CHAPTER 15

PROMOTING OOCYTE MATURATION, OVULATION AND SPAWNING IN FARMED FISH

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1. REPRODUCTIVE DYSFUNCTIONS OF FISH

Aquaculture, especially of marine species, is quite a new agricultural activity in relation to domestic animal production. With the exception of carp culture (family Cyprinidae) in Asia which started many centuries ago, and rainbow trout (*Oncorhynchus mykiss*) farming in Europe and North America which was

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commercialized in the last century, aquaculture as we know it is being practiced for only a few decades (Kirk, 1987). As a result, it is doubtful that a "domestic" fish species exists today, at least according to the interpretation of the word in terrestrial animal husbandry. In addition, even carp and rainbow trout, which are considered highly domesticated, do not reproduce readily in captivity.

In order to establish aquaculture as a successful and efficient agricultural activity, there is a need to control reproductive processes in fish, in order to obtain high-quality seed (i.e. eggs and sperm) and produce juveniles for grow-out without the need to obtain them from the wild. Surprising as it may appear, the aquaculture industry of species such as the freshwater eels (*Anguilla* spp.), the yellowtail and greater amberjack (*Seriola* spp.), groupers (*Epinephelus* spp.) and the bluefin tuna (*Thunnus thynnus*), is based almost exclusively on the collection of juveniles or adults from the wild (Ottolenghi et al., 2004). In some fish species, it is sometimes possible to control reproduction by manipulating environmental parameters, such as photoperiod, water temperature, tank depth and/or volume, spawning substrate, etc. Even then, the existence of the artificial environment with the associated human presence is by default an inhibiting factor on reproduction, whereas it is often impractical or even impossible in some fishes to simulate the environmental parameters accompanying reproductive maturation in the wild (i.e. spawning migration, depth, riverine hydraulics, etc.). Therefore, hormonal therapies have been employed in the past decades in order to control reproduction in cultured fishes and induce or synchronize oocyte maturation (OM), ovulation, and spawning. In some species, hormonal manipulations are the only way to produce fertilized eggs, whereas in other fishes exogenous hormones are used only as a management tool to enhance the efficiency of egg production and facilitate hatchery operations.

For example, in salmonids, which require insemination *in vitro* for the production of fertilized eggs, OM, and ovulation are often induced with hormones. This is to synchronize egg collection and fry production, thereby minimizing handling and stress to the fish, and reducing labor requirements (Goren et al., 1995; Haffray et al., 2005). In Pacific salmon (*Oncorhynchus* spp.), hormonal therapies can also advance ovulation by a few weeks (Goren et al., 1995), thus reducing losses due to pre-spawning mortality (Slater et al., 1995). Hormonal therapies in fishes are also employed for the collection of gametes for interspecific hybridization and chromosome set manipulation. Finally, genetic selection also requires hormonal therapies to enable proper maturation and timely collection of gametes for artificial fertilization. Therefore, hormonal therapies have an important role in broodstock management, and will continue to be a necessary tool even after fish become properly "domesticated" and reproduce spontaneously in captivity.

1.1. Failure of Vitellogenesis, Oocyte Maturation, and Ovulation

Although there is great variation among fishes, it is true that all cultured species today exhibit some degree of reproductive dysfunction, necessitating routine or occasional hormonal therapies to induce OM, ovulation, and spawning (Zohar

and Mylonas, 2001), whereas at times it is also necessary to induce gametogenesis (Miura et al., 1991a; Ohta et al., 1997). The most easy to address reproductive problem in females is observed in salmonids (*Onchorhynchus* and *Salmo* spp.). For example, whereas fish do undergo vitellogenesis, OM, and ovulation, they fail to spawn their eggs when reared in captivity (Bromage and Cumaranatunga, 1988; Zohar, 1989), presumably because they are not offered the gravel substrate necessary to build a nest for depositing their eggs (Liley and Kroon, 1995). Failure of spawning poses management problems in the hatchery, because ovulation is not synchronized among females, and it takes 6–10 weeks for all females in a broodstock to undergo ovulation. To obtain the eggs, all nonovulated females are checked manually for ovulation two or three times a week during the spawning season. Such handling is laborious and can result in stress, injury, disease, and often high mortalities, especially in Pacific and Atlantic salmon kept at sea (Slater et al., 1995).

The most common reproductive dysfunction of captive fishes is the unpredictable occurrence or failure of OM, and hence ovulation or spawning. Some examples of fishes exhibiting this type of dysfunction include various flatfishes (Berlinsky et al., 1996; Berlinsky et al., 1997; Larsson et al., 1997; Mugnier et al., 2000), members of the Serranidae family (Tucker, 1994; Watanabe et al., 1995; Watanabe et al., 1998b), the striped bass (*Morone saxatilis*) and white bass (*M. chrysops*) (Mylonas and Zohar, 2001a), the fugu (*Takifugu* spp.) (Yang and Chen, 2004; Chen, 2005), the shi drum (*Umbrina cirrosa*) (Barbaro et al., 2002; Mylonas et al., 2004c), the dusky grouper (*Epinephelus marginatus*) (Marino et al., 2003), and the bluefin tuna (Mylonas et al., 2005), to name a few. Fishes exhibiting this type of dysfunction undergo vitellogenesis, but with the onset of the spawning season the oocytes fail to undergo OM. This type of reproductive dysfunction may often diminish over the years, after many generations of fish are produced and reared in culture conditions.

The final and most severe form of reproductive dysfunction is the failure to undergo or complete vitellogenesis, which is exemplified by the freshwater eels, as well as by most captive populations of greater amberjack (*Seriola dumerili*) in the Mediterranean (Garcia et al., 2000) and the Mekong River catfish (*Pangasius bocourti*) (Donaldson, 1996). In the Japanese eel (*Anguilla japonica*), the current approach to the artificial manipulation of vitellogenesis involves weekly treatments with gonadotropin (GtH) preparations for a period of 7–17 weeks (Ohta et al., 1997). OM and ovulation is induced by a combination treatment of pituitary extracts (which contain GtHs) and the maturation inducing steroid 17, 20β-dixydroxy-progesterone (see Chapter 11), and fertilization is done *in vitro* after manual stripping of the eggs.

1.2. Endocrine Causes of Reproductive Dysfunctions

The lack of vitellogenesis, OM, and ovulation or spawning in cultured fishes is presumably due to the absence of the appropriate environmental stimuli, and the existence of stressors imposed by captivity (Schreck et al., 2001). For example,

many of the commercially important cultured fishes migrate hundreds of kilometers to reach the environmental niches where conditions are optimal for the survival of their offspring. During this migration or with the arrival at the spawning grounds, the fish may experience significant environmental changes – e.g. water salinity or chemistry, temperature, depth, or substrate. In addition, the maintenance of fish in small tanks due to space and cost considerations, in combination with the existence of unnatural stimuli (e.g. mechanical sounds and human presence) may have negative effects on the reproductive function of fish. As a result, captive broodstocks often become arrested in advanced stages of vitellogenesis, followed by follicular atresia (Zohar, 1989).

The first suggestions as to the endocrine nature of the failure of fish to undergo OM and ovulation came from the finding that pituitaries of reproductively mature fish contained a factor able to induce ovulation when injected to another mature individual (Houssay, 1930). Presumably, the GtH present in the pituitary of reproductively mature fish acted on the gonads of the recipient fish and induced OM and ovulation. These results suggested that perhaps the reason reproductively mature fish do not undergo OM, ovulation, and spawning in captivity is due to a failure of the pituitary GtH stores to be released in the circulation. Later studies in cultured gilthead sea bream (*Sparus aurata*) demonstrated that levels of luteinizing hormone (LH) in the pituitary increased during vitellogenesis and peaked with the approach of the spawning season (Zohar et al., 1988, 1995a). However, plasma levels of LH in most females remained undetectable and oocytes underwent atresia. On the other hand, in females that spawned spontaneously, OM and ovulation were preceded by a distinct surge of LH in the plasma. These data further indicated that in fish failing to ovulate, LH was produced and accumulated in the pituitary, but was not released to the bloodstream in order to trigger OM and ovulation.

More recent and conclusive evidence of the effect of captivity on the brain–pituitary–gonad axis came from a series of studies in striped bass. In these studies, the levels of various reproductive hormones were compared between wild fish captured on their spawning grounds and cultured fish during the spawning season (Mylonas et al., 1997b, 1998; Steven, 2000; Steven et al., 2000; Mylonas and Zohar, 2001a). In wild females, a plasma LH surge accompanied OM and ovulation, whereas plasma LH levels in females reared in captivity remained low and unchanged at the completion of vitellogenesis. However, levels of LH and its mRNA in the pituitary did not differ between wild and captive females (Steven, 2000), demonstrating again that LH was synthesized and stored in the pituitary during vitellogenesis, but not released into the circulation of captive fish. In addition, mRNA levels of the pituitary receptor for the major LH-releasing hormone, gonadotropin-releasing hormone (GnRH), were similar between wild and captive females. This further suggests that the disruption in LH release from the pituitaries of captive fish is not due to a dysfunction in pituitary responsiveness, but may originate in the hypothalamic control of pituitary function. In fact, differences were observed between wild and

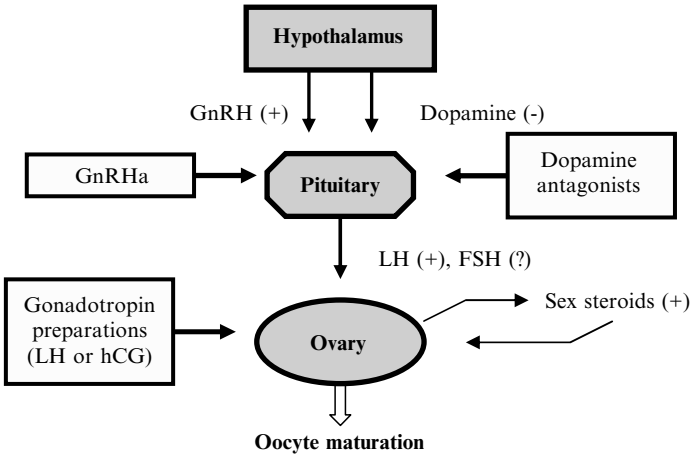

Figure 1. Schematic representation of the brain–pituitary–gonad axis and its endocrine control, along with the sites of intervention using hormonal therapies for the induction of oocyte maturation. (*See Color Plates*).

captive females undergoing OM, when comparing the pituitary content of the endogenous GnRH peptides. The GnRH mRNA levels within the brain, however, were similar between the two groups, indicating that the altered pituitary content of GnRH in captive fish may be a result of altered release from the hypothalamus, rather than deficient synthesis (Steven, 2000; Steven et al., 2000). Considered together, the above results strongly suggest that the failure of many cultured fish to undergo OM, ovulation and spawning in captivity is the result of a lack of LH release from the pituitary to the circulation, most probably due to captivity-induced altered release of the relevant and necessary GnRH(s) from the hypothalamus. This is the reason manipulations of OM, ovulation, and spawning in cultured fishes utilize either GtH preparations that act directly at the level of the gonad, or GnRH agonists (GnRHa) that act at the level of the pituitary to induce release of the endogenous LH stores (Figure 1). Endogenous LH, in turn, acts at the level of the gonad to induce steroidogenesis and the process of OM, ovulation, and spawning.

2. HORMONAL THERAPIES

Hormonal therapies to induce OM, ovulation, and spawning have been employed in fish hatcheries well before our understanding of the hormonal failure in the reproductive axis. The first applications employed the method of "hypophysation," by which ground pituitaries or pituitary extracts (containing LH) obtained from broodfish during the spawning season were injected into reproductively mature individuals of the same or different species (Von Ihering, 1937; Migita et al., 1952;

Ball, 1954). With the advent of protein isolation techniques, purified LH of piscine origin became available (Donaldson, 1973; Yaron, 1995), together with human chorionic gonadotropin (hCG) which has a high degree of structure homology with LH (Ludwig et al., 2002). In the last two decades, after the discovery of GnRH (Schally, 1978) and the synthesis of highly active agonists of GnRH (Crim and Bettles, 1997), spawning induction therapies shifted from the use of GtHs. This was partly due to the fact that by acting at a higher level of the brain–pituitary–gonad axis and stimulating the release of the fish's own LH, GnRHas may provide for a more integraded stimulation of reproductive processes and other physiological functions. Today, both GnRHas and GtHs are used extensively in spawning induction therapies, GtHs having the advantage that their effectiveness does not rely on the existence of an active or responsive pituitary.

2.1. Gonadotropin (GTH) Preparations

Hypophysation as a method to induce OM, ovulation, and spawning is still employed in freshwater aquaculture (i.e. Cyprinidae), especially in developing countries or remote areas where access to expensive purified hormones is limited. This is because pituitaries from mature fish may be readily available on-site or from local slaughterhouses, whereas purified GtHs or synthetic GnRHas are less widespread and relatively expensive (Thalathiah et al., 1988). Of more worldwide use are commercial preparations of carp pituitary extract (CPE) and purified salmon GtH (Ohta et al., 1997; Brzuska, 2004). For hypophysation, a ratio of one pituitary from a donor fish for a recipient fish of similar weight has been used for males, whereas the ratio is 1.5:1 in the case of females (Fontenele, 1955). Treatment is usually split into a small priming dose (10–20% of total) and a larger resolving dose given 12–24 h apart. Effective doses range from 2 to 10 mg of pituitary per kilogram body weight of the recipient fish (Thalathiah et al., 1988; Parauka et al., 1991; Kucharczyk et al., 1997; Chen, 2005). The major drawbacks of the use of hypophysation are (a) the potential for transmission of diseases from donor to recipient fish and (b) the variation of LH content in donor pituitaries. The latter may vary according to body weight, sex, and age of donor fish, the time of year the pituitaries were collected, and the period of time elapsed from the death of the fish to the collection and preservation of the pituitary (Yaron, 1995). Purified preparations of salmon and carp LH of standardized potency have also been available for some time (Donaldson, 1973; Yaron, 1995). Similar to hypophysation, treatment with purified LH is done in two steps, utilizing a priming and resolving dose, whereas effective doses may range between 3 and 5 mg kg^{-1} (Kucharczyk et al., 1997; Ohta et al., 1997; Brzuska, 2004; Leonardo et al., 2004). Due to species specificity of fish LHs, the commercially available preparations are usually limited to phylogenetically related fish species.

Although the problems of biosecurity and dose variation were solved by the purification of LH from fish pituitaries, availability remained restricted and the cost very high. On the other hand, hCG has been available in clinical grade, standardized preparations throughout the world. Consequently, hCG has been employed in spawning induction trials of many species in culture. Unlike GtH preparations of piscine origin, hCG is often given in a single dose, ranging between 100 and 4,000 international units (IU) kg^{-1}. The effectiveness of hCG after a single treatment is probably due to this GtH's relatively long residence time in circulation (Ohta and Tanaka, 1997). This is not related to the fact that it is a heterologous hormone, since it has been shown to have a significantly longer half-life compared to the GtHs of pituitary origin – i.e. follicle stimulating hormone (FSH) and LH – both in fish (Fontaine et al., 1984) and humans (Ludwig et al., 2002). Characteristic of the long half-life of hCG in circulation and its prolonged effect on gonadal maturation is the induction of spermatogenesis and spermiation of Japanese eel after a single injection (Miura et al., 1991a, b). However, this hormone is not as effective in inducing vitellogenesis even after multiple treatments, whereas salmon GtH is more effective (Ohta et al., 1997; Ijiri et al., 2003). After years of trials, an hCG preparation has been recently approved for commercial utilization in spawning induction therapies in fish (CHORULON, Intervet International bv, The Netherlands).

One of the main advantages of the use of GtH preparations in hormonal therapies is that they act directly at the level of the gonad and can be effective even if pituitary LH stores are low, or the pituitary gonadotrophs are not responsive to GnRHa. In such situations, GnRHa may not be effective at all or may require a long time to elicit a response. In the case of using gravid wild fish for the production of seed, or the transport of cultured broodstock from outdoor ponds/cages to indoor hatchery facilities, a long period between treatment and spawning may result in pre-spawning mortalities, due to stress induced by capture and transportation. Under these circumstances, piscine LH or hCG may be more appropriate in inducing OM, ovulation, and spawning (Hodson and Sullivan, 1993; García et al., 2001).

2.2. Gonadotropin-Releasing Hormone Agonists (GnRHa)

The use of GnRHas for spawning induction therapies has important advantages over the use of GtH preparations. For example, GnRHas are synthesized and obtained in pure form and, therefore, do not pose a disease transmission threat. Also, the use of GnRHas is generic due to the structural similarity of native GnRHs among fishes (Lethimonier et al., 2004). The most important advantage, however, is that by acting at a higher level of the brain–pituitary–gonad axis, GnRHas may provide for a more intergraded stimulation of reproductive processes by directly or indirectly stimulating the release of other hormones involved in OM, such as growth hormone (Le Gac et al., 1993), insulin-like growth factors (Negatu et al., 1998), prolactin (Weber et al., 1995), and thyroid hormones (Cyr and Eales, 1996).

Agonists of GnRH instead of the native peptides are employed in hormonal therapies, because native GnRHs are degraded quickly in circulation by endopeptidases located in the pituitary, liver, and kidney (Zohar et al., 1990). Substitutions of the GnRH decapeptide at position 6 with a dextrorotatory (D) amino acid and at position 10 with an ethylamide group (Figure 2), produce superactive agonists which are resistant to enzymatic degradation (Goren et al., 1990; Weil et al., 1992; Ulloa-Aguirre and Timossi, 2000), thus being cleared from circulation much slower than the native GnRHs (Gothilf and Zohar, 1991; Forniés et al., 2003; Haffray et al., 2005). As a result, GnRHas remain in the circulation much longer and stimulate a stronger release of LH from the pituitary. Furthermore, due to their modified polarity and tertiary structure, some of these GnRHas also exhibit increased binding affinity to the pituitary GnRH receptors (De Leeuw et al., 1988; Habibi et al., 1989; Pagelson and Zohar, 1992). Increased resistance to enzymatic cleavage and higher receptor binding affinity results in GnRHas which are 30–100 times more potent than the native GnRHs in inducing LH release (Peter et al., 1988; Zohar et al., 1989; Crim and Bettles, 1997). Recently, the GnRHa Azagly-nafarelin (GONAZON, Intervet International bv, The Netherlands) has been approved for use in hormonal therapies in aquaculture fish, and its efficacy has been so far documented in salmonids (Haffray et al., 2005), finally making GnRHa officially available to the aquaculture industry.

2.3. Other Pharmacological Therapies

Primarily in freshwater fishes, dopamine antagonists (DA) have often been used in combination with GnRHas in hormonal therapies (Peter et al., 1993). In these species, dopamine inhibits the basal release of LH and reduces or inhibits

	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 2. Amino acid sequences of common native gonadotropin-releasing hormone (GnRH) variants in fish, and two synthetic agonists used for hormonal therapies in fish. 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 (ET) substitution at position 10.

GnRH-induced LH release (Peter and Yu, 1997). Administration of DA (e.g. domperidone, pimozide, reserpine, or metoclopramide) prior to the injection of GnRHa removes the inhibition on the gonadotrophs and enhances the stimulatory effect of GnRHa on LH release. In salmonids, catfishes, and cyprinids (Saligaut et al., 1999; Silverstein et al., 1999; Yaron et al., 2003) the inhibiting role of dopamine is well documented, but with the exception of the mullets (*Mugil* spp.) (Glubokov et al., 1994; Aizen et al., 2005), it appears to be absent in most commercially important marine fishes (Copeland and Thomas, 1989; King et al., 1994; Zohar et al., 1995a; Prat et al., 2001; Kumakura et al., 2003b). Currently, hormonal manipulations of reproduction using a combined GnRHa/DA treatment are used mostly in cyprinids (Yaron, 1995; Mikolajczyk et al., 2003; Mikolajczyk et al., 2004), catfishes (Silverstein et al., 1999; Brzuska, 2001; Wen and Lin, 2004), and mullets (Glubokov et al., 1994; Aizen et al., 2005).

In search of the optimal hormonal therapy for the induction of spawning, some researchers have also examined the efficacy of combinations of GtHs together with GnRHas, though the results do not seem to be better compared to treatments using only one of the hormones (Wen and Lin, 2004).

2.4. Tailoring the Therapy: Acute Injections vs. Sustained-Release Delivery

The use of GtHs and, especially, GnRHas in aquaculture has indeed revolutionized broodstock management, as predicted almost three decades ago (E.M. Donaldson, FAO World Conference on Aquaculture, Kyoto, Japan, 1976; unpublished), and with the recent approval of commercial hCG and GnRHa preparations for use in the aquaculture industry, it is sure that hormonal therapies will become established as practical and often indispensable tools for hatchery managers. Administration of these hormones has been done mostly via injections in saline, but it was recognized even from the first hypophysation experiments that administration of a hormone would be more efficacious in the form of a sustained-release treatment (Fontenele, 1955; Aida et al., 1978). Also, some work has been carried out examining the potential of oral delivery of GnRHa (Thomas and Boyd, 1989; Solar et al., 1990; McLean et al., 1991; Sukumasavin et al., 1992; Schep et al., 1999; Roelants et al., 2000; Mikolajczyk et al., 2002), but this method has not progressed adequately. On the contrary, more effort has been invested in the past two decades in the development and application of injectable or implantable sustained-release delivery systems for GnRHas, for the control of reproductive processes in cultured fishes (see reviews by Zohar, 1996; Mylonas and Zohar, 2001b). Much less work has been done in developing GtH-delivery systems, partly due to the high cost and species specificity of highly purified piscine LHs (Sato et al., 1995; Sato et al., 1997; Zohar and Mylonas, 2001).

The interest in developing GnRHa-delivery systems stems from both the reproductive physiology of fish and the need to optimize broodstock management practices. For example, although GnRHas resist enzymatic degradation in

the blood circulation compared to the native peptides, their maximum half-life does not exceed 23 min *in vivo* (Gothilf and Zohar, 1991). Plasma GnRHa levels after a single injection are elevated for a period of a few hours to a few days, depending on the specific GnRHa, initial dose, fish species, and water temperature (Crim et al., 1988; Harmin and Crim, 1993; Zohar et al., 1995a; Mylonas and Zohar, 2001b). The brief residence time of GnRHa in circulation is probably the reason why a single injection of GnRHa does not always result in 100% OM and ovulation (Mikolajczyk et al., 2003; Kaminski et al., 2004), and in species with asynchronous ovarian development and daily spawning rhythms, long-term ovulation is effected in only a small percentage of the broodstock (Zohar et al., 1995a; Zohar and Mylonas, 2001). Therefore, GnRHa-based hormonal therapies for many fishes involve multiple injections of GnRHa, given over the course of hours or days (Pankhurst et al., 1986; Mylonas et al., 1992; Dabrowski et al., 1994; Slater et al., 1995). Also, in species like the European sea bass (*Dicentrarchus labrax*), the barramundi (*Lates calcarifer*), and the silver perch (*Bidyanus bidyanus*), which have a multiple-batch group-synchronous ovarian development, one or two GnRHa injections were necessary to induce ovulation of the first batch of oocytes (Almendras et al., 1988; Carrillo et al., 1995; Levavi-Sivan et al., 2004), while further ovulations could only be obtained with additional injections (Mylonas et al., 2003). Repetitive handling of broodstock requires substantial labor, time, and monitoring. Especially in situations where the broodfish are kept outdoors – in ponds or cages, it is very time-consuming and labor intensive to crowd, capture, anaesthetize, and inject the fish with hormones. Furthermore, repetitive handling is stressful to the fish and can often result in injury, disease, and prespawning mortalities, or at the very least it can adversely affect the progression of OM (Pankhurst and Van Der Kraak, 1997).

The striped bass, a species which completes vitellogenesis but rarely undergoes spontaneous OM in captivity (Mylonas and Zohar, 2001a), exemplifies the ineffectiveness of even multiple GnRHa injections to induce ovulation. Treatment of post-vitellogenic females under ambient water temperature (7–18°C) with two GnRHa injections resulted in elevations of plasma GnRHa for at least 7 days (Figure 3). The resulting increases in plasma LH, 17β-estradiol (E_2), and testosterone (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 17, 20β, 21-trixydroxy-progesterone – the maturation inducing steroid, and although 60% of the females initiated the early stages of OM (germinal vesicle migration) (Mylonas et al., 1997a), none progressed further than the peripheral germinal vesicle stage. As a result, no ovulation was observed.

Obviously, more than two consecutive GnRHa injections are necessary to support a slow OM in striped bass. Sustained-release GnRHa-delivery systems were used to provide the long-term elevation of GnRHa, required to stimulate the necessary changes in the profile of all reproductive hormones

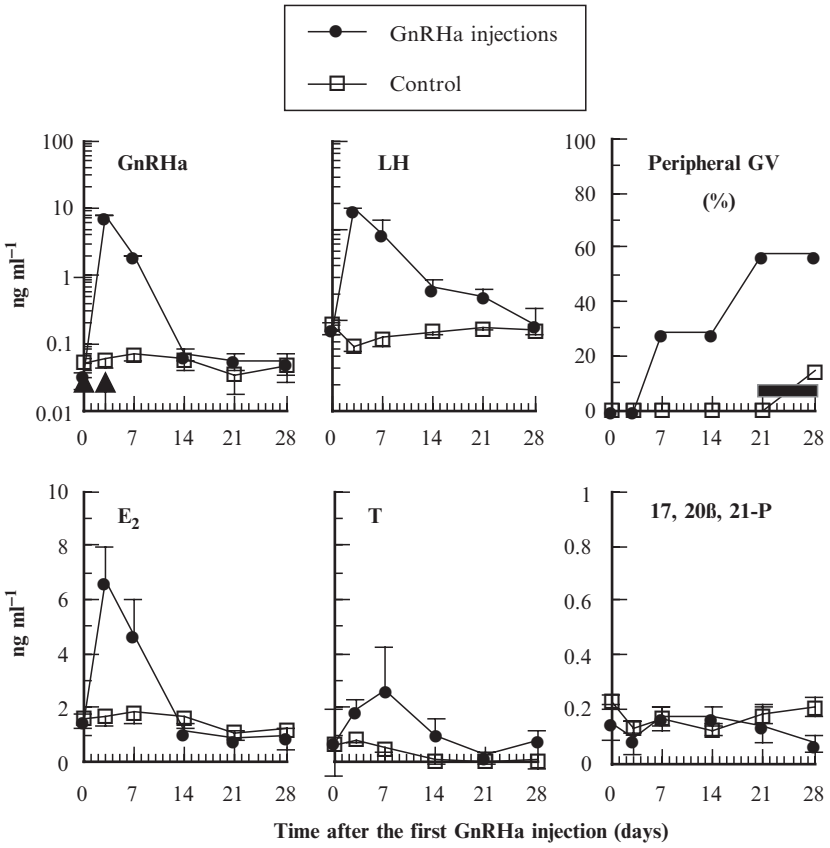

Figure 3. Mean (±SEM) plasma hormone levels of cultured striped bass ($n = 7$) and cumulative percentage of females having oocytes at the peripheral germinal vesicle (GV) stage after two injections (arrows) of GnRHa (15 μg kg^{-1}) during the spawning season (April). With the exception of the 17, 20β, 21-trixydroxy-progesterone, all plasma hormone profiles (LH, E_2 and T) were significantly changed by the GnRHa treatment (ANOVA, $P \leq 0.05$). The horizontal bar indicates the time when oocytes in atresia were observed. None of the females in the study completed OM and no ovulation was observed. (From Mylonas and Zohar, 2001b.)

for the appropriate duration, in order to result in complete OM and successful ovulation (Figure 4).

In mammals, synthesis and release of LH is regulated by the frequency and amplitude of the GnRH pulses released into the hypothalamus–pituitary portal system (Gharib et al., 1990). In fish, it has not been possible to document the natural mode and rate of GnRH release, since unlike all other vertebrates most fishes do not possess 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 in rainbow trout

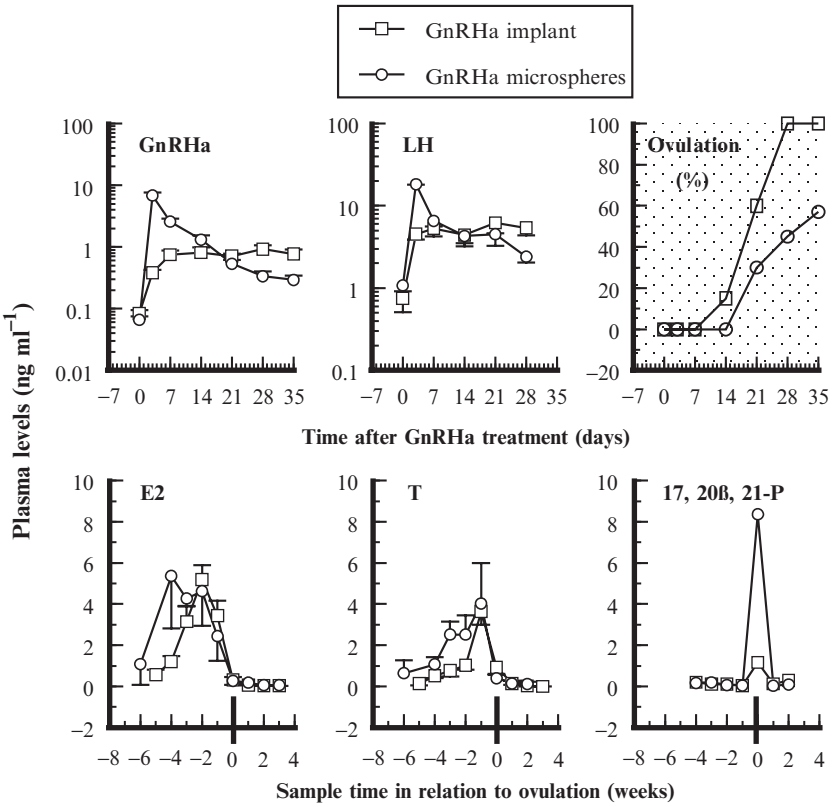

Figure 4. Mean (±SEM) plasma hormone levels and cumulative ovulation percentage of cultured striped bass (n = 7) after treatment with different GnRHa-delivery systems (15–20 µg GnRHa kg^{-1}) during the spawning season (April). Plasma data for the steroid hormones (n = 1–7) were plotted vs. the sampling time when the fish were found to have already ovulated (Ov). Changes in plasma 17, 20β, 21-trixydroxy-progesterone were observed in two females only, which happened to be sampled 12 h before ovulation. (From Mylonas and Zohar, 2001b.)

(Zohar et al., 1986) suggests that a pulsatile GnRH release may also be functional in some fishes. However, contrary to the situation in mammals (Ulloa-Aguirre and Timossi, 2000), treatment with GnRHa-delivery systems in all fish species tested so far does not result in pituitary desensitization and inhibition of LH release, but a sustained elevation of LH for many days or weeks (Weil and Crim, 1983; Sokolowska et al., 1984; Breton et al., 1990; Mylonas et al., 1997c, 1998). 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.

In some fishes, nevertheless, GnRHa injections in single or multiple applications, may be an equally appropriate or even better approach compared to GnRHa-delivery systems. For example, in the group-synchronous multiple-spawning shi drum, although GnRHa-delivery systems induced ovulation or spawning in 95% of the treated females compared to 69% of females given a single injection (Barbaro et al., 2002), multiple spawnings were not induced (Mylonas et al., 2000; Barbaro et al., 2002). Multiple cycles of OM, ovulation, and spawning in this species could be induced only with multiple GnRHa injections (Mylonas et al., 2004c). In the also group-synchronous multiple-spawning European sea bass, although different types of GnRHa-delivery systems induced multiple spawns, spawning was not synchronous among females and egg quality was low compared to other studies (Forniés et al., 2001), suggesting that constantly high plasma LH levels may not induce the appropriate maturational events for multiple spawning of viable eggs. On the other hand, multiple injections spaced 7–14 days apart induced up to four consecutive spawns (Figure 5), producing eggs of high fecundity, as well as percentage fertilization, hatching, and larval survival (Mylonas et al., 2003). So, the advantage of

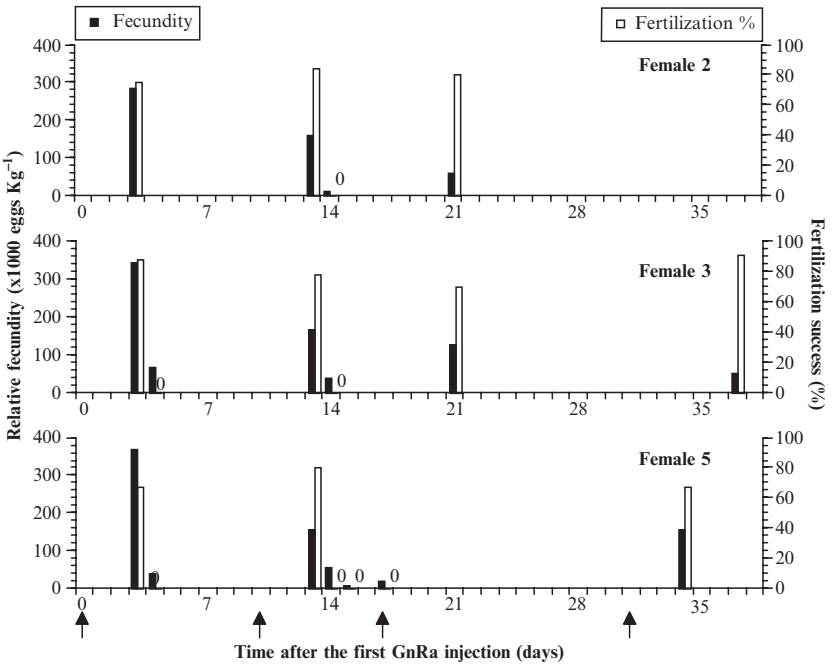

Figure 5. Relative fecundity and fertilization success of individual European sea bass spawns after treatment with multiple injections (arrows) of GnRHa (10 μg kg^{-1}). The zeros (0) refer to fertilization percentages of residual eggs released the days immediately after the major spawning events. (From Mylonas et al., 2003.)

GnRHa-delivery systems in this species is only the increased reliability in inducing OM of the most advanced batch of post-vitellogenic oocytes, but not the stimulation of multiple spawnings.

It is unclear, why the European sea bass and shi drum do not respond as well to sustained GnRHa treatment compared to other fishes with multiple-batch group-synchronous or asynchronous patterns of OM (reviewed by Mylonas and Zohar, 2001b). One explanation may be the greater interval between spawning events observed in these species. For example, the ovulation/spawning interval is 1 day for the white bass (Mylonas et al., 1997c), barramundi (Almendras et al., 1988), summer flounder (*Paralichthys dentatus*) (Berlinsky et al., 1997), and the gilthead sea bream (Barbaro et al., 1997); 2 days for turbot (*Scophthalmus maximus*) (Mugnier et al., 2000) and the dusky grouper (*E. marginatus*) (Marino et al., 2003); and 2–4 days for the striped trumpeter (*Latris lineata*) (Morehead et al., 1998) and American shad (*Alosa sapidissima*) (Mylonas et al., 1995). In the case of the European sea bass and shi drum, the spawning interval appears to be about 7 days (Asturiano et al., 2002; Mylonas et al., 2003, 2004c). Completion of vitellogenesis and recruitment of subsequent batches of oocytes to undergo OM between spawning events may require a period of relatively low plasma LH in these species, while OM and ovulation requires acute increases in plasma LH, as described in other fishes (Peter and Yu, 1997).

3. APPLICATIONS OF HORMONAL THERAPIES

The hormonal therapies employed in female broodfish can be categorized according to the reproductive process that they attempt to induce or facilitate, as well as according to the mode of ovarian development of the fish. Reproduction is separated into the stages of oocyte proliferation and growth (i.e. vitellogenesis) (see Chapters 1 and 2), and OM and ovulation (see Chapters 11 and 12). Ovulation/spawning can occur in a single or multiple times. The stage of reproduction to be induced, as well as the mode of ovulation and spawning may influence the choice of hormonal therapy.

3.1. Vitellogenesis

Hormonal therapies for the stimulation of vitellogenesis are relatively rare, especially using GnRHa, since most fishes undergo vitellogenesis in captivity. Also, the effectiveness of GnRHa is usually limited to individuals well undergoing vitellogenesis; therefore, the process may be enhanced but not initiated. For example, gonadal development was not stimulated in the Pacific herring (*Clupea harengus pallasi*) when fish were treated with GnRHa-delivery systems in September during early vitellogenesis (Carolsfeld et al., 1988). In the ayu (*Plecoglossus altivelis*) (Aida, 1983) and winter flounder (*Pleuronectes americanus*) (Harmin et al., 1995), GnRHa treatment of immature fish prior to the onset of vitellogenesis was ineffective in inducing gonadal development. Finally,

treatment of pubertal striped bass with GnRHa-delivery systems did not induce vitellogenesis (Holland et al., 1998a). On the other hand, GnRH emulsified in Freund's adjuvant induced vitellogenesis in the ayu, in individuals that were at the early stages of oogenesis and were maintained under photoperiodic conditions inhibiting to gonadal development (Aida, 1983). Also, treatment of winter flounder with a GnRHa-delivery system during vitellogenesis induced elevations in plasma T and E_2 levels, and increased oocyte diameter and gonadosomatic index (Harmin et al., 1995). Finally, in the milkfish (*Chanos chanos*), multiple treatments with GnRHa-delivery systems enhanced vitellogenesis and advanced spawning up to 2 months (Lee et al., 1986a), and in the grey mullet (*Mugil cephalus*) a combination of sustained-release delivery systems for both GnRHa and T enhanced vitellogenesis to the stage that females could be induced to spawn (de Monbrison et al., 1997). In the latter species, it was also recently shown that use of a DA alone or together with a sustained-release GnRHa treatment was effective in enhancing vitellogenesis to the point that females could be induced to spawn with another hormonal therapy (Aizen et al., 2005).

The only exception to the almost universal failure of GnRHa in inducing (i.e. initiating and promoting) vitellogenesis is the red sea bream (*Pagrus major*), where treatment of sexually mature, but reproductively inactive females (Matsuyama et al., 1995; Gen et al., 2001), as well as sexually immature, prepubertal females (Kumakura et al., 2003b) with a GnRHa-delivery system induced vitellogenesis, followed by OM, ovulation, and spawning within 3 weeks. These studies showed that a single treatment with a GnRHa-delivery system induces elevations in pituitary GnRH-receptor mRNA, FSHβ- and LHβ-mRNA, and plasma LH, E_2 and T, resulting in induction of vitellogenesis, as well as OM and ovulation.

Surprisingly, little is known today regarding the general inability of GnRHa treatments to induce vitellogenesis. This is related to the limited knowledge of the endocrine function of the brain and pituitary during this part of the reproductive cycle, compared to their better known function during OM and ovulation. Similar to other vertebrates, it has been well demonstrated in fish, that the pituitary synthesizes and releases both FSH and LH (see review by Yaron et al., 2003). In females, FSH is thought to be responsible for the regulation of vitellogenesis, while LH is responsible for OM and ovulation. Unfortunately, with the exception of salmonids, there are no assays available to measure FSH, due to difficulties in producing anti-FSH antibodies from the very small FSH amounts contained in fish pituitaries. As a result, almost all studies in non-salmonid fishes investigating the role of FSH during vitellogenesis are based only on measurements of FSHβ-mRNA levels in the pituitary, and not of the released protein in the blood.

Based on the available research, some speculation is warranted on the reason GnRHa treatments do not usually stimulate vitellogenesis, whereas they are quite successful in inducing OM, ovulation, and spawning (section 3.2). For example, GnRH may not be strongly involved in the regulation of this stage of

the reproductive cycle or, perhaps, FSH is not stimulated by the administration of GnRHa at this stage (Mateos et al., 2002; Kumakura et al., 2004). Also, LH release may be under a strong dopaminergic inhibition, thus preventing the response to exogenous GnRHa (Aizen et al., 2005). A very likely reason could also be that the pituitary may be unresponsive to the administered GnRHa at this stage, and as a result FSH and/or LH is not released (Crim and Evans, 1983; Breton et al., 1998). For example, in the Southern flounder (*P. lethostigma*) (Berlinsky et al., 1996) and summer flounder (Berlinsky et al., 1997), although GnRHa-delivery systems were ineffective in inducing OM in females in early or mid-vitellogenesis, daily injections of CPE induced vitellogenesis followed by OM. Synthesis and release of LH, as well as gametogenesis may be achieved in some fishes in response to GnRHa, after priming with T (Crim et al., 1988), or after co-treatment of GnRHa with T (Lee et al., 1986b; de Monbrison et al., 1997; Henry et al., 1998), suggesting that the pituitary must first be exposed to gonadal steroids before GnRHa can stimulate release of LH. Presumably, combined GnRHa and T treatments are more effective than GnRHa alone, because T stimulates pituitary synthesis of LH, which is then released in response to the GnRHa treatment (Crim et al., 1988). In striped bass, however, although GnRHa and T stimulated LH release, the gonads were unresponsive and vitellogenesis was not induced (Holland et al., 1998a). Finally, hormones other than FSH or LH may be involved in the initiation and progression of vitellogenesis, and these hormones may not be under the control of GnRHa.

GtH preparations have been much more successful in inducing vitellogenesis, the best example being that of the freshwater eels, which do not undergo any gonadal development in captivity. In the Japanese eel, for example, treatment with purified salmon pituitary extract (SPE) which contains LH, can induce complete gametogenesis (Ohta et al., 1997; Suetake et al., 2002; Saito et al., 2003). However, for the SPE treatment to be effective, the therapy must be given on a weekly basis over the course of many months. The same long-term therapy with SPE (11–29 weekly injections) was required for the induction of gametogenesis in the more recent attempts to control reproduction in the European eel (*A. anguilla*) (Pedersen, 2003, 2004). Similarly in the Mekong catfish (*P. bocourti*), 2–10 daily injections of 500 IU kg^{-1} hCG were necessary to stimulate oocyte growth to the point that a resolving treatment with two injections of hCG spaced 8–10 h could induce OM and ovulation (Cacot et al., 2002). Finally in the grey mullet, vitellogenesis was induced, but not completed, with pregnant mare's serum gonadotropin (PMSG) using three injections per week (Kuo, 1995). These procedures are obviously cumbersome, labor intensive, and potentially injurious to the fish, and a better approach using a GtH-delivery system has been investigated in order to reduce handling and treatment of the fish (Sato et al., 1996; Sato et al., 1997). Such a delivery system has been developed using an emulsion of lipophilized gelatin (LG), loaded with purified salmon LH (sLH) and was shown in the Japanese eel to produce a sustained elevation of plasma sLH for 24 days after treatment. Compared to immature females treated

weekly with sLH in saline, females given the LG emulsion attained a higher gonadosomatic index after 9 weeks and developed gonads with oocytes at the germinal vesicle migration stage (Sato et al., 1997).

A unique and very promising way to induce gametogenesis in the European eel through the elevation of the endogenous FSH and LH is currently being developed (van den Thillard and Spaink, unpublished data). The method utilizes genetically engineered eel cell lines, which contain the genes of eel LH and FSH, controlled by a constitutive promoter. Once implanted subcutaneously to immature eels, such cells are expected to produce continuously the two endogenous eel GtHs, thus stimulating gonadogenesis. Preliminary work has showed encouraging results.

3.2. Oocyte Maturation, Ovulation, and Spawning

The most common reproductive dysfunction in cultured fish is the failure of females to undergo OM, ovulation, and spawning after the completion of vitellogenesis. Hence, most of the applications of hormonal therapies have focused on this aspect of reproduction control. Three modes of ovarian development have been identified in fish: synchronous, group-synchronous, and asynchronous (Wallace and Selman, 1981; Tyler and Sumpter, 1996). Synchronous ovarian development is characteristic of semelparous species like the Pacific salmons and freshwater eels, which reproduce only once in their lifetime. Group-synchronous fish are further separated into single-batch and multiple-batch spawners. In these fishes, 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 with single-batch group-synchronous ovarian development undergo OM and ovulate only once (e.g. striped bass and rainbow trout). Species like the European sea bass, shi drum and greater amberjack have a multiple-batch group-synchronous ovarian development and undergo multiple ovulations within the course of a few weeks. Finally, species with asynchronous ovarian development, such as the gilthead sea bream (Zohar et al., 1995a), red porgy (*P. pagrus*) (Mylonas et al., 2004a), and red sea bream (Watanabe and Kiron, 1995) have a population of primary oocytes and a heterogeneous population of vitellogenic oocytes, from which several batches are recruited and undergo OM, ovulation and spawning during the annual spawning season, in daily or almost-daily intervals. For the purpose of hormonal therapy applications, fish are separated into two classifications: single-time spawners (synchronous and single-batch group-synchronous) and multiple spawners (multiple-batch group-synchronous and asynchronous).

3.2.1. Single-time spawners

As mentioned earlier, GtH preparations are still widely used for the manipulation of reproduction in cultured fishes. Specific information on doses and treatment

protocols using LH of piscine origin or hCG can be found in earlier extensive reviews (Donaldson, 1973; Lam, 1982; Donaldson and Hunter, 1983; Zohar and Mylonas, 2001). Since then, many more species or studies have been added to the list. For example in the European catfish (*Silurus glanis*), carp pituitary homogenate (4 mg kg^{-1}) was successful in inducing ovulation, though in a smaller percentage of females compared to a combined GnRHa/DA treatment (Brzuska, 2001). In the Japanese catfish (*S. asotus*), a single injection of hCG (10,000 IU kg^{-1}) was effective in inducing OM and ovulation in yearling females for an extended period of time from June to September (Kumakura et al., 2003a). In yet another catfish species, the Brazilian "cachara" (*Pseudoplatystoma fasciatum*), CPE and hCG were both effective in inducing ovulation, whereas a combined CPE/hCG treatment was ineffective (Leonardo et al., 2004). Also, a single injection of hCG at 1,000 or 2,000 IU kg^{-1} was effective in inducing ovulation with similar fertilization and hatching percentages in the Korean spotted sea bass (*Lateolabrax maculatus*) (Lee and Yang, 2002). Finally, in wild-caught ocellated puffer (*Takifugu ocellatus*), both single- and double-injections of extracted pituitaries (6 mg kg^{-1}) or hCG (2500 IU kg^{-1}) were 100% effective in inducing OM and ovulation, resulting in high fertilization success (Chen, 2005), while in photoperiod-manipulated pikeperch (*Sander lucioperca*), single or multiple injections of hCG (200 IU kg^{-1} per injection) were effective in inducing ovulation after 66–71 h (Zakes and Szczepkowski, 2004).

One of the first applications of GnRHa, either in the form of injections or sustained-release delivery systems, has been for the synchronization of ovulation in salmonids (Donaldson et al., 1981; Crim and Glebe, 1984; Breton et al., 1990). Traditionally, synchronization of ovulation using GnRHa injections (10–100 μg kg^{-1}) involved two injections spaced 3 days apart, given around 2 weeks before the expected date of maturation of the broodstock. If the treatment is given too early, a third injection may be required to successfully complete OM and ovulation in all females. Given at the expected date of maturation, a single GnRHa injection may also induce ovulation in 80–90% of the females within 2–4 weeks (Breton et al., 1990; Haraldsson et al., 1993; Pankhurst and Thomas, 1998; Haffray et al., 2005), whereas the two-injection protocol induces 100% ovulation (Van Der Kraak et al., 1985; Sullivan et al., 1989; Mylonas et al., 1992).

Single or multiple injections of GnRHa are also widely employed in other single-time spawning fishes. As in salmonids, when a two-injection protocol is used, GnRHa is given in a priming (5–10%) and resolving dose (95–90%), and if a DA is also employed it is given with the priming dose. For example, in the ocellated puffer both a single and double injections of GnRHa (50 μg kg^{-1}) were effective in inducing OM and ovulation, without any difference in egg fertilization success (Chen, 2005), while similar results were obtained using 2–4 injections of GnRHa (total of 40–300 μg kg^{-1}) in the obscure puffer (*T. obscurus*) (Yang and Chen, 2004) and the bullseye puffer (*Spoeroides annulatus*) (Duncan et al., 2003). In the grey mullet, two injections of GnRHa (30 μg kg^{-1}) spaced 22.5 h apart, along with the DA metoclopramide (15 mg kg^{-1}) were very effective in

inducing spawning within 24 h (Aizen et al., 2005). Similarly, two injections of GnRHa (20 μg kg^{-1}) spaced 6 or 12 h apart, together with the DA pimozide (5 mg kg^{-1}) induced ovulation in 95% of treated common carp (*Cyprinus carpio*) (Mikolajczyk et al., 2004). In the same fish, two injections of GnRHa (80 μg kg^{-1}) spaced 6 h apart, without a DA, induced ovulation of only 45% of treated females (Mikolajczyk et al., 2003). Two injections of GnRHa in combination with a DA have been used successfully also in the koi carp (*C. carpio*) (Arabaci et al., 2004), lake mullet (*Chalcalburnus tarichi*) (Arabaci and Sari, 2004), and wild catfish (*S. asorus*) (Wen and Lin, 2004). Finally, a single injection of GnRHa (20 μg kg^{-1}) induced ovulation in 1-year-old tench (*Tinca tinca*) (Rodríguez et al., 2004). GnRHas in the form of injections will continue to be used widely in aquaculture, partly due to their greater availability and lower cost compared to the GnRHa-delivery systems, which are not widely available commercially and/or are not yet approved for use in aquaculture applications.

The use of GnRHa-delivery systems begun in salmonids and has been extensive, both in research and commercial situations (under experimental licenses), as they offer considerable advantages compared to the multiple injection method. Similar to a well-timed two-injection protocol, a single application of a GnRHa-delivery system (10–50 μg kg^{-1}) may stimulate OM and ovulation of 100% of treated females within 2 weeks (Breton et al., 1990). More importantly, however, due to the sustained-release 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., 1983; Crim and Glebe, 1984; Goren et al., 1995). Advancement of ovulation by a few weeks is important in preventing pre-spawning mortalities in Pacific salmon, especially if fish are kept at sea.

The superiority of GnRH-delivery systems over the use of injections has been demonstrated also in various other species with single-batch group-synchronous ovarian development. For example, emulsified GnRH induced ovulation in Japanese plaice (*Limanda yokohamae*) and goby (*Acanthogobius flavimatus*), whereas the same dose given as an injection was ineffective (Aida et al., 1978). In the yaqui catfish (*Ictalurus pricei*), a GnRHa-delivery system was the only hormonal preparation able to induce spawning, whereas hCG, catfish pituitary extract, and combined sGnRHa/DA treatments were ineffective (Mylonas and Zohar, 2001b). In the tiger puffer (*T. rubripes*), GnRHa-delivery systems (400 μg kg^{-1}) induced ovulation in 18 and 10 days in fish with mean oocyte diameter of 800–900 μm and 900–1,000 μm, respectively (Matsuyama et al., 1997). This latter result underscores a very important advantage of GnRHa-delivery systems compared to injections, which is the flexibility in the timing of application. Whereas a single GnRHa injection would fail in females which have not fully completed vitellogenesis, a GnRHa-delivery system is able to induce OM and ovulation in fish of lesser maturity, albeit requiring a longer period of time. An extensive review of applications of GnRHa-delivery systems in inducing OM, ovulation, and spawning in single-batch group-synchronous species has been

published recently (Mylonas and Zohar, 2001b), and since then more species have been added to the list, including the bullseye puffer (Duncan et al., 2003), cobia (*Rachycentron canadum*) (Kilduff et al., 2002), devil stinger (*Inimicus japonicus*) (Takushima et al., 2003), and common carp (Brzuska and Bialowas, 2002).

3.2.2. Multiple spawners

As for single spawners, single or multiple injections of GnRHa are also employed in the induction of OM, ovulation, and spawning of multiple spawning fish. For example, it was already mentioned earlier (section 2.4) that multiple injections are more appropriate than delivery systems for the induction of multiple spawning in European sea bass (Mylonas et al., 2003), whereas GnRHa injections (Mylonas et al., 2004c) and GnRHa-delivery systems (Mylonas et al., 2000; Barbaro et al., 2002) were equally effective in the shi drum. In three other members of the Sciaenidae family, the red drum (*Sciaenops ocellatus*), spotted seatrout (*Cynoscion nebulosus*), and orangemouth corvina (*C. xanthulus*), a single injection of 100 μg GnRHa kg^{-1} induced OM, ovulation, and spawning within 30–35 h, whereas a second injection 3 weeks later induced further spawnings in the spotted seatrout (Thomas and Boyd, 1988). Also, a single injection of GnRHa was successful in inducing OM and tank spawning in the silver perch (Levavi-Sivan et al., 2004), while multiple GnRHa injections induced ovulation in the Reeves shad (*Tenualosa reevesii*) (Wang et al., 2003), though only a single spawning event was observed in both species.

Sustained treatment with GnRHa has been used effectively in a variety of multiple-batch group-synchronous fish, which either do not undergo OM and ovulation in captivity or do so unpredictably. For example, GnRHa-delivery systems induced two consecutive spawns within 3 days in white bass (Mylonas et al., 1997c) and greater amberjack (Mylonas et al., 2004b), five spawns within 7 days in the barramundi (Almendras et al., 1988), five ovulations within 2 weeks in striped trumpeter (Morehead et al., 1998), one to four ovulations within 7 days in the black sea bass (*Centropristis striata*) (Watanabe et al., 2003), and seven ovulations within 10 days in the dusky grouper (Marino et al., 2003). Similar results have been also obtained in other fishes with a similar mode of ovarian development (see Mylonas and Zohar, 2001b; Shein et al., 2004; Berlinsky et al., 2005).

The greatest potential, however, of sustained-release GnRHa-delivery systems is in the induction of OM, ovulation, and spawning in multiple spawning females with a daily or almost daily ovulation/spawning frequency. For example, the red porgy, red sea bream, and gilthead sea bream (Sparidae family) have an asynchronous mode of ovarian development and are capable of undergoing OM and spawning on a 24 h cycle for periods up to 4 months (Watanabe and Kiron, 1995; Zohar et al., 1995a; Mylonas et al., 2004a). In the early days of the aqauculture of the gilthead sea bream, when spontaneous spawning occurred rarely, 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. On the contrary, >70% of the females given a GnRHa-delivery system continued spawning on a daily basis. Similar results have been obtained with the other two sparids (Matsuyama et al., 1995; Zohar and Mylonas, 2001). In this way, GnRHa-delivery systems result in a significant increase in the total number of eggs produced in a hatchery, by increasing the number of broodfish undergoing OM, and the number of ovulations per spawning season (Barbaro et al., 2002). Also, a GnRHa-delivery system has been examined recently in Atlantic bluefin tuna, a species posing a great challenge to the aquaculture industry (Ottolenghi et al., 2004). A GnRHa-delivery system (40–80 μg kg^{-1}) was administered underwater to untranquilized fish swimming in a rearing cage, and was shown to induce OM, ovulation, and spawning, with production of viable larvae both after spontaneous spawning and after *in vitro* fertilization (Mylonas et al., 2006).

Members from various flatfish families also present significant difficulties in undergoing OM in captivity, and application of GnRHa-delivery systems has proven very successful in inducing multiple spawnings, often of improved quality compared to the few naturally spawning females. For example, in captive-reared Southern flounder a GnRHa-delivery system induced up to three ovulations in all females with mean oocyte diameter of >500 μm (Berlinsky et al., 1996). In wild-caught summer flounder, the same treatment induced daily ovulations for 8 days (Berlinsky et al., 1997), whereas in fish maintained for more than a year in captivity, the same treatment induced not only ovulation, but also tank spawning (Watanabe et al., 1998a). Similarly in turbot, treatment with a GnRHa-delivery system induced multiple ovulations in 100% of treated fish compared to 50% of controls, and it reduced the interovulation period (Mugnier et al., 2000). Also, in the yellowtail flounder, two different GnRHa-delivery systems induced an average of eight consecutive ovulations, compared to three in control fish, resulting in the production of twice as many eggs and of higher fertilization and hatching percentage than control females (Larsson et al., 1997). The same two GnRHa-delivery systems have also induced daily spawnings for up to 2 weeks in the Senegal sole (*Solea senegalensis*), though with very limited fertilization success (Agulleiro et al., 2006; Howell et al., 2006). Finally, GnRHa-delivery systems were shown to stimulate daily ovulations in the greenback flounder (*Rhombosolea tapirina*) (Poortenaar and Pankhurst, 2000).

The use of GtH preparations for the induction of multiple spawning in fish is somewhat limited, compared to single spawning species. Nevertheless, in the greenback flounder a single injection of hCG produced daily ovulations, in a similar fashion to a GnRHa-delivery system (Poortenaar and Pankhurst, 2000). In the whitemouth croaker (*Micropogonias furnieri*) a single injection of hCG was effective in inducing the first ovulation within 2 days after treatment, but it was not reported if the females continued ovulating (Berois et al., 2004). Similarly, in chub mackerel (*Scomber japonicus*) a single injection of hCG induced ovulation 36 h later, but since the fish were sacrificed for histological evaluations it was not known if more cycles of OM and ovulation could have

been induced by the treatment (Shiraishi et al., 2005). Also in the yellowtail (*S. quinqueradiata*), both a single and a double-injection protocol of hCG was effective in inducing spawning, though only for a single time (Chuda et al., 2001; Chuda et al., 2002). Finally, in the tropical catfish (*Heterobranchus longfilis*), a single injection of hCG induced 100% ovulation and the same treatment could be repeated successfully after 6–8 weeks (Nguenga et al., 2004).

4. EGG QUALITY

Egg quality is an important parameter for commercial fish hatcheries, as it can limit the number, as well as the quality of fry produced from a broostock. However, our knowledge of either the intrinsic factors or the environmental parameters influencing egg quality is limited (Kjørsvik et al., 1990; Bromage, 1995; Brooks et al., 1997). Recent studies have made significant advances towards this direction (Lahnsteiner, 2000; Nocillado et al., 2000; Lahnsteiner et al., 2001; Lahnsteiner and Patarnello, 2003; Shin et al., 2003). Still, the criteria for the assessment of good quality eggs used in commercial hatcheries are limited to egg buoyancy (Lahnsteiner and Patarnello, 2003), number of oil droplets and blastomere morhphology (Shields et al., 1997), or even simply fertilization and hatching success (Mylonas et al., 1992). The purpose of this section is not to review the current knowledge of the factors that determine egg quality in general, but rather to point out some considerations one should have in mind when employing hormonal therapies to induce OM, ovulation, and spawning.

In the case of species which do not spawn spontaneously after ovulation in captivity and fertilization is achieved artificially after stripping, it has been shown that the amount of time during which the eggs remain in the ovarian or abdominal cavity after ovulation and before stripping is directly related to loss of egg quality, a process referred to as "overripening" (Bromage et al., 1994). This period varies among fishes from many days in salmonids (Lahnsteiner, 2000); to <6 h in chub mackerel (Shiraishi et al., 2005), 4–6 h in Atlantic halibut (*Hippoglossus hippoglossus*) (Bromage et al., 1994), <3 h in snapper (*P. auratus*) (Hobby and Panhurst, 1997), 2 h in tiger puffer (Chuda et al., 1998), and 1 h in tilapia (*Oreochromis niloticus*) (Bromage et al., 1994); to only 30 min in the white bass (Mylonas et al., 1996). Therefore, in order to ensure high egg quality and fertilization success, the time of ovulation after hormonal therapy (i.e. latency period) must be predicted to a high degree of accuracy in most species (Chen, 2005), in order to limit the interval between ovulation and stripping (i.e. overripening). When hormonal therapies for the induction of OM and ovulation are tried for the first time in a species, an overripening period of 1–2 h may be considered a good starting point, subject to further evaluation. The latency period depends on both intrinsic (Wendling et al., 2000) and environmental parameters (Brzuska, 1999), some of which include species, water temperature (Yaron, 1995), type of hormone and dose (Wen and Lin, 2004), as well as the history of

fish in the preceding period (e.g. low vs. high temperatures) (Van Der Kraak and Pankhurst, 1996; Tveiten et al., 2001) and the stage of ovarian maturity at the time of the hormone treatment (Matsuyama et al., 1997). The stage of ovarian maturation is usually determined after obtaining an ovarian biopsy and (a) measuring the mean or maximum oocyte diameter (Garcia, 1989; Mylonas et al., 2004b; Shiraishi et al., 2005), (b) determining the position of the germinal vesicle (Lutes et al., 1987; Billard et al., 1995; Mylonas et al., 1995; Yaron, 1995), or (c) identifying the onset of coalescence of the lipid droplets (Mylonas et al., 1997a, 2003; Fauvel et al., 1999). A more sophisticate method for verifying that females have reached the appropriate developmental stage at which hormonal induction should be applied, has been developed recently in the striped bass on the basis of the *in vitro* OM after hormonal stimulation of biopsied oocytes from prospective females (Weber et al., 2000). Finally, the occurrence of >5–10% full-grown oocytes undergoing atresia, in some fishes it is an indication that a female may not respond successfully to a hormonal therapy (Cerdá et al., 1997). Specific guidelines must be developed for each particular species and hormonal therapy, with modifications made according to the specific stock and hatchery conditions.

One question often posed in regards to hormonal therapies is their effect on egg quality, compared to naturally ovulating or spawning broodfish (Slater et al., 1995). It is assumed that hormonal therapies are used only if a broodstock is not reproducing normally in captivity, or for management purposes, such as to increase synchronization of maturation, or to implement interspecific hybridization or genetic selection programs. In that respect, it makes little practical difference if the resulting egg quality is slightly, yet significant statistically, reduced compared to naturally spawning populations. Nevertheless, appropriate hormonal therapies do not usually have a negative effect on egg quality (Gillet et al., 1996; Barbaro et al., 1997; Mugnier et al., 2000; Duncan et al., 2003; Haffray et al., 2005), whereas at times they can even improve egg fecundity (Barbaro et al., 1997; Morehead et al., 1998; Mikolajczyk et al., 2004) and quality (Larsson et al., 1997; Mylonas et al., 2003) compared to spontaneously maturing populations.

Fertilization success may be sometimes reduced if very high doses of a hormone are used (Billard et al., 1984; Mylonas et al., 1992), though the mechanisms for such an effect are not clear. One explanation may be found in the slight asynchrony of the processes of OM and ovulation, and the effect an exogenous hormone stimulation may over-impose. Hormonal therapies induce OM via synthesis of the maturation inducing steroids, whereas ovulation is stimulated via synthesis of prostaglandins (see Chapter 12). One of the events that take place during OM is the resumption of meiosis, and its progression from the first meiotic prophase to the second meiotic anaphase at the end of OM, at which time the oocyte is considered fertilizable. It has been suggested that meiotic maturation may not be completed in synchrony with ovulation (Goetz, 1983; Nagahama, 1983). Therefore, some eggs may not be ready to be fertilized

immediately after ovulation, even if ovulation occurs naturally. Completion of meiotic maturation may take place soon after ovulation – depending on water temperature, while the eggs remain in the ovarian or abdominal cavity. This hypothesis is supported by experiments in salmonids, which reported a lower fertilization success of eggs stripped immediately after ovulation, compared to eggs striped a short time after ovulation (Hirose et al., 1977; Springate et al., 1984). Similarly, based on biochemical evaluations of gilthead sea bream eggs, it was recently suggested that some eggs do not reach full maturation prior to ovulation, which results in their failure to be fertilized (Lahnsteiner and Patarnello, 2003). It is possible that due to a very strong, rapid and unnatural stimulation provided by an excessive dose of a hormonal therapy, the processes of OM and ovulation become greatly out of phase, resulting in the ovulation of unfertilizable eggs, which can not complete meiotic or biochemical maturation in the ovarian or abdominal cavity. It is important, therefore, to establish empirically the appropriate hormone dose in each species of interest.

Although at times differences in egg quality between hormonal treatments have been reported in the same species, there is no established trend between GtH and GnRHa preparations, or between injection and sustained-release treatments. For example in the European catfish, though both treatments were effective in inducing ovulation, fish treated with a combination of GnRHa/DA produced eggs of higher fertilization and hatching success than those obtained from females treated with CPE (Brzuska, 2001). On the contrary in carp, although a double-injection therapy with CPE and a single injection of GnRHa were equally effective in inducing ovulation and producing fertilized eggs, 2-day larval survival was higher for the CPE treatment (Brzuska, 2004). Finally, in the ocellated puffer there were no differences in egg quality between females induced to ovulate with pituitary extract, hCG, or GnRHa in a single or double-injection protocol (Chen, 2005). Obviously, the effect of a hormonal treatment on egg quality may vary and it is difficult to make general recommendations in favor of one treatment over another based on the produced egg quality. Nevertheless, one criterion to chose one hormonal therapy over another would be the degree of synchronization of the response time to ovulation or spawning among different broodfish, both because greater synchrony – i.e. less variation – has significant management advantages for the hatchery, and because it may also indicate a physiologically more appropriate response of the females (Wen and Lin, 2004).

5. FUTURE DIRECTIONS

As described in this chapter, GnRHa-based spawning induction technologies have been successfully applied to numerous commercially important farmed species. It is also clear, that recent progress in understanding the GnRH system in farmed fish will lead to more efficient and cost-effective approaches.

The current GnRHa-based spawning induction therapies were developed before it was discovered that many commercially important Perciform fishes

(such as sea breams, freshwater and seawater basses, groupers, etc.) have three forms of GnRHs in their brain (Powell et al., 1994; Gothilf et al., 1995; Alok and Zohar, 2005). While two of these forms, salmon (s) and chicken (c) GnRH-II, were previously found in many species, including salmonids and cyprinids, the third form named sea bream (sb) GnRH was demonstrated to be a novel GnRH peptide present in more evolved fishes. Further studies led to the conclusion that sbGnRH is the principle endogenous LH releaser and the most physiologically relevant form to the completion of OM, ovulation, and spawning in gilthead sea bream and other perciform species (Gothilf et al., 1997; Holland et al., 1998b; Senthilkumaran et al., 1999; Rodríguez et al., 2000; Okuzawa et al., 2003). This suggests that in these species, sbGnRH is the GnRH form that should be targeted in future studies on the effects of confinement on the GnRH system and the failure to spawn in captivity. In addition, cGnRH-II has been shown to be the most potent LH releaser in gilthead sea bream and other fishes (Zohar et al., 1995a, b; Bosman et al., 2000). Since both the sbGnRH and cGnRH-II forms are present in the pituitary of fish undergoing final gonadal development (Holland et al., 1998b), and a peak in their synthesis is observed 8 h before ovulation in gilthead sea bream (Gothilf et al., 1997), a combined administration of sbGnRH and cGnRH II agonists should also be considered for spawning induction. The recent cloning and studies of the pituitary GnRH receptors in a number of farmed fishes (Alok et al., 2000; Lethimonier et al., 2004) will help in identifying the most potent GnRHa combination(s) and in determining their doses and mode of application to optimize physiologically tailored GnRHa administration, and in turn achieve optimal ovulation and spawning (Alok and Zohar, 2005).

As described in section 1.1.2, it is clear that the failure of captive fish to undergo OM, ovulation, and spawning is the result of the lack of LH secretion from the pituitary. Consequently, most spawning induction related research and development efforts have focused on the use of exogenous GnRHs to trigger the release of LH and the chain of events leading to final gonadal development. However, it is also evident that the endocrine system upstream from the pituitary is impaired in captive fish, which adversely affects the normal functioning of the endogenous GnRH system in captive broodstocks. The next generation of spawning induction technologies will thus be based on understanding the nature of the captivity-induced alterations in the GnRH system, and on developing strategies to correct them. Studies in the striped bass demonstrated that captivity results in altered secretion patterns of the relevant sbGnRH from the brain to its final destination in the pituitary (Steven et al., 2000). Understanding the mechanisms involved in regulating the synthesis and secretion of the relevant GnRHs will no doubt lead to the development of new strategies to simulate in captive broodstock the natural patterns of GnRH synthesis and release occurring in wild fish. The recent cloning and characterization of multiple GnRH genes and their cDNAs from a variety of fish species (Sherwood et al., 1997; Alok and Zohar, 2005) paves the way for a more complete elucidation of

the regulation of GnRH gene expression and synthesis, and for a better understanding of the effect of confinement on the function of the GnRH system. Moreover, the recent development of transgenic zebrafish overexpressing a GnRH promoter that drives a green fluorescent reporter gene (Abraham, Du, Knight, and Zohar, unpublished data) introduces a very powerful tool for unveiling the environmental and endocrine mechanisms involved in stimulating, inhibiting, or altering GnRH production. Those recently developed tools and emerging studies may lead in the future to simple strategies to manipulate the expression of specific GnRH genes and/or their release, thereby overcoming the adverse effect of confinement on the GnRH system and leading to successful completion of oogenesis, OM, and ovulation.

Finally, optimizing the success of spawning induction therapies and the quality of the eggs produced requires a better understanding of the recently discovered complex hormonal processes occurring inside the gonad. In addition to the well-documented patterns of gonadal steroidogenesis, a number of additional endocrine factors such as growth factors and catecholamines have also been shown to play a critical role in the acquisition of ovarian maturational competence (Patiño et al., 2001; Patiño and Sullivan, 2002; Vidal et al., 2004; Dufour et al., 2005). The recent discovery of the existence of a full GnRH–GtH–Steroid axis in the ovaries of the gilthead sea bream (Wong and Zohar, 2004) establishes a new paradigm in the endocrine regulation of reproduction in fish, which may have implications in the manipulation of ovulation and spawning in aquaculture. Studying the functional significance and patterns of the local gonadal endocrine system is important in determining the timing of spawning induction and in tailoring the GnRH-based induction therapy, in order to ensure optimal spawning success and quality of eggs.

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