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Induced Sexual Maturation of Herring Using GnRH 'Quick-Release' Cholesterol Pellets

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ABSTRACT

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An analogue of gonadotropin-releasing hormone, [D-Ala^6, des Gly^{10}] mammalian GnRH ethylamide (mGnRH-A) was effective in advancing maturation of Pacific herring (*Clupea harengus pallasi*) provided the fish had matured to Hay's modified Hjort stage IV (gonads are two-thirds of mature size; germinal vesicles have not migrated). The analogue was administered in new 'quick-release' cholesterol pellets made of cholesterol and cellulose (4:1, *w/w*). In vitro perfusion studies indicated 94% of the 100 μg of mGnRH was released from such pellets during 12 h. Implanted 'quick-release' mGnRH-A pellets resulted in more mature fish; the advancement over control fish was maintained for at least 3 weeks. Also, a higher percentage of fish reached a more mature Hjort's stage in response to mGnRH in pellets containing 100 μg (dose approximately 800–1000 μg/kg) compared with 20 μg (dose approximately 170–220 μg/kg). The response to different GnRH analogues may be species specific because [D-Ala^6, des Gly^{10}] salmon GnRH ethylamide was not effective in herring, but is known to be as effective as mGnRH-A in milkfish. A potential application of mGnRH-A is to advance maturation of stage IV herring kept in captivity (impounded) for commercial production of spawn-on-kelp.

INTRODUCTION

Schools of Pacific herring (*Clupea harengus pallasi*) normally spawn en masse during the spring (Rounsefell, 1930; reviewed by Hay, 1985). However, synchrony of sexual maturation and spawning within schools is poorly understood. Once factor which probably controls these reproductive events is gonadotropin-releasing hormone (GnRH). This hormone is thought to trigger the pituitary to release gonadotropin which, in turn, induces gonadal matura-

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tion. GnRH-like material is present in the brains of herring as in other teleosts (Sherwood, 1986).

Application of synthetic GnRH may provide clues about the role of the native hormone in herring reproduction. Studies on the effects of GnRH in other teleosts have been reviewed recently (Lam, 1982; Peter, 1982, 1983; Sherwood, 1987) and a technical paper on the use of GnRH-A in herring has appeared (Kreiberg et al., 1987). Synthetic preparations of mammalian and salmon GnRH on their analogues are widely effective in releasing gonadotropin and accelerating ovulation, but the effects vary with the nature of the molecule and the species of fish (Crim, 1984; Donaldson et al., 1984; MacKenzie et al., 1984; Peter et al., 1985). In general, GnRH analogues are more effective than native GnRH. Also, multiple injections of GnRH and implantation of preparations from which GnRH is released more slowly, such as a cholesterol pellet, have been effective for induction of reproductive responses in fish (Peter, 1980; Breton et al., 1983; Crim et al., 1983a,b; Weil and Crim, 1983; Crim and Glebe, 1984; Van Der Kraak et al., 1985).

In Pacific herring, injection of a GnRH analogue has been shown to advance the onset of the stage in which sperm and ova can be released by the application of gentle pressure (Kreiberg et al., 1987). However, spawning did not result from this treatment and the minimum stage of maturity at which herring can respond to GnRH-A is not known. We therefore decided to test a sustained-release preparation, higher doses of GnRH-A, and a teleost-based GnRH-A in herring to see if the effectiveness of the treatment could be extended to induce spawning and advance the sexual maturation of fish earlier in the year. Our first objective was to determine the rate of release of mGnRH-A in pellets with a cellulose–cholesterol matrix. Our second aim was to observe the stage of sexual maturation induced in adult herring treated at different stages of sexual development with different doses of a superactive GnRH analogue embedded in a pellet.

MATERIALS AND METHODS

Pellet preparation and evaluation

The GnRH analogues were [D-Ala^6, des Gly^{10}] mammalian GnRH ethylamide (mGnRH-A; Syndel) and [D-Ala^6, des Gly^{10}] salmon GnRH ethylamide (sGnRH-A; Bachem). Cylindrical pellets of 3×3 mm were made of cholesterol/cellulose (4:1, w/w) using a pellet press (Parr Instrument Co., Moline, IL). The analogue was mixed with cholesterol/cellulose powder prior to pelletization. The final weight of each pellet was approximately 30 mg (28.0 ± 0.2, mean $\pm$ SE).

To determine the in vitro release of GnRH, we placed pellets in a 25-ml glass flow-through chamber with a Sylgard (Dow Corning) lined bottom. An inlet

arm was present at the bottom of the vessel and an outlet arm at the top. Phosphate-buffered saline (1.25 g Na_2HPO_4, 0.15 g KH_2PO_4, 8.5 g NaCl, 0.1 g ethylmercurithiosalicylate sodium salt/l) with gelatin (10 g/l), pH 7.6, was pumped into the chamber with a perfusion pump. The outflow was collected using a fraction collector. A control sample of medium was collected just before the pellet, held in a nytex net, was placed on the bottom of the chamber by pinning the net to the Sylgard bottom. Gentle bubbling of air through a needle was used to constantly mix the medium in the chamber. Ten-min fractions of about 1–2 ml were collected for 4, 6, 8, or 12 h. The volume of each fraction was measured and an aliquot removed for radioimmunoassay (RIA). Pellets used for the in vitro studies contained native mammalian GnRH because our antiserum, B-6, detects native GnRH, but not the analogue. The experiments were carried out in a Percival environment chamber at 9 °C to match the water temperature in which herring were held. At the end of the experiment, each pellet was placed in 1 ml of 0.01 *N* HCl, pulverized, vortexed, rehydrated for 1 h and centrifuged at 10 000*g* for 30 min. An aliquot of the supernatant was taken for RIA.

Pellets with matrix only ($n=5$) or with 100 μg of native GnRH hormone ($n=16$) were implanted into herring ($n=21$) in October 1984. Ten pellets with hormone were removed 1 week later; the remainder of the hormone pellets and the control pellets were removed 2 weeks after implantation. GnRH was extracted from the pellets as outlined above for the in vitro studies. An aliquot of the supernatant was taken for RIA.

GnRH in the pellet studies was measured by RIA as described by Sherwood et al. (1983, 1986). Synthetic mammalian GnRH (LHRH, Peninsula Labs.) was used as standard and as ^{125}I-tracer. Antiserum B-6 at a final dilution of 1:5000 was used for the assays. This resulted in 49–64% binding of the total ^{125}I-labelled GnRH (B_0) added to each reaction tube. A level of 20% inhibition of this binding was obtained at 3–8 pg of mammalian GnRH.

Biological response and evaluation

Pacific herring (*Clupea harengus pallasi*) were taken from wild-caught stock held in netpens at the Pacific Biological Station in Nanaimo. B.C., in September 1984, February 1985, and March 1985; they were used in the experiments referred to as September, February and March. The parent stock spawns annually during the latter part of March. The fish we used averaged 114.7 ± 0.3 g (mean $\pm$ SE) in September, 99.1 ± 0.4 g in February, and 76.3 ± 0.3 g in March (the latter group was selected for smaller size to avoid early maturation).

Twelve groups of adult herring (sex unknown) were used: four groups of 20 each in the September and February experiments and four groups of 10 each in the March experiments. In the September and February experiments, the groups were as follows: (1) control untreated, (2) control implanted with blank

pellets, (3) experimental implanted with pellets containing 20 μg mammalian GnRH analogue (low dose), and (4) experimental implanted with pellets containing 100 μg mammalian GnRH analogue (high dose). On the basis of body weight, the doses of hormone with the 20 μg and 100 μg pellets, respectively, were 172 μg/kg and 943 μg/kg (September), 220 μg/kg and 862 μg/kg (February) and 202 μg/kg and 1006 μg/kg (March). Half of groups 2, 3 and 4 received a second identical pellet 2.5 weeks later; the other half were sacrificed for determination of gonadosomatic index. In the March experiment, the four groups were the same as listed above for the September and February experiments, except group 3 received pellets containing 100 μg salmon GnRH analogue instead of the low-dose mammalian analogue. Second pellets were not implanted in the March groups. The fish were sacrificed at the end of all three experiments.

The herring were immobilized by placing them in chilled sea water (0.5–1.0°C) prior to implantation. The pellets were pushed through the peritoneum via a stainless steel tube inserted through a 4-mm incision medial to the pelvic fin. Each group was then placed in a separate 3-m tank of filtered running sea water maintained at 9–11°C, and fed ad libitum with a modified commercial salmon grower diet.

Sexual maturity of the males and females was evaluated by the International (Hjort) Scale (Bowers and Holliday, 1961) as modified for Pacific herring by Hay (1985). In this scale sexual maturity of the herring can be divided into several stages. Hjort stage I refers to 'undeveloped' or thread-like gonads. Stage II herring have 'starting' or ribbon-shaped gonads with a breadth of less than 5 mm; some of the oocytes are developing yolk vesicles. The 'developing' (stage III) and 'maturing' (stage IV) gonads are in a growth phase, but are distinguished from each other primarily by gonad size resulting in a considerable difference in the gonadosomatic index (GSI). Stage V 'mature' eggs become transparent, possibly due to the coalescence of lipid droplets (Bowers and Holliday, 1961). The eggs are also adhesive and can be extruded with abdominal pressure although ovulation has probably not occurred (D.E. Hay, personal communication, 1987); milt will flow under pressure from the testes. Stage VI 'ripe' females have ovulated; the distinction from stage V is seen externally as easier extrusion of 'running' eggs and greater adhesiveness of the eggs; sperm flows without pressure. Stage VII 'spent' fish have spawned and both sexes have 'slack' or 'baggy' gonads from which blood is expelled with abdominal pressure. Ranges of oocyte diameters at each stage of the modified Hjort maturity scale have been determined (Hay et al., 1987), but were not used in this study to assess maturity as egg size varies with female body size (Hay, 1985). In our experiment, sexual maturity as defined above was assessed on a weekly basis by gentle squeezing of the abdomen. Evaluation was based on transparency, stickiness, and ease of extrusion of the eggs. Also, maturity was judged by the gonadosomatic index (GSI = 100 × gonad weight/whole body weight)

measured 2.5 weeks after the first or second pellet implantation, that is, at the end of each experiment.

RESULTS

Most of the GnRH was released from the cholesterol–cellulose pellets by 10–12 h in vitro leaving only 3–4 μg of the initial 100 μg after 12 h of perfusion

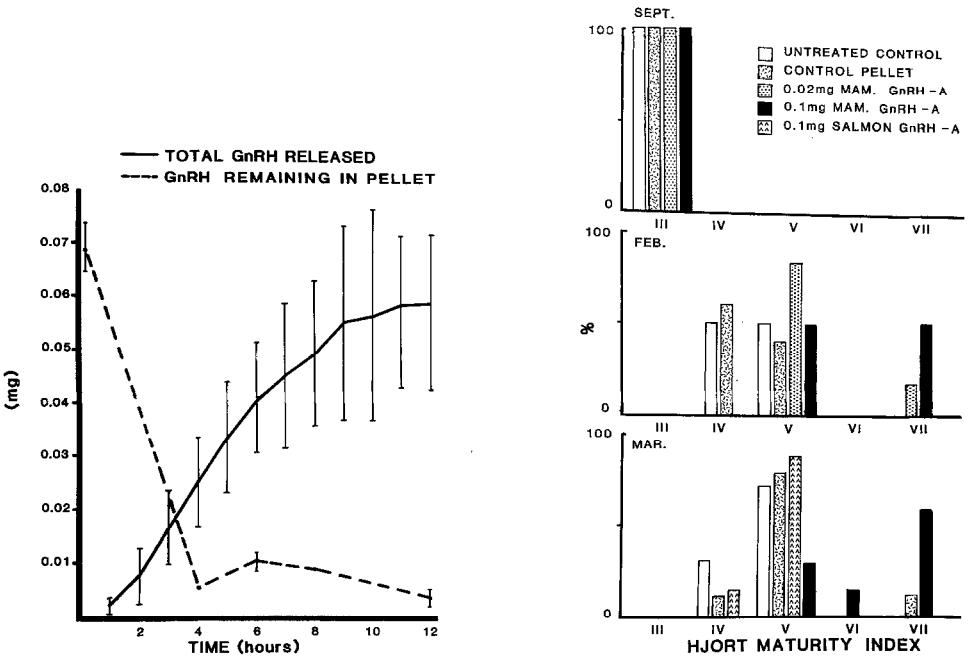
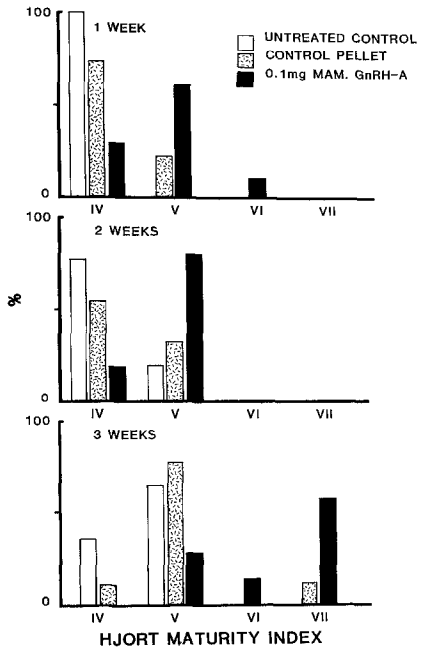


Fig. 1. In vitro release of GnRH from "quick-release" cholesterol/cellulose pellets. The solid line indicates the total amount released; the dashed line shows the amount (mean ± SE) remaining in the pellet with time.

Fig. 2. Effect of GnRH analogue implants on sexual maturity in herring. The maturity of the fish at the end of three experiments is indicated on the horizontal axis. Hay's modified Hjort scale refers to the following stages: developing (III), maturing (IV), mature (V), ripe (VI) and spent (VII). Percentage of surviving fish in each treatment group is shown on the ordinate. Fish were treated with one pellet on 17 September and one on 1 October (top), one pellet on 22 February and one on 14 March (middle) and a single pellet on 14 March (bottom). The first two experiments were terminated 2.5 weeks after the second pellet while the last experiment was ended 3 weeks after the single pellet. The numbers of fish at the end of the three experiments were 10, 10, and 10 untreated controls; 5, 5, and 9 controls with blank pellets; 6 and 6 fish with 20 μg mammalian GnRH analogue, 5, 8, and 7 fish with 100 μg of mammalian GnRH analogue for the top, middle, and bottom graph, respectively, and 7 treated with 100 μg salmon GnRH analogue (bottom graph).



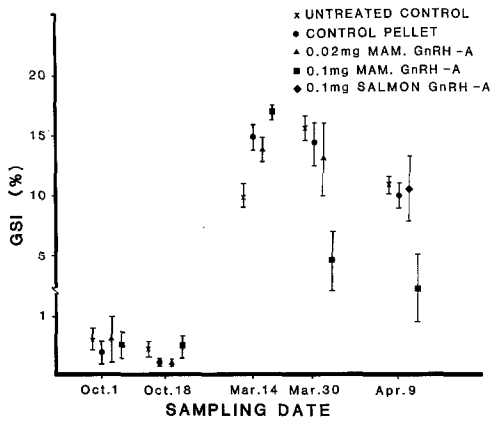


Fig. 3. The effect of a single mammalian GnRH analogue pellet on sexual maturity at 1 week (top), 2 weeks (middle) and 3 weeks (bottom) after treatment. Fish were implanted on 14 March. Maturity is indicated as a percentage of the surviving fish in each group at the time of sampling. Group sizes at top, middle, and bottom were 10, 10 and 10 untreated controls; 9, 9, and 9 controls with a pellet; and 10, 10 and 7 fish with a 100-μg mammalian GnRH analogue pellet, respectively. Maturity of the fish is classified by the Hjort scale as in Fig. 2.

Fig. 4. Maturation of herring after GnRH analogue treatment as assessed by gonadosomatic index (GSI) (mean $\pm$ SE). In the first experiment fish were implanted with one pellet on 17 September; half of these fish were sampled on 1 October; the other half of the fish were given a second pellet on 1 October and sampled on 18 October. In the second experiment fish were given one pellet on 22 February; half of the fish were sampled on 14 March; the other half were given a second pellet on 14 March, and sampled on 30 March. In the third experiment fish were given a single pellet on 14 March and sampled on 9 April.

(Fig. 1). Equivalent pellets implanted into herrring retained 0.17 ± 0.02 μg (mean $\pm$ SE) after 1 week and 0.12 ± 0.02 μg (mean $\pm$ SE) after 2 weeks.

In fish treated in September, neither the maturity based on the Hjort scale (Fig. 2) nor the GSI (Fig. 4) changed relative to control groups. However, the pellets were effective in fish implanted in February and March. The high dose of mammalian GnRH analogue markedly advanced sexual maturity; 50% of the February fish and 60% of the March fish had released their gametes at 5 weeks and 3 weeks, respectively (Fig. 2). In contrast, only one of the 10 fish with control pellets in the March experiment and none of the February con-

trols or March untreated controls advanced beyond the mature stage (Hjort V).

Sexual maturation of the February fish was advanced less by the low dose of the analogue compared with the high dose although the low dose was still effective; 17% of the low-dose fish were spent 5 weeks later (Fig. 2). The salmon analogue, administered only as a high dose in March, did not advance sexual maturation compared with controls implanted with a blank pellet (Fig. 2).

To follow the time course of the maturational response to hormone treatment, the fish in the March experiment were monitored on a weekly basis using Hjort scale criteria. The majority of fish treated with the high-dose mammalian GnRH pellets were more sexually advanced compared with the control groups and the advancement was distinguishable within 1 week of treatment (Fig. 3). This difference was maintained throughout the 3-week observation period even though all groups showed gonadal development during this period (Fig. 3). One treated fish appeared to regress between the first and second weeks from Hjort stage VI to V, but the distinction between these two stages depends on the ease of gamete expression and hence is less reliable compared with criteria for other stages. Although three treated fish died between the second and third weeks, 60% of the treated fish were spent and all treated fish were at least stage V. Only one out of 10 pelleted controls and none of the untreated controls were spent at the end of the experiments.

The gonadosomatic index increased more than 10-fold in all fish between October and March as would be expected due to normal maturation. Differences in the GSI among the treatment groups were present on the final sampling dates in March and April; the difference was between group 4 receiving a high dose of 100 μg mGnRH-A and the other three groups (Fig. 4). The low gonadosomatic index values on the 30 March and 9 April sampling dates are because the post-spawned fish in group 4 have a GSI near zero.

DISCUSSION

An analogue of GnRH applied in a cholesterol/cellulose pellet is clearly effective in advancing sexual maturation and subsequent spawning in herring. We found that the effectiveness of the treatment depended on several factors: (1) the dose of the analogue, (2) the type of analogue, and (3) the sexual maturity of the fish at the time of treatment.

The present experiment is the first to use cholesterol/cellulose pellets and to test in vitro release of GnRH from these pellets. The results indicate that the administration of a GnRH analogue embedded in a cholesterol/cellulose matrix can trigger sexual maturation culminating in gamete shedding in herring. The maturation response in herring was dose-dependent in that 100 μg (800–1000 μg/kg) was more effective than 20 μg (170–220 μg/kg) of the mammalian GnRH analogue (mGnRH-A). These doses were compared with the

same analogue and stimulus duration, and in fish of the same reproductive phase. Studies in teleosts other than herring have also shown that mammalian GnRH analogues can induce ovulation or spermiation (Donaldson et al., 1982; Sower et al., 1982; Van Der Kraak et al., 1983, 1985); the results are dose-dependent in some studies (Aida, 1983; Van Der Kraak et al., 1985). Signs of inhibition by the high dose were not observed. Fish exposed to GnRH-A at high levels or for prolonged periods of time appear to be less inhibited compared with mammals; neither rapid desensitization of gonadotropes nor rapid inhibition of gonadotropin secretion was observed in fish pituitary cultures treated with GnRH-A (Levavi-Zermonsky and Yaron, 1987). Also, prolonged exposure of milkfish and sea bass to GnRH-A induced spawning without signs of inhibition (Marte et al., 1987; Almendras et al., 1988).

The cholesterol/cellulose pellets in the present experiment were designed to improve the effect of a single injection of analogue. In a pilot experiment in 1983, we found a single injection of 3–4 μg of a mammalian GnRH analogue into mature (Hjort stage V) or ripe (stage VI) herring (about 100 g average weight) did not induce spawning. Kreiberg et al. (1987) recently found that a single injection of 20 μg/kg or 200 μg/kg of the same mammalian GnRH analogue as in the present study advanced maturation from maturing (stage IV) to mature (stage V) or ripe (stage VI, i.e., induced ovulation), but did not result in spawning during the 6-day observation period. Thus, in the present experiment it seemed desirable to increase the dose and duration of GnRH release. Others have shown that multiple injections or slow-release preparations of mammalian GnRH analogues produced a gonadotropin (GtH) release of greater magnitude or prolonged duration compared with a single injection in salmon (Van Der Kraak et al., 1985), carp (Breton et al., 1983), and male goldfish (Peter, 1980; Chang and Peter, 1983). Crim et al. (1983b) embedded GnRH analogues in 100% cholesterol matrix pellets and produced a sustained release of GtH in trout that lasted 12 weeks. After the completion of the present study, we showed that GnRH-A cellulose/cholesterol pellets produce release of GtH that lasts longer than an injection in trout (Crim et al., 1988) and induce multiple spawnings in individual sea bass (Almendras et al., 1988). In the present experiment, cellulose was added to the cholesterol base to reduce the duration of release compared with 100% cholesterol pellets: (1), to determine if a shorter duration stimulus is sufficient to produce a reproductive response, and (2) to avoid possible inhibitory effects on the gonads by prolonged GnRH administration. We have subsequently tested in vitro release in pellets with varying ratios of cellulose and cholesterol (Sherwood et al., 1988). Otherwise the rate of release has only been estimated from in vitro release of fluorescein dye (Kent et al., 1981) or from biological responses (Crim et al., 1983b; Weil and Crim, 1983). In vitro, our pellets released 79% of the hormone by 6 h and 94% by 12 h, while the in vitro release of fluorescein dye from 100% cholesterol pellets indicated 50% was released after 100–600 h.

The mode of action of GnRH-A may be (1) to give a 'head start' to fish by triggering early maturation that continues at a normal rate independent of further GnRH stimulation or (2) to accelerate maturation as long as the hormone is present in elevated amounts. To date, no well controlled experiment that addresses these options has been published. Our evidence shows that sexual maturation is triggered with a relatively short period of stimulation. Our pellets release their hormone in vitro within 12 h; precocious maturation is observed at 1 week after treatment, but is not greater than that induced by a single injection (Kreiberg et al., 1987). In the present experiment this 'head start' was maintained for at least 3 weeks at which time spawning occurred in several groups. Thus our evidence supports the 'head start' hypothesis, but does not indicate whether the subsequent rate of development is normal or accelerated. A comparison of several preparations with differing release rates in both in vivo and in vitro systems is needed to determine the optimum duration of GnRH stimulation.

The mammalian GnRH analogue used in this study was clearly effective in herring while the salmon analogue at the same dose was not. In goldfish the same mammalian analogue was also more effective in releasing GtH compared with the same salmon analogue (Peter et al., 1985). In contrast, the same two analogues were equally effective in milkfish (Marte et al., 1987) and salmon (Donaldson et al., 1984). In the milkfish study, pellets identical to those in the present experiment were used. The two types of analogues (mGnRH-A and sGnRH-A) have the same modification of their structures, but one is based on the mammalian and the other on the salmon GnRH molecule. This suggests that phylogenetic differences in the GnRH molecule or its receptors are important in determining the response to an analogue in a given species. The receptors or post-receptor events may be the basis of the species response as the same form of GnRH is present in the brain of these teleost species (Sherwood et al., 1984; Sherwood, 1986; Sherwood and Harvey, 1986).

Herring that are in an early vitellogenic and spermatogenic stage (Hjort stage III) are not responsive to treatment by a GnRH analogue of the dose and duration used here. Only after a 10-fold or more increase in gonadosomatic index (Hjort state IV; GSI 10%) did the herring respond to mammalian GnRH analogue; even at this stage, vitellogenesis may not be complete (Bowers and Holliday, 1961). In a number of studies by other workers, GnRH treatment was also successful only after fish had matured naturally to a 'threshold' reproductive stage. In trout and carp, the release of GtH in response to GnRH was low or absent in early maturation stages (Crim and Cluett, 1974; Weil et al., 1975, 1978); in ayu, landlocked salmon and sea bass, ovulation and spawning were incomplete or did not occur in treated immature fish (Aida, 1983; Crim et al., 1983a; Harvey et al., 1985). The criteria for judging 'immature' fish are not always clear, although egg diameter is often used. Also, it is not known if the lack of sensitivity of immature fish to GnRH analogues is absolute or if

a different dose, analogue or duration of treatment (such as a pellet with more than 80% cholesterol) could accelerate maturation.

Our results also indicate that spawning or gamete release can occur in individual herring in the absence of spawning pheromone from other herring; spawning pheromone is thought to be important for synchronized spawning in the wild (Stacey and Hourston, 1982). In the present study, only 50–60% of the herring treated in February or March with the 100-μg pellet of mammalian GnRH analogue spawned while the remaining ripe fish did not. Those that spawned did so one or two at a time, i.e., the spawning was not synchronized. Thus, maturation induced by a GnRH analogue can lead to spent fish who have not been exposed to spawning pheromone from other members of the school. The milt of such fish in a school of ripe herring may be a source of pheromone that eventually triggers synchronous mass spawning.

Our results from the application of GnRH analogues suggest that native GnRH is important in maturation of Pacific herring. A synthetic GnRH analogue artificially accelerates sexual maturation provided the correct analogue and dose are used in fish that have matured sufficiently. The greater success we achieved in producing spent fish as compared to Kreiberg et al. (1987) may be due to an extended observation period rather than a difference between effectiveness of our pellet compared with an injection of a similar dose. GnRH analogues could be useful for accelerating maturation in fish which are at least Hjort stage IV. However, pellets with a more prolonged release may be needed to stimulate fish at Hjort stage III. Practical aspects of these findings are pertinent for the commercial production of 'spawn-on-kelp' in which newly captured herring may have delayed or asynchronous spawning and a decrease in commercial value (as well as in egg and larva viability) if spawning occurs more than 2 weeks after ovulation (Hay, 1986). Slow-release GnRH pellets might be useful in producing a core group of milt-releasing males to trigger mass spawning.

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