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New technologies for the control of gamete maturation in marine fishes, as tools in broodstock management

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SUMMARY - The plasma levels of gonadotropin (GtH) II, the major pituitary hormone controlling final gamete maturation in fish were compared between wild and captive broodstocks of striped bass (*Morone saxatilis*) in order to gain an understanding of the failure of captive fish to undergo final oocyte maturation (FOM) and spermiation. Captive fish, both male and female, had lower plasma GtH II levels during the spawning season, and this seems to be the cause of the reproductive dysfunction in striped bass, and presumably in other fishes reared in captivity. Injections of an agonist of gonadotropin-releasing hormone (GnRHa) were effective in inducing pituitary GtH II release, which in turn can initiate FOM and spermiation. However, a prolonged presence of elevated GtH II in the circulation was necessary to induce complete FOM, and long term production of milt. A sustained elevation of plasma GtH II was achieved using two polymeric, sustained-release GnRHa-delivery systems, a treatment which resulted in 100% FOM, ovulation and spawning of viable eggs, and production of good quality milt for up to 21 days after treatment. This novel technology was shown to be effective in a variety of commercially important marine fishes.

Key words: *Morone*, induced spawning, ovulation, spermiation, gonadotropin, GnRHa.

RESUME - *"Nouvelles technologies pour le contrôle de la maturation des gamètes chez les poissons marins, comme instruments pour la gestion des reproducteurs". Les niveaux de gonadotropine (GtH) II dans le plasma, principale hormone pituitaire qui contrôle la maturation finale des gamètes chez les poissons, ont été comparés entre des populations sauvages et marines de bar d'Amérique (*Morone saxatilis*) afin de mieux comprendre pourquoi les poissons captifs ne présentaient pas la maturation finale des ovocytes et la spermiation. Les poissons captifs, aussi bien mâles que femelles, avaient des niveaux plus faibles de GtH II dans le plasma pendant la saison de reproduction, et ceci semble être la cause du dysfonctionnement reproductif chez le bar d'Amérique, et probablement chez d'autres poissons élevés en captivité. Des injections d'un agoniste de l'hormone de libération de la gonadotropine (GnRHa) ont été efficaces pour l'induction de la libération de GtH II pituitaire, qui à son tour peut déclencher la maturation finale des ovocytes et la spermiation. Cependant, une présence prolongée de GtH II élevée dans la circulation était nécessaire pour induire une maturation finale des ovocytes complète, et une production à long terme de laitance. Une élévation soutenue de GtH II dans le plasma a été obtenue en utilisant deux systèmes polymériques de libération soutenue de GnRHA, un traitement qui a donné 100% de maturation finale des ovocytes, d'ovulation et de ponte d'oeufs viables, ainsi que la production de laitance de bonne qualité jusqu'à 21 jours après le traitement. Cette technologie novatrice s'est montrée efficace pour plusieurs poissons marins d'importance commerciale.*

Mots-clés : Morone, *ponte induite, ovulation, spermiation, gonadotropine, GnRHa.*

Introduction

Enhancement of the quality of farmed species can be achieved by various genetic

manipulations. Apart from selective breeding, techniques such as hybridization, chromosome manipulation and monosex cultures can be employed to provide animals of greater stress and disease resistance, higher growth rate and supreme flesh quality (Thorgaard, 1995). These manipulations require that mature gametes are collected manually from the broodstock and fertilization is done artificially. Successful fertilization requires that gametes are properly matured and collected at a precise time, otherwise there is a loss of fertilization ability. Practically all marine fishes exhibit some degree of reproductive dysfunction when reared in captivity, the most common being absence of final oocyte maturation (FOM) and ovulation in the female and diminished milt production in the male (Zohar, 1989). The necessary handling involved in artificial fertilization protocols adds an additional stressor, which often results in failure to complete final gamete maturation and release. It is important, therefore, to identify the site of hormonal failure on the reproductive axis of fish reared in captivity, and devise spawning induction therapies which will induce FOM and spermiation reliably.

The striped bass (*Morone saxatilis*) is an anadromous perciform fish native to the Atlantic coast of North America (Setzler *et al.*, 1980) that has traditionally supported important commercial and recreational fisheries. Dramatic declines in landings during the 1970s had prompted significant efforts by state and federal agencies to develop methods for the artificial spawning and larval production of striped bass, using the limited number of available wild broodstock (see Harrell *et al.*, 1990). In addition to its culture potential, the striped bass has been used as a model in our studies, because: (i) females do not undergo FOM spontaneously when reared in captivity; (ii) captive males exhibit diminished milt production and low sperm quality and; (iii) wild fish during FOM and spermiation are highly accessible at known spawning areas, and blood samples can be easily acquired for the identification of hormonal changes associated with final gamete maturation.

The objectives of our study were to: (i) identify the hormonal failure in captive striped bass responsible for the absence of FOM and reduction in milt production, based on hormone profile comparisons between wild and captive fish during the spawning season; (ii) develop an efficient hormonal therapy for the induction of FOM and spermiation, based on the use of the appropriate hormone and application method; and (iii) test the effectiveness of the spawning induction therapy on striped bass, as well as other commercially important marine species.

Gametogenesis in fish

The reproductive cycle of fish is separated into two phases. The mitotic multiplication and growth of the gametes constitutes the first phase, while the preparation for and release of the mature gametes constitutes the second phase (Nagahama, 1994). The first phase in the females (Fig. 1) is referred to as vitellogenesis, since the major event is the production of the yolk-protein precursor vitellogenin and its accumulation into the growing oocyte (Specker and Sullivan, 1994). In the males (Fig. 2), the first stage of the reproductive cycle is referred to as spermatogenesis and is completed with the production of flagellated, though still immotile, spermatozoa, i.e., spermiogenesis (Billard, 1986; Callard, 1991).

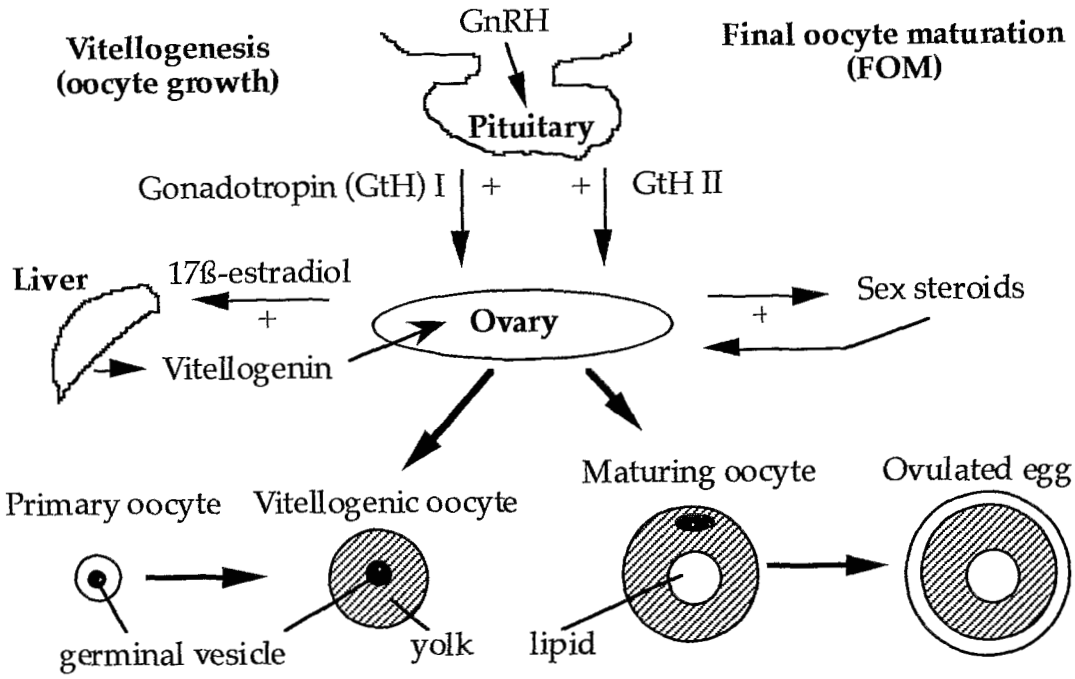

Fig. 1. Schematic representation of the hormonal control of the hypothalamus-pituitary-gonad axis of female fish and the morphological changes associated with vitellogenesis and final oocyte maturation.

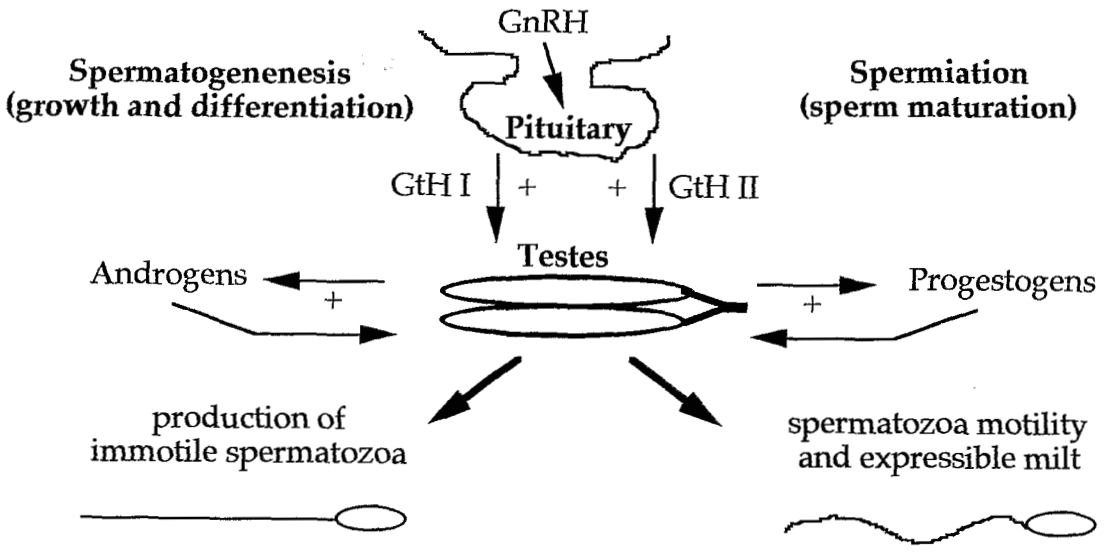

Fig. 2. Schematic representation of the hormonal control of the hypothalamus-pituitary-gonad axis of male fish and the major events in the testes during spermatogenesis and spermiation.

The second phase of the cycle is referred to as final maturation. Final oocyte maturation in the female includes a number of cytological, biochemical and nuclear changes that prepare the oocyte for ovulation and fertilization (Nagahama, 1983; Jalabert *et al.*, 1991). Final oocyte maturation begins with the migration of the nucleus or germinal vesicle (GV) to the periphery of the oocyte, at the area below the sperm entry site, the micropyle (Guraya, 1986). Coalescence of the lipid-droplets and yolk-globules occurs soon after or during GV migration (Wallace and Selman, 1981; Goetz, 1983). Once the GV acquires a peripheral position (pGV), the GV membrane breaks down (GVBD), the spindle formation follows, and the process of meiotic maturation is completed with the emission of the first polar body. At this time the oocyte becomes arrested once again at metaphase II, and meiosis is re-activated and completed upon fertilization.

The process of final testicular maturation includes the acquisition of motility capacity by the spermatozoa and the production of expressible milt, also referred to as spermiation (Nagahama, 1994; Billard *et al.*, 1995). In some fishes, spermatogenesis and spermiation are temporally separated, with spermiation occurring after the conclusion of spermatogenesis. As a result, during the spawning season the testes contain exclusively spermatozoa (Billard, 1987; Malison *et al.*, 1994). In other fishes, the two processes can occur simultaneously, although most of spermatogenesis takes place prior to the spawning season, whereas spermiation occurs during the spawning season (Berlinsky *et al.*, 1995; Jackson and Sullivan, 1995; Matsuyama *et al.*, 1995). Although morphologically complete spermatozoa can be present within the testes for many months prior to the spawning season, the capacity for motility upon contact with water is acquired during the spermiation period, in response to hormone-induced changes in the pH of the seminal fluid (Miura *et al.*, 1992; Miura *et al.*, 1995).

Endocrine control of FOM and spermiation

Reproduction in vertebrates is regulated by the brain, via the release from the hypothalamus of gonadotropin-releasing hormone (GnRH), which stimulates the release of gonadotropin (GtH) from the pituitary (Zohar, 1989; Trudeau and Peter, 1995). All vertebrates except placental mammals possess two forms of GnRH (Sherwood *et al.*, 1994). Teleost fish posses a universal form, cGnRH II and a specific form, most often sGnRH. In striped bass and gilthead sea bream, three forms of GnRH have been identified, cGnRH II, sGnRH and sea bream GnRH (sbGnRH) (Gothilf *et al.*, 1996). Although all three forms are potent GtH releasers (Zohar *et al.*, 1995a), only one of these forms, i.e., sbGnRH, reach the pituitary and is, presumably the form regulating pituitary GtH release.

Similar to other vertebrates, it is now recognized that teleost fishes have two forms of GtH, referred to as GtH I and II (Swanson, 1991; Schulz, 1995). Of the two forms, GtH II is either the prominent form during the whole reproductive cycle, or it correlates with the onset of FOM and spermiation. The other form, GtH I, is mostly involved in vitellogenesis and spermatogenesis.

Gonadotropic stimulation of the ovary (Fig. 1) during vitellogenesis results in the production of testosterone (T) by the theca cell and its aromatization to 17ß-estradiol

(E_2) by the aromatase enzyme in the granulosa cells (Nagahama, 1994). In response to stimulation by E_2, the liver produces vitellogenin, the lipoprotein precursor of yolk (Specker and Sullivan, 1994), which is taken up by the growing oocytes in the ovary via receptor-mediated endocytosis. At the completion of vitellogenesis, a surge in plasma GtH II is accompanied by a drop in plasma E_2, a transient increase in plasma T during GV migration (mGV) and a dramatic elevation in the plasma levels of the maturation inducing steroid (MIS), a progestogen. The MIS acts at the level of the oocyte to induce FOM, followed by ovulation and spawning (Nagahama, 1994; Nagahama *et al.*, 1994). The steroidogenic shift that occurs during FOM is induced by the GtH II surge prior to FOM.

The onset of spermatogenesis (Fig. 2) is associated with an elevation in plasma androgen levels (Borg, 1994). Although differences among species do exist, the most important fish androgen is 11-ketotestosterone (11-KT), which is produced from T. Spermiation is better correlated with plasma elevation of progestogens, most often the female MIS (Scott *et al.*, 1984; Liley and Rouger, 1990; Pankhurst, 1990), and milt production can be induced by exogenous treatment with these steroids (Pankhurst, 1994). Similar to the situation in female fish undergoing FOM, it appears that an increase in plasma GtH II levels at the beginning of the spawning season causes the shift in the steroidogenic production of androgens by the testes to the production of progestogens, i.e., the MIS (Nagahama, 1994). This elevation of GtH II and MIS during spawning (Kyle *et al.*, 1985; Kobayashi *et al.*, 1986; Liley and Kroon, 1995) induce elevations in expressible milt volume by stimulating seminal fluid production (Baynes and Scott, 1985; Marshall *et al.*, 1989), whereas the MIS stimulates the acquisition of motility capacity of the stored spermatozoa (Miura *et al.*, 1992).

Hormonal manipulations of FOM and spermiation

Unfortunately, when reared in captivity, many fish species exhibit some degree of reproductive dysfunction. Most commercially important fishes do reach reproductive maturity under culture conditions, but although vitellogenesis occurs normally, female broodstocks fail to complete the reproductive cycle and do not spawn (Zohar, 1988; Zohar, 1989), while male fish exhibit a diminished production or low quality of milt (Billard, 1989). Therefore, it is necessary to intervene, most often using pharmacological approaches, in order to induce FOM, ovulation or spawning in females, and enhance milt production in males.

Reliable and efficient therapies for inducing spawning of commercially cultured fishes have been made possible in the last two decades due to advances in endocrinology and peptide chemistry (Zohar, 1988; Zohar, 1989; Zohar, 1996). Usually a homologous or heterologous hormone is injected to stimulate gonadal maturation resulting in ovulation/spermiation at predictable times. Such interventions can be made at various levels of the hypothalamus-pituitary-gonad axis, and hormonal treatments can be grouped into two categories according to the anatomical target upon which they act. The first category includes GtH preparations that act on the gonads. The second category includes various GnRH agonists (GnRHa) which act at the level of the pituitary gland.

Recent spawning induction methods focus on the development of therapies employing highly potent GnRHa (Crim *et al.*, 1987; Zohar, 1988; Peter *et al.*, 1993), which trigger the secretion of the fish's own GtH II, thus activating its pituitary-gonad axis. There are significant advantages in using GnRHa over GtH preparations. First, GnRH agonists are small decapeptides that apparently do not trigger an immune response and can be reused with no reduction in their effectiveness. Second, because they act at a higher level of the hypothalamus-pituitary-gonad axis, GnRHa can provide a more balanced stimulation of reproductive events and possibly a better integration of these events with other physiological functions in the fish, by directly or indirectly affecting the release of other hormones necessary for successful FOM, spermiation and spawning. Such hormones may include growth hormone (Le Gac *et al.*, 1993), prolactin (Weber *et al.*, 1995) and thyroid hormones (Cyr and Eales, 1996). Third, due to their resistance to enzymatic degradation, GnRH agonists have a many-fold increase in potency with a concomitant decrease in cost per kg of body weight of fish treated compared to GtH II preparations. Finally, being synthetic peptides, they do not carry the risk of transmitting diseases to the treated broodstock, a danger always associated with the use of piscine pituitary extracts. These advantages have attracted researchers and practising aquaculturists to the use of various GnRHa molecules and much effort has been recently channelled towards improving this method of induced spawning by establishing optimum dosages and application regimes specific for targeted species and rearing conditions.

Despite their increased resistance to enzymatic degradation (Zohar *et al.*, 1990a), GnRHas are being cleared from the circulation rapidly (Gothilf and Zohar, 1991) and a single injection is often ineffective in inducing FOM, ovulation or sustained spermiation, as it induces only a transient elevation in plasma GtH II levels. This situation leads to repeated injections of GnRHa, a manipulation which is stressful to the broodstock and results in mortalities. Avoiding the need for multiple injections, long-term administration of GnRHa via sustained-release formulations (Crim *et al.*, 1988; Zohar, 1988; Zohar *et al.*, 1994; Zohar, 1996) was demonstrated to be far more efficient in inducing FOM, spermiation and spawning in a variety of farmed fishes. Such spawning induction methods are much less labour- and management-intensive since they require only a single treatment. Most importantly, they obviate the need for repetitive handling of valuable and sensitive broodstock. The effectiveness of these GnRHa-delivery systems is due to the sustained elevation of plasma GtH II levels which they induce, as shown in Atlantic salmon (*Salmo salar*) (Weil and Crim, 1983), rainbow trout (*Oncorhynchus mykiss*) (Breton *et al.*, 1990) and gilthead sea bream (*Sparus aurata*) (Zohar *et al.*, 1995b). Unlike the situation in mammals, birds and reptiles (Chang and Jobin, 1994), continuous *in vivo* treatment of most fishes with GnRHa does not result in down-regulation of GtH II release. Therefore, sustained-administration of GnRHa via delivery-systems appears to be a promising way of inducing FOM, spermiation and spawning in farmed fishes. We have prepared various polymer-based, sustained-release GnRHa-delivery systems, and developed a hormonal therapy which could be used in striped bass, as well as many other commercially important marine species.

Materials and methods

Endocrine control of FOM in wild striped bass

Mature male and female striped bass were obtained by electrofishing at a spawning site of the Nanticoke River, Maryland, USA, during the 1995 April spawning season (Mylonas *et al.*, 1997d). The females ranged in length and weight from 90 to 130 cm and 15 to 35 kg, respectively. Ovarian biopsies were obtained via a 3-mm internal-diameter glass catheter inserted into the ovary via the ovipore and were stored on ice until examined at the end of the day. Blood was collected from the caudal vasculature and was kept in heparinized vials on ice until separated by centrifugation at the end of the day. The separated plasma was stored at -80°C until assayed for hormone content.

The diameter of the oocytes was determined using a stereoscope, and the stage of maturation was evaluated macroscopically and histologically as described previously (Mylonas *et al.*, 1997e). Fish were classified into five maturation stages as follows: Vg, vitellogenic oocytes prior to any lipid-droplet coalescence, with a centrally located GV; mGV, oocytes undergoing lipid-droplet coalescence and GV migration; pGV, the lipid-droplets were completely coalesced and the GV was located on the periphery of the oocyte; GVBD, GV breakdown had occurred and the oocytes were undergoing yolk-globule coalescence; Spent, recently spawned females with flat abdomens giving a few ovulated eggs when biopsied.

Endocrine profiles of captive striped bass females

Female striped bass produced from wild Chesapeake Bay broodstock in 1983 and 1985, were reared to reproductive maturity at the Crane Aquaculture Facility, Baltimore, Maryland. Females were individually tagged with passive integrated transponders (Destron/IDI, Boulder, Colorado) and were kept in 50-m^3, circular tanks supplied with ambient Chesapeake Bay water. To examine the endocrine profiles of non-hormone-induced females during post-vitellogenesis and the spawning season, beginning on 13 March 1992, eight females (mean weight 6.6±1.1 kg) were anaesthetized in a 0.04 ml/l solution of Quinaldine (Eastman Kodak Company, Rochester, New York) and sampled weekly or biweekly for ovarian biopsies (Mylonas *et al.*, 1997e) and blood (Mylonas *et al.*, In Review-b). Changes in oocyte morphology and the position of the GV were examined as above.

Induction of FOM using GnRHa treatments

Five-year-old females (mean weight 4.5±0.6 kg) produced from wild Chesapeake Bay broodstock were allocated into four treatment groups (n = 5) and were given either a GnRHa-implant (40 µg GnRHa/kg body weight) on day 0, GnRHa-microspheres (40 µg/kg) on day 0, two injections of GnRHa (20 µg/kg) on days 0 and 3, or saline. This last group also served as a control. The GnRHa-implants were fabricated from Ethylene-Vinyl Acetate copolymer (EVAc, Elvax, DuPont Chemical Company, Wilmington, Delaware) as solid 2 mm disks (Zohar *et al.*, 1990b), and the

GnRHa-microspheres were produced from biodegradable poly-[fatty acid dimer-sebasic acid] (Mylonas *et al.*, 1995a). Both delivery systems were loaded with the agonist [D-Ala^6, Pro^{9}NEt]-GnRH (Bachem Bioscience Inc., King of Prussia, Pennsylvania). At the time of treatment, females contained vitellogenic oocytes (800 to 900 µm in diameter) with no signs of FOM, such as lipid-droplet coalescence or GV migration. After treatment, all females were kept together, and isolated from males, in a 4.5-m^3 circular tank supplied with recirculated water at controlled temperatures (17.2 - 20.1°C) considered to be optimal for FOM and spawning of striped bass (Secor and Houde, 1995) . All females were sampled for blood and gonadal biopsy prior to and at various times after treatment, depending on their ovarian stage of maturation.

Induction of spermiation using GnRHa-delivery systems

Male striped bass were maintained in 12.4 m^3 circular tanks supplied with recirculated water under simulated natural photoperiod and thermoperiod. On 9 May 1994 (day 0), a group of 18 spermiating males with mean body weight of 1.13±0.18 kg, were selected from the population, were individually tagged with P.I.T. tags and were treated with either a saline injection, GnRHa-microspheres (40 µg GnRHa/kg) or a GnRHa-implant (44 µg GnRHa/kg fish). All fish were placed together, without any females, in a 2.4 m^3 circular tank supplied with recirculated water under simulated natural photoperiod but constant water temperature (18°C). Prior to treatment (day 0) and on days 2, 4, 7 and 14, total expressible milt was collected from all fish by applying gentle abdominal pressure. At the same times, blood was collected from the caudal vasculature using a heparinized syringe fitted with a 21-gauge needle. The separated plasma was stored at -80 °C until assayed for various hormones (Mylonas *et al.*, 1997c).

Hormonal and statistical analyses

Although in our studies the plasma levels of various reproductive hormones were measured, only the results of exogenous GnRHa and endogenous GtH II are presented here. The description of the radioimmunoassays used here have been reported elsewhere (Mylonas *et al.*, 1997c; Mylonas *et al.*, 1997d). Data were analysed statistically by Analysis of Variance (ANOVA) followed by Duncan's New Multiple Range (DNMR) test, using a linear statistics software (SuperAnova, Abacus Concepts Inc., California). Data are presented as mean ± standard error, unless otherwise indicated.

Results and discussion

The role of GtH II in inducing FOM is well established in fish (Nagahama *et al.*, 1994) . Plasma GtH II levels are often non detectable during the pre-FOM period, they increase gradually during FOM and show a dramatic peak just prior to ovulation (Zohar *et al.*, 1986; Aida, 1988; Kobayashi *et al.*, 1988). The plasma GtH II profiles of wild striped bass during FOM are in agreement with this model of pituitary control of

ovarian development (Fig. 3a). The onset of early-FOM (mGV) was not associated with significant increases in plasma GtH II, although by the completion of this process (pGV stage) plasma GtH II levels were significantly higher than in the Vg stage. The late-FOM phase (GVBD), however, was associated with a marked increase in plasma GtH II, which resulted in a dramatic elevation in plasma levels of the MIS (Mylonas *et al.*, 1997c). After spawning, plasma GtH II declined, but in accordance with observations in other teleosts (Stacey *et al.*, 1984; Breton *et al.*, 1988), it remained elevated compared to the Vg stage. In captive females, only the oocytes of one untreated female underwent FOM and ovulated, while in the rest of the fish the oocytes underwent atresia, i.e., cell degeneration (Fig. 3b). Plasma GtH II of the non-maturing females remained unchanged during the monitoring period. On the contrary, in the female that ovulated without any hormonal treatment, FOM was associated with a surge in plasma GtH II. These results demonstrate that the hormonal failure responsible for the absence of FOM, ovulation and spawning in captive striped bass is located at the level of the pituitary, specifically on the synthesis and/or release of GtH II after the completion of vitellogenesis. As a result of the lack of GtH II release the necessary steroidogenic shift from estrogen to MIS production does not take place in captivity, and FOM is absent (Mylonas *et al.*, in Review-b). Male striped bass reared in captivity also appeared to have lower plasma GtH II levels (Mylonas *et al.*, 1997c) compared to fish in the wild (Mylonas *et al.*, 1997d), a phenomenon which may be responsible for the diminished milt production observed in captivity (see later). Based on the findings of lower plasma GtH II levels in captive striped bass, we next examined the potential of GnRHa in stimulating the release of GtH II, and inducing FOM and spermiation.

Two injections of GnRHa on days 0 and 3 produced significant ($P \leq 0.01$) elevations of plasma GnRHa and GtH II at least until day 7 (Fig. 4). Of the GnRHa-injected fish, 67% initiated FOM by day 7, but only one of the fish completed FOM and ovulated by day 35 (data not shown). Atretic oocytes were observed in some of the biopsies of females from both treatments on day 21, and in the biopsies of all fish by day 35. It was concluded, that although GnRHa injections are effective in inducing pituitary GtH II release, which in turn can initiate FOM in captive striped bass, a prolonged presence of elevated GtH II in the circulation is necessary to induce complete FOM and ovulation. These results formed the basis for our efforts in developing sustained-release, delivery systems for GnRHa to induce FOM, ovulation and spawning in striped bass (Zohar *et al.*, 1990b; Mylonas *et al.*, 1995a).

Both GnRHa-delivery systems resulted in high GnRHa levels in the circulation for at least 13 days (Fig. 5a). Plasma GtH II levels were undetectable prior to GnRHa treatment and increased significantly (ANOVA, DNMR, $P \leq 0.05$) thereafter (Fig. 5b). Plasma GtH II followed the plasma profiles of GnRHa, increasing continually during the first 13 days after treatment and declining by day 25. Sustained elevations of GtH II in response to GnRHa-delivery systems have been documented in Atlantic salmon (Weil and Crim, 1983), rainbow trout (Breton *et al.*, 1990), and gilthead sea bream (Zohar *et al.*, 1990b), among others. The present results demonstrate in yet another fish species the absence of desensitization of GtH II release in response to continuous GnRHa stimulation, unlike what has been observed in the goldfish (*Carassius auratus*) (Habibi and Peter, 1991). In fact, plasma GtH II in striped bass increased continually for the first 13 days after treatment, in the presence of unchanged (GnRHa-microspheres) or declining (GnRHa-implant) plasma GnRHa

levels. The continually increasing GtH II levels observed in GnRHa-induced females during FOM are equivalent to what was observed in wild females (Mylonas *et al.*, 1997c). The latter study demonstrated that FOM in the wild occurs under increasing plasma GtH II, with maximal levels observed around the GVBD stage. Therefore, the continuous GnRHa stimulation provided by the delivery systems appears to result in a physiological GtH II profile. All females treated with the GnRHa-delivery systems initiated FOM and ovulated within 3 and 10 days after treatment, respectively (Fig. 5c). Both GnRHa-delivery systems were also effective in inducing spawning of viable eggs from captive broodstock in a commercial facility (Mylonas *et al.*, in Review-b), with fecundity ranging between 120 and 170 thousand eggs/kg body weight, and fertilization % between 38 and 47%. The fecundity and response time after treatment were comparable to those obtained with other GnRHa-delivery systems (Woods and Sullivan, 1993).

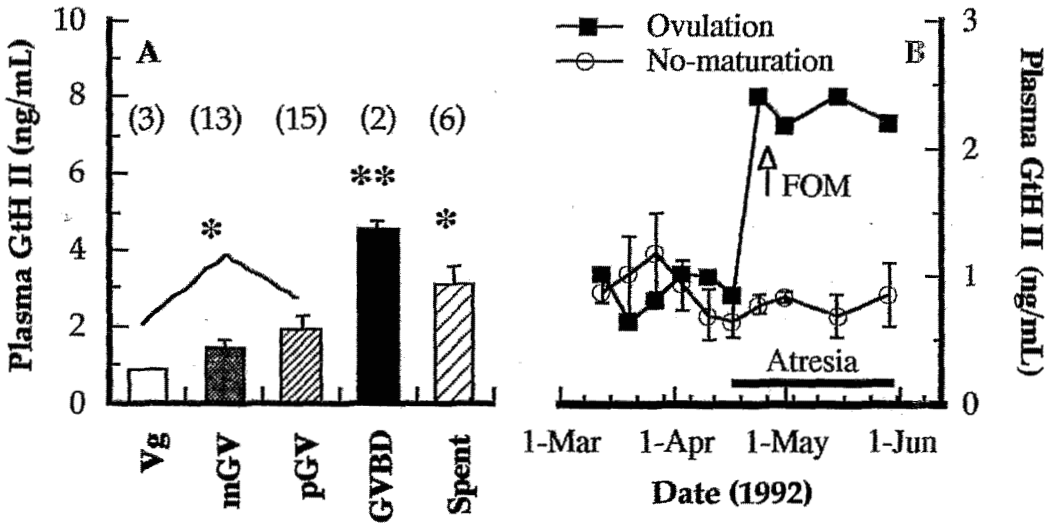

Fig. 3. Mean (+ s.e.m.) plasma GtH II levels of female striped bass (n shown in parentheses) from the wild during final oocyte maturation (A), and of captive females (n=8) during the corresponding spawning period (B). Asterisks indicate significant differences from the previous sample mean, unless otherwise indicated (ANOVA, DNMR, *P≤0.05, **P≤0.01). The data from the only captive female which underwent FOM and ovulation is shown separately (B). There were no significant differences among sample means collected at different times from non-maturing captive females. The time FOM begun in the ovulating female, and the onset of atresia in the non-maturing captive females are also shown. Figure A is from Mylonas *et al.* (1997d), and B from Mylonas *et al.* (In Review-b).

In early spawning experiments in 1992 (data not shown), we observed that when males were removed from the large holding tanks where they were kept during the year and were placed in smaller spawning tanks, total expressible milt was diminished when evaluated after 14 days. Treatment with a GnRHa-implant prior to placement in the spawning tanks enhanced milt volume compared to a saline or

GnRHa injection (Mylonas *et al.*, in Review-a). The reduction in milt volume after placement in spawning tanks with gravid females was surprising, given that physical interactions between gravid females and spermiating males can significantly increase milt volume (Rouger and Liley, 1993; Liley and Kroon, 1995). In striped bass, it appears that handling or transfer to a spawning tank, which is smaller and has fewer fish, had an inhibitory effect on milt production and this inhibition was not overcome by the presence of a gravid female. Confinement-induced chronic stress or treatment with cortisol can affect GtH synthesis or release, as well as gonadal steroidogenesis (Pickering *et al.*, 1987; Carrangher *et al.*, 1989).

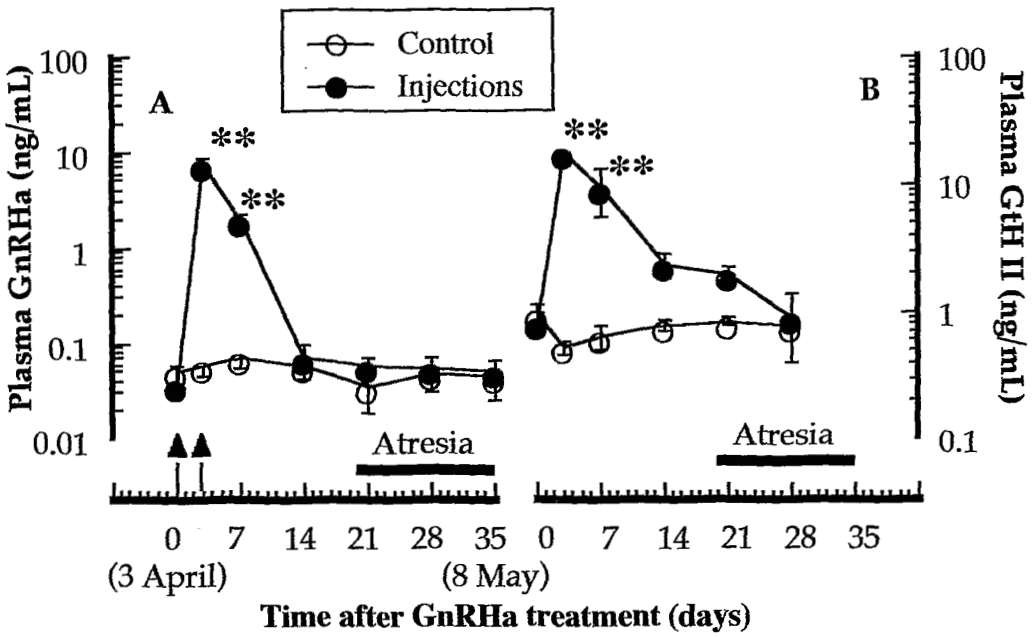

Fig. 4. Mean (± s.e.m.) plasma GnRHa and GtH II levels of captive female striped bass (n=6) after two injections of 15 µg GnRHa/kg (arrows) at the end of vitellogenesis. Asterisks indicate means which were significantly different from the day 0 mean (ANOVA, DNMR, P≤0.01). The time when atretic oocytes could be seen in the biopsies is indicated by a horizontal bar.

The experiments described here were carried out to further examine the potential of GnRHa-delivery systems in enhancing milt production in fish that were striped of their milt frequently (Mylonas *et al.*, 1997d). Both GnRHa-delivery systems induced a significant elevation in total expressible milt (P≤0.01) compared to saline-treated controls (Fig. 6). Fourteen days after GnRHa-treatment, total expressible milt of GnRHa-treated fish was still elevated (6.3 ± 0.7 ng/mL) and was significantly higher than the control group (P≤0.01). Total expressible milt from the control group diminished significantly on day 7 (P≤0.05) and day 14 (P≤0.01), compared to the amount collected on day 0. When the data was expressed as total number of spermatozoa produced per kg body weight, i.e., taking into account the sperm density of the milt, there were also significant (P≤0.01) increases in response to the

GnRHa treatment (Fig. 6). This observation suggests that increased milt production in response to treatment with the GnRHa-delivery systems was not due to increased seminal fluid production alone, but there was a true increase in the production or availability of spermatozoa. Single injections of GnRHa, on the other hand, result in a decrease in sperm density which is often proportional to the induced increase in milt volume (Takashima *et al.*, 1984; García, 1991). As a result, the total amount of spermatozoa produced may not differ from saline-treated fish. In the present study, the total amount of milt produced by GnRHa-treated fish over the duration of the study was 420% higher than untreated controls, while total spermatozoa production was 394% higher.

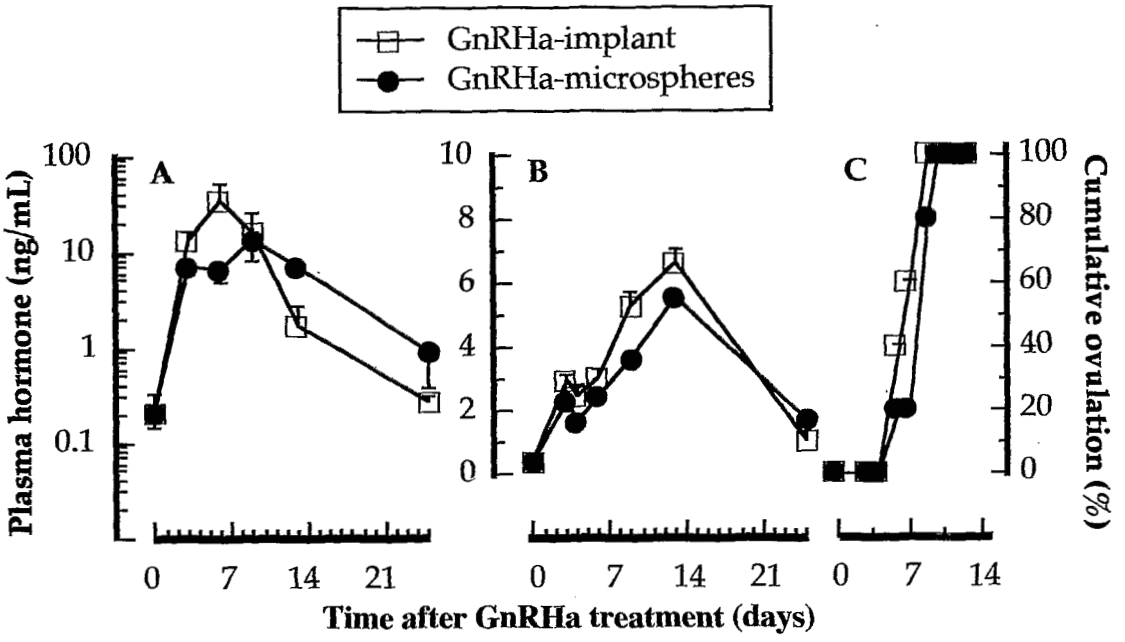

Fig. 5. Mean (± s.e.m.) plasma GnRHa and GtH II levels (A and B) and cumulative % ovulation (C) of captive striped bass females (n = 5) treated with two sustained-release GnRHa-delivery systems. There were significant increases in plasma levels of both hormones after treatment with the GnRHa-delivery systems (ANOVA, DNMR, P≤0.05). From Mylonas *et al.* (In Review-b) .

The effectiveness of the two GnRHa-delivery systems in inducing FOM, ovulation and spawning has been evaluated in other cultured species. In the white bass, both delivery systems were effective and induced ovulation within 35 to 82 h after treatment (Mylonas *et al.*, 1996). An average of 294,500 eggs/kg were produced with a mean fertilization success of 81.2% and 24h-survival of 46.5%. In addition, treatment with a GnRHa-delivery system induced multiple spawns in white bass females, resulting in a overall egg production of two- to eight-fold higher than previously reported from captive fish, and similar to annual values reported for wild fish (Mylonas *et al.*, 1997b). American shad (*Alosa sapidissima*) given a GnRHa-implant started spawning 2 days after treatment and continued spawning for the next 9 days, producing a mean total of 50,100 eggs/kg with mean fertilization of 62%

(Mylonas *et al.*, 1995b). In Atlantic salmon, 100% of females treated with the GnRHa-implants ovulated within 14 days, compared to only 10% of saline-injected controls, producing eggs of >80% survival to the eyed stage (Mylonas *et al.*, 1993). Similarly in the Pacific coho salmon (*Oncorhynchus kisutch)*, both delivery systems induced 100% ovulation of females up to six weeks prior to the natural ovulation time, without any negative effects on egg quality (Mylonas *et al.*, 1993). In the yellowtail flounder (*Pleuronectes ferrugineus*), GnRHa-microspheres induced eight consecutive spawns, resulting in a more that two-fold increase in total egg production compared to controls (Larsson *et al.*, 1997). GnRHa treated females produced eggs of 68% and 60% fertilization and hatching success, respectively, compared to 39% and 25% fertilization and hatching success, respectively, observed in eggs collected from control females. Obviously, sustained-administration of GnRHa via delivery-systems is an effective and efficient therapy for the control of FOM, ovulation and spawning in fish, especially in species with asynchronous ovarian development. The resulting sustained elevation of GtH II provides a continuous gonadal stimulation resulting in multiple spawning. This therapy obviates the need for repetitive handling to administer multiple GnRHa injections, which in addition to interfering with the FOM and spawning processes, they can often damage valuable broodstock and result in pre- or post-spawning mortalities.

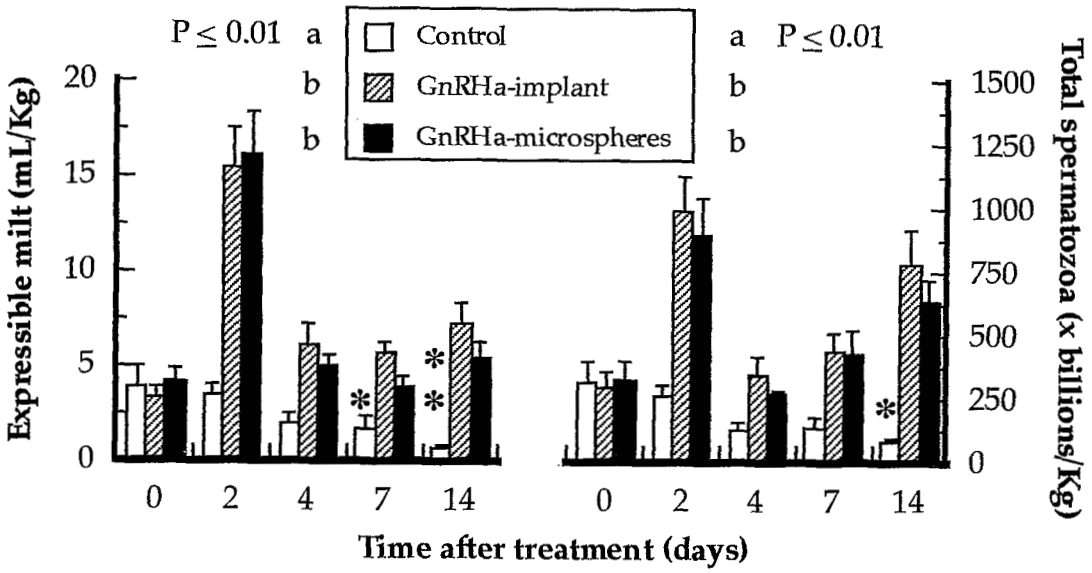

Fig. 6. Mean (+ s.e.m.) total expressible milt and total spermatozoa production in striped bass (n = 6) given GnRHa-delivery systems during the spawning season. Treatment groups that were significantly different during the study (two-way ANOVA, DNMR) are indicated by different letters next to the significance level on the side of the legend. Within the control group, sample-time means which were significantly different from the day 0 mean (one-way ANOVA) are indicated by asterisks (*P≤0.05; **P≤0.01). From Mylonas *et al.* (1997c).

The two GnRHa-delivery systems have also been evaluated in inducing long-term increases in milt production in various commercially important species. For example, treatment of white bass with GnRHa-loaded microspheres induced a significant elevation in milt volume for at least 7 days (Mylonas *et al.*, 1997a). Sperm density, as well as sperm motility and fertilization success were not affected by the GnRHa treatment. In the European sea bass (*Dicentrarchus labrax*), GnRHa-implants and microspheres induced elevations in total expressible milt for 20 and 35 days, respectively, without a reduction in sperm density or motility (Sorbera *et al.*, 1996). Finally, in yellowtail flounder, GnRHa-microspheres induced elevations in milt volume for 30 without affecting sperm density or fertilization success (Clearwater and Crim, 1995). Therefore, sustained-administration of GnRHa via delivery-systems is a very effective management tool for enhancing the production of physiologically normal milt in cultured fishes. Further studies are necessary, however, to better document the quality of the produced milt, not only in terms of sperm density and fertilizing ability, but also in terms of viability during cryo-storage (Rana, 1995), since a major benefit of such enhancement in milt production would be the ability to collect and store milt for later use, without the need for frequent handling of, or access to males. This is especially true in striped bass and white bass, and possibly other hybridization programs, that require artificial fertilization of eggs collected from females with asynchronous rate of final oocyte maturation and ovulation.

Based on the results of our studies on the use of GnRHa-delivery systems for the induction of FOM and spermiation, both delivery systems were equally effective. Therefore, the basis for selecting one delivery system over the other, lays solely on their differences in chemical nature, morphology and application procedure. The implants are fabricated in the form of 2 or 3 mm disks using a non-degradable polymer, whereas the microspheres (5 - 200 µm) are prepared as a powder using a biodegradable polymer. These characteristics make one system more desirable than the other for some purposes, or under certain conditions. For example, the implants are easily handled, they do not need any in-the-field preparation and their administration is very fast using a syringe-type implanting device. However, since the GnRHa load in the implants is fixed for a particular preparation, the effective dose will vary depending on fish weight. Implants of different GnRHa content have to be specially prepared for fish varying greatly in size (for example, striped bass versus white bass). On the other hand, it is possible to use the GnRHa-microspheres to treat broodfish varying from a 20 kg Atlantic salmon to a 20 g ornamental fish without modifying the preparation. The fish are simply injected with the appropriate amount of vehicle-suspended microspheres based on their body weight. The GnRH-microspheres have also the major advantage of being biodegradable and over time they are eliminated from the fish's flesh. This may prove to be an important consideration in species were broodstock may eventually be sold for human consumption.

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