. japonicus* females and reared the resulting larvae to subadults (Hudinaga 1942). The capture of wild females with mature ovaries for immediate spawning in captivity, known as "sourcing", was the only method known and practiced for inducing penaeid females to spawn in captivity until the

early 1970s. As discussed earlier, shrimp eyestalk ablation was not used in commercial shrimp maturation until the early 1970s.

The sourcing of gravid female shrimp is still widely practiced today in many countries with an abundant supply of wild brood in nearby waters. In the past, Japan had a total output of 600 to 700 million PL shrimp annually using sourced female *P. japonicus*. About 80% were used to restock coastal fisheries and the rest were used in commercial culture (Liao and Chao 1983). Sourcing, or obtaining ready-to-spawn females from the wild, has been used worldwide for experimental and commercial culture of numerous other species. This is particularly true in Southeast Asia where a single *P. monodon* female can sell for US$500-$2,000 or more. Sourcing, however, limits culturists to the use of indigenous species that may or may not be the best, or even a suitable, culture species and is dependent on seasonal availability, migratory movements, weather, natural rhythms, and diseases in feral populations. Efforts to induce penaeid reproduction in captivity continued so that a consistent, reliable source of PL seedstock could be obtained to support commercial culture operations and establish the basis for genetic selection to develop ideal domestic stocks with strong growth and survival characteristics (resistance to diseases). Annie Laubier-Bonichon and L. Laubier at the Centre Oceanologique de Bretagne in Brest, France, developed the "Laubier method" of shrimp maturation which involves maturation of *P. japonicus* using temperature and photoperiod manipulation, without ablation or the removal of one eye (Laubier-Bonichon and Laubier 1976; Laubier-Bonichon 1978). The Laubier method worked for *P. japonicus* on a small scale but was not dependable for commercial use. The French made other important advances in shrimp maturation (Aquacop 1977a, 1977b, 1979, 1984). Additionally, the Southeast Asian Fisheries Development Center (SEAFDEC) in the Philippines made very important contributions to our present knowledge of shrimp maturation (Primavera 1978, 1979; Primavera et al. 1980). Good literature reviews of maturation and reproduction in penaeid shrimp were done by Primavera (1985), Harrison (1990), and Bray and Lawrence (1992). In the 1990s, shrimp viruses forced the industry to rely less on feral populations, adopt biosecurity measures, and look more closely at perfecting domestication of species. The shrimp aquaculture industry has followed similar steps taken earlier by the poultry and swine industries in an attempt to control diseases.

Maturation and Spawning Research Highlights in the United States

Johnson and Fielding (1956) reported the first successful maturation and spawning (with fertilized eggs) of *P. setiferus* in the US, but this was in ponds. In 1959, the National Marine Fisheries Service (NMFS) had begun to adopt and use a modification of the Japanese culture technique to assist with closing the cycle of important species for the shrimp fishery in Texas. Cummings (1961) described maturation and spawning in the pink shrimp *P. duorarum*. Dr. Fujinaga visited the NMFS Laboratory in Galveston, Texas, in 1963 with the intention of scouting the area for a shrimp mariculture facility. Instead, a facility was later built in the state of Florida. Some of the other research publications from the NMFS lab were Brown and Patlen (1974), Brown et al. (1979, 1980), and Duronslet et al. (1975). The Galveston Laboratory, serving as an important demonstration and training center for maturation-hatchery biologists worldwide, continued to refine maturation, hatching and larval-rearing methods throughout the 1970s. The methods utilized by the NMFS researchers are still widely known as the intensive method or "the Galveston Laboratory Technique" (Klima 1978; McVey 1983) sometimes referred to as the clearwater method. The methods used today are basically modifications of this intensive method, and methods developed in Asia and other parts of the world. NMFS continued research and training at the Galveston laboratory in the early 1980s, and later under Texas A&M University (Lawrence et al. 1980) the research continued along similar lines. Similar research occurred at Texas A&M University (TAMU) main campus (Chamberlain and Gervais 1984) and the TAMU Texas Agricultural Experiment Station labs in Corpus Christi and Port Aransas, Texas. Other institutions

like The Oceanic Institute in Hawaii (Oyama et al. 1988) worked with shrimp maturation and spawning. Since the late 1980s, research and development by the US Marine Shrimp Farming Program has contributed to the success of the US shrimp aquaculture industry. Commercial trials with the domestication of *P. vannamei* have resulted in disease resistant strains using the HHGI concept and animals are provided to the US industry that have been tested commercially and selected from numerous families.

Hybridization of penaeid shrimp was attempted with *P. setiferus* + *P. stylirostris* and other species at TAMU in the 1980s, but their offspring were sterile (Lawrence et al. 1984; Bray et al. 1990). Reproduction of penaeid species was detailed by Bray and Lawrence (1992) but little to no work has been done since then on hybridization.

Fecundity

Martosubroto (1974) showed that there is a direct correlation between size of the shrimp and the number of eggs per spawn. Other references showing higher egg numbers with larger animals are Emmerson (1980), Ottogalli et al. (1988), Hansford and Marsden (1995), and Beard et al. (1977). Evidence indicates multiple spawning of unablated *P. setiferus* (five spawns per lifetime) and at least two spawns per season from *P. setiferus, P. duorarum, P. japonicus* and *Metapenaeus affinis*. Multiple spawning of unablated *P. japonicus* and pond-raised *P. vannamei* has been shown in captivity and in one case, an unablated female spawned 19 times in 7 mo. Unablated *P. merguiensis* have been noted to spawn an average of 2.6 mo in captivity compared with an average 2.8 mo for *P. japonicus*.

There are conflicting data, but wild *P. vannamei* generally produce average spawns of between 55 000 and 150 000 eggs, whereas pond-raised females of the same species and size produce 22 000-100 000 eggs. Larger species such as *P. monodon* can produce 700 000 to over 1 million eggs/spawn. For example, a 290-g (10.2-oz) female *P. monodon* might spawn 700 000 eggs, whereas a 454-g (1-LB) female might spawn 1.4 to 1.8 million eggs each spawn (personal experience in Indonesia). Some data for wild *P. vannamei* spawners from the Ecuadorian coast might contradict the above (Roeland Wouters, CENAIM/ESPOL, Ecuador, The following equation was calculated from 612 spawns (some of them were repeat spawns) from spawners with weights ranging from 27 to 80 g: $y = 3665 x + 22\,660$ with R-squared = 0.1892 [eggs per spawn = (3665 x spawner weight in g) + 22 660]. The largest spawn in this group was 621 000 eggs from a 45-g female. Pond shrimp (n=51) give similar results to wild spawners. A significant positive correlation between fecundity and spawner weight ($P<0.05$; $P<0.001$) has been shown in most batches of wild *P. vannamei* spawners. When filling in data in the previously listed equation, it can be noticed that this equation is okay for wild broodstock, while domesticated animals produce 30% less than predicted. The latter observation is only based on 25 samples, which is not enough to draw solid conclusions, but similar results have been reported by numerous hatcheries. This could indicate that lower fecundity of domesticated broodstock is not only due to lower spawner weight, but that other factors, such as inadequate feed, could be involved as well. Some managers feed their animals excellent feeds (artificial broodstock diets and fresh diets) and often overcome this problem in ponds. Most hatchery operations report that it generally takes three to four generations to obtain pond broodstock of equal or better quality than wild broodstock. Operations which depend upon wild broodstock should start serious breeding programs as soon as possible and over time the benefits will become apparent. Fecundity is just one of a long list of traits to select for in a breeding program. Spawning without ablation, rapid growth, and resistance to disease might be others.

Other data available on fecundity (Peter Larkins, personal communication) are: domesticated stocks from Colombia (Wt 37 g) eggs/female 105,000 (n=25); wild stocks from Panama (pond raised from wild nauplii kept in maturation tanks with Wt 29.5 g) eggs/female 116,000 (n=25); and Ecuadorian (wild broodstock kept in maturation tanks with Wt 60 g) eggs/female 230 000 (n=130).

Other data (Dr. Henry C. Clifford, personal communication) indicate that the pond-

reared, domesticated, *P. vannamei* broodstock raised and maintained under normal maturation conditions, naturally mated, unablated and ablated, averaging around 45 g (females) typically produce in the range of 120,000-160,000 viable nauplii/spawn. It has been more than 15 yr since Clifford measured the percent fertilization (per cent of fertile eggs in each spawn), but he recalled it generally varied from 60-90% in viable spawns. Natural spawning (percent mating) generally were on the order of 7-12% of the female population per d (when the maturation system was "healthy"). The author's experience has been that the percentage of fertile eggs of ablated female *P. vannamei* is in the 90% + range shortly after ablation and tapers off with time. After 3 mo, the animals had to be replaced.

In contrast, Preston et al. (1999) showed that wild kuruma shrimp broodstock produced about the same number of eggs as equal-sized domesticated kuruma shrimp broodstock, but the survival of larvae from the domesticated stock was half that of larvae from wild broodstock. They found that it would take 12 domesticated brood to produce enough PL to stock a 1-ha pond, whereas, it would only take six wild brood to stock the same pond. However, the costs of postlarvae production using wild broodstock is Aus$851 per pond compared to Aus$390 using domesticated broodstock to stock a 1-ha pond with postlarvae. The high cost of sourcing wild brood contributed to the difference. See Magarelli (1981) for further information, primarily with reference to *P. stylirostris* production and the importance of nutrition.

Male Reproductive System

The male genital system was thoroughly discussed by Motoh (1981). References on the male shrimp spermatophore and spermatozoa are Jeri (1998); Bauer (1986); Heitzman and Diter (1993); Bauer and Cash (1991); Pascual et al. (1998). The spermatozoa are non-motile and have been described as resembling a golf ball on a tee. Leung-Trujillo (1990) found that the number of spermatozoa is directly related to the size of the male. She found that a 35-g male might carry 70 million sperm per compound spermatophore. Methods for assessing male sperm quality have been reported by Bray et al. (1985) and Leung-Trujillo and Lawrence (1987).

Female Reproductive System

The female reproductive system consists of paired ovaries, paired oviducts and a single thelycum; the first two are internal and the last is an external organ and was thoroughly discussed by Motoh (1981).

Description of Current Technology in Shrimp Maturation and Spawning

Almost all hatcheries require availability of oceanic-quality water on a 24-h basis. Salinity and temperature are the most important water parameters impacting production of shrimp in the hatchery, and must be maintained in a narrow range, between 27 and 36 ppt salinity and 28 C (82 F) plus or minus two degrees for most penaeids. These and other important factors in the maturation and spawning of penaeid shrimp are discussed in detail by Treece and Fox (1993).

Parameters for Tropical Shrimp Maturation and Allowable ranges/24 hr.

Salinity
27-36 ppt +/- 0.5
Temperature
28 C +/- 2 (80.5-84.2 F)
pH
7.8 +/- 0.2
Light
14 L, 10 D
D.O.
5 ppm

Other parameters to consider in the maturation of penaeids are nitrogen levels in the water (especially ammonia and nitrites) which should be very low to non-existent. Average sea water has: 0.02-0.04 mg/L (ppm) NH_4-N = ammonium ion (total ammonia nitrogen), 0.01-0.02 mg/L (ppm) NO_2-N (nitrite), and 0.1-0.2 mg/L (ppm) NO_3-N (nitrate). Chen and Chin (1988) found that 0.1 mg/L (ppm) nitrite or above can affect reproduction.

Nutrition of broodstock is another important aspect of shrimp maturation. Middleditch et al. (1980) showed that *P. vannamei*

grown in captivity reached sexual maturity when fed diets similar in fatty acid profiles to that of marine bloodworms. Bloodworms have a high n-3 and n-6 PUFA ratio and this variable is thought by some to be the key factor necessary for a maturation diet. Lytle and Lytle (1989) looked at the fatty acid composition and variations in individual bloodworms. Dechan and Chen (1975) described the process required for the culture of a similar Lugworm species, which could be modified for bloodworms. Results indicate that squid, oysters and a diet supplement made from *Artemia* called Marilla appear to best match the fatty acid profile of bloodworms. A large hatchery in Panama reported that Marilla, when added to the maturation diet, saved approximately US$27 000/yr by increasing the frequency of females spawning, the total number of viable eggs spawned and the survival rate of the nauplii produced. Magarelli (1981) also found sex-specific nutritional requirements for crude protein and fat in cultured *P. stylirostris* broodstock. He found that female shrimp required a higher protein level, a lower fat level, a higher protein/calorie ratio and a much higher protein/fat ratio than males. Again, a combination diet is most often used so that all of the essential requirements for both males and females are met.

It has also been demonstrated that marine polychaetes can be replaced successfully with *Artemia* biomass for shrimp maturation and reproduction and that the culture of *Artemia* biomass can be done under intensive or extensive conditions (Naessens et al. 1997). Roeland Wouters of CENAIM/ESPOL in Ecuador and researchers at the Laboratory of Aquaculture and *Artemia* Reference Center at the University of Gent, Belgium, have replaced polychaetes with *Artemia*, and CENAIM reports to have successfully replaced both polychaetes and *Artemia* with an experimental artificial diet.

Shrimp Biosecurity

Biosecurity measures are a must now that serious diseases such as the White Spot Syndrome Virus (WSSV) and others have plagued the industry since the early 1990s. The serious reader should obtain the "Proceedings of the U.S. Marine Shrimp Farming Biosecurity Workshop (February 1998)" edited by Shaun Moss, Shrimp Program Manager with The Oceanic Institute US Marine Shrimp Farming Program (or see www.oceanicinstitute.org). The proceedings provide a good overview of the US Marine Shrimp Farming Program biosecurity strategy and an interesting glimpse at some private sector shrimp farms in the United States.

Biosecurity Measures and Suggested Criteria For Countries Importing Live Shrimp

The following are suggested for maintaining biosecurity within a country or area and suggested criteria for importing live shrimp. The criteria should have a sunset of 1 yr and should be reviewed and modified, if necessary, yearly.

All imports should be restricted to closed cycle hatcheries with at least 2 yr experience with this larval production method.

Hatcheries should have at least a 2 yr performance and health record of producing shrimp (broodstock, nauplii, and postlarvae) preferably with past imports to the country, which could verify in a practical way the health status of the larval production centers. Hatcheries should be clear of Taura Syndrome Virus (TSV) and WSSV for the past 6 mo.

Selected hatcheries should be asked as early as possible to perform a general health certification using polymerase chain reaction (PCR) assay for WSSV, and *in situ* hybridization for TSV in a diagnostic lab, for their broodstock tanks or ponds and maturation tanks in production. A reputable diagnostic lab should do such certification once a month before the season begins, and during stocking season postlarvae produced should be spot-tested for WSSV and TSV using 6-day-old PL to 30-day-old PL samples. Hatcheries should practice the amplification method on brood (if brood die, then body parts are fed to other brood to amplify the effects).

If needed selected hatcheries should be visited at least two mo prior to shipments into the

country, by designated representatives of the industry and regulatory authority to collaborate biosecurity procedures and general health status.

Sampling methods in the broodstock farms, maturation and hatchery tanks, for viral diagnostics, should be standardized and conducted by a reputable diagnostic laboratory.

The country of Australia has published on the Internet a report detailing the country's animal quarantine policy, a description of the import risk analysis process for shrimp, and categorization of shrimp disease agents (http://www.aqis.gov.au/docs/anpolicy/98-086b.doc).

Mating of Open-Thelycum Shrimp

The mating of open-thelycum shrimp was discussed by Aquacop (1977b), Primavera (1979) and De Saint-Brisson (1985). Many other authors have deduced the presence of sex pheromones in decapod crustaceans (see Dunham 1978).

Mating of Closed-Thelycum Shrimp

Mating of closed-thelycum penaeids was described by Hudinaga (1942), Primavera (1979, 1985), Yano (1987) among others. Spawning lasts from 2 to 7 min.

Maturation Research On Hormonal Control

Researchers in the 1980s were able to isolate and characterize hormonal systems involved in maturation/reproduction of the spiny lobster (Quackenbush and Herrnkind 1983). Researchers later undertook similar work with penaeid shrimp (Chan et al. 1988; Bradfield et al. 1989). The pink shrimp *P. duorarum* was the first penaeid to be researched and researchers at Texas A&M University looked into the elimination of some of the husbandry problems associated with maturation (egg fertility, decreased spawning rate over time), but progress has been slow.

Panouse (1943) was the first to recognize that removal of the X organ/sinus gland complex by eyestalk ablation often results in premature or nonseasonal gonadal hypertrophy. This effect has been attributed to removal of gonad inhibiting hormone (GIH), which is neither sex- nor species-specific (Otsu 1963; Bomirski et al. 1981). Research on other aspects of hormonal control over maturation has been done by: Bliss (1966); Kamemoto et al. (1966); Adiyodi and Adiyodi (1970); Fingerman (1970); Silverthorn (1975); Van Herp et al. (1977); Bollenbacher et al. (1978); Chang and O'Conner (1978); Highnam (1978); Kleinholz (1978); Andrew and Saleuddin (1979); Kulakovskii and Baturin (1979); Emmerson (1980); Bellon-Humbert et al. (1981); Faure et al. (1981); Adiyodi and Subramoniam (1983); Quackenbush and Herrnkind (1983); Quackenbush and Keeley (1986); Laufer et al. (1986, 1987) and Bradfield et al. (1989).

According to Caillouet (1972), Aquacop (1975), and Duronslet et al. (1975), ova in female shrimp are typically reabsorbed without subsequent spawning. These problems were alleviated by the ablation of only one eyestalk (unilateral eyestalk ablation), which provided moderate hormonal stimulus without reabsorption of ova or excessive mortality (Arnstein and Beard 1975; Wear and Santiago 1977). Consequently, unilateral eyestalk ablation rapidly emerged worldwide as a simple procedure for inducing reproduction of numerous species of penaeid shrimp reared in captivity. Some researchers have even used ablation to improve growth rate of shrimp (Hameed and Dwivedi 1977).

Eyestalk ablation has been performed using a variety of methods described by Duronslet et al. (1975) and Primavera (1978,1985) and is summarized by Fox and Treece (2000). Shrimp that are ablated as they prepare to enter their reproductive peak are more conditioned to yield a reproductive (as opposed to molting) response than those entering a reproductively dormant period (Bliss 1966). Within a molt cycle, ablation performed during premolt leads to molting; ablation immediately after molting causes death; and ablation during intermolt leads to maturation (Adiyodi and Adiyodi 1970).

The fecundity and viability of spawns from ablated females have sometimes been inferior to spawns from females matured in the wild (Adiyodi and Adiyodi 1970; Beard and Wickins 1980; Emmerson 1980; Lumar 1981). Other important works dealing with crustacean hormonal control, ablation and crustacean

reproduction can be found in the following references: Otsu (1963); Fingerman (1970); Silverthorn (1975); Laubier-Bonichon and Laubier (1976); Santiago (1977); Van Herp et al. (1977); Dunham (1978); Kleinholz (1978); Andrew and Saleuddin (1979); Aquacop (1979); Kulakovskii and Baturin (1979); Primavera et al. (1980); Bellon-Humbert et al. (1981); Bomirski et al. (1981); Faure et al. (1981); Adiyodi and Subramoniam (1983); and Bray and Lawrence (1992).

Ablation of Male Penaeid Shrimp

Male ablation causes precocious maturation of *P. monodon* and *P. merguiensis* (Alikunhi et al. 1975); however, it has also been shown to increase gonad size and double mating frequency of smaller (25-30 g) *P. vannamei* in comparison to similar-size, unablated control shrimp (Chamberlain and Lawrence 1981). Eyestalk ablation of male shrimp has rarely been considered useful and the author does not recommend its use under practical culture conditions.

Further information on ablation and shrimp reproductive physiology can be found in Treece and Yates ([1990] 1993), Treece and Fox (1993) and Fox and Treece (2000).

Broodstock Diseases

Consult Brock and Main (1995) if working with *P. vannamei* and Alday de Graindorge and Flegel (1999) if working with *P. monodon*. An Internet web site where shrimp diseases are discussed is http://www.aqis.gov.au, and a 50+ page document can be downloaded at http://www.aqis.gov.au/docs/anpolicy/98-086b.doc.

High health broodstock are available from commercial hatcheries. One example is at Internet web site http://www.hihealthshrimp.com.

According to Itami et al. (1998), establishment of disease-resistant shrimp strains is one possible solution to the disease problems now threatening the world shrimp industry. Because artificial breeding techniques of kuruma shrimp have not been sufficiently developed, this will be an important area for future research. Recent developments in molecular biology such as identification of cDNA markers and microsatellite markers for growth performance and viral disease resistance, may lead to a higher rate of genetic improvement in shrimp (Acacia et al. 1997).

Other Research on Maturation and Spawning

A few researchers and few commercial hatcheries in Venezuela, Ecuador, Australia and Mexico have been able to select families in a breeding program and obtain egg development, mating and spawning of captive penaeids without ablation. Temperature and photoperiod manipulation alone have not produced sustainable commercial operations without ablation. As a rule, hatcheries have not been able to base a long-lasting, profitable, highly-productive commercial operation without ablation. The nonablation approach is the desired route for hatcheries in the future, but presently only makes up a very small percentage. Some of the research toward this end was discussed by Benzie (1997) and Browdy (1998). Most of the research deals with endocrinology, particularly the research aiming at isolating and identifying substances that promote maturation.

A shrimp maturation unit or tank is described in detail by Treece and Fox (1993) and the procedures for operation are covered in that publication.

Research on broodstock nutrition often focuses on developing diets that enhance maturation (Harrison 1990, 1997). From the literature, personal experience, and discussions with maturation managers, it appears to be much easier to mature domesticated shrimp than wild shrimp. Progress has been made toward domesticating shrimp or pond-raised stock and a growing number of hatcheries are doing this without eyestalk ablation. Mendoza et al. (1997) discussed the influence of squid extracts on triggering of maturation.

United States: Texas A&M University has just completed a maturation facility study dealing with closed filter types and concluded that the column filter developed by John Ogle at the Gulf Coast Marine Research Laboratory in Ocean Springs, Mississippi, improved shrimp maturation

and increased the number of females spawning each night compared to the bead filters used and on the open market (Bray, personal communication).

Researchers at Texas A&M University have been working on isolating and characterizing the hormones in *P. vannamei* using monoclonal antibodies, and have synthesized and injected brood animals (Dr. Larry Keeley personal communication). The commercial hatchery in Texas is not interested in the injected form because shrimp do not handle the stress of injection well. A few years before, Dr. Scott Quackenbush (one of Dr. Keeley's colleagues) isolated and characterized the hormones in the pink shrimp *P. duorarum* after leaving TAMU to join a Florida University.

Ecuador: CENAIM in Ecuador is presently conducting a study entitled "Improvement of reproduction and egg quality of penaeid shrimp by induction of ovary maturation by neuropeptides and by diet" (http://www.cenaim.espol.edu.ec). This project contains two parts: (1) endocrinology, coordinated by Julie Nieto (julianieto@latinmail.com) who is also doing some research at the Catholic University of Leuven (Belgium), and (2) nutrition. In the endocrinology study, the identification and purification of 30 novel pure peptides from shrimp central nervous system is the major outcome of the project thus far. However, it is important to stress that these peptides were not purified based on a maturation assay due to the difficulty encountered in establishing a reliable assay. Nevertheless, these novel peptides are present in the nervous system during the maturation process and therefore may be directly or indirectly related to such physiological processes. Purified peptides need to be characterized, determining their physiological pathway of action and their kinetics during the maturation process. The major limitation encountered during this project was the establishment of a homologous bioassay which proved a role of the peptide in maturation. The bioassay is the key to the success in the purification of peptides. *In-vivo* experiments had no positive results since *P. vannamei* are sensitive to manipulation. Working with *in-vitro* assays was the second option used and is presently underway.

Australia: Dr. Michael Hall at the Australian Institute of Marine Science (AIMS) has been working on replacement of eyestalk ablation in *P. monodon* for 5 yr (m.hall@aims.gov.au).

Mexico: Eduardo Figueras, Hatchery Director, Industrias Pecis, Merida, Yucatán, (http://www.pecis.com; EFigueras@pecis.com) has been conducting research on the domestication of *P. vannamei* broodstock and found that maturation improves with each generation. Some organisms were kept unablated and their offspring normally spawned without ablation. He reports that some hatcheries in Mexico are seeing excellent results with unablated females, after their third domesticated generation. At present, their hatchery had a second generation of broodstock and almost 30% of their females were spawning without ablation. The interesting thing about this is that the average number of females spawning has improved from 5% daily to 12% and the spawns are about the same size as the ablated controls. They have also found that an unablated female can produce high quality nauplii through almost 200 d in compared to 120 d for the ablated females.

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TEMPERATURE TOLERANCE AND OXYGEN CONSUMPTION RATES FOR JUVENILE SOUTHERN FLOUNDER *PARALICHTHYS LETHOSTIGMA* ACCLIMATED TO FIVE DIFFERENT TEMPERATURES

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ABSTRACT

Critical thermal maximum (CTM), lethal thermal tolerance (LT), and oxygen consumption rates (μg O_2/g fish/min) of juvenile southern flounder (average weight 5.5 g) were determined for acclimation temperatures (AT) of 13, 17, 21, 25, and 29 C. A total of 75 fish were used to measure CTM and LT in salinities of 0, 12, and 34 ppt. Three replicates of two fish each were used to measure oxygen consumption rates in 34 ppt. Salinity had a significant effect on both CTM and LT ($P<0.05$). The mean CTM for 0 ppt was 0.46 C lower than the mean CTM for 12 ppt and 0.84 C lower than the mean for 34 ppt. The mean LT for 0 ppt was 0.40 C lower than the mean LT for 12 ppt and 0.61 C lower than the mean for 34 ppt. The LT was 20.39 C higher than acclimation temperature at 13 C but only 9.85 C higher at 29 C. The highest LT was 38.85 C for fish acclimated to 29 C. The oxygen consumption rate increased from 1.26 to 4.53 μg O_2/g fish/min as temperature increased from 13 to 29 C. The highest Q_{10} values, for oxygen consumption, occurred between 21 C and 25 C. Between 13 C and 17 C the Q_{10} was 2.37, between 17 C and 21 C the Q_{10} was 2.50, between 21 C and 25 C the Q_{10} was 2.68, and between 25 C and 29 C the Q_{10} was 1.29. Based on the relationship between LT and preferred temperature, and the observed decline in Q_{10} for oxygen consumption, we calculated that the final preferred temperature (FP) for juvenile southern flounder is between 25 C and 29 C in salinities from 0 to 34 ppt.

INTRODUCTION

The southern flounder *Paralichthys lethostigma* has potential as a species for aquaculture (Waters 1999). Little is known about the effect of acclimation temperature on thermal tolerance and oxygen consumption, and knowledge of the thermal tolerance for juvenile southern flounder will aid in grow-out facility site selection. Likewise, oxygen consumption data will help in the development of oxygen management strategies.

Southern flounder grow well in fresh and salt water (Lasswell et al. 1977; Daniels et al. 1996). This euryhaline ability has generated interest in their potential for aquaculture. Several studies have been done to develop captive spawning techniques (Arnold et al. 1977; Henderson-Arzapalo et al. 1988; Berlinsky et al. 1996) and larviculture methods (Daniels et al. 1996; Denson and Smith 1997; Jenkins and Smith 1997; Smith et al. 1999). However, little information is available on the environmental requirements for grow-out.

The natural geographic range of the southern flounder in the wild extends from the Albemarle Sound of North Carolina to Jupiter Inlet, Florida, USA, on the Atlantic Coast and from Caloosahatchee Estuary Florida, USA, to northern Mexico in the Gulf of Mexico (Ross 1980). Adult southern flounder can be found in coastal water systems in salinities ranging from full-strength sea water to fresh water. During the months of December through January they migrate to the open ocean to spawn (Smith et al. 1975). The pelagic eggs hatch at sea and the developing larvae typically reenter the estuaries

just as they are completing metamorphosis (Burke et al. 1991).

Preferred temperature is commonly determined through the use of either a vertically or horizontally arranged temperature gradient tank (Tsuchida and Fukataki 1991). These test tanks, however, are inadequate for flounder as this species has a strong natural desire to stay hidden by not moving rather than to seek out a preferred temperature within a tank (Jun Kita personal communication, Marine Ecology Research Institute, Chiba, Japan). Recent studies by Tsuchida (1995) and Kita et al. (1996) have reported an indirect method of determining the preferred temperature of fish. Tsuchida (1995) reported a linear relationship between final preferred temperature and lethal tolerance. The final preferred temperatures of 14 marine fish species were plotted against their lethal temperatures yielding a linear relationship with an r^2 of 0.981 (LT = 0.741FP + 17.549). This relationship can be used to calculate the final preferred temperature of other species, given the relationship between lethal temperature and acclimation temperature (LT = aAT + b). This calculation is possible because the final preferred temperature is defined as the temperature where preferred temperature equals acclimation temperature. Thus, by replacing acclimation temperature with final preferred temperature, the formulas for the two equations can be combined leaving the final preferred temperature to be solved (aFP + b = 0.741FP + 17.549).

The final preferred temperature coincides with optimum temperature for growth (Brett 1971; Kellogg and Gift 1983). The point where the Q_{10} for oxygen consumption starts to decrease with increasing acclimation temperature also corresponds to the optimal temperature for growth (Kita et al. 1996). Thus, the final preferred temperature may be determined indirectly, based on the relationship between oxygen consumption and acclimation temperature.

The purposes of this study were 1) to determine how acclimation temperature affects thermal tolerance and oxygen consumption rates and 2) to indirectly determine the final preferred temperature for juvenile southern flounder.

MATERIALS AND METHODS

Hatchery-raised juvenile southern flounder from the Tidewater Research Station (TRS) hatchery in Plymouth, North Carolina, USA, weighing 5.5 g ± 1.9 g, were shipped to the Marine Ecology Research Institute in Onjuku-machi, Chiba, Japan on 9 June 1999. Two wk prior to the experiments, juveniles were acclimated to the five test temperatures (13, 17, 21, 25 and 29 C) and fed frozen krill and moist pellets.

Thermal Tolerance

Fish at each temperature were given 3 d to acclimate to salinities of 0, 12, and 34 ppt. Fifteen thermal tolerance tests were run, one for each temperature and salinity combination. A total of 75 fish were transferred to the experimental tanks 1 h prior to the initiation of the tests. The experimental tanks were closed systems controlled by a ceramic heater and cooling device (Matsushita Electric NU-301 AHD) and a programmable thermostat (Shimaden FP21). From the acclimation temperatures, the test water was heated in increments of 5 C/h. The critical thermal maximum (CTM), where the fish lost their equilibrium, and lethal temperature (LT), where opercular movement ceased, were recorded as described by Tsuchida (1995). After the experiments, water samples were taken to confirm salinities via an inductively coupled salinometer (601 MK-III Yo-KAL Environmental Electronics).

Oxygen Consumption

Five oxygen consumption tests were run, one for each acclimation temperature at 34 ppt salinity. A total of 10 fish were transferred to the respirometry chamber (Fig. 1) approximately 20 h prior to the start of the experiment. The respirometry chamber was sunk in a water bath maintained at the desired acclimation temperature (± 0.2 C) by a water heater and chiller (AQUA C101A-5). The flow into the respirometry chamber was cut off and the dissolved oxygen concentration was measured every 20 sec with an oxygen probe (TOA DO-25A with OE-211 oxygen electrode TOA Electronics Ltd.). Measurements continued until the oxygen

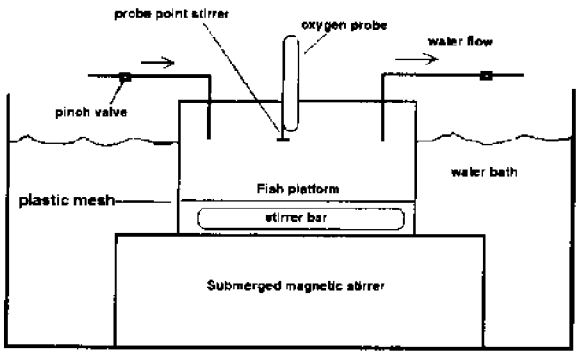

Figure 1. Respirometry Chamber used to measure oxygen consumption of southern flounder *Paralichthys lethostigma*.

concentration decreased from 100% to 80% saturation over a period ranging from 2-4 h. Oxygen consumption rate (μg O_2/g fish/min) was calculated according to Kita et al. (1996). From the oxygen consumption rates, Q_{10} was calculated as:

$$Q_{10} = (\text{Rate 1}/\text{Rate 2})^{(10/(\text{Temp 2}-\text{Temp 1}))}$$

where,

Rate 1 = the oxygen consumption rate at temperature 1

Rate 2 = the oxygen consumption rate at temperature 2

Temp 1 = the lower of the two temperatures used to determine oxygen consumption

Temp 2 = the higher of the two temperatures used to determine oxygen consumption

RESULTS

Temperature Tolerance

The salinity varied as follows: 0.20 ± 0.15, 12.10 ± 3.43 and 34.24 ± 0.09 ppt. Salinity had a significant ($P<0.05$) effect on both CTM and LT. The mean CTM for 0 ppt was 0.46 C lower than the mean CTM for 12 ppt and 0.84 C lower than the mean for 34 ppt. The mean LT for 0 ppt was 0.40 C lower than the mean LT for 12 ppt and 0.61 C lower than the mean for 34 ppt. The difference between the CTM at 0 ppt and the CTM at 34 ppt was greatest at 13 C. A similar trend was found for LT (Table 1). The mean LT was 20.39 C higher than acclimation temperature at 13 C but only 9.85 C higher at 29 C (Fig. 2). A similar trend was found for CTM (Fig. 3). The highest mean LT was 38.85 C for fish acclimated to 29 C.

Although salinity had a statistically significant effect on LT, the magnitude of this effect is on average less than 0.5 C. Thus, a single function across all salinities was developed to calculate the final preferred temperature (LT = 0.336AT + 29.54). Using the function for this line,

Table 1. Effect of salinity on critical thermal maximum (CTM) and lethal temperature (LT) at five different acclimation temperatures for juvenile southern flounder *Paralichthys lethostigma*. Values are means (± sd) for five fish. Means followed by a different letter are statistically different ($P<0.05$).

	Critical thermal maximum Temperature (C)				
Salinity (ppt)	13	17	21	25	29
0	32.56 ± 0.36a	34.78 ± 0.08a	36.36 ± 0.26a	37.18 ± 0.13a	38.04 ± 0.18a
12	32.92 ± 0.08a	35.36 ± 0.09b	36.72 ± 0.18b	37.66 ± 0.21b	38.50 ± 0.25b
34	33.74 ± 0.75b	36.00 ± 0.12c	37.08 ± 0.8c	37.78 ± 0.05b	38.58 ± 0.00b
	Lethal temperature Temperature (C)				
Salinity (ppt)	13	17	21	25	29
0	32.88 ± 0.57a	34.98 ± 0.13a	36.80 ± 0.14a	37.98 ± 0.05a	38.70 ± 0.14a
12	33.14 ± 0.05a	35.84 ± 0.26b	37.16 ± 0.08b	38.12 ± 0.05b	38.94 ± 0.06b
34	34.08 ± 0.80b	36.10 ± 0.00c	37.22 ± 0.14b	38.18 ± 0.08b	38.96 ± 0.06b

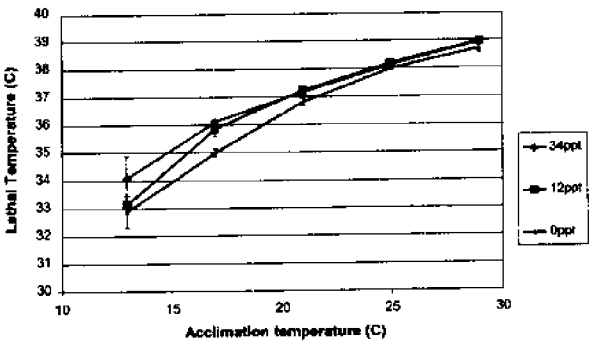

Figure 2. Lethal temperature vs. acclimation temperature for juvenile southern flounder *Paralichthys lethostigma* at three different salinities.

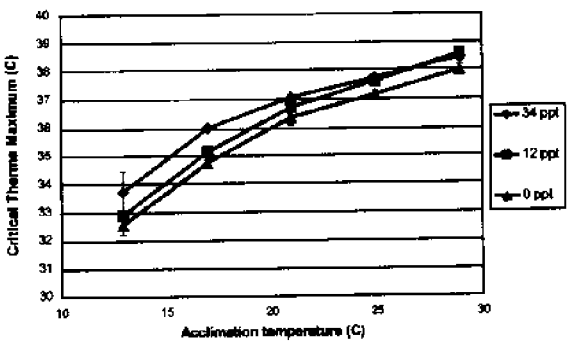

Figure 3. Critical thermal maximum vs. acclimation temperature for juvenile southern flounder *Paralichthys lethostigma* at three different salinities.

the calculated final preferred temperature is 29.61 C. This corresponds to the point where the line for lethal tolerance vs. acclimation temperature for southern flounder crosses the line for lethal temperature vs. final preferred temperature for 14 marine species (Tsuchida 1995; Fig. 4).

Oxygen Consumption

The oxygen consumption rate increased with increasing temperature and ranged from 1.26

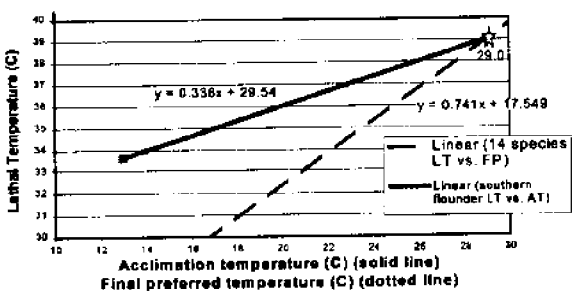

Figure 4. Lethal temperature vs. acclimation temperature for southern flounder *Paralichthys lethostigma* and lethal temperature vs. final preferred temperature as described by Tsuchida (1995).

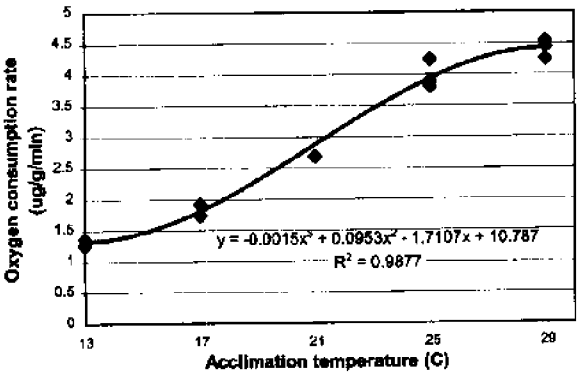

Figure 5. Juvenile southern flounder Paralichthys lethostigma oxygen consumption rate (µg/g/min) at five different temperatures.

to 4.53 µg O_2/g fish/min (Fig. 5). The highest Q_{10} values occurred between 21 C and 25 C. Between 13 C and 17 C the Q_{10} was 2.37, between 17 C and 21 C the Q_{10} was 2.50, between 21 C and 25 C the Q_{10} was 2.68, and between 25 C and 29 C the Q_{10} was 1.29. Using the point where a drop in the Q_{10} becomes apparent (Kita et al. 1996), these results suggest that the final preferred temperature of juvenile southern flounder is between 25 and 29 C.

DISCUSSION

Although salinity has a significant effect on the thermal tolerance of juvenile southern flounder, the magnitude of this effect is so small (<0.5 C) that it is of little practical significance to culturists. Furthermore, salinity has no effect on juvenile southern flounder growth and survival (Daniels and Borski 1998). Therefore, southern flounder can be considered to thrive in a wide range of salinity. This provides the following advantages to culturists: 1) flexibility in water source selection and 2) the ability to use varying salinities to combat pathogens.

Compared to the 14 species examined by Tsuchida (1995) the southern flounder has a relatively high thermal tolerance, similar to the sea bass *Lateolabrax japonicus* and the black sea bream *Acantopagnus schlegeli*. This high tolerance is necessary for outside culture in eastern North Carolina as water temperatures can reach the low to middle 30 C range during the summer months.

Oxygen consumption rates were found to be temperature dependent for juvenile southern flounder. However, oxygen consumption rate varies with fish weight. Burke (1998) has determined the effect of weight on southern flounder oxygen consumption. By combining the relationship between temperature, weight and oxygen consumption, culturists will be able to predict the oxygen needs of their stock and thus manage oxygen input accordingly.

The preferred temperature for juvenile southern flounder, weighing 5.5 g ± 1.9 g, as calculated by the combination of the thermal tolerance tests and the oxygen consumption rate tests, falls within the range of 25-29 C. Davis (1998) found that the optimal temperature for growth of juvenile southern flounder was 25 C. This correlation, between final preferred temperature and optimum temperature, supports previous studies that show that fish prefer to live in the temperature where growth is optimized (Brett 1971; Kellogg and Gift 1983).

This study was completed with southern flounder raised from eggs collected from a single female. Additional replications of this work are needed to account for possible variations in the thermal tolerance and oxygen consumption rates that may exist within the North Carolina population and between the different strains that occur over the entire natural range.

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RECENT PROGRESS IN CONTROLLED REPRODUCTION OF SOUTHERN FLOUNDER *PARALICHTHYS LETHOSTIGMA*

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ABSTRACT

Hormone-induced spawning of southern flounder *Paralichthys lethostigma* has produced substantial numbers of viable eggs, but wide variations in percent fertilization and percent hatching have been reported. Recently, sustained natural spawning of southern flounder broodstock, without hormone induction, has been achieved at the Center for Marine Science, University of North Carolina at Wilmington (UNCW), USA. Adults (avg. wt. = 1.12 kg; n = 25) were stocked in two 4.8-m^3 controlled-environment tanks in October 1998 and held under natural photothermal conditions until January 1999, when an artificial winter photoperiod of 10 L: 14 D was maintained through April 1999. Natural spawning was observed in early December 1998 and increased in frequency to a peak in March 1999, before declining in late April. Water temperature ranged from 13.9 to 24.5 C during the spawning period. Natural spawns over 142 d produced a total of 18.3 x 10^6 eggs, with an average percent fertilization of 28.0% (range = 0-100%), yielding 4.94 x 10^6 viable eggs. The percentage of eggs that remained buoyant in full-strength sea water averaged 41.3% (0-99%), while the percent hatching of buoyant eggs averaged 37.3% (0-100%) and survival of yolksac larvae to the first-feeding stage averaged 30.2% (0-100%). A preliminary comparison suggests that natural spawning may produce higher egg quality than hormone-induced spawning in terms of fertilization and hatching success. These results suggest that natural and hormone-induced spawning of photothermally conditioned fish will help produce the large numbers of eggs required to support commercial production. Additional research is needed to address the problem of variable egg quality.

INTRODUCTION

The southern flounder *Paralichthys lethostigma* is a high-value recreational and commercially harvested flatfish found in estuarine and shelf waters of the Atlantic and Gulf coasts from North Carolina, USA, to Mexico (Gilbert 1986). With the implementation of fishery quotas for the summer flounder in the early 1990s, landings of southern flounder have increased. Today, the southern flounder is the number one flatfish species landed in North Carolina (Copeland et al. 1999).

Interest in the southern flounder as an aquaculture candidate in the southeastern United States is related to the wide range of temperature and salinity tolerance of this species; 50-d-old juveniles can tolerate salinities as low as five ppt, while older juveniles can tolerate fresh water (Smith et al. 1999a). This suggests that this species could potentially be cultivated in inland fresh and brackishwater ponds as well as in coastal areas (Berlinsky et al.1996; Daniels et al.1996; Jenkins and Smith 1999; Smith et al.1999a). At present, reliable methods for controlled breeding and production of high quality eggs are needed to accelerate hatchery research and development of commercial grow-out systems.

In Japan, commercial farmers rely mainly on photothermal conditioning and natural spawning of Japanese flounder *P. olivaceus* broodstock to supply the large numbers of high quality eggs needed to support commercial hatchery operations (Ijima et al. 1986; Min 1988; Tsujigado et al. 1989). In the United States, natural spawns of the southern flounder have been rare, and researchers have therefore focused on hormone-induced spawning. Intramuscular implantation of a cholesterol-cellulose pellet containing gonadotropin releasing hormone-analogue (GnRH-a) has produced repetitive spawning and substantial numbers of viable eggs

in Southern flounder, but wide variations in percent fertilization and percent hatching have been reported (Berlinsky et al. 1996).

Natural spawning without hormone induction of captive southern flounder broodstock was recently achieved in our laboratory. The objectives of this paper are to describe the environmental and culture conditions and the reproductive performance associated with natural spawning. A preliminary comparison of natural spawning and hormone-induced spawning is also made.

MATERIALS AND METHODS

Natural Spawns

The southern flounder broodfish used in this study originated from two sources. One group (laboratory-reared) originated from fish collected as juveniles at 100-105 mm SL in the summer of 1993 near Beaufort, North Carolina, USA. A second group (wild-caught) originated as adults captured by commercial fishermen during September 1998 in Pamlico Sound, North Carolina, USA. Wild-caught fish were held in 20-m diameter outdoor concrete tanks supplied with flow-through brackish water (12-20 g/L salinity) for 6 wk before transport to the Center for Marine Science, University of North Carolina at Wilmington (UNCW), where this study took place between December 1998 and April 1999. Fish were individually tagged and held for 3 wk in flow-through sea water (34 ppt) tanks under ambient conditions before stocking into a controlled-environment broodfish system.

The controlled-environment broodfish system consisted of two circular fiberglass tanks (diam. = 2.46 m; depth = 1 m; vol. = 4.76 m^3). Situated out of doors, the broodfish tanks were insulated and provided with a conical fiberglass cover fitted with a timer-controlled, fluorescent fixture, containing two 20-W daylight bulbs. Average light intensity at the water surface was 234 lux.

Both tanks were supported by a water-recirculating system, consisting of a high-rate sandfilter, fluidized bed biofilter, foam fractionater, and ultraviolet sterilizer. Water from each tank drained through an egg collector (diam. = 0.76 m; depth = 0.76 m; vol. = 0.24 m^3) before entering a reservoir tank (diam. = 1.54 m; depth = 1 m; vol. = 1.86 m^3), from which water was pumped to the biofilter system. Water flow to each tank was approximately 38 L/min and water was exchanged at a rate of approximately 10%/d. Immersion heaters placed in the reservoir tank controlled water temperature.

In October 1998, one broodfish tank (tank 1) was stocked with 13 fish (2.73/m^3) consisting of six laboratory-reared and seven wild-caught adults with an average weight of 0.948 kg (2.59 kg/m^3). At stocking, the sex ratio was 8 female: 3 males: 2 unknown. In November 1998, the second broodfish tank (tank 2) was stocked with twelve fish (2.52/m^3), consisting of only wild-caught adults, with an average weight of 1.28 kg (3.23 kg/m^3). The sex ratio was unknown at the time of stocking.

Gonadal maturity of individual brooders was assessed periodically by biopsy of anaesthetized (0.3 g/L 2-phenoxyethanol) fish, using a polyethylene cannula (1.57 mm o.d. x 1.14-mm i.d.) (Shehadeh et al. 1973). Ovarian samples were fixed in a solution of 10% formalin in sea water. General stage of oocyte development (i.e, pre-vitellogenic, cortical vesicle, vitellogenic and atretic) was determined from the microscopic appearance, and males were identified by the presence of milt when pressure was applied to the gonadal area.

Fish were fed to satiation once daily (approximately 0900), a diet that consisted primarily of Atlantic silversides *Menidia menidia* supplemented with squid, krill, and commercially-prepared diets containing 45% (INVE Aquaculture, Grantsville, Utah, USA) and 55% protein (Corey Feed Mills Ltd., New Brunswick, Canada) and 16% fat. The feeding rate of the broodstock averaged about 1% BW/d.

To obtain gonadal maturation and spawning in January, presumed to be the natural reproductive period of southern flounder in North Carolina waters (Berlinsky et al. 1996), fish were exposed to ambient light and temperature conditions until 9 January 1999, when timers were used to maintain a constant winter photoperiod of 10 L: 14 D, and a water temperature that did not fall below approximately 14.5 C (Fig. 1).

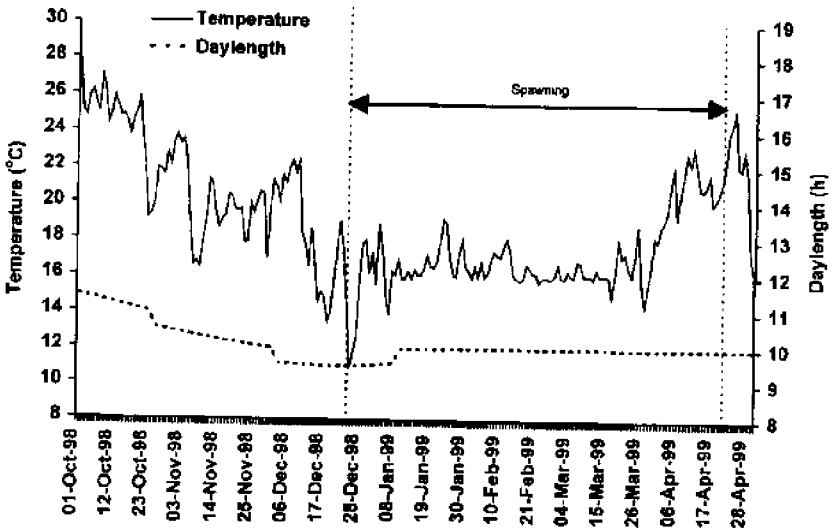

Figure 1. Photoperiod and temperature conditions during natural spawning of Southern flounder broodstock in 4.76 m^3 tanks.

Egg collectors were checked daily for spawned eggs. Once daily, eggs were siphoned from the collector, transferred to a separatory funnel in sea water (32-37 ppt), and buoyant eggs ("floaters") were separated from sinking eggs ("sinkers"). The numbers of eggs in each fraction were estimated using volumetric methods.

Floaters were transferred to 15-L airlift "in-tank" incubators placed inside the reservoir tank or situated in an indoor laboratory. In-tank incubators were stocked at a density of 1,000 eggs/L, while indoor incubators were stocked at densities of 300-600 eggs/L. Indoor incubators were provided with 1-µm-mesh filtered sea water (sterilized by ultraviolet light) at 16-19 C and supplied with diffused aeration. Using volumetric methods, survival of embryos were monitored through hatching (d 2 to d 3 after fertilization) and at the first-feeding stage (d 6 to d 7 after hatching), when 100% of the larvae possessed functional (fully pigmented) eyes, mouth, and alimentary tract.

Percent fertilization was determined as the percentage of viable embryos, while percent hatching was determined as the percentage of viable larvae hatched from fertilized eggs. Percent fertilization and percent hatching were expressed as percentages of total eggs and of buoyant eggs. Survival to the first-feeding stage was expressed as a percentage of total and of buoyant eggs.

Water Quality

Temperature, salinity, and dissolved oxygen were monitored daily, while pH, total ammonia-nitrogen, nitrite and nitrate were monitored weekly. Average daily values (and ranges) were as follows: salinity, 35.2 (32-37) g/L; dissolved oxygen, 7.64 (6.01-9.01) mg/L; pH 8.12(7.8-8.4); total ammonia-nitrogen, 0.029 (0-0.08) mg/L, nitrite-nitrogen, 0.022 (0.003-0.051) mg/L; nitrate-nitrogen, 2.75 (0.6-9.4) mg/L. Temperature, salinity, dissolved oxygen, and pH in the incubators were monitored once at the end of the incubation period. Average values (and ranges) were as follows: salinity, 35.5 (34-38) g/L; dissolved oxygen, 8.50 (7.59-9.27) mg/L; pH, 8.36 (8.2-8.5); temperature, 17.4 (16-18.6) C.

Hormone-induced Spawns

Hormone-induced spawning trials were conducted at the Tidewater Research Station (North Carolina State University) in Plymouth, North Carolina, USA. Adult Southern flounder (avg. wt. 1.2 kg) originated from the same source as the wild-caught brooders used for natural spawning trials at UNCW. Fish were stocked into tanks (diam. = 3.0 m; depth = 1.0 m; vol. = 7.4 m^3) supplied with recirculating sea water. Broodfish were exposed to artificial photoperiod and temperature conditions simulating ambient, reaching 9 h L:15 h D and 16 C by 15 December. Beginning in January 1999, females with

maximum oocyte diameters of 500 µm were selected for hormone-induced spawning. To induce spawning, females were implanted with a 95% cholesterol and 5% cellulose pellet (Sherwood et al. 1988) containing [D-Ala^6 Des-Gly^{10}] LHRH ethylamide (GnRH-a, Sigma Chemical Co., St. Louis, Missouri, USA) at a dose of 100 µg/kg (Berlinsky et al. 1996).

In some hormone-induced spawning trials, females were allowed to spawn volitionally in the tanks ("tank spawns"). In others trials, ovulated females were strip-spawned by applying gentle pressure to the abdomen. Eggs from a single female were collected in a glass beaker and mixed with the sperm from two males (Berlinsky et al. 1996), then left undisturbed in at least 100 ml of sea water for 1 h. The floating eggs were separated from the sinking eggs in a separatory funnel. Embryos were incubated in a 70-L round fiberglass tank containing 34 ppt filtered sea water at 16 C and at a maximum density of 500 eggs/L. Percent fertilization and percent hatching were determined as described above.

RESULTS

Natural Spawns

On 3-5 December 1998, fully hydrated ova were first observed in the egg collector from tank 1, consisting of both laboratory-reared and wild-caught adults. None of these individuals had been treated with hormones, indicating that natural spawning had occurred. Spawning increased in frequency to a peak in March 1999 before declining in mid-April. Fertilized eggs were first observed in tank 1 on 12 January 1999.

On 5 February 1999, fully hydrated ova were first observed in the egg collector from tank 2, consisting entirely of wild-caught adults. Natural spawning in tank 2 increased in frequency to a peak in late March and early April before declining in late April and fertilized eggs were first observed on 2 March 1999.

During a 142-d spawning period from 3 December 1998 to 23 April, eggs were collected on 70 days in tank 1 and on 53 days in tank 2 (Table 1). Numbers of eggs collected/d from each tank ranged from 5,490 to 601,250, averaging 159,596 in tank 1 and 133,104 in tank 2. For the duration of the 142-d spawning period, a total of 11,331,319 eggs were collected from tank 1 and 7,054,489 were collected from tank 2, for a total of 18,385,808 eggs from both tanks.

Egg buoyancy, Fertilization, and Hatching

Percent fertilization of naturally spawned eggs varied day to day from 0 to 97.2%, averaging 30.6% in tank 1 and 24.9% in tank 2, with an overall average of 28.0% for both tanks (Table 1). A total of 3,470,516 fertilized eggs were produced in tank 1, while 1,472,577 were

Table 1. Summarized data on natural spawning of southern flounder broodstock in two 4.76-m3 tanks (3 December 1998 to 23 April 2000). Each tank was stocked with 12-13 adults. Data are presented for each tank and for both tanks combined.

Tank No.	No. of days eggs observed	Total eggs spawned (No. spawned per day)	No. of floaters (No. per day)	Floaters (%) (range)	Fertilization rate (% overall) (range)	Fertilization rate ((% of floaters) (range)	No. of fertilized eggs (range)	Hatching rate (% of floaters) (range)
1	70	11,331,319 (5,490-601,250)	5,737,902 (0-412,750)	46.6 (0-97.5)	30.6 (0-99.0)	50.2 (0-100)	3,470,516 (0-177,300)	41.9 (0-87.1)
2	53	7,054,489 (22,750-419,000)	2,199,867 (0-244,000)	34.2 (0-96.5)	24.9 (0-95.2)	50.6 (0-100)	1,472,577 (0-233,996)	31.9 (0-99.1)
1 + 2	123	18,385,808	7,937,769	41.3	28.0	50.4	4,943,093	37.3

Estimated as percent hatching (% overall) = floaters (%) x percent hatching (% of floaters).
Estimated as survival to first-feeding (% overall) = floaters (%) x survival to first-feeding (% of floaters).

produced in tank 2, for a total of 4,943,092 for both tanks combined. On days that spawning was observed, an average of 34,811 fertilized eggs were collected from each tank.

During incubation of eggs in full-strength sea water (32-36 g/L), the percentage of eggs that remained buoyant (i.e.,"floaters") varied among spawns from 0 to 99.2%, with an average of 41.3% for both tanks (Table 1). For both tanks 1 and 2, percent hatching averaged 37.3% of floaters (15.4% overall), while survival of yolk-sac larvae to the first-feeding stage averaged 30.2% of floaters (12.5% overall).

Thermal Regime

From 5 January to 23 April 1999, the period during which the majority of spawns occurred, water temperatures varied over a wide range of 13.9 C to 24.5 C (Fig. 1). Fertilized eggs were also obtained under this temperature range, although availability of fertilized eggs increased to a peak during March and early April, while water temperature averaged around 17.5 C. The number of eggs spawned decreased as water temperatures increased steadily in April. Spawning appeared to be stimulated by a change in weather conditions; a warming or cooling trend was followed by an increase in egg release.

Growth and Sex Ratios of Broodstock

A gonadal examination of all broodstock made on 11 January 1999 revealed the following sex ratios: 8 females: 3 males: 2 unknown in tank 1 and 5 females: 2 males: 5 unknown in tank 2. A gonadal examination of all broodstock made on 27 April 1999 revealed four individuals in tank 1 and two individuals in tank 2 with atretic, hydrated eggs, indicating probable spawners. Assuming that these six individuals participated in spawning, an average of 3,064,301 eggs was released per female during the study. Two individuals in tank 1 and one individual in tank 2 from which ovarian tissue was sampled were also observed to emit a milky fluid from the urogenital opening on the dorsal (ocular) side when pressure was applied to the abdominal region. Microscopic examination of this fluid revealed highly motile 1-2 μm particles, presumably spermatozoa, and indicating hermaphroditic individuals.

Hormone-induced Spawns

In 1999, a total of 31 hormone-induced spawning trials were conducted, producing a total of 1,101,000 eggs of which 62% were floaters (Table 2). Of the floaters, 19% were fertilized (12% overall). The percent hatching of floaters was 16.5% (9.9% overall).

Of the 31 hormone-induced spawning trials, 14 tank spawns yielded 345,000 eggs, of which 55% were floaters, but no fertilization was obtained under this method (Table 2). Seventeen strip-spawning trials yielded 756,000 eggs, of which 68% were floaters, with 40% of these floaters being fertilized (27% overall). Percent hatching of the floaters averaged 30% (20.4% overall).

DISCUSSION

Natural Spawning

Through sustained, natural spawning of captive southern flounder broodstock, commercially significant quantities of viable embryos were produced in this study. Previous attempts to obtain natural spawning of this species met with limited success. Using photoperiods and

Table 2. Summarized data on hormone-induced spawns of southern flounder (*Paralichthys lethostigma*) at the Tidewater Research Station (North Carolina State University) in 1999, including eggs collected from both tank and strip spawns. Average percent hatching (range) was determined within 24 h of first observed hatch for floating eggs only.

Method of spawning	Batches	Total eggs spawned	Floaters	Floaters (%)	Fertilization rate (% overall)	Fertilization rate (% of floaters)	No. of fertilized eggs	Hatching rate (% of floaters)	Hatching rate (% overall)
Tank	14	345,000	190,000	55	0	0	0	0	0
Strip	17	756,000	514,000	68	27	40	206,000	30	20.4
Total	31	1,101,000	704,000	62	12	19	134,000	16.5	9.9

temperatures which simulated natural seasonal changes, Arnold et al. (1977) obtained natural spawning from 3 of 6 females for 13 consecutive days in 30-m^3 tanks producing 120,000 eggs with a percent fertilization of 30-50% and a percent hatching of 6-35% of the fertilized eggs. Controlled photoperiod and temperature were also effective in stimulating release of 200,000 eggs from 5 females over 2 spawning seasons, but no fertilization was obtained (Henderson-Arzapalo et al. 1988).

The results of this study demonstrate that wild-caught southern flounder adults, conditioned through photothermal manipulation for only 8 wk, can be spawned successfully without hormone induction during their first season in captivity. This is important to avoid a prolonged period of acclimation to captivity. In wild-caught turbot *Scopthalmus maximus*, efficient spawning occurred only after 2 yr of habituation to captivity (Devauchelle et al. 1988). In southern flounder, only females > 2 kg spawned naturally in captivity (Arnold et al. 1977) and successful hormone-induced tank spawning was attained with broodstock held in captivity for at least 1.5 yr and receiving photothermal conditioning for a minimum of 12 wk prior to spawning (Smith et al. 1999b).

The natural spawning period for southern flounder is believed to be December and January (Henderson-Arzapalo 1988; Berlinksy et al. 1996; Smith et al. 1999b). In this study, viable embryos were produced from January through late April 1999, indicating that the extended photoperiod regime was effective in extending the spawning season at least 2 mo beyond the natural spawning period for this species. Using a combination of photothermal conditioning and GnRH-a implants, southern flounder broodstock spawned volitionally in tanks over an extended period of 99 d from January to late April (Smith et al. 1999b).

It is likely that natural spawning of Southern flounder in this study was promoted by a number of factors. The fish were received from fishermen in good health and water quality parameters in broodtanks were maintained within optimal ranges throughout the study. The fish fed well and showed little or no evidence of disease for the duration of the study. In addition to the photothermal conditions, which maintained fish in a reproductive state from December through April, factors such as diet, tank size and color, and low light intensities apparently minimized stress and were conducive to natural spawning.

Based on our observation that egg release increased following a change in weather conditions, pulsatile temperature conditions may have been an important stimulus for sustained natural spawning in this study. Smith et al. (1999b) reported that a drop in water temperature from 17 to 14 C inhibited spawning in southern flounder, which resumed when temperature was returned to 17 C. In red drum, spawning is stimulated by gently raising or lowering the water temperature (Roberts 1990).

Sustained release GnRH-a pellet implants are highly effective in inducing ovulation of successive batches of eggs in the southern flounder (Berlinksy et al. 1996), allowing repetitive strip spawning. These workers produced substantial numbers (1.6×10^6) of eggs from multiple strip spawns of 12 females over a 2-wk period, but percent fertilization varied considerably (7-95%) between females and between spawns from individual fish. As an alternative to strip-spawning, photothermal conditioning coupled with GnRH-a implants have also resulted in successful tank spawning of southern flounder (Smith et al. 1999b). From a broodstock consisting of 3 females, these workers collected an average of 277,844 eggs during a 99-day period for a total of 17,782,000 of which 32.8% were fertilized (range = 0-82%), although survival through hatching was not reported. A combination of photothermal conditioning and GnRH-a implants apparently reduced stress and resulted in higher egg production and an extended spawning period (Smith et al., 1999b).

Results of this study demonstrate that photothermal conditioning of southern flounder can produce natural spawning, without the use of hormones, resulting in relatively high spawning success in terms of egg production and quality and the duration of the spawning period. Natural spawning of southern flounder broodstock produced a total of 18.4 million eggs, from 5 females, with an overall percent fertilization of

28% and an overall percent hatching of 9%. In comparison, 31 hormone-induced spawning trials produced 1,101,000 eggs, with lower overall percent fertilization and percent hatching of 12% and 9.9%, respectively. While spawning success varied widely under both natural and hormone-induced spawning trials, the data suggest that natural spawns generally resulted in higher egg quality.

Higher egg quality under natural spawning may be related to minimal handling of the broodfish, since stress can reduce spawning success (Kjesbu 1989). Inappropriate hormone dosages can also affect egg quality (Lam 1994), and strip spawning may cause varying egg quality due to over ripening or disturbances in the ovulation process (Bromage 1995).

A major disadvantage of natural spawning is the inability to time the spawns to suit the needs of the fish culturist. Both natural and GnRH-a induced spawns will help supply the large numbers of eggs necessary to support commercial hatcheries. Additional research is needed on hormonal, nutritional and environmental effects on egg quality.

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REPRODUCTIVE MECHANISMS IN THE GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* AND COOPERATIVE RESEARCH TO IMPROVE SEED PRODUCTION TECHNOLOGY IN THE MEKONG DELTA REGION OF VIETNAM

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ABSTRACT

The giant freshwater prawn, *Macrobrachium rosenbergii*, is a commercially important species of crustacean cultured extensively throughout Southeast Asia. In Vietnam, where Japan International Research Center for Agricultural Sciences (JIRCAS) is currently implementing a comprehensive project entitled "Evaluation and improvement of farming systems combining agriculture, animal husbandry and fisheries in the Mekong Delta," *M. rosenbergii* is considered to be an important target species by the Vietnamese Government, and its aquaculture is being actively promoted. Farmers have traditionally depended on wild sources to obtain seed for aquaculture but are now faced with dwindling resources and a shortage of natural spawners. Development of improved means of artificial seed production for *M. rosenbergii* in the Mekong Delta is thus essential. JIRCAS is currently implementing basic studies on the reproductive endocrinology of *M. rosenbergii* as part of the Mekong Delta project. Biochemical and molecular biological research is being conducted both at the project site, Cantho University's College of Agriculture, in Cantho Province, Vietnam, in collaboration with Vietnam counterparts, and on JIRCAS Tsukuba premises. As part of our on-site studies in Vietnam, we are conducting an assessment of typical feeds utilized in freshwater prawn culture in Vietnam, and evaluating the effects of this feed on reproduction. This research is expected to be relevant in controlling female reproduction in captivity and improving seed production technology.

STATUS OF FRESHWATER PRAWN CULTURE IN VIETNAM

The Mekong Delta of Vietnam possesses more than four million hectares of natural land area, of which water bodies excluding rivers comprise 954,350 ha. Freshwater bodies total 641,350 ha or 67.2% of total water surface, and brackishwater areas comprise 313,000 ha (Tien 1993). The flat lowland, moderate climate, and rich natural aquatic resources of the Mekong Delta provide favorable conditions for agricultural and aquacultural development in the region (NEDECO 1991). Aquacultural activity is often integrated with other farming enterprises such as rice cultivation, or is implemented on a mono-culture basis, in ponds or in garden canals. Several species of fish, including common carp, tilapia, and silver barb are cultured in the Mekong Delta. Among crustacean species, the giant freshwater prawn, *Macrobrachium rosenbergii*, is widely targeted in freshwater areas.

In Vietnam, annual production of *M. rosenbergii* varies from 5,000 to 8,000 t (Lin and Lee 1992). However, these production statistics are based on total harvest from both aquaculture and capture fisheries; actual production due to aquaculture is estimated to be as low as 2,000-3,000 t (Hien et al. 1998). Compared to an annual production of 50,000 t of saltwater *Penaeus*

monodon or black tiger prawn (Aqua Farm News 1996; Nien and Lin 1996), *M. rosenbergii* culture appears to be a much less developed industry. The Vietnamese Government aims to promote the culture of *M. rosenbergii* because of its high export value and potential to contribute to increased income levels among farmers in the Mekong Delta.

Freshwater prawn culture in the Mekong Delta is greatly dependent on the use of prawn juveniles collected from natural bodies of water. Most farmers engaged in *M. rosenbergii* culture stock prawns at low density; thus, productivity levels are typically low, being about 250-300 kg/ha in monoculture, and 150-180 kg/ha in rice-prawn combined farming systems in which prawn culture is integrated with rice cultivation (Dung 1991; Tuan and Phuong 1994). Much of the freshwater prawn culture is practiced in the Phung Hiep District of Cantho Province (Sanh et al. 1993), and several districts in Angiang, Tiengiang, Vinhlong and Travinh Provinces (Lin and Lee 1992). This may be related to the natural distribution of *M. rosenbergii*. In the wild, *M. rosenbergii* is distributed from Nhatrang in the central part of the country to the South, but is found mostly in Cantho, Vinhlong, Travinh, and Soctrang Provinces (Thang 1993) (Fig. 1).

At a current market price of approximately US $10/kg, *M. rosenbergii* is a high-priced species and incentive among farmers to engage in its culture is significant (Hien et al. 1998). Income levels per capita in the Mekong Delta vary with province, but a value of US $130-200/yr may be fairly typical (Danida Report 1996). In contrast, many prawn farmers earn up to US $1,000/yr in terms of net income, especially those conducting rice-prawn farming (Hung 1992; Tuyen 1993).

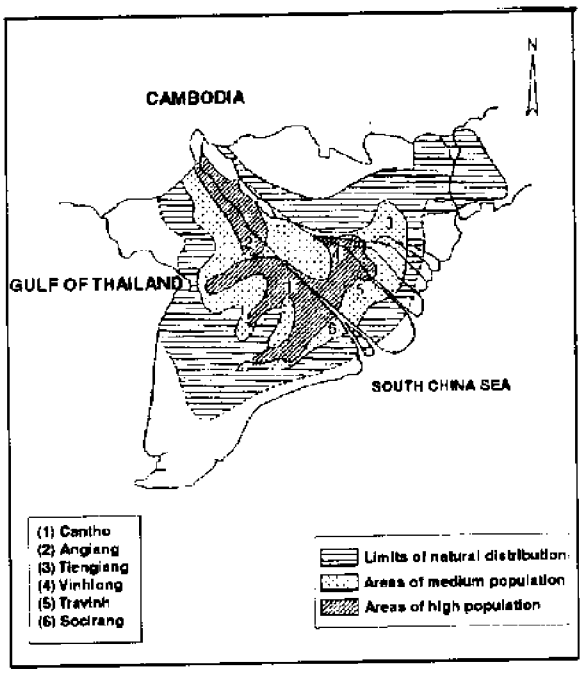

Figure 1. Distribution of *Macrobrachium rosenbergii* populations in the Mekong Delta (redrawn from Thang, 1993). *M. rosenbergii* is found in most freshwater areas of the Mekong Delta, but populations are especially concentrated in central regions of the Delta. Horizontally-shaded areas show limits of natural distribution, while dotted areas and diagonally-shaded areas show areas of medium and high populations, respectively. Numbers indicate approximate locations of provinces where *M. rosenbergii* is found.

Rice-prawn combined farming systems, in which freshwater prawns juveniles are released into rice fields and are allowed to forage, has become a familiar site in the Mekong River Delta. A schematic diagram of the rice-prawn system is shown in Fig. 2. This type of culture is extensive. Canal area, which is used for prawn culture, covers about 15-20% of the total area of the rice field itself. Water levels are controlled to be 1-1.2 m in depth in the canal and 0.2-0.3 m depth on the rice field. Juveniles are stocked often at a

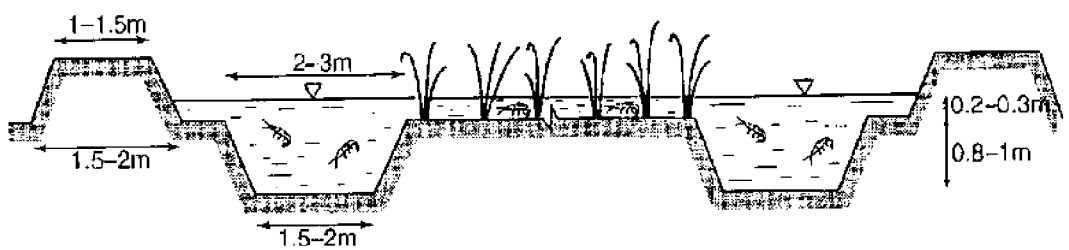

Figure 2. Transect drawing of a rice-prawn field. Canal area comprises 15-20% of total area as described in the text. Prawns are initially stocked in the canals, but can also be found foraging within the rice fields as well.

size of 5-10 g with a stocking density of 0.5-2 individuals/m^2. Stocking activities are usually conducted from December to February. Many farmers do not provide feed, allowing prawns to forage only on natural feed sources occurring in the rice fields (Tuyen 1990). Some farmers, however, utilize agro-byproducts such as rice bran, broken rice, cassava, and coconut pulp as a means of feed.

The culture period in rice-prawn systems varies from 4 to 8 mo depending on farming practices and available capital of the farming households. Yield differs from place to place within a specific region. For instance, in the Thotnot District of Cantho Province, average production reaches 268 kg/ha. In Phunghiep District also in Cantho Province, production is about 100-200 kg/ha (Hien et al. 1998). The major obstacle to further development of rice-prawn farming is not the system itself; it is rather due to the lack of a stable supply of seed and the inability of national hatcheries to meet farmers' demands, as described in the following section.

Natural Sources of Seed; Status of National Hatcheries

As described above, much of the commercial culture of *M. rosenbergii* is dependent on the use of juvenile prawns collected from natural sources. Fishing gear such as brushwood, stow nets, straw nets, or shelter traps are commonly used to obtain juvenile prawns (Hien et al. 1998). Brushwood gear (Fig. 3a) consists of a bundle of tree branches placed in the river, allowing juvenile prawns to be entrapped. Using stow net gear, a net is placed across the river in a zig-zag fashion (Fig. 3b), while straw net gear consists of an enclosed net which is pulled by a boat (Fig. 3c). Shelter traps, square-shaped structures consisting of bamboo frames enclosed with netting (Fig. 3d), are placed along the edges of rivers, and periodically checked for prawn juveniles trapped inside (Lin and Lee 1992). Persons engaging in the above activity are usually fisher folk who sell their catch to middlemen, through which juvenile prawn seed is made available to farmers. Although there are five national hatcheries in Vietnam for the production of *M. rosenbergii*, many farmers prefer to use prawn juveniles obtained in the above manner, as hatchery output is insufficient to meet demands in both quality and quantity.

The Vungtau hatchery, located southeast of Ho Chi Minh City, was the first national hatchery to be established for *M. rosenbergii* seed production, and was built by the Mekong River

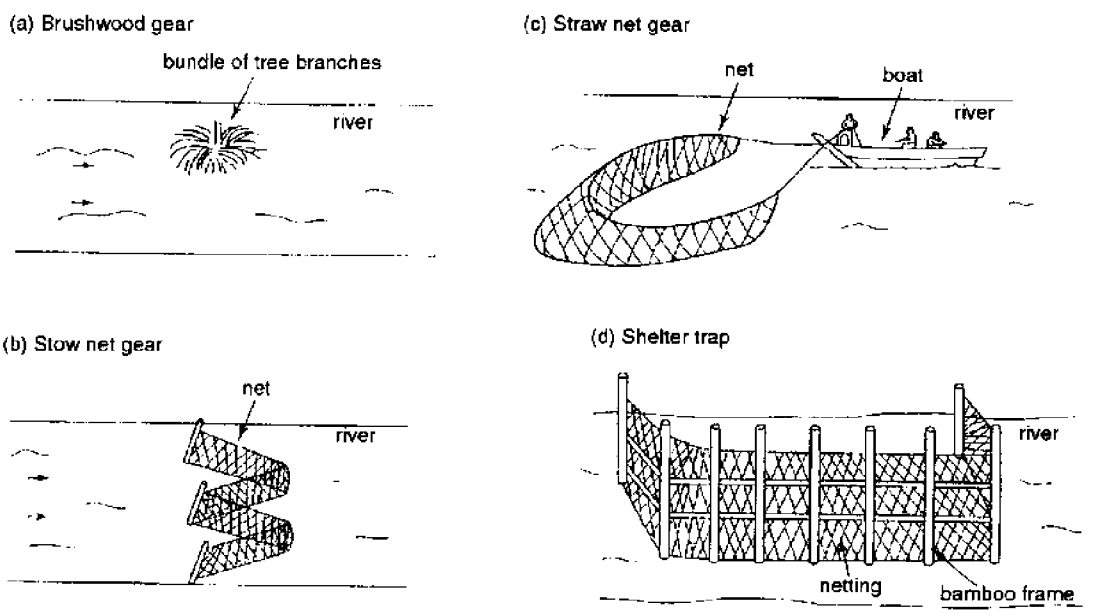

Figure 3. Schematic diagrams of fishing gears used for obtaining juvenile *M. rosenbergii*. (a) Brushwood gear; (b) stow net gear; (c) straw net gear; (d) shelter traps. More detailed explanations are given in the text.

Commission and the Vietnamese Government. It has been in operation since 1987 (Hien et al. 1998). There are four other national hatcheries for *M. rosenbergii* in Vietnam: Nhabe outside of Ho Chi Minh City, Gocong in Tiengang Province, Long My in Cantho Province, and Travinh in Travinh Province. The Long My hatchery is the newest of the five and is still under construction. Annual production capacities for these national hatcheries range from 4,000,000 to 5,000,000 post-larvae/yr at the Vungtau and Nhabe hatcheries, and 2,000,000 to 3,000,000 post-larvae/yr at the other hatcheries; however, most hatcheries are not operating at full capacity due to low survival and technical constraints, which include securing good-quality broodstock and controlling the outbreak of disease (Hien et al. 1998). There is, however, a significant demand for high quality artificially-produced seed, thus there is an urgent need to improve seed quality and decrease price in order to decrease the country's dependence on the use of wild juveniles.

According to personal communications from personnel at the National University, Faculty of Fisheries (Ho Chi Minh City); Research Institute for Aquaculture No. 2 (RIA2), Ministry of Fisheries; and research counterparts at the College of Agriculture, Cantho University, the control of female maturation under captive conditions is the most significant obstacle in establishing a stable means of broodstock cultivation in Vietnam. In the wild, female prawns first mature after reaching a size of 20-40 g; eggs obtained from these females are of good quality and their larvae show high percent survival after hatching. However, females of hatchery origin which are cultured as broodstock often mature while only 7-10 g BW. Use of such precociously mature females results in eggs and larvae of poor quality; offspring of these females may mature even more precociously. For the above reasons, broodstock collected from wild sources, 20-50 g individuals, are presently employed in most hatchery operations. However, this is causing resources of spawners to decline due to over-exploitation, and the dependence of such wild broodstock in hatchery operations is limiting the ability of hatcheries to meet the needs of the aquaculture industry (Hien et al. 1998).

Outline of Cooperative Research

In research between JIRCAS and Cantho University, we are examining how rearing conditions in the hatchery may contribute to precocious reproductive development in female *M. rosenbergii*. Preliminary work has focused on the effects on reproduction and growth of typical feeding regimens practiced in the Mekong Delta. We have conducted an analysis of nutritional content of locally-available feed resources, and are considering how manipulation of diet may be used to control growth and reproductive development (Hien et al. in preparation). In a second phase of these joint studies, we are investigating the effects of water temperature and salinity on reproductive development by simulating typical conditions of culture sites found in the Mekong Delta. Basic studies at JIRCAS are being conducted to elucidate the physiological mechanisms of reproduction in *M. rosenbergii* in parallel to on-site studies in Vietnam. In particular, research at JIRCAS is focused on the hormonal control of vitellogenin (yolk-protein) synthesis and uptake in relation to ovarian development (Wilder et al. 1994) and mechanisms of osmoregulation in relation to salinity adaptation (Wilder et al. 1998). It is expected that a more fundamental understanding of reproductive function at the molecular level will enable better interpretation of how environmental and nutritional conditions influence maturation, and will be applicable in the on-site control of maturation. The research at both JIRCAS and Cantho University is described below.

Basic Research on Reproductive Mechanisms

In *M. rosenbergii* and most other species of decapod Crustacea, reproduction is thought to be under the control of various hormones, including vitellogenesis inhibiting hormone (VIH) and vitellogenesis stimulating hormone (VSH). The presence of VIH in the eyestalk has been well-established, but less is known about VSH which is thought to originate in the brain and thoracic ganglia. Thus, many aspects of reproductive function in Crustacea, including regulatory mechanisms of vitellogenesis, remain unclear. This is due in great part to the fact that the

biochemical nature of vitellogenin (yolk protein) is not fully known. The chemical characterization of vitellogenin and the elucidation of regulatory hormones responsible for mediating reproduction in crustaceans are highly urgent.

In most crustacean species, vitellogenin exists as the precursor of yolk protein and has a molecular mass of more than 200 kDa. In *M. rosenbergii*, vitellogenin is thought to be first synthesized in the hepatopancreas and thereafter secreted into the hemolymph. Subsequently, during vitellogenesis, vitellogenin is taken into the ovary and processed into several subunits to serve as an important source of nutrients during the processes of ovarian and embryonic development. These subunits are known as vitellin.

In research at JIRCAS, we are currently examining the primary structure of vitellin and vitellogenin in *M. rosenbergii*, and are attempting to elucidate the site of vitellogenin synthesis. In order to do so, we first extracted vitellin from a mature ovary and filtered the extract with microconcentrators to cut off low molecular weight proteins. The filtrate was then subjected to reversed-phase high performance liquid chromatography (HPLC), and four major proteins (fractions A, B, C and D) were separated using a linear gradient of acetonitrile/trifluoroacetic acid (TFA) (Fig. 4). The results of Western blotting suggested that the four fractions were vitellins. Using TOF (time-off-flight) mass spectrometry, it was observed that the four fractions recovered from HPLC exhibited protonated molecular ion peaks at m/z 89560.7, 88721.1, 88963.6 and 88900.9, respectively, indicating molecular weights of approximately 90 kDa for all vitellins.

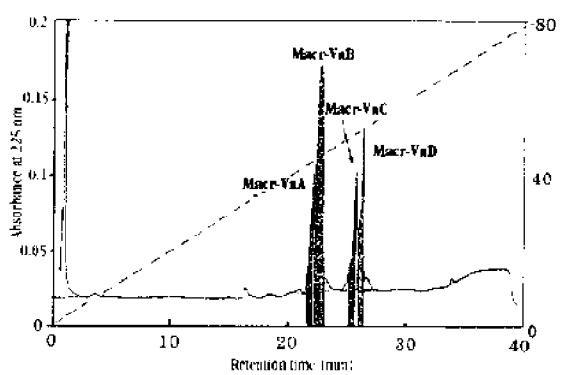

Figure 4. Reversed-phase HPLC elution profiles of M. rosenbergii ovarian extract, showing 4 peaks (Macr-VnA, VnB, VnC and VnD) of purified vitellin.

The four fractions were initially subjected to N-terminal amino acid sequence analysis, and we were able to identify more than 30 amino acid residues. To obtain more information about the amino acid sequences, the four fractions were digested with lysyl endopeptidase and the digested fragments were separated by reversed-phase HPLC on an ODP-50 column with a linear gradient of acetonitrile/TFA. A total of 48, 53, 57 and 46 fragments (for fractions A, B, C and D, respectively) were thus obtained and amino acid sequences for several of these fragments were determined.

In order to clone the four cDNA-encoding fractions (fractions A, B, C and D), total RNA isolated from the ovary and hepatopancreas were subjected to reverse transcription (RT) reaction in order to synthesize cDNA. The resultant cDNAs were then subjected to polymerase chain reaction (PCR) using degenerate oligonucleotide primers. The PCR products were subcloned into a plasmid vector and analyzed to determine the DNA sequences. The complete DNA sequences of the four vitellin cDNAs were determined and the conceptually translated amino acid sequences were identical to those of the N-terminal and lysyl endopeptidase fragment sequences. In subsequent research, this will enable us to determine the full DNA sequences of the four vitellins, and thus their complete amino acid sequences. This will provide important structural information whereby it will be possible to more fully understand the process of vitellogenesis in *M. rosenbergii*.

In addition, to identify the synthetic site of vitellogenin, we are analyzing the specific expression of mRNA using Northern hybridization. The site of expression of mRNA for fractions C and D has already been identified as the hepatopancreas. Furthermore, in order to obtain a complete picture of the dynamics of vitellogenin synthesis, expression of mRNA in various tissues (hepatopancreas, ovary, hemocytes, subepidermal adipose tissue and muscle) are being analyzed at different stages of reproduction.

The above research will serve as a basis for developing a bioassay system which will

enable us to identify VIH and VSH. Using such a bioassay system, the role of VIH and VSH, as well as of other hormonal factors in regulating vitellogenesis, may be examined. In turn, we expect that this will result in an understanding of how various environmental and nutritional factors affect the onset of maturation. Details of the above research are reported in Yang et al. (In press).

On-site Studies in Vietnam

Initial research was conducted to address how rearing conditions practiced in the Mekong Delta affect reproductive development in *M. rosenbergii*. We selected three representative feeding regimens, consisting of commercial pellets, hand-prepared pellets made from rice bran, fish meal, and vitamin supplements, and chopped trash fish. Treatments were analyzed for proximate composition (Table 1), as well as amino acid and fatty acid composition (data not shown). Results differed among treatments, although commercial and hand-prepared pellets showed similar profiles. A large-scale experiment, in which 30 male and female prawns for each treatment were reared together in 1.3-t tanks, was designed to look at the effects of differing feeds at saturated and unsaturated levels. A small-scale experiment, in which females were reared individually in 60-L tanks, was designed to examine the effects of feed and compare reproductive development between prawns of wild and hatchery origin.

In both the large- and small-scale experiments, prawns were examined for ovarian maturation by observing the size of ovaries through the carapace. In the small-scale experiment, prawns were initially blood-sampled, and extent of maturation was assessed by measuring hemolymph vitellogenin levels via enzyme immunoassay (EIA).

In both experiments, prawns were an initial size of 5 g, and were reared for up to 20 wk. In the large-scale experiment, prawns fed trash fish matured for the first time at a larger size, more than 15 g BW, while prawns fed with commercial and hand-prepared pellets matured at a size of approximately 12 g, indicating that trash fish were effective in obtaining mature prawns of larger size. In the small-scale experiment, prawns of hatchery origin showed elevated hemolymph vitellogenin levels and matured in 4-7 wk after the start of experimentation while those of wild origin required 16-20 wk. This revealed that the phenomenon of precocious maturation in hatchery-reared prawns is related to an earlier onset of vitellogenin synthesis.

Total essential amino acid (EAA) contents were higher in trash fish than in other feeds, including lysine, an amino acid known to strongly stimulate growth. On the other hand, fatty acids of the n-6 (Σn-6) family and linoleic contents in trash fish were very low compared to commercial and hand-prepared pellets, while fatty acids of the n-3 (Σn-3) family and linolenic acid were high in trash fish and commercial pellets. It is suggested that the balance of amino acid and fatty acid composition present in diets employed by farmers in the Mekong Delta is a main factor affecting the dynamics of growth and reproduction in *M. rosenbergii*. Differing nutritional conditions to which wild and hatchery-reared prawns are exposed during the early life stages may be a factor in determining the onset of vitellogenin production, and thus ovarian maturation.

The connection between nutritional condition and reproductive function is unclear, but this research suggests a link between diet and hormonal makeup. However, a better understanding of the role of nutrition in reproduction can only be established after basic hormonal mechanisms in Crustacea are more fully elucidated.

In a second phase of study, we are currently evaluating the effects of salinity and water temperature on growth and reproductive

Table 1. Proximate composition of representative diets used in freshwater prawn culture in the Mekong Delta. Results are shown as percent (%) dry weight and moisture content (%).

Component	Commercial pellets	Hand-prepared pellets	Trash fish
Crude protein	37.7	26.0	60.0
Crude lipid	6.3	7.2	10.6
Ash	11.1	18.9	13.0
Carbohydrate	43.4	43.7	19.2
Fiber	11.6	4.2	-
Moisture	9.5	9.3	77.8

development using a similar experimental protocol. In addition, we are conducting studies on the development of larval feeds using locally-available resources. Based on the results of this research, we plan to conduct trial investigations in freshwater prawn seed production at a mini-hatchery established by Cantho University.

The above is a partial summary of collaborative research on freshwater prawn culture conducted as the fisheries component of an international comprehensive project between the Japan International Research Center for Agricultural Sciences (JIRCAS), Cantho University, and the Cuu Long Delta Rice Research Institute entitled "Evaluation and improvement of farming systems combining agriculture, animal husbandry, and fisheries in the Mekong Delta." The first phase of the project, initiated in 1994, was concluded in 1999. The authors continue to engage in collaborative research on this topic under the framework of the second phase project, "Development of new technologies and their practice for sustainable farming systems in the Mekong Delta" which is scheduled to run from 1999 to 2003.

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RELATIONSHIP BETWEEN EXTERNAL AND INTERNAL MORPHOLOGICAL CHANGES AND FEEDING HABITS IN THE FRY STAGE OF JAPANESE CATFISH *SILURUS ASOTUS*

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ABSTRACT

The prevention of sibling cannibalism during the early stages of development is a very important consideration for the successful seed production of the Japanese catfish *Silurus asotus*. Although several methods have been tested for this purpose, none thus far has reliably reduced cannibalism-induced mortality to acceptable levels. To clarify the phenomenon of sibling cannibalism, a study was carried out on the relationship between external and internal morphological changes and feeding habits during the fry stage of Japanese catfish. The results show that the growth of body height and mouth width were temporarily arrested when the fry attained a total body length of 40 mm and at the time of disappearance of the mandibular barbels, respectively. Furthermore, the relationships between the length of the stomach and intestine *versus* the total body length (TL) were best described by sigmoid curves.

INTRODUCTION

The Japanese catfish *Silurus asotus* is well known as a carnivorous fish and is widely distributed in Japan (Miyadi et al. 1976). In seed production of this species, biting and cannibalistic behavior were recognized about 40 d after hatching, inducing high mortality. Although several methods have been tested for the prevention of sibling cannibalism, for example, localization by shelter (Fukuda 1974; Umezawa et al. 1994a) and dispersion by aeration (Fukuda 1974), none have reliably reduced cannibalism-induced mortality to acceptable levels. According to a very recent study, the observed cannibalism is related to the feeding (quality and quantity) and the density of the larvae (Umezawa et al. 1994b; Kuruma and Nomura 1997; Tejima et al. 1996, 1997). It has also been reported that mortalities due to cannibalism can be effectively reduced if larvae are raised quickly at an optimum temperature to shorten the developmental periods of when cannibalism occurs. However, despite these technical developments, little is known about the relationship between the morphology and feeding habits during the early growth stages of Japanese catfish.

In general, it is well known that the Japanese catfish fry have an interesting external morphological change involving the disappearance of a pair of mandibular barbels. Regarding this point, Atoda (1935) reported that the fry of the Japanese catfish possessed two pairs of mandibular barbels. One was permanent and the other was temporary. Sato and Katagiri (1966) reported that the temporary mandibular barbels showed signs of degeneration at 35 mm TL when Japanese catfish were reared in the laboratory. These fell off spontaneously in fishes ranging in size of 60-70 mm TL, and ultimately disappeared (Atoda 1935).

Regarding internal morphological changes, Suyehiro (1942) and Ishida and Sato (1960) reported the development of the digestive system in detail. However, their body sizes were not clarified and the individuals investigated were more than 200 mm TL. There is little information on the relationship between the morphology and

feeding habits during the fry stages of the Japanese catfish.

In this study, we describe the relationship between the external and internal morphological changes and feeding habits during the fry stage of Japanese catfish reared in the laboratory in an attempt to clarify the phenomenon of sibling cannibalism.

MATERIALS AND METHODS

Capture of Mature Fish

During the spawning season of June 1998, mature Japanese catfish were captured with a small set-net "Fukube ami" from an irrigation creek in the tributary of the Omonogawa River, Akita, Japan. The captured fish were transferred to the laboratory and kept in a stock pond until collection of eggs and sperm. Mature fish were selected by body size, palpation, and the shape of the caudal fin. It was easy to distinguish the sex of the fish using these criteria.

Collection of Eggs and Sperm

The collection of eggs and sperm was done according to the methods described by Kuge et al. (1989), Kanazawa and Tasaki (1989), and Nomura (1996). To induce final maturation selected individuals were given an intraperitoneal injection with human chorionic gonadotropin (HCG) at a dosage of 10,000 IU/kg BW for females and 5,000 IU/kg BW for males, respectively. Oocytes underwent final maturation and ovulation between 16 h and 20 h after injection. Determination of male maturation was very difficult, and the quantity of collected sperm was very poor. Therefore, the testes were removed and homogenized with 0.6% Ringer solution, and a sperm suspension was prepared for use in artificially fertilizing the eggs.

Fertilization and Incubation

Artificial fertilization was conducted using the dry-method technique and the percent fertilization was high, ranging from 80 to 99%. Fertilized eggs were incubated at 20 C on hatching trays with a continuous water exchange. The eggs hatched out after 96 h of incubation and the percent hatch ranged from 80 to 93%.

Rearing of Larvae

A brood of Japanese catfish larvae was reared at 20 C in a 100-L polycarbonate tank equipped with a sponge filter. Feed was given three times daily at 0900, 1300, and 1700 and was supplied at about 40% of the body weight with *Daphnia* sp. for the first 14 d after hatching. Switching from the live feed to a formulated feed used for marine fish larvae (crude protein content: 52%, Nippon Nosan Kogyo K.K.) took place gradually from 8 to15 d after hatching. The formulated feed was given daily in three equal rations at 0900, 1300, and 1700, and providing the formulated feed to satiation of the larvae began from16 to 60 d after hatching. The Japanese catfish fry was later weaned on to a commercial carp feed (crude protein content: 39%, Nippon Formula Feed Mgf Co., Ltd.) and resulted in good growth (Fig. 1.).

Sampling and Measurements

The experimental fish were sampled periodically and fixed in 10% formalin for external and internal morphological investigations. The body weight (wet weight) for the preserved specimens was measured 6 mo after fixation. The following body parts were also measured: total body length (TL), body height (BH), mouth width (MW), and the number of barbels were recorded.

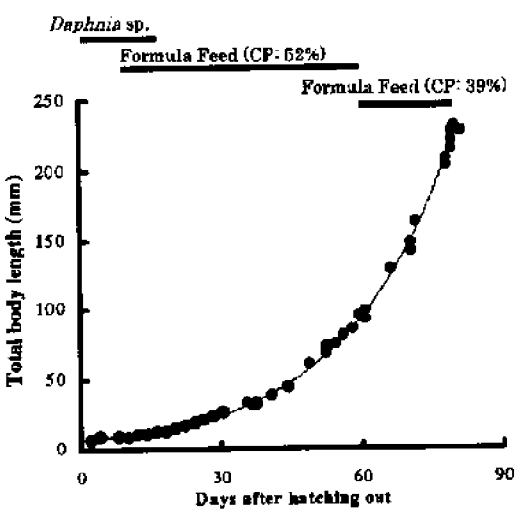

Figure 1. Increase in total body length of Japanese catfish *Silurus asotus* reared at 20 C. Feeding schedule is illustrated at the top of the figures.

Regarding the internal morphology, the coiling of the digestive tract was observed by first removing it from the body cavity and sketching it from the ventral side. The digestive tract was divided into the stomach and intestine parts. Distinguishing between the esophagus and stomach was difficult and the thinnest portion of the tract between the esophagus and the stomach was used as the division point for measurements of the length of the stomach parts (SPL; S-1, S-2, S-3, Fig. 2.). The intestine part length (IPL) was carefully measured using calipers as described by Suyehiro (1942).

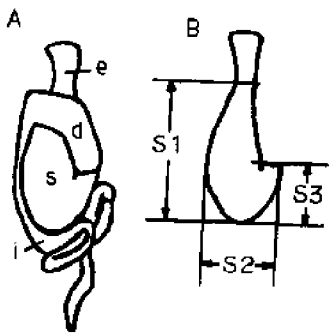

Figure 2. Digestive tract in Japanese catfish *S. asotus*. A, ventral view of digestive tract; B, measuring points of stomach parts (ventral view). e, esophagus; s, stomach; d, duodenum; i, intestine.

RESULTS

External Morphological Changes

The relationship between the body height and mouth width versus total body length are shown in Fig. 3. Although the BH and MW increased in proportion to the increase in TL, their continued development was temporarily arrested when the fry attained a size of 40 mm TL. At this size (age) the disappearance of the mandibular barbels also occurred. Moreover, the catfish larvae acquired the adult body shape when at 40 mm TL, and possessed three pairs of barbels. Cannibalistic behavior was observed when fry ranged in size from 20 mm to 80 mm TL.

Internal Morphological Changes

A diagram of the stomach and intestine of the Japanese catfish are shown in Fig. 2. Suyehiro (1942) previously reported the I-shape of the stomach. However, from specimens

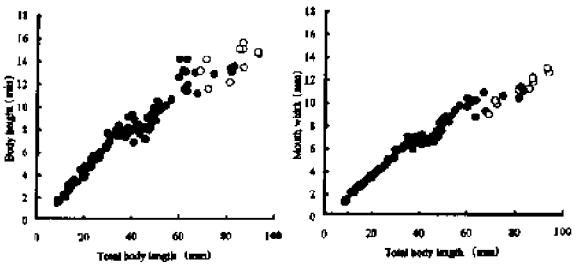

Figure 3. Relationship between body height and mouth width versus total body length in Japanese catfish *S. asotus*. Closed symbols, 3 pairs of barbels; Open symbols, 2 pairs of barbels.

examined during the current examination reveal a structure more like a V-shape. Ohara (1987) also reported that the stomach was generally short, and was closely related to a V-shape. The stomach and intestine were easily discerned by a valve and from their outward appearance. Although measuring the stomach length was attempted, accurate measurements could not be made due to the expansion and contraction of the stomach.

Illustrations of the digestive tract in Japanese catfish are shown in Fig. 4. The morphological changes in the intestines are indicated according to the method described by Kafuku (1952). They were as follows: body length of 22 mm (A), the intestine continues from the pyloric region and is very thick, crossing the esophagus to the lower right part of the fish's body in an arc. It then bends back again to the pyloric region of the stomach and reaches the anus.

At a body length of 44 mm (B), the intestine moves upwards, turns to the left, and bends back at the crossing point of the esophagus. After that, it moves downward along the stomach

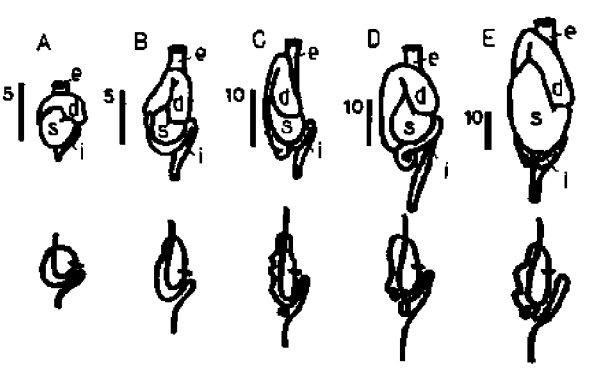

Figure 4. Illustrations of the digestive tract in Japanese catfish *S. asotus* at different stages during growth. A, 22 mm TL; B, 44 mm TL; C, 108 mm TL; D, 169 mm TL; E, 195 mm TL.

surface and upward while again drawing an arc before reaching the anus.

At a body length of 108 mm (C), the intestine was twice as small going toward the body cavity back from the stomach surface. It bends back once or twice at the lower end of the stomach and once at the pyloric region before reaching the anus. Although the coiling patterns of the intestine were similar to each other, they became more complex after the disappearance of the temporary mandibular barbels (Fig. 4. (C), (D), (E)).

Relationship Between SPL and TL

The relationship between the stomach parts length (SPL) and total body length (TL) is shown in Fig. 5 and is best described as a sigmoid curve. After the disappearance of the temporary mandibular barbels, the growth of the stomach parts were temporarily arrested at a body length ranging from 70 to150 mm. The change in stomach part (S1) was greater in comparison with the other parts (S2, S3).

Relationship Between IPL and TL

The relationship between the intestine parts length (IPL) and total body length (TL) is shown in Fig. 6, can also be best described as a sigmoid curve. The growth of the intestine was temporarily arrested at a body length ranging from 100 to150 mm. The changes in the intestine occurred later than those observed in the stomach.

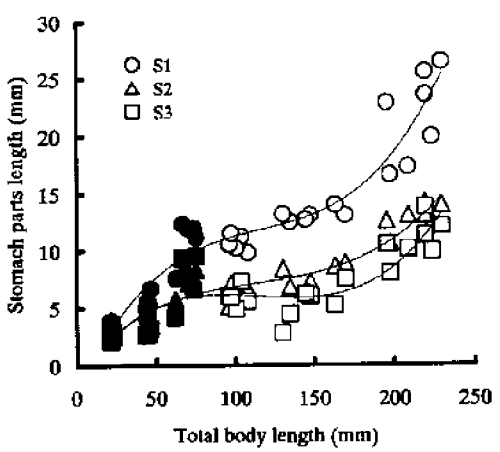

Figure 5. The relationship between stomach parts length and total body length in Japanese catfish *S. asotus*. Three parts of stomach (S1, S2, S3) are shown in Fig. 2. Closed symbols, 3 pairs of barbels; Open symbols, 2 pairs of barbels.

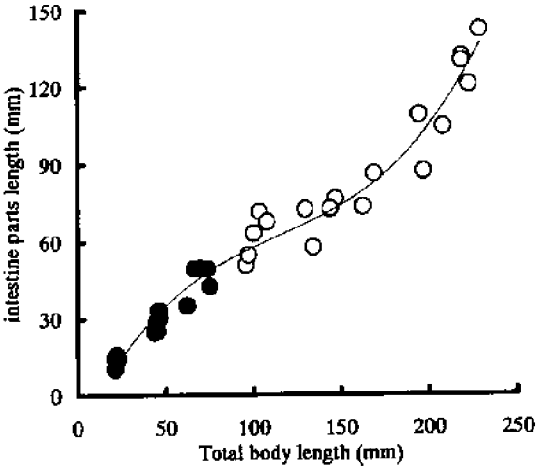

Figure 6. The relationship between the intestine parts length and total body length in Japanese catfish *S. asotus*. Closed symbols, 3 pairs of barbels; Open symbols, 2 pairs of barbels.

Although the temporary mandibular barbels were just recognizable at a body length of 70 mm, they almost disappeared at the body length of 100 mm.

DISCUSSION

The Japanese catfish *Silurus asotus* is well known as a carnivorous fish during its early developmental stages. During the culture process, the larvae actively shook their bodies continuously both to the right and left. When their barbels touched the body or caudal fin of another individual, a biting behavior was observed. From the recorded culture data, the mortality was high between 6 and 20 d after hatching. The percent survival at 30 d after hatching was 0.5% (initial, 12,045; final, 56; dead, 7,095; unknown, 4,894). Mutual biting was perceived as the main cause of mortalities and the "unknowns" were attributed to cannibalism. Judging from the observations made during the rearing process, the mouths that were suitable to hunt and swallow smaller fish were very large during the larval stage. Moreover, the temporary arrestment of BH and MW occurred at the same time as the when the adult form was attained and the mandibular barbels disappeared.

Regarding the relationship between body size and cannibalism, Hirakawa et. al. (1997) reported that cannibalism was occurring when larvae ranged between 10 and 15 mm to 70 mm TL, and not at the first feeding stage. Moreover,

Umezawa et. al. (1994a) observed a cloudiness from the tail to hind body portions of the bodies of some individuals to the exposure of caudal vertebra as early as 10 d after hatching.

According to reports from the Saitama Prefectural Fisheries Station (1992), cannibalism did not occur in fish more than 7.8 g in weight. To confirm this report, a rearing experiment was conducted in 1999 using fry that were more than 8.0 g in body weight. Percent survival after 3 mo in culture was 84% which is consistent with what was reported previously.

The disappearance (degeneration and disappearance) of the temporary mandibular barbels was the most noticeable external morphological change that was observed. The catfish apparently used the barbels to search for food, and when touching another individual instinctively tries to swallow the other individual from the head. It is also known that the barbels function as taste buds reacting to mechanical and chemical stimulation (Bardach et al. 1967). The function(s) of the temporary mandibular barbels are of great interest and an obvious area of future investigations.

Cannibalism in the Japanese catfish may also be related to external and internal morphological changes during the early stages of growth. The mouth and stomach were very large and although there is no valve between the esophagus and stomach, the sphincter does not restrict the possibility for larger catfish fry to swallow smaller catfish fry easily. Cannibalistic activity increased in fry above 20 mm TL and decreased after they ranged in size from 70 to 100 mm TL. This response was also coincident with the disappearance of the temporary mandibular barbels. In contrast, the internal morphological changes were expressed later than the external changes (Fig. 3).

In general, it is well known that the ratio of intestine length and total body length (relative length of gut, RLG) approximates a value of one or less (Takeuchi 1991). In the current investigation, the RLG in catfish fry was from 0.6 when individuals were 100 mm TL, 0.25 when 100-150 mm TL, and then 0.6 again when ranging from 150 to 200 mm TL (Fig. 6).

These results indicate that the relationship between external and internal morphological changes and feeding habits in Japanese catfish fry may be closely related and the feeding habits were different between fry and early larval stages in this species. It is still not clear as to the significance of the temporary curtailment in growth of body height, mouth width, or length of stomach parts and intestine parts during development. An examination into Japanese catfish fry bred at high temperatures and fed the same diet is currently underway to shed some light on this observation. Furthermore, research on the barbels, digestive enzymes, reproductive endocrine systems, and genetic factors are being investigated in order to clarify the phenomenon of cannibalism in the Japanese catfish *Silurus asotus*.

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STUDIES ON THE "COBALT" VARIANT OF RAINBOW TROUT

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ABSTRACT

The "cobalt" variant of rainbow trout *Oncorhynchus mykis* lacks most of the pars intermedia of the pituitary in which melanocyte-stimulating hormone (MSH) cells are distributed. An excessive accumulation of fat in the abdominal cavity is also observed in variant. In this study, the role of MSH on lipid metabolism of the trout and the possible relation to fat accumulation in the cobalt variant were investigated. In the light muscle, dark muscle, liver, and mesenteric fat, the cobalt variant showed higher contents of triacylglycerol (TG) than the normal trout. When α-MSH with an unacetylated -terminus (N-Des-Ac-α-MSH) was administered to the normal trout, circulating levels of fatty acid and lipolytic activities in the liver increased. N-Des-Ac-α-MSH also stimulated TG lipolysis in the cultured liver slices from both the normal and the cobalt variant of the trout. Those findings indicate the importance of MSH as a lipotropic hormone in the trout, and the lack of MSH cells in the pituitary remnant of the cobalt variant appears to be related to the abnormal accumulation of fat. Studies of the cobalt variant of trout may provide valuable information for pituitary research, for example, in studies of the functions of hormones produced by the pars intermedia and the early development of the pituitary.

INTRODUCTION

A blue-colored variant of rainbow trout *Oncorhynchus mykiss* appears rarely at trout experimental stations and commercial trout farms in Japan. This variant is often referred to as the "cobalt trout" because of the cobalt-blue body color. Another characteristic of this variant is the excessive accumulation of fat in the abdominal cavity, suggesting an abnormality in lipid metabolism (Kaneko et al. 1993).

Anatomical and histological studies reveal that the cobalt variant of rainbow trout has an irregularly-shaped pituitary which is completely detached from the hypothalamus (Fig. 1), and situated in the region ventral to the usual location of the pituitary. A normal adenohypophysis is divided into three regions: rostral pars distalis, proximal pars distalis, and pars intermedia. In the pituitary of the cobalt trout, there are few of the somatolactin and melanocyte-stimulating hormone (MSH) cells usually distributed in the pars intermedia of a normal

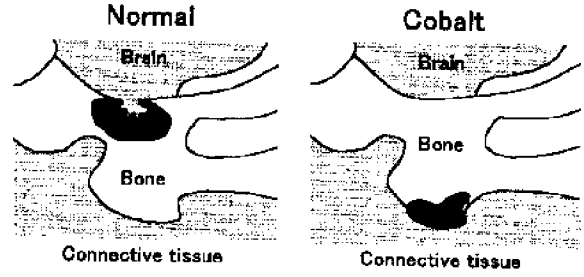

Figure 1. Location of the pituitary in the midsagittal plane of the normal and cobalt variant of the rainbow trout. Black area indicates the pituitary.

pituitary (Kaneko et al. 1993). In higher vertebrates, MSH show a weak, but significant, lipolytic activity (Ramachandran et al. 1976). This study investigates the role of MSH on lipid metabolism of the rainbow trout and its possible relation to the accumulation of fat in the cobalt variant.

MATERIALS AND METHODS

Cobalt-variant were collected from Shiga Prefectural Samegai Trout Farm (Maibara, Shiga)

and from Shizuoka Prefectural Fuji Trout Farm (Fujinomiya, Shizuoka). Normal rainbow trout were hatched and reared at the National Research Institute of Aquaculture, Nikko Branch. Both the cobalt variant and normal trout were reared separately in indoor rectangular tanks supplied with spring water (10 C). The light muscle, dark muscle, liver, and mesenteric fat were quickly removed, and lipids were extracted from these tissues using the method of Folch et al. (1957). Lipid classes were separated by thin-layer chromatography on CHROMATOROD S III (Iatron Laboratories, Tokyo, Japan), and triacylglycerol (TG) contents were quantified by a IATROSCAN MK-5 flame-ionization detector (Iatron Laboratories).

To investigate the effect of MSH on circulating fatty acid levels, normal trout were cannulated via the dorsal aorta, and a saline solution containing salmon α-MSH, α-MSH with an unacetylated –terminus (N-Des-Ac-α-MSH), β-MSH, or β-endorphin was injected via the cannula. Blood samples were then taken from the cannula into a syringe and centrifuged at 3000 x g for 5 min. Concentrations of plasma fatty acid were measured using a commercial kit; NEFA C-TestWako (Wako, Osaka, Japan).

In vivo and *in vitro* experiments for lipolysis of TG essentially followed the protocol of Plisetskaya et al. (1989). For the *in vivo* experiment, fish were injected intraperitoneally with saline containing N-Des-Ac-α-MSH. Three h after injection, the liver was removed, frozen on dry ice and stored at -80 C. For the *in vitro* experiment, the liver slices were incubated in RPMI 1640 (pH 7.8) containing N-Des-Ac-α-MSH at 20 C. After a 3-h incubation period, the liver slices were frozen on dry ice and stored at -80 C. TG lipase activity was determined as breakdown of ^{14}C-triolein to ^{14}C-oleic acid *in vitro* (Khoo and Steinberg 1981; Sheridan et al. 1985).

The ssignificance of differences between the two groups was analyzed by ANOVA followed by Duncan's multiple range test or Mann-Whitney U-test. Calculations were performed using the computer program STATISTICA (Design Technologies Incorporation, Tokyo, Japan).

RESULTS AND DISCUSSION

Triacylglycerol contents in the light muscle, dark muscle, liver, and mesenteric fat of the cobalt trout were significantly (P <0.01) higher than those of the normal trout (Fig. 2). They were also higher than the TG contents in tissues of the same species (steelhead trout) during parr-smolt transformation (Sheridan et al. 1983). These results suggest decreased lipolytic activities (hydrolysis of TG) in the cobalt variant of the trout.

Our previous study shows that the number of MSH cells in the pituitary of the cobalt trout is fewer than in the pituitary of the normal trout (Kaneko et al. 1993). MSH is derived from the precursor molecule, pro-opiomelanocortin (POMC), as isolated and identified from salmon pituitary tissue (Kawauchi and Muramoto 1979; Kawauchi et al. 1984). In rat and rabbit, MSH

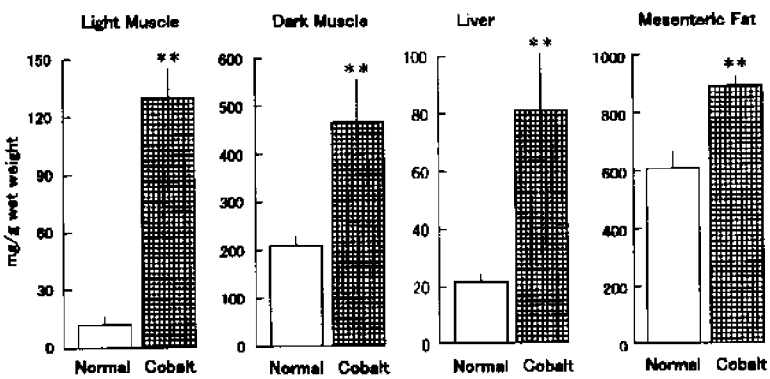

Figure 2. Triacylglycerol contents in the light muscle, dark muscle, liver and mesenteric fat of the normal and cobalt variant of the rainbow trout. Data are expressed as means ± SEM (n = 6). **Significantly different from the normal trout at $P < 0.01$.

cells show weak but significant lipolytic activity (Ramachandran et al. 1976). In salmon MSH cells, POMC is thought to be processed to α-MSH, N-Des-Ac-α-MSH, β-MSH and β-endorphin (Kawauchi et al. 1984). Changes in plasma levels of fatty acids after a single intra-arterial administration of these four peptides derived from POMC are shown in Fig.3. Administration of N-Des-Ac-α-MSH showed a significant ($P < 0.01$) increase in plasma levels of fatty acids, while the other 3 peptides showed no significant effect. The *in vivo* effect of various doses of N-Des-Ac-α-MSH on TG lipolysis in the liver are shown in Fig. 4. Intraperitoneal administration of N-Des-Ac-α-MSH stimulated TG lipase activity in the liver significantly ($P < 0.05$) at dosages of 10 and 100 ng/g BW. In isolated rabbit adipocytes, salmon N-Des-Ac-α-MSH shows the highest lipolytic activity among 7 peptides derived from salmon POMC; 3 types of α-MSH, 2 types of β-MSH, corticotrophin (ACTH), and N-terminal peptide of pro-opiocortin (Kawauchi et al. 1984). On the other hand, lipolytic activity of homologous POMC-derived peptides on salmonids is not known. This study revealed that salmon N-Des-Ac-α-MSH revealed a high potential to stimulate lipolysis in the rainbow trout.

As shown in Fig. 5, *in vitro* administration of N-Des-Ac-α-MSH also stimulated TG lipase

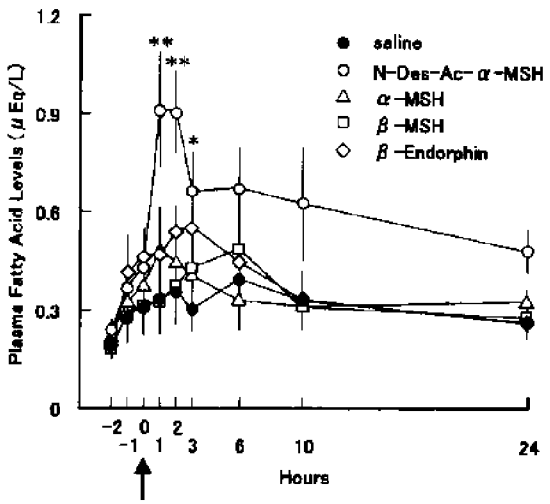

Figure 3. Effect of intra-arterial injection of POMC-derived peptides (1 ng/g body weight) on plasma fatty acid levels in the normal rainbow trout. Data are expressed as means ± SEM (n = 4). *,**Significantly different from control at $P < 0.05$, 0.01, respectively.

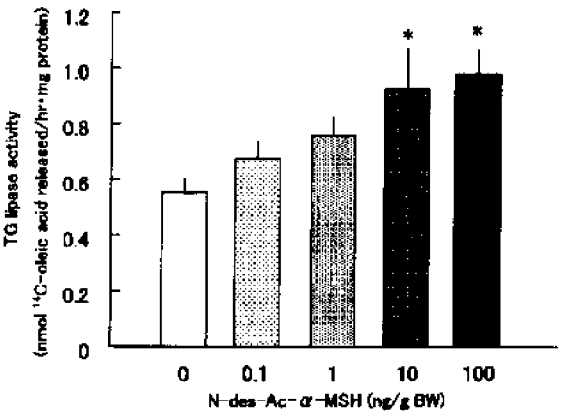

Figure 4. Effect of *in vivo* administration of N-des-acetyl-α-MSH on TG lipase activity in the liver of the normal rainbow trout 3 h after intraperitoneal injection. Data are expressed as means ± SEM (n = 5). *Significantly different from control at $P < 0.05$.

activity in the liver slices from the trout, suggesting the direct effect of this peptide on hepatocytes. Stimulation of lipolysis of TG by N-Des-Ac-α-MSH was observed in the normal and the cobalt variant of the trout. This result shows that the cobalt variant still has a lipolytic response to MSH, at least in the liver, and suggests that the lack of this hormone secreted from the pituitary is one reason for the significant obesity in the cobalt variant. Recent studies in higher vertebrates revealed several specific sequences which are necessary for pituitary organogenesis (Takuma et al. 1998; Treier et al. 1998; Lin et al. 1999). Further studies on the cobalt variant may provide valuable information of the pituitary

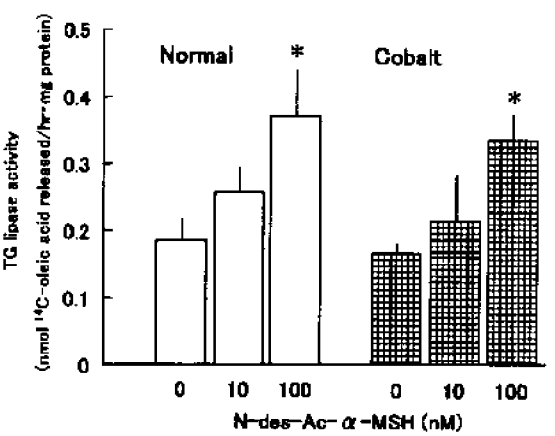

Figure 5. Effects of *in vitro* administration of N-des-Ac-α-MSH on TG lipase activity in the liver slices from the normal and cobalt variant of the rainbow trout. Data are expressed as means ± SEM (n = 4-5). *Significantly different from the control at $P < 0.05$.

ogranogenesis and the possible application of hypophyseal hormones to aquaculture.

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APPENDIX

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