

Induced Spawning of Milkfish, *Chanos chanos*, by a Single Application of LHRH-Analogue

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ABSTRACT

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Current procedures for induced spawning in milkfish, *Chanos chanos*, involve the injection of pituitary homogenate and human chorionic gonadotropin followed by hand stripping of the hydrated eggs. This procedure results in the loss of valuable broodstock, low fertilization rates and unpredictable time of spawning. A reliable method for inducing spawning is essential.

In this study, luteinizing hormone-releasing hormone analogue (LHRH-a) was tested for its effectiveness as an ovulatory and spawning agent. A single administration of LHRH-a via pellet implantation or liquid injection induced spontaneous spawning approximately 48 or 24 h after application, respectively. The average dosage used was $41.7 \pm 3.3 \mu\text{g}/\text{kg}$ body weight for pellet implant and $58.7 \pm 9.3 \mu\text{g}/\text{kg}$ body weight for injection. The spontaneous release of eggs was achieved in all spawning attempts when the initial egg diameter was at least $800 \mu\text{m}$ on average and had a single mode distribution. Seven of 15 attempts with natural fertilized spawns were successful and resulted in an average of 83.8% fertilization.

INTRODUCTION

In many cases, fish kept under captive conditions fail to proceed through their normal reproductive cycle. It has been assumed that culture conditions do not provide an environment conducive to completing maturation of the gonads and spawning. In some cases, changing the culture environment has proven sufficient alteration for fish to resume their normal reproductive activities. In other cases, intervention via hormonal therapies is required at some point along the hypothalamus—pituitary—gonadal axis which controls reproductive activities in teleost fishes (Lam, 1982; Donaldson and Hunter, 1983).

The formulation of a standardized method to induce milkfish (*Chanos chanos*) to spawn has eluded investigators for over a decade (Lam, 1984; Kuo, 1985). Initially, spawning attempts have involved administering piscine pituitary extracts (salmon or carp), plus human chorionic gonadotropin (HCG)

(Vanstone et al., 1977; Juario et al., 1979; Kuo et al., 1979; Liao et al., 1979). More recently, HCG alone has been used to bring about the final maturation of ova (Tseng and Hsiao, 1979; Lin, 1982, 1984). In all past attempts, however, fertilized eggs were obtained by manually stripping both females and ripe males for their gametes. This action in most cases resulted in the loss of broodstock, in only a single spawning during a season, and a low fertilization rate (0–60%). These factors clearly underscore the need for a more reliable method of inducing milkfish to spawn. Survival of milkfish broodstock is very important because of the length of time it takes for milkfish to reach sexual maturity—at least 4 years for the male and 5 for the female (Liao and Chen, 1984). In addition, milkfish are capable of spawning more than once during a single spawning season (Lee et al., 1986a). A reliable method should result in a higher fertilization rate, survival of spawners after spawning, and multiple spawnings.

A limiting factor in trying to influence the activity of the hypothalamus—pituitary—gonadal axis is the availability and biological activity of the chemical messenger employed. Gonadotropins and pituitary extracts used in past spawning attempts on milkfish were obtained from other vertebrate species and may lack the specificity and/or activity required to produce the number of fertilized ova obtained with natural spawnings.

The discovery and characterization of the peptide that controls the synthesis and release of gonadotropins by the pituitary in mammals has provided a new approach by which control over the reproductive activities of fishes may be obtained (Lam, 1982; Donaldson and Hunter, 1983). This peptide, luteinizing hormone-releasing hormone (LHRH), and more recently, its superactive analogues (LHRH-a), have been used to induce ovulation in a number of fish species (Breton and Weil, 1973; Hirose and Ishida, 1974; Lam et al., 1976; Chan, 1977; Crim and Glebe, 1984; Fitzpatrick et al., 1984; Barnabé and Barnabé-Quest, 1985).

In this report, we examine the effectiveness of LHRH-a as an ovulatory and spawning agent for milkfish; the effectiveness of LHRH-a administered in either pellet implants or injections; the stage of maturity at which LHRH-a is most effective in inducing ovulation and spawning in milkfish and, lastly, we demonstrate that the spontaneous release and natural fertilization of eggs may be achieved for milkfish.

MATERIALS AND METHODS

The experimental fish used in the spawning trials were 7–8 years of age and in a weight range of 3.2–5.4 kg. These fish were either undergoing or had undergone chronic hormonal therapies to enhance their maturation. The details and results of the maturation experiments will be presented in other reports (Lee et al., 1986b). Fish used in induced spawning experiments were first anesthetized using 2-phenoxyethanol at a concentration of 0.3 ml per liter of seawater.

The state of maturity of both males and females was then assessed as described by Lee et al. (1985). Ova were obtained by cannulation, fixed in 10% formalin, and their diameters were measured with a compound microscope to $50\ \mu\text{m}$ (Shehadeh et al., 1973). Induced spawning was attempted when a female had an average egg diameter of $650\ \mu\text{m}$ or more. The maturity of the males was assessed by exerting pressure on the abdomen and observing whether or not milt could be extruded.

In each spawning attempt, LHRH-a was only administered once through either intramuscular pellet implantation or injection, in contrast to two or more injections in conventional spawning trials. LHRH-analogue (LHRH-a), des Gly¹⁰ [D-Ala⁶] LHRH ethylamide, used in the induced spawning experiments was purchased from either Sigma Chemical Company, U.S.A. or from Oriental Scientific Instruments and Export Corporation, Shanghai, China. The LHRH-a from Sigma was incorporated into cholesterol pellets with each pellet containing between 200 and $250\ \mu\text{g}$ of LHRH-a. The preparation and administration of the cholesterol implant are described elsewhere (Lee et al., 1985). The LHRH-a purchased from China was dissolved in sterile 0.6% saline just prior to use and administered via an intramuscular injection just below the dorsal fin. A dose of $250\ \mu\text{g}$ per fish was administered to all females and males receiving hormones. Control fish were either injected with normal saline or not treated at all.

These experiments were conducted in a variety of holding facilities, including 30 000-l round fiberglass tanks, 10 000-l wooden tanks, and 5000-l round fiberglass tanks. All holding tanks were provided with running seawater and aeration. Initially, no attempt was made to manipulate the sex ratio of the individuals used in a spawning trial from April to June. In the latter stages (July — November), however, spawning attempts were conducted with a 2 : 1 male to female ratio.

The criterion used to define a successful spawn was the release of ovulated and hydrated eggs into the water column. The number of eggs released in a tank was estimated by taking the average number of eggs found in ten 1-liter subsamples and multiplying by the volume of the spawning tank. Spawning eggs were fixed in 10% formalin and their egg diameters measured as above.

RESULTS

A total of 50 induced spawning attempts was conducted by use of intramuscular pellet implantation ($N=17$) and via injection ($N=33$). During these experiments the average temperature ($26.4 \pm 0.8^\circ\text{C}$) and salinity ($36.3 \pm 1.5\text{‰}$) were relatively stable.

Induced spawning attempts via pellet implants all occurred in fish undergoing chronic hormonal therapies to enhance maturity. During the months of March through July, these fish were receiving LHRH-a pellet implants on a

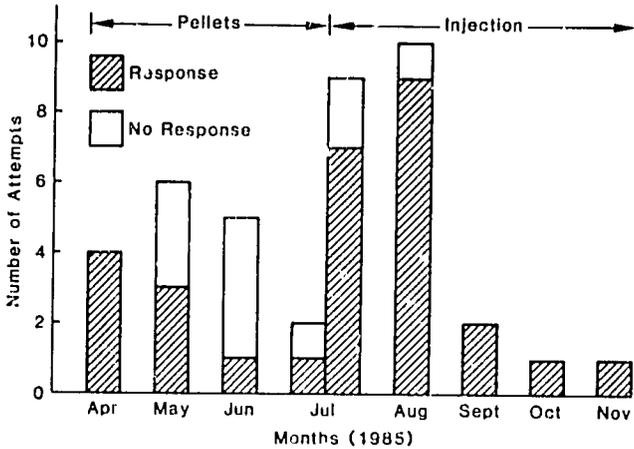


Fig. 1. The frequency of successful and unsuccessful induced spawnings of milkfish, attempted in April through November, 1985. Two strategies were employed in inducing final maturation and spawning: (1) LHRH-a cholesterol pellet implants and (2) LHRH-a liquid injections.

monthly basis. In April, all of four mature fish that possessed large eggs ($790\text{--}950\ \mu\text{m}$) received their regularly scheduled LHRH-a pellet implants. Consequently, they spawned consistently approximately 48 h after being implanted. In following months, similar results were also observed. The frequency of successful spawns, however, decreased dramatically during the months of May through July (Fig. 1). Only one placebo-implanted control fish ever reached the maturation state defined for an induced spawning trial. She was found in the month of July.

The dosages per kilogram body weight in spawning attempts using either implants or injections were $41.7 \pm 3.3\ \mu\text{g}$ or $58.7 \pm 9.3\ \mu\text{g}$ for successful spawning and $51.9 \pm 9.1\ \mu\text{g}$ or $62.5 \pm 12.4\ \mu\text{g}$ for unsuccessful spawning, respectively. The data are presented only for fish which were weighed at the time of spawning. There was no statistical difference ($P > 0.05$) in terms of the amount of LHRH-a administered by either implants or injection between successful and unsuccessful spawns.

The numbers of successful and unsuccessful spawns versus the initial egg diameters at which the implants were administered are illustrated in Fig. 2. None of the successful spawns occurred at an initial egg diameter less than $750\ \mu\text{m}$. Successful spawns occurred when average initial egg diameters fell within or over the range of $750\text{--}800\ \mu\text{m}$. However, there were quite a few unsuccessful spawns in this size range of eggs. Egg size distribution for unsuccessful spawns showed two modes. Overall, a 53% spawning success rate was obtained using the pellet implant as a spawning agent.

Nineteen of 33 attempts using LHRH-a administered via an injection resulted in successful spawns (58% success rate). In contrast to the induced spawns

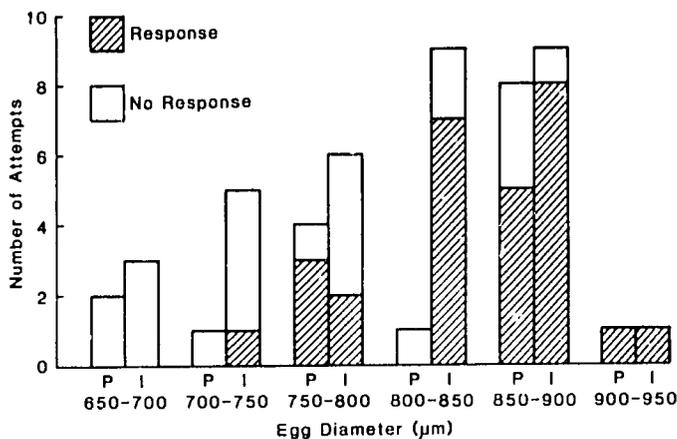


Fig. 2. The number of successful and unsuccessful induced spawns versus the average egg diameters at which hormonal therapies were initiated. The results for two different modes of administration (P = pellet and I = injection) are presented.

with pellets, successful spawns from fish injected with LHRH-a occurred within 20–26 h of the injections. The number of successful spawns was related to the average size of the eggs at the time the LHRH-a injections were administered (Fig. 2). The number of successful spawns appeared to correlate with initial egg diameters between 700 and 950 μm . There were only 16% or three spawning attempts that failed when average egg diameters were between 800 and 950 μm . The egg size frequency distribution from each of the three spawning attempts that failed revealed a bimodal frequency distribution. One mode could be found between 400 and 500 μm and the other peaked between 300 and 900 μm . The egg size frequency distribution from all of the spawning attempts with initial mean oocyte diameters of 750 μm or greater consistently exhibited a similar pattern (Fig. 3). Successful spawns occurred in fish that possessed single modal distribution of egg size and in some fish that possessed bimodal distributions. In the latter case, the smaller clutch of eggs did not exceed 350 μm in size. Spawning attempts in fish that possessed bimodal distribution of egg sizes did not succeed. Notably, the same two females (DTC-7 and DBW-9) were induced to spawn, successfully or not, in accordance with the frequency distribution of egg sizes (Fig. 3).

One female was sampled five times over a period of 26 days. The changes in the egg size frequency distribution and in the eggs themselves are presented in Fig. 4. The first sample represents the state of maturity of the ovary before a single injection of LHRH-a was given. Subsequently, the female spawned within 24 h after receiving the injection. A sample 8 days later revealed that the clutch of eggs that were initially between 250 and 300 μm had grown to an average egg diameter of 421 μm . This sample also possessed some atretic eggs. The eggs continued to grow in size and when they had attained an average diameter of

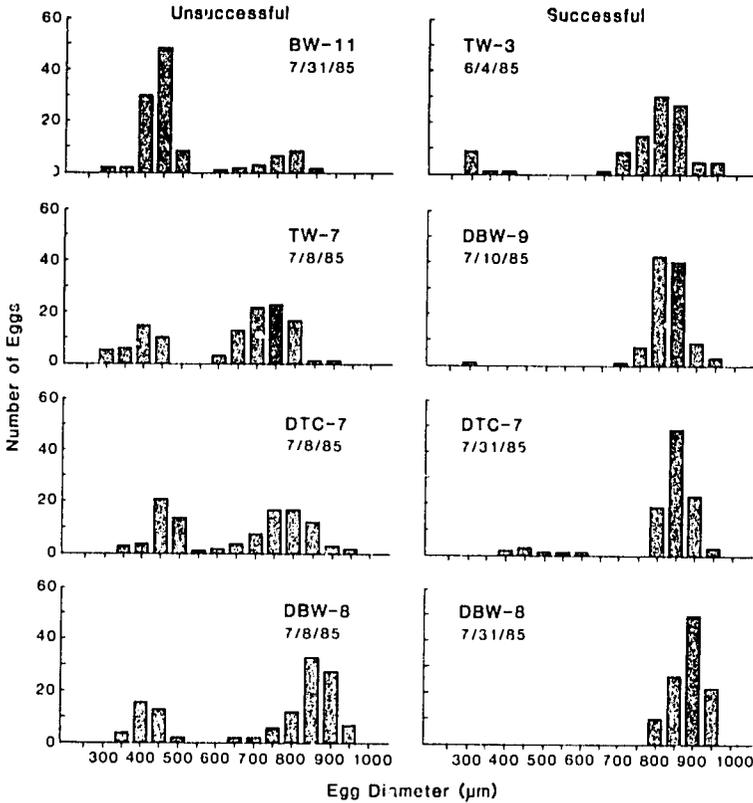


Fig. 3. The observed egg size frequency distribution from individuals in which final maturation and spawning were attempted utilizing a single LHRH-a injection (250 μg). Successful spawns are presented in the right column and unsuccessful in the left.

816 μm , the female was given another injection of LHRH-a and once again spawned within 24 h.

The rate and pattern of growth of the eggs from four individual milkfish are presented in Fig. 5. First, the growth rate of the oocytes appears to be linear between 250 and 800 μm and ranges between 21.4 and 27.4 μm per day. After 800 μm , there was a dramatic reduction in the rate of growth; eggs exhibited little or no change in size. This state could be maintained for at least 5 days, at which time the females were induced to spawn. All four fish were successfully induced to spawn with a single injection of LHRH-a at the final average egg sizes shown in Fig. 5.

Natural fertilization of eggs did not occur in all of the successful spawnings. In some cases, no running ripe males were present when the females spawned. Taking these situations into account, only one in five induced spawnings via pellet implants resulted in fertilized eggs. Concerning spawnings induced via injection, only females were given the LHRH-a initially. Five trials did not

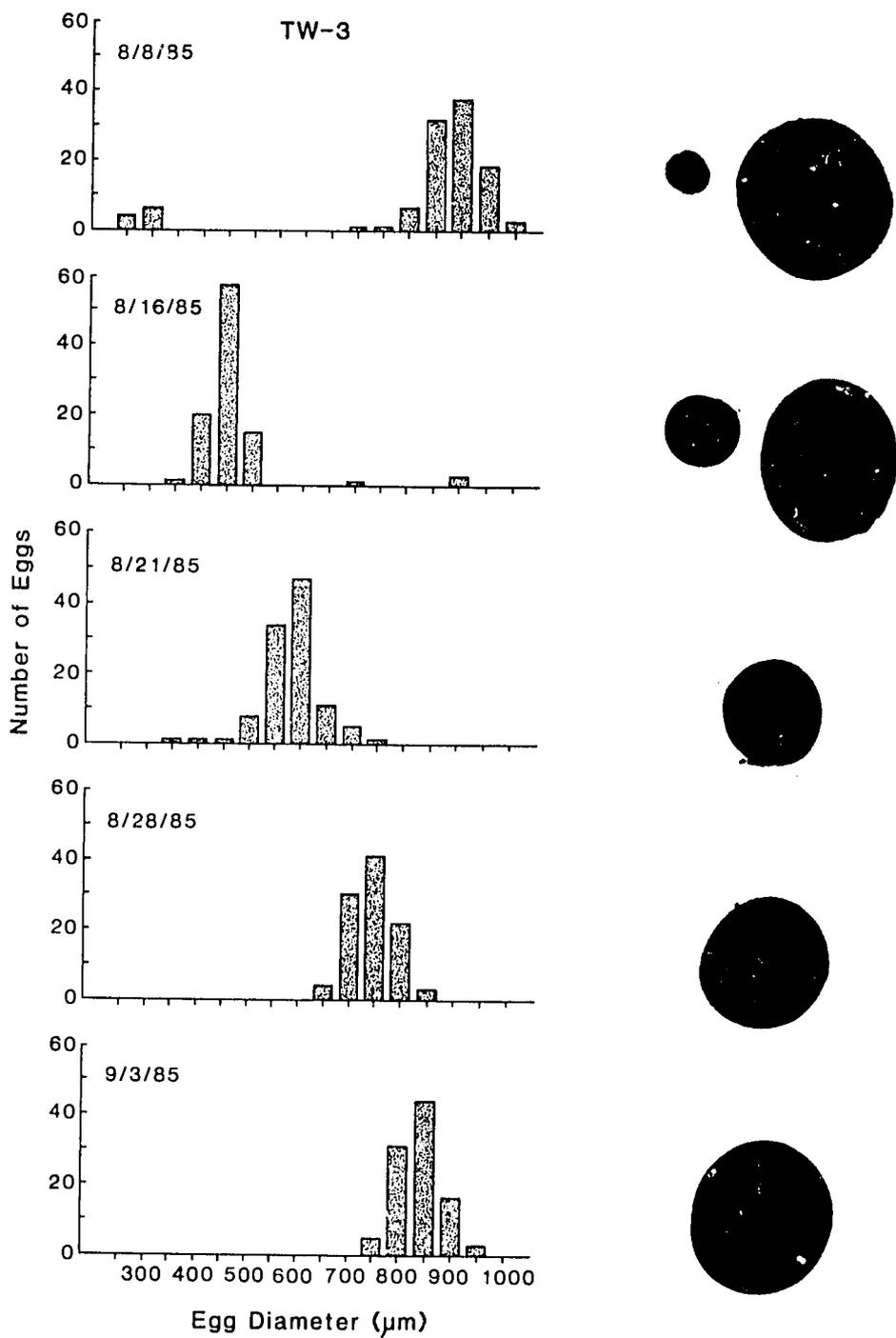


Fig. 4. Changes in egg size frequency distribution of a female milkfish (TW3) sampled over a period of 26 days. The morphology of oocytes sampled via cannulation is also presented.

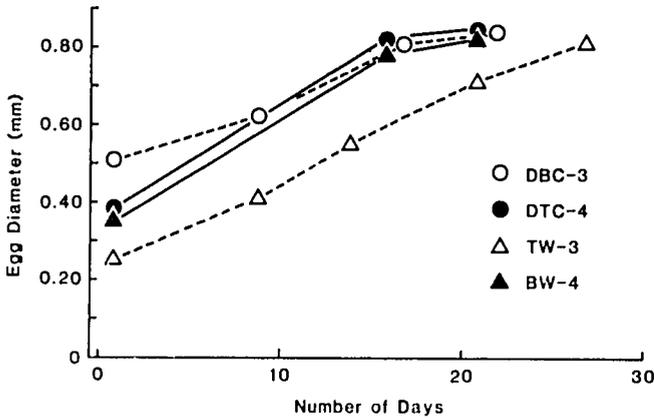


Fig. 5. The growth rate of eggs from four individual milkfish. Each point represents the mean egg diameter from at least 100 ova. The standard errors for each point are approximately 0.05 mm.

result in fertilized spawns even with running males present. We adopted a new strategy which entailed injecting both the females and males at the same time and placing the treated individuals in a smaller holding facility. This method resulted in several "natural" fertilized spawns — seven of 15 attempts.

Table 1 summarizes fertilization rates and number of eggs produced during the spontaneous release of ova and natural fertilization by the males. The fertilization rates are very high, averaging 83.8% and ranging between 14% and 97.4%. The number of eggs released in a fertilized spawn averaged 400 000, about four times the average number of eggs released in an unfertilized spawn. This difference in the number of eggs released is very significant. There is no significant difference ($P > 0.05$) between fertilized and unfertilized spawnings with respect to the diameters of either pre-spawned or spawned eggs.

DISCUSSION

In the early phase of the spawning season, fish that possessed large eggs spawned after receiving their scheduled implants. The spawning of hydrated eggs is the culmination of ovarian maturation regulated by the slow release of LHRH-a from the pellet implants. The decrease in the number of successful spawnings as the natural spawning season approached raises some critical questions. An evaluation of the pellets themselves (i.e., length of time stored before use, ratio of pellet weight:length, etc.), showed no appreciable differences between those related to successful spawnings and those that were not. The decrease in successful spawns via pellets is viewed therefore as a biological phenomenon rather than a technical artifact. Further investigations are required to fully understand the nature of this problem.

Apparently, this problem may be solved by providing a surge of LHRH-a in

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)	No. 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 \pm S.E.			0.839 \pm 0.035	1.248 \pm 0.045	400,000	
Unfertilized spawns						
TW-3*	68.5	5.20	0.785	1.202	59,000	0
DBC-8	52.0	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 \pm S.E.			0.839 \pm 0.053	1.194 \pm 0.054	104,700	

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

the form of an injection, as demonstrated by the spawnings achieved via injections once the proper stage of maturity was reached. This implies that the problem may be one of dosage and of timing the administration for the most receptive state of maturity. The total dosage given to the individual did not differ by form (pellet or injection). The LHRH-a profile that does enter the systemic circulation differs significantly, however, depending upon the mode of administration (Crim, 1985). This difference probably accounts for the temporal variation in spawning occurrence between the two modes of administration (approximately 24 h for injection and 48 h for implantation). The minimal effective dosage for successful spawning is still under investigation.

The stage at which a female will respond to a single administration of LHRH-a (pellet or injection) can be crudely estimated on average egg diameters determined from cannulated oocytes. The information on ovarian maturation reported herein indicates a critical period of approximately a week in which the probability of success of induced spawning with LHRH-a is optimal. This period is determined when average egg diameters are $\geq 800 \mu\text{m}$ and oocytes distributed by size exhibit a single mode. In two modes, the mode with smaller ova should not exceed $350 \mu\text{m}$. The data indicate that this period represents completion of vitellogenesis since eggs exhibit no growth. In addition, induced

spawnings were all successful in this period regardless of whether the eggs had just reached the 800 μm average egg diameter or had remained at this stage for 5 days.

The lack of success in spawning fish having a bimodal frequency distribution of eggs implies that the new clutch of eggs is undergoing a period of rapid growth. The hormonal fluxes which control this event are probably inhibiting response to the surge in LHRH-a. A similar situation has been reported for the curimbata, *Prochilodus scrofa* (Fenerich-Verani et al., 1984). Females that possessed bimodal distribution of eggs did not respond to HCG injections, but when fish having unimodal distributions of the right size oocytes were injected, successful spawnings resulted.

We have made progress in defining conditions that bring about the spontaneous release of hydrated eggs and the natural fertilization of these eggs by the males. The resulting fertilization rates are usually very high and the sacrifice of valuable broodstock is no longer necessary.

One possible explanation for the fertilized spawns is that both males and females were synchronized with LHRH-a injections. Initially, only the females received injections. Spawning was observed but no fertilization occurred, 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). This dribbling of eggs has also been used as a cue to begin stripping eggs from the female (Juarico and Duray, 1983; Juarico et al., 1984; Lam, 1984). The number of eggs released during this kind of activity is about a quarter the number of eggs found in fertilized spawns. The average egg diameters in fertilized versus unfertilized spawns differ little (Table 1), suggesting that the eggs have reached a similar state of hydration. The number of eggs released in fertilized spawns agrees closely with the fecundity estimates used in these experiments (Lee et al., 1986b). Also, the observed numbers of oocytes spawned in our experiments are comparable to the numbers obtained from treated fish that have been stripped (Lin, 1982, 1984; Liao and Chen, 1984). Thus, ovulation rates appear to have approached 100% and the dribbling of eggs may be aberrant behavior. Further evidence supporting this theory is presented in another report describing spawning behavior observed during fertilized and unfertilized spawns (Lee et al., unpubl.).

Another factor to be considered is the isolation of the treated fish 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 taking treated individuals and spawning them in this size tank lends itself to a feasible hatchery design as well as to stock improvement by selective breeding. These results are encouraging. At present, however, fertilized spawns occur only half of the time, even when all spawning conditions are optimal.

In summary, LHRH-a is clearly a potent method for inducing ovulation and spawning in milkfish when administered during a specific state of maturity. The mode of administration does affect spawning success. Although natural fertilization occurred, all factors necessary for a reliable method of producing fertilized eggs are not yet identified and will be the subject of future investigations.

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