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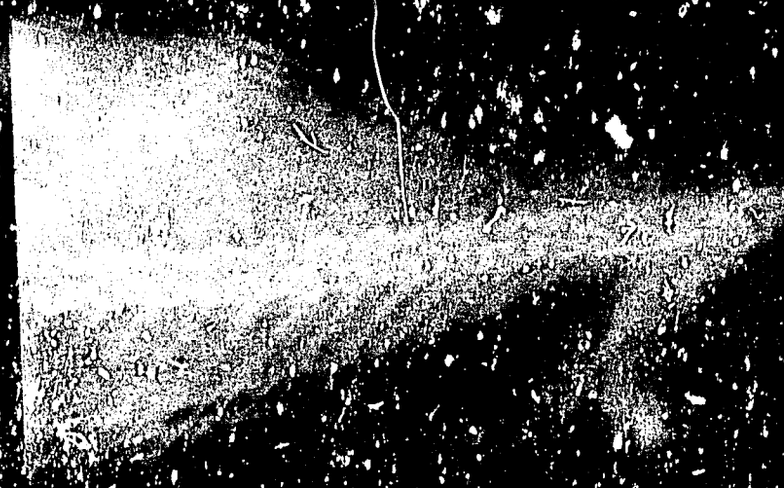
STUDIES ON THE MATURATION AND SPAWNING OF MILKFISH IN CAPTIVITY

PROGRESS REPORT
OCTOBER 1984 - DECEMBER 1988



A PROGRAM OF
THE AGENCY FOR INTERNATIONAL DEVELOPMENT
BUREAU FOR SCIENCE AND TECHNOLOGY
OFFICE OF AGRICULTURE
RENEWABLE NATURAL RESOURCES DIVISION

CONTRACT NO. DAN-4161-A-00-4055-00
WITH THE
OCEANIC INSTITUTE, HAWAII

Oceanic INSTITUTE

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Cheng-Sheng Lee
(Principal Investigator)

Ellen Antill	John Ginoza	R. an Murashige
Jessie Banno	Philip Giriodi	Yoshioki Oozeki
Paul Bass	Phyllis Ha	Vernon Sato
Anita Belanger	Atsushi Hagiwara	Christina Swanson
Christine Bingham	James Hendee	Clyde Tamaru
Robert Bourke	Christopher Kelley	William Walsh
Alex Bocek	Patricia Martinez	Laura Wallace
Mary Clarke	Garret Miyamoto	Gregory Weber
Bryan Eastham	Aaron Moriwake	Mark Yoder
Hiroki Eda	Mark Morioka	

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Dr. I.-C. Liao	Tungkang Marine Laboratory, Taiwan
Mr. Mao-Sen Su	Tungkang Marine Laboratory, Taiwan
Mr. Robin Chen	Tungkang Marine Laboratory, Taiwan
Dr. Sofyan Ilyas	Research Coordinating Center for Fisheries, Jakarta, Indonesia
Dr. Fuad Cholik	Research Institute for Coastal Aquaculture, Maros, South Sulawesi, Indonesia
Mr. Zafril Iman Azwar	Gondol Research Station, Bali, Indonesia
Mr. Agus Prijono	Gondol Research Station, Bali, Indonesia
Mr. Gede S. Sumiarsa	Gondol Research Station, Bali, Indonesia
Dr. Flor Lacanilao	Southeast Asian Fisheries Development Center, Tigbaujan, Philippines
Dr. Clarissa Marte	Southeast Asian Fisheries Development Center, Tigbaujan, Philippines
Dr. Alfredo Santiago	Southeast Asian Fisheries Development Center, Tigbaujuan, Philippines
Mr. Salvador Pamplona	Najuan Substation, SEAFDEC, Philippines
Mr. Rodolfo Mateo	Najuan Substation, SEAFDEC, Philippines
Dr. Lawrence Crim	Memorial University, Newfoundland, Canada
Dr. Malcolm Gordon	University of California, Los Angeles
Dr. John Hunter	National Marine Fisheries Service, La Jolla
Ms. Sue Nakamura	Department of Zoology, University of Hawaii
Ms. Nancy Clark	Sun Productions/Graphic Design, Honolulu

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EXECUTIVE SUMMARY

The goal of the project "Studies On the Maturation and Spawning of Milkfish" is development of a complete technological resource base to transfer to milkfish farmers and researchers in lesser developed countries. Integration of new findings with existing culture practices will ultimately contribute to high and reliable production of a relatively inexpensive source of animal protein. The primary objective is to develop techniques to control reproduction of this important teleost in captivity. A related objective is to evaluate the applicability of these techniques to other cultured species. Suitable intensive and semi-intensive hatchery procedures are also targets of development.

Some of the program's accomplishments since its inception four years ago are:

1. International workshops on the reproduction and culture of milkfish, and the control of spawning in teleosts.

2. Produced a State-of-the-Art document summarizing available information on milkfish culture.

3. An effective chronic hormone therapy to influence maturation in milkfish. Administration of a combination of LHRH-a and 0.25 mg 17-MT in long-term release capsules increases the number of female milkfish that complete maturation. When combined with manipulation of photoperiod, maturation of the female can be induced out of season.

4. A chronic LHRH-a + testosterone treatment to accelerate egg growth in striped mullet. A single female can be spawned twice within the same spawning season without apparent loss of egg quality.

5. New hormone implant technology to improve delivery of 17-MT which induces testicular maturation in striped mullet.

6. Correlation between average temperature and daylength over an annual cycle and the percentages of maturing milkfish and mullet. This information provided the critical limits of each environmental parameter at which maturation was initiated naturally, and guidelines of which levels to test experimentally.

7. Description of the fluxes in serum gonadal steroids that naturally accompany ovarian maturation in milkfish and mullet. The profiles indicated the serum levels that chronic treatments mimic. In addition, they are integral to interpreting response to a particular chronic hormonal treatment.

8. Improved method to induce milkfish to spawn in captivity. Spawning can be induced with an acute administration of LHRH-a; this results in a high number of fertilized eggs without the loss of valuable broodstock. A complete description of the female milkfish maturation process was a direct result of this research.

9. Natural spawnings of milkfish broodstock have taken place in two 500 m^2 dirt ponds from June through July, 1988. These spawnings provide a rare opportunity for assessing egg quality of induced- versus naturally-spawned eggs.

10. The most cost-effective method to date for inducing striped mullet to spawn in captivity. During the 1987-88 mullet spawning season, this method induced a record 125 mullet to spawn, and resulted in an estimated total production of 92 million fertilized eggs.

11. Hatchery techniques for mass culture of milkfish larvae. Eight intensive larval rearing trials during the 1987-88 milkfish season resulted in average survival of 32.7% (range: 19-46%). The average density of milkfish juveniles at harvest was 5.8 individuals/liter (range: 4-7 individuals/liter).

12. Improved hatchery techniques for mass rearing of mullet larvae. During the 1987-88 mullet season, average larval survival from 12 intensive larval rearing trials conducted in 5000-liter tanks was 34.3% (range 19-52.2%). The average density of juveniles at harvest was 6 individuals/liter (range: 3-11 individuals/liter). These results are unprecedented in the mass culture of this species.

13. Field testing of developed hormone technologies in the Philippines, Taiwan and Indonesia.

14. Project's research results published and/or presented in refereed journals, newsletters, workshops, and various conferences and/or meetings.

PROJECT RATIONALE

Traditional milkfish aquaculture has been practiced in Southeast Asia for centuries. It is one of the most extensively farmed fish species in the world. An estimated 724 million pounds are produced annually in the Philippines, Indonesia, and Taiwan. At present,

fishponds in Indonesia, the Philippines and Taiwan are 184,600, 176,000 and 15,000 ha, respectively. Per hectare yields of milkfish range from about 360 kg in Indonesia to 2,000 kg in Taiwan. The governments of these countries wish to expand the culture of milkfish to 1) offset protein deficiencies, and 2) increase employment.

Traditional milkfish culture begins with wild-caught fry grown to fingerling stage and stocked in growout ponds which have been organically enriched to improve productivity. The prime market size ranges from 300-400 grams body weight and is usually reached within a year. This method is still widely practiced today. Because of dependence on seed from the wild, the industry is affected by seasonal and annual variations in fry availability. As milkfish culture technology improves, (e.g. deepwater culture system in Taiwan) the demand for milkfish fry increases.

Although demand is difficult to document, reports at the 2nd International Milkfish Aquaculture Conference, Iloilo, Philippines (October, 1983) indicated that approximately 1.6 billion fry were required annually in the Philippines alone. During that year, less than 70% of the required fry were available. The majority of conference participants agreed that the major problem facing the industry was the lack of a consistent milkfish fry supply. If that could be resolved, milkfish aquaculture would provide inexpensive, high quality animal protein to the peoples of Southeast Asia and the Pacific Basin.

A solution to the problem of fry availability would be to develop an artificial milkfish propagation program whose focal point was the control of the species' reproductive processes. Early studies indicated however, that, although extensively cultured, very little was known about the basic biology of this species despite its extensive culture. In addition, traditional practices did not require production of sexually mature and active individuals. The Philippine conference concluded that propagating milkfish in captivity depended on research to control their sexual maturation and spawning. Total production levels could then be improved by extending the spawning season, selective breeding, etc. analogous to terrestrial species. The result would be a continuous supply of seed stock on demand, thereby eliminating dependence on wild stocks.

PROJECT OBJECTIVES

The Milkfish Research Project began in October, 1984, and its primary objective was to develop the ability to mature and spawn milkfish in captivity. Once methods to control reproduction in milkfish were established, development and testing of suitable cost-effective intensive and semi-intensive hatchery techniques were to be pursued (1986). Technologies developed for both reproduction and larval rearing would then be expanded for use with other finfish species. Finally, the feasibility of adapting new technologies to lesser developed countries (LDCs) was to [illegible] mi[illegible].

The project progressed [illegible] timate goals by carrying out specific [illegible]

1) **Workshops:** To [illegible] orkshops to promote exchange of knowledge between world experts on milkfish reproduction and culture, and controlled spawning of fish in general. Workshops were sponsored by USAID and sometimes co-sponsored by other funding agencies. Workshop proceedings were published either as special issues of refereed journals or as books by the Oceanic Institute.

2) **State-of-the-Art Document:** To provide a complete, up-to-date summary of work in milkfish reproduction and culture. The book, published by the Oceanic Institute, was a compilation of chapters written by milkfish experts who were chosen by the senior scientific editor, Dr. C.-S. Lee, in consultation with USAID staff and members of the project's Technical Advisory Group (TAG) Each chapter was refereed by other scientists familiar with the subject matter.

3) **Chronic Hormonal Treatments:** To design long-term hormonal treatments to induce or enhance maturation of male and female milkfish and mullet.

4) **Acute Hormonal Treatments:** To improve the technique of hormonally inducing milkfish and mullet to spawn in captivity.

5) **Enhance Knowledge Concerning The Reproductive Biology and Physiology Of Milkfish And Mullet:** To determine annual fluxes in serum steroid profiles that accompany maturation of both sexes in milkfish and mullet.

6) **Technology Transfer:** To train individuals to produce and administer hormone implants, and assist in field testing the technology in other countries (i.e., Philippines, Indonesia, and Taiwan). Experiment results have been reported and submitted to recognized refereed journals for publication.

The majority of our objectives have been realized and the results are summarized in this report. Field testing

of developed hormonal technologies continues at the Tungkang Marine Laboratory, Taiwan, and the Gondol Research Station, Bali, Indonesia. The identification and manipulation of environmental cues which moderate the onset of maturation are being investigated. These experiments are being conducted by manipulating environmental parameters alone, or in combination with chronic hormonal treatments. Results should provide means of extending (i.e., controlling) the maturation season for both mullet and milkfish. Further work targets development of mass culture systems for mullet and milkfish larvae.

PROJECT MANAGEMENT

A cooperative agreement between the United States Agency for International Development (USAID) and the Oceanic Institute (OI) was formed so that the technical capabilities, facilities, and resources of both organizations could be directed to solving problems associated with controlling reproduction of milkfish. Project performance required substantial involvement by USAID's Bureau for Science and Technology, Office of Agriculture (S&T/AGR) primarily by the Technical Advisory Group (TAG) formed by the S&T/AGR. Members of the TAG include milkfish researchers from Indonesia, Philippines, Taiwan, and others deemed appropriate by S&T/AGR. This group has convened periodically to advise and guide the project manager and S&T/AGR on achieving the goals of the cooperative agreement. Members of the TAG have been:

1. Dr. Lamarr Trott (Senior Advisor, USAID)
2. Dr. Malcolm Gordon (University of California, Los Angeles, UCLA)
3. Dr. John Hunter (National Marine Fisheries Service, California)
4. Dr. Alfredo Santiago (South East Asian Fisheries Development Center, SEAFDEC, Philippines)
5. Dr. I.-C. Liao and Mr. Mao-Sen Su (Tungkang Marine Laboratory)
6. Dr. Fuad Cholik (Research Institute for Coastal Aquaculture, Maros, South Sulawesi, Indonesia)

Specialists in specific fields, such as Dr. Edward D. Houde, University of Maryland, and Dr. Lawrence Crim, Memorial University, Canada, have been invited to participate in the TAG meetings.

It is OI's responsibility to plan, organize and conduct the program's research based on recommendations of the TAG. At regular intervals suggested by the TAG, a group of qualified experts known as the External Evaluation Panel (EEP) is invited to conduct an in-depth analysis of the project to determine its progress, its current direction and offer suggestions for its future.

ACCOMPLISHMENTS TO DATE

* Workshop on the Reproduction and Culture of Milkfish held April 22-24, 1985 at the Tungkang Marine Laboratory, Taiwan and proceedings published by the Oceanic Institute and Tungkang Marine Laboratory, ISBN 0-9617016-1-7, 226 pp.

* State-of-the-Art Document: Aquaculture of Milkfish (*Chanos chanos*). Published 1986, by the Oceanic Institute, HI, USA. ISBN 0-9617016-0-9, 284 pp.

* Singapore Workshop for the Induction of Spawning of Teleosts. Proceedings to be published as a special issue in *Aquaculture*, Vol. 74 (1 and 2), Nov./Dec. 1988.

* Redefined the annual reproductive cycle of milkfish and mullet in captivity:

  Determined steroid profiles that accompany maturation in milkfish.

  Correlated temperature and photoperiod with onset of maturation in milkfish.

  Determined steroid profiles that accompany egg growth and induced final maturation in striped mullet.

  Correlated temperature and photoperiod with the onset of maturation in female striped mullet.

* Development of chronic hormone treatments for influencing maturation in the milkfish and mullet:

  Tested the effectiveness of various LHRH-a and/or 17α-methyltestosterone combinations on the maturation of milkfish.

  Determined the response of ovarian steroids to the various chronic LHRH-a and/or 17-MT combinations.

  Examined the effectiveness of chronic LHRH-a + 17-MT treatment administered at different times prior to the start of the spawning season.

Determined the effect of chronic LHRH-a + testosterone and LHRH-a + 17-MT treatment on egg growth in striped mullet.

Demonstrated the effectiveness of 17-MT administered either orally or by implantation.

* Improved methods used to induce spawning of milkfish and mullet:

  Induced spawning of milkfish with a single application of LHRH-a.

  Defined the stage of maturation most receptive to an acute LHRH-a treatment for the induction of spawning in milkfish.

  Determined the minimum effective dose of LHRH-a.

  Induced spawning of mullet with LHRH-a alone or in combination with carp pituitary homogenate.

  Determined the most cost-effective method of hormonally inducing mullet to spawn.

* Hatchery techniques for the mass culture of milkfish and mullet larvae.

  Developed a mass culture technique for the intensive rearing of mullet larvae.

  Developed hatchery techniques for the mass culture of milkfish larvae.

  Described rates of oxygen consumption accompanying development of mullet embryos and larvae. Assessed abiotic factors (i.e. temperature and salinity) on oxygen consumption.

* Technology Transfer

  Field-tested developed chronic hormonal techniques at stations in the Philippines, Taiwan, and Indonesia.

SUMMARY OF PROGRAM ACTIVITIES

A summary of the activities undertaken to attain the stated objectives is provided in the following sections.

MILKFISH

Workshop: Reproduction And Culture Of Milkfish.

The TAG recommended that world experts in milkfish reproduction and culture be invited to a workshop where they could meet to exchange ideas and information regarding their progress in milkfish culture, and share solutions to some of the problems they had encountered. The goal was accelerated achievement in this area. The invited speakers, their institutions and their presentation subjects follow:

1) Dr. Lawrence Crim, Marine Sciences Research Laboratory, Memorial University of Newfoundland. Methods for acute and chronic hormone administration in fish.

2) Dr. T.J. Lam, Department of Zoology, National University of Singapore. Induced spawning in fish.

3) Dr. Ching-Ming Kuo, International Center for Living and Aquatic Resources Management. A review of induced breeding of milkfish.

4) Dr. Isao Hanyu, Department of Fisheries, Faculty of Agriculture, Tokyo University. Environmental factors in fish reproduction.

5) Dr. Cheng-Sheng Lee, Oceanic Institute. Environmental factors in milkfish reproduction and the preparation of a luteinizing hormone-releasing hormone cholesterol pellet and its implantation in milkfish.

6) Dr. A. Kanazawa, University of Kagoshima. Nutritional factors in fish reproduction.

7) Dr. K. Fukusho, National Research Institute of Aquaculture, Japan. Status of marine larval culture in Japan.

8) Mr. S. Pamplona, Najuan Research Substation, Southeast Asian Fisheries Development Center (SEAFDEC). Milkfish farming in the Philippines.

9) Dr. I.-C. Liao, Tungkang Marine Laboratory. Milkfish culture in Taiwan.

10) Mr. Clyde S. Tamaru, Oceanic Institute. Population structure of the milkfish *Chanos chanos*: past and proposed analysis.

11) Mr. L.T. Lin, commercial fish farmer in Taiwan. My experiences in artificial propagation of milkfish studies on natural spawning of pond reared broodstock.

12) Mr. F.H. Chen, commercial fish farmer in Taiwan. My experience in traditional milkfish culture.

13) Mr. C.C. Chiu, commercial fish farmer in Taiwan. Deepwater pond system for milkfish culture.

The proceedings of this workshop, entitled *Reproduction and Culture of Milkfish*, were published by the Oceanic Institute and the Tungkang Marine Laboratory.

State-Of-The-Art Document

The cooperative agreement between USAID and OI required that the latter prepare a state-of-the-art document to 1) review the biological, economic, social, and geographic base of the milkfish production industry, and 2) provide a complete and current (1985) summary of the species and the industry. The contributors to the document and their topics follow:

1) Dr. Malcolm S. Gordon and Ms. Ly-Quang Hong, University of California at Los Angeles. Biology.

2) Mr. Clyde S. Tamaru, Oceanic Institute. Population Structure.

3) Dr. Cheng-Sheng Lee, Oceanic Institute. Reproduction.

4) Mr. Christopher Kelley and Dr. Cheng-Sheng Lee, Oceanic Institute. Artificial Propagation.

5) Dr. Wade O. Watanabe, Carribean Marine Research Center, U.S.A., Larvae and Larval Culture.

6) Dr. A.C. Villaluz, Aquaculture Dept., SEAFDEC, Philippines. Fry and Fingerling Collection and Handling.

7) Dr. Corazon B. Santiago, Binangonan research Station, SEAFDEC, Philippines. Nutrition and Feeds.

8) Dr. I-Chiu Liao and Ms. Tzyy-Ing Chen, Tungkang Marine Laboratory, Taiwan. Milkfish Culture Methods in Southeast Asia.

9) Mr. Ming-Chen Tung and Mr. Guang-Hsiung Kou. National Pingtung Institute of Agriculture and National Taiwan University, respectively. Pathology.

10) Dr. Yung C. Shang, University of Hawaii, U.S.A. Economic Aspects of Milkfish Farming in Asia.

After peer review, the state-of-the-art document was published by the Oceanic Institute. Copies may be obtained by writing to the Oceanic Institute.

IDRC - Oceanic Institute Workshop On Fish Breeding

A number of projects in Asia have focused on controlling fish reproduction. Many of these programs have received support by either IDRC, International Development Research Centre, Canada or USAID. An IDRC - USAID co-sponsored workshop on fish breeding took place in Singapore, April 7-10, 1987. Presentations encompassed freshwater and marine species from diverse locations (e.g., China, Indonesia, Japan, Malaysia, Philippines, Singapore, Sri Lanka, Taiwan, Thailand, Brazil, Hawaii, and Canada). The objectives of the workshop were to:

1) Review the current status and trends in research on fish breeding in Asia.

2) Compare new and traditional techniques for practicality and cost-effectiveness for a variety of tropical finfish species.

3) Recommend new research approaches and ways to encourage production groups to use experimental breeding technology.

4) Exchange information among researchers and practical fish breeders.

The papers presented at this workshop consisted of reviews and original research which have been peer reviewed for publication in a special issue of *Aquaculture*. A list of the contributors and the titles of their papers are presented below:

1) R.E. Peter et al. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GNRH analogues and dopamine antagonists.

2) K. Aida. A review of hormone changes during spawning in cyprinid fishes.

3) T. B. Saidin et al. Induced breeding techniques practiced on Batu Berendam, Melaka, Malaysia.

4) S. Ngamvongchon et al. Effectiveness of LHRH analogue for the induced spawning of fresh water fish in the Northeast of Thailand.

5) N. Zonneveld et al. Induced breeding with carp pituitary suspension and egg incubation of the Asian catfish, *Clarias batrachus*.

6) J. Carolsfeld et al. Analysis of protocols for application of an LHRH analog for induced spawning of female pacu (*Colossoma mitrei* Berg 1895).

7) W.S.A.A.L. Kumarasiri and P. Seneviratne. Induced multiple spawnings of Chinese carps in Sri Lanka.

8) C.-S. Lee and C.S. Tamaru. Advances and future prospects in controlled maturation and spawning of grey mullet (*Mugil cephalus* L.) in captivity.

9) N. Sherwood et al. Sustained hormone-release: I. Characteristics of *in vitro* release of gonadotropin-releasing hormone analogue (GnRH-a) from pellets.

10) L.W. Crim, N. Sherwood and C.E. Wilson. Sustained hormone release: II. Influence of an LHRH analog implant on plasma LHRH analog profiles and plasma gonadotropin levels in a bioassay model fish, the rainbow trout.

11) J.M. Almendras et al. Sustained hormone release: III. Use of GnRH analogues to induce multiple spawnings in sea bass, *Lates calcarifer*.

12) L.C. Lim et al. Recent developments in the spawning of sea bass, *Lates calcarifer* (Bloch), in Singapore.

13) C.-M. Kuo, et al. Induced sex reversal and breeding of blue-spotted grouper, *Epinephelus fario*.

14) A. Prijino and I.N.A. Giri Natural spawning of milkfish in captivity and its larval rearing.

15) C.L. Marte, L.W. Crim and N.M. Sherwood. Induced gonadal maturation and rematuration in milkfish: Limited success with chronic administration of testosterone and gonadotropin-releasing hormone analogues (GnRH-a).

16) C.S. Tamaru et al. Characterizing the stage of maturity most receptive to an acute LHRH-analogue therapy for inducing milkfish (*Chanos chanos*) to spawn.

Annual Reproductive Cycle Of Milkfish

Until recently, relatively little was known about milkfish reproductive biology and physiology (Kelley and Lee, 1986; Lee, 1986; Marte, 1987). Natural spawnings of captive broodstock have occurred in both sea cages and earthen marine ponds (Lin, 1985; Marte and Lacanilao, 1986). These events are significant achievements in milkfish culture, but the critical parameters which led to them remain elusive (Gordon and Lee, 1986; Kelley and Lee, 1986).

A complete understanding of the reproductive biology and physiology of the milkfish permits effective use of the chronic hormonal treatments that have and will be developed. Clearer descriptions than those presented in the state-of-the-art document were required for environmental parameters (i.e., temperature and photoperiod) under which maturation occurred. In addition, annual fluxes in sex steroids from individual males and females were monitored and correlated with the numbers of individuals maturing in a single spawning season. This information provides insight into the natural levels of serum sex steroids during maturation and the limits of the reproductive season. Its value in understanding the effectiveness of chronic hormonal treatments will be described in a later section.

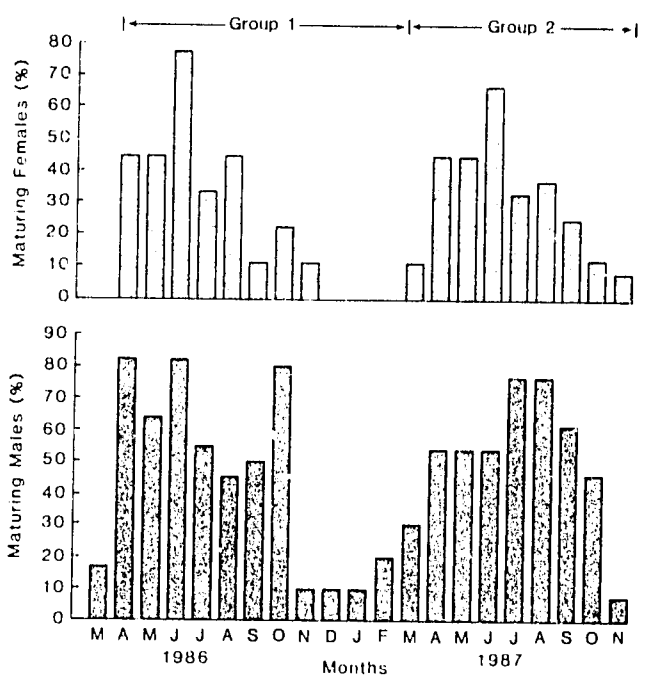

Figure 1. *The percentage of maturing male and female milkfish from groups #1 and #2 during two consecutive reproductive seasons are presented. Group 1 fish consisted of 9 females and 12 males while group 2 fish consisted of 8 females and 5 males. Both groups were kept in different holding facilities. Monitoring of gonadal maturity was done in the same way.*

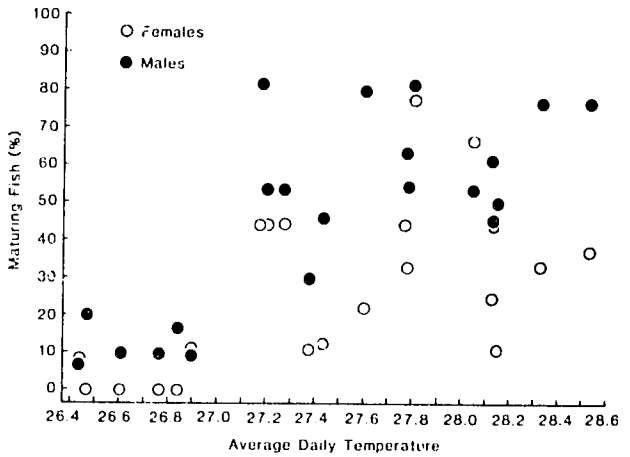

Figure 2. *The percentage of males and females that were maturing is plotted against the observed average monthly temperatures. Maturation of both sexes was positively correlated with the increase in temperature. (r = 0.62 and 0.60 (P<0.05) for females and males respectively.*

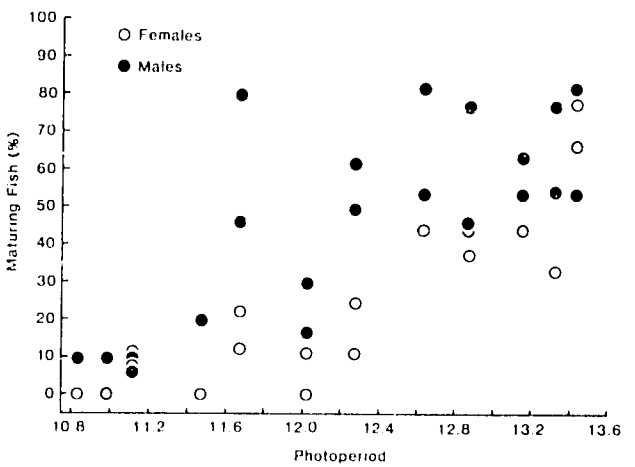

Figure 3. *The percentage of males and females that were maturing versus the calculated daylength observed during the two reproductive seasons is presented. Increases in the percentage of maturing individuals for both sexes were positively correlated with the increase in daylength. (r = 0.75 and 0.86 (P<0.01) for males and females respectively).*

Temperature and photoperiod are the primary environmental factors influencing temporal changes in reproductive activity (De Vlaming, 1974; 1975; Billard and Breton, 1979; Crim, 1982; Wooton, 1982; Lam, 1983). The relative importance of each factor varies between species. Data on fry seasonality, maturation in sea cages, outdoor and indoor tanks (Wainright, 1982; Lacanilao and Marte, 1980; Liao and Chen, 1979; Lee et al., 1987c) indicate maturation of both sexes occurs at temperatures above 20°C (Lee, 1986). The data presented in our studies suggest that maturation began when water temperature exceeded 27°C. The wide range of temperatures at which milkfish have been known to mature suggests that temperature may not be as significant as photoperiod. Higher correlation was obtained between the percentage of maturing fish and fluxes in daylength (see Figs. 2 and 3). Although further study is required to confirm this hypothesis, preliminary results from experiments under artificial lighting conditions support it (Lee et al., 1987c).

Steroid profiles from captive males and females of this species suggest a much broader reproductive period than was previously reported for Hawaiian waters (Kuo and Nash, 1979). Estradiol 17β (E_2) and testosterone levels from females showed multiple peaks from the same individuals during a single reproductive season (see Fig. 4). These peaks reflect the species' ability for multiple spawning within a season, (Lee et al., 1986a). Serum E_2 and testosterone, however, were poorly correlated with the percentage of females maturing during the same period. This suggests lack of synchrony in maturation between females during the reproductive season. Lowest levels of serum E_2, testosterone, and 11-ketotestosterone in males occurred from November through February, delineating a period of reproductive inactivity.

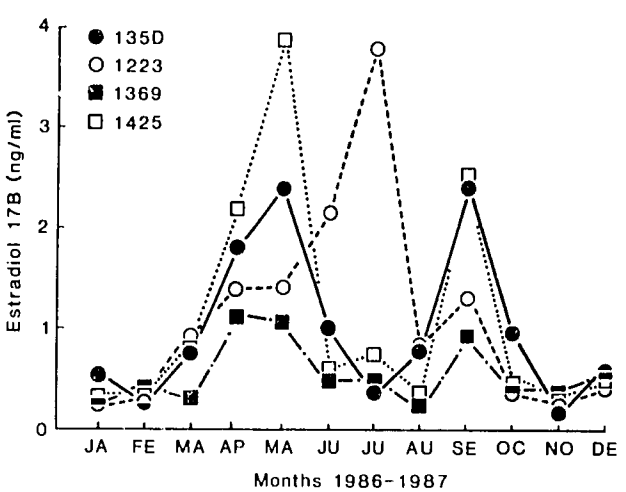

Figure 4. *The monthly changes in E_2 observed from four individual group #1 females during the 1986-1987 season are represented. The observed peaks and valleys in serum E_2 coincide with the increase in the percentage of maturing females. Multiple peaks observed in the same females suggest that the milkfish is a multiple spawner. No significant differences in E_2 levels could be detected between individuals but there were significant variation (P<0.01) detected between the months sampled.*

Influence Of Chronic Administration Of LHRH-Analogue And/Or 17α-Methyltestosterone On Maturation In Milkfish, *Chanos chanos*

Hormonal induction of the maturation process requires a prolonged period (weeks to months) during which circulating levels of the administered hormone or hormone analogue must remain elevated. Until recently, the only effective method to chronically administer the hormone or analogue of choice was by multiple injection (Yamamoto et al., 1974; Lam, 1982). Initial attempts to induce maturation in captive milkfish using similar strategies were unsuccessful (Lam, 1982; Lacanilao et al., 1985). Handling stress can reportedly compromise the efficacy of any hormone treatment (Billard et al., 1981; Lam, 1984).

Improvements in slow hormone-releasing devices provide an alternative means of chronically administering hormones with minimum trauma (Crim, 1985). Luteinizing hormone-releasing hormone analogue (LHRH-a) cholesterol implants and silastic tube capsules containing steroid were used in research to find a chronic hormone therapy to trigger or enhance the maturation of captive milkfish (Lee et al., 1986b). Examples of the implanter and of the hormone pellets are presented in Plate II.

The effectiveness of the LHRH-a cholesterol pellet produced at the Oceanic Institute was tested on landlocked salmon. With the assistance of Dr. Lawrence Crim, Memorial University of Newfoundland, Canada, serum gonadotropin was assayed in landlocked salmon implanted with cholesterol pellets containing LHRH-a from Sigma Chemical Company, and from China. Two dosages, 100 μg and 200 μg, were tested. The results demonstrated that the LHRH-a cholesterol implants effectively elevated serum gonadotropin levels for an extended period of time, presumably by the chronic stimulation of its release from the pituitary. Both dosages tested indicated that the implant was effective for at least 4 weeks (see Fig. 5).

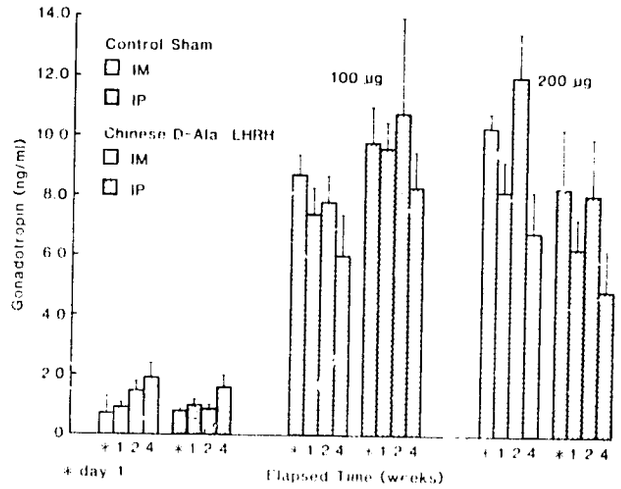

Figure 5. *Serum gonadotropin levels in rainbow trout in response to a sham or a LHRH-a cholesterol implant. IM = intramuscular, IP = intraperitoneal denote locations in which implants were administered.*

The release of steroids from the silastic implants were tested both *in vitro* and *in vivo* on juvenile milkfish. The results demonstrated that a single silastic implant could effectively elevate serum steroid levels for an extended period of time. Implant release rates are affected by several factors, e.g., the type of steroid contained in the implant (see Fig. 6). The release profiles for all steroids tested were very similar, however. The data also demonstrated that serum steroid levels result from implant release and metabolic clearance.

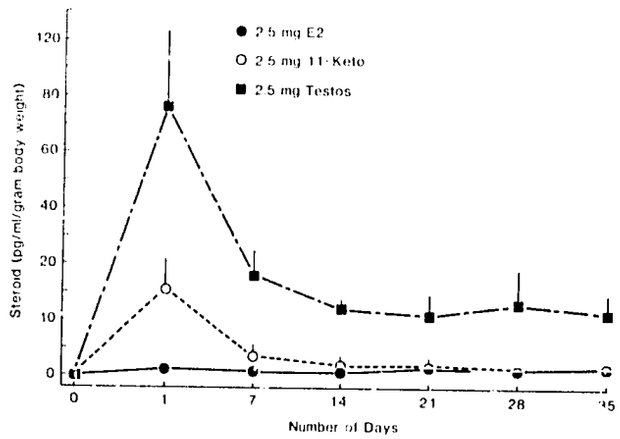

Figure 6. *Three serum steroid profiles resulting from intramuscular implants of the same dosage. In vivo profiles were obtained from implanted juvenile milkfish. Each point represents the average value from five fish. The bars designate standard errors.*

The five chronic hormone therapies tested were: cholesterol pellets containing 200 μg of LHRH-a (LHRH-a pellet); tubing packed with either 250 μg of dissolved 17α-methyltestosterone (liquid 17-MT capsule) or 10 mg crystalline 17α-methyltestosterone (crystalline 17-MT capsule); the combinations of LHRH-a pellets plus a liquid 17-MT capsule; or LHRH-a pellets plus a crystalline 17-MT capsule. Experimental groups of 20 milkfish each received one of these five therapies. A sixth control group received placebo implants. Results suggested that the combination of LHRH-a pellet + (0.25 mg) 17-MT capsules was the most effective hormone therapy (Fig. 7).

The combination of LHRH-a and 17-MT, at 10 mg and 0.25 mg respectively, appears to enhance maturation of milkfish. The combination of LHRH-a pellet + 0.25 mg 17-MT capsule induced maturation of males earlier and maintained their sperm production for the duration of the experiment. This, coupled with the number of females that matured and the times at which they did so, clearly established this treatment as the most effective.

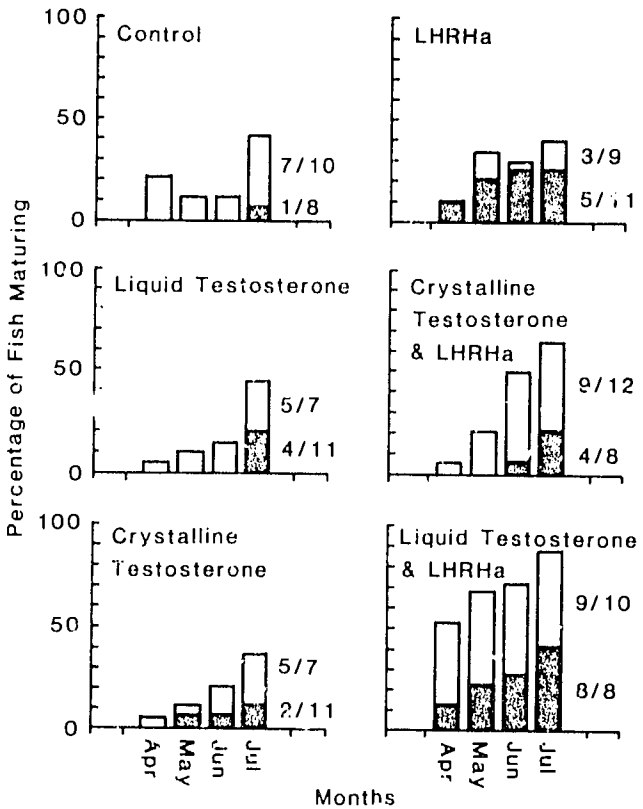

Figure 7. *The percentage of maturing male and female milkfish in response to various LHRH-a and/or 17-MT combinations is presented. Solid bar indicates females and blank bar indicates males. Fractions represent the number of mature fish to the total number of sexed fish observed at the end of the experiment (i.e., July).*

The following season (1986), the effectiveness of the chronic LHRH-a + 0.25 mg 17-MT therapy was re-evaluated using a large number of individuals. Milkfish response to hormone therapy when treatment was initiated at different times prior to the spawning season was also investigated. In this study, 100 milkfish were divided into two groups. In each group, 40 individuals were chronically exposed to the LHRH-a + 17-MT therapy and the remaining 10 were implanted with placebos. The first group (Experiment I) was implanted in March, 1986; the second (Experiment II) in April, 1986.

There was no significant difference between the percentage of males that matured in the treated and control groups. However, a significantly higher percentage of hormone-implanted females matured by the onset of the natural spawning season (Fig. 8).

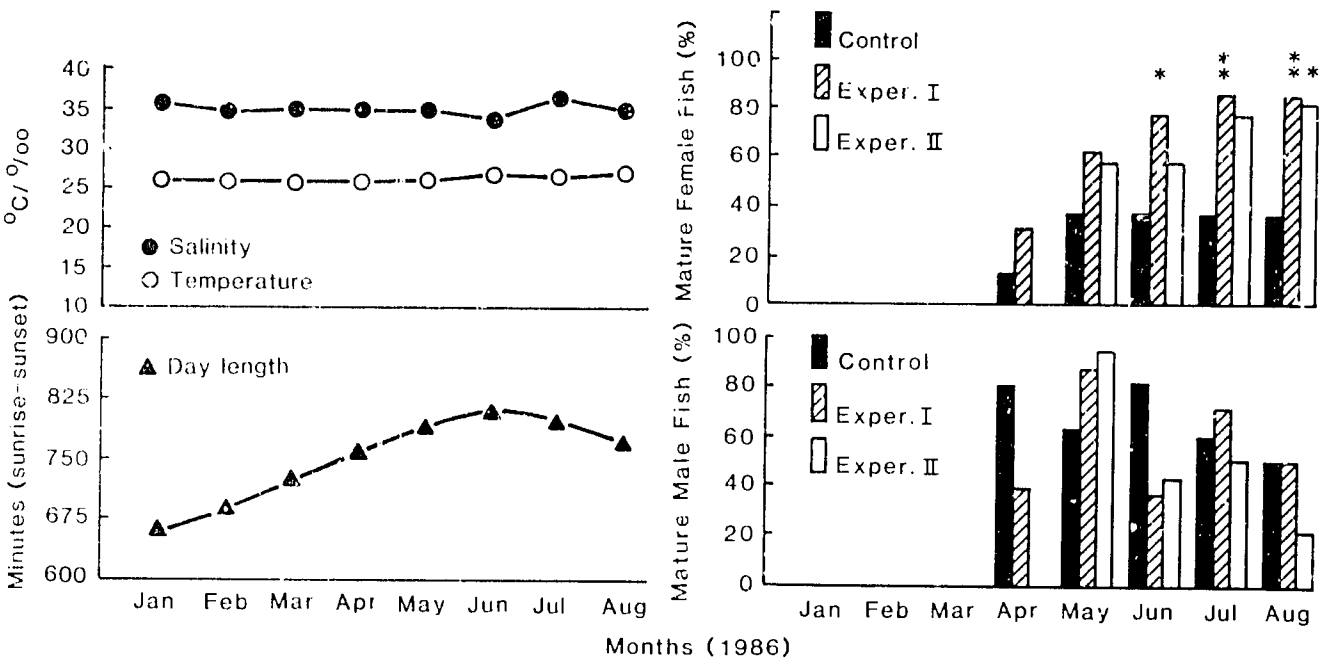

Figure 8. *The observed salinity, temperature, photoperiod and cumulative percentage of females and males observed during the 1986 maturation study. Experiment I was initiated on March 4, 1986 and Experiment II was initiated on April 2, 1986. The only difference between the two experiments was when treatment was initiated.* * = $p<0.05$, ** = $p<0.01$.

Eighty-five percent of the hormone-implanted females compared to 33.3% of the placebo-implanted females reached the maturation stage at which induced spawning trials could be initiated. These results confirmed the treatment's effectiveness for stimulating female maturation; further investigation on stimulating male maturation was required.

The data also suggested that individual response to therapy varies seasonally. Chronic exposure to the hormonal therapy appeared more effective when initiated near the start of the natural breeding season. This indicated that combining chronic hormonal treatments with environmental manipulation (i.e. photoperiod) may effectively induce maturation out of season. Initial investigations have been encouraging. When milkfish were implanted with the most effective chronic hormonal treatment and subjected to a long photoperiod from October through February, maturation of the female was successfully induced, but only a partial response by the males was achieved (Fig. 9).

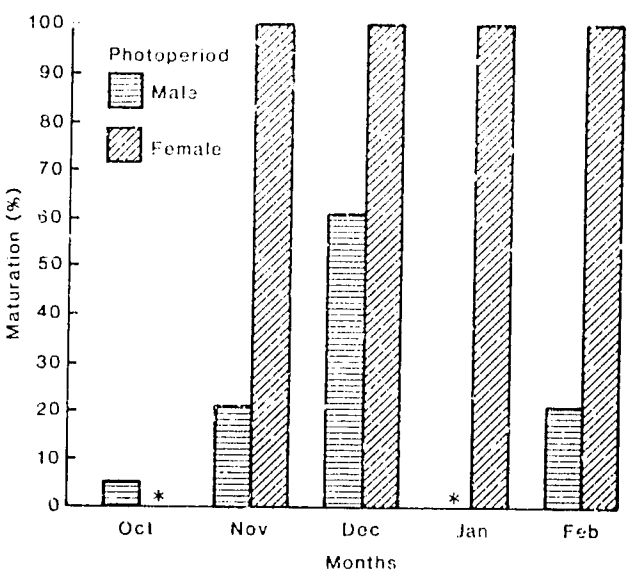

Figure 9. *The response of male and female milkfish exposed to a long daylength and administered LHRH-a + 17-MT implants is presented. Treatment was initiated in October which is naturally a period of gonadal inactivity. Female maturation was induced successfully, however, only a marginal response was achieved with males. * = zero percent maturation. No maturation of either sex was observed in controls.*

Three factors thought to contribute to the treatment's effectiveness are: 1) The long-term release of the respective hormones circumvents handling stress associated with old techniques of chronically administering exogenous chemical messengers; 2) The combination of the two hormones is important. 17-MT has a stimulatory effect on the pituitary in producing gonadotropin(s) in fish (Crim & Evans, 1979, 1982, 1982). This effect, combined with the actions of LHRH-a, provides a continuous release of the native gonadotropin(s) at the elevated circulating levels required for a response by the gonad; 3) As suggested by the different response of both sexes to the LHRH-a + 0.25 mg 17-MT, as opposed to LHRH-a + 10 mg 17-MT, an optimal titer for both steroid and gonadotropin(s) in the blood is required for maturation (Figs. 10 and 11).

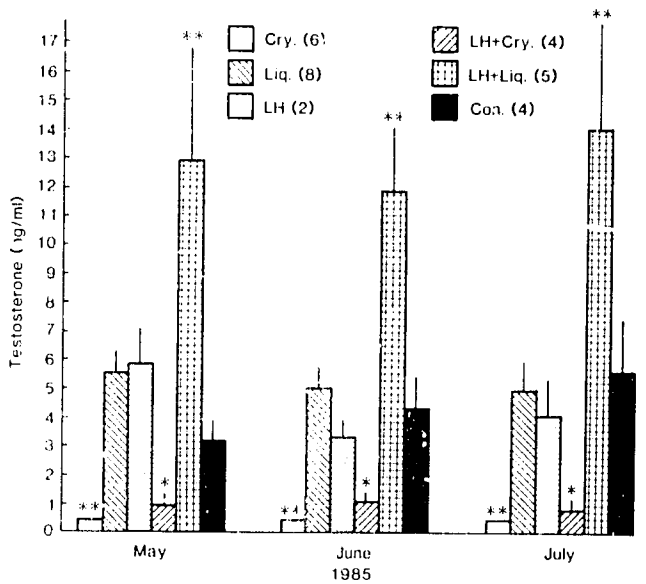

Figure 10. *Average monthly serum testosterone values from females undergoing the various LHRH-a and/or 17-MT treatments during 1985. The number in parentheses represents the number of individuals sampled and the bars represent the SEM. Significant variation is detected between treatments (P<0.001) but not between the months sampled.* * = p<0.05, ** = p<0.01 versus controls.

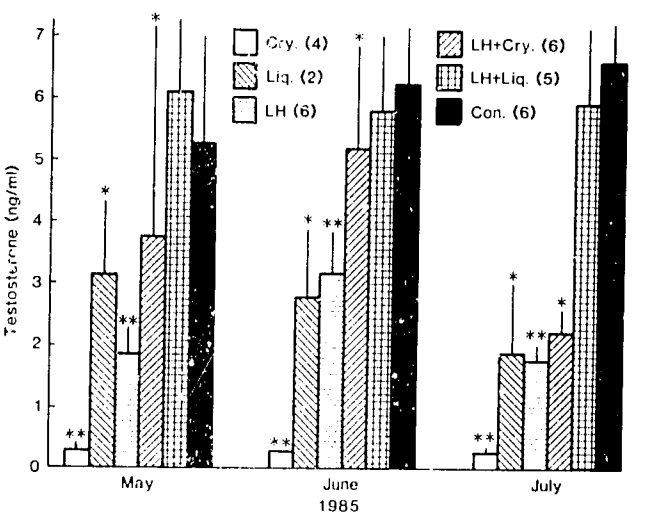

Figure 11. *Average monthly serum testosterone levels from male milkfish undergoing various chronic LHRH-a and/or 17-MT treatments during 1985. Numbers in parentheses represent the number of males sampled and the bars represent SEM. No significant differences are observed between the months in which values were obtained but there is significant (P<0.001) variation between treatments.* * = p<0.05, ** = p<0.01 versus controls.

The dosage of the LHRH-a pellets was the same, but the amount of steroid used in silastic capsules was different. Both sexes of milkfish exposed to the high dose (10 mg) of 17-MT, administered alone, or in combination with LHRH-a, responded with a substantial decrease in circulating levels of sex steroids. Negative feedback by 17-MT may compromise the efficacy of the hormone combination, particularly at the higher dosage. The 0.25 mg 17-MT dosage appeared appropriate because no significant differences were found when comparing the serum steroid profiles of testosterone and E_2 accompanying oocyte growth of treated and untreated females (Figs. 12 and 13).

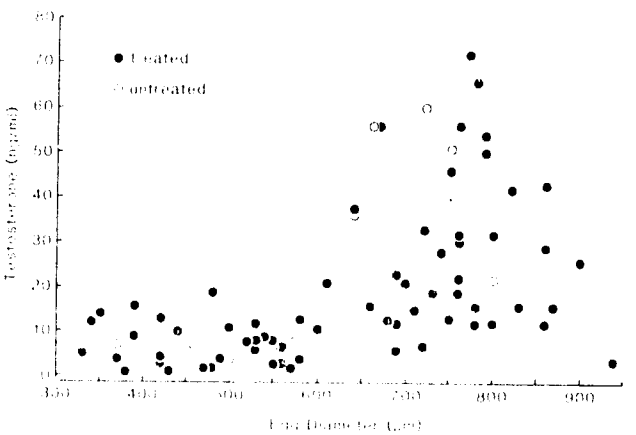

Figure 12. *Serum testosterone values from control and LHRH-a + 0.25 mg treated females plotted against the average egg diameters which they possessed when sampled. After log transformation of the data, no significant differences can be found between treated and control levels.*

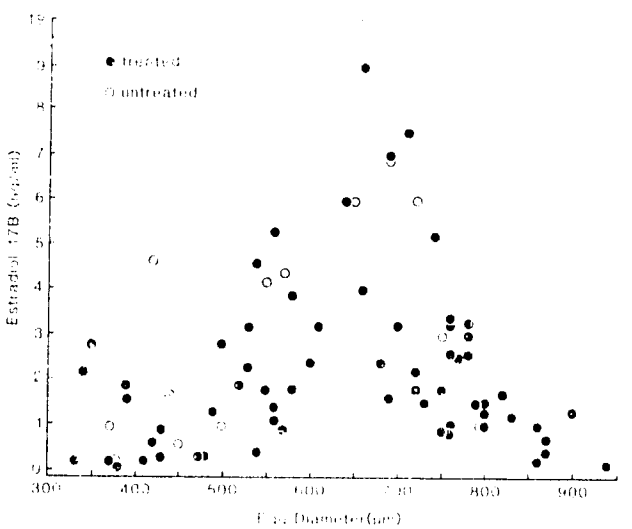

Figure 13. *Serum E_2 levels from control and LHRH-a + 0.25 mg 17-MT treated females plotted against the average egg diameters that they possessed when sampled. After log transformation of the data, there are significant differences ($P < 0.01$) in E_2 observed between egg sizes, but no differences could be detected between the treated and untreated females.*

The treatment of LHRH-a + 0.25 mg 17-MT is far superior in stimulating maturation of female milkfish, which suggests that at the 0.25 mg dosage, it is still stimulatory at the level of the pituitary, but is not inhibitory at the level of the ovary.

Induced Spawning Of Milkfish, *Chanos chanos*, By A Single Application Of LHRH-analogue.

A standardized method to induce milkfish to spawn has eluded investigators for over a decade (Lam, 1984; Kuo, 1985). Initially, spawning attempts involved administering piscine pituitary extracts (salmon or carp), plus human chorionic gonadotropin (HCG) (Vanstone et al., 1977; Liao et al., 1979; Juario et al., 1979; Kuo et al., 1979). More recently, HCG alone has been used to induce final maturation of ova (Tseng & Hsiao, 1979; Lin, 1982; 1984). In all past attempts, however, fertilized eggs were obtained by manually stripping females and ripe males for their gametes. This usually resulted in loss of broodstock, a single spawning during a season, and low fertilization (0-60%). These factors clearly emphasized the need for a more reliable method to induce milkfish to spawn.

The discovery and characterization of the peptide that controls the synthesis and release of gonadotropins by the pituitary in mammals has provided a new method to control reproductive activities of fishes (Lam, 1982; Donaldson & Hunter 1983). This peptide, luteinizing hormone-releasing hormone (LHRH), and its superactive analogues (LHRH-a), have been used to induce ovulation in several fish species (Breton & Weil, 1973; Hirose & Ishida, 1974; Lam et al., 1975; Chan, 1977; Crim & Gebe, 1984; Fitzpatrick et al., 1984; Barnabe & Barnabe-Quet, 1985). When using any hormonal treatment two critical points must be identified: 1) the maturation state at which the individual will respond to a particular therapy and 2) the appropriate hormone and dosage to be administered. These factors were investigated in developing an acute LHRH-a therapy for inducing milkfish to spawn.

The stage at which a female will respond to a single administration of LHRH-a can be crudely estimated with average egg diameters determined from cannulated oocytes. This stage is determined when average egg diameters are ≥ 750 μm and oocytes distributed by size exhibit a single mode. If there are two modes, the mode with smaller ova should not exceed 400 μm (Figs. 14 and 15).

How and when this stage appears is described in the following sections.

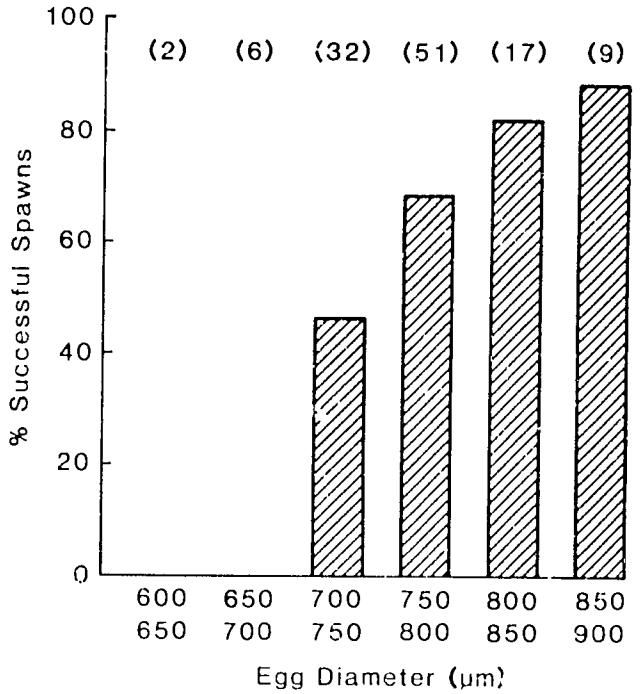

Figure 14. *The rate of spawning success compared to the average oocyte diameter that a female possessed when given a single injection of LHRH-a. The number in parentheses represents the number of spawning attempts.*

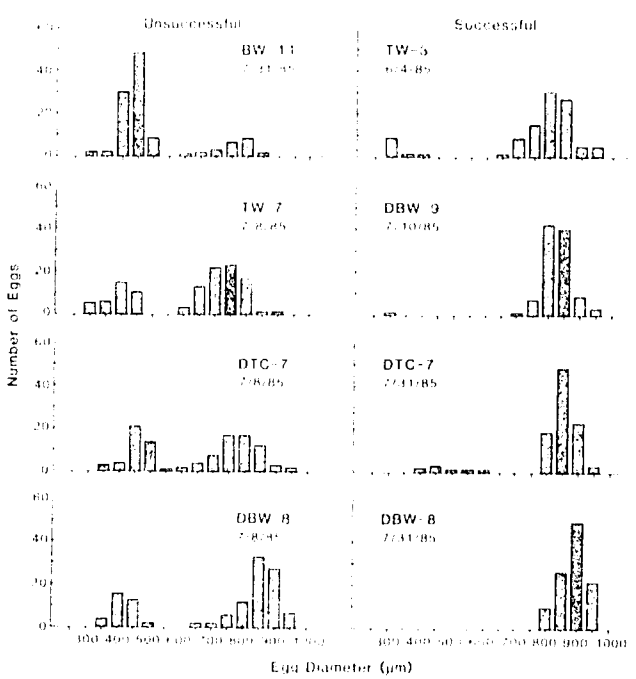

Figure 15. *The observed size frequency distribution from female milkfish in which final maturation and spawning were attempted utilizing a single LHRH-a (250 ug) injection. Successful spawns are presented in the right column and unsuccessful spawns are in the left column.*

The milkfish's ovary undergoes a series of group synchronous type maturations (Wallace and Selman, 1981). At some point, the ovary possesses a fairly uniform clutch of large oocytes and a more variable clutch of smaller oocytes from which larger eggs are recruited. It takes approximately a month for a clutch of eggs to develop (Fig. 16).

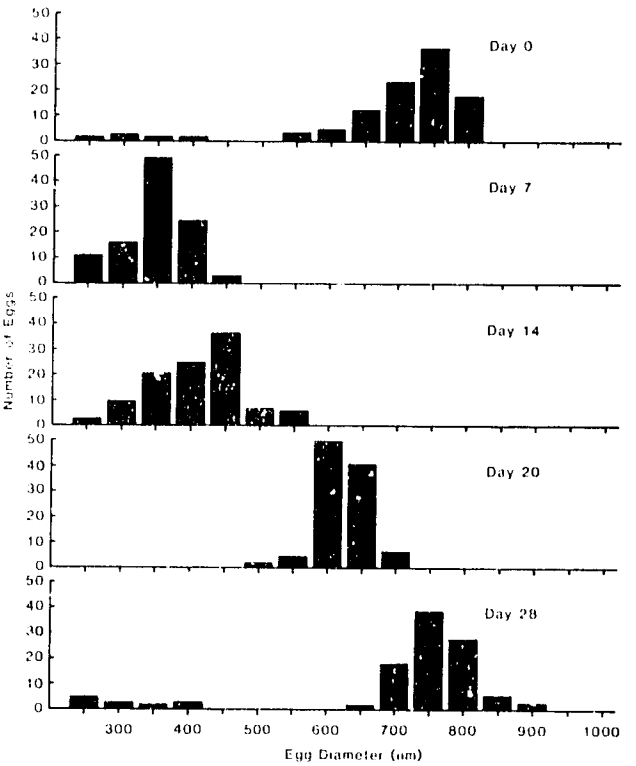

Figure 16. *Changes in the size frequency distribution of cannulated oocytes obtained from a female milkfish (#152E) over a period of 28 days. Note the reappearance of a bimodal size frequency distribution at the latter part of the sampling period.*

The *in vivo* pattern of oocyte growth is consistent between and within individuals. A predictive model of egg growth, i.e., egg size = 359.077 + 28.796 (days) - .475 (days^2) was formulated from the current data and validated with growth rates from a 1985 study (Fig. 17).

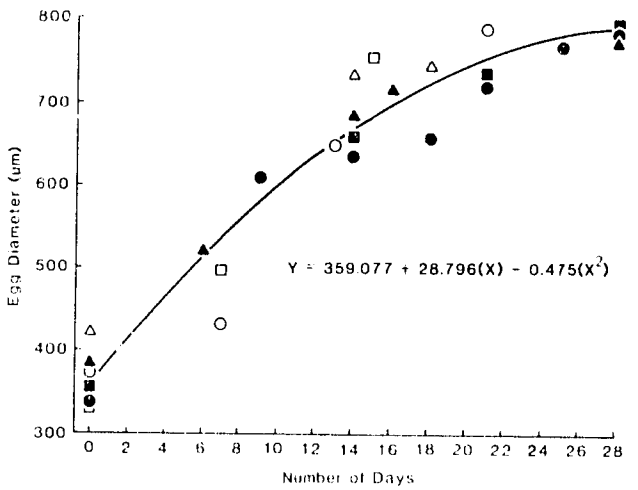

Figure 17. *The predicted growth curve and the observed growth rates of oocytes from two individuals (#152E and #167A) (r2 = 0.942). The growth data were obtained from three successive maturations from each female during the 1986 spawning season.*

The formula is a quadratic relationship that has an asymptote at approximately 800 µm.

Histologically, the initiation of the vitellogenic stage is characterized by the presence of primary yolk granule stage oocytes (Tan, 1985).

This is followed by the accumulation of yolk, the major activity accompanying oocyte growth and a consistent feature of egg maturation in teleosts (Wallace and Selman, 1981). Examples of milkfish oocytes during their growth phase are shown in Plate 5.

Changes in serum levels of E_2 and testosterone are highly correlated with egg growth. The steroid profiles observed at the end of egg growth are consistent with those indicating the cessation of vitellogenesis: the decrease in serum levels of E_2 and the concomitant increase in testosterone (Fig. 18).

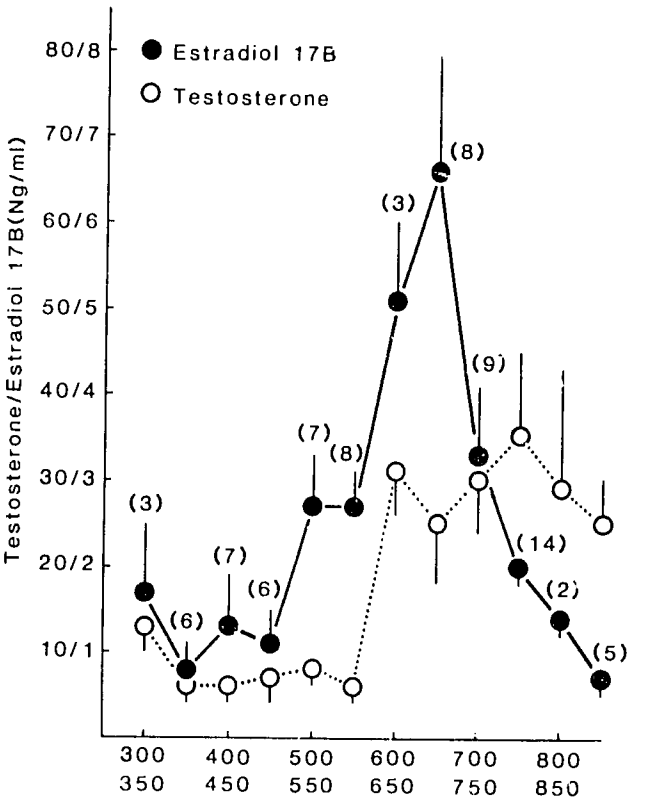

Figure 18. *E_2 and testosterone profiles that accompany the growth of oocytes in the milkfish. Numbers in parentheses represent the number of observations within a 50 µm grouping of oocytes. The bars represent SEM.*

Information on ovarian maturation indicates a critical period of about a week when the probability of success of induced spawning with LHRH-a is optimal. The data indicate that this period represents completion of vitellogenesis. The eggs exhibit no growth and the serum steroid profile is consistent with those observed from several other teleosts. All induced spawnings were successful in this period whether the eggs had just reached the 750 µm average egg diameter or remained at this stage for five days.

Dosage

The spawning success rate was compared to thirteen 5 µg/kg body weight dosage classes administered. Various dosages of LHRH-a were also administered to males to determine if fertilized spawns were dose dependent. The minimum effective dose of LHRH-a ranges from 1-5 µg/kg body weight (Fig. 19).

MILKFISH

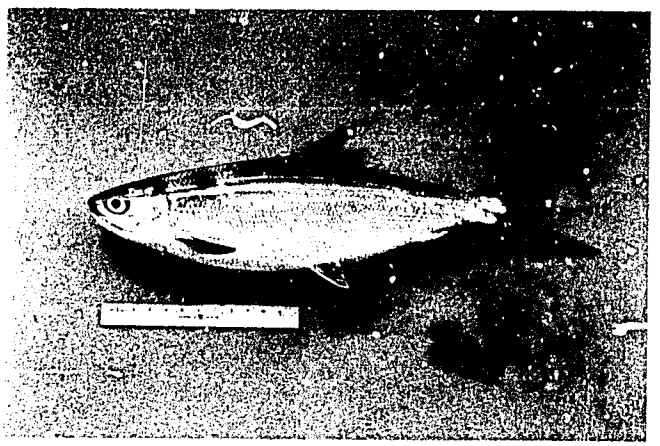

Plate 1. *A typical milkfish broodstock specimen used in experiments on controlling maturation and spawning.*

Plate 3. *Gregory Weber is about to use a polyethylene canula inserted through the milkfish's oviduct to retrieve an ovarian biopsy for staging the maturational stage of the ovary.*

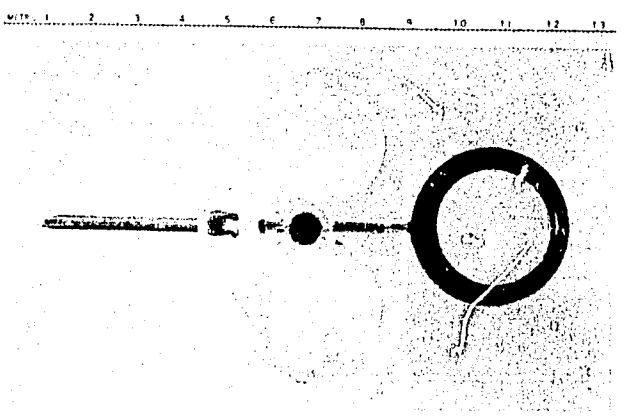

Plate 2. *The device used to implant the cholesterol and silastic tubing implants used in the experiments. This type of implanter was originally used to implant E_2 pellets into the necks of chickens. The cholesterol implant is made to fit into the bore of the needle of the implanter.*

Plate 4. *Clyde Tamaru and Jessie Banno are obtaining a blood sample from the caudal vein of a milkfish. The blood sample will be used to determine circulating serum gonadal steroid levels that will aid in evaluating a hormonal treatment.*

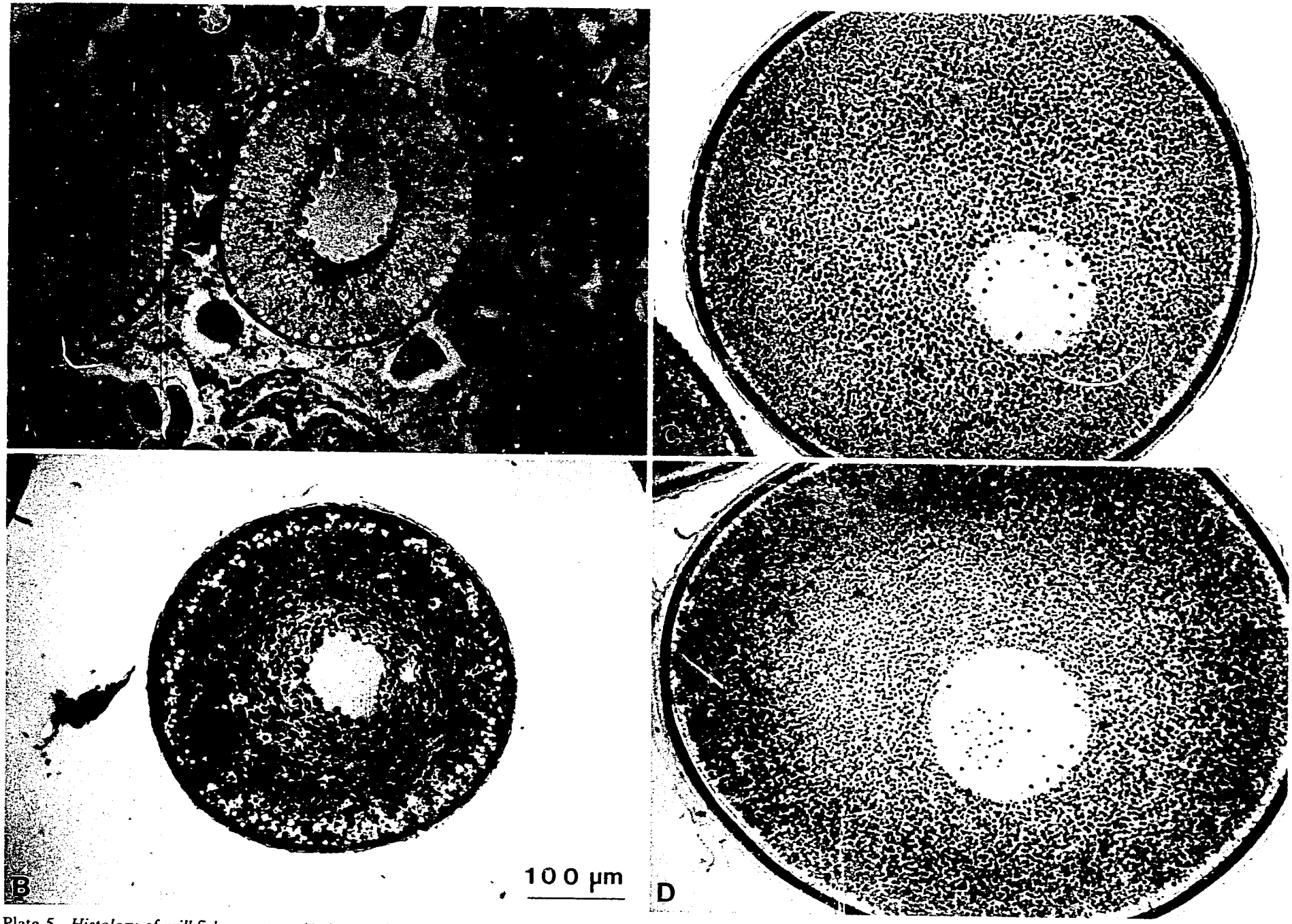

Plate 5. *Histology of milkfish oocytes.* *A) A cannulated ovarian sample showing primary yolk globule and perinucleolar stage oocytes. Stained with Mallory's triple stain. 40X.*
B) A 400 µm oocyte exhibiting incorporation of yolk into discrete yolk globules. Stained with Mallory's triple stain. 40X.
C) Tertiary yolk globule stage oocyte that is 600 µm in diameter. Stained with Mallory's triple stain. 40X.
D) An oocyte taken from a female just prior to receiving an injection of LHRH-a. The tertiary yolk globule stage oocyte is 750 µm in diameter. Stained with Mallory's triple stain. 40X.

MULLET

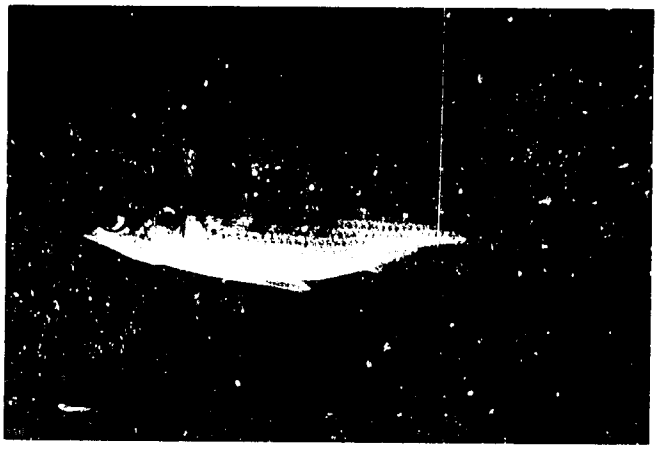

Plate 6. *A specimen of the striped mullet, Mugil cephalus.*

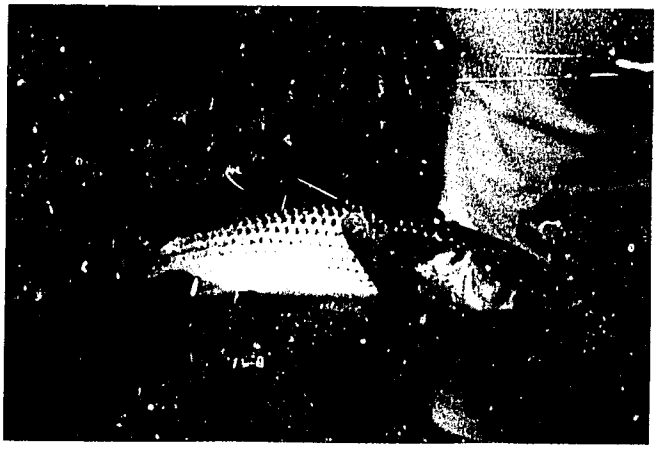

Plate 8. *A chronic hormonal treatment about to be administered to a striped mullet.*

Plate 7. *A striped mullet being scanned for its identification number. The intramuscular tag provides for a permanent means of identification and allows investigators to follow an individual's performance.*

Plate 9. *A spermiating male striped mullet that has resulted from implants of 17-MT.*

Plate 10. *Invited speakers and workshop organizing committee members of the Reproduction and Culture of Milkfish workshop held between April 22-24, 1985 at the Tungkang Marine Laboratory, Taiwan. See the workshop proceedings for names of participants.*

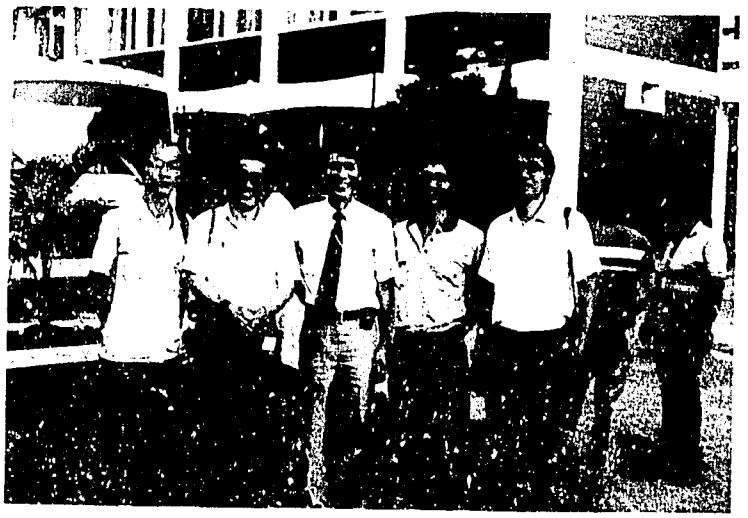

Plate 11. *People associated with the milkfish program at Tungkang Marine Laboratory, Taiwan are, from left to right, Mr. Robin Chen, Dr. I.-C. Liao (director of TML), fourth from left is Dr. C.-S. Lee (principal investigator, Oceanic Institute), and Dr. M. S. Su (present director of TML).*

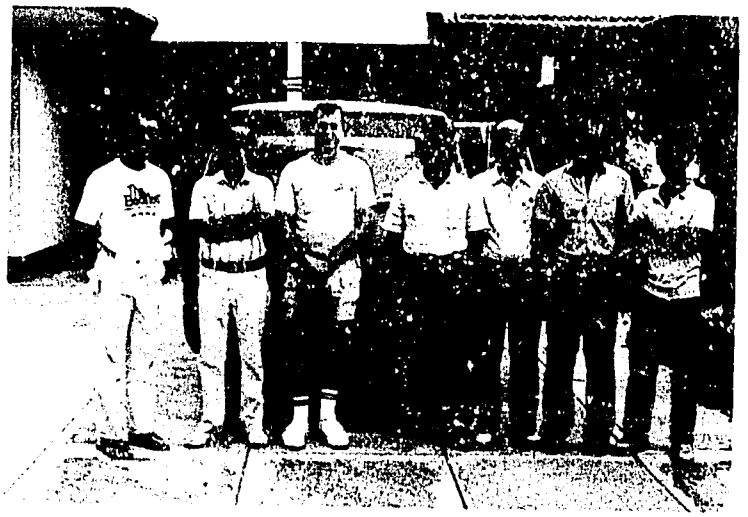

Plate 12. *People associated with the milkfish program at the Gondol Research Station, Bali, Indonesia are Dr. Fuad Cholik (second from left), Dr. Lamar Trott, USAID Senior Advisor (third from left), Mr. Zafril Iman Azwar (fourth from left), Mr. Agus Prijono (fifth from left) and Mr. Gede S. Sumiarsa (seventh from left).*

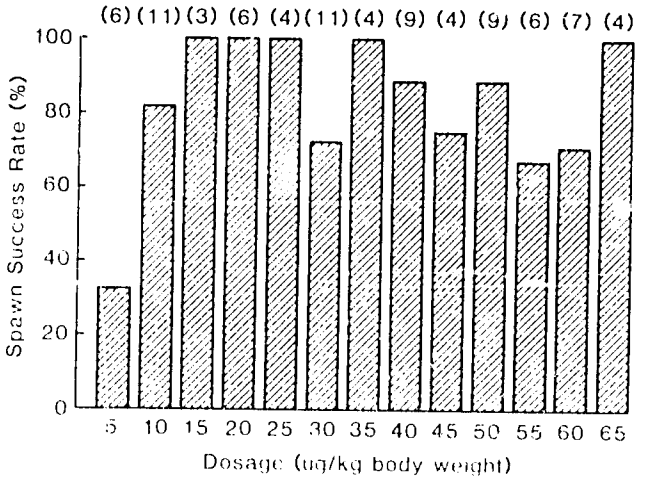

Figure 19. *The observed spawning success rate in response to varying dosages of LHRH-a. The number in parentheses represent the number of spawning attempts. The minimum effective dosage of LHRH-a to induce spawning in milkfish ranges between 1-5 µg/kg bodyweight.*

Within the range of dosages tested (1-65 µg/kg body weight), there were no significant effects on fecundity, fertilization rates, spawned egg diameters, or number of fertilized spawns. Dosages administered to male milkfish (20-65 µg/kg body weight) were not correlated with the occurrence of fertilized spawns.

Obtaining Fertilized Spawns Without Stripping

Initially, only females received injections. The spontaneous release of eggs was achieved in all spawning attempts when treatment was initiated at the stage of maturity previously described. Spawning occurred but fertilization was sporadic, even in the presence of running males. In these cases, the females dribbled their eggs over a period of hours, a phenomenon routinely observed in past spawning attempts (Lam, 1984; Kuo, 1985), and used as a cue to begin stripping eggs from the female (Juario & Duray, 1983; Juario et al., 1984; Lam, 1984).

In an attempt to improve the number of fertilized spawns, males and females were injected at the start of an induced spawning experiment. The treated fish were then isolated from nontreated fish. Natural fertilized spawns were achieved in seven of 15 attempts and resulted in an average of 83.8% fertilization. The spawning data are summarized in Table 1 found in the Appendix.

Possible explanations for the fertilized spawns are that 1) both males and females were synchronized with LHRH-a injections, and/or 2) the treated fish were isolated in a holding facility that does not inhibit their behavior but concentrates the visual and/or pheromonal cues that lead to synchronizing the mating process. The smallest volume of water in which a fertilized spawn occurred was 5000 liters. The practice of selectively spawning treated individuals in a tank of this size is a feasible hatchery design. Although results are encouraging, even when spawning conditions are optimal, fertilization presently occurs in only 50% of the spawns. Work is in progress to establish means to increase the number of induced fertilized spawns.

STRIPED MULLET

The striped mullet, *Mugil cephalus*, is a foodfish cultured in many areas of the world (Nash and Kuo, 1975; Nash and Shehadeh, 1980). Because of its economic importance, extensive research has been conducted on controlling reproductive stages, such as the hormonal induction of final maturation and spawning (Tang, 1964; Liao et al., 1971; Kuo et al., 1973; 1974a; Kuo, 1982; Lee et al., 1987a; 1987b), environmental induction of female maturation (i.e. vitellogenesis) (Kuo et al., 1974b); and hormonal induction of spermatogenesis and spermiation (Weber and Lee, 1985; Lee and Weber, 1986). Although mullet is extensively cultured, little is known about its reproductive biology and physiology. Like milkfish, such information is essential for efficient use of hormonal treatments to stimulate the reproductive processes.

In teleosts, temperature and photoperiod are the two most important environmental parameters which moderate activity along the hypothalmo-pituitary-gonadal axis (Billard and Breton, 1979; Crim, 1982; Wooton, 1982; Lam, 1983). The relative importance of each factor may vary by species. It has been reported that photoperiod was important for initiation of mullet maturation while temperature was important for the completion of vitellogenesis (Kuo et al., 1974). However, minimum daylength and temperatures that stimulate the onset of vitellogenesis have yet to be defined. Correlating fluxes in daylength and temperature with the onset of maturation indicates that when daylength falls below 11.3 hours, female mullet begin to possess vitellogenic oocytes (Fig. 21). A high degree of correlation is observed between the decrease in temperature and the increase in egg growth. Females begin to mature naturally when average daily temperatures fall below 27°C (Fig. 20). It is therefore apparent that previous work manipulating environmental parameters to induce maturation (Kuo et al., 1974) needs to be re-evaluated. The highest temperature employed in those experiments was 26°C, within the range that mullet mature naturally.

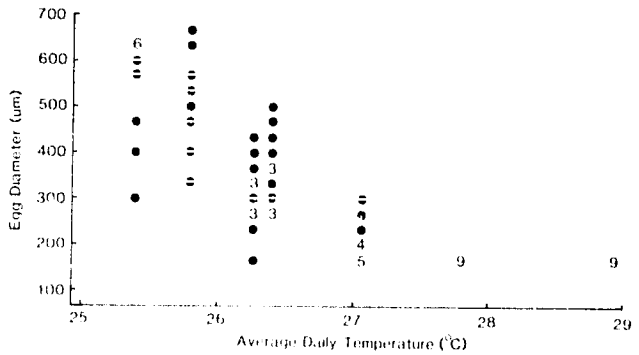

Figure 20. *Correlation between the observed change in average egg diameters from mullet females and the average daily water temperature during between September, 1986 - March, 1987.*

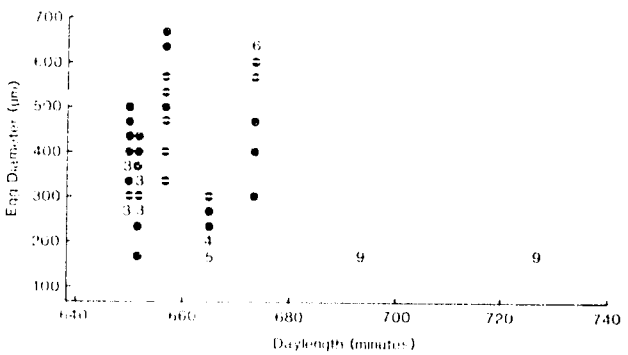

Figure 21. *Correlation between calculated daylength and the observed changes in egg diameters from mullet females between September, 1986 - March, 1987.*

E_2 and testosterone serum profiles were highly correlated to egg growth. Serum profiles from mullet were similar to those of most teleosts that have been examined. Serum E_2 increases as oocytes grow in size and begins to fall at completion of vitellogenesis (Fig. 22). In contrast, testosterone also increases with egg growth but continues to increase as the oocytes reach their peak in size. Serum E_2 levels are relatively high when treatment for induced spawning is initiated, a possible reason that hormone dosages required for mullet spawning are among the highest reported. Alternatively, the high serum testosterone levels that accompany the completion of vitellogenesis may be an internal signal of the state of ovarian maturation.

That serum E_2 levels of mature and immature females are not significantly different at the very early stages of egg growth is understandable because of the key role E_2 plays in the vitellogenic process (Fig. 23). However, failure in maturation may occur at the level of the pituitary or at the ovary and cannot be differentiated by the E_2 levels alone. This information is still useful because it provides insight to the levels at which various hormones might be used to stimulate maturation.

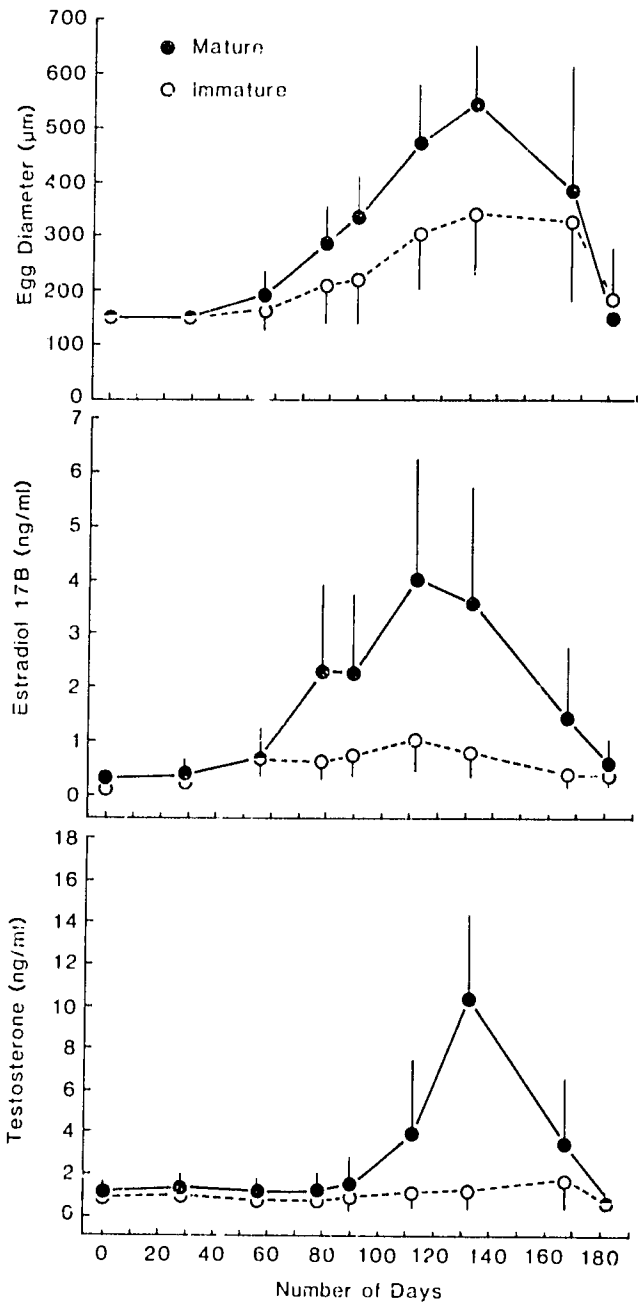

Figure 22. *Egg growth, E_2, and serum testosterone profiles from maturing females and also from females that did not complete vitellogenesis. Symbols are means and bars are SEM. Sampling of the female mullet was conducted between September, 1986 - March, 1987. Mature group N = 13; Immature group N = 8.*

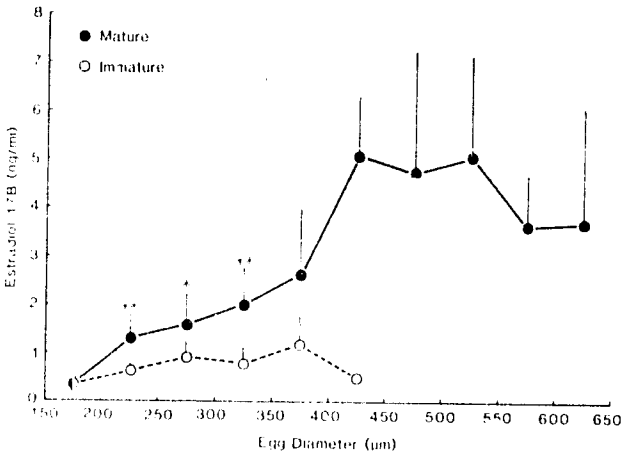

Figure 23. *Relationship between E_2 and average egg diameter possessed by female mullet that completed vitellogenesis (mature) and from those that did not mature. Symbols are averages and bars represent SEM. * = $p<0.05$, ** = $p<0.01$.*

Effects Of Chronic Hormonal Treatment On Egg Growth In The Mullet

The effects of LHRH-a + testosterone (T) or LHRH-a + 17-MT implants on maturation of female striped mullet were investigated with the same chronic release implants used in the milkfish experiments. Response to treatment was evaluated by monitoring *in vivo* oocyte growth rates. E_2 and testosterone profiles of treated and control females were also obtained to determine the implant's mode of action.

The pattern and rate of oocyte growth observed for most of the placebo-implanted controls was consistent with past observations of the mullet reproductive cycle in Hawaiian waters (Kuo and Nash, 1975). Numbers of mature females peaked in January and February and in most cases, only one spawning could be induced per female (Fig. 24). Oocyte growth profiles from fish in the control group revealed some natural individual variability. Some females apparently undergo and complete vitellogenesis earlier than others. These females will normally maintain their oocytes throughout the season (unpublished data). When induced to spawn, however, a small percentage remature. To our knowledge, multiple spawnings from an individual female mullet during the same season have never been reported. They may remature after being induced to spawn because the environmental cues which initiate vitellogenesis are still present. There appears to be a "window" during the mullet spawning season in which both photoperiod and temperature are optimal for initiating and completing vitellogenesis. This "window" is, to a large degree, open for a fixed period of time. If oocyte growth can be accelerated, the opportunity for multiple spawnings should increase.

When the treatment was initiated when oocytes were in the early stages of vitellogenesis, the LHRH-a + testosterone effectively accelerated growth rate over the placebo control group. The treated group had the highest percentage females that were induced to spawn twice within the same season (Kelley et al., 1987). Initial results from larval rearing trials with the larvae of the first and second spawnings from the same individual did not reveal any loss of egg quality. There are obvious practical uses for these chronic hormone treatments; reproductive potential can be doubled from the same broodstock.

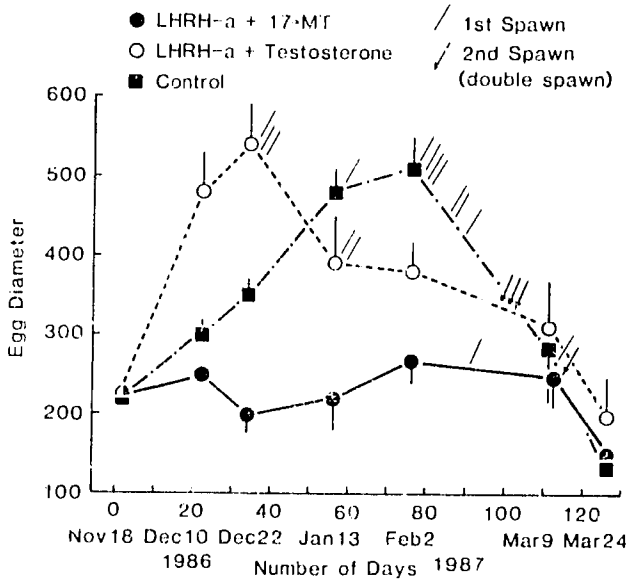

Figure 24. *Profiles of egg growth from control, LHRH-a + 17-MT, and LHRH-a + testosterone treated female mullet are presented. The experiment was initiated on November 18, 1988. Symbols represent averages and bars are SEM. Lines indicate when a female was induced to spawn and arrows indicate times a female was spawned for a second time.*

The LHRH-a + 17-MT treatment resulted in inhibited egg growth. The dosages of the hormones used were the same in both treatments, but the results were quite different. The steroid profiles of T and E_2 from females that underwent the LHRH-a + 17-MT treatment provide a possible explanation for the poor response. Circulating levels of T and E_2 fell when 17-MT was used in the treatment (Figs. 25 and 26). Levels were even lower than those observed for fish that did not mature naturally. Steroid profiles from the different groups suggest that the poor performance of the LHRH-a + 17-MT group may be due to an inhibition of E_2 and T synthesis.

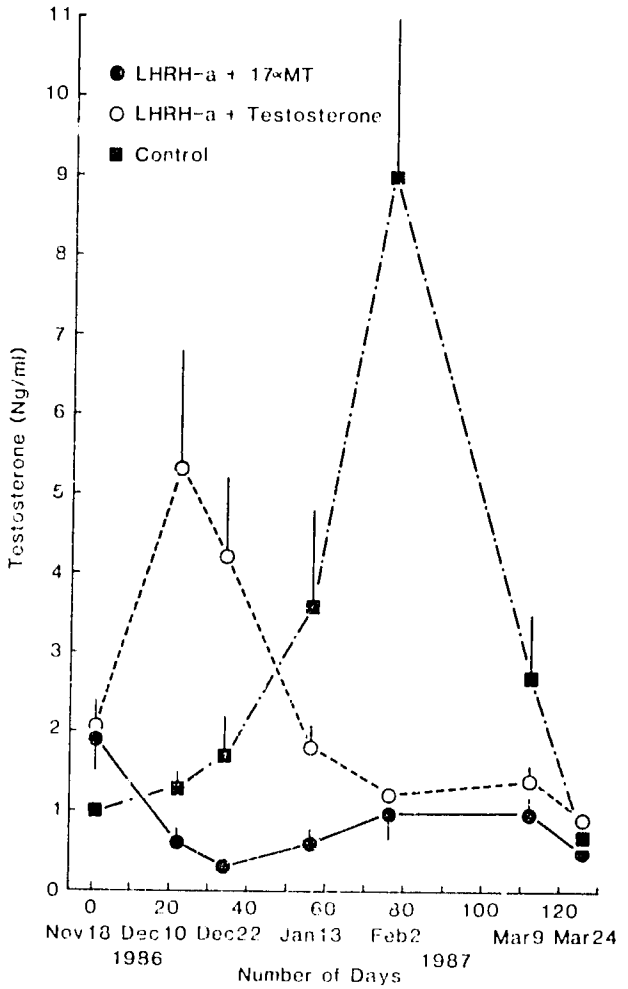

Figure 25. *Serum testosterone values from placebo and hormone-implanted females are presented. Symbols are averages and bars represent SEM. Note the sharp drop in serum testosterone levels in the LHRH-a + 17-MT treated females.*

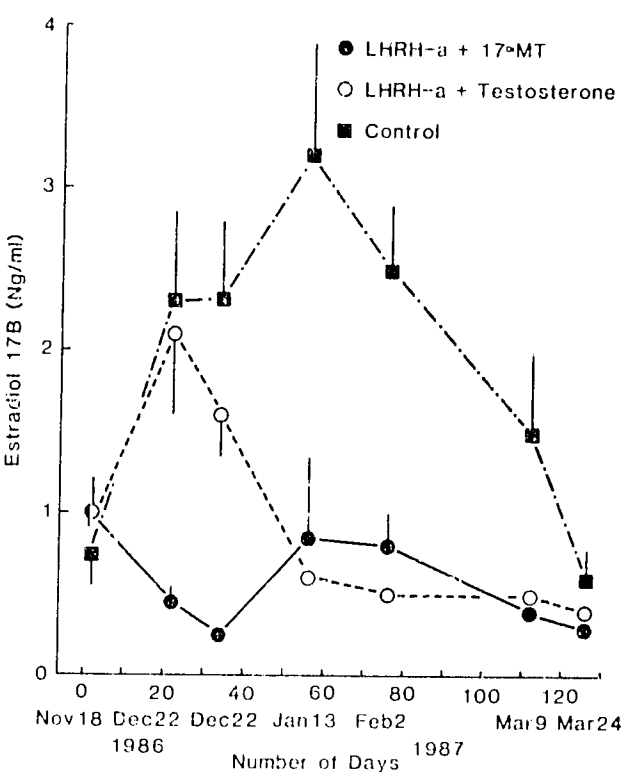

Figure 26. *E_2 values from controls and the two hormone therapies tested for their effects on vitellogenesis in the mullet. Note the drop in serum E_2 levels in response to LHRH-a + 17-MT.*

Although we have no direct evidence, the depressed values appear to be the result of negative feedback exerted by the dosage of 17-MT. To conclude that negative feedback is occurring at the level of the ovary will require determination of gonadotropin(s) levels in the mullet in response to the steroid treatment. The resulting serum steroid values after treatment demonstrate that the steroid used can influence the effectiveness of the treatment. Future work will evaluate whether LHRH-a or other steroids are more effective when used alone or in combination. Chronic hormonal therapies combined with the manipulation of environmental parameters (i.e., photoperiod and/or temperature) need to be evaluated.

Testicular Maturation In The Mullet

As mentioned previously, the steroid profiles accompanying gonadal maturation provide insight to the limits of the period of reproductive activity, as well as the hormones involved in the mediation of the maturation process. Correlating steroid profiles with testicular maturation reveals that serum androgen (testosterone and 11-ketotestosterone) titers increase prior to the peak in the percentage of running ripe males (Fig. 27). This suggests that these androgens may play a significant role in mediating spermatogenesis. The peak of serum levels of the androgens found in the mullet can be mimicked by using the 10 mg implant (see previous section).

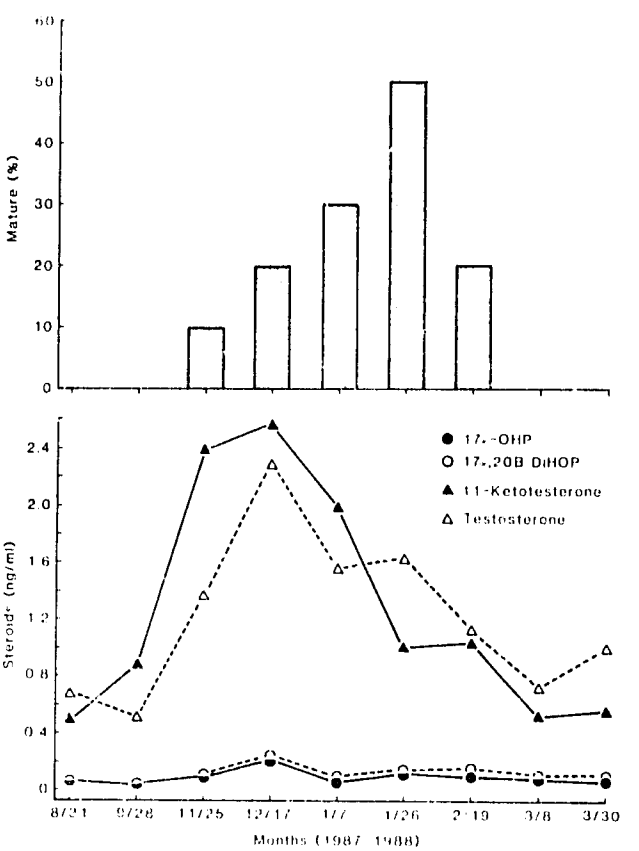

Figure 27. *The profile of testicular steroids detected in the serum of male mullet and the percentage of milting males just prior to and during the 1987-88 spawning season.*

Induced Testicular Maturation

Administration of an intramuscular injection of 17-MT every other day is an effective but time-consuming method to induce spermatogenesis and spermiation in male mullet (Shehadeh et al., 1973). Orally-administered 17-MT is a more efficient means of administration, and the induction of testicular maturation is independent of salinity and photoperiod (Lee and Weber, 1986).

Spermiation can be maintained for at least 55 weeks (Weber and Lee, 1985). Although administration does not involve the repeated handling common to a multiple injection protocol, the treatment does not discriminate between sexes, however, and depends upon the fish feeding on the treated diet. The silastic implants have been demonstrated to effectively elevate serum steroid levels *in vivo* in juvenile milkfish.

Following implantation with 17-MT capsules during the "off-season," male mullet were induced to mature within three weeks. The minimum effective implant dose resulting in maturation was 4.7 ± 1.2 μg/g body weight. The recommended implant dose to ensure a high percentage of maturation is 19.7 ± 17.6 μg/g body weight. Males implanted only once with the latter dose have been spermiating for at least a year with no apparent effect on their ability to fertilize spawned oocytes (Fig. 28). This is an example of controlled maturation: efficient administration of an effective hormone resulting in the maturation of the male mullet on a *demand basis*, and is the first step in the domestication process.

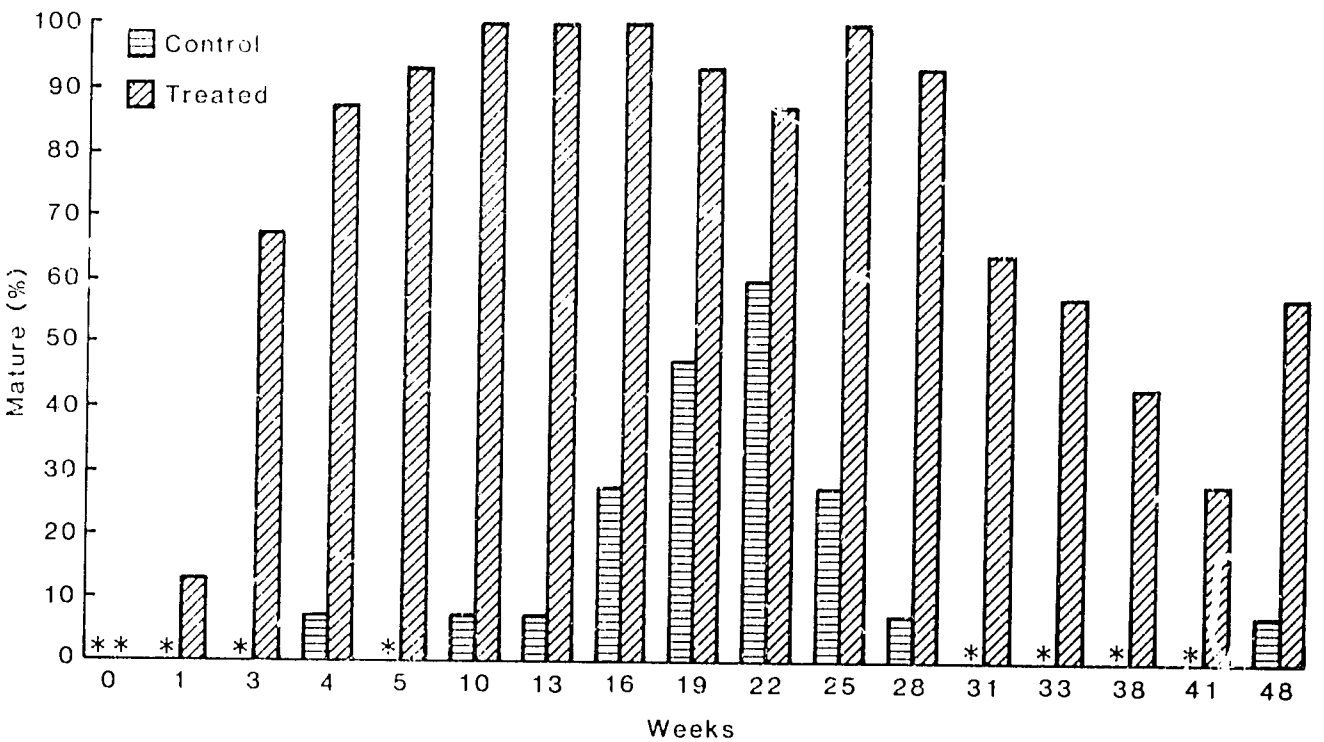

Figure 28. *A comparison of the percentages of milting males obtained naturally or from a group of males implanted with a single 10 mg capsule of 17-MT. The data were obtained from August, 1987 through July 1988.*

Improvement In The Method For Induced Spawning In The Mullet

Control of reproductive processes in commercially-important teleost fishes has always been a goal of fish culturists. Techniques for particular species continuously evolve as more efficient and cost-effective methods are developed. As noted, one of the most recent is the use of high potency analogues of luteinizing hormone-releasing hormone (LHRH-a) to induce final maturation and spawning in fish (see reviews by Lam, 1982 and Donaldson and Hunter, 1983).

The application of a high potency synthetic analogue of luteinizing hormone-releasing hormone (LHRH-a), des Gly^{10} [D-Ala^{6}] LHRH ethylamide to induce final maturation and spawning in grey mullet was investigated. Females with average egg diameters of 600 µm or more were administered a priming injection of carp pituitary homogenate (CPH), followed by a resolving injection of LHRH-a 24 hours later; this resulted in a high spawning success rate (94.1%). Fertilization and hatching rates, and qualitative changes in egg morphology were similar to those reported with traditional methods. In this study, 20 mg/kg CPH followed by 200 µg/kg LHRH-a were the minimum dosages required for fertilized spawns. This is the most cost-effective method yet developed to induce mullet to spawn. The performance of this treatment in comparison to other hormone regimens is presented in Table 2 found in the Appendix.

LARVAL REARING OF MULLET

Progress with larval rearing has not advanced as far as the control of reproduction. The most successful rearing trials have resulted in survival of 2-5 juveniles/liter at 60 days post- hatching (Nash et al., 1977; Kraul, 1983). However, results have not been consistent enough to demonstrate a suitable technology.

During the 1986-87 season, nine intensive larval rearing trials were conducted in 5000-liter fiberglass tanks. The results of those trials are summarized in Table 3 found in the Appendix.

Results demonstrate that mullet survival above 10% at 60 days posthatching can be consistently achieved. Juvenile densities at harvest were the highest reported for this species (Kraul, 1983). The food regimen initially consisted of rotifers, followed by *Artemia* nauplii, and finally artificial food (Fig. 29).

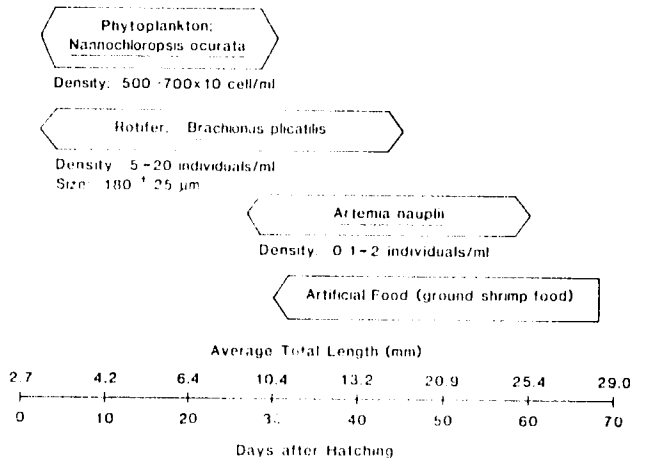

Figure 29. *A schematic representation of the feeding regimen used during the 1986-87 mullet larval-rearing trials.*

The relationship between initial stocking density and survival was negatively correlated and statistically significant ($r = -0.68$, $p < 0.05$) suggesting optimal initial stocking densities to be used in mullet rearing trials. The results indicate 10-30 larvae/liter as an optimal range when using 5-10 rotifers/ml and 700,000-1,000,000 cells/ml of phytoplankton.

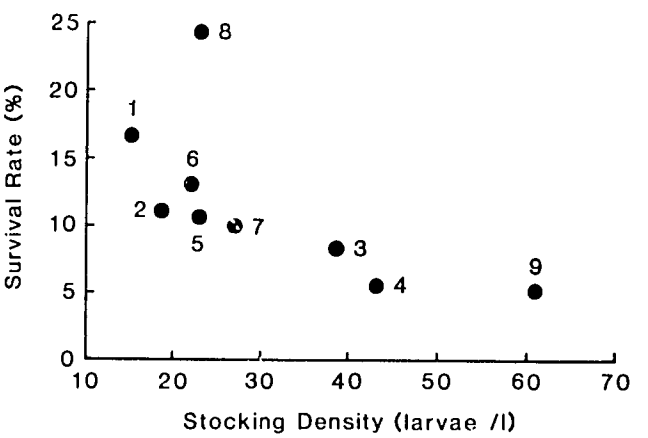

Figure 30. *The correlation between the percent survival and initial stocking densities from nine larval-rearing trials during the 1986-87 mullet larval-rearing trials. Numbers represent trial numbers.*

Mullet larvae open their mouths by the second day after hatching and begin feeding as early as 70 hours after hatching, well before completion of yolksac absorption, i.e., Day 5 (Fig. 31). Initial effects of withholding food can be detected as early as 84 hours (3.5 days) after hatching, contrary to previous reports. The data suggest that larvae should be offered rotifers at least by Day 2.

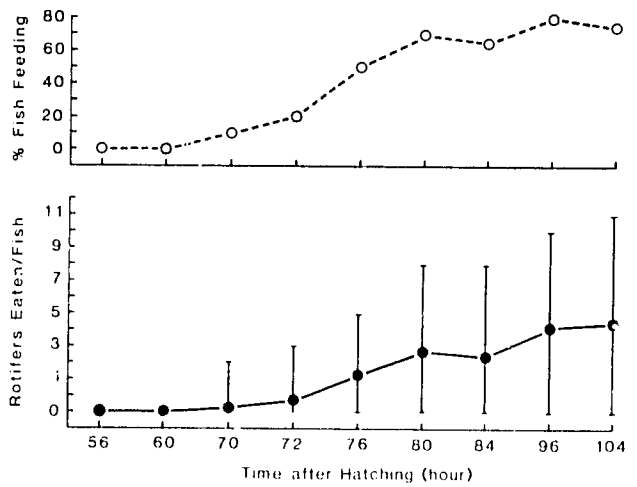

Figure 31. *The percentage of fish feeding and the number of rotifers eaten per larvae during the first 104 hours after hatching. Each point represents 20 individuals and bars represent observed ranges.*

During the 1987-88 mullet spawning season, trials were run in eleven 5000-liter tanks stocked with 10-30 larvae/liter, the optimal initial stocking density suggested by 1986-87 data. Survival rates at 50 days posthatching averaged an unprecedented 34.3% (range: 19.1%-52.2%) and clearly demonstrated the possibility of intensively rearing striped mullet.

Food selectivity experiments suggested preference for larger food organisms from Day 8 to Day 10. Earlier addition of *Artemia* nauplii during the 1987-88 season resulted in increased growth rate compared to the previous year; this reduced the hatchery phase to 45-50 days (Fig. 32). Future work will investigate whether manipulation of environmental factors (i.e., salinity, daylength, etc.) can also enhance growth and shorten the hatchery phase.

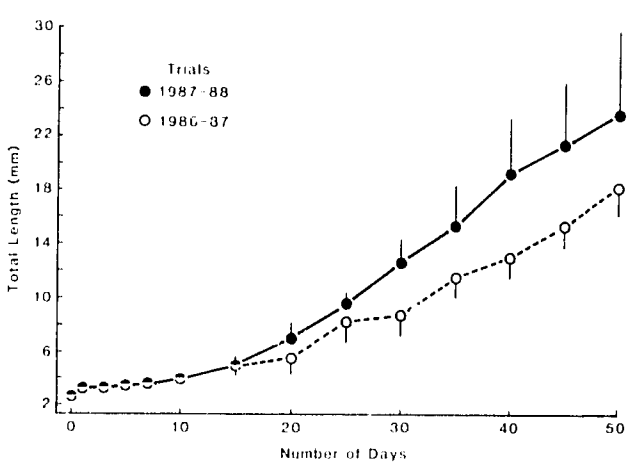

Figure 32. *The average growth observed for mullet larvae during the 1986-87 and 1987-88 rearing seasons (N = 9 and 11, respectively). Bars represent SEM.*

Effects Of Temperature And Salinity On Development And Metabolic Rates

Figure 33 summarizes the effects of temperature and salinity on embryonic development. Total egg mortality was observed at 18°C and more than 95% of newly-hatched larvae were abnormal at 32°C. These temperatures represent thermal tolerance limits for early development. Egg mortality and decreased normal hatch in 15 ppt indicated that this salinity was close to the lower tolerance limit. Mortality at the gastrula stage increased in 45 ppt. Although deleterious effects were apparent at these extreme salinities, the results demonstrate that striped mullet eggs have greater salinity tolerance than was previously known. There was also a significant inverse relationship between spawned egg diameter (0.87-0.96 mm) and hatching. Normal hatch was expressed by:

$$y = -5.65001 + 0.63164x_1 + 0.03021x_2 + 0.00143x_1x_2 - 0.01342x_1^2 - 0.00089\,x_2^2 - 2.23659\,x_3;$$

y = proportion of normal hatch
x_1 = temperature (°C)
x_2 = salinity (ppt)
x_3 = spawned egg diameter

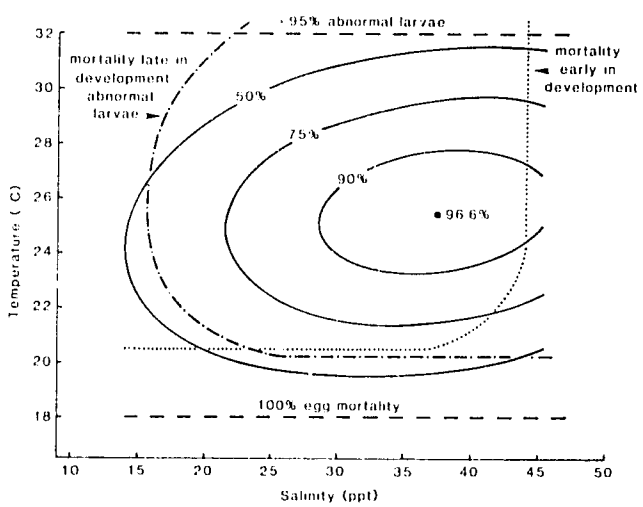

Figure 33. *Effects of temperature and salinity on development and normal hatch of striped mullet eggs. Normal hatch isopleths calculated by multiple regression. Dashed lines indicate deleterious effects.*

The calculated optimum for normal hatch (96.6%) is located at 25.5°C and 37.4 ppt.

Oxygen consumption rates during embryonic stages and the first 38 days of larval development were measured at 24°C by differential respirometry. Experiments were conducted at the blastula, gastrula and four embryonic stages, and the yolksac, preflexion, flexion and postflexion larval stages.

Oxygen uptake rates of eggs increased linearly from .024 $\mu l\ O_2\ egg^{-1}\ h^{-1}$ (.323 $\mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$) by blastulae to .177 $\mu l\ O_2\ egg^{-1}\ h^{-1}$ (2.516 $\mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$) by embryos prior to hatching (Fig. 34). Respiration rates did not vary significantly among four salinities (20, 25, 30, 35 ppt).

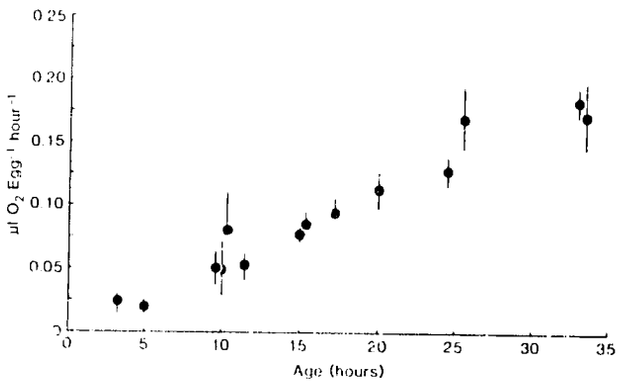

Figure 34. *Oxygen consumption rates of striped mullet eggs in relation to age (hours after fertilization) at 24°C.*

Larval oxygen consumption increased from .243 $\mu l\ O_2$ $larva^{-1}\ h^{-1}$ shortly after hatching to 18.880 $\mu l\ O_2\ larva^{-1}$ h^{-1} on Day 38 (Fig. 35). This increase was expressed by:

$$\ln y = -1.7693 + 0.0078\ x_1 - 0.1595\ x_2 - 0.000003\ x_1^2$$

$y = \mu l\ O_2\ larva^{-1}\ h^{-1}$
x_1 = larval age in hours after hatching
x_2 = the gradient between the rearing and experimental temperatures

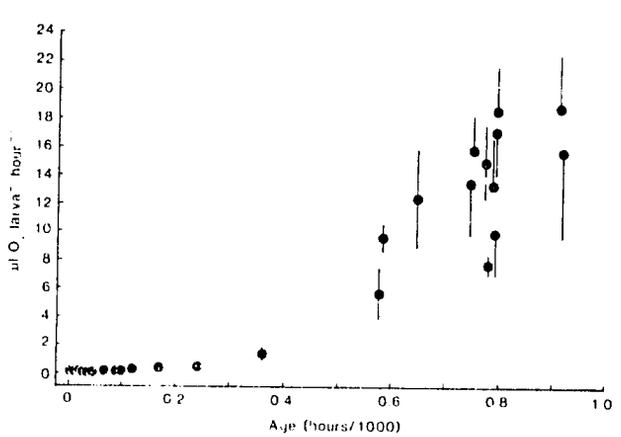

Figure 35. *Oxygen consumption rates of striped mullet larvae in relation to age (hours after hatching) at 24°C.*

Oxygen consumption varied in direct proportion to dry weight, according to the functional regression:

$$\ln y = 2.314 + 0.983 \ln x$$

$y = \mu l\ O_2\ larva^{-1}\ h^{-1}$
x = mg dry wt

Mass-specific oxygen uptake remained constant from the preflexion through postflexion stages (10.838 $\mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$).

Larval oxygen consumption rates did not vary significantly among salinities from 10-35 ppt. Acute temperature increases elicited significant increases in oxygen consumption by yolksac (Q_{10} = 2.75) and postflexion larvae (Q_{10} = 1.40).

LARVAL REARING OF MILKFISH

Larval-rearing trials used a protocol similar to that used in mullet rearing. The majority of larval milkfish were feeding by 88 hours after hatching. Early effects of starvation on growth were detectable by 136 hours after hatching. Eight larval-rearing trials conducted during the 1988 season resulted in survival rates of 19.2-46.4% at Day 30. Density range at harvest was 4.1-6.8 juveniles/liter which was comparable to mullet results.

Exposing milkfish embryos to direct salinity changes reveals that their salinity tolerance occurs very early. Although hatching occurred at all salinities tested (5-40 ppt) the highest percent of normal larvae resulted at 20-40 ppt. The range of salinities that can be tolerated increased with age. For two-day-old larvae, the highest was 30 ppt. Information obtained from the salinity tolerance experiments may be used to improve survival and growth during intensive rearing of milkfish larvae.

Effects Of Temperature And Salinity On Development And Metabolic Rates

A series of 12 incubation trials was conducted during the 1988 season. Results are summarized in Table 4 found in the Appendix. The initial three experiments were run as a factorial design using levels of 23, 28 and 33°C and 15, 25, 35 and 45 ppt. Normal development to the late embryonic stages occurred in all temperature combinations, but the levels of aeration necessary to maintain egg buoyancy in low salinities apparently inhibited hatching.

Nine experiments were conducted to determine the effects of temperatures from 20-36°C (35 ppt) on development. Eggs were transferred to the experimental temperatures as blastulae and after embryo formation.

Results indicated that 23°C is near the lower thermal tolerance limit because total mortality occurred in three trials at this temperature. The upper tolerance limit for development was exceeded by 34.5°C when the transfers were conducted at the blastula stage. Within the tolerable range, hatching varied among spawnings, was highest in 25.5-28°C.

Oxygen consumption rates during the embryonic development of milkfish was measured at 28°C. Oxygen uptake increased linearly from .074 $\mu l\ O_2\ egg^{-1}\ h^{-1}$ (.644 $\mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$) to .402 $\mu l\ O_2\ egg^{-1}\ h^{-1}$ (3.916 $\mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$) prior to hatching. There were no significant differences in oxygen consumption rates among salinities from 20-35 ppt.

Yolksac larvae were maintained at 23, 28, and 33°C. Larvae appeared capable of feeding (open mouth and pigmented eyes) at 100 h, 57 h, and 40 h at 23, 28 and 33°C respectively. Starvation mortality commenced by 150 h at 23°C, 100 h at 28°C and 85 h at 33°C.

Oxygen consumption rates of yolksac larvae varied directly with temperature. The mean rates were .328 $\mu l\ O_2\ larva^{-1}\ h^{-1}$, .435 $\mu l\ O_2\ larva^{-1}\ h^{-1}$ and .634 $\mu l\ O_2\ larva^{-1}\ h^{-1}$ at 23, 28, and 33°C respectively. The Q_{10} for the 23-33°C range was 1.93. The effect of temperature was expressed by:

$$y = 0.00058\ x^2$$
$y = \mu l\ O_2\ larva^{-1}\ h^{-1}$
x = temperature (°C)

Mass-specific oxygen consumption rates ($Q\ O_2$) varied directly with both age and temperature. These effects were expressed by:

$$y = .00680_{x1} + .00767_{x2}$$
$y = Q\ O_2$
x_1 = temperature (°C)
x_2 = age in hours after hatching

Oxygen consumption measurements were also obtained following acute increases of temperature from 28 - 33°C. Experiments were conducted early and late in embryonic development, and on Day 0 and Day 1 with larvae.

The effect of the acute increases, corrrected for the effect of development, was greater early in embryonic development (Q_{10} = 3.55) than among late embryos (Q_{10} = 1.72). Similarly, acute increases elicited greater responses among Day 0 larvae (Q_{10} = 2.99) than on Day 1 (Q_{10} = 1.86).

The effects of temperature and age on the dry mass of larvae were expressed by:

$$y = 0.9881 - .00001_{x1x2}$$
y = dry weight (mg)
x_1 = age in hours after hatching
x_2 = temperature (°C)

ROTIFER RESTING EGG PRODUCTION

Rotifers are essential for larval rearing of many marine teleosts. Like brine shrimp, *Artemia*, they produce resting eggs (cysts) which can remain dormant for several years and be hatched on demand. Rotifer cysts are not used as food but can serve as inoculum for their mass culture, thereby reducing start up time of traditional culture methods. Mass production of resting eggs from two stocks of rotifers was investigated.

Several factors influenced the number of resting eggs produced. Past research has demonstrated that the number and sizes of resting eggs produced varies between rotifer stock. L-type (Japanese stock) and S-type (Hawaiian stock) exhibit different salinity optima for producing resting eggs (Fig. 36). These are 8 ppt and 32 ppt for the L- and S-type, respectively. After a week in culture, 10,000 rotifers of S- and L-type, fed the same amount of food and maintained at optimal salinities for resting egg production, will produce 10,500 and 2,300 resting eggs, respectively.

Resting eggs from different stocks will produce newly-hatched rotifers of different sizes. The S-type rotifer (106-210 μm lorica length) will produce smaller resting eggs and hatched rotifers than the L-type (130-340 μm). The potential use of hatched rotifers from resting eggs as a food source at initial stages of feeding when sizes of food particles are critical has yet to be fully realized.

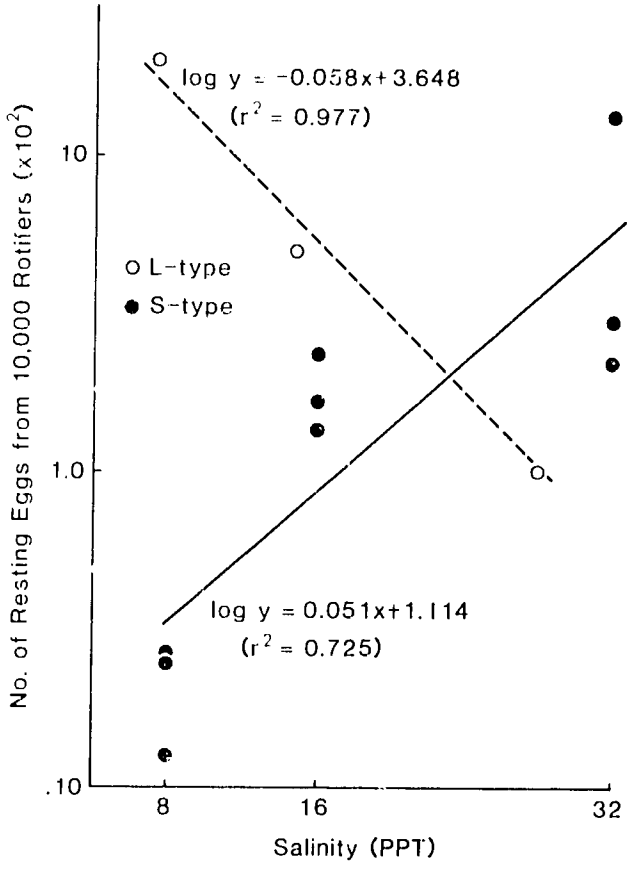

Figure 36. *The observed number of resting eggs produced from the L-type (Japanese stock) and S-type (Hawaiian stock) rotifers in response to varying salinities.*

TECHNOLOGY TRANSFER

Memoranda of Understanding (MOU) on milkfish research have been signed between the Oceanic Institute and three countries (Indonesia, Philippines, and Taiwan). Representatives from these countries who have been trained in the production and implantation of developed hormonal treatments are:

1) Jessie Banno, Southeast Fisheries Development Center, Philippines
2) Agus Prijono, Gondol Research Station, Indonesia
3) Robin Chen, Tungkang Marine Laboratory, Taiwan

The feasibility of transfering developed hormone technology is currently being assessed in coordination with the Oceanic Institute.

The following scientists from the Peoples' Republic of China received training in several aspects of the control of reproduction of striped mullet and milkfish:

Fang Yongqiang*	Third Institute of Oceanography, National Bureau of Oceanography
Li Jiaer*	South China Sea Fisheries Institute, Chinese Academy of Fisheries Science
Zheng, Chen-Wei*	Institute of Oceanography, Academic Sinica, Qingdao
Wang, You-Huai*	East China Sea Fisheries, Research Institute
Su, Ping-Ren	Nanjing University
Sun, Guo-Ying	East China Normal University
Cen, Feng	Bureau of Aquatic Products, Ministry of Agriculture, Animal Husbandry and Fisheries.
Mazid, M. A.	Fisheries Research Institute Bangladesh

* Funding provided by NOAA, U.S.A. All other funding provided by the Peoples' Republic of China.

The results of the work on reproduction and larval rearing of mullet and milkfish have been summarized and pertinent articles have been published by a variety of refereed journals. A list of the publications that have been, or are in the process of, being published are presented in Appendix A. Workshops and meetings are another form of technology transfer. A list of Finfish Program staff who have attended and presented papers of conducted research is also included in the appendix.

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TABLES

Table 1. *Egg size before injection, spawned eggs and number of eggs released in fertilized and unfertilized spawns.*

Fish ID	FL(cm)	Weight(kg)	Egg Size(mm) (before)	Egg Size(mm) (spawned)	# of eggs released	Fertilization Rate (%)
			FERTILIZED SPAWNS			
DBC-4 *	67.0	4.90	0.790	1.269	770,000	95.0
BW-4	66.0	4.85	0.804	1.214	380,000	97.4
DBW-8	66.0	4.00	0.862	1.189	390,000	94.0
DBC-3	62.5	4.55	0.843	1.296	268,000	97.0
BW-4	66.0	4.85	0.826	1.225	520,000	94.0
DBW-9	62.0	3.85	0.859	1.232	184,000	14.0
TW-3	68.5	5.20	0.891	1.311	555,000	95.0
Mean ± S.E.			0.839±0.035	1.248±0.045	400,000	
			UNFERTILIZED SPAWNS			
TW-3 *	68.5	5.20	0.785	1.202	59,000	0
DBC-8	62.0	4.10	0.887	1.264	236,000	0
DTC-7	62.0	3.65	0.830	1.147	61,500	0
TW-3	68.5	5.20	0.861	1.243	89,000	0
TW-12	64.0	4.40	0.806	1.125	90,000	0
TW-3	68.5	5.20	0.816	1.181	152,000	0
DBC-6	59.5	3.15	0.791	N/A	<100	0
DBW-8	66.0	4.00	0.939	N/A	150,000	0
Mean ± S.E.			0.839±0.053	1.194±0.054	104,700	

* = Spawned with pellet implant. All others spawned with injections.

Table 2. *Estimated production cost of mullet larvae in the hatchery.*

Treatment	Fertilized Eggs	Number of Hatched Larvae	Hormone Cost per 100,000 larvae produced (US$)
CPH/CPH	372,750	186,375	4.51
CPH/LHRH-a	782,370	391,185	0.97 ~ 1.78
LHRH-a/CPH	499,000	249,500	1.53 ~ 2.79
LHRH-a/LHRH-a	409,500	204,750	0.99 ~ 4.05
500C IU HCG/LHRH-a	536,000	268,000	2.82 ~ 3.98
10000 IU HCG/LHRH-a	548,114	274,057	5.16 ~ 6.29

Table 3. *Results of nine larval-rearing trials conducted in 5000-liter tanks during the 1986-87 mullet season.*

Trial #	Culture Days	Stocking Number	Number Harvested	Percent Survival	Average T.L.(mm.)
1	60	62,100	10,474	16.9	23.0
2	60	73,650	8,340	11.3	21.1
3	60	153,800	13,069	8.5	18.2
4	60	172,500	9,644	5.6	19.8
5	70	93,700	10,048	10.7	23.4
6	77	87,750	11,517	13.1	30.8
7	75	107,250	10,700	10.0	34.0
8	70	90,750	22,251	24.5	29.0
9	65	243,000	12,400	5.1	26.2

Total number of mullet juveniles produced = 108,443
Average percent survival = 11.7%

Table 4. *Percentages of total and normal hatch of eggs transferred to experimental temperatures during two developmental stages.*

Temperature (°C)	Blastula Total	Blastula Normal	Embryo Total	Embryo Normal
20.0°	0.0	0.0	0.0	0.0
21.5°	0.0	0.0	83.5	48.1
23.0°	53.9	41.6	85.1	60.6
25.5°	72.1	52.7	83.4	60.6
28.0°	63.4	48.7	70.1	53.0
30.5°	50.6	40.4	37.2	29.7
33.0°	53.5	36.3	55.8	41.9
34.5°	39.6	0.0	83.2	35.8
36.0°	13.9	0.0	20.8	0.0