

## **Influence of Chronic Administration of LHRH-Analogue and/or 17 $\alpha$ -Methyltestosterone on Maturation in Milkfish, *Chanos chanos***

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### ABSTRACT

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Five chronic hormone therapies; cholesterol pellets containing 200  $\mu$ g of LHRH-a (LHRH-a pellet); silastic tubing packed with either 250  $\mu$ g of dissolved 17 $\alpha$ -methyltestosterone (liquid 17 $\alpha$ -MT capsule) or 10 mg crystalline 17 $\alpha$ -methyltestosterone (crystalline 17 $\alpha$ -MT capsule); or the combinations of LHRH-a pellets plus a liquid 17 $\alpha$ -MT capsule or LHRH-a pellets plus a crystalline 17 $\alpha$ -MT capsule, were tested to determine the best treatment for inducing maturation in captive milkfish (*Chanos chanos*). Experimental groups of 20 milkfish each received one of these five therapies. A sixth control group received placebo implants. LHRH-a pellets were administered monthly; crystalline 17 $\alpha$ -MT capsules were administered once, and liquid 17 $\alpha$ -MT capsules were administered twice, at the beginning of the experiment and 3 months later.

Results show that the combination of LHRH-a pellets plus liquid 17 $\alpha$ -MT capsules is the most effective hormone therapy for enhancing the maturation of both sexes. Fifty percent of these fish matured in April, 1 month after implantation, and close to 90% of the fish in this treatment matured by July. The combination of LHRH-a pellets plus crystalline 17 $\alpha$ -MT capsules enhanced the maturation of male milkfish but not female. LHRH-a alone was also effective in the maturation of females, but was the least effective of all treatments in maturing males. 17 $\alpha$ -MT capsules alone, in either form did not induce maturation in female milkfish.

### INTRODUCTION

It is well known that environmental cues play an important role in regulating reproduction in teleost fishes (Crim, 1982; Lam, 1983; Stacey, 1984). Environmental cues mediate secretion of hormones by the brain and pituitary which synchronize the activities of various organs involved in reproduction into an orchestrated physiological and biochemical response. Under culture conditions, the required environmental stimuli may be lacking and this may pose a

physiological barrier to commencement of reproductive processes. Under such conditions hormonal therapies need to be found to bypass this block (Lam, 1982).

The milkfish (*Chanos chanos*), an important food fish in Southeast Asia, the Philippines, and Taiwan, provides a good example of these problems. Milkfish rarely mature and spawn in captivity. Development of a reliable method for controlling maturation and spawning of milkfish has been attempted for a number of years (Lam, 1984; Kuo, 1985) without success. Natural maturation of milkfish has occurred at a number of locations, in different kinds of rearing facilities, and in a variety of environments (Lam, 1984; Kuo, 1985; Lee, 1985). Natural spawning has occasionally taken place in both sea cages (Lacanilao and Marte, 1980; Marte et al., 1984) and in marine ponds (Lin, 1985). Despite this work, a thorough understanding of the maturation process in milkfish has yet to be attained (Kelley and Lee, 1986). Such an understanding is crucial to stabilizing the supplies of mature milkfish for the purpose of fry production.

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 mode of chronically administering the hormone or analogue of choice was through a multiple injection protocol (Yamamoto et al., 1974; Lam, 1982). Initial attempts at inducing maturation in captive milkfish using similar strategies have met with little success (Lam, 1982; Lacanilao et al., 1984). 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 mode of chronically administering hormones and decrease the amount of handling (Crim, 1985). LHRH-analogue cholesterol pellets implanted into landlocked salmon (Crim et al., 1983a), rainbow trout (Crim et al., 1983b), and Atlantic salmon (Crim and Glebe, 1984) accelerated the reproductive cycles of these fish. Gonadotropic hormone production in the juvenile trout pituitary was triggered by testosterone in a silastic capsule implanted in the peritoneal cavity (Crim and Evans, 1982). However, initial attempts to utilize these hormone pellet implants for inducing maturation in milkfish have been unsuccessful (Lacanilao et al., 1984). In these experiments, exogenous gonadotropins such as crude salmon pituitary homogenate (SPH), partially purified salmon gonadotropin (SG-G100), and human chorionic gonadotropin (HCG), were administered via pellet implants.

In this study, our aim was to find a chronic hormone therapy to trigger or enhance the maturation of captive milkfish. If a hormone therapy can circumvent maturational problems, a stable supply of mature milkfish can be obtained.

## MATERIALS AND METHODS

### *Broodstock acquisition*

In 1979, approximately 1000 milkfish (about 1–2 years old) were stocked into two fish ponds on the Kona coast of the island of Hawaii. The fish fed exclusively on the natural flora found within these ponds and were exposed to salinities averaging 7‰ throughout the year. During the months of October through November 1984, 150 individuals from a shipment of 177 fish survived barge transport to the Oceanic Institute on the island of Oahu. These milkfish were completely acclimated to full strength seawater (35‰) and an artificial diet of Purina trout chow before the experiment began.

In January 1985, ten individuals were randomly stocked into each of twelve, 6.1 m diameter, 30 000-l fiberglass tanks. The fork lengths of the fish ranged between 54 and 79 cm. Sex ratio of the fish was not determined prior to stocking because of the lack of any sexual dimorphism.

The fork length, total body weight, and gonad weight were recorded for each of the 27 fish that died while being transported from the island of Hawaii to Oahu. The gonadosomatic index (GSI) was calculated as

$$\text{GSI} = \frac{\text{Gonad weight}}{\text{Body weight}} \times 100$$

The GSIs derived from these fish were used as baseline data for the experimental fish.

### *Hormone preparation*

The two kinds of hormones examined in this study are luteinizing hormone-releasing hormone analogue, des-Gly<sup>10</sup>-(D-Ala<sup>6</sup>)-LHRH ethylamide (LHRH-a), and 17 $\alpha$ -methyltestosterone (17 $\alpha$ -MT). Both were purchased from Sigma Chemical Company, U.S.A., and were incorporated into vehicles designed for chronic, as opposed to acute, hormone release. The LHRH-a was incorporated into a cholesterol pellet as described by Lee et al. (1985). Each pellet weighed approximately 23 mg and had an average length and diameter of 5.5 and 2.4 mm, respectively. A single pellet, produced in this manner, contains approximately 200  $\mu$ g of LHRH-a. The 17 $\alpha$ -MT was incorporated into two types of silastic capsules by modifying the method of Dziuk and Cook (1966). The preparation of these capsules is described in detail elsewhere (Lee et al., 1986b).

### *Hormone treatments*

Five kinds of hormone treatments were tested including LHRH-a pellets and two forms of 17 $\alpha$ -MT capsules, individually or in combination. To account

for differences in tank conditions, the control fish were distributed among the treatment tanks. Thus, the fish in 10 of the 12 tanks were treated in the following manner: eight fish were treated while the remaining two fish were implanted with placebos. Each treatment had a replicate tank. The remaining two tanks were representative of all treatments except controls. A total of 20 fish received each treatment.

The implantation of hormone pellets was carried out following the procedure described by Lee et al. (1985). Milkfish were anesthetized using 2-phenoxyethanol at a concentration of 0.3 ml/l of seawater. Each fish was finclipped for individual identification and implanted intramuscularly on the right side. Lastly, the maturation state of the individual was assessed. The above procedures were carried out once each month.

LHRH-a pellets were replaced on a monthly basis while the crystalline  $17\alpha$ -MT capsules were given only once. The liquid  $17\alpha$ -MT capsules were implanted at the beginning of the experiment and replaced after the 14th week of treatment. The reason for the difference in treatment periods between the two  $17\alpha$ -MT capsules is that the crystalline  $17\alpha$ -MT capsule may be effective for as long as a year (Moore, 1981). The liquid  $17\alpha$ -MT capsule contains a much lower dosage of  $17\alpha$ -MT, however, and required another capsule.

The criterion used to score a male's maturity was expulsion of sperm from the urogenital pore after exerting slight pressure on the abdomen. The quantity of sperm was rated at 0 for no sperm, +1 for little sperm, to a maximum of +3 when copious amounts of sperm were produced. Eggs were sampled via polyethylene cannulae (Shehadeh et al., 1973). When egg diameter reached at least 0.7 mm, the female was scored as mature. This is the minimum size that eggs must attain before spawning can be induced in milkfish (Kuo, 1985).

To estimate fecundity and to determine if cannulation provides a sample of ova representative of the maturational state of the ovary, three female gonads with vitellogenic eggs were fixed in 10% formalin. The total weight of the gonads was measured and three subsamples of at least 1 g each were taken from the anterior, middle, and posterior portions of the ovary. The subsamples were then teased to free the individual eggs for counting. The total number of eggs per ovary was then estimated as

$$\text{Total number of eggs} = \frac{(\text{No. of eggs from subsample}) \times (\text{Ovary weight})}{\text{Weight of subsample}}$$

The egg diameters of at least 100 eggs from each subsample were measured to 50  $\mu\text{m}$  and the mean diameters of the subsamples were then tested for significant differences using Student's *t*-test and Chi square analysis (Sokol and Rohlf, 1969).

## RESULTS

The autopsies of the 27 fish that died during transport are summarized in Table 1. Sixteen (59%) were males while 11 (41%) were females. There were

TABLE 1

Size and gonadosomatic index (gonad weight/body weight  $\times 100$ ) of milkfish in November when they were collected for maturation experiments (numbers in parentheses indicate the range)

	Fork length (cm) mean $\pm$ SE	Body weight (kg) mean $\pm$ SE	Gonad weight (g) mean $\pm$ SE	GSI mean $\pm$ SE
Male N = 16	53.8 $\pm$ 6.20 (41.0-61.0)	2.18 $\pm$ 0.55 (1.05-3.05)	1.06 $\pm$ 0.67 (0.15- 2.69)	0.048 $\pm$ 0.035 (0.020-0.158)
Female N = 11	51.6 $\pm$ 5.11 (46.5-59.5)	2.15 $\pm$ 0.38 (1.60-3.00)	6.19 $\pm$ 4.30 (1.49-15.16)	0.269 $\pm$ 0.136 (0.093-0.505)

no significant differences between the sexes with respect to fork length and body weight. In addition, all fish possessed immature gonads.

During the course of this experiment, water temperature ranged from 24 to 26°C and salinity ranged from 33 to 37‰ in all tanks.

Since the sex of the individual fish could not be determined at the beginning of the experiment, the percentage of maturing fish was used to evaluate the efficacy of the hormonal therapies (Fig. 1). The contributions of the particular sexes were included as they became known. Four males in the control group were found to possess milt as early as April. This number declined steadily until the month of July, when there was a dramatic increase. Only one female of the control group reached maturity (possessed 0.7 mm oocytes) during the course of the experiment, attaining this state in July.

There was a steady increase in the number of mature males in both the crystalline 17 $\alpha$ -MT capsule and liquid 17 $\alpha$ -MT capsule treatments. In July, this number increased to its highest point. The first mature female in the crystalline 17 $\alpha$ -MT capsule treatment was found in May, while the first in the liquid 17 $\alpha$ -MT capsule treatment was found in July.

The LHRH-a pellet treatment was the least effective therapy for producing mature males but appeared to enhance the total number of females that reached maturity. Furthermore, mature females were found as early as April.

The combination of LHRH-a pellet and 17 $\alpha$ -MT capsule, in either crystalline or liquid form, resulted in a significantly higher number of mature individuals by July when compared to all the other treatments. The combination of LHRH-a pellet and liquid 17 $\alpha$ -MT capsule, however, deserves special attention. As the experiment progressed and the fish were sexed, it was found that 80% of the males matured in April. They remained in that condition during the course of the experiment. This is in contrast to the males found in the control group where the number of mature males fluctuated during the same time period.

Under the LHRH-a pellet and liquid 17 $\alpha$ -MT capsule therapy, two mature females were also found in April. The number of mature females increased

TABLE 2

Oocyte size distribution and estimated fecundity in three milkfish ovaries

Fish ID	Sampling site	Range of oocyte diameters ( $\mu\text{m}$ )															$\bar{X}$	SD	N	Estimated fecundity
		300-350	350-400	400-450	450-500	500-550	550-600	600-650	650-700	700-750	750-800	800-850	850-900	900-950	950-1000	1000-1050				
BC	A								1	2	19	30	24	15	8	1	853	66	100	581 000
	B							1	1	2	13	31	27	18	6	1	856	67	100	
	C							1	2	4	14	31	28	12	7	1	848	71	100	
TW	A						1	2	7	15	27	20	20	4	3	1	800	81	100	657 000
	B						1	5	7	23	31	26	12	1			774	68	100	
	C							4	8	22	31	22	6	6	1		778	72	100	
TC	A	1	3	11	14	7	11	8	19	20	6						677	62	100	264 000
	B	4	5	8	17	5	12	15	18	12	4						659	60	100	
	C	4	7	11	8	13	11	8	19	16	6						673	62	103	

$\bar{X}$  = Mean oocyte diameter; SD = 1 standard deviation; N = total number of oocytes.

Sites sampled: A = anterior, B = middle, and C = posterior regions of the right ovary. Total body weights of the fish sampled are 5.30, 5.05, and 4.25 kg for BC, TW, and TC, respectively.

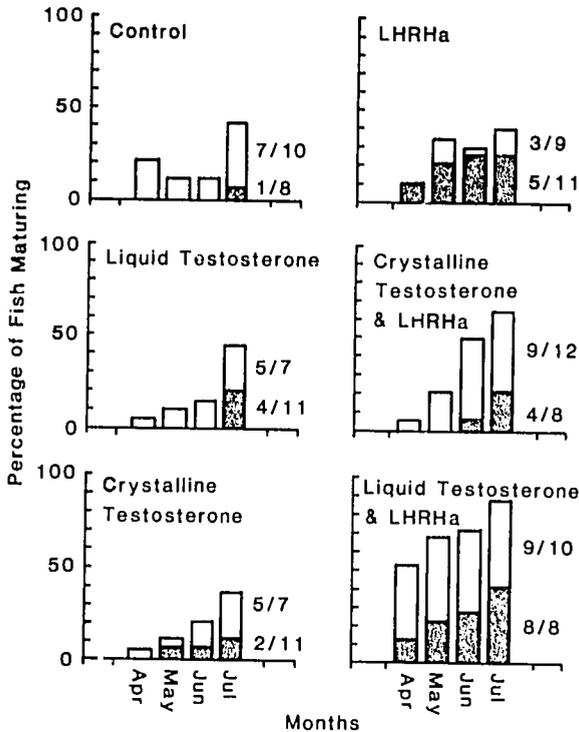


Fig. 1. Percentage of fish that reach maturity in response to different hormone therapies. Solid bar indicates females and blank bar indicates males. Fractions represent the number of mature fish to the total number of sexed fish.

steadily during the course of the experiment. By July, almost 90% of the individuals were found to be mature.

The LHRH-a pellet plus crystalline  $17\alpha$ -MT capsule therapy produced a high percentage of running ripe males by June, but a relatively low number of mature females was found in this treatment group. Overall, 65% of the individuals treated with LHRH-a and crystalline  $17\alpha$ -MT matured by the end of the experiment.

The egg size frequency distribution of three subsamples taken from the same ovary indicates a uniform distribution of oocytes (Table 2). This trend is consistently demonstrated in samples taken from three individual fish. Thus, a sample obtained by cannulation should represent the maturational state of the ovary.

An interesting observation emerged during the routine cannulation of females. A recurrence in the appearance of large-sized ova was observed within certain individuals. This strongly suggests that milkfish are capable of multiple spawnings. Females undergoing the LHRH-a pellet and liquid  $17\alpha$ -MT capsule therapy possessed egg diameters  $> 700 \mu\text{m}$  several times during April through August (Table 3). During the early stages of the experiment (weeks

TABLE 3

Correspondence between the times when females possessed average egg diameters of  $700\ \mu\text{m}$  or more and the lunar cycle

Fish ID	Approximate phases of the moon						Total
	Full moon	Half moon	Full moon	Half moon	Full moon	Half moon	
	Weeks of treatment						
	5	9	13	18	21	24	
DBC-4	*	-	-	*	-	-	2
TW-3	*	*	*	*	*	*	6
DTC-4	-	-	-	*	-	*	2
BW-4	-	*	-	*	-	*	3
DTW-9	-	*	-	*	-	*	3
DBW-9	-	-	*	*	-	*	3
DBC-8	-	-	-	*	-	*	2

End of  
experiment

\* = Female possessed egg diameters of  $700\ \mu\text{m}$  or greater.

5-13), no clear pattern between the individuals and the egg sizes was observed. Different females attained egg diameters  $> 700\ \mu\text{m}$  at varying points in time. There were, however, two instances of synchronous appearance of females with large eggs. Both of these coincided with the appearance of the first quarter moon as the natural spawning season approached (weeks 18-24). On average, each fish attained egg diameters  $> 700\ \mu\text{m}$  at least three times, with a range of between two to six times. In contrast, the control fish that matured possessed oocytes  $> 700\ \mu\text{m}$  only once during the same period.

## DISCUSSION

The GSI of the experimental milkfish, in October and November, was similar to the data reported for individuals found in Hawaiian waters at the same time (Kuo and Nash, 1979). If this group of milkfish is representative, they were in a refractory period of their reproductive cycle.

As the months passed, the number of individuals that matured in the control group typified past experiences with milkfish maturation. A relatively low number of females matured spontaneously, even during their peak spawning season. A variable number of running ripe males could be found at any one time.

The limited number of mature fish observed in the liquid and crystalline

$17\alpha$ -MT capsule treatments suggests that this therapy alone is ineffective for stimulating the maturation process in milkfish. This is contrary to other reports utilizing this particular hormone.  $17\alpha$ -MT is a potent androgen capable of inducing spermatogenesis and spermiation in mullet, stickleback and fundulus (Lofts et al., 1966; Borg, 1981; Weber and Lee, 1985). This also takes into account that when  $17\alpha$ -MT was administered orally to milkfish at various concentrations, the best dosage brought about testicular development only at the histological level (Lee et al., 1984, 1986d). In this study, mature males were not induced significantly before the spawning season (Fig. 1). One must consider that the dosages used may be inappropriate or that  $17\alpha$ -MT alone may not be sufficient for stimulation of gonadal development in this species.

Until recently, it was common practice to influence vitellogenesis in fish by stimulating the ovary with exogenous gonadotropins. The availability and species-specificity of these proteins are two common problems.

LHRH-a acts at the level of the pituitary and causes the release of gonadotropin, which in turn stimulates maturation of the gonads (Coy et al., 1975; Crim and Evans, 1983; Crim et al., 1983b). Combining the use of this neuropeptide and incorporating it into hormone implants reduces the amount of handling and consequent stress. The appearance of mature females in April is 3 months prior to the appearance of mature control females and at least 2 months ahead of the normal reproductive season of milkfish (Kuo and Nash, 1979). An accelerated maturational process has also been reported in rainbow trout and Atlantic salmon when treated with LHRH-a pellets (Crim et al., 1983b; Crim and Glebe, 1984).

In our study, male milkfish exhibited only a limited response to the LHRH-a pellet treatment. A similar observation of limited male response to chronic LHRH-a treatment has been reported for other fish and mammals as well (Vickery, 1981; Crim et al., 1983a). This so-called "paradoxical" effect of LHRH-a on testicular development may be limited to a particular phase during the male reproductive cycle. When LHRH-a was given to male salmon at various maturational stages, either testicular development was reduced or the milt volume and duration of spermiation increased, depending on the initial maturation state in which therapy was begun (Crim et al., 1983a). If there are particular phases in which the testes in teleost fishes are sensitive to chronic LHRH-a exposure, then such a "window of activity" needs to be defined. This mode of therapy could then be expanded for use in other fish species.

The combination of LHRH-a and  $17\alpha$ -MT, in both crystalline and liquid forms, appears to enhance maturation of milkfish. The combination of LHRH-a pellet and liquid  $17\alpha$ -MT capsule, however, induced maturation of males at an earlier time and maintained their sperm production for the duration of the experiment. This finding, coupled with the number of females that matured and the times at which they did so, clearly establishes this treatment as supe-

rior. Further study is required to understand the lower response of females to the combination of LHRH-a pellet and crystalline  $17\alpha$ -MT capsule.

$17\alpha$ -MT has recently been demonstrated to have a stimulatory effect on the pituitary in producing gonadotropin(s) in fish (Crim and Evans, 1979, 1982, 1983). 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. An optimal titer for both steroid and gonadotropin(s) in the blood is required for maturation. This was suggested by the different responses of both sexes to the LHRH-a plus liquid  $17\alpha$ -MT vs. LHRH-a plus crystalline  $17\alpha$ -MT. The LHRH-a pellets are the same. The silastic tubing capsules contained different amounts of steroid in different states. Obviously, knowledge of the characteristics of the silastic implants (i.e., releasing rates, duration, etc.), is required to fully understand the action of this particular treatment and this will be part of our future investigations.

An important outcome of following the ovarian changes of individual fish during this experiment is documentation that milkfish are capable of multiple spawnings during a single spawning season. This capacity has only recently been reported for the milkfish (Lee et al., 1986a,c). The validation of the cannulation method provides confidence that the ovarian changes recorded are not the result of sampling error but reflect the state of the ovary. The rate at which the ova grow and whether or not the different therapies influence the rate of growth or recruitment are topics of future investigations.

Of interest is the synchrony in the possession of large oocytes exhibited by the females as the peak spawning period approached (Table 3). The timing of these events appeared to coincide with the phases of the moon (i.e., quarter moon) as previously reported for the spawning activity of wild milkfish in Philippine waters (Kumagai, 1981, 1984). Note that this observation was made as the hormone treatments were being terminated, raising some obvious questions. What physiological or environmental cue(s) creates the observed results if not hormone therapy? Is it possible that the termination of the therapy produced this result? Will re-initiating the hormone therapy maintain the observed rematuration of the ovary and effectively extend the spawning season? Many questions remain to be answered. However, the strategy we employed for enhancing the maturation of milkfish works. The implantation of LHRH-a pellet plus liquid  $17\alpha$ -MT capsule has produced the most promising results thus far. Further investigation is required to fully understand the mode of action for this particular hormonal therapy.

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## REFERENCES

- Billard, R., Bry, C. and Billet, C., 1981. Stress, environment, and reproduction in teleost fish. In: A.P. Pickering (Editor), *Stress and Fish*. Academic Press, New York, pp. 185-208.
- Borg, B., 1981. Effects of methyltestosterone on spermatogenesis and secondary sexual characteristics in three-spined stickleback (*Gasterosteus aculeatus*, L.). *Gen. Comp. Endocrinol.*, 44: 177-180.
- Coy, D.H., Schally, A.V., Vilchez-Martinez, J.A., Coy, E.J. and Arimura, A., 1975. Stimulatory and inhibitory analogs of LHRH. In: M. Motta, P.G. Crosignani and L. Martini (Editors), *Hypothalamic Hormones*. Academic Press, New York, NY, pp. 1-12.
- Crim, L.W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. *Can. J. Fish. Aquat. Sci.*, 39: 17-21.
- Crim, L.W., 1985. Methods of acute and chronic hormone administration in fish. In: C.-S. Lee and I.C. Liao (Editors), *Reproduction and Culture of Milkfish*. Oceanic Institute, Hawaii, and Tungkang Marine Laboratory, Taiwan, pp. 1-13.
- Crim, L.W. and Evans, D.M., 1979. Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.*, 37: 192-196.
- Crim, L.W. and Evans, D.M., 1982. Positive testosterone feedback on gonadotropic hormone in the rainbow trout. In: C.J.J. Riechter and H.J. Th. Goos (Editors), *Proc. Int. Symp. Reprod. Physiol. Fish*, Pudoc, Wageningen, p. 23.
- Crim, L.W. and Evans, D.M., 1983. Influence of testosterone and/or luteinizing hormone-releasing hormone analogue on precocious sexual development in the juvenile rainbow trout. *Biol. Reprod.*, 29: 137-142.
- Crim, L.W. and Glebe, B.D., 1984. Advancement and synchrony of ovulation in Atlantic salmon with pelleted LHRH analog. *Aquaculture*, 43: 47-56.
- Crim, L.W., Evans, D.M. and Vickery, B.H., 1983a. Manipulation of the seasonal reproductive cycle of the landlocked Atlantic salmon (*Salmo salar*) by LHRH analogues administered at various stages of gonadal development. *Can. J. Fish. Aquat. Sci.*, 40: 61-67.
- Crim, L.W., Sutterlin, A.M., Evans, D.M. and Weil, C., 1983b. Accelerated ovulation by pelleted LHRH analogues treatment by spring-spawning rainbow trout (*Salmo gairdneri*) held at low temperature. *Aquaculture*, 35: 299-307.
- Dziuk, P.J. and Cook, B., 1966. Passage of steroids through silicone rubber. *Endocrinology*, 78: 208-211.
- Kelley, C.D. and Lee, C.-S., 1986. Artificial propagation. In: C.-S. Lee, M.S. Gordon and W.O. Watanabe (Editors), *Aquaculture of Milkfish (Chanos chanos): State of the Art*. Oceanic Institute, Hawaii, pp. 83-116.
- Kumagai, S., 1981. Ecology of milkfish with emphasis on reproductive periodicity. Terminal Report submitted to the SEAFDEC Aquaculture Department and JICA, 106 pp.
- Kumagai, S., 1984. The ecological aspects of milkfish fry occurrence, particularly in the Philippines. In: J.V. Juario, R.P. Ferraris and L.V. Benitez (Editors), *Advances in Milkfish Biology and Culture*. Island Publishing House, Inc., Metro Manila, Philippines, pp. 53-68.
- Kuo, C.M., 1985. A review of induced breeding of milkfish. In: C.-S. Lee and I.C. Liao (Editors), *Reproduction and Culture of Milkfish*. Oceanic Institute, Hawaii, and Tungkang Marine Laboratory, Taiwan, pp. 57-78.
- Kuo, C.M. and Nash, C.E., 1979. Annual reproduction cycle of milkfish, *Chanos chanos* (Forsskal) in Hawaiian waters. *Aquaculture*, 16: 247-252.

- Lacanilao, F.L. and Marte, C.L., 1980. Sexual maturation of milkfish in floating cages. *Asian Aquaculture*, 3(8): 4-6.
- Lacanilao, F., Marte, C.L. and Lam, T.J., 1984. Problems associated with hormonal induction of gonad development in milkfish (*Chanos chanos* Forsskal). *Proc. 9th Int. Comp. Endocrinol. Symp.*, Hong Kong, December 1980, pp. 135-143.
- Lam, T.J., 1982. Applications of endocrinology to fish culture. *Can. J. Fish. Aquat. Sci.*, 39: 111-137.
- Lam, T.J., 1983. Environmental influences on gonadal activity in fish. In: W.S. Hoar, D.J. Randall and E.M. Donaldson (Editors), *Fish Physiology*, Vol. IX, Part B. Academic Press, New York, NY, pp. 65-116.
- Lam, T.J., 1984. Artificial propagation of milkfish: present status and problems. In: J.V. Juario, R.P. Ferraris and L.V. Benitez (Editors), *Advances in Milkfish Biology and Culture*. Island Publishing House, Inc., Metro Manila, Philippines, pp. 21-39.
- Lee, C.-S., 1985. Environmental factors in the reproduction of milkfish. In: C.-S. Lee and I.C. Liao (Editors), *Reproduction and Culture of Milkfish*. Oceanic Institute, Hawaii, and Tung-kang Marine Laboratory, Taiwan, pp. 99-114.
- Lee, C.-S., Weber, G.M. and Thomas, J., 1984. Effects of  $17\alpha$ -methyltestosterone on gonad development in immature milkfish, *Chanos chanos* Forsskal. Abstracts, Second International Milkfish Aquaculture Conference, Iloilo, Philippines, 4-8 October 1983. p.45.
- Lee, C.-S., Tamaru, C.S. and Crim, L.W., 1985. Preparation of a luteinizing hormone-releasing hormone cholesterol pellet and its implantation in the milkfish (*Chanos chanos* Forsskal). In: C.-S. Lee and I.C. Liao (Editors), *Reproduction and Culture of Milkfish*. Oceanic Institute, Hawaii and Tung-kang Marine Laboratory, Taiwan, pp. 215-226.
- Lee, C.-S., Tamaru, C.S., Banno, J.E., Kelley, C.D., Bocek, A. and Wyban, J.A., 1986a. Induced maturation and spawning of milkfish, *Chanos chanos* Forsskal, by hormone implantation. *Aquaculture*, 52: 199-205.
- Lee, C.-S., Tamaru, C.S. and Kelley, C.D., 1986b. Technique for making chronic-release LHRH-a and  $17\alpha$ -methyltestosterone pellets for intramuscular implantation in fishes. *Aquaculture*, 59: 161-168.
- Lee, C.-S., Tamaru, C.S., Kelley, C.D. and Banno, J.E., 1986c. Induced spawning of milkfish, *Chanos chanos*, by a single application of LHRH-analogue. *Aquaculture*, 58: 87-98.
- Lee, C.-S., Weber, G.M. and Tamaru, C.S., 1986d. Effects of orally administered  $17\alpha$ -methyltestosterone on spermatogenesis in immature milkfish, *Chanos chanos* Forsskal. *J. Fish Biol.* (in press).
- Lin, L.T., 1985. My experience in artificial propagation of milkfish - studies on natural spawning of pond-reared broodstock. In: C.-S. Lee and I.C. Liao (Editors), *Reproduction and Culture of Milkfish*. Oceanic Institute, Hawaii, and Tung-kang Marine Laboratory, Taiwan, pp. 185-203.
- Lofts, B., Pickford, G.E. and Atz, J.W., 1966. Effects of methyltestosterone on the testes of a hypophysectomized cyprinodont fish, *Fundulus heteroclitus*. *Gen. Comp. Endocrinol.*, 221: 74-88.
- Marte, C.L., Lacanilao, F.J. and Juario, J.V., 1984. Completion of the life cycle of milkfish, *Chanos chanos* (Forsskal) in captivity. Abstracts, Second International Milkfish Aquaculture Conference, Iloilo, Philippines, 4-8 October 1983, p.21.
- Moore, F.L., 1981. Technique for making small hormone-filled capsules. *Gen. Comp. Endocrinol.*, 43: 409.
- Shehadeh, Z.H., Kuo, C.M. and Milisen, K., 1973. Validation of an in vivo method for monitoring ovarian development in the grey mullet (*Mugil cephalus*). *J. Fish Biol.*, 5: 489-496.
- Sokol, R.R. and Rohlf, F.J., 1969. *Biometry*. W.S. Freeman and Co., San Francisco, 776 pp.
- Stacey, N.E., 1984. Control of the timing of ovulation by exogenous factors. In: G.W. Potts and R.J. Wooten (Editors), *Fish Reproduction: Strategy and Tactics*. Academic Press, London, pp. 207-222.
- Vickery, B.H., 1981. Physiology and antifertility effects of LHRH and agonistic analogs in male

- animals. In: G.I. Zatuchni, J. D. Shelton and J.J. Sciarra (Editors), LHRH Peptides as Female and Male Contraceptives. Harper and Row, Philadelphia, PA, pp. 275-290.
- Weber, G.M. and Lee, C.-S., 1985. Effects of  $17\alpha$ -methyltestosterone on spermatogenesis and spermiation in the grey mullet, *Mugil cephalus* L. *J. Fish Biol.*, 26: 77-84.
- Yamamoto, K., Morioka, T., Hiroi, O. and Omori, M., 1974. Artificial maturation of female Japanese eels by the injection of salmon pituitary. *Bull. Jpn. Soc. Sci. Fish.*, 40: 1-7.