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Use of GnRHa-delivery systems for the control of reproduction in fish

Constantinos C. Mylonas[1] & Yonathan Zohar[2]
[1]*Institute of Marine Biology of Crete, P.O. Box 2214, Heraklion, Crete 71003, Greece (Phone (30) 81-285443; Fax (30) 81-241882; E-mail: mylonas@imbc.gr);* [2]*Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt, Baltimore, MD 21202, USA (E-mail: zohar@umbi. umd.edu)*

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Abstract

The most commonly observed reproductive dysfunctions in cultured fish are the unpredictability of final oocyte maturation (FOM) in females, and the diminished volume and quality of sperm in males. Gonadotropin-releasing hormone agonists (GnRHa) have been used extensively in order to stimulate the release of pituitary luteinizing hormone (LH) required to induce FOM, ovulation and spermiation. Because multiple hormonal treatments are often necessary for a successful response, fish must be monitored and handled extensively, which is labor intensive, stressful to the fish and can often result in broodstock mortalities. To ameliorate this problem, sustained-release delivery systems for GnRHa have been developed during the last two decades and have been increasingly applied in controlling reproduction of a variety of cultured fish. Solid implants of cholesterol or poly[ethylene-vinyl acetate], and biodegradable microspheres of poly[lactide-glycolide] or poly[fatty acid dimer-sebasic acid] release GnRHa

for a period of time (from a few days to many weeks.) GnRHa-delivery systems do not cause desensitization of the pituitary gonadotrophs in fish, and by stimulating a sustained elevation of plasma LH they induce the natural progression of plasma steroid increases associated with FOM and spermiation. This method has been used with very encouraging results in females of more than 40 cultured species and has been effective in inducing FOM, ovulation or spawning in fish with synchronous, group-synchronous and asynchronous ovarian development. In males, GnRHa-delivery systems have been tested in more than 20 species, producing significant increases in milt production for up to 5 weeks. Future research should focus on the optimization of this technology in terms of (a) using the most potent GnRHa, (b) identifying the most appropriate GnRHa release kinetics according to the reproductive biology of different species, and (c) determining minimum effective doses. Developments in these areas will greatly enhance the effectiveness and efficiency of GnRHa-delivery systems, while at the same time reducing their cost thus making them more affordable to the aquaculture industry.

Introduction

The culture of fish at an industrial level has a history of less than a century. As a relatively new area of farming compared to terrestrial animal production, aquaculture often resorts to rather primitive production methods. Not surprisingly, only a decade ago it was said that "... the weight of books and papers on various aspects of marine fish and shellfish culture is well in excess of the actual product ..." (Kirk, 1987). Although the industry has progressed tremendously since then, aquaculture is still far behind the sophistication of livestock and poultry production. For example, even today seed acquisition of some fish species relies either on the collection of juveniles from the wild, e.g., freshwater eel (*Anguilla* spp., Anguillidae), milkfish (*Chanos chanos*, Chanidae), gray mullet (*Mugil cephalus*, Mugilidae), yellowtail and Mediterranean amberjack (*Seriola* spp., Carangidae), and bluefin tuna (*Thunnus thynnus*, Scombridae); or on the artificial induction of ovulation of gravid wild broodstock, e.g., summer flounder (*Paralichthys dentatus*, Paralichthyidae), striped bass (*Morone saxatilis*, Moronidae), Australian bass (*Macquaria novemaculeata*, Percichtyidae) and sturgeon (*Acipenser* spp., Acipenseridae). Development of domesticated broodstocks can provide a year-round seed supply via photothermal manipulations (Munro et al., 1990), and the growth, survival and flesh quality characteristics of the fish can be enhanced via genetic selection (Thorgaard, 1995).

Unfortunately, when reared in captivity most fish exhibit some degree of reproductive dysfunction. Many species of captive fish are able to reach reproductive maturity in aquaculture conditions and gonadal growth occurs normally. However, very often females fail to undergo final oocyte maturation (FOM) and do not spawn (Zohar, 1989b; Peter et al., 1993), while males exhibit diminished production or low quality of milt (Billard, 1986, 1989). Such problems can sometimes be ameliorated using elaborate and costly manipulations of environmental parameters, such as temperature, photoperiod, salinity, tank volume and depth, substrate or vegetation, etc., (Zohar, 1989b; Munro et al., 1990; Yaron, 1995). In some species, however, including many highly priced marine fish, hormonal treatments are the only means of controlling reproduction reliably. A variety of hormonal approaches have been used successfully so far, but there are various problems associated with these methodologies. The most important of which are: (a) fish have to be at an advanced stage of gonadal development at the time of treatment; and (b) multiple hormonal applications are very often necessary for a successful response. Consequently, the fish must be monitored and handled extensively, which is not only labor intensive but also stressful to the fish and often leads to mortalities of valuable and painstakingly reared broodstock (Lee and Tamaru, 1988; Bry et al., 1989; Harmin and Crim, 1992). There is a need for maturation induction technologies that minimize handling while maximizing the percentage of broodstock that responds successfully to hormonal therapy.

Sustained-release delivery systems for gonadotropin-releasing hormone (GnRH) agonists have been increasingly employed in various culture situations during the past two decades, in order to control the reproduction of commercially important fish. We review the development of this technology, its physiological relevance to the commonly observed reproductive dysfunctions in fish, and its applications in broodstock management.

The reproductive cycle in fish

The reproductive cycle can be divided into two major phases. The proliferation, growth and differentiation of the gametes constitute the first phase (vitellogenesis and spermatogenesis), while the maturation of oocytes and spermatozoa and preparation for release constitutes the second phase (FOM and spermiation).

Vitellogenesis and final oocyte maturation

The major event during vitellogenesis is the production of the yolk protein precursor (vitellogenin) and its sequestration into the growing oocyte (Tyler and Sumpter, 1996). Final oocyte maturation occurs at the completion of vitellogenesis and includes a number of cytological and nuclear changes that prepare the oocyte for ovulation and fertilization (Goetz, 1983; Nagahama, 1983). During FOM, the nucleus or germinal vesicle (GV) migrates to the periphery of the oocyte at the area below the sperm entry site, the micropyle (Gilkey, 1981). During or soon after GV migration, coalescence of the lipid droplets and yolk globules occurs, followed by the breakdown of the GV membrane (GVBD) and the re-initiation of meiosis, which was arrested in prophase I during vitellogenesis (Jalabert et al., 1991). The oocyte is ovulated with its chromosomes arrested once again, at metaphase II, and meiosis is reactivated and completed upon fertilization (Goetz, 1983; Nagahama et al., 1994).

Three patterns of ovarian development are recognized in teleosts: synchronous, group-synchronous and asynchronous (Wallace and Selman, 1981; Tyler and Sumpter, 1996). Synchronous development is characteristic of semelparous species like the Pacific salmons (*Oncorhynchus* spp.) and freshwater eels, which reproduce only once in their lifetime. In group-synchronous fish, two or more distinct populations of oocytes at different stages of development are present in the ovary. There is a population of primary oocytes and one or more populations of developing oocytes. During the annual spawning season, females of single-batch group-synchronous species undergo FOM and ovulate only once (e.g., striped bass and trout). Species like the European seabass (*Dicentrarchus labrax*, Moronidae), yellowtail flounder (*Pleuronectes ferrugineus*, Pleuronectidae) and rabbitfish (*Siganus guttatus*, Siganidae) have a multiple-batch group-synchronous ovary and undergo multiple ovulations within the course of a few days or weeks. Finally, species with asynchronous ovarian development have a population of primary oocytes and a heterogeneous population of vitellogenic oocytes, from which several batches are recruited and undergo FOM during the annual spawning season, in regular or semi-regular intervals. The distinction between multiple-batch group-synchronous or asynchronous ovary is based on the occurrence of distinct populations of oocytes undergoing development in a synchronous fashion. As such, it is sometimes difficult to differentiate between the two modes of ovarian development, especially in species with a moderate spawning frequency (i.e., three to five times).

Spermatogenesis and spermiation

Spermatogenesis is the first phase of the male reproductive cycle and is completed with the production of flagellated spermatozoa, i.e., spermiogenesis (Billard, 1986). Testicular maturation or spermiation occurs during the spawning season and includes the acquisition of motility capacity by the spermatozoa, the hydration of the testes with seminal plasma and the production of expressible milt (Nagahama, 1994; Billard et al., 1995). In some fish, spermatogenesis and spermiation are temporally separated, with spermiation occurring after the conclusion of spermiogenesis. As a result, during the spawning season the testes exclusively contain spermatozoa (Billard, 1986; Malison et al., 1994). However, in most species there is significant overlap between the two processes, with spermatogenesis as well as spermiation taking place during the spawning season (Jackson and Sullivan, 1995; Matsuyama et al., 1995b).

Endocrinology of fish reproduction

Reproduction is regulated by the brain via the release of GnRH from the hypothalamus (Yu et al., 1997). This decapeptide stimulates the release of gonadotropin (GtH) from the pituitary (Kobayashi et al., 1997). In some fish, mostly freshwater species, dopamine provides a negative control of pituitary GtH release (Peter and Yu, 1997). Fish possess two or three different variants of GnRH (Gothilf et al., 1996; Holland et al., 2000a), and 14 variants have been so far identified from various vertebrates (Figure 1). Within each species, all GnRH forms are potent GtH releasers (Zohar et al., 1995a), and one or two forms may be present in the pituitary (Holland et al., 1998a; Holland et al., 2000b). Pituitary control of reproduction is via a dual GtH system (Schulz, 1995), with

follicle stimulating hormone (FSH or GtH I) regulating vitellogenesis and spermatogenesis, and luteinizing hormone (LH or GtH II) regulating FOM and spermiation (Slater et al., 1994; Moberg et al., 1995). In some species, LH is the prominent form during the entire reproductive cycle (Van Der Kraak et al., 1992; Yaron, 1995).

Females

During vitellogenesis, FSH or LH stimulate the production of testosterone (T) by the theca cells and its aromatization to 17β-estradiol (E_2) in the granulosa (Nagahama, 1994). In response to stimulation by E_2 the liver produces vitellogenin, which is sequestered by the oocytes in a receptor-mediated process enhanced by FSH. At the completion of vitellogenesis a surge in plasma LH stimulates a drop in plasma E_2, a transient increase in plasma T during GV migration, and a dramatic elevation in the plasma levels of the maturation inducing steroid (MIS), which acts at the level of the oocyte membrane to induce FOM (Nagahama, 1994; Nagahama et al., 1994; Peter and Yu, 1997). The most common MISs are 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P). The elevation of LH prior to FOM also induces the maturational competence of the oocytes (Kagawa et al., 1998), a process by which the *de novo* synthesis of MIS receptors enables the oocyte to respond to the MIS and undergo maturation (Thomas and Ghosh, 1995). In addition to LH, insulin-like growth factor I (IGF-I) has been shown to induce maturational competence and FOM (Negatu et al., 1998). Depending on the species, the steroidogenic shift described above and the process of FOM may take place over the course of a few hours (Zohar et al., 1988), a few days (Mylonas et al., 1998a), or more than a week (Scott et al., 1983).

Males

Gonadotropins regulate spermatogenesis via the production of androgens by the testes, mainly 11-ketotestosterone (11-KT; Borg, 1994). Since T is the precursor of 11-KT, the levels of the two androgens co-vary during most of the reproductive season. Plasma 11-KT levels peak during spermiogenesis and decline just prior to, or during the spermiation period. In the immature testis of the Japanese eel (*Anguilla japonica*, Anguillidae) it has been shown *in vitro* that GtH-induced 11-KT production by the Leydig cells stimulates activin B production by the Sertoli cells, which in turn induces spermatogenesis (Miura et al., 1991). Similar to the females, an increase in plasma LH levels at the onset of the spawning season, causes a shift in the steroidogenic production of androgens by the testes to the production of MIS (Nagahama, 1994). Luteinizing hormone and the MIS induce increases in expressible milt volume by stimulating production of seminal plasma (Marshall et al., 1989; Pankhurst, 1994), and the MIS stimulates motility capacitation of the stored spermatozoa via an increase in the pH of the seminal plasma (Miura et al., 1995; Ohta et al., 1997).

Reproductive dysfunctions in cultured fish

Although the term "domestic" is often used in reference to cultured broodstocks, it is doubtful that a domestic fish species exists today, at least according to the interpretation of the word in terrestrial animal husbandry. As mentioned earlier, most cultured species exhibit some degree of reproductive dysfunction when reared in captivity. Problems are more widespread in female broodstock and can vary from inconsistent spawning only, to the complete failure of oogenesis. For example, salmonids undergo vitellogenesis, FOM and ovulation, but fail to spawn their eggs when reared in captivity (Bromage and Cumaranatunga, 1988; Zohar, 1989a). Since ovulation is not synchronized among females, the whole broodstock needs to be checked manually for ovulation two or three times a week during the 6-week-long spawning season. Such handling of the fish is laborious and can result in stress, injury, disease and high mortalities, especially in Pacific salmon, and in Atlantic salmon (*Salmo salar*, Salmonidae) kept at sea. Maturation induction techniques using exogenous hormones have been developed in order to synchronize the ovulation of the broodstock to within one or two weeks, thus making egg collection more efficient and less stressful to the broodstock (Donaldson and Hunter, 1983; Zohar, 1988).

The most commonly observed reproductive dysfunction in captive fish, especially marine species, is the unpredictable occurrence or absence of FOM. Just about every marine fish culturist has encountered this problem, at least during the first few generations of rearing a species in captivity. Some examples include the gilthead seabream (*Sparus aurata*, Sparidae) (Zohar et al., 1995b), the striped bass (Mylonas et al., 1997e), various flatfish (Berlinsky et

	1	2	3	4	5	6	7	8	9	10
Native peptides										
Mammal (mGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-NH_2
Sea bream (sbGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Ser-	Pro-	Gly-NH_2
Salmon (sGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly-NH_2
Chicken II (cGnRH II)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Gln-	Pro-	Gly-NH_2
Synthetic agonists										
Mammal (mGnRHa)	pGlu-	His-	Trp-	Ser-	Tyr-	DAla-	Leu-	Arg-	Pro-	NEt
Salmon (sGnRHa)	pGlu-	His-	Trp-	Ser-	Tyr-	DArg-	Trp-	Leu-	Pro-	NEt

Figure 1. Amino acid sequences of common GnRH variants in fish, and two hyperactive agonists used for maturation induction. Variants have been named after the organism from which they were first identified. Differences in the primary structure of native variants compared to the mammalian form are underlined. Agonists are synthesized by a D-amino acid substitution at position 6 and an ethylamide (NEt) substitution at position 10.

al., 1997; Larsson et al., 1997) and many members of the Serranidae family (Tucker, 1994; Watanabe et al., 1998a). Studies in the gilthead seabream and striped bass showed that the failure of captive fish to undergo FOM in captivity is due to the absence of LH release during the spawning season, even though the hormone accumulates in the pituitary during oogenesis (Zohar, 1988; Mylonas et al., 1997d; Mylonas et al., 1998b). Fish that exhibit this type of dysfunction undergo normal vitellogenesis, but with the onset of the spawning season the developing oocytes fail to initiate FOM; instead they undergo atresia. Treatment of such broodstock with exogenous GtH or GnRHa at the completion of vitellogenesis stimulates gonadal steroidogenesis, FOM and ovulation. Spawning does not always result from such hormonal manipulations, in which case the eggs are stripped manually and fertilized artificially. The stimuli for the female to spawn are probably not only of an endocrine nature and may also depend on (a) the existence of the right environment, e.g., water depth, water flow, nest substrate or vegetation, or (b) the presence of the right number of males exhibiting the appropriate spawning behavior.

The final and most severe form of reproductive dysfunction of captive female broodstock is the failure to undergo vitellogenesis. Such reproductive failure is observed in the freshwater eels, in most captive Mediterranean amberjack populations (*Seriola dumerilii*, Carangidae) (Garcia et al., 2000) and in the Mekong River giant catfish (*Pangasius gigas*, Pangasiidae) (see Donaldson, 1996). In the Japanese eel, for example, the current approach to the artificial manipulation of vitellogenesis involves the weekly treatment with GtH preparations for a period of 7–17 weeks (Ohta et al., 1997). Final oocyte maturation and ovulation is induced by a combination treatment of pituitary extract and 17,20β-P, and fertilization is done artificially after stripping. This method is partially successful, but it is obviously very labor-intensive.

The reproductive dysfunctions observed in captive male broodstocks are less severe in nature compared to the females, but still require special manipulations. Except for the freshwater eels mentioned earlier, all male fish reared in captivity undergo spermatogenesis and spermiation without the need for pharmacological intervention. The problem that is often observed is diminished milt production (Billard, 1989; Berlinsky et al., 1997). For example, yellowtail flounder and turbot (*Scophthalmus maximus*, Bothidae) produce $<$1 ml of milt per fish (Suquet et al., 1992; Clearwater and Crim, 1998), the European catfish (*Silurus glanis*, Siluridae) produces only 0.4 ml kg^{-1} body weight (Linhart and Billard, 1994), and certain genetically selected strains of Atlantic salmon produce $<$0.1 ml kg^{-1} body weight (Zohar, 1996). An additional problem is that of milt consistency and sperm density. Frequently, milt from species exhibiting reduced milt volume in captivity is very viscous and fails to disperse when released in the water or mixed with sperm diluents during artificial fertilization (Mylonas et al., 1998a; Vermeirssen et al., 1998), especially when the fish undergo repeated stripping (Lee and Tamaru, 1988). Similar to female brood-

stock, hormonal therapies using exogenous GtH or GnRHa have been employed to increase milt volume in cultured, as well as wild-caught spermiating males.

Historical overview of maturation-induction methods

The development of pharmacological approaches for the induction of maturation in fish sprung out of the pioneering work of Professor B.A. Houssay, a winner of the 1947 Nobel Prize in Physiology or Medicine. In his studies on the functions of the pituitary, he observed that treatment of an Argentinean livebearer (*Cnesterodon decemmaculatus*, Poeciliidae) with heterologous pituitary homogenates induced females to ovulate (Houssay, 1930). Presumably, the LH present in the pituitary glands acted on the gonads to induce FOM and ovulation. This technique, referred to as hypophysation, was used extensively in other cultured species (Donaldson and Hunter, 1983), but was later replaced by methods employing either human chorionic gonadotropin (hCG) extracted from the urine of pregnant women, or piscine pituitary extracts and purified LH obtained through chromatographic separation (Lam, 1982; Donaldson and Hunter, 1983; Zohar, 1989b). Of the gonadotropin preparations, hCG is the most commonly used today due to its wider availability, higher purity and standardized activity (Lee et al., 1988). It has been tested with variable success in many commercially important fish, often in combination with GnRHa. Major drawbacks of all GtH preparations is their high cost and the fact that fish may become refractory to similar treatment in subsequent spawning seasons (see discussion in Van Der Kraak et al., 1989; Watanabe et al., 1998b). It has been suggested that due to the large molecular size and heterologous nature of GtH preparations, fish develop an immune response and attempts to use it for more than one reproductive season either fail or require an increasingly higher dose (Zohar, 1989b; Peter and Yu, 1997; Zohar and Mylonas, 2001).

The next generation of maturation induction methods focused on the use of synthetic GnRH and its hyperactive agonists (Figure 1), which trigger the secretion of the fish's own GtH, thus activating its pituitary-gonad axis (Zohar, 1988; Crim and Bettles, 1997). There are significant advantages in using GnRHa instead of GtH preparations. First, GnRHa is a small decapeptide that apparently does not trigger an immune response. Second, because GnRHa acts at a higher level in the hypothalamus-pituitary-gonad axis, it can provide a more balanced stimulation of reproductive events and possibly a better integration with other physiological functions, by directly or indirectly affecting the release of other hormones necessary for successful FOM, spermiation and spawning. Such hormones may include growth hormone and IGFs (Le Gac et al., 1993; Negatu et al., 1998), prolactin (Weber et al., 1995) and thyroid hormones (Cyr and Eales, 1996). Finally, GnRHa is synthesized chemically and does not carry the risk of transmitting diseases to the broodstock, a danger always associated with the use of pituitary extracts. These advantages have attracted researchers and practicing aquaculturists to the use of GnRHa, and much effort has been channeled in the last two decades towards improving this method of induced maturation by establishing optimum dosages and application regimes specific for targeted species and rearing conditions.

Dopamine (DA) antagonists are sometimes used together with GnRHa in maturation induction protocols (Peter et al., 1993). The presence of dopaminergic inhibition has been decisively demonstrated in many cyprinid fish and the African catfish (*Clarias gariepinus*, Clariidae), but appears to be absent or weak in most commercially important marine fish (Copeland and Thomas, 1989; King et al., 1994; Zohar et al., 1995b). Also, the significance of DA inhibition appears to change during the reproductive cycle, and although in the white sturgeon (*Acipenser transmontanus*) it is stronger during the spawning season (Pavlick and Moberg, 1997), in other species it may be minimal during this stage (Linard et al., 1995) . As a result, spawning induction protocols using a combined GnRHa and DA antagonist treatment are used extensively only for cyprinid fish (Peter et al., 1993; Yaron, 1995).

Experimental basis for the need for long-term GnRHa treatment

The development and utilization of synthetic GnRHa in fish culture has greatly increased the level of sophistication and control of hatchery production (Zohar, 1988; Crim and Bettles, 1997), and contributed substantially to the growth and, perhaps more importantly, diversification of the aquaculture industry. However, injection of GnRHa does not always result in 100% ovulation, and in species with asynchronous ovarian development and daily spawning rhythms,

long-term ovulation is affected in only a small percentage of the treated females (Zohar et al., 1995b). The synthesized GnRHas were designed to resist enzymatic degradation in circulation, thus increasing their residence time in circulation compared to the native peptides (Zohar et al., 1989; Zohar et al., 1990a). Unfortunately, even the GnRHa most resistant to enzymatic degradation has a half-life of only 23 min *in vivo*, compared to 5 min of the native sGnRH (Gothilf and Zohar, 1991). The failure of single injection protocols to reliably induce maturation is probably the result of this short residence time of GnRHa in circulation, which ranges from a few hours to a few days depending on GnRHa, initial dose, fish species and water temperature. For example, injected [d-Ala^6-Pro^9-NEt]-mGnRH is cleared from the circulation within 8 h in rainbow trout (*Oncorhynchus mykiss*, Salmonidae) maintained at 10–12 °C (Crim et al., 1988), within 12 h in gilthead seabream kept at 23 °C (Zohar et al., 1995b), and within 3 d in winter flounder (*Pleuronectes americanus*, Pleuronectidae) maintained at 0.5 °C (Harmin and Crim, 1993). As mentioned earlier, FOM may require many days to be completed, and GnRHa must be maintained elevated in the circulation throughout this time in order to induce the necessary elevations in plasma LH. Also, fish do not begin FOM immediately after GnRHa injection, even though plasma LH or steroid levels may begin to increase within hours after treatment. This latency period can vary from a few days to weeks, depending on: (a) species sensitivity to handling stress; (b) water temperature; and (c) stage of gonadal development and closeness to maturation at the time of GnRHa treatment. As a result of the short residence time of GnRHa in the circulation, GnRHa-based maturation induction protocols for many fish require multiple injections of GnRHa, given over the course of hours or days. For example, induction of ovulation of walleye (*Stizostedion vitreum*, Percidae) requires two GnRHa injections given 24 h apart (Pankhurst et al., 1986a), the yellow perch (*Perca flavescens*, Percidae) requires two injections given 48 h apart (Dabrowski et al., 1994), while synchronization of ovulation in salmonids is achieved with two injections spaced 3 d apart (Mylonas et al., 1992; Slater et al., 1995). In the winter flounder, ovulation can be achieved within 2–4 weeks after injections of GnRHa three times per week (Harmin et al., 1995). Also, in species like the European seabass and the barramundi (*Lates calcarifer*, Centropomidae), which have a multiple-batch group-synchronous ovarian development, two GnRHa injections are often necessary to induce ovulation of the first batch of oocytes (Almendras et al., 1988; Carrillo et al., 1995), while further ovulations can be obtained only with additional injections (Y. Zohar, unpublished data and Almendras et al., 1988).

A very good example of the ineffectiveness of even multiple GnRHa injections to induce ovulation is the striped bass, a species that completes vitellogenesis but rarely undergoes FOM in captivity (Mylonas et al., 1997e). Treatment of post-vitellogenic females under ambient water temperature (7–18 °C) with two GnRHa injections spaced 3 d apart resulted in elevations of plasma GnRHa for at least 7 d (Figure 2). The resulting increases in plasma LH, E_2 and T followed the profile of plasma GnRHa, and once GnRHa was cleared from the circulation LH decreased significantly and plasma E_2 and T returned to pre-treatment levels. There was no plasma elevation of the MIS, and although 60% of the females initiated the early stages of FOM, none progressed further than the peripheral GV stage. Obviously, more than two consecutive GnRHa injections are necessary to support a slow FOM in striped bass, indicating that long-term GnRHa-delivery systems can be important tools in controlling maturation of this species.

The short residence time of injected GnRHa in circulation poses a problem in male broodstock management as well. As mentioned earlier, male fish in captivity often exhibit reduced milt production. A single injection of GnRHa increases milt volume by stimulating seminal plasma production, but often with a proportional decrease in sperm density (Takashima et al., 1984; Garcia, 1991). The end effect is that the total number of spermatozoa produced per kg body weight may not differ between GnRHa-treated and untreated males. Although reduction of sperm density and hydration of the testes facilitates sperm release, and is part of the testicular maturation process during spermiation (Ueda et al., 1985), successful milt enhancement protocols are expected to result in an overall increase in both expressible milt and spermatozoa production.

A more serious drawback of single-injection GnRHa treatments in males is that the spermiation enhancement effect is rather short-lived, lasting only a few days. Such a brief increase in available milt may be adequate if the females mature and ovulate in relative synchrony or only once during the spawning season, e.g., carp (*Cyprinus carpio*, Cyprinidae). However, in situations where females spawn at multiple times or during a prolonged reproductive

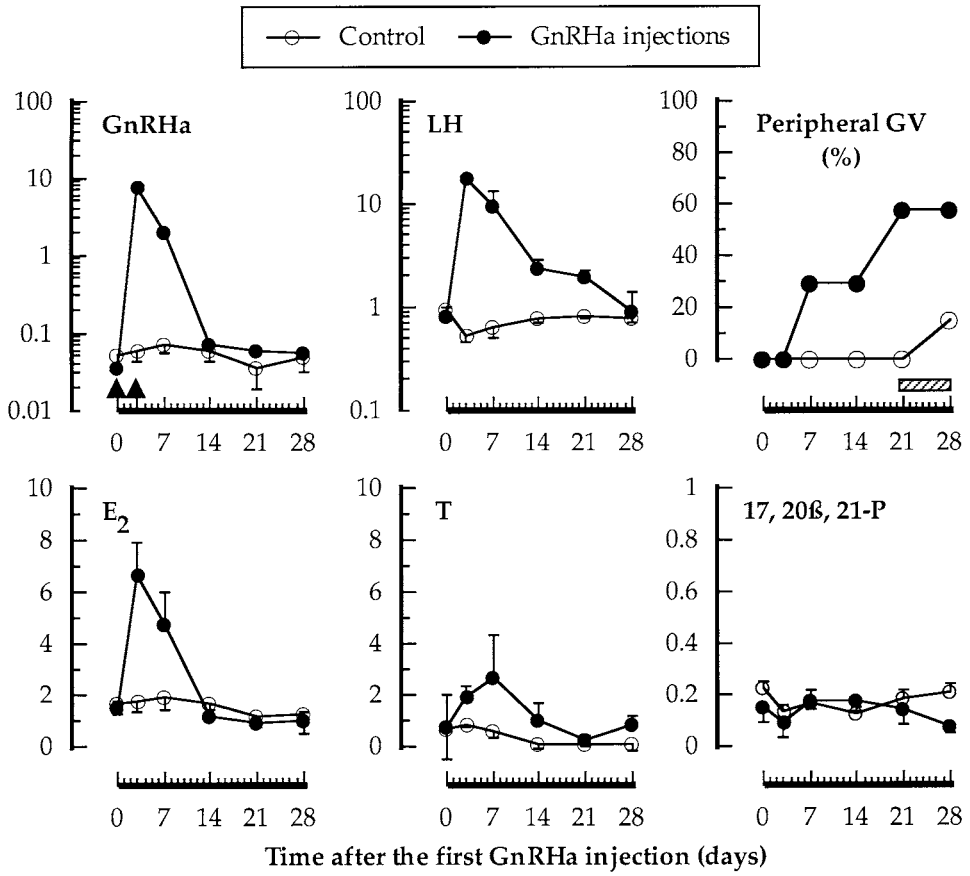

Figure 2. Mean (± s.e.m.) plasma hormone levels (ng ml^{-1}) and cumulative % oocyte maturation of cultured striped bass (n = 7) after two injections (arrows) of GnRHa (15 μg kg^{-1}) or saline during the spawning season (April). Fish (4–8 kg) were maintained in a flow-through system supplied with Chesapeake Bay water (4–2 g l^{-1} salinity; 7–18 °C) and had a mean oocyte diameter of 850 (± 20) μm at the start of the experiment. With the exception of plasma 17,20β,21-P, all hormone profiles were significantly changed by the GnRHa treatment (ANOVA, $P \leq 0.05$). The horizontal hatched bar indicates the time when atretic oocytes could be seen in the biopsies. Most GnRHa-injected females reached the peripheral GV stage, but none ovulated within the duration of the study.

season, e.g., salmon, yellowtail flounder and gilthead seabream, a long-term increase in milt production must be induced. Also, where artificial fertilization is employed, large volumes of expressible milt are often required, and individual males may be handled and stripped repetitively for days or even weeks. Increases in milt production for such long periods of time necessitate treating the males with multiple injections of GnRHa. For example in carp, daily injections of GnRHa resulted in continually increasing amounts of expressible milt for 5 d, but once treatment was discontinued, milt volume returned to pre-treatment levels within 3 d (Takashima et al., 1984). In Atlantic salmon, GnRHa injections given every 2 d were necessary to induce spermiation and maintain milt volume for 12 d (Weil and Crim, 1983), and in the barramundi weekly injections of GnRHa were necessary to maintain long-term spermiation (Garcia, 1993). Finally, in the winter flounder, two injections of GnRHa within 48 h induced a more than three-fold elevation in expressible milt, whereas a single injection failed to significantly increase milt production (Harmin and Crim, 1993). Repeated handling for GnRHa injections is cumbersome, can be stressful and damaging to the fish, and may disrupt spawning behavior in species that are expected to undergo volitional tank spawning over a long spawning season.

Development of GnRHa-delivery systems

The need for the development of a hormonal formulation that does not require repeated applications was recognized early in the development of spawning induction therapies (Fontenele, 1955). Later, in evaluating the effectiveness of different vehicles, it was found that GnRH emulsified in Freud's adjuvant was more effective in inducing ovulation of Japanese plaice (*Limanda yokohamae*, Pleuronectidae) and goby (*Acanthogobius flavimanus*, Gobiidae; Aida et al., 1978). Although the release kinetics of GnRH from the adjuvant were not evaluated, the greater success of the preparation was attributed to a prolonged GnRH release from the oil emulsion.

Cholesterol pellets

After the demonstration of a sustained release of GnRHa from a cholesterol matrix in mammals (Kent et al., 1980), studies were undertaken to evaluate its efficacy in inducing maturation and spawning in cultured fish. Initial trials with Atlantic salmon (Weil and Crim, 1983) and barramundi (Harvey et al., 1985) indicated that treatment with a GnRHa-delivery system was much more effective in advancing gonadal maturation, and inducing ovulation and spermiation compared to a single injection of GnRHa dissolved in saline. Thus, the cholesterol matrix was the first to be thoroughly evaluated and developed as a sustained-release GnRHa-delivery system for fish (Carolsfeld et al., 1988; Crim et al., 1988; Sherwood et al., 1988). Combining cholesterol, cellulose and GnRHa in powder form and compressing the resulting mixture in a pellet press, sometimes after addition of cocoa butter, prepares this delivery system. The cylindrical pellets (3 mm diameter $\times$ 3 mm long, 30 mg) are loaded with 25–250 μg of GnRHa and can be applied intraperitoneally or intramuscularly, via a small incision or using a syringe-type applicator.

Once implanted into the fish, the entrapped GnRHa is released in a diffusion-controlled process, but depending on the cholesterol:cellulose ratio, matrix erosion may also facilitate GnRHa release. However, release kinetics can be only grossly controlled by adjusting the matrix composition: pellets of $<$95% cholesterol ("fast") release 90% of their GnRHa content within 24 h *in vitro*, whereas pellets with $>$95% cholesterol ("slow") release 20% of their GnRHa content in the first 24 h, and only an additional 20% during the next 5 weeks (Carolsfeld et al., 1988; Sherwood et al., 1988). The same general response is observed *in vivo*, with fast pellets resulting in high plasma GnRHa levels during the first 2 d after treatment, while slow pellets exhibit a many-fold lower rate of GnRHa release, resulting in moderate elevations in the plasma for up to 4 weeks (Crim et al., 1988). An inefficiency of GnRHa release from pellets with very high cholesterol content was demonstrated recently in the red seabream (*Pagrus major*, Sparidae) where 100% cholesterol pellets released only 6% of their GnRHa load during 70 d (Matsuyama et al., 1995a). As far as stimulating GtH release, the fast cholesterol pellets can maintain high plasma levels for at least 8 d, while the slow pellets can elevate plasma GtH for at least 8 weeks (Crim et al., 1983b). Cholesterol pellets are inexpensive and can be prepared at the hatchery, but making large numbers of them is labor-intensive, since each pellet is pressed individually and the amount of matrix/GnRHa necessary for each pellet must be weighed separately. Application is also very simple and does not damage the fish, and GnRHa pellets can be stored at room temperature for at least 4 months without losing their bioactivity (Garcia, 1996). An important disadvantage of the cholesterol delivery system is the significant variation in GnRHa release from individual pellets (Carolsfeld et al., 1988). Also, cholesterol is an active biomolecule and a precursor to steroid hormone synthesis, posing some concern as to the influence it may have in gonadal function.

Ethylene-vinyl acetate implants

Ethylene-vinyl acetate copolymer (EVAc) is a non-degradable copolymer of ethylene and vinyl acetate monomers, and delivery systems can be manufactured in the form of microspheres or solid implants (Rhine et al., 1980; Brown et al., 1986). The EVAc delivery system is prepared by adding the solvent-dissolved polymer to a mixture of inulin (from tubers of the flower Dahlia) and bovine serum albumin (i/bsa), and the appropriate amount of GnRHa in powder form. The solvent is evaporated, resulting in a solidified sponge-like matrix consisting of polymer and i/bsa with GnRHa. The GnRHa-loaded EVAc systems used in fish are rubbery 2 mm-long cylindrical implants of 2 or 3 mm diameter (5 or 15 mg), are usually loaded with 25–250 μg of GnRHa and are given intramuscularly using a syringe-type applicator. The i/bsa mixture is used as a bulking agent, creating channels within the solidified polymer. Upon application and contact with body fluids, the i/bsa mixture slowly dissolves

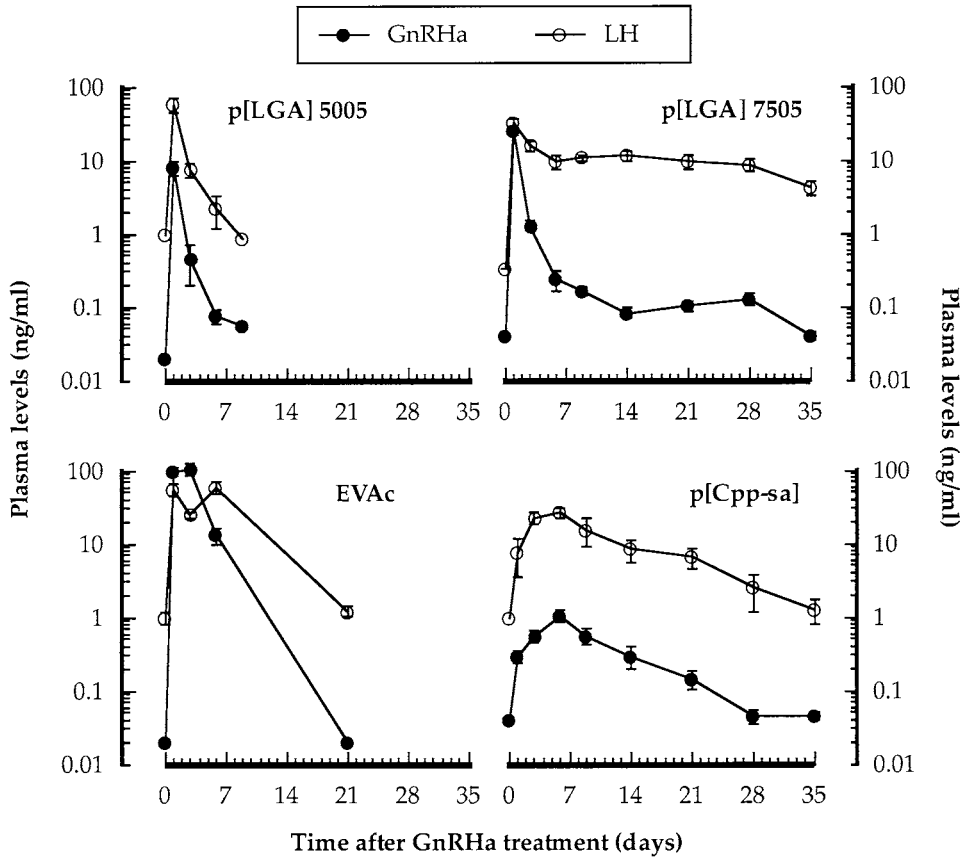

Figure 3. Mean (± s.e.m.) plasma GnRHa and LH levels of cultured female gilthead seabream after treatment with GnRHa-delivery systems during the spawning period (March). Fish (n = 6) were maintained in 0.5-m^3 tanks supplied with water of ambient temperature (21 °C) and were treated with 100–200 μg GnRHa kg^{-1} using microspheres produced with poly[lactide-glycolide] at 50:50 molar ratio, 5000 molecular weight (p[LGA] 5005) or p[LGA] at 75:25 molar ratio, 5000 molecular weight (p[LGA] 7505); and implants fabricated with poly[1,3-bis(p-carboxyphenoxypropane)-sebasic acid] (p[Cpp-sa]), or poly[ethylene-vinyl acetate] (EVAc) (modified from Gothilf, 1990).

along with the entrapped GnRHa. The release kinetics from this delivery system are diffusion-controlled and EVAc implants used in fish typically release GnRHa for about 15 d (Figure 3). However, GnRHa release for more than 5 weeks can be achieved by manipulations of the percentage loading with i/bsa, the i:bsa ratio, or by changing the geometry of the implants and applying a permeability controlling coating (Zohar, 1996).

GnRHa-delivery systems using EVAc are simple to fabricate and large batches of 200–500 implants can be produced in a single preparation, thus minimizing production cost and GnRHa-content variation between individual implants. Application is very simple and is not damaging to the fish, and the implants maintain their effectiveness for at least 3 years if stored desiccated at −20 °C. Manufacturing does require technical expertise and specialized equipment, making it unfeasible for on-site preparation. One concern regarding the EVAc implants is their use of BSA, a large heterologous protein, which has the potential for inducing an immune response in subsequent uses. However, in more that a decade of trials, there is no report to this effect.

Biodegradable microspheres

Biodegradable polyesters of lactic acid and glycolic acid were first developed as suture material in surgery applications, but in the early 1970s copolymers of lactide-glycolide (p[LGA]) were evaluated as drug-delivery matrices (Lewis, 1990). Another family of

biodegradable polymers developed more recently for peptide delivery is the polyanhydrides, which are synthesized as copolymers using fatty acid dimer and sebasic acid (p[Fad-sa]), or bis-carboxyphenoxy propane and sebasic acid (p[Cpp-sa]) (Chasin et al., 1990). Biodegradable GnRHa microspheres with diameters 5–200 μm can be prepared by a solvent evaporation method using a double emulsion, and are usually loaded with GnRHa at a 3% ratio (Okada et al., 1994a; Mylonas et al., 1995a). The peptide release from biodegradable microspheres is the combined result of peptide diffusion and polymer degradation. Based on the monomer types, monomer ratio and length of the polymer (i.e., molecular weight) the relative influence of the two release parameters can be controlled, thus affecting the release kinetics (Chasin and Langer, 1990).

Release of GnRHa from p[LGA] and polyanhydride microspheres is characterized by an initial burst immediately after application and a sustained or continuously declining release until depletion of the microspheres (Figure 3). Some of the long-term preparations can release GnRHa for up to 8 weeks *in vivo* (Mylonas et al., 1995a), resulting in immediate and sustained elevations of plasma LH that can vary in duration depending on the stage of gonadal development of the fish (see later discussion). Compared to the previously described GnRHa-delivery systems, microsphere manufacturing requires the highest level of expertise and specialization of equipment, which is outside the realm of a commercial hatchery. Apart from being biodegradable, the major advantage of microspheric delivery systems is that they can be administered as a liquid suspension on a volume to weight basis, enabling the use of the same microspheric preparation for treating individuals ranging in weight from 10 g to 10 kg. This characteristic makes the GnRHa microspheres the method of choice for applications in spawning induction of broodstock of ornamental fish species.

Other delivery systems

Implants of silicone rubber or silastic tubing constituted some of the earliest attempts to prepare a GnRHa-delivery system. However, a highly hydrophilic GnRHa does not penetrate easily through the hydrophobic matrix, making silicone ineffective (Pankhurst et al., 1986a; Pankhurst et al., 1986b; Zohar, 1988) or inefficient, at best, as a GnRHa delivery system (Weil and Crim, 1983; Billard et al., 1984; Harvey et al., 1985; Henry et al., 1998). Osmotic pumps can result in highly controlled release of GnRHa and have been shown to be effective in various fish (Marte et al., 1987; Almendras et al., 1988; Matsuyama et al., 1997), but they are extremely expensive for use in large scale hatchery operations and their large size (7×10 to 8×32 mm) makes them difficult to apply. Finally, non-degradable solid implants of methacrylate copolymer have been shown to release GnRH *in vitro* for up to 10 weeks and were effective in inducing ovulation in the ayu (*Plecoglossus altivelis*, Osmeridae) (Hirose et al., 1990), and North Sea plaice (*Pleuronectes platessa*, Pleuronectidae) (Scott et al., 1999), and induced spermatogenesis and vitellogenesis in the red seabream (Matsuyama et al., 1993).

Applications of GnRHa-delivery systems in fish culture

During the last two decades, GnRHa-delivery systems have been tested in a wide variety of cultured and wild fish. Through increases in circulating LH levels, these delivery systems can synchronize ovulation, induce FOM in species that fail to do so in captivity, and in some instances enhance vitellogenesis. In males, they advance the onset of spermiation, increase the amount of expressible milt and spermatozoa production, and increase milt fluidity by increasing seminal plasma production.

Induction of GtH release

In mammals, synthesis and release of FSH and LH is regulated by the pulsatile release of GnRH from the hypothalamus (Gharib et al., 1990). Changes in GnRH release pulsatility are well documented during the reproductive cycle, and the frequency and amplitude of the GnRH pulses are instrumental in regulating the amount of gonadotropins released in the circulation. However, high doses of GnRHa administered in a sustained fashion induce down-regulation of pituitary GnRH receptors, resulting in a decrease in gonadotropin release (Okada et al., 1994b). In fact, GnRHa-delivery systems are used extensively for the suppression of the reproductive system in mammals (Heinrichs et al., 1994). In fish, it has not been possible yet to document the natural mode and rate of GnRH release in the pituitary, since unlike all other vertebrates, most fish do not posses a hypothalamus-pituitary portal system, and hypothalamic GnRH is

secreted directly in neuronal synapses at the pituitary gonadotrophs. The presence of an episodic release of LH suggests that a pulsatile GnRH release may also be functional in rainbow trout (Zohar et al., 1986). Contrary to the situation in most mammals, however, treatment with GnRHa-delivery systems results not in pituitary desensitization and inhibition of GtH release, but in a sustained elevation of LH for many weeks (Breton et al., 1990). Sustained elevations of plasma GtH in response to GnRHa-delivery systems have been documented in all fish examined so far, including gilthead seabream (Figure 3), striped bass (Figure 4), goldfish (*Carassius auratus*, Cyprinidae) (Sokolowska et al., 1984), Atlantic salmon (Weil and Crim, 1983), rainbow trout (Breton et al., 1990) and white bass (*Morone chrysops*, Moronidae) (Mylonas et al., 1997b). So, it appears that in fish, unlike the situation in mammals, birds and reptiles (Chang and Jobin, 1994), sustained GnRHa treatment does not produce anti-gonadotropic effects.

The question, however, remains as to the physiological relevance of sustained GnRHa stimulation, and the soundness of GnRHa-delivery systems as tools for inducing maturation in fish. A definitive answer cannot be given unless it becomes possible to measure "real-time" neurosecretory activity of GnRH neurons in the fish pituitary. We can, however, evaluate the GnRHa-delivery system approach on the basis of its induction of physiological profiles of GtH, or by its efficacy in inducing maturation and production of viable gametes. In comparing striped bass females undergoing maturation in the wild (Mylonas et al., 1997d) with cultured fish induced to ovulate using GnRHa-delivery systems and kept at constant temperature (Mylonas et al., 1998b), similar plasma LH profiles were observed during FOM. In both wild and cultured females, there was an initial LH increase during GV migration, followed by a further increase during GVBD. In cultured females, this second increase in plasma LH occurred in the presence of constant, or even declining GnRHa levels. Also, treatment of rainbow trout with GnRHa-delivery systems induced a gradual increase in plasma LH during FOM (Breton et al., 1990), a profile that paralleled the LH changes observed in spontaneously maturing females (Breton et al., 1998). These observations suggest that the pituitary of rainbow trout and striped bass did not simply respond to the sustained GnRHa stimulation with a dose-dependent LH release. Instead, the gonadotrophs appear to be controlling LH release in the presence of a continuous and powerful GnRHa stimulus, perhaps through changes in pituitary responsiveness to GnRH (Peter and Yu, 1997).

However, the situation may be different in other fish or under different environmental conditions. For example, in the gilthead seabream, a daily spawner, a circadian cycle of LH of a precisely 24-h periodicity controls FOM and ovulation (Gothilf et al., 1997), but when treated with GnRHa-delivery systems, females have constantly elevated plasma LH, paralleling the GnRHa levels in circulation (Figure 3). In spite of the constantly high plasma LH levels, females undergo the usual daily rhythm of vitellogenesis, FOM and ovulation (Zohar et al., 1995b), although the cycle may be slightly shorter than 24 h (Barbaro et al., 1997). Fecundity is improved by the GnRHa treatment, while fertilization and hatching % is similar to eggs obtained from spontaneously spawning individuals. Also, in striped bass maintained under ambient temperature conditions, the constantly elevated plasma LH in response to treatment with GnRHa-delivery systems induced the expected successions of plasma E_2, T and 17,20β,21-P elevations characteristic of FOM and ovulation (Figure 4). Perhaps ovarian responsiveness to LH changes during FOM, so that even constantly high levels provide the appropriate gonadal stimulation for the necessary changes in gonadal steroidogenesis to occur. Therefore, although the sustained presence of GnRHa from a delivery system may not reflect the natural situation, in fish it appears to stimulate the appropriate hormonal changes for inducing gonadal maturation and the production of viable gametes.

Final oocyte maturation, ovulation and spawning

Synchronous and single-batch group-synchronous fish

Since the most common reproductive dysfunction in captivity involves the absence or the unreliable occurrence of FOM and ovulation, the majority of studies with GnRHa-delivery systems have focused on the female. One of the better established applications is in the culture of salmonids, especially salmon, where commercially available p[LGA] and EVAc GnRHa-delivery systems are used to synchronize ovulation (Breton et al., 1990; Goren et al., 1995). Traditionally, synchronization of ovulation involves the use of GnRHa injections spaced 3 d apart, administered within 2 weeks before the expected date of maturation of the broodstock. Timed well, a single GnRHa injection may induce ovulation in 80–90% of the females within 2–4 weeks (Breton et al., 1990; Haraldsson et

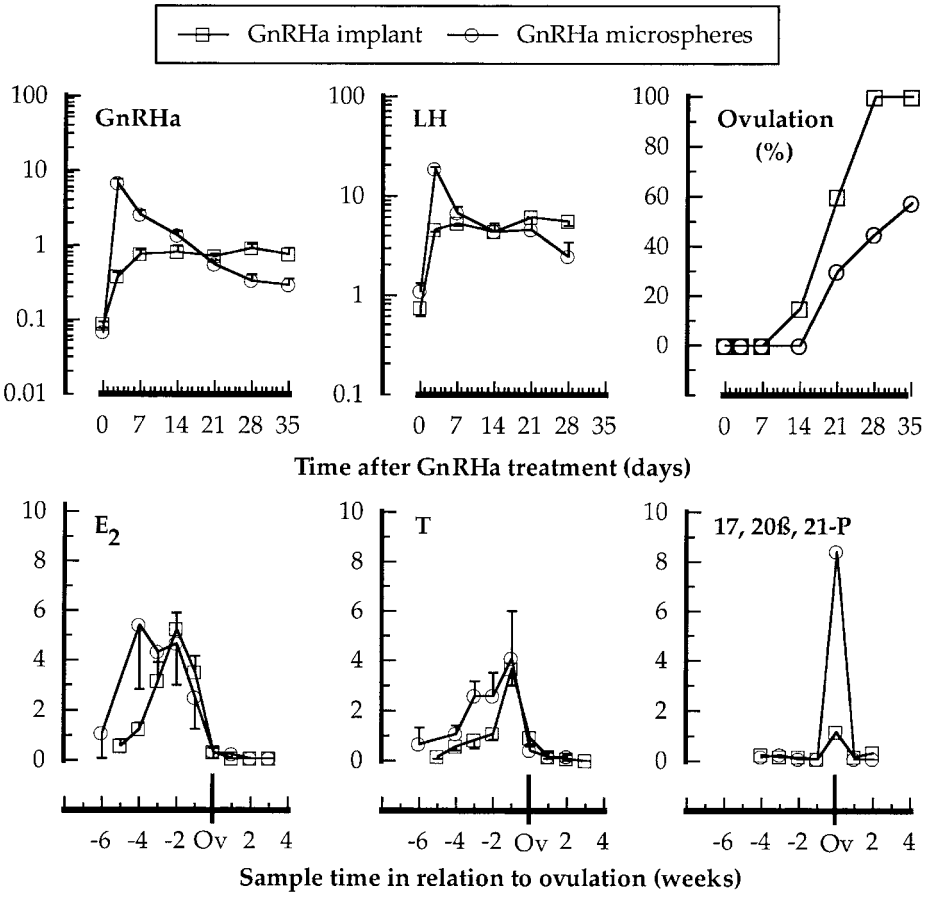

Figure 4. Mean (± s.e.m.) plasma hormone levels (ng ml^{-1}) and cumulative % ovulation of cultured striped bass (n = 7) after treatment with an EVAc implant (20 μg GnRHa kg^{-1}) or p[Fad-sa] microspheres (15 μg GnRHa kg^{-1}) during the spawning season (April). Experimental conditions were as described for Figure 2. Plasma data for the steroid hormones (n = 1 to 7) were plotted versus the sampling time when the fish were found to have already ovulated (0v). Changes in plasma 17,20β,21-P were observed in two females only, which happened to be sampled 12 h before ovulation.

al., 1993; Solar et al., 1995; Pankhurst and Thomas, 1998), whereas a two-injection protocol can effectively induce ovulation within 1–2 weeks (Van Der Kraak et al., 1985; Sullivan et al., 1989; Mylonas et al., 1992). If the treatment is given too early, a third injection may be required to successfully complete FOM in all females. Another complication from poorly timed GnRHa injection protocols is that of partial ovulations. Within the salmonid ovary, ovulation is a progressive process and very often females ovulate only part of their oocytes after an inadequate hormonal stimulation. Because the eggs in the case of the Pacific salmons are collected after sacrificing the fish, unovulated, and thus infertile, eggs constitute a great financial loss.

GnRHa-delivery systems offer considerable advantages for salmonid hatcheries, compared to the multiple injection method. For example, in response to a single application, between 80–100% of treated females should be expected to ovulate within 2 weeks after treatment. Due to the long-term delivery of GnRHa, the timing of application does not have to be as precisely determined and applications can be initiated up to 6 weeks prior to the expected onset of ovulation (Crim et al., 1983a; Crim and Glebe, 1984; Goren et al., 1995). Such acceleration of gonadogenesis, and advancement of ovulation and spermiation by a period of a few weeks to months may prevent the substantial pre-spawning mortalities often observed in Pacific salmon broodstocks, which rapidly

deteriorate in health during the reproductive season, and can ensure optimal egg collection, especially if fish are kept at sea. Also, the occurrence of partial ovulations is limited when using sustained GnRHa treatments (Y. Zohar, unpublished data) and there are no negative effects on oocyte size, fertilization success or embryo survival (Crim et al., 1983b; Breton et al., 1990; Haraldsson et al., 1993; Mylonas et al., 1993). Finally, whereas GnRHa injections have limited success in broodstocks maintained in seawater (Sower et al., 1982; Slater et al., 1995), GnRHa-delivery systems have produced promising results in inducing ovulation reliably. Successful applications of GnRHa-delivery systems have been reported for various members of the family Salmonidae (Table 1), including the rainbow trout (Breton et al., 1990), brown trout (*Salmo trutta*) (Goren et al., 1995), Atlantic salmon (Crim et al., 1983a; Goren et al., 1995; Mylonas et al., 1995a), chinook salmon (*Oncorhynchus tshawystcha*) (Solar et al., 1995), coho salmon (*O. kisutch*) (Zohar et al., 1990b; Mylonas et al., 1993; Goren et al., 1995), sockeye salmon (*O. nerka*) (P. Swanson and Y. Zohar, unpublished data) and Arctic char (*Salvelinus alpinus*) (Haraldsson et al., 1993). A recent study suggests that sustained GnRHa treatment may also influence reproductive behavior in fish, since treatment of both male and female sockeye salmon with a GnRHa-delivery system shortened significantly the duration of their migration to the spawning area (Sato et al., 1997; Kitahashi et al., 1998).

GnRH-delivery systems have also been used successfully in non-salmonid species with single-batch group-synchronous ovarian development. For example, emulsified GnRH induced ovulation in Japanese plaice and goby, whereas the same dose given as a saline injection was ineffective (Aida et al., 1978). In the Yaqui catfish (*Ictalurus pricei*, Ictaluridae), GnRHa given in an EVAc implant was the only hormonal preparation able to induce spawning, whereas hCG, catfish pituitary extract, and a liquid preparation of sGnRHa and pimozide were ineffective (M. Ulibarri and Y. Zohar, unpublished data). In the tiger puffer (*Takifugu rubripes*, Tetraodontidae), an important emerging candidate for aquaculture in Japan, GnRHa administered via a cholesterol pellet or an osmotic pump induced ovulation in 18 and 10 d in fish with mean oocyte diameter of 800–900 μm and 900–1000 μm, respectively (Matsuyama et al., 1997). This latter result underscores a very important advantage of GnRHa-delivery systems compared to injections: flexibility in the timing of application. Whereas a single GnRHa injection will probably fail in females that have not fully completed vitellogenesis, a long-term GnRHa-delivery system is able to induce FOM and ovulation in fish of lesser maturity, albeit requiring a longer period of time. Other single-batch group-synchronous species in which GnRHa-delivery systems have been used successfully include the winter flounder (Harmin and Crim, 1992), sablefish (*Anoplopoma fimbria*, Anoplopomatidae) (Solar et al., 1992) and grey mullet (de Monbrison et al., 1997), among others (Table 1).

Multiple-batch group-synchronous and asynchronous fish

The application of GnRHa-delivery systems with perhaps the greatest potential in aquaculture is the induction of FOM in females with multiple-batch group-synchronous or asynchronous ovarian development. For example, an EVAc implant induced two consecutive spawns within 3 d after treatment in white bass, with about 50% of the eggs released in each spawn (Mylonas et al., 1997b). In the barramundi, an osmotic pump delivering 1.5–4.5 μg of GnRHa kg^{-1} day^{-1} induced up to five consecutive spawns within a 7-d period (Almendras et al., 1988). Similarly, females given GnRHa embedded in a >95% cholesterol matrix spawned for 3 to 4 consecutive days (Almendras et al., 1988; Garcia, 1989). Fecundity was higher for the first one or two spawns, while fertilization % was similar among spawns. Also, GnRHa given in a delivery system induced vitellogenesis and FOM in more than 96% of wild female striped trumpeter (*Latris lineata*, Latridae), resulting in up to five consecutive ovulations in a period of 2 weeks, compared to only 33% of control fish ovulating for a maximum of three consecutive times (Morehead et al., 1998). Fecundity was highest for the first ovulation and declined steadily in subsequent batches, while fertilization % was unchanged. The declining fecundity in subsequent spawns observed in the above mentioned studies is characteristic of the reproductive biology of some group-synchronous species in captivity, and is not likely a reflection of failing GnRHa-delivery systems. The same profile of diminishing fecundity in the course of repeated spawnings after treatment has been observed in the American shad (*Alosa sapidissima*, Clupeidae) (Mylonas et al., 1995b), the Florida pompano (*Trachinotus carolinus*, Carangidae) (Hicks, 1998), the shi drum (*Umbrina cirrosa*, Sciaenidae) (Mylonas et al., 1999), the European seabass

(Forniés et al., 2000) and the turbot (*Scophthalmus maximus*) (Mugnier et al., 2000).

Some of the most spectacular results from GnRHa-delivery systems have been obtained recently from various flatfish and from members of the Sparidae family. For example, in captive-reared Southern flounder (*Paralichthys lethostigma*, Paralichthyidae), GnRHa in a cholesterol matrix induced one to three ovulations in all females with mean oocyte diameter of >500 μm (Berlinsky et al., 1996). In the congener wild summer flounder, the same treatment induced daily ovulations for up to eight consecutive days (Berlinsky et al., 1997). In fish maintained for more than a year in captivity the same treatment induced not only ovulation but also tank spawning (Watanabe et al., 1998b). Fecundity and fertilization % were highest on the first ovulation and decreased subsequently (Berlinsky et al., 1997). In the turbot, treatment with EVAc implants induced multiple ovulations in 100% of treated fish compared to 50% of controls, it significantly reduced the duration of the spawning season by reducing the inter-ovulation period, and significantly increased the fecundity per batch, though there was no increase in total fecundity (Mugnier et al., 2000). Finally, in the cold-water congener yellowtail flounder, both p[Fad-sa] microspheres and EVAc implants induced an average of eight consecutive ovulations, compared to three for control fish, resulting in the production of twice as many eggs and of higher fertilization and hatching % than control females (Larsson et al., 1997).

Members of the Sparidae family are capable of undergoing FOM and spawning repetitively on a 24-h cycle for periods of up to 4 months. In captivity, however, very often only a small percentage of the broodstock undergoes FOM spontaneously and hormonal treatments induce ovulation for only a short period of time. In the gilthead seabream, for example, a single GnRHa injection at the onset of the spawning season induced spawning in the majority of females, but only 20% continued spawning on a daily basis (Zohar, 1988). On the contrary, all females given GnRHa in a polymeric delivery system initiated spawning 3 d after treatment and >70% of the females continued spawning on a daily basis. In another experiment, gilthead seabream treated with GnRHa entrapped in p[LGA] or p[Fad-sa] microspheres spawned daily for 96 and 137 d, respectively, and produced a greater number of eggs, with slightly higher fertilization% and equal hatching success compared to control females (Y. Zohar, unpublished data). In the Pacific Ocean congener red seabream, treatment with cholesterol pellets or osmotic pumps containing GnRHa induced the typical daily rhythm of ovulation (Matsuyama et al., 1993; Matsuyama et al., 1995a). Fertilization % was very low, however, probably due to low milt volumes produced by the males and the fact that fertilization was evaluated only during the first 2 weeks of spawning, when fertilization success is low even in naturally spawning fish. Finally, in another congener, the Mediterranean red porgy (*Pagrus pagrus*, Sparidae), a single GnRHa injection induced daily ovulations for only 5 d, whereas treatment with GnRHa-microspheres initiated daily ovulations that lasted for 32 d (Zohar and Mylonas, 2001). GnRHa-delivery systems can significantly enhance the total number of eggs produced by multiple-spawning fish, by increasing the number of broodfish undergoing FOM, increasing the number of ovulations per spawning season and, frequently, by increasing fecundity, fertilization and hatching success.

Spermiation and milt production

As mentioned earlier, it is very common for captive males to produce only small amounts of milt, often of extremely high sperm density (>90%) and low sperm motility. Similar to the females, long-term treatment of males with GnRHa via delivery systems can result in sustained elevations of plasma LH, as shown in the Atlantic salmon (Weil and Crim, 1983), white bass (Mylonas et al., 1997a) and striped bass (Figure 5 and Mylonas et al., 1998a). In striped bass, p[Fad-sa] microspheres and EVAc implants induced plasma elevations of LH lasting for at least 14 d, resulting in a slight, but significant, elevation of plasma 17,20β-P (Figure 5), a steroid controlling sperm maturation in fish. Total expressible milt increased dramatically 2 d after treatment, and milt production was maintained at significantly higher levels than untreated control fish for at least 14 d (Figure 5). The initial increase in milt volume was associated with a decrease in sperm density during the first 4 d, but for the remaining 10 d sperm density was similar to pre-treatment levels (Mylonas et al., 1997c). As a result, total spermatozoa production per kg body weight during the study was four-fold higher in fish treated with GnRHa-delivery systems, compared to controls. A moderate reduction in sperm density due to increased seminal plasma production is one of the processes associated with final testicular maturation, and is a desirable effect of GnRHa treatments, especially in

Table 1. Applications of GnRHa-delivery systems for the induction of vitellogenesis (vg), ovulation (ov), spawning (sp) and homing migration (migr) in females, and the induction of spermatogenesis (sr), and enhancement of seminal fluid (sfl) and milt production (ml) in male cultured fishes. Doses have been rounded up for simplification and where an "x" follows the dose it indicates multiple applications

Species (by family)	Common name	Delivery[1] system	Dose $\mu g\ kg^{-1}$	GnRH[2]	Effect	Reference
Perciformes						
Centropomidae						
Lates calcarifer	Barramundi	Chol	9–24	ET_2	sp	Harvey et al., 1985
		Osmo	60–160	A	sp	Almendras et al., 1988
		Chol	30–200	A, R	sp	Almendras et al., 1988
		Chol	40–75	A	sp	Garcia, 1989
Centropomus undecimalis	Snook	Chol	100	B, C, M, S	ov	Skapura et al., 2000
Moronidae						
Dicentrarchus labrax	European seabass	EVAc	100	A	ml	Sorbera et al., 1996
		p[Fad-sa]	50	A	ml	Sorbera et al., 1996
		EVAc	75	A	sp	Forniés et al., 2000
		p[Fad-sa]	50	A	sp	Forniés et al., 2000
Morone chrysops	White bass	EVAc	60	A	ov	Mylonas et al., 1996
		p[Fad-sa]	40	A	ov	Mylonas et al., 1996
		p[Fad-sa]	40	A	sp	Mylonas et al., 1997b
		p[Fad-sa]	25	A	ml	Mylonas et al., 1997a
Morone saxatilis	Striped bass	Chol	30–110	A[4]	ov, sp	Hodson and Sullivan, 1993
		Chol	20	A	sp	Woods and Sullivan, 1993
		EVAc	45	A	ml	Mylonas et al., 1997c
		p[Fad-sa]	40	A	ml	Mylonas et al., 1997c
		EVAc	30–50	A	ov, sp	Mylonas et al., 1998b
		p[Fad-sa]	40	A	ov, sp	Mylonas et al., 1998b
Serranidae						
Epinephelus aeneus	White grouper	EVAc	25	A	ov, ml	Hassin et al., 1997
Epinephelus marginatus	Dusky grouper	EVAc	15–80	A	ov	Marino et al., 2000
Epinephelus striatus	Nassau grouper	Chol	50	A	ov	Watanabe et al., 1995
Siganidae						
Siganus fucescens	Rabbitfish	EVAc	25	A	ov, ml, sp	A. Elizur, personal communication
Siganus guttatus	Rabbitfish	Chol	5500	Nal	sp	Harvey et al., 1985
Sparidae						
Acanthopagrus schlegeli	Black porgy	p[LGA]	120	T	vg, sr	Chang et al., 1995
Dentex dentex	Common dentex	EVAc	25–130	A	ov	Greenwood et al., 2001
Pagrus major	Red seabream	Met	70	A	vg, sp	Matsuyama et al., 1993
		Met	70	A	sr	Matsuyama et al., 1993
		Chol	210	A	vg, sp	Matsuyama et al., 1995a
		Chol	180	A	sr	Matsuyama et al., 1993
		Osmo	100	A	vg, sp	Matsuyama et al., 1993
		Osmo	100	A	sr	Matsuyama et al., 1993
Pagrus pagrus	Red porgy	p[Fad-sa]	20	A	sp	Canario et al., 1997
		p[Fad-sa]	40	A	sp	Zohar and Mylonas, 2001
Sparus aurata	Gilthead seabream	EVAc	230	A, R	sp	Zohar et al., 1990b
		p[LGA]	230	T	sp	Zohar et al., 1990b
		p[LGA]	40	L	sp	Barbaro et al., 1997
		p[Fad-sa]	100	A	sp	Unpublished[3]
Various perciform families						
Acanthogobius flavimanus	Goby	Fa	7500	M	ov	Aida et al., 1978
Cheilodactylus spectabilis	Banded morwong	Chol	100–200	A	ov	Ritar, 1999
Glaucosoma hebraicum	Australian dhufish	Chol	5–125	A	sp	Pironet and Neira, 1998
		"	30–110	A	ml	Pironet and Neira, 1998
Latris lineata	Striped trumpeter	Chol	1000	A	vg, ov	Morehead et al., 1998
		Chol	100–200	A	ov	Ritar, 1999
Lutjanus argentimaculatus	Mangrove jack	EVAc	25	A	ov, ml, sp	A. Elizur, personal communication
Trachinotus carolinus	Florida pompano	EVAc	50	A	sp	Hicks, 1998
Umbrina cirrosa	Shi drum	p[Fad-sa]	40	A	sp	Mylonas et al., 1999

Table 1. Continued

Species (by family)	Common name	Delivery[1] system	Dose μg kg^{-1}	GnRH[2]	Effect	Reference
Non Perciformes						
Clupeidae						
Alosa sapidissima	American shad	EVAc	80	A	sp	Mylonas et al., 1995b
		EVAc	120	A	ml	Mylonas et al., 1995b
Clupea harengus pallasi	Pacific herring	Chol	900	A	ov, ml	Carolsfeld et al., 1988
Paralichthyidae						
Paralichthys dentatus	Summer flounder	Chol	100	A	ov	Berlinsky et al., 1997
		Chol	100	A	sp	Watanabe et al., 1998b
		p[Fad-sa]	50	A	ov	Unpublished[3]
		EVAc	50	A	ov	Unpublished[3]
Paralichthys lethostigma	Southern flounder	Chol	200	A	ov	Berlinsky et al., 1996
Pleuronectidae						
Hippoglossus hippoglossus	Atlantic halibut	EVAc	25	A	sfl	Vermeirssen et al., 2000
		EVAc	20	A	ov	Mazorra de Quero et al., 2000a
		EVAc	25	A	sfl	Mazorra de Quero et al., 2000b
Limanda yokohamae	Japanese plaice	Fa	1000	M	ov	Aida et al., 1978
Pleuronectes platessa	North Sea plaice	p[Fad-sa]	15–45	A	ml, sfl	Vermeirssen et al., 1998
		Met	80	A	ov	Scott et al., 1999
		Coco	80	A	ov	Scott et al., 1999
Pleuronectes ferrugineus	Yellowtail flounder	Chol	225	A	ov	Larsson et al., 1997
		p[Fad-sa]	75	A	ov	Larsson et al., 1997
		Chol	45–200	A	ov	Clearwater and Crim, 1998
		p[Fad-sa]	30	A	ov	Clearwater and Crim, 1998
Pseudopleuronectes americanus	Winter flounder	Chol	80–240	A	ov, sp	Harmin and Crim, 1992
		Chol	200	A	vg, sr	Harmin et al., 1995
Rhombosolea tapirina	Greenback flounder	Chol	100	A	ov	Poortenaar and Pankhurst, 2000
Scophthalmidae						
Scophthalmus maximus	Turbot	EVAc	25	A	ov	Mugnier et al., 2000
Salmonidae						
Salmo salar	Atlantic salmon	Chol	1700	T	sr	Crim et al., 1983a
		"	1700	Nal	ml	Crim et al., 1983a
		"	700	T	vg, ov	Crim et al., 1983a
		Chol	270	Nal	sr, ml	Weil and Crim, 1983
		Sil	1600	A	sr, ml	Weil and Crim, 1983
		Chol	30–50	T	ov	Crim and Glebe, 1984
		EVAc	15	A	ml	Mylonas et al., 1993
		p[Fad-sa]	75	A	ov, ml	Mylonas et al., 1995a
		EVAc	15–35	A	ov, ml	Goren et al., 1995
		EVAc	5	A	ml	Zohar, 1996
		EVAc	145	A	sr	Henry et al., 1998
Salmo trutta	Brown trout	EVAc	125	A	ov	Goren et al., 1995
Salvelinus alpinus	Arctic charr	EVAc	40	A	ov	Haraldsson et al., 1993
		p[LGA]	20	T	ov	Gillet et al., 1996
Oncorhynchus kisutch	Coho salmon	EVAc	15–75	A	ov	Zohar et al., 1990b
		p[LGA]	75	A	ov	Mylonas et al., 1993
		p[Fad-sa]	75	A	ov	Mylonas et al., 1993
		EVAc	75	A	ov	Goren et al., 1995
Oncorhynchus mykiss	Rainbow trout	p[LGA]	10–50	T	ov	Breton et al., 1990
		Chol	25–125	T	ov	Crim et al., 1983b
Oncorhynchus nerka	Sockeye salmon	EVAc	400	A	migr	Sato et al., 1997
		p[LGA]	75	A	ov, sr	Unpublished[3]
Oncorhynchus tshawytscha	Chinook salmon	EVAc	35	A	ov	Solar et al., 1995
Various non-perciform families						
Anoplopoma fimbria	Sablefish	Chol	100	A	ov	Solar et al., 1992
Carassius auratus	Goldfish	Chol	125	T	ov	Sokolowska et al., 1984
Chanos chanos	Milkfish	Chol	90×	A[5]	vg, sr	Lee et al., 1986a
		Chol	40	A	sp	Lee et al., 1986c
		Osmo	70	A, R	sp	Marte et al., 1987
		Chol	30	A, R	sp	Marte et al., 1988

Table 1. Continued

Species (by family)	Common name	Delivery[1] system	Dose μg kg^{-1}	GnRH[2]	Effect	Reference
Ictalurus pricei yaqui	Catfish	EVAc	45–80	A	sp	Unpublished[3]
Mugil cephalus	Grey mullet	p[Fad-sa]	30	A[5]	vg	de Monbrison et al., 1997
		EVAc	35	A	sp	de Monbrison et al., 1997
Plecoglossus altivelis	Ayu	Met	2200	M	ov	Hirose et al., 1990
		"	1100	A	ov	Hirose et al., 1990
		Fa	3000	M	sr	Aida, 1983
		"	25000	M	vg, ov	Aida, 1983
Takifugu rubripes	Tiger puffer	Chol	400	A	vg	Chuda et al., 1997
		Osmo	700	A	ov	Matsuyama et al., 1997
		Chol	400	A	ov	Matsuyama et al., 1997
Tinca tinca	Common tench	EVAc	40	A	ml	Linhart et al., 1995

[1] Chol = cholesterol; Coco = coconut oil; EVAc = poly[ethylene-vinyl acetate]; p[Fad-sa] = poly[fatty acid dimer-sebasic acid]; p[LGA] = poly[lactide-glycolide]; Met = polymethacrylate; Osmo = osmotic pump; Sil = Silicone rubber; Fa = Freund's adjuvant.
[2] A = D-Ala^{6}Pro^{9}NEt-mGnRH; M = native MGnRH; B = native sbGnRH; Nal = [6-(D-2-Naphthylalanine)]-mGnRH; C = native CGnRH II; R = D-Arg^{6}Pro^{9}NEt-sGnRH; ET$_2$ = D-hArg (ET$_2$)^{6}Pro^{9}NEt-mGnRH; S = native sGnRH; L = D-Leu^{6}Pro^{9}NEt-mGnRH; T = D-Trp^6-mGnRH.
[3] Unpublished results of the authors and various collaborators.
[4] In combination with an hCG injection.
[5] In combination with a T-delivery system.

species with extremely dense and viscous milt (Clearwater and Crim, 1998; Vermeirssen et al., 1998; Mazorra de Quero et al., 2000b; Vermeirssen et al., 2000). Similar treatment of white bass with GnRHa-loaded p[Fad-sa] microspheres also induced a significant elevation in total expressible milt, which had similar sperm density and fertilizing capacity as milt from control fish (Figure 6). In the European seabass, whereas a single GnRHa injection increased milt production for 7 days, two types of GnRHa-delivery systems increased total milt volume for up to 35 days after treatment and prolonged the natural spawning period by at least 14 days (Sorbera et al., 1996). Sperm density was unaffected and sperm motility was equally high among all fish. In the yellowtail flounder, GnRHa-loaded cholesterol pellets and p[Fad-sa] microspheres induced significant elevations in milt volume for at least 29 d without a decrease in sperm density (Clearwater and Crim, 1998). Both the percentage of sperm activated and the swimming duration were significantly enhanced by the GnRHa treatments, while no negative effect was observed on fertilization and hatching % of the eggs inseminated. The latter is an important finding, demonstrating that sustained-treatment with GnRHa enhances the production of mature, viable spermatozoa with a good ability to fertilize eggs and produce normal progeny. Finally, in the Australian dhufish (*Glaucosoma hebraicum*, Glaucosomatidae), a GnRHa injection failed to induce spermiation, whereas a GnRHa-delivery system resulted in an increased production of milt of normal sperm density and high motility (Pironet and Neira, 1998) and in the protogynous hermaphrodite white grouper (*Epinephelus aeneus*, Serranidae), an EVAc implant induced spermiation for 6 months (Hassin et al., 1997).

GnRHa-delivery systems have been employed also for the enhancement of sperm production in salmonids. In fact, the potential of sustained-treatment with GnRHa was first evaluated in male Atlantic salmon (Crim et al., 1983a; Weil and Crim, 1983). Treatment with a cholesterol GnRHa pellet induced spermiation in 100% of the males, producing up to four-fold more milt than untreated fish for a period of at least 12 d (Weil and Crim, 1983). The amount of milt produced was similar to the one produced by fish given GnRHa injections every 2 d. In large scale commercial trials with EVAc implants, treatment of Atlantic salmon increased total expressible milt from 0.3 ml kg^{-1} to 1.2 and 4.8 ml kg^{-1}on days 15 and 18, respectively, after treatment, without reducing sperm density or motility (Goren et al., 1995). Finally, the same GnRHa-delivery system produced remarkable results in males of a genetically selected strain of Atlantic salmon which, although more than 17 kg in body weight, produced an abnormally low volume (2 ml) of expressible milt if left untreated. Ten days after implantation with a GnRHa, average milt production increased by 20-fold (Zohar, 1996).

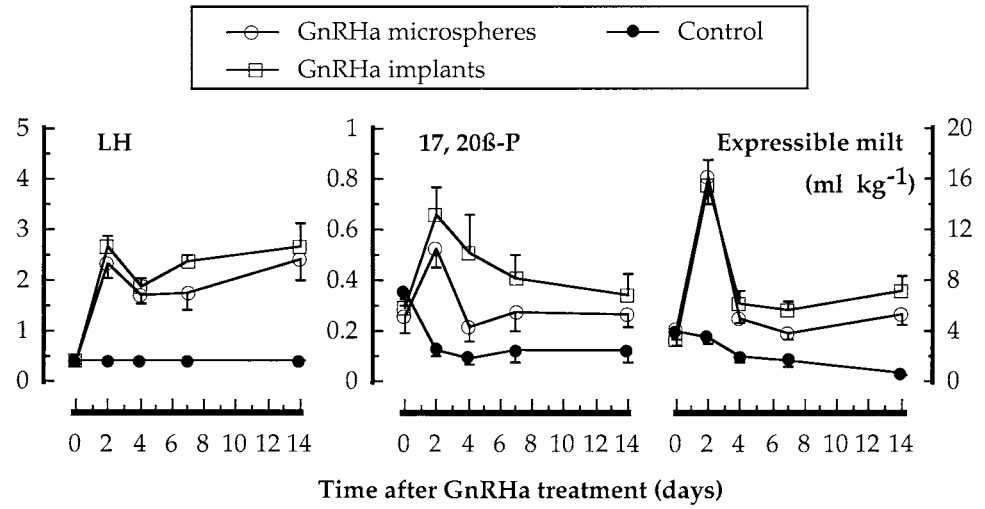

Figure 5. Mean (± s.e.m.) plasma hormone levels (ng ml^{-1}) and total expressible milt of cultured striped bass (n = 6) in response to treatment with a GnRHa-loaded EVAc implant (44 μg GnRHa kg^{-1}) or p[Fad-sa] microspheres (15 μg GnRHa kg^{-1}) during the spermiation period (May). All males (mean weight = 1.1 kg) were maintained in a 2.4-m^3 tank supplied with recirculated water at 18 °C. Both GnRHa-delivery systems induced significant increases (ANOVA, DNMR) in plasma LH ($P \leq 0.01$) and 17,20β-P ($P \leq 0.05$), and in total expressible milt ($P \leq 0.01$) during the experiment, compared to controls (modified from Mylonas et al., 1997c).

In some fish, however, GnRHa-delivery systems may be limited in their ability to increase spermatozoa and milt production, and are unable to induce spermiation, even if fish are treated during their natural spawning season. For example, in the common tench (*Tinca tinca*, Cyprinidae) treatment with an EVAc implant induced a two-fold increase in milt volume for 5 d, whereas a single GnRHa injection induced more than a three-fold increase (Linhart et al., 1995). In Atlantic halibut (*Hippoglossus hippoglossus*, Pleuronectidae), when males producing small milt volumes with low sperm motility were treated with an EVAc implant at the end of the spawning season, milt volume and sperm motility were enhanced slightly, but with a concomitant decrease in spermatocrit (Mazorra de Quero et al., 2000b; Vermeirssen et al., 2000). Similarly, in the North Sea plaice GnRHa-loaded p[Fad-sa] microspheres increased only seminal plasma production (Vermeirssen et al., 1998), and in the common dentex (*Dentex dentex*, Sparidae) induced only a decrease in spermatocrit from >90% to 60% (Greenwood et al., 2001). In a much worse situation, GnRHa-cholesterol pellets were ineffective in inducing or enhancing sperm production both in Southern and summer flounder (Berlinsky et al., 1996; Berlinsky et al., 1997). Although a consistent stimulation of spermiation did not result, GnRHa-delivery systems can still be of use to the commercial production of the above two species. For example, an increase in the fluidity of the milt enhances its rapid mixing with culture water in the case of tank-spawning, or with ovarian fluid and sperm diluents in the case of artificial insemination. The application of GnRHa-delivery systems for inducing spermiation warrants further investigation in these fish, in order to determine if a different mode of GnRHa application or higher doses are required for a successful enhancement of milt production.

Vitellogenesis, spermatogenesis and advancement of maturation

In addition to their success in inducing ovulation and sperm production, GnRHa-delivery systems have been demonstrated in limited situations to induce vitellogenesis and the early stages of spermatogenesis, thus advancing the onset of FOM and spermiation. The first such demonstration was made in the ayu, where mGnRH emulsified in Freund's adjuvant induced spermatogenesis and vitellogenesis in fish that were at the early stages of gonadogenesis and were maintained under photoperiodic conditions inhibiting to gonadal development (Aida, 1983; Shimizu, 1996). The pituitary gonadotrophs were hypertrophied and appeared to have enhanced synthetic and secretory activity (Aida, 1983). Later, treatment of winter

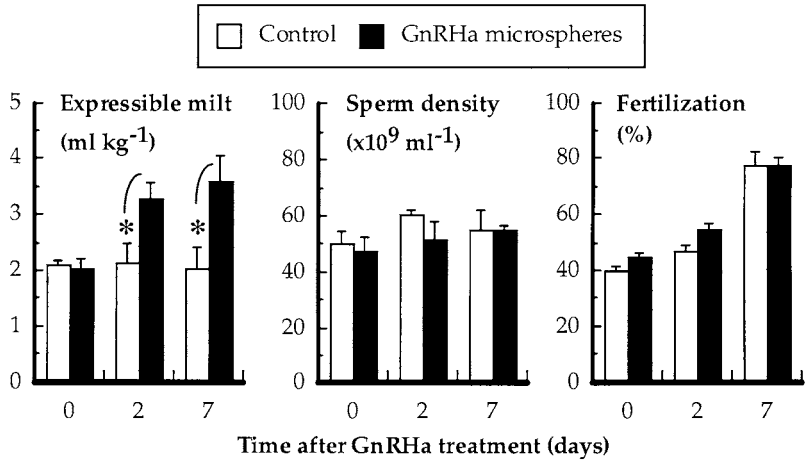

Figure 6. Mean (± s.e.m.) total expressible milt and sperm density (n = 6), and fertilization success (n = 4) of cultured white bass males treated with p[Fad-sa] GnRHa microspheres (25 μg GnRHa kg^{-1}) during the spermiation period (March). Males (mean weight = 0.65 kg) were maintained in 0.5-m^{3}tanks supplied with recirculated water at 18.5–20.5 °C. There were significant increases in expressible milt (ANOVA, $P \leq 0.05$) in response to the GnRHa treatment, whereas sperm density and fertilization % were unaffected (modified from Mylonas et al., 1997a).

flounder with GnRHa in cholesterol pellets during early vitellogenesis induced elevations in plasma T and E_2 levels, and increased oocyte diameter and gonadosomatic index (Harmin et al., 1995). Also, in the black porgy (*Acanthopagrus schlegeli*, Sparidae), a single treatment of p[LGA] microspheres induced spermatogenesis and advanced the onset of spermiation by 5 weeks, and resulted in significantly higher volumes of milt for at least 8 weeks (Chang et al., 1995). In the milkfish, multiple treatments with GnRHa in cholesterol pellets enhanced vitellogenesis and spermatogenesis, resulting in a one to two month advancement of the spawning season (Lee et al., 1986a). A much greater percentage of fish underwent advanced maturation when the GnRHa treatment was combined with a long-term 17α-methyltestosterone (MT) treatment. Similarly, in the grey mullet a combination of GnRHa and MT given in the form of microspheric delivery systems induced vitellogenesis to the stage that females could be induced to spawn with further hormonal manipulations, producing more than 2×10^6 eggs kg^{-1} (de Monbrison et al., 1997). Presumably, combined GnRHa/T treatments are more effective than GnRHa alone, because testosterone stimulates pituitary synthesis of LH, which is then released in response to the GnRHa treatment (Crim et al., 1988).

In a great many cases, however, treatment of fish early during gonadogenesis with GnRHa-delivery systems does not accelerate gonadal development and, therefore, such applications are unsuccessful in inducing FOM and spermiation. In studies with the ayu and the winter flounder (Aida, 1983; Harmin et al., 1995), GnRHa treatment of immature fish prior to the onset of spermatogenesis or vitellogenesis was completely ineffective. Also, in the case of the Pacific herring (*Clupea harengus pallasi*, Clupeidae) FOM, spermiation and spawning was effected in fish treated in February when their gonads were in the advanced stages of vitellogenesis and spermatogenesis (Carolsfeld et al., 1988). However, no advancement of gonadal development was observed when fish at the early stages of vitellogenesis and spermatogenesis were treated in September. Similarly, in the Southern flounder (Berlinsky et al., 1996) and summer flounder (Berlinsky et al., 1997) GnRHa-cholesterol pellets were ineffective in inducing FOM in females in early or mid-vitellogenesis, whereas daily injections of carp pituitary extract (CPE) induced oocyte growth and, subsequently, FOM. Finally, treatment of pubertal striped bass with GnRHa-delivery systems did not induce vitellogenesis, even in combination with T treatment (Holland et al., 1998b). All these results suggest that unlike their almost universal effectiveness in inducing FOM and spermiation, GnRHa-delivery systems are mostly ineffective in inducing vitellogenesis and early spermatogenesis. Furthermore, in the few species where they have been shown to enhance vitellogenesis and spermatogenesis, they do so only after these processes are already well under way. One

notable exception is the red seabream, where treatment of completely immature fish (ovaries at the perinucleolar oocyte stage and testes at the spermatogonial stage) 6 months prior to the reproductive season with GnRHa-delivery systems resulted in FOM, spermiation and spawning within 12–18 days (Matsuyama et al., 1993; Matsuyama et al., 1995a). The factors that contribute to the general failure of GnRHa to induce vitellogenesis and spermatogenesis in fish are probably multiple. Firstly, the pituitary may not be mature enough to respond to GnRHa and, therefore, GtHs are either not synthesized or not released (Crim and Evans, 1983; Breton et al., 1998). The effectiveness of co-treatment of GnRHa with T in inducing GtH synthesis/release and gonadogenesis in some fish (Lee et al., 1986b; de Monbrison et al., 1997; Henry et al., 1998) suggests that the pituitary must first be exposed to gonadal steroids before GnRHa can stimulate the release of GtH. Secondly, even though GtH may be released in response to GnRHa stimulation, the gonads may be unresponsive and steroidogenesis is not induced (Holland et al., 1998b). Thirdly, and most likely, hormones other than the GtHs may be involved in the initiation and progression of vitellogenesis and spermatogenesis, and these hormones may not be under the direct control of GnRHa. Further research is necessary to effectively employ GnRHa-delivery systems in applications regulating vitellogenesis and spermatogenesis in fish that fail to do so in culture.

Future directions for research

Controlled-release delivery systems for GnRHa have proven to be an important broodstock management tool, and have contributed to the species diversification of the aquaculture industry in the last decade. Large, commercial-scale application trials of GnRHa-delivery systems have been carried out in salmonids (Goren et al., 1995), but in most other species there is need for further development. One area of future research includes: (a) the establishment of minimum and optimal treatment doses for each species; and (b) the identification of the most appropriate GnRHa or mixture of GnRHas for inducing LH release and, thus, FOM, ovulation, spermiation and spawning. Reduction of the amount of GnRHa required to successfully induce spawning will decrease the cost of the treatment, and the avoidance of unnecessarily high concentrations of GnRHa may improve spawning success. In the barramundi, for example, GnRHa doses of >150 μg kg^{-1} in a cholesterol matrix induced less spawnings and resulted in the production of eggs of lower fertilization success (Garcia, 1989). It has also been shown in other fish that high doses of GnRHa may result in diminished egg quality, perhaps by inducing ovulation too rapidly (Billard et al., 1984; Crim and Glebe, 1984; Mylonas et al., 1992; Mugnier et al., 2000). GnRHa-delivery systems made with different agonists were similarly effective in the few cases examined (Marte et al., 1987; Almendras et al., 1988). There is enough literature, however, demonstrating that the LH-releasing potencies of different GnRHas vary significantly (Zohar, 1988; Crim and Bettles, 1997; Peter and Yu, 1997). The GnRHas used so far in spawning induction experiments were based on the mGnRH or sGnRH primary structure. Although both forms can be found in fish – mGnRH is common in ancient Actinopterygii, while sGnRH is very common in Teleostei – these are not necessarily the most relevant GnRH forms in all fish. This is particularly true in the highly evolved Perciformes, which is the vertebrate order with the largest number of species (Nelson, 1994). For example, recently it has been shown in gilthead seabream and striped bass that cGnRH II is much more potent in inducing LH release than the other two endogenous forms (Zohar et al., 1995a; Mylonas et al., 2000), while the most abundant form in the pituitary seems to be sbGnRH (Holland et al., 1998a; Holland et al., 2000b). Future delivery systems using synthetic agonists of these variants, or combinations of them (D. Alok and Y. Zohar, unpublished data), may prove more potent inducers of LH synthesis/release, and of FOM, spermiation and spawning.

Another area where research is needed relates to the optimal time of treatment with GnRHa-delivery systems, especially of female broodstock. As discussed earlier, because delivery systems release GnRHa for long periods of time, treatment does not have to be timed as precisely as when using simple GnRHa injections. In salmonids, for example, induction of ovulation using GnRHa-delivery systems can be initiated 3–6 weeks before the onset of the natural period, without any negative effects on the quality of the eggs (Goren et al., 1995). Such early treatment, however, may not be possible in other fish, and each case must be examined separately. It is a fact that compared to a single GnRHa injection, GnRHa-delivery systems can be used effectively in inducing FOM in females of lesser maturity, but there is obviously a limit to how early the fish can be

treated, without resulting in diminished % ovulation and lower egg quality (Crim and Glebe, 1984; Woods and Sullivan, 1993; Mylonas et al., 1996).

The success of the delivery systems in inducing complete FOM and ovulation may also depend on the release rate of GnRHa, and this is also an area where research must focus. For example, fast-releasing implants are effective in inducing ovulation only once in barramundi, but slow-releasing implants induce multiple spawnings (Almendras et al., 1988). In the European seabass, a GnRHa-delivery system which produces an initial burst of GnRHa followed by a secondary elevation induces two spawns of good quality eggs, while a delivery system that produces constantly elevated plasma GnRHa also produces multiple spawns, but of lower quality (Forniés et al., 2000). On the other hand, multiple GnRHa injections given after each subsequent ovulation induce multiple spawnings of good quality eggs (S. Hassin and Y. Zohar, unpublished). The latter results point to the need for pulsatile release of GnRHa for European seabass, as well as some other species. Such GnRHa release from the delivery system perhaps better mimics the endogenous GnRHa and LH release profiles controlling multiple spawning in this fish (Asturiano et al., 2000), similar to what has been shown in other multiple spawners (Gothilf et al., 1997). Polymer-based delivery systems that produce pulsatile release have been developed for human applications and were proposed for use in vaccinations, in order to eliminate the need for secondary booster treatments (Heller et al., 1992). Such polymers may potentially be used to design delivery systems that release GnRHa in pulses, according to the reproductive endocrinology of the fish.

To apply all the delivery systems described in this manuscript, broodfish must be captured, anaesthetized and then treated intramuscularly or intraperitoneally. Handling of broodstock is a time- and labor-intensive operation, especially when dealing with large species kept in outdoor ponds, offshore cages or large tanks. During the reproductive period, fish become more susceptible to stress and infectious diseases (Pankhurst and Van Der Kraak, 1997; Schreck et al., 2001), and there is an increased risk of post-handling mortalities due to disease or injury. Moreover, the handling can negatively influence the spawning success of the fish and the quality of the gametes. Compared to multiple injections, sustained-release GnRHa-delivery systems reduce the necessary handling to a minimum, but there are situations where handling of the fish must be avoided entirely. In such instances, oral delivery of GnRHa may offer an alternative treatment method, and some efforts have be made in the last decade to examine this possibility (Solar et al., 1990; Thomas and Boyd, 1989; McLean et al., 1991; Breton et al., 1995; Sukumasavin et al., 1992; Roelants et al., 2000). Such methods can find important applications throughout the aquaculture industry, but may prove indispensable for the future cultivation and domestication of large pelagic species like the amberjacks and the tunas, which are maintained in large offshore cages and can be very sensitive to handling. To date, there is no oral GnRHa-delivery system available and much work is required to address issues such as: (a) determination of the appropriate time for treatment without relying on the examination of an ovarian biopsy; (b) delivery of the hormone to broodstock of species that cease feeding during the spawning period; (c) treatment of all fish in a tank population according to their body weight; and (d) bioavailability of the ingested GnRHa. Still, the GnRHa-delivery systems described in this manuscript offer an important "assisted reproduction" management tool and we expect them to be used by increasingly more commercial hatcheries in the coming years.

Acknowledgments

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