Asian Fisheries Science **9** (1996): 9-20.
Asian Fisheries Society, Manila, Philippines

https://doi.org/10.33997/j.afs.1996.9.1.002

Hormonal Induction of Maturation in Striped Mullet, *Mugil cephalus*[1]

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Abstract

The effectiveness of chronic release hormone implants for stimulating testicular and ova-rian maturation in striped mullet, *Mugil cephalus*, was examined. Ten milligram 17α-methyltestosterone silastic capsules induced a significant increase in milt production in males for up to 11 months. Two hundred microgram luteinizing hormone releasing hormone analog pellets accelerated vitellogenesis, which led to an increase in the number of females that devel-oped an additional clutch of oocytes during the spawning season. Hormone implants therefore provide a means for mullet hatcheries to increase broodstock performance.

Introduction

Understanding and being able to manipulate endocrine control of fish reproduction is critical to the development of hatchery technology. The removal of fish from their natural environment to a hatchery tank or pond environment for breeding can have a significant effect on hormonal regulation of gonad function. The most common problem in hatchery broodstock is inhibition of the final stages of oocyte and sperm development and release, collectively referred to as spawning. A number of species also show inhibition of earlier stages of oocyte and sperm development (collectively called maturation) under hatchery conditions.

A number of techniques are used to induce spawning when it is inhibited by captivity. These will be reviewed in another paper presented at this forum. In brief, the actual physiological processes involved are usually triggered by an acute increase in endogenous hormone level. A common and effective method to duplicate this naturally occurring surge is to administer an intraperitoneal or intramuscular injection. Maturation, on the other hand, is stimulated by a chronic and smaller increase in hormone levels. Inducing these processes requires a continuous delivery of hormone into the fish for up to several months. Although the most common method has been to add hormone to the feed (Lam 1982; Weber and Lee 1985), this means of administration has several drawbacks. First, only hormones which are not susceptible to enzymatic degradation in the digestive tract can be used (Crim 1985). Secondly, due to loss of hormone while the feed is in water, or from failure to be absorbed across the

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[1]A revised version of the paper presented at the Symposium "Hormones in Aquacultures," Third Asian Fisheries Forum, Singapore, October 1992.

wall of the intestines, an excessive quantity of hormone is required. Thirdly, the actual dosage of the hormone each fish receives is dependent on their feeding rate, which can be quite variable.

Recent advances in chronic hormone delivery vehicles have produced a variety of pellets and capsules which slowly release hormones when implanted into the musculature or abdominal cavity (reviewed by Crim 1985). Since release is directly into the body fluid, any hormone can be administered using this method and, because there is no loss of hormone, the treatment dosage can be significantly reduced. In addition, precise dosages can be more effectively administered since the amount of hormone reaching the circulatory system is not dependent on feeding rate.

These implants have recently been tested for their effectiveness in stimulating maturation in striped mullet, *Mugil cephalus*. The percentage of male broodstock which mature naturally in captivity is often less than 50% (Lee et al. 1992) and, due to handling stress, many cease milt production after only a single spawning trial. A high percentage of female broodstock will naturally mature each season, but produce only a single clutch of eggs (Greeley et al. 1987; Kelley et al. 1987).

Experiments were conducted to determine whether implants can induce male testicular maturation and thereby increase the percentage of broodstock available for spawning trials. Experiments with females tested the effectiveness of implants for inducing maturation and stimulating an increase in individual egg production. This paper summarizes the results of these experiments.

Materials and Methods

General Methods

The fish were maintained at the Oceanic Institute in Hawaii in either 29,000-l rubber-lined dirt ponds or 5,000-l fiberglass round tanks fitted with continuous water exchange and aeration. To obtain measurements and samples, or to administer hormones, the fish were removed from the ponds by seine net and placed into an anesthesia tank containing 300 ppm 2-phenoxy ethanol. After data and samples had been obtained, the fish were revived in a recovery tank and returned to the ponds until the next scheduled sampling date. Individual fish were identified with pittags (Biosonics Inc.) implanted into the dorsal musculature.

Testicular maturation in males was assessed by milt production. The abdomen of each male was massaged in an anterior to posterior direction, and the quantity of milt extruded from the gonopore was subjectively rated between zero and three. Ovarian maturation in females was assessed by determining the stage of oocyte growth and the mean vitellogenic oocyte diameter. Oocyte samples were obtained by cannulation and examined under a microscope to determine whether the oocytes were previtellogenic (i.e., primary growth or cortical alveoli stage) or vitellogenic. Vitellogenic oocyte samples were then saved in 10% formalin, and examined under a microscope fitted with an ocular micrometer. Mean oocyte diameters were calculated from a

measurement of 100 oocytes from each sample. Samples having a mean diameter of 200-599 μm were recorded as vitellogenic or maturing. Samples having a mean diameter of 600 μm or over were recorded as postvitellogenic or fully mature.

Hormones were administered in one of two types of chronic release implants. The peptide hormone, Des Gly^{10} $D\text{-}Ala^{6}$ Luteinizing Hormone Releasing Hormone analog (LHRH), was incorporated into cholesterol pellets containing approximately 200 μg. Steroid hormones, specifically testosterone (T),17α-methyltestosterone(MT), 11-ketotestosterone (KT) or 17β-estradiol (E2), were loaded into 2-cm long silastic capsules sealed with an elastomer. A complete description of how the implants were made is provided in Lee et al. (1986). Both types of implants were injected into the dorsal musculature.

Data were subjected to chi-square analysis and one-way Anova.

Male Experiments

EXPERIMENTS 1 AND 2: MT CAPSULE DOSE RESPONSE

In experiment 1, conducted in June-September 1985, 25 fish were divided into three treatment groups and one control group. Half of the controls (n=10) were implanted with an empty silastic capsule. Treatment group 1 (n=5) received a capsule containing 0.25 mg MT dissolved in castor oil. Treatment group 2 (n=5) received a capsule containing 2.5 mg MT dissolved in castor oil, and treatment group 3 (n=5) received capsules containing 2.5 mg of undissolved MT. Testicular maturation was assessed at 2-, 3- or 4-week intervals.

In all subsequent experiments, only undissolved MT was used. In experiment 2, conducted in August-October 1986, 15 fish were divided into three groups of five. Those in the control group were implanted with an empty silastic capsule. Treatment group 1 fish were implanted with a 2.5 mg MT capsule, and treatment group 2 fish were implanted with a 10 mg MT capsule. MT in both treatment groups was in crystalline form. Testicular maturation was assessed weekly for 5 weeks.

EXPERIMENT 3: MT CAPSULE DURATION OF EFFECTIVENESS

In this experiment, conducted between August 1987 and July 1988, 30 males were divided into two groups of 15. The fish in the control group were not implanted. The fish in the treatment group were implanted with a 10 mg MT capsule. Testicular maturation was assessed at 4-week intervals.

EXPERIMENT 4: COMPARISON OF MT, T AND KT CAPSULES

In this experiment, conducted between August 1989 and May 1990, 40 males were divided into four groups of 10. The fish in the control group were not implanted. Treatment group 1 fish were implanted with a 10 mg T capsule. Treatment group 2 fish were implanted with a 10 mg KT capsule, and treatment group 3 fish were implanted with a 10 mg KT capsule. Testicular maturation was assessed at 4-week intervals.

Female Experiments

EXPERIMENT 1: EFFECTIVENESS OF LHRH AND MT IMPLANTS DURING THE OFF-SEASON

In this experiment, conducted in June-December 1985, 64 females were divided into eight groups of eight which included three types of controls, two sources of LHRH, two sources of LHRH plus MT and an MT group. Since there was no significant difference between the controls, or between the two sources of LHRH with or without MT, these groups were combined for the purpose of this paper into four main groups. The fish in the control group (n=24) were implanted with either blank pellets or empty silastic capsules. Treatment group 1 fish (n=16) were implanted with a single 200 µg LHRH pellet. Treatment group 2 fish (n=16) were implanted with both a 200 ug LHRH pellet and a 0.25 mg MT capsule. Treatment group 3 fish (n=8) were implanted with a 0.25 mg MT capsule. Ovarian maturation was assessed monthly starting in July.

EXPERIMENT 2: EFFECTIVENESS OF LHRH, T AND MT IMPLANTS DURING THE ON-SEASON

In this experiment, conducted between November 1986 and March 1987, 46 females, all of which had initiated vitellogenesis, were divided into two treatment groups and one control group. Treatment group 1 (n=11) received two implants: an LHRH pellet and a 0.25 mg MT capsule. Treatment group 2 (n=11) also received two implants: an LHRH pellet and a 0.25 mg T capsule. The fish in the control group (n=24) received a placebo pellet and a capsule. Ovarian maturation was assessed at 3-week intervals.

EXPERIMENT 3: COMPARISON OF LHRH, T AND E_2 IMPLANTS DURING THE ON-SEASON

In this experiment, conducted between November 1987 and March 1988, 46 females, all of which had initiated vitellogenesis, were divided into five groups. Treatment group 1 (n=9) received a 2.5 mg E_2 capsule. Treatment group 2(n=9) received a 2.5 mg T capsule. Treatment group 3 (n=8) received an LHRH pellet. Treatment group 4 (n=8) received an LHRH pellet and a 2.5 mg E_2 capsule. Treatment group 5 (n=8) received an LHRH pellet and a 2.5 mg T capsule. Ovarian maturation was assessed at 3-week intervals.

Results

Male Experiments

The results of experiments 1 and 2 are provided in Tables 1 and 2. Neither controls nor males implanted with 0.25 mg MT capsules matured during the first experiment. Of the males implanted with 2.5 mg MT capsules, 20-60% matured in both experiments starting at 3 weeks after implantation. There was no significant difference between castor oil-dissolved and undissolved MT. Males implanted with 10 mg MT capsules started maturing after week 2 of the second experiment. All the males were mature by week 4. Therefore, among the dosages tested, 10 mg of MT produced the most effective response.

Table 1. Percentage of control and treated males which were mature at each sampling period during experiment 1.

Treatment	Week 0	4	7	9	11
Control	0	0	0	0	0
0.25 mg MT in castor oil	0	0	0	0	0
2.50 mg MT in castor oil	0	0	40	60	60
2.50 mg MT in crystalline form	0	0	40	40	60

Table 2. Percentage of control and treated males which were mature at each sampling period during experiment 2.

Treatment	Week 0	2	3	4	5
Control	0	0	0	0	0
2.50 mg MT	0	0	30	30	60
10.0 mg MT	0	20	40	100	100

The results of experiment 3 are shown in Fig. 1. From 2 weeks after implantation in August until April, 60% or higher of the males implanted with 10 mg MT capsules were mature. This percentage was consistently higher than that of the controls throughout the entire 11 months of the experiment ($P<0.05$, 1-way ANOVA), suggesting that 10 mg MT capsules have an effective duration of close to 1 year.

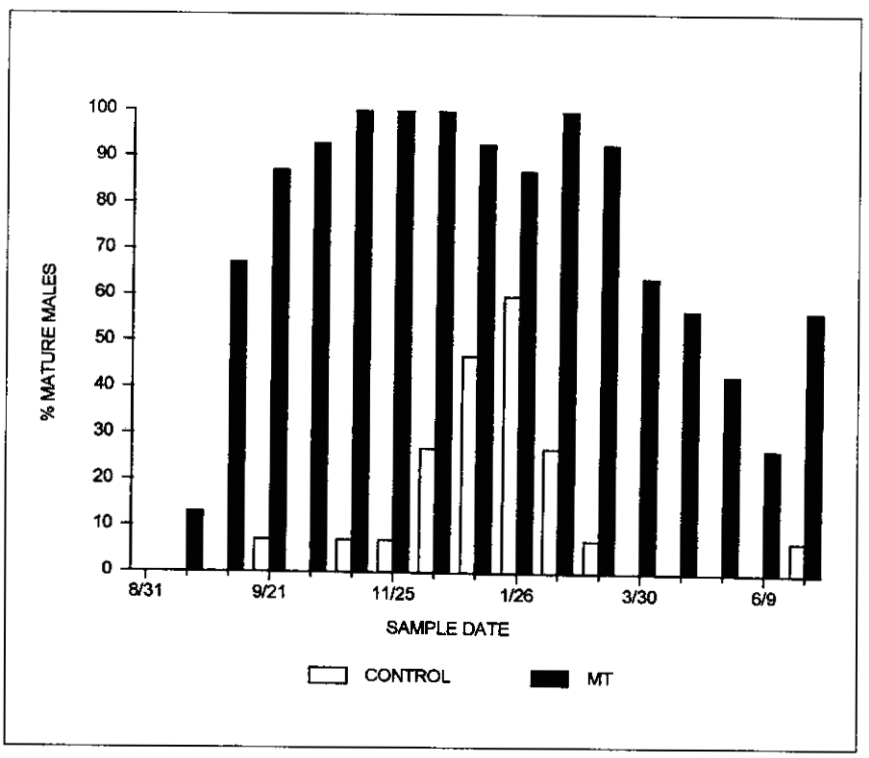

Fig. 1. Effects of a single MT implant on male mullet maturation.

Table 3 summarizes the individual spawning frequency data of both control and implanted males. Sixty-five percent of the MT implanted males versus 17% of the control males were used in more than four spawning trials. By increasing the milt production, MT capsules therefore increased the usefulness of the implanted males.

Table 3. Percentage of control and MT-implanted males in male experiment 2 which were repeatedly used in different spawning trials during the season.

	Number of spawns				
Treatment	1-4	5-8	9-12	13-16	17-20
Controls	83	10	7	0	0
10 mg MT	35	32	24	6	3

The results of experiment 4 are presented in Fig. 2. The average monthly percentage of mature males in the control group was lower than that of all three treatment groups. KT and MT capsules produced the highest percentage of mature males and both also had similar durations of at least 8 months. Since MT is far less expensive than KT, MT is a more economically feasible treatment.

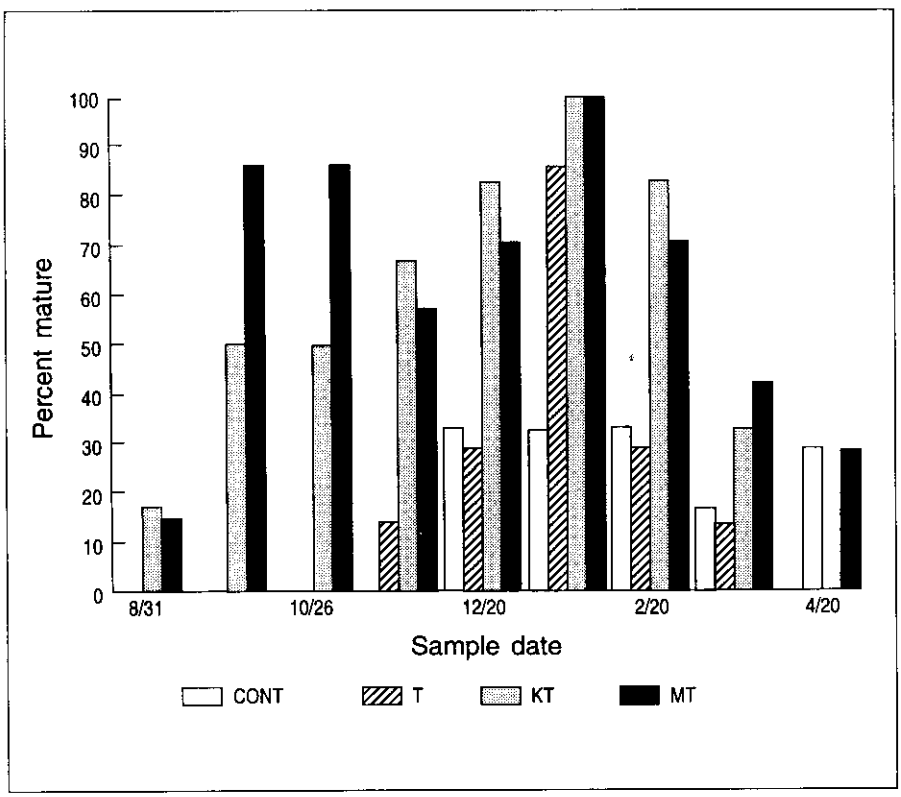

Fig. 2. Comparison of MT, T and KT capsules on male mullet maturation.

Female Experiments

The results of experiment 1 are presented in Fig. 3. There was no significant difference between control and treatment groups with respect to the onset of vitellogenesis. By end of October, 20-40% of the females in each group had vitellogenic eggs, while none of the groups had females undergoing vitellogenesis at the end of September. Therefore, none of the three treatments, LHRH pellets, MT capsules, or both, accelerated the onset of maturation, nor specifically altered the pattern of maturation in comparison to controls.

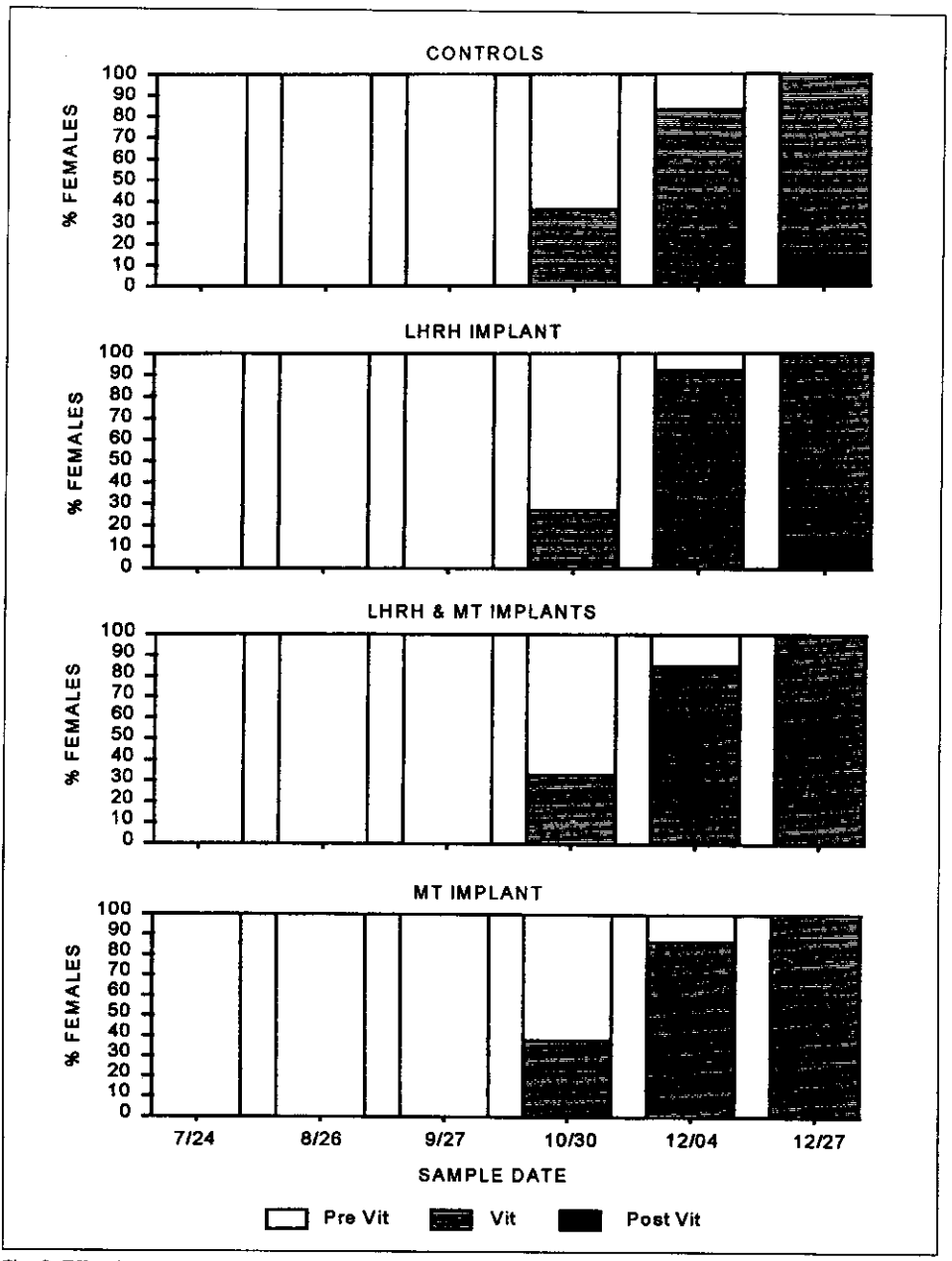

Fig. 3. Effectiveness of LHRH and MT implants during the off-season on female mullet maturation.

Table 4 shows the mean daily increase in oocyte diameter for each of the three groups in experiment 2. The mean oocyte growth rate for the control group was 4.0 $\mu m \cdot day^{-1}$. In contrast, the rate for the LHRH+T group was significantly higher (11.3 $\mu m \cdot day^{-1}$), while the rate of the LHRH+MT group was significantly lower (1.8 $\mu m \cdot day^{-1}$).

Table 4. Mean oocyte growth rates for the control and treatment groups in female experiment 2.

		Mean oocyte diameter (μm)		
Treatment	N	Day 0	Day 22	Mean daily increase (μm)
Control	10	216	304	4
LHRH+T	9	216	465	11.3
LHRH+MT	10	220	260	1.8

Table 5 shows the cumulative percentage of females which had completed vitellogenesis during the first 2.5 months of the experument. In correlation to the oocyte growth rate data, a significantly higher percentage of females in the LHRH+T group were postvitellogenic by 22 December in comparison to controls, while none of the LHRH+MT females had completed vitellogenesis by 2 February. Therefore, a combination of LHRH and T implants accelerated maturation, while a combination of LHRH and MT implants inhibited maturation.

Table 5. Cumulative percentage of females in the control and treatment groups which completed vitellogenesis at each sampling period during female experiment 2.

	Date				
Treatment	11/18	11/25	12/16	1/6	1/27
Control	0	0	0	8	29
LHRH+T	0	9	36	73	73
LHRH+MT	0	0	0	0	0

Table 6 shows the mean daily increase in oocyte diameter for each of the five treatment groups in experiment 3. There was no significant difference between the E_2 and T groups (7.3 and 6.6 $\mu m \cdot day^{-1}$, respectively) nor between LHRH, LHRH+E_2 and LHRH+T groups (10.5, 9.9 and 11.0 $\mu m \cdot day^{-1}$, respectively). Females implanted with LHRH, with or without E_2 or T, had a significantly higher oocyte growth rate than either the E_2 or T groups. These results suggest that LHRH pellets, rather than T or E_2 capsules, are capable of significantly accelerating oocyte growth rates.

Table 6. Mean oocyte growth rates for each group in female experiment 3.

Treatment	N	Mean oocyte diameter (μm) Day 0	Day 28	Mean daily increase (μm)
E_2	9	267	421	7.3
T	9	302	439	6.6
LHRH	8	287	508	10.5
LHRH+E_2	8	290	499	9.9
LHRH+T	8	308	538	11

Similar to Table 5, Table 7 shows the cumulative percentage of females which had completed vitellogenesis during the first 2-3 months of experiment 3. In correlation to the oocyte growth rate data, a significantly higher percentage of females in groups implanted with LHRH pellets were postvitellogenic by 16 December, in comparison to the groups implanted with steroids alone. Fully mature and spawnable females were found 3 weeks after implantation in the LHRH, LHRH + E_2 and LHRH+T groups, but not in the E_2 and T groups.

Table 7. Cumulative percentage of females in each group which completed vitellogenesis at the first five sampling periods during female experiment 3.

Treatment	Date 11/4	11/25	12/16	1/6	1/27
E_2	0	0	22	56	78
T	0	0	40	70	80
LHRH	0	40	70	80	90
LHRH+E_2	0	22	67	78	89
LHRH+T	0	25	75	88	88

The combined results of experiments 2 and 3 suggest that LHRH pellets, either alone or in combination with E_2 or T capsules, accelerate maturation; while LHRH pellets in combination with MT capsules inhibit maturation. Table 8 shows the percentage of females in each of the groups from the two experiments which spawned either zero, one or two times during the season. These data suggest that by accelerating maturation, LHRH pellets stimulated multiple spawning in mullet. Secondly, by inhibiting maturation, a combination of LHRH and MT implants inhibited spawning. Third, E_2 and T capsules alone had little or no effect on maturation and spawning.

Table 8. Summary of female experiments 2 and 3: Percentage of females in each group which spawned either zero, one, or two times during the season.

Treatment	Number of spawns 0	1	2
Control	34	62	4
E_2	11	78	11
T	10	80	10
LHRH	10	50	40
LHRH+E_2	11	33	56
LHRH+T	17	44	39
LHRH+MT	73	27	0

Discussion

The types of hormone implants used in these studies are not new. Dziuk and Cook (1966) described silastic implants containing steroids. Moore (1981) and Shelton (1982) both described testosterone capsules which had an effective duration of 1 year or longer. Parkes (1942) demonstrated the effectiveness of cholesterol as an excipient for peptide hormones, while Kent et al. (1980) and Crim et al. (1983) described the use of LHRH cholesterol tablets in mammals and fish, respectively.

Since a radioimmunoassay for MT is not presently available, the release rate of the 10 mg MT capsule has not been measured. Release rates of T and KT capsules have been measured and both show an initial transient spike followed by a sustained low release for a period of at least 1 month (Tamaru et al. 1990). The release rate of the 200 μg LHRH pellet also has not been measured. However, Crim (unpubl. data) assayed the pellet by measuring serum Gth levels, and found that it causes a significant increase in Gth when implanted either in the intraperitoneal cavity or intramuscularly.

Our results show that these implants are an effective method for stimulating maturation in both male and female mullet. Males implanted with a single 10 mg MT capsule produced milt continuously for up to 11 months. In females which had initiated vitellogenesis, a single LHRH pellet accelerated the process, thereby reducing the length of the maturation period. This ultimately led to an increase in the number of fish which developed and matured a second clutch of eggs before the end of the reproductive season.

LHRH pellets did not stimulate females to initiate maturation when implanted during the off-season. At this time of year, mullet ovaries are characterized by previtellogenic oocytes undergoing primary growth (Kelley et al. 1991). Studies have shown that primary growth is Gth-independent and that oocytes reach a "critical size" prior to initiating the cortical alveoli stage, which is the first Gth-dependent stage (Barr 1968; Khoo 1979; Wallace and Selman 1981). Furthermore, it has been shown that the pituitary gonadotropic cells undergo seasonal morphological changes (Kaneko et al. 1986). Therefore, the failure of the LHRH pellets during the off-season may have been due either to the pituitary being unresponsive to LHRH, the ovary being unresponsive to Gth, or both.

Off-season maturation in females can be stimulated by shortening the photoperiod and lowering water temperature (Kuo et al. 1974; Kelley et al. 1991). Once the oocytes initiate vitellogenesis, an LHRH pellet can be used to insure the completion of the process and stimulate multiple clutch formation. Using a combination of environmental manipulation and LHRH pellets has resulted in up to four spawns by individual females during a single year (Kelley et al., unpubl. data).

Combining an E_2 or T capsule with an LHRH pellet did not increase the rate of vitellogenesis. However, combining an MT capsule decreased the rate, which ultimately inhibited the oocytes from completing maturation during the season. Females receiving this treatment showed significantly lower serum levels of E_2 and T (Tamaru et al. 1989). Kelley et al. (1987) suggested that these

results could be explained if T levels are regulated by negative feedback and MT is a non-aromatizable. MT capsules would then cause a significant decrease in E_2 levels which would inhibit vitellogenesis.

In contrast, MT capsules appear to stimulate testicular maturation at any time of the year. There are several possibilities which could explain this result. First, histological studies have shown that the mullet off-season testis contains only spermatogonia (Grier 1981). It has been shown in Japanese eel that both human chorionic gonadotropin (HCG) and KT can stimulate spermatogonia to initiate and complete all stages of spermatogenesis in vitro (Miura et al. 1991). Therefore, unlike females, males may not have either a gonadotropin-independent nor a steroid-independent stage during their annual cycle. Second, if a steroid-independent stage does exist, it is asynchronous and does not create an unresponsive period. Once spermatogenesis is initiated, all post-spermatogonial stages can be found at the same time since intra- but not intercystic maturation is synchronous (Grier 1981). However, spermatogonia are not enclosed in cysts, which form at the onset of spermatogenesis. To our knowledge, the appearance of different stages of spermatogonia (i.e., types A and B) in mullet testes has not been studied. Finally, MT may stimulate spermatogenesis directly rather than indirectly through the release of Gth from the pituitary. Therefore, even if the gonadotropic cells were in a "regressed state," testicular maturation could still be induced.

Conclusion

Chronic release hormone implants are an effective means of correcting stress-related inhibition of maturation in mullet hatcheries. They also appear to improve broodstock performance by stimulating milt production beyond the natural season in males, and multiple clutch formation in females. Coupled with environmental control techniques, year-round production of mullet fry is a clear possibility in the near future.

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Manuscript received 15 January 1991; accepted 15 February 1996.