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Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality

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ABSTRACT

This article reviews the use of hormonal treatments to enhance sperm production in aquaculture fish and the methods available for evaluating sperm quality. The different types of testis development are examined and a brief review is presented of the endocrine regulation of spermatogenesis in fishes, including the increasing evidence of the existence of spermatozoa subpopulations. Hormonal manipulations are employed to induce spermatogenesis in species such as the freshwater eels, to synchronize maximal sperm volume to ovulation for *in vitro* fertilization and to enhance sperm production in species with poor spermiation. The hormones that are employed include gonadotropins (GtHs) of piscine or mammalian origin, and gonadotropin-releasing hormone agonists (GnRHa) administered by injections or controlled-release delivery systems, with or without dopaminergic inhibitors. Pheromones in the culture water and hormones added to the sperm *in vitro* have also been employed to enhance spermiation and sperm quality, respectively, in some fishes. Hormonal therapies usually do not affect sperm quality parameters, except in cases where fish fail to spermiate naturally or produce very small volumes of high-density sperm. Different parameters have been used to evaluate fish sperm quality, including sperm volume and density, spermatozoa motility and morphometry, and seminal plasma composition. The development of Computer-Assisted Sperm Analysis (CASA) systems made possible the estimation of a higher number of sperm motion parameters using an objective, sensitive and accurate technique. The development of Assisted Sperm Morphology Analysis (ASMA) software has introduced a new approach for sperm evaluation studies, demonstrating changes in the spermatozoa related to reproductive season, hormonal treatments or the cryopreservation processes, and how these may be related to changes in sperm motility and fertilization capacity. The article concludes with a few practical protocols for the enhancement of sperm production in aquaculture species.

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1. Introduction

Production of high quality eggs and sperm is a prerequisite for the sustainable expansion of aquaculture. In captivity, control of reproductive function begins with the manipulation of the environment, in order to provide the necessary conditions and information - such as photoperiod and thermal cyclicity where they exist, and at times spawning substrate - in order to condition the fish and stimulate them to undergo gametogenesis (oogenesis and spermatogenesis), maturation and spawning. However, in many commercially produced species, there are important reproductive dysfunctions that hinder the efficient and reliable production of fertilized eggs (Mylonas et al., 2010). Reproductive dysfunctions are most often seen in females, with the failure of oocyte maturation, ovulation and/or spawning being the most common. As an exception to the above rule of females being the problematic sex in aquaculture, various flatfishes produce very small amounts of sperm (also referred to as semen or milt) during the spawning period (Agulleiro et al., 2007; Guzmán et al., 2011b; Vermeirssen et al., 1998, 2004). Furthermore, hatchery-produced males (F1 generation) of some fishes do not exhibit any breeding behavior and fail to spawn with the females, even if females complete ovulation and spawning. Such an example is the Senegalese sole (*Solea senegalensis*) (Norambuena et al., 2012). In many other fishes where males usually complete spermatogenesis and spermiation in captivity, it is often observed that the amount of good quality sperm produced may be diminished. Hormonal manipulations using a variety of exogenous hormones have been used in many fishes, in order to address the problems exhibited by male breeders. The objective of this article is to summarize the current knowledge on the reproductive function of male fish in aquaculture, broodstock management and methods to enhance spermatogenesis and sperm production. In addition, the article provides an extensive review of the available sperm quality evaluation methods.

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2. Spermatogenesis and spermiation

The process of gametogenesis in male fishes has been separated into three phases (Schulz et al., 2010). In phase one, type A spermatogonia proliferate and differentiate into early B spermatogonia, which then undergo multiple mitotic divisions – their total number being species-specific and genetically determined – that result in late B spermatogonia. In phase two, after their last mitotic multiplication, late B spermatogonia undergo meiotic division into spermatocytes I and then spermatocytes II, eventually becoming haploid spermatids. In phase three – referred to as spermiogenesis – the spermatids differentiate into flagellated spermatozoa and are released in the testicular lumen during spermiation (reviewed by Billard, 1986; Miura and Miura, 2001; Miura et al., 2002; Schulz and Miura, 2002; Schulz et al., 2010; Vizziano et al., 2008).

The spermatozoa are released from the spermatocysts into the sperm ducts where maturation - the process that renders them capable of vigorous motility and fertilization (Schulz et al., 2010) - takes place prior to sperm release, during the spawning season. Fish ejaculate sperm spontaneously during spawning, and with the exception of catfishes (Kazeto et al., 2008; Mansour et al., 2004; Viveiros et al., 2002), sperm can also be expressed easily from the sperm ducts after application of gentle abdominal pressure (referred to as "stripping"). Sperm release can be synchronized with female spawning *via* tactile or pheromonal communications (Stacey, 2003).

2.1. Different types of testis development

Testicular development, *i.e.* the changes in the testis structural morphology during the reproductive cycle, has been reviewed extensively (Billard, 1986; Mañanos et al., 2009; Schulz and Miura, 2002; Schulz et al., 2010). Testis development has many common features among fishes, as well as vertebrates in general, such as for example the progress through the different developmental stages of germ cells. The main differences in testis development among different species relate to: (a) the timing of the progress through the different developmental stages of the germ cells in relation to the seasonality of spawning of the fish species, (b) the testis structure and migration of the spermatocysts, and (c) the stage of development of the germ cells that leave the spermatocysts.

Different species of fish present different seasonality in spermatogenesis and spermiation, ranging from the production of sperm all-year-round, to the production of sperm for a short or long reproductive period each year or to sperm production once in a life-time. Spermatogenesis can be considered an asynchronous type of maturation, as most species present periods of development with all stages of germ cells. The different seasonality in spermatogenesis among species results in differences in the presence and abundance of the different developmental stages of germ cells observed among species. Guppies (Poecilidae) that produce sperm all-year-round were described to have an asynchronous testis all-year-round, which was also termed continuous, as sperm was produced continually (Billard, 1986). Rainbow trout (*Oncorhynchus mykiss*), on the other hand, has a seasonal production of spermatozoa in separate annual cycles of spermatogenesis to produce releasable sperm for a short reproductive period. In general, much of the annual spermatogenesis cycle in fish is asynchronous, with all stages of germ cell development present at the same time. Nevertheless, there is a clear progression through the different germ cell stages and at a given time of the reproductive cycle, the testis is dominated by a certain stage of germ cells. Towards the end of the spawning period the testis is almost entirely full of spermatozoa, with a limited presence of earlier stage germ cells. Therefore, germ cells appear to be synchronized to achieve sperm availability for a short fixed period (Billard, 1986, 1992).

The different structural types of testis (*e.g.* anastomosing tubular and lobular) have been ordered by phylogeny (Parenti and Grier, 2004) with clearly defined structural criteria, based on the tubule or lobule network and the position of spermatogonia and spermatocysts (Grier et al., 1980; Grier, 1993; Parenti and Grier, 2004). A tubule is defined as a tube with both ends open and connecting to other tubular structures, while a lobule was defined as essentially a tube with one end being blind, forming a lobe (Grier, 1993). When observed in a two-dimensional histological section, these tubules and lobules can appear similar, resulting in considerable confusion in the literature. The lobular structure of the testis has been further divided into restricted and unrestricted (Grier et al., 1980). Restricted lobular testes contain the different stages of spermatogonia and spermatocysts in different restricted areas. The spermatogonia are situated at the distal section (blind end) of the lobule, and away from the lumen (open end), while spermatocysts with spermatids are principally observed close to the lumen. As the germ cells undergo the different stages of spermatogenesis, the spermatocysts appear to move towards the lumen, so that the spermatozoa are released into the lumen when the spermatocysts rupture. Unrestricted lobular testes have spermatogonia and spermatocysts at different stages of development throughout the lobules, and spermatocysts may move only slightly towards the lumen prior to the release of the spermatozoa.

Important aquaculture species have both tubular and unrestricted lobular testis type. Tubular type testes are found in various fishes (Parenti and Grier, 2004), ranging from the primitive non-teleost order Acipenseriformes (which includes paddlefish and sturgeons) to early teleost orders such as Cypriniformes (carps), Siluriformes (catfishes), Salmoniformes (salmons and trouts) and Esociformes (pikes). Lobular testes are found in Neoteleostei, with unrestricted lobular testes present in the orders Perciformes and Mugiliformes. The different testis structures (tubular or unrestricted lobular testes) do not appear to be related to the amount of sperm that can be produced, as very different volumes of sperm can be collected from species from orders classified with the same testis structure. For example, sperm collection from rainbow trout (a Salmoniformes species with tubular type testes) (Billard, 1992) and European seabass (*Dicentrarchus labrax*, a Perciformes species with unrestricted lobular type testes) (Asturiano et al., 2001; Sorbera et al., 1996) is easy and large volumes can be collected. On the contrary, sperm collection from African catfish (*Clarias gariepinus*, a Siluriformes species with tubular type testes) (Viveiros et al., 2002) and spotted rose snapper (*Lutjanus guttatus*, a Perciformes species with unrestricted lobular type testes) (Ibarra-Castro and Duncan, 2007) is difficult and the amount of sperm collected is usually not sufficient for aquaculture purposes. In the case of catfishes, the problem with low volumes of sperm collected with stripping is related to the presence of seminal vesicles in the efferent duct (Viveiros et al., 2002).

Testicular development can also be classified as cystic or semicystic. In cystic development, when the spermatocysts rupture they release spermatozoa, while in semicystic development they release spermatids, which then differentiate into spermatozoa and complete development in the lumen. Cystic development is the most prevalent among fishes, but a growing number of species from various taxonomic orders and families have been identified to have semicystic development, including Perciformes–Bleniidae (Lahnsteiner and Patzner, 1990), Ophiliiformes-Ophiliidae (Mattei et al., 1993), Scorpaeniformes–Scorpaenidae (Muñoz et al., 2002) Pleuronectiformes–Soleidae (Garcia-Lopez et al., 2005), Siluriformes–Callichthyidae (Spadella et al., 2007), Syngnathiformes–Syngnathidae (Biagi et al., 2010) and Gymnotiformes–Gymnotidea (Vergílio et al., 2013). The most notable aquaculture species with semicystic development is Senegalese sole (Garcia-Lopez et al., 2005), which has a small testis (gonadosomatic index of <0.15%) and very low-volume sperm production all-year-round (<80 μl per fish) (Garcia-Lopez et al., 2005), which have frustrated aquaculture practices (Morais et al., 2014). However, it is unclear if low sperm production is an aspect associated to semicystic development, as sperm production in other species with semicystic development was either not quantified or the species were of a small size (at reproductive maturity) and small sperm volumes would be expected. Senegalese sole appear to have low sperm volume requirements for successful reproduction due to the paired spawning behavior, where the male and

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female spawn with genital ducts held closely together so that the released sperm is immediately next to the released eggs (Morais et al., 2014).

2.2. Endocrine control

Hormonal regulation of fish spermatogenesis and spermiation has been described previously (Billard et al., 1990; Miura and Miura, 2001; Miura et al., 2002; Peñaranda et al., 2010a; Schulz and Miura, 2002; Schulz et al., 2010; Vizziano et al., 2008; Watanabe and Onitake, 2008). Spermatogenesis and spermiation are regulated by the two pituitary gonadotropins (GtHs), namely Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), whose synthesis and release is controlled by hypothalamic hormones, the major one being gonadotropin-releasing hormone (GnRH). Kisspeptin is also a hormone involved in the onset of puberty and the stimulation of reproductive function, and it has been shown recently to influence both GnRH expression in the brain and FSH/LH in the pituitary, and kisspeptin antagonists reduced sperm volume in spermiating striped bass (*Morone saxatilis*) (Zmora et al., 2015). A dopamine inhibition of the stimulatory effect of GnRH exists in some fishes, mainly freshwater species (Peter et al., 1993). Recently a gonadotropin release inhibiting hormone (GnIH) has been identified also in fish, and was shown to be involved in the regulation of LH/FSH production, though both stimulatory and inhibitory effects have been documented (Biran et al., 2014; Moussavi et al., 2013; Tsutsui and Ubuka, 2014; Wang et al., 2015). The two GtHs regulate the function of the gonads through their action on the production of sex steroid hormones (androgens, estrogens and progestins), as well as other growth factors (Miura and Miura, 2001; Schulz et al., 2010). The FSH is mainly regulating the mitotic phase of spermatogenesis, while LH is mainly involved in the phase of spermiogenesis and spermiation (Schulz et al., 2010), though it has been recently shown that spermatogenesis and production of viable spermatozoa can take place in the complete absence of LH stimulation in zebrafish (*Danio rerio*) (Zhang et al., 2015). Before the onset of spermatogenesis, spermatogonial stem cell renewal seems to be regulated by 17β-estradiol (E_2) acting on Sertoli cells (Miura and Miura, 2003). The androgen 11 ketotestosterone (11-KT) is the major regulator of spermatogenesis and spermiogenesis, while 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and, in some species, 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P or 20β-S) function as the maturation inducing steroid (MIS) and regulate spermiation and sperm maturation (Miura and Miura, 2003), at which stage the spermatozoa acquire the capacity for forward motility and fertilization upon spawning. Regarding the function of the two GtHs, LH is mainly involved in the stimulation of androgen production in Leydig cells, whereas FSH seems to exert more complex functions in the male testes, stimulating androgen production from the Leydig cells as well, but also regulating Sertoli cell activity during spermatogenesis (Ohta et al., 2007). Other possible functions of FSH in the testes include the stimulation of Sertoli cell proliferation and differentiation, and the synthesis of certain growth factors that act as autocrine and paracrine factors involved in Sertoli cell proliferation and differentiation, and germ cell development (Miura and Miura, 2001; Schulz and Miura, 2002; Schulz et al., 2010).

2.3. Sperm maturation

Sperm maturation (*i.e.* spermiation) is the phase of male gamete development during which the spermatozoa change from nonfunctional, flagellated gametes to fully matured spermatozoa, able to initiate forward motility and fertilize eggs, and includes physiological but no morphological changes (Schulz et al., 2010). Sperm maturation in fish occurs in the sperm duct (Miura et al., 1992; Morisawa and Morisawa, 1988), and the progestin 17,20β-P together with changes in pH and ionic composition of seminal plasma are involved in this process (see Section 6.2.)

In salmonids and Japanese eel (*Anguilla japonica*) the sperm is not capable of motility initiation if it is extracted from the testis without passing through the sperm duct, but that can be reverted if it is incubated in a solution with HCO^{3-} and a high pH (Morisawa and Morisawa, 1988; Ohta et al., 1997a, 1997b). Progestins mediate the process of sperm maturation in male fish (Scott et al., 2010). In the Japanese eel, 17,20β-P regulates sperm maturation through the increase of pH of seminal plasma, which induces an increase of sperm cAMP, allowing its motility (Miura et al., 1992). Miura and Miura (2003) proposed a model in the Japanese eel in which the 17,20β-P acts at the receptor at the spermatozoa membrane and activates the carbonic anhydrase, causing an increase of the seminal plasma pH.

Depending on the species, 17,20β-P and/or 20β-S have been identified as the active progestin(s). However, the limitations in the analysis of these progestins and their low plasma levels could explain at least in part why some of these progestins have not been identified as active steroids. The classically accepted role of progestins is their participation in the control of gamete maturation, both eggs and spermatozoa, and has been related with the development of the hydration mechanisms that are part of the germinal vesicle breakdown in oocytes and the increase of sperm volume (and decrease of sperm density) during spermiation. Cyclical shifts in gonadal steroidogenesis have been described in group-synchronous spawning species, causing waves of progestins in the plasma, related with each spawning event or with peaks of sperm production (Asturiano et al., 2002).

3. Need for the use of exogenous hormones in male breeders

Although spawning induction therapies are more often targeted towards females in aquaculture situations, hormonal therapies in male broodstock may be needed for the stimulation or enhancement of sperm production. Even in species that do produce releasable sperm, it is at times necessary or useful to enhance spermiation and increase sperm volume, for example in order to facilitate sperm collection or increase availability during *in vitro* fertilization protocols.

3.1. Hormonal induction of spermatogenesis and spermiation

Under culture conditions, Japanese and European eel (genus *Anguilla*) males have immature testes containing only type A and early-type B spermatogonia (Miura et al., 1991b, 1991c, 2002; Peñaranda et al., 2010a) and fail to produce any sperm. To continue spermatogenesis, spermiogenesis and spermiation, it is necessary to administer a long-term (*i.e.* during several weeks) hormonal treatment, usually with a GtH (Asturiano et al., 2005, 2006; Pérez et al., 2000). This treatment promotes plasma increases of 11-KT, which is the major androgen in most male fishes, including the freshwater eel (Miura et al., 1991a). Leydig cells are considered the major source of androgens, while androgen receptors are mainly expressed in Sertoli cells and in interstitial cells. However, androgen receptors are also expressed in Leydig cells, where androgens change the expression/activity of steroidogenic genes (Miura et al., 2006), suggesting that androgens develop biological activity *via* the testicular somatic cells (Schulz et al., 2010). Sertoli cells produce different growth factors during spermatogenesis, and their expression or repression seems to regulate spermatogonial mitosis and germ cell differentiation (Baudiffier et al., 2012; Schulz et al., 2010). So, as a result of the exogenous GtH hormonal treatment, spermatogenesis proceeds and spermiation is achieved.

Another example of a fish that fails to undergo normal spermatogenesis is the golden rabbitfish (*Siganus guttatus*) in Okinawa (Japan), which does not attain full gonadal maturation during the first spawning season, even though its testes have well-developed spermatogenic germ cells, including spermatozoa (Komatsu et al., 2006b). On the contrary in the Philippines, males and females in captivity are sexually mature at the age of 10 and 12 months, respectively (Juario et al., 1985). Implants loaded with GnRH agonists (GnRHa) were used to induce

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complete maturation of these fish (Komatsu et al., 2006a). The treatment promoted spermatogenesis, inducing the development and maturation of spermatogenic germ cells, and leading to spermiation in under-yearling male testes.

In Siluridae *spp*, various hormonal therapies – though producing variable results – have been assessed for their ability to increase seminal volume and produce releasable sperm. These include the Mekong catfish (*Pangasius bocourti*) (Cacot et al., 2003), employing heterologous GtH preparations or GnRHa combined with dopamine antagonists (DA) and the African catfish, where several protocols were tested (Viveiros et al., 2002). However, even in those treatments that enabled collection of sperm by stripping, sperm showed lower quality and was of less quantity than that obtained directly from the testis. So, the problem of collecting sperm by stripping in catfishes could not be solved by the administration of exogenous hormones.

3.2. Enhancement of sperm production in poorly spermiating fishes

In general, male fish do complete spermatogenesis in captivity and produce sperm of adequate quality (*i.e.* motility characteristics). However, the amount of sperm produced is often reduced, either due to inadequate rearing conditions (*e.g.* small or shallow tanks not allowing proper breeding behavior, lack of salinity changes during the spawning season, and excessive handling during the spawning season) or as a result of selective breeding for fast growth (Mylonas and Zohar, 2001b; Zohar, 1996; Zohar and Mylonas, 2001). In these situations, males produce some sperm in the beginning of the female spawning season, but the amount is reduced rapidly and towards the end of the season either no sperm or a very small amount is available. This phenomenon is problematic in fish species with a rather long female spawning season, and with batch spawning females with asynchronous or group-synchronous ovarian development. An extreme situation is observed in flatfishes, where there is often very small sperm production when fish are reared in captivity, especially towards the end of the reproductive season (Vermeirssen et al., 1998, 2000). This sperm may be of very high spermatocrit (>85% spermatozoa) and the spermatozoa may be immotile or the sperm may not disperse readily in the tank water upon release, resulting in very low fertilization success.

The reason for the low sperm production or the very high spermatocrit (which are due to low seminal fluid production) has been shown in some species to be the low levels of plasma LH during the period of spermiation (Mylonas and Zohar, 2001b). In fact, treatment of fish with GnRHa or GtH preparations has a marked effect on seminal fluid production (Clemens and Grant, 1964, 1965; LaFleur and Thomas, 1991; Takashima et al., 1984). Furthermore, it has been observed that in many seasonal spawning fishes, the testes at the beginning of the spermiation season contain most of the spermatozoa that they have available for the spawning season (Billard et al., 1995), although they also contain some spermatocysts at earlier stages of development. These spermatocysts may undergo further development and produce more spermatozoa during the spawning season, especially in response to exogenous hormonal treatment (Rainis et al., 2003). So, the poor production of sperm by some species is due to (a) low seminal fluid production, which prevents the release of the fully developed spermatozoa from the testes or (b) the lack or low rate of further spermatogenesis and production of spermatozoa during the spawning season. Therefore, exogenous hormones may stimulate further the process of spermatogenesis, although the main effect may be mainly an increased seminal fluid production, which is necessary to allow the spermatozoa to be "washed" out of the testes (Fig. 1).

3.3. Synchronization of sperm production to ovulation for in vitro fertilization

The practice of *in vitro* fertilization (also called artificial fertilization or strip spawning) is a fairly simple husbandry procedure, where the gametes are removed manually from each sex and fertilized *in vitro* as has been already described and reviewed (Duncan et al., 2013; Mylonas et al., 2010; Urbanyi et al., 2009). This practice is common in the mass production of freshwater species (Salmonids and Cyprinids, among others), and there is interest in applying it to marine fishes in order to facilitate the genetic crosses required for selective breeding programs (Fauvel et al., 1999, 2012). The most difficult aspect of *in vitro* fertilization is the prediction of ovulation time after hormonal therapy, since eggs become overripe after ovulation and lose their fertilization potential (Bromage and Roberts, 1995; Duncan et al., 2013; Mylonas et al., 2010; Urbanyi et al., 2009). Therefore, most research – and thus scientific literature and reviews – have so far focused on females. However, an essential prerequisite of *in vitro* fertilization is that maximal sperm production in the males is synchronized with the time of ovulation and the collection of the ripe eggs from the females. As explained earlier in Sections 3.1 and 3.2, exogenous hormones may be needed for some fishes to ensure that adequate volumes of sperm are produced during the reproductive season. Especially in *in vitro* fertilization applications, hormonal therapies must be timed appropriately in order to ensure that maximal sperm production coincides with ovulation. The time of availability of sperm after the application of exogenous hormones depends on the type and method of hormone used, the

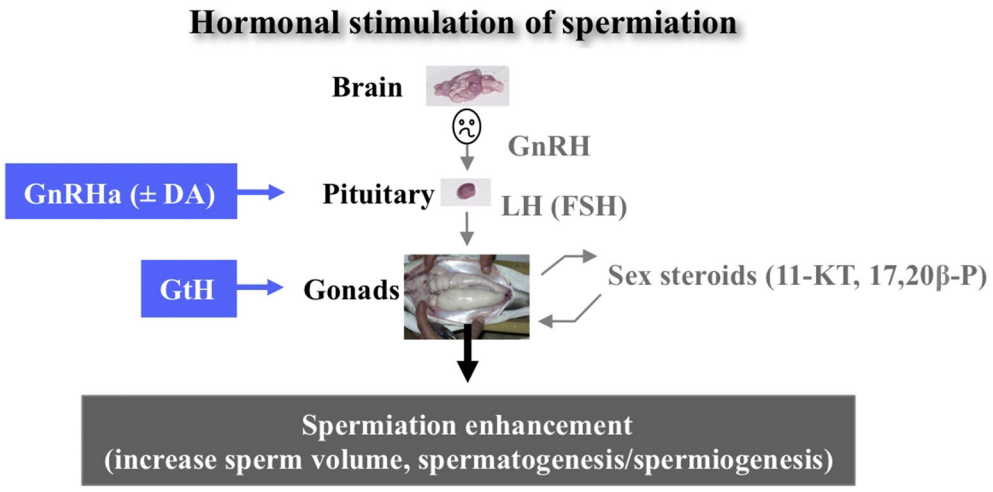

Fig. 1. Schematic representation of the dysfunction in the reproductive axis of cultured male breeders, and the exogenous hormonal interventions for the enhancement of spermiation. DA = dopamine antagonist, FSH = follicle stimulating hormone, GnRH = gonadotropin-releasing hormone, GnRHa = GnRH agonist, GtH = gonadotropin, LH = luteinizing hormone, 11-KT = 11 ketotestosterone, 17,20β-P = 17,20β-dihydroxy-4-pregnen-3-one.

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species and temperature (these aspects will be considered in Section 5). In males, the latency time from hormone application to spermiation is generally shorter than the latency time in females from hormone application to ovulation. For example, European seabass males produced significantly higher sperm production 2 days after GnRHa application (Sorbera et al., 1996) compared to females that ovulated 3–4 days after GnRHa application (Mylonas et al., 2003). Therefore, when both sexes must be induced with exogenous hormones to undergo spermiation/ovulation for *in vitro* fertilization, the therapies must be timed appropriately in males and females, in order to ensure maximum sperm availability at the time of ovulation. This appropriate timing in the case of males is more flexible, because the spermatozoa released at spermiation are stored in the lumen of the testes (Grier, 1993; Grier and Taylor, 1998) until spontaneously released during spawning or artificially collected by stripping. Therefore, maximum sperm availability actually extents for a period after maximum spermiation has been achieved. In many species these characteristics facilitate the management of mixed sex stocks, and protocols have been developed where both sexes are treated with exogenous hormones at the same time to ensure maximum sperm availability before and at the time of ovulation.

4. Environmental factors influencing spermatogenesis and spermiation

Environmental factors (*e.g.* photoperiod, temperature, nutrition, lunar cycle, ocean currents, and rains), as well as social context determine the timing and progress of reproductive development and so have a critical influence on spermatogenesis and the availability of spermatozoa for release during spawning (Bromage et al., 2001; Mañanos et al., 2009). When these environmental factors are not optimal, reproductive dysfunctions compromise gametogenesis and, therefore, sperm production (Duncan et al., 2013; Mylonas et al., 2010; Zohar and Mylonas, 2001). The environmental factors that improve sperm production by avoiding dysfunctions are particular to each species and can be considered limiting factors that should be met to ensure the progress of gametogenesis. These factors are (in order of significance): holding conditions > nutrition > environment during gametogenesis > environment during spawning (Duncan et al., 2013). The holding environment must avoid the adverse effects of stress on reproductive development (Schreck, 2010) by maintaining high fish welfare (Duncan et al., 2013). For example, rainbow trout that were left briefly without water at random points in the 9 months before the reproductive period had reduced sperm density compared to undisturbed controls (Campbell et al., 1992). An adequate diet is also essential to ensure the production of high quality sperm in the quantities necessary (see reviews by Fernández-Palacios et al., 2011; Izquierdo et al., 2001) and sperm production has been improved by varying the content of dietary poly-unsaturated fatty acids (PUFA, Eicosapentaenoic, EPA; Docosahexaenoic, DHA and Arachidonic acid, ARA) and anti-oxidants (vitamin E and selenium) (Asturiano et al., 2001; Beirão et al., 2015). Temperature during spermatogenesis or the spawning period has been shown to affect sperm volume and motility, and spermatozoa velocity and morphology (Adriaenssens et al., 2012; Breckels et al., 2014; Breckels and Neff, 2013; Gallego et al., 2012b; Williot et al., 2000). Consequentially, temperature has been used in some fishes to predict the spawning period (Baynes et al., 1993; Rothbard and Yaron, 1995) and to manipulate an advance, delay or elongation of the spawning period (Brauhn, 1971; Hall et al., 2002; Lang and Tiersch, 2007).

Social environment is another factor that affects sperm production, and various social parameters have been demonstrated to affect sperm quality parameters in different species. These parameters include the presence of females (Cabrita et al., 2010), hierarchical position of dominant *versus* subordinate fish (Leach and Montgomerie, 2000; Rudolfsen et al., 2008a; Vladić, 2001), presence of dominant fish on subordinate fish (Kustan et al., 2012), number of subordinate fish together or increasing competition for female (Candolin and Reynolds, 2011; Pilastro et al., 2002; Stoltz and Neff, 2006). Therefore, the environmental parameters that can be controlled in the culture system should be adjusted to provide optimal conditions for sperm production and once this has been achieved then the need for further measures (*i.e.* exogenous hormones) to improve sperm production can be assessed.

5. Hormonal methods to enhance sperm production

As with artificial induction of ovulation in fish, hormonal therapies for the enhancement of spermiation and sperm production have been tried and employed in aquaculture before the exact nature of the endocrine control of spermatogenesis and spermiation was known (see reviews by Mylonas and Zohar, 2001a; Zohar and Mylonas, 2001). Later on, evidence had suggested that the diminished sperm production could be the result of low LH release from the pituitary during the spermiation period (Mylonas et al., 1997a, 1997b, 1998b; Mylonas and Zohar, 2001b). Although recent data indicates that FSH alone may be able to induce spermatogenesis and spermiation in some fishes (Chu et al., 2015; Mazón et al., 2014; Zhang et al., 2015), methods to enhance spermiation have traditionally focused on the use of exogenous LH preparations that act directly at the level of the gonad, or the use of GnRHas – with or without a DA – that release the endogenous LH stores from the pituitary (Fig. 1). The endogenous LH, in turn, acts at the level of the gonad to induce steroidogenesis, resulting mainly in spermiation and seminal fluid production – and thus the increase in sperm volume. The hormonal treatments in some cases also induce the process of spermatogenesis, and spermiogenesis and spermiation, but this effect may be through stimulation of other hormones (Schulz et al., 2010), especially in the case of using GnRHas.

5.1. GtH preparations of piscine origin

The injection of pituitary extracts (PE) from mature fish into breeders of the same or different species – referred to as "hypophysation" – was the first method used to control reproductive function in aquaculture fishes (Von Ihering, 1937) and has been used widely in a variety of species, especially cyprinids (see reviews by Mañanos et al., 2009; Zohar and Mylonas, 2001). Pituitary extracts contain mainly LH and less FSH, since the pituitaries are collected from fish during the spawning season, and stimulate oocyte maturation and enhance spermiation. Hypophysation is still used extensively today, especially in developing countries or remote areas where access to expensive purified hormones is limited. The advantages of this approach (apart from being inexpensive and easy to prepare) include (a) rapid action, as the exogenous LH acts directly on the gonad, (b) it does not require an active pituitary containing large amounts of LH and (c) it may be more effective in stimulating gametogenesis as well, as it contains also FSH and other hormones that may have some involvement in gametogenesis. On the other hand, the use of PEs has significant drawbacks. The most evident disadvantage is the great difficulty of calculating the doses of administered hormones due to the great variability in the LH content of the pituitaries, which depends on the sex and reproductive stage of the donor fish at the time of sampling. In addition, PEs are a heterogeneous mix of different compounds than can have adverse physiological effects, as well as cause immunoreactions – due to the large molecular weight of the GtHs, especially in species needing long-term hormonal treatments with repeated administration of these extracts. Finally, PEs can transmit diseases to very expensive and genetically selected broodstock. Thus, the chromatographic purification or partial purification of LH from pituitaries of mature fish was the following approach, and for some time salmon (*Oncorhynchus spp*) and carp (Cyprinidae family) GtHs have been commercialized, although species-specificity of fish LH can limit their use only to phylogenetically-related fishes.

Still, males of a variety of fish species have been treated with PEs, in order to enhance spermiation (Table 1). For example, carp PE (CPE, 1.5 mg kg^{-1} body weight, b.w.) enhanced sperm production 1 d after

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Table 1
Representative applications of hormonal manipulations for the enhancement of spermiation using various gonadotropin (GtH) preparations of piscine origin.

Species	Common name	Treatment[a]	Enhancement period (h)[b]	Reference
Abramis brama	Bream	CPE	18–24	(Kucharczyk et al., 1997)
Acipenser brevirostrum	Shortnose sturgeon	CPE	24	(Horváth et al., 2005)
Acipenser ruthenus	Sterlet	CPE	24, 48, 72 /12–42	(Alavi et al., 2012; Dzyuba et al., 2012; Psenicka et al., 2008)
Anguila japonica	Japanese eel	Salmon PE	42 days[c]	(Kagawa et al., 2009)
Brycon amazonicus	Yamú	CPE	24	(Velasco-Santamaría et al., 2006)
Brycon insignis	Tiete tetra/piabanha	CPE	8	(Viveiros et al., 2012b)
Brycon opalinus	Pirapitinga-do-sul	CPE	8	(Orfão et al., 2011)
Brycon orbignyanus	Piracanjuba	CPE	5	(Maria et al., 2006)
Clarias gariepinus	African catfish	CPE, Clarias PE	24	(Viveiros et al., 2002)
Colossoma macropomum	Tambaqui	CPE	10	(Maria et al., 2015)
Cyprinus carpio	Common carp	CPE	24	(Li et al., 2013)
Huso huso	Beluga	CPE	48	(Linhartova et al., 2013)
Odontesthes bonariensis	Pejerrey	CPE, salmon PE	24	(Miranda et al., 2005)
Piaractus brachypomus	Cirapitinga	CPE	12	(Nascimento et al., 2010)
Prochilodus argenteus	Curimatã-pacu	CPE	–	(Arantes et al., 2011)
Prochilodus lineatus	Curimba	CPE	8	(Viveiros et al., 2009, 2010)
Silurus glanis	European catfish	CPE	24–48	(Linhart et al., 2004)
Tinca tinca	Common tench	CPE	24	(Linhart et al., 1995)

[a] Ground pituitaries or pituitary extracts, mainly Carp Pituitary Extract (CPE), or pituitary extracts (PE) from other fishes.
[b] The enhancement period is in days (d) after treatment. The absence of a number indicates that this parameter was not reported in the article.
[c] Through the use of a controlled-release osmotic pump loaded with salmon PE.

treatment in common tench (*Tinca tinca*) (Linhart et al., 1995) and higher sperm volume and motility was obtained from common bream (*Abramis brama*) treated with homologous PE (2.5 mg kg^{-1}) or CPE (2 mg kg^{-1}) (Kucharczyk et al., 1997). Male European catfish (*Silurus glanis*) were treated with CPE at a dose of 5 mg kg^{-1} and injected at four times (Day 0, 6, 12 and 18) resulting in higher sperm volumes after the 3rd treatment (Linhart et al., 2004). African catfish males were treated with CPE or *Clarias* PE, but even in those treatments that resulted in sperm collection using manual stripping, sperm volume was less and quality was lower compared to sperm obtained directly from the testis (Viveiros et al., 2002). Sperm volume of pejerrey (*Odontesthes bonariensis*) was increased by the injection of 30 mg kg^{-1} CPE (13.5-fold increase) or salmon PE (12.8-fold increase), with sperm concentration remaining unchanged as either more spermatozoa were produced in response to the treatment or more became available for stripping (Miranda et al., 2005). Spermatozoa motility and fertilization success of sperm from hormone treated males were not statistically different compared to controls (Miranda et al., 2005).

In South American fishes, a single injection of CPE (dose not reported) was used to improve the sperm quality of streaked prochilod (*Prochilodus lineatus*) (Viveiros and Godinho, 2009; Viveiros et al., 2010). Ovulation and spermiation of curimatã-pacu (*Prochilodus argenteus*) was induced with CPE (Arantes et al., 2011) and out-of-season sperm production was induced in pirapitinga (*Piaractus brachypomus*) after a single intramuscular injection (Nascimento et al., 2010). Finally in the tambaqui (*Colossoma macropomum*), intramuscular injections of 2 mg kg^{-1} of CPE increased sperm volume and decreased viscosity (Maria et al., 2015), as a routine method for Characiformes (Viveiros and Godinho, 2009). Also, various studies have been carried out to induce spermiation and improve the sperm cryopreservation techniques in several species of the genus *Brycon* (Characidae family) that comprises >70 fish species distributed in Central and South America. A dose of 4 mg kg^{-1} CPE was used for yamú (*Brycon amazonicus*) males (Velasco-Santamaría et al., 2006), and for evaluating cooling and freezing effects on piracanjuba (*Brycon orbignyanus*) sperm (Maria et al., 2006). A single intramuscular dose of 5 mg kg^{-1} of CPE has been used to increase sperm volume and decrease viscosity in already running males of pirapitinga-do-sul (*Brycon opalinus*) (Orfão et al., 2011; Viveiros et al., 2012a), while a lower dose of 3 mg kg^{-1} of CPE was effective in the case of piabanha (*Brycon insignis*) (Viveiros et al., 2012b).

Finally, CPE has been used in various sturgeons, and a single dose as low as 1 mg kg^{-1} was effective to obtain sperm (with 77% motility) 24 h later in male shortnose sturgeon (*Acipenser brevirostrum*) (Horváth et al., 2005). Male sterlet (*Acipenser ruthenus*) were treated with a single dose of CPE at 4 mg kg^{-1} to evaluate the effect of repeated stripping on sperm production, its fertilization ability and cryo-resistance (Dzyuba et al., 2012). The highest numbers of spermatozoa per individual were collected 15–42 h post CPE treatment; the sperm motility ranged between 26 and 100% depending on the stripping sequences; higher total volumes were obtained making sequential sperm collections, and fertilization was 90–100% with fresh sperm and 13–76% using frozen-thawed samples. In the same species, CPE injection of 4 mg kg^{-1} was used to evaluate the post-cryopreservation effect of different cryoprotectants (methanol and DMSO) on the acrosome of the spermatozoa (Psenicka et al., 2008), and to test different techniques to measure the changes on the spermatozoa volume during the motility period, in comparison with other species (Bondarenko et al., 2013). Finally in the beluga (*Huso huso*), a study of the morphology and ultrastructure of spermatozoa in comparison with related sturgeons was undertaken after treating males with a single injection of 4 mg kg^{-1} of CPE (Linhartova et al., 2013). The study concluded that spermatozoan ultrastructure and morphology were similar among sturgeon species, and beluga may be closely related to the genus *Acipenser*, even though it has been assigned to the genus *Huso*.

So, PEs and the hypophysation technique is used widely in various fishes, especially in developing countries where access to expensive purified hormones is limited, whereas pituitaries from mature fish are easily available. However, most of these works are not reported in the peer-reviewed literature, but can be found as conference abstracts, PhD theses, and manuals or books written in local languages.

5.2. Recombinant GtHs of piscine origin

Recently, studies have examined the production and use of recombinant (re) LH and FSH of some fish species. Recombinant proteins are produced by introducing the protein DNA sequence into a plasmid, which transfers the sequence into cultured cells of another organism, referred to as the "expression system". Large glycoproteins, such as GtHs that require glycosylation, are produced in yeast, mold, insect or mammalian cell expression systems, to ensure correct glycosylation. Incorrect glycosylation reduces *in vivo* half-life and biological activity/receptor binding of the reGtH and for these reasons mammalian cells are the most popular for producing recombinant mammalian glycosylated proteins (Demain and Vaishnav, 2009). Homologous reFSH and reLH have been produced for zebrafish (So et al., 2005), channel catfish

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(*Ictalurus punctatus*) (Zmora et al., 2007), goldfish (*Carassius auratus*) (Hayakawa et al., 2008), Japanese eel (Kobayashi et al., 2010), European seabass (Molés et al., 2011), Senegalese sole (Chauvigné et al., 2012), cinnamon clownfish (*Amphiprion melanopus*) (Kim et al., 2012) and European eel (*Anguilla anguilla*) (Peñaranda et al., 2015). All these reGtHs were shown to stimulate GtH receptors *in vitro*. Assays *in vitro* found that homologous reFSH of zebrafish (So et al., 2005) and Senegalese sole (Chauvigné et al., 2012) stimulated only the FSH receptors, while reLH was promiscuous and stimulated both FSH and LH receptors. However, the *in vitro* effect of reGTHs was not always the same as the *in vivo* effect. In the Japanese eel reGtH stimulated spermatogenesis *in vitro*, but had little effect *in vivo* (Kazeto et al., 2008). On the other hand, reGtH administered *in vivo* to male Senegalese sole (Chauvigné et al., 2012) and European seabass (Mazón et al., 2013, 2014) stimulated testosterone (T) and 11-KT release, and reFSH applied *in vivo* induced the early stages of spermatogenesis in immature Japanese eel (Kamei et al., 2006) and immature European seabass (Mazón et al., 2014).

Spermiation was enhanced in mature goldfish with the application of reGtH (Hayakawa et al., 2008; Kobayashi et al., 2006). Mature goldfish injected with a single injection of 20 μl g^{-1} of superworm hemolymph containing either reFSH or reLH increased sperm volume >4× after 24 h compared to control fish. Full spermatogenesis and the production of spermatozoa were induced with reGtH in Japanese eel (Hayakawa et al., 2009) and European eel (Peñaranda et al., 2015). Japanese eel were treated with superworm hemolymph containing undetermined concentrations of goldfish reGtH. Treatments (volume of hemolymph per gram of eel) of either 3 μl g^{-1} reLH, or 3 μl g^{-1} reFSH + 3 μl g^{-1} reLH administered eight consecutive times at 2–5 day intervals induced complete spermatogenesis with the production of spermatozoa (Hayakawa et al., 2009). However, spermatozoa were not released from the spermatocysts into the lumen as would be expected and as observed with other GtH therapies. More recently, full spermatogenesis and the production of spermatozoa with spermiation into the lumen was achieved using homologous reFSH and reLH (synthesized with Chinese hamster ovary-CHO expression system by Rara Avis Biotec S.L., Valencia, Spain) in European eel (Peñaranda et al., 2015). The treatment that induced spermatogenesis and spermiation was weekly injections during six weeks of reFSH at a dose of 2.8 μg $fish^{-1}$ combined with increasing doses of reLH during the last three weeks as follows: 1 μg $fish^{-1}$ in week four, 2 μg $fish^{-1}$ in week five and 6 μg $fish^{-1}$ in week six. The sperm quality was variable and not all the spermiating males produced samples with high sperm quality. However, the sperm had motilities ≥50%, densities around 7×10^9 cells ml^{-1} and sperm volumes of approximately 0.4 ml. Thus, for the first time in teleosts, homologous reGTHs have produced good quality sperm, demonstrating that the half-life of these hormones is long enough to induce *in vivo* effects (Kobayashi et al., 2006). A different approach using this type of biotechnology was to inject a plasmid with the LH sequence directly into the muscle of mature European seabass (Mazón et al., 2013). Mature males were administered two injections on day 0 and 3 of 200 μg $fish^{-1}$ of plasmid, and sperm was collected on five occasions at weekly intervals from day 2 onwards. Circulating LH in plasmid-injected fish was significantly higher than in control fish. Total sperm produced and sperm density was doubled by the plasmid treatment compared to control groups, and was similar to a group injected with reLH. The use of reGtH clearly has great promise for future applications in aquaculture, but there remains considerable work to identify the most adequate expression system to produce biologically active reGtH with a long half-life.

5.3. GtH preparations of mammalian or human origin

A wide range of GtH preparations of mammalian or human origin have been tested in some fishes, including mammalian FSH and LH, ovine LH, Pregnant Mare Serum Gonadotropin (PMSG) and human Chorionic Gonadotropin (hCG) (Donaldson and Hunter, 1983). However, only hCG – which is purified from the urine of pregnant women – has been used routinely in aquaculture. The comparative success of the use of hCG in stimulating increases in sperm volume, compared to the lack of success of other mammalian GtH preparations (Donaldson and Hunter, 1983) has resulted in just a few isolated reports on the use of other mammalian GtHs (Billard, 1977). The advantages of hCG include (a) a very rapid response, as it acts directly on the gonad – similarly to CPE, (b) world-wide availability due to its use in human Assisted Reproductive Technologies, (c) sterile, pure and bioassayed activity and (d) longer half-life in circulation compared to pituitary GtHs (Zohar and Mylonas, 2001). A drawback of hCG that is often quoted by aquaculturists is that the treatment becomes less effective with repeated use (*i.e.* over repeated reproductive seasons) due to the development of an immune response. However, studies examining the antigenicity of hCG have produced contradictory results (Zohar and Mylonas, 2001). For example, hCG antibodies were observed in circulation in striped bass after a single treatment of hCG (Zohar and Mylonas, 2001), but no antibodies against hCG were detected in silver carp (*Hypophthalmichthys molitrix*) and goldfish, in the latter even after three repeated treatments (Van Der Kraak et al., 1989).

The first studies with hCG administered a dose from 100 to 10,000 IU kg^{-1} and resulted in a dose dependent increase in the occurrence of spermiating goldfish (Yamazaki and Donaldson, 1968). This early success, in addition to the long half-life of hCG in circulation (Ohta and Tanaka, 1997; Zohar and Mylonas, 2001) has resulted in the use of hCG to enhance sperm production in a wide range of fish species (Table 2). In all species used, increased sperm volume may be achieved after a single injection of hCG and within a period of a few hours, but the effect is rather short lived and most studies found higher sperm volume for a period of only 1–2 days after administration. In the Japanese eel, a single hCG injection can induce the whole process of spermatogenesis resulting in sperm production (Miura et al., 1991b), but only weekly administrations guarantee high volume and adequate quality for *in vitro* fertilization (Asturiano et al., 2006). The most common dose applied in fishes is 1000 IU kg^{-1}, but doses may range from 150 IU kg^{-1} in pikeperch (*Sander lucioperca*) (Falahatkar and Poursaeid, 2014) and 312 IU kg^{-1} in pejerrey (Miranda et al., 2005) to 10,000 IU kg^{-1} in goldfish (Yamazaki and Donaldson, 1968) and 50,000 IU kg^{-1} in silver perch (*Leiopotherapon plumbeus*) (Denusta et al., 2014). When the latter extremes are not considered, the mean (±SD) hCG dose applied across all remaining species is 1200 ± 458 IU kg^{-1}.

Recombinant hCG (rehCG) is also available commercially, but has not been used much in fish so far. In a recent trial comparing hCG and rehCG in the European eel, rehCG was more effective in increasing sperm volume, density, motility and kinetic features, throughout most weeks of the treatment (Gallego et al., 2012b).

5.4. GnRHa injections and delivery systems

The commercial synthesis and widespread use of GnRHa for human medicine (Ulloa-Aguirre and Timossi, 2000), made a number of different GnRHas available for spawning induction therapies in fish. Compared to the use of GtH preparations, GnRHa offers some important advantages. For example, GnRHa treatments are less species-specific, due to the high structural similarity of native GnRHs among fishes (Lethimonier et al., 2004). Being of synthetic nature, a GnRHa offers absolute biosecurity against disease transmission threats compared to PEs, and a better calculation of effective doses. The most important advantage is that GnRHa acts at a higher level of the brain-pituitary-gonad axis and stimulates the release of the endogenous GtHs (mainly LH, but also FSH), as well as other pituitary hormones that may be involved in the regulation of reproduction (Cyr and Eales, 1996; Le Gac et al., 1993; Negatu et al., 1998; Weber et al., 1995). In species such as cyprinids (Yaron, 1995), catfishes (Brzuska, 2001) and mullets (Aizen et al., 2005), where there is a strong dopaminergic inhibition of GtH release,

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Table 2
Representative applications of hormonal manipulations for the enhancement of spermiation using gonadotropin (GtH) preparations of mammalian origin.

Species	Common name	Treatment[a]	Enhancement period (d)[b]	Reference
Abramis brama	Common bream	hCG	–	(Kucharczyk et al., 1997)
Acipenser ruthenus	Sterlet	hCG	1	(Rzemieniecki et al., 2004)
Anguilla anguilla	European eel	hCG[c]	35	(Asturiano et al., 2006)
Anguilla japonica	Japanese eel	hCGc	35	(Ohta and Tanaka, 1997)
Carassius auratus	Goldfish	hCG	1	(Yamazaki and Donaldson, 1968)
Dicentrarchus labrax	European seabass	hCG	7	(Schiavone et al., 2006)
Leiopotherapon plumbeus	Silver perch	hCG	1	(Denusta et al., 2014)
Leuciscus leuciscus	Dace	hCG	2	(Cejko et al., 2012)
Odontesthes bonariensis	Pejerrey	hCG	2	(Miranda et al., 2005)
Pagrus auratus	New Zealand snapper	hCG	1	(Pankhurst, 1994)
Pangasius bocourti	Mekong catfish	hCG	1	(Cacot et al., 2003)
Rhynchocypris oxycephalus	Chinese minnow	hCG	1	(Park et al., 2002)
Sander lucioperca	Pikeperch	hCG	1	(Falahatkar and Poursaeid, 2014)
Siganus argenteus	Forktail rabbitfish	hCG	2	(Rahman et al., 2003)

[a] Human chorionic gonadotropin (hCG).
[b] The enhancement period is in days (d) after treatment. The absence of a number indicates that this parameter was not reported in the article.
[c] Treatment applied a minimum of 5 times at weekly intervals.

GnRHa treatments are usually combined with DA (*e.g.* domperidone, pimozide, reserpine or metoclopramide). Another advantage of GnRHa is that it is a small decapeptide with a low molecular weight and is effective in μg doses, enabling its incorporation in controlled-release delivery systems (Mylonas and Zohar, 2001a). Various GnRHas are available that are based on the native structure of either the mammalian or salmon GnRH, all modified structurally with synthetic amino acid substitutions at positions 6 and/or 10 of the peptide (Zohar and Mylonas, 2001), which makes them more active and resistant to enzymatic degradation (Zohar et al., 1990).

The potential of long-term administration of GnRHa *via* delivery systems was recognized from the early beginnings of commercial aquaculture (Fontenele, 1955). This is because oocyte maturation and spermiation often require prolonged hormonal treatments, given in multiple injections (Dabrowski et al., 1994; Slater et al., 1995). Handling stress from such repetitive manipulations may be damaging to the brood fish or affect negatively their reproductive performance (Schreck, 2010). This may be especially true when broodfish are very large (*e.g.* groupers, amberjacks or tunas) or kept outdoors – in ponds or sea cages (Corriero et al., 2009; Mylonas et al., 2007) – and it is also very time consuming and labor intensive to capture and inject the fish with multiple treatments. Therefore, GnRHa-delivery systems have been used extensively in the last decades, mainly for controlling oocyte maturation in females, but also for enhancing spermiation in males (Mañanos et al., 2009; Mylonas and Zohar, 2001a; Mylonas et al., 2010).

The available GnRHa delivery systems may be in the form of solid implantable pellets of cholesterol (Weil and Crim, 1983), Ethylene-Vinyl Acetate (EVAc) (Mylonas et al., 2007; Zohar, 1996) or other material (*e.g.* "Ovopel") (Horváth et al., 1997), or in the form of biodegradable microspheres (Mylonas et al., 1995). Both solid/implantable and microspheric/injectable systems have been used effectively in controlling reproductive function, each method having specific advantages. For example, the injectable systems can be administered to fish of great variations in size without any modification of the hormone preparation. On the other hand, solid implants are easier to use by aquaculturists in the field, but they offer less accuracy on treatment dose when breeders are of different sizes. Delivery systems for GnRHa may release for periods of 1 to 5 weeks, depending on the matrix type and water temperature (Crim et al., 1988; Mañanos et al., 2002; Mylonas et al., 1998a; Mylonas and Zohar, 2001a; Vermeirssen et al., 2004; Zohar, 1996).

As mentioned above, a single injection of GnRHa has a short-term effect on the reproductive function, due to the transient increase in plasma LH, which lasts for a few hours or days. For example, in the barbel (*Barbus barbus*) a single injection of GnRHa (with a DA) increased sperm volume for a period of only 12 to 24 h after treatment (Cejko et al., 2014). Similarly in the golden rabbitfish, a single injection of GnRHa induced an increase in sperm volume for only 24 h, but after 48 h from treatment, spermiation was similar to non-treated controls (Garcia, 1991). Repeated weekly injections of GnRHa in the same species could sustain elevations in sperm volume for 4 weeks, underlining the potential of controlled-release delivery systems for a more long-term stimulation of sperm production (Garcia, 1993). Longer stimulation was effected in the European seabass, where a single GnRHa injection induced a significant increase in plasma LH for 3 days (Mañanos et al., 2002), which was associated with an increase in expressible sperm for 3 days (Rainis et al., 2003) or 7 days (Sorbera et al., 1996). After plasma LH levels returned to pretreatment levels, the amount of sperm produced was similar to non-treated controls (Mañanos et al., 2002). Examples of the use of GnRHa injections for the enhancement of spermiation are given in Table 3.

On the other hand, the use of controlled-release delivery systems for GnRHa can result in a very long-term stimulation of sperm production for up to many weeks (Table 3). For example, in yellowtail flounder (*Pleuronectes ferrugineus*) both GnRHa implants and microspheres induced sustained elevations in sperm production for >29 days (Clearwater and Crim, 1998). Similarly in the European seabass, GnRHa implants and microspheres induced significantly elevated plasma LH levels for 28–42 days after treatment (Mañanos et al., 2002), resulting in the collection of significantly higher sperm volumes for at least 35 days after treatment (Sorbera et al., 1996). In fact, the amount of sperm produced after 35 days was almost 2× higher than the amount produced prior to the GnRHa treatment, even after repeated weekly stripping of all expressible sperm. In another study with European seabass, sperm production was stimulated for only 3 days after a GnRHa injection, whereas GnRHa implants enhanced spermiation for at least 27 days (Fig. 2). Finally, in striped bass both GnRHa microspheres and implants enhanced sperm production for at least 14 days, with treated fish producing >4× as much sperm than non-treated controls over the course of the study (Mylonas et al., 1997b). So, it is clear from all the studies undertaken so far, that GnRHa delivery systems offer an important tool for the long-term enhancement of sperm production in aquaculture (Table 3).

6. Other approaches to enhance sperm production

In addition to the use of hormones administered to male breeders in order to induce spermatogenesis and enhance sperm production, there are also other approaches that have been employed in some fishes. These methods may stimulate spermiation or improve sperm quality.

6.1. Pheromone applications

The importance of pheromones in fish reproduction is generally accepted and often insinuated, but perhaps surprisingly the identification

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Table 3
Representative applications of hormonal manipulations for the enhancement of spermiation using injections of agonists of gonadotropin-releasing hormone (GnRHa) and sustained-release GnRHa-delivery systems (implants or microspheres).

Species	Common name	Method[a]	GnRHa type[b]	Enhancement period (d)[c]	Reference
Acanthopagrus australis	Yellowfin bream	Inj	Trp	2	(Black and Pankhurst, 2009)
Acipenser baerii	Siberian sturgeon	Inj	Phe	1.5	(Williot et al., 2002)
Acipenser ruthenus	Sterlet	EVAc, OP	Ala	7	(Alavi et al., 2012)
Barbus barbus	Barbel	Inj	Arg[d]	1	(Cejko et al., 2014)
		OP	Ala[d]	0.5	(Cejko et al., 2014)
Carassius carassius	Crucial carp	Inj	Arg[d]	1	(Cejko et al., 2013)
Coregonus lavaretus	European whitefish	Inj	Naf	11	(Wojtczak et al., 2005)
Cyprinus carpio	Common carp	Inj	Ala	2	(Takashima et al., 1984)
		Inj	Ala[d]	2.5	(Roelants et al., 2000)
Dicentrarchus labrax	European seabass	Inj	Ala	3, 7	(Rainis et al., 2003; Sorbera et al., 1996)
		EVAc, FA	Ala	27, 35	(Rainis et al., 2003; Sorbera et al., 1996)
Gadus morhua	Atlantic cod	Chol	Arg	27	(Garber et al., 2009)
Hippoglossus hippoglossus	Atlantic halibut	EVAc	Ala	30, 40	(Vermeirssen et al., 2000, 2004)
Lates calcarifer	Asian seabass	Inj	Ala[d]	2	(Hilomen-Garcia et al., 2002)
Leuciscus leuciscus	Dace	Inj	Arg[d]	2	(Cejko et al., 2012)
		OP	Ala[d]	2	(Cejko et al., 2012)
Morone chrysops	White bass	FA	Ala	7	(Mylonas et al., 1997c)
Morone saxatilis	Striped bass	EVAc, FA	Ala	14	(Mylonas et al., 1997b)
		FA	Ala	14	(Mylonas et al., 1995)
Oncorhynchus mykiss	Rainbow trout	Inj	Ala	7	(Heyrati et al., 2010)
Osmerus eperlanus	Smelt	Inj	Ala[d]	3	(Kowalski et al., 2012)
		Inj	Ala[d]	1	(Krol et al., 2009)
Pagrus auratus	New Zealand snapper	Inj	Ala	1	(Pankhurst, 1994)
Pangasius bocourti	Mekong catfish	Inj	Arg	2	(Cacot et al., 2003)
Perca flavescences	Yellow perch	Inj	Ala	4	(Dabrowski et al., 1994)
Platichthys stellatus	Starry flounder	Chol	Ala	35	(Moon et al., 2003)
Pleuronectes americanus	Winter flounder	Inj	Ala	12	(Harmin and Crim, 1993)
		Chol	Ala	35	(Shangguan and Crim, 1999)
Pleuronectes ferrugineus	Yellowtail flounder	Chol, FA	Ala	29	(Clearwater and Crim, 1998)
Rhombosolea tapirina	Greenback flounder	Chol	Ala	35	(Lim et al., 2004)
Rhynchocypris oxycephalus	Chinese minnow	Inj	Ala	2	(Park et al., 2002)
Salmo salar	Atlantic salmon	FA	Ala	10	(Mylonas et al., 1995)
		EVAc	Ala	10	(Zohar, 1996)
		Inj	Arg[d]	8	(King and Young, 2001)
Siganus guttatus	Golden rabbitfish	Inj	Ala	1	(Garcia, 1991)
				7	(Garcia, 1993)
Solea senegalensis	Senegalese sole	EVAc	Ala[d]	25	(Guzmán et al., 2011a, 2011b)
Tinca tinca	Common tench	Inj	Arg	5	(Linhart et al., 1995)
		EVAc	Ala	5	(Linhart et al., 1995)

[a] Chol = cholesterol/cellulose; EVAc = poly[ethylene-vinyl acetate]; FA = poly[fatty acid dimer-sebasic acid]; Inj = Injection(s); OP = Ovopel, pellet containing GnRHa (Ala) and metoclopromide.
[b] Ala = D-Ala^{6}Pro^{9}NEt-mGnRH; Arg = D-Arg^{6}Pro^{9}NEt-sGnRH; Naf = Azagly Nafareling (Gonazon); Phe = D-Phe^{6}NH$_2$-mGnRH; Trp = D-Trp^6-mGnRH.
[c] The enhancement period is in days (d) after treatment.
[d] Combination treatment with a dopamine antagonist (DA) such as pimozide, domperidone, reserpine or metoclopramide, or steroids.

of pheromones and the mechanisms of production/reception and biological function have been described in just a few species, such as the goldfish (DeFraipont and Sorensen, 1993; Stacey et al., 1989), masu salmon (*Oncorhynchus masou*) (Yambe et al., 2006) and Mozambique tilapia (*Oreochromis mossambicus*) (Keller-Costa et al., 2014). In Mozambique tilapia the male is the signaling-sex, while in goldfish and masu salmon the female was identified as the emitter of pheromones, and the male as the receptor. However, of these three species only male goldfish were described to increase GtH secretion, sperm production and motility in response to the female MIS that functions also as a pheromone (*i.e.* 17,20β-P). Holding male goldfish in a flow through aquarium with a concentration of 10^{-10} M declining to 10^{-11} M of 17,20β-P increased sperm volume >3× (from 22 μg prior to exposure to 77 μg after exposure) compared to 21 μg of sperm in control fish exposed to the ethanol carrier (DeFraipont and Sorensen, 1993). It has been shown that female goldfish produce and release 17,20β-P (also androstenedione and 17,20β-P-sulfate) in the pre-ovulatory period and electro-olfactogram recordings (EOGs) have shown that males can detect low pmol concentrations of these substances (Sorensen et al., 1999; Sorensen and Stacey, 2004). During ovulation, females produce and release prostaglandin $F_{2\alpha}$ (PGF), which stimulates male spawning behavior and spawning. It is also interesting to note that sperm from male goldfish exposed to 17,20β-P fertilized more eggs than sperm from control males (Zheng et al., 1997). This was observed both with *in vivo* competition between two males for an ovulated female and with *in vitro* fertilization using the stripped sperm.

Following this work, 17,20β-P has been used to increase sperm production and motility in common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*). Precocious male common carp increased significantly their sperm volume (4×) when exposed to a 5×10^{-10} M concentration of 17,20β-P (Stacey et al., 1994) and Nile tilapia exposed to a 5×10^{-9} M concentration of 17,20β-P increased significantly their sperm volume (2×) and motility duration compared to controls and pre-exposure values (Pinheiro et al., 2003). In salmonids, PGF have been identified as probable pheromones that stimulate sperm production. Both Atlantic salmon (*Salmo salar*) (Moore and Waring, 1996) and brown trout (*Salmo trutta*) (Moore et al., 2002) mature parr were shown to increase significantly (2×) sperm volume when exposed to 10^{-8} M concentration of either $PGF_{2\alpha}$ or $PGF_{1\alpha}$. These studies demonstrate that pheromones enhanced significantly sperm production and suggest that pheromones require further research especially in species that produce small amounts of sperm even in the wild, since behavior and pheromones may be the mechanism that increases sperm volume in the moment that sperm is required for spawning.

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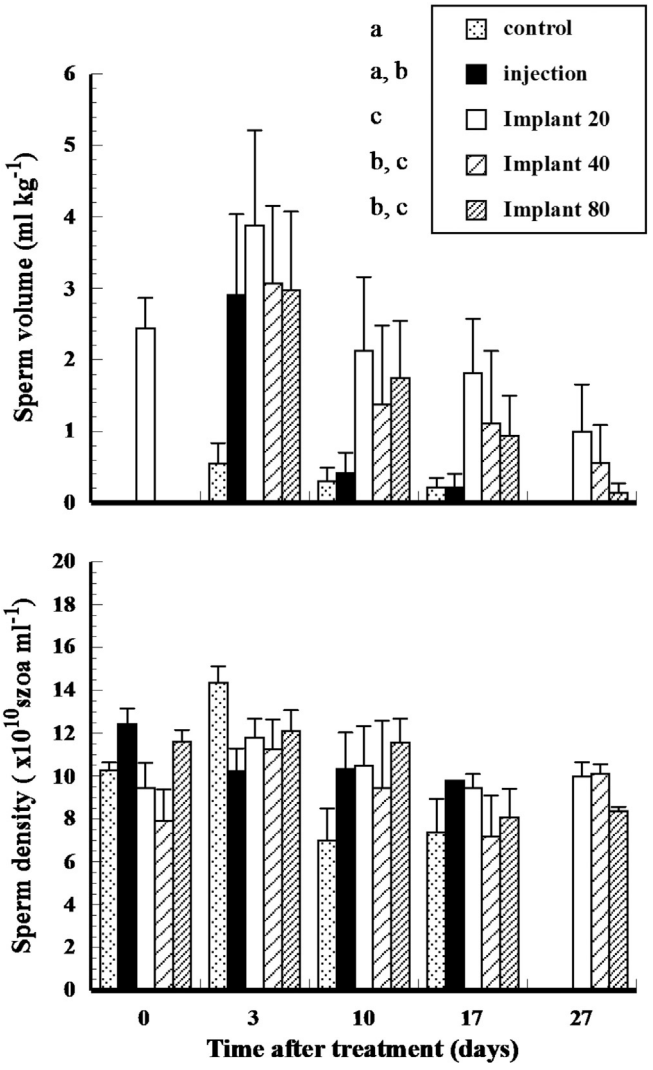

Fig. 2. Mean (+SEM) volume of expressible sperm and spermatozoa density over a 27-day period, from European seabass (*Dicentrarchus labrax*, n = 6) administered different hormonal treatments of GnRHa: control = saline, Inj = a single injection of GnRHa, Implant = GnRHa delivery system – implant at different doses (20–80 μg kg^{-1}). Different letters in the legend indicate significant differences ($P \leq 0.05$) in sperm volume between treatments throughout the study (two-way ANOVA, P = 0.010, DNMR $P \leq 0.05$). There were no significant differences among treatments in spermatozoa density. (From the experiments reported by Rainis et al., 2003).

6.2. Hormones added to sperm in vitro to increase sperm quality

After spermiation, spermatozoa undergo maturation (or capacitation), a process that renders them capable of forward motility upon ejaculation and competent to fertilize an oocyte (Schulz et al., 2010). The process is regulated through the actions of the MIS (17,20β-P or 20β-S). Their physiological roles have been explored in the last two decades (reviewed by Asturiano et al., 2002; Scott et al., 2010) and a two phase process of maturation has been proposed in the females of species where both hormones are found (Mylonas et al., 1998a), with a first phase (early maturation) controlled by 17,20β-P, followed by a second phase (final maturation) in which 20β-S predominates (Asturiano et al., 2000). The existence of several types of progestin membrane receptors in fish cells has been documented and its presence has been related with oocyte and testis/sperm function (reviewed by Morini et al., 2014; Thomas et al., 2004). The binding of progestin to spermatozoa membrane progestin receptors (mPRα, mainly localized on the mid piece of the spermatozoa) results in rapid activation of intracellular signaling pathways by a non-genomic mechanism involving some G proteins, causing an increase in cAMP production and of intracellular calcium levels, and improving spermatozoa motility (Tubbs and Thomas, 2009). The *in vitro* progestin stimulation of sperm motility has been described in different sciaenid species (Tubbs and Thomas, 2008), such as the spotted sea trout (*Cynoscion nebulosus*), Atlantic croaker (*Micropogonias undulatus*) and red drum (*Sciaenops ocellatus*), and in the southern flounder (*Paralichthys lethostigma*) (Tan et al., 2014).

Sperm motility has been improved by incubating sperm samples with 20β-S (10–200 nM) for 1–5 min in the spotted sea trout (Tubbs and Thomas, 2008), the Atlantic croaker (Thomas et al., 2004) or the southern flounder (Tubbs et al., 2011). In the Atlantic croaker, sperm motility increases were obtained out of the peak of the spawning season with such treatment (Thomas et al., 2005), a method that could be used to obtain high quality sperm for longer periods. And in the southern flounder, 20β-S treatment caused an increase of spermatozoa motility and fertilization capacity (Tan et al., 2014). Moreover, a single GnRHa administration (100 μg kg^{-1}) induced an increase of the concentration of membrane mPRα receptors that was correlated with higher sperm motility and fertilization success (Tubbs et al., 2011). Altogether, these results underline that *in vitro* treatment of fish sperm with 20β-S has a good potential for practical applications in aquaculture production or research, in cases where *in vitro* fertilization is used.

7. Effect of hormonal therapies on sperm quality

While treatment of male breeders with exogenous hormones enhances consistently sperm production (*i.e.* volume) in fish, the effect of the treatment on various sperm quality parameters is variable. For the majority of examples, hormonal treatments do not affect sperm quality or they reduce spermatozoa density (Table 4) through the stimulation of testicular hydration and the production of seminal fluid. In some cases – such as cultured flatfishes that produce very thick sperm in captivity (up to 80% spermatocrit), the increase in seminal fluid production induced by the hormonal therapy results also in an improvement in sperm quality parameters (Vermeirssen et al., 2004), as explained below.

7.1. Changes in spermatozoa density

A common result of all hormonal therapies for the enhancement of spermiation is the hydration of the testes through the production of seminal fluid (Clemens and Grant, 1965), which is effected by the MIS (Scott et al., 2010). The increased fluid content of the testes allows the stripping of more spermatozoa, which are often already present within the testes (*i.e.* intra-testicular sperm) but cannot be released. As a result, hormonal therapies "wash out" more available spermatozoa from the testes, and over time the spermatozoa density of the collected sperm decreases significantly (Table 4). In Atlantic halibut (*Hippoglossus hippoglossus*) (Vermeirssen et al., 2004), sperm volume decreases towards the end of the spawning season, with a concomitant dramatic increase in viscosity, making it difficult to collect sperm. Treatment at this time with GnRHa in controlled-release implants increases seminal fluid production – through increases in testicular MIS synthesis (Vermeirssen et al., 2000), which results in a significant increase in sperm volume, and a decrease in sperm density for a period of 40 days (Vermeirssen et al., 2000, 2004). The resulting reductions in spermatozoa density in various fishes range between 20 and 300%, but coupled with the significant increases in expressible sperm, the end result is that there is an increase in total number of spermatozoa collected in response to hormonal stimulation. Only in extreme situations, where exogenous hormone stimulation of sperm production lasts for a very long time through repeated treatments, sperm concentration may be reduced by up to 400× compared to normal values, as observed in meagre (*Argyrosomus regius*) after 17 weeks of spermiation induction with the multiple use of

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Table 4
Representative effects of various hormonal manipulations on sperm quality parameters.

Species	Common name	Hormone treatment[a]	Motility %[b]	Motility duration[b]	Sperm density[b]	Sperm velocity[b]	Reference
Abramis brama	Common bream	GtH	(+)				(Kucharczyk et al., 1997)
		hCG	(+)				(Kucharczyk et al., 1997)
Acanthopagrus australis	Yellowfin bream	GnRHa		(=)	(+)		(Black and Pankhurst, 2009)
Acipenser ruthenus	Sterlet	GnRHa, GtH	(+)			(+)	(Alavi et al., 2012)
Barbus barbus	Barbel	GnRHa	(=)		(=)	(=)	(Cejko et al., 2014)
Carassius carassius	Crucian carp	GnRHa	(=)		(=)	(=)	(Cejko et al., 2013)
Coregonus lavaretus	European whitefish	GnRHa	(=)	(=)	(=)	(+)	(Wojtczak et al., 2005)
Cyprinus carpio	Common carp	GnRHa			(−)		(Takashima et al., 1984)
Dicentrarchus labrax	European seabass	GnRHa	(=)	(=)	(=)		(Rainis et al., 2003)
		hCG	(=)	(=)	(=)		(Schiavone et al., 2006)
Esox masquinongy	Muskellunge	GtH			(−)		(Lin and Dabrowski, 1996)
Esox lucius	Pike	GtH			(−)		(Billard and Marcel, 1980)
Gadus morhua	Atlantic cod	GnRHa	(+)		(+)		(Garber et al., 2009)
Hippoglossus hippoglossus	Atlantic halibut	GnRHa			(−)	(+)	(Vermeirssen et al., 2000; Vermeirssen et al., 2004)
Lates calcarifer	Seabass	GnRHa				(−)	(Hilomen-Garcia et al., 2002)
Leuciscus leuciscus	Dace	GnRHa	(=)		(−)	(=)	(Cejko et al., 2012)
		GtH	(=)		(−)	(=)	(Cejko et al., 2012)
Morone chrysops	White bass	GnRHa	(=)		(=)		(Mylonas et al., 1997c)
Morone saxatilis	Striped bass	GnRHa			(±)		(Mylonas et al., 1997b)
		GnRHa			(+/=)		(Mylonas et al., 1995)
Oncorhynchus mykiss	rainbow trout	GnRHa		(=)	(=)		(Heyrati et al., 2010)
Osmerus eperlanus	smelt	GnRHa	(+)		(+/=)	(+)	(Kowalski et al., 2012)
		GnRHa	(=)	(=)	(−)	(=)	(Krol et al., 2009)
Pangasius bocourti	Mekong catfish	GnRHa			(=)		(Cacot et al., 2003)
		hCG			(=)		(Cacot et al., 2003)
Platichthys stellatus	Starry flounder	GnRHa	(=)		(+/=)	(=)	(Moon et al., 2003)
Pleuronectes americanus	Winter flounder	GnRHa			(=)		(Harmin and Crim, 1993)
		GnRHa	(±)		(−)		(Shangguan and Crim, 1999)
		GtH	(=)	(=)	(−)		(Shangguan and Crim, 1999)
Pleuronectes ferrugineus	Yellowtail flounder	GnRHa	(+)	(+)	(=)		(Clearwater and Crim, 1998)
Rhombosolea tapirina	Greenback flounder	GnRHa		(=)	(−)	(=)	(Lim et al., 2004)
Rhynchocypris oxycephalus	Chinese minnow	GnRHa			(−)		(Park et al., 2002)
		hCG			(−)		(Park et al., 2002)
Salmo trutta	Brown trout	GnRHa	(=)	(=)	(−)		(Mousavi et al., 2011)
Siganus guttatus	Golden rabbitfish	GnRHa			(−)		(Garcia, 1991; Garcia, 1993)
Silurus glanis	European catfish	GtH			(=)		(Linhart and Billard, 1994)
Solea senegalensis	Senegalese sole	GnRHa			(−)		(Guzmán et al., 2011b)

[a] GnRHa = gonadotropin-releasing hormone agonists (w/without DA); human Chorionic Gonadotropin = hCG; Pituitary extracts or piscine GtHs (mainly LH) = GtH.
[b] (+) = increase; (−) = decrease; (=) = not affected. The absence of any symbol indicates that this parameter was not reported in the article.

GnRHa implants (Fig. 3), and participation in two spawning events each week (unpublished data).

In many fishes, however, sperm density may be unaffected or may even increase in response to hormonal treatment (Table 4), especially with the use of GnRHa delivery systems. In these situations, it is obvious that the hormonal therapy functions not only in increasing seminal fluid production and the facilitation of stripping of existing spermatozoa from the testes, but also in stimulating spermatogenesis, spermiogenesis and spermiation (Schulz et al., 2010). In European seabass for example (Rainis et al., 2003), the testes of spermiating males towards the end of the spawning period contain large numbers of spermatocysts at different stages of development (Fig. 4A), in addition to the tubules being full of spermatozoa (Fig. 4B). Seven days after treatment with a GnRHa implant that induced a significant increase in sperm volume without a significant decrease in spermatozoa density (Fig. 2B), the testes had tubules filled with a large number of spermatozoa in the central part of the gonad (Fig. 4C), whereas the cortical area still contained a significant number of spermatocysts with spermatogonia, spermatocytes and spermatids (Fig. 4D). On day 21, males treated with a GnRHa implant still produced significantly more sperm than non-treated controls (Fig. 2A), but at this time the whole testis contained only free spermatozoa (Fig. 4E and F). The somatic cells were greatly hypertrophied - which is a sign of the end of the spermiation period - and there were no spermatocysts with spermatocytes or spermatids, suggesting that all spermatocysts available at the beginning of the hormonal induction had undergone spermiogenesis and spermiation (spermatozoa release). So, in many fishes the resulting increase in sperm volume after hormonal stimulation involves the combination of (a) increased seminal fluid production that facilitates the release/stripping of existing spermatozoa and (b) development and spermiation of spermatocysts already at different stages of spermatogenesis.

7.2. Changes in motility parameters

Hormonal enhancement of spermiation does not usually influence sperm quality parameters such as sperm motility percentage, motility duration or spermatozoa velocity (Table 4). An exception may be seen in species that either spermiate very poorly in captivity – such as a number of flatfishes, or fish that do not produce any expressible milt in captivity, although their testes may contain small amounts of intratesticular sperm. For example in sterlet (Alavi et al., 2012), GnRHa and CPE induced spermiation in non-spermiating males in <24 h after treatment, an effect that was no doubt related to a stimulatory effect on seminal fluid production alone and the maturation of existing intratesticular spermatozoa, and not to any enhancement of spermatogenesis and/or spermiogenesis. Such increase in seminal fluid, together with increases in testicular steroid production (Miura et al., 1992; Vermeirssen et al., 1998, 2000) and changes in pH (Alavi and Cosson, 2005; Genz et al., 2014; Woolsey and Ingermann, 2003), have a significant positive effect on sperm quality, such as motility percentage and spermatozoa velocity. Similarly in common bream (Kucharczyk et al., 1997), PEs and hCG induced 2× increases in sperm volume within <36 h after treatment, resulting in a highly significant improvement in motility percentage (from 22 to 84%). As mentioned earlier in Atlantic halibut (Vermeirssen et al., 2004), sperm volume decreases significantly towards the end of the spawning season, with a concomitant dramatic

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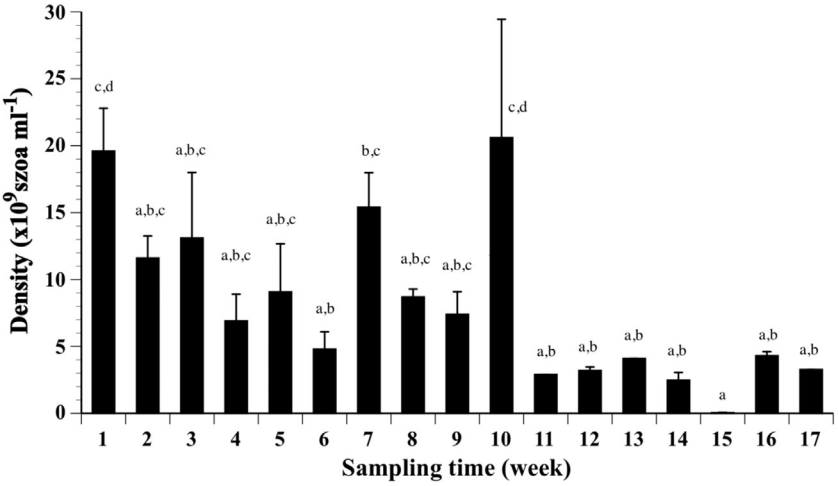

Fig. 3. Mean (+SEM) sperm density of meagre (*Argyrosomus regius*, n = 1–4) at weekly samplings during a spawning induction experiment (2014). All males were given a GnRHa implant at the beginning of the experiment, and were treated again as needed when sperm production was considered inadequate (approximately every 2–3 weeks). Statistically different means are indicated by different letter superscripts (one-way ANOVA, P = 0.012; DNMR, P ≤ 0.05).

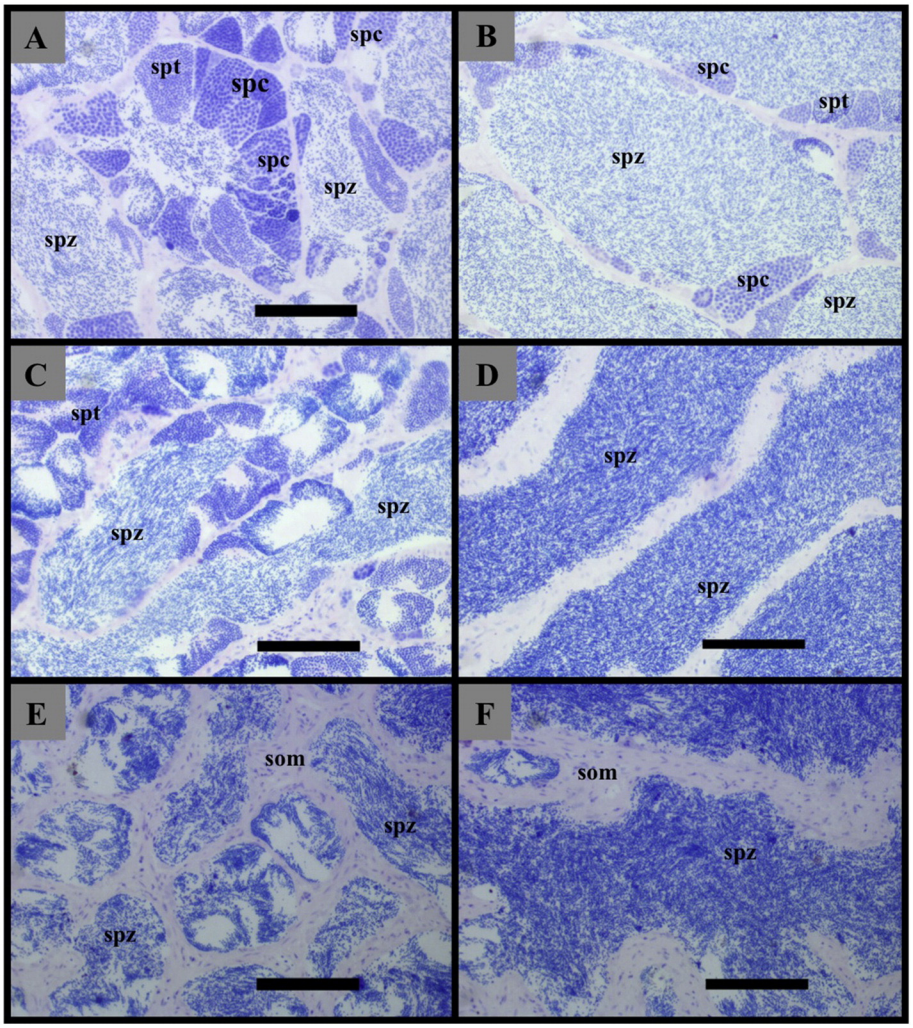

Fig. 4. Histological sections of testes from European seabass at different times after treatment with a GnRHa implant. At Day 0, the cortex of the testis (A) contained free spermatozoa, but also a large number of spermatocysts with spermatids and spermatocytes at various stages, while the central part (B) contained mostly spermatozoa. At Day 7 after GnRHa implantation, the cortical area of the testes (C) contained less intact spermatocysts with more advanced-stage gametes (spt), while the central part (D) contained exclusively spermatozoa. At Day 21, both the cortical (E) and central area (F) of the testes contained exclusively spermatozoa, without any intact spermatocysts with gamete cells at earlier developmental stages. In addition, the somatic cells lining the tubules became hypertrophied. Somatic cells (som), spermatocytes (spc), spermatids (spt) and spermatozoa (spz). The bar in all sections is 100 μm (fFrom the experiments reported by Rainis et al., 2003).

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increase in viscosity and a decrease in sperm motility. Treatment with GnRHa implants increased sperm volume, with a concomitant decrease in sperm density and a long-term increase in motility percentage (Vermeirssen et al., 2000, 2004) allowing the fertilization of eggs with good results. So, in general hormonal therapies for the enhancement of spermiation have positive effects on sperm quality characteristics only in species that have very viscous sperm in captivity or that do not produce any releasable sperm. In the vast majority of fishes, however, they have neither positive nor negative effects on sperm quality parameters.

7.3. Changes in sperm composition

7.3.1. Fatty acids

Polyunsaturated fatty acids are the main components of fish sperm membranes (Wathes et al., 2007). *In vitro* testis treatment with hCG in goldfish demonstrated that the T production stimulated by hCG was inhibited by n-3 series fatty acids such as EPA and DHA (Wade et al., 1994). Asturiano et al. (2001) found similar results in European seabass males, indicating that PUFA are capable of regulating prostaglandin and androgen production. In European eel males that were treated weekly with hCG injections to induce sexual maturation, levels of EPA, DHA and ARA remained unchanged during spermiation, suggesting an important role of PUFA in the development of spermatozoa membranes (Baeza et al., 2014). The latter study also showed significant correlations between different fatty acids and sperm quality parameters, especially between spermatozoa velocity and the consumption of ARA present in the testis (Baeza et al., 2015a). Also, in the same hormonally treated animals, correlations were found between sex steroids and fatty acids, suggesting that EPA in the testis may act as a modulator of androgen synthesis (Baeza et al., 2015b). In another study in European eel males, it was shown that after 10 weeks of hormonal treatment to induce sexual maturation, animals fed previously with different diets reached the same levels of PUFA in the sperm, highlighting the important role of fatty acids in sperm viability (Baeza, 2015; Butts et al., 2015). After the hormonal treatment, the sperm showed >60% of motile spermatozoa, with PUFA being the fatty acid class with the highest concentration in sperm, and DHA having the highest concentration among PUFA.

7.3.2. Seminal plasma proteins

During the last few years, the importance of seminal plasma proteins in relation to sperm quality has been demonstrated (Lahnsteiner, 2007; Peñaranda et al., 2010b). In Eurasian perch (*Perca fluviatilis*) the seminal plasma contains proteins that are involved in membrane trafficking, organization, spermatozoa motility and oxido-reductase activity (Shaliutina et al., 2012). In the same regard, the high presence of Cu/Zn superoxide dismutase (SOD) and Zn in Japanese eel sperm provided a higher resistance to reactive oxygen species (ROS) (Celino et al., 2011). In brown trout, lysozyme activities were higher in samples with high motility, whereas spermatozoal immunoglobulin concentrations were higher in samples with low motility (Lahnsteiner and Radner, 2010). Both results probably are related to antibacterial activity of the seminal plasma. Furthermore, anti-trypsin and anti-proteinase activity has been found in seminal plasma of freshwater species (Ciereszko et al., 1998; Dabrowski and Ciereszko, 1994). Although their function has not been elucidated, it seems that they have protective role against proteolysis. In fact, multiple forms of proteolytic enzymes have been found in seminal plasma of teleost fishes (Kowalski et al., 2003). Finally, Fe metabolism also seems to play a role in sperm quality, since transferrin polymorphism observed in seminal plasma of common carp was related to sperm motility parameters (Wojtczak et al., 2007b).

7.3.3. Ion concentrations and pH

Several ions (*e.g.* K^+, Na^+, Cl^-, Ca^{2+} and Mg^{2+}) can be found in the seminal plasma of fish, and Ca^{2+} and K^+ have been proposed to be the main ions involved in sperm motility activation in marine fish (Cosson et al., 2008; Morisawa, 2008; Pérez et al., 2016), but the exact mechanism through which this happens is still unknown. Moreover, there are only a few works studying the changes of ion concentrations during sperm capacitation. In hCG-treated European eel, the ionic composition of seminal plasma varied through the spermiation period in relation to sperm motility (Asturiano et al., 2004). The K^+ concentration increased with increasing sperm motility, while Ca^{2+} and Mg^{2+} concentrations decreased as sperm motility increased. The concentration of Na^+ showed a slight decreasing tendency and pH values had no significant differences, being constant between pH 8.4 and 8.6 during the whole assay. Miura et al. (2013) observed that sperm motility in Japanese eel decreased 24 h after injection of 17α-hydroxyprogesterone (a precursor of the MIS) in correlation with a decrease in seminal plasma K^+, while seminal plasma pH and Na^+ concentration were not affected by the hormonal treatment.

As mentioned earlier (Section 2.3) sperm maturation (*i.e.* capacitation) occurs in the sperm duct (Miura et al., 1992; Morisawa and Morisawa, 1988), and in salmonids and Japanese eel if the sperm is extracted from the testes without passing through the sperm duct, it becomes unable to acquire motility, unless it is incubated in a solution with HCO_3^- and high pH, or elevated concentrations of K^+ (Morisawa and Morisawa, 1988; Ohta et al., 1997a). In hCG-treated European eel, incubating sperm in extender solution with low pH (6.5) induced a reversible motility inhibition, which was not observed in sperm maintained at physiological pH of 8.5 (Peñaranda et al., 2010c). Using flow cytometer, it was shown for the first time that intracellular Ca^{2+} and K^+ levels increased with the hyperosmotic activation of sperm motility in the European eel (Gallego et al., 2014a). So, internal Ca^{2+} and K^+ fluctuations seem to participate in the initiation of motility in European eel sperm (Pérez et al., 2016), in agreement with the proposed model of sperm motility activation in marine fish (Morisawa, 2008). In the latter work, baseline levels of intracellular pH (pH_i) remained constant 30 s post-activation, and a gradual decrease was observed 60 s post-activation, in agreement with other published data (Oda and Morisawa, 1993).

7.4. Relationships between motility parameters, and fertilization and hatching rates

The use of high quality gametes from both males and females is an essential prerequisite to achieve high fertilization success and hatching, both for aquaculture and scientific purposes. With regards to sperm, motility percentage has been the most commonly used parameter to predict quality and fertilization potential. However, for many years the conventional method of motility evaluation has been subjective, and the more recent utilization of Computer-Assisted Sperm Analysis (CASA) systems (See Section 8 below) has made it possible to estimate a higher number of sperm parameters by an objective, rapid and accurate technique (Gallego et al., 2013b). As a result, today total motility percentage (TM, which includes all spermatozoa showing any movement) and progressive motility percentage (PM, which includes only spermatozoa that swim in an essentially straight line) estimated using CASA are recognized as important sperm parameters related to male fertility and sperm competition in fish (Rurangwa et al., 2004), and high correlations ($r > 0.7$) have been found between these parameters, and fertilization and hatching success in some marine fishes such as the pufferfish (*Takifugu niphobles*), Atlantic halibut (Ottesen et al., 2009) and red seabream (*Pagrus major*) (Liu et al., 2007). However, similar studies on the existence of such a relationship in other fishes have produced conflicting results, as negligible correlations were found between TM or PM and fertilization success (Bozkurt and Secer, 2006). In this respect, it is worth highlighting that fertilization trials should be carried out with an optimal sperm/egg ratio for each species - limiting to the fertilization process, and not in excess, which will allow differences in sperm quality to be reflected on fertilization success. In addition, such trials should be using a wide range of sperm motility

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values, to avoid masking the real correlations between the motility values, and fertilization and hatching success.

In addition to TM or PM being good indicators of fertilization ability, spermatozoa velocities may also predict the fertilization potential of spermatozoa. In pufferfish, it was shown that the coefficients of correlation between fertilization success and CASA-obtained curvilinear velocity (VCL), straight-line velocity (VSL) or angular path velocity (VAP) were higher than with TM and PM (Gallego et al., 2013a). Similar data has been reported in other marine species such as Atlantic salmon, Atlantic cod (*Gadus morhua*) and green swordtail (*Xiphophorus helleri*), in which spermatozoa velocity seems to be the major component that determines fertilization success and the proportion of the paternity through sperm competition (Gage et al., 2004; Gasparini et al., 2010; Rudolfsen et al., 2008b). Based on this information, new approaches for male broodstock selection through evaluation of sperm kinetics can be used in some fishes, which could optimize the reproductive efficiency in fish farms, making rational use of gametes possible and limiting the number of breeding males and, thus, reducing production costs.

8. Monitoring sperm quality parameters

Different parameters have been used to evaluate fish sperm quality, including sperm volume, aspect and color, sperm density, spermatozoa motility and morphometry and seminal plasma composition. However, new techniques are being developed and have improved the assessment methods (Bobe and Labbé, 2010; Cabrita et al., 2009; Cosson et al., 2008; Pérez et al., 2009; Valdebenito et al., 2015), although an evident lack of standardization can compromise the comparison of results between different laboratories (Rosenthal et al., 2010).

8.1. Sperm density

Very different methods have been used to evaluate sperm density, from the classical use of haemocytometers to spermatocrit determinations, flow cytometry and spectroscopy measurements. Sørensen et al. (2013) evaluated different techniques, demonstrating their strong and weak points and made emphasis on the need for protocol standardization (Table 5). The most common counting method for human spermatozoa is the use of a haemocytometer, which is classified by the World Health Organization as the 'gold standard' (WHO, 1999). However, this method is time consuming (Suquet et al., 1992), and precision relies on skilled personnel. Thus, several studies have been carried out to develop faster and more automated counting methods (reviewed in Fauvel et al., 2010).

Spermatocrit (*i.e.* the percentage of spermatozoa volume after centrifugation of sperm in a capillary tube) is a fast and easy method, and correlations between spermatocrit and haemocytometer estimations of sperm density have been reported for several species (reviewed previously by Sørensen et al., 2013). However, spermatocrit results can be influenced by the spermatozoa sedimentation capacity reported in some fish species (Fauvel et al., 2010), as well as spermatozoa head size changes that occur in some marine fishes during the spawning season (Asturiano et al., 2006; Butts et al., 2011c).

Flow cytometry may also be employed to determine the number of spermatozoa by measuring one or more fluorescent stains. The method features high precision, sensitivity, accuracy and speed (Cordelli et al., 2005). Sørensen et al. (2013) reported that "measurements made with flow cytometer had the strongest relationship to haemocytometer counts, demonstrating the usefulness of this technique". Computer-Assisted Sperm Analysis also allows the quantification of sperm density (Ehlers et al., 2011) and, finally, some researchers have applied also spectrophotometric techniques (Fauvel et al., 1999).

8.2. Computer-assisted sperm analysis (CASA)

Initially, evaluation of sperm motility was done by a subjective assessment of only two sperm motion traits: the percentage of motile spermatozoa and the duration of spermatozoa movement. Then, sperm samples were classified into arbitrarily selected scales of criteria usually comprising 3–5 categories (*e.g.* low, medium and high; I, II, III, IV and V; or 20, 40, 60, 80 and 100%). The development of CASA systems made possible the estimation of a higher number of sperm motion parameters using an objective, sensitive and accurate technique. These systems evolved from the multiple photo-micrographic exposure and video-micrographic techniques for spermatozoa tracks, using a computer equipped with imaging software (Rurangwa et al., 2004). This technique was first introduced in the 1980′s in mammalian sperm and much later modern CASA systems were adapted for fish spermatozoa (Kime et al., 1996; Van Look and Kime, 2003; Wilson-Leedy and Ingermann, 2007).

To use CASA, the spermatozoa movement is video-recorded after activation, and then a short sequence of the video file is analyzed (Figueroa et al., 2014; Kime et al., 1996; Wilson-Leedy et al., 2009). The CASA analysis consists of the determination of a high number of spermatozoa movement parameters using an objective, sensitive and accurate technique. The TM and PM parameters can provide a general overview of the quality of the sperm sample. However, for some authors the most useful parameters are the specific spermatozoa velocities (Gallego et al., 2013a; Kime and Tveiten, 2002; Rurangwa et al., 2004). The most commonly used parameters include VCL, VSL, VAP and beating cross frequency (BCF). Several companies produce CASA equipment, but the parameters recorded by each system are similar. The improvement of CASA techniques to evaluate sperm motility parameters that could not be assessed by the human eye, allowing the tracking of many individual spermatozoa and hundreds of motion tracks analyzed per sample in an objective and repetitive way, has permitted an increasing number of applications in a wide range of aquaculture-related research fields (Table 6). It is noteworthy that most of the parameters evaluated by CASA systems have been correlated positively with fertilization potential of the sperm, thus CASA is a very useful tool for assessing sperm quality in fish reproduction research, although it is not yet used widely in commercial production. However, CASA systems must be calibrated to each species of interest, trying to follow previously

Table 5
Resource requirements, advantages and disadvantages for the different quantitative methods used to determine fish sperm density (modified from Sørensen et al., 2013).

Quantification method	Requirements	Advantage	Disadvantages
Neubauer improved haemocytometer	Microscope, haemocytometer and trained personnel	Inexpensive, precise, and well described	Time consuming in the literature
Spermatocrit	Centrifuge, microhaematocrit tubes tube sealant, and microhaematocrit tube reader	Fast, precise, and low level of training	Inaccurate, and sperm sedimentation
Computer-Assisted Sperm Analysis (CASA)	Microscope, computer with frame grabber, CASA software, software calibration and specific training	Fast, additional measures of sperm quality may be obtained	Low precision, inaccurate, and trained personnel required
Flow cytometry	Flow cytometer, and specific training	Precise and accurate	Trained personnel required, need to extrapolate by equation, and expensive if fluorospheres are required

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Table 6
Representative applications of Computer-Assisted Sperm Analysis (CASA) in some aquaculture related areas.

Research area	Species	Common name	Reference
CASA development	*Acipenser ruthenus*	Sterlet	(Boryshpolets et al., 2013)
	Anguilla anguilla	European eel	(Gallego et al., 2013b)
	Cyprinus carpio	Common carp	(Boryshpolets et al., 2013)
	Danio rerio	Zebrafish	(Wilson-Leedy et al., 2009)
	Oncorhynchus mykiss	Rainbow trout	(Boryshpolets et al., 2013)
Sperm cryopreservation	*Acipenser baerii*	Siberian sturgeon	(Judycka et al., 2015; Sieczynski et al., 2015)
	Anguilla anguilla	European eel	(Asturiano et al., 2004, 2007)
	Brycon orbignyanus	Piracanjuba	(López et al., 2015)
	Clarias gariepinus	African catfish	(Rurangwa et al., 2001)
	Cyprinus carpio	Common carp	(Warnecke and Pluta, 2003)
	Dicentrarchus labrax	European seabass	(Cabrita et al., 2011)
	Gadus morhua	Atlantic cod	(Butts et al., 2011a)
	Perca fluviatilis	Eurasian perch	(Bernáth et al., 2015)
	Salmo salar	Atlantic salmon	(Dziewulska et al., 2011)
	Sparus aurata	Gilthead seabream	(Beirão et al., 2011b; Cabrita et al., 2010)
	Thymallus thymallus	Grayling	(Horváth et al., 2015)
Sperm chilled storage	*Anguilla anguilla*	European eel	(Peñaranda et al., 2010c)
	Hippoglossus hippoglossus	Atlantic halibut	(Babiak et al., 2006)
	Oncorhynchus mykiss	Rainbow trout	(Lahnsteiner et al., 2004)
	Salmo trutta	Brown trout	(Formicki et al., 2015)
	Steindachneridion parahybae	Surubim-do-Paraíba	(Sanches et al., 2015)
Activation media tests	*Coregonus lavaretus*	European whitefish	(Dziewulska et al., 2015)
	Cyprinus carpio	Common carp	(Zarski et al., 2015)
	Oncorhynchus mykiss	Steelhead	(Kanuga et al., 2012)
	Perca fluviatilis	Eurasian perch	(Lahnsteiner, 2011)
Broodstock management	*Dicentrarchus labrax*	European seabass	(Felip et al., 2006)
	Solea senegalensis	Senegalese sole	(Beirão et al., 2011a)
Spermiation induction	*Acipenser ruthenus*	Sterlet	(Alavi et al., 2012)
	Anguilla anguilla	European eel	(Asturiano et al., 2005; Gallego et al., 2015)
	Hippoglossus hippoglossus	Atlantic halibut	(Vermeirssen et al., 2004)
	Osmerus eperlanus	Smelt	(Kowalski et al., 2012)
In vitro fertilization trials	*Anguilla anguilla*	European eel	(Butts et al., 2014)
	Hippoglossus hippoglossus	Atlantic halibut	(Ottesen et al., 2009)
	Oncorhynchus mykiss	Rainbow trout	(Tuset et al., 2008c)
	Pagrus major	Red seabream	(Liu et al., 2007)
Diet evaluation	*Anguilla anguilla*	European eel	(Baeza et al., 2015a; Butts et al., 2015)
	Dicentrarchus labrax	European seabass	(Martínez-Páramo et al., 2012a, 2012b)
	Gadus morhua	Atlantic cod	(Butts et al., 2011c)
	Solea senegalensis	Senegalese sole	(Beirão et al., 2015)
Comparative physiology	*Acipenser gueldenstaedtii*	Russian sturgeon	(Li et al., 2012)
	Gadus morhua	Atlantic cod	(Butts et al., 2010c)
	Hippoglossus hippoglossus	Atlantic halibut	(Alavi et al., 2011)
	Oncorhynchus mykiss	Rainbow trout	(Wojtczak et al., 2007a)
	Oncorhynchus tshawytscha	Chinook salmon	(Lehnert et al., 2012)
	Salvelinus namaycush	Lake trout	(Galvano et al., 2013)

standardized methods, and details of the set-up must be reported together with the obtained results to guarantee repetitiveness of the process.

8.3. Assisted sperm morphometry analysis (ASMA)

The development of ASMA software for the study of spermatozoa morphometry has introduced a new approach for sperm evaluation studies, demonstrating that changes in the spermatozoa head and/or flagella are related with the reproductive season, the effect of hormonal treatments or the cryopreservation processes, and how this can be related to changes in sperm motility and fertilization capacity. These aspects have been explored in an increasing number of aquaculture studies (Table 7).

Spermatozoa ultrastructure and morphology were studied previously by transmission electron microscopy, scanning electron microscopy or laser light-scattering spectroscopy and stroboscopic illumination (reviewed by Pérez et al., 2009). However, these techniques are subjective and time-consuming, and when spermatozoa morphology is analyzed by these visual methods, the intra- and inter-observer laboratory variations are usually very large (Soler et al., 2003). The ASMA has introduced a higher repeatability and validity in these morphological evaluations. Moreover, a high number of spermatozoa can be measured using ASMA, allowing fast and accurate studies of sperm samples, with much less expensive equipment required, in comparison with electron microscopy techniques. As a result, ASMA has been used increasingly in mammalian research since the 1990′s, but its application to fish is much more recent. However, fish sperm differs in many aspects from that of mammals (Kime et al., 2001) and the ASMA methodology used for livestock production animals needs set-up adjustments to be applicable to fish (Van Look and Kime, 2003). One of the first applications of ASMA methodology in fish was carried out in the European eel, comparing the results obtained by ASMA to those obtained by scanning electron microscopy (Marco-Jiménez et al., 2006a). Later, ASMA was used to describe spermatozoa morphology changes during sperm maturation under hormonal induction (Asturiano et al., 2006), as well as evaluating the osmotic effects suffered by spermatozoa and the cryoprotectant effects during cryopreservation (Asturiano et al., 2007; Garzón et al., 2008; Marco-Jiménez et al., 2006b; Peñaranda et al., 2010c).

Several studies have evaluated the relationships between morphology, motility and fertilization capacity in European eel (Asturiano et al., 2006), rainbow trout (Tuset et al., 2008a) and Atlantic cod (Butts et al., 2010b; Tuset et al., 2008b). Other studies have tried to improve the ASMA techniques. For example, different staining techniques were compared for the morphometric study of rainbow trout sperm (Tuset et al., 2008c), two techniques were compared for the morphometric study of gilthead seabream spermatozoa (Gallego et al., 2012a), and the use of an ASMA open-source software was explored (Butts

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Table 7
Representative applications of assisted sperm morphometry analysis (ASMA) software in some aquaculture related areas.

Research area	Species	Common name	Reference
Reproductive strategies	*Gadus morhua*	Atlantic cod	(Butts et al., 2010b)
	Oncorhynchus tshawytscha	Chinook salmon	(Flannery et al., 2013)
Sperm chilled storage and/or cryopreservation	*Anguilla anguilla*	European eel	(Marco-Jiménez et al., 2006b; Peñaranda et al., 2010c) (Asturiano et al., 2007; Garzón et al., 2008)
	Dicentrarchus labrax	European seabass	(Peñaranda et al., 2008)
	Gadus morhua	Atlantic cod	(Butts et al., 2010a)
	Sparus aurata	Gilthead seabream	(Gallego et al., 2012a)
Relation with sperm motility	*Anguilla anguilla*	European eel	(Asturiano et al., 2006)
	Gadus morhua	Atlantic cod	(Butts et al., 2010b; Tuset et al., 2008a)
	Oncorhynchus mykiss	Rainbow trout	(Tuset et al., 2008c)
Technique development	*Anguilla Anguilla*	European eel	(Asturiano et al., 2006)
	Gadus morhua	Atlantic cod	(Butts et al., 2011c)
	Oncorhynchus mykiss	Rainbow trout	(Tuset et al., 2008b)
	Oncorhynchus tshawytscha	Chinook salmon	(Butts et al., 2011b)
Comparative physiology	*Anguilla anguilla*	European eel	(Gallego et al., 2014a; Peñaranda et al., 2010b)
	Diplodus puntazzo	Sharpsnout seabream	(Marco-Jiménez et al., 2008)
	Gadus morhua	Atlantic cod	(Butts et al., 2010b, 2011c)
	Sparus aurata	Gilthead seabream	(Marco-Jiménez et al., 2008)
	Takifugu niphobles	Pufferfish	(Gallego et al., 2014b)

et al., 2011b). During the last years, ASMA techniques are becoming relatively common in studies of fish reproductive strategies or fish comparative physiology (Table 7).

8.4. Fluorescent staining

Cell viability (evaluated based on cell-membrane integrity) and mitochondrial functionality (*i.e.* ATP synthesis to maintain sperm motility) have also been used as indicators of fish sperm quality, using simple and rapid protocols based on the use of fluorescent staining (He and Woods, 2004; Ogier de Baulny et al., 1999; Rurangwa et al., 2004; Segovia et al., 2000). To assess the non-viable cells, membrane-impermeable nucleic acid stains can be used, which identify positively dead spermatozoa by penetrating into cells with damaged membranes. An intact plasma membrane will prevent these products from entering the spermatozoa and staining the nucleus. Phenanthridines, such as propidium iodide (PI, Garner et al., 1994), SYBR14 (Garner et al., 1994; Segovia et al., 2000) and bisbenzimidazole Hoechst 33258 (De Leeuw et al., 1991; Garzón et al., 2008) have been used commonly. The use of sperm viability kits, combining the SYBR Green and PI stains, has become popular in fish research during the last years because they allow classification of spermatozoa as dead when nuclei show red fluorescence over spermatozoa head and as alive when they show green fluorescence (Gallego et al., 2012a). Mitochondrial function can be assessed using rhodamine 123 (Segovia et al., 2000) or 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), allowing a distinction between spermatozoa with poorly and highly functional mitochondria (Asturiano et al., 2006; Graham, 2001).

8.5. Other techniques

The cryopreservation of the sperm can cause different damages to the spermatozoa. For example, it can induce DNA fragmentation and changes in proteins profile (Zilli and Vilella, 2012), as well as increases on the reactive oxygen species (ROS) inducing alterations at the DNA level (Martínez-Páramo et al., 2012a; Thomson et al., 2009). Thus, some techniques have been specifically developed during the last years to evaluate the effects of the freezing-thawing processes. For example, the Comet assay is used for the measurement of DNA damage, evaluating the grade of DNA fragmentation (Cabrita et al., 2005; Riesco et al., 2011). The TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end-labeling) and SCSA (sperm chromatin structure assay) assays are flow cytometry-based techniques used as well to evaluate DNA breaks and fragmentation (Bungum et al., 2011; Chohan et al., 2006). Finally, during the last years, new techniques providing specific information about damages in specific genes have been developed using qPCR approaches. For example, after gilthead seabream sperm cryopreservation, the lesions generated in nuclear genes (having important roles in embryo development) and mitochondrial genes have been recently quantified (Cartón-García et al., 2013). It is expected that these and other new techniques will be used more widely in the field of sperm quality in the future.

8.6. Spermatozoa subpopulations

Computer-Assisted Sperm Analysis systems are able to track a very large number of spermatozoa per capture/frame, which means hundreds of motion tracks analyzed per sample. Despite working with such extensive databases, sperm motility analyses often show the mean parameter values, considering the whole sperm sample as homogeneous. However, it has been pointed out that the sperm of some species is not a homogeneous mixture, and different spermatozoa subpopulations coexist in the same sperm sample (Gallego, 2013). Thus, the spermatozoa can be classified into different subpopulations within each sample according to their kinetic characteristics (Holt et al., 2007) making use of multivariate statistical methods such as cluster analysis (Gallego et al., 2015; Martínez-Pastor et al., 2011). Using these kinetic parameters, the samples are characterized considering not only the mean values of CASA parameters, but also the relative proportions of each subpopulation (Holt and Harrison, 2002; Martinez-Pastor et al., 2005).

Sperm subpopulation analysis has been studied mostly in mammals (Dorado et al., 2010, 2011; Quintero-Moreno et al., 2003), but it has been applied in a few studies in fish (reviewed by Gallego, 2013) demonstrating the coexistence of 3–4 motility-based subpopulations of spermatozoa in the sperm of different species, such as Senegalese sole (Beirão et al., 2009; Martínez-Pastor et al., 2008), gilthead seabream (*Sparus aurata*) (Beirão et al., 2011b), three-spined stickleback (*Gasterosteus aculeatus*) (Le Comber et al., 2004), steelhead trout (*Oncorhynchus mykiss*) (Kanuga et al., 2012) and European eel (Gallego et al., 2015).

The spermatozoa subpopulations found in the different species have several combinations of velocity and linearity, but all the studies have described a 'fast and linear' subpopulation that apparently includes the best-quality spermatozoa (Beirão et al., 2009; Martínez-Pastor et al., 2008) and has been correlated positively with total motility, implying that the samples with the highest proportion of these spermatozoa tended to show the highest motility (Gallego et al., 2015). The rest of the subpopulations could be related with exhausted spermatozoa near cessation of swimming, and probably unable to fertilize an egg

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(Gallego et al., 2012b), or even being germ cells forced out during the stripping process (Marco-Jiménez et al., 2006a).

The number, the proportion and the motility characteristics of the subpopulations present in the sperm comprise useful information whose biological meaning can be analyzed in relation to reproductive strategies, hormonal treatments, individual male differences, sperm aging, sperm storage or cryopreservation effects, testis physiology and male fertility (Figueroa et al., 2014; Gallego et al., 2014b, 2015; Martínez-Pastor et al., 2008).

9. Representative practical protocols of the use of hormones for the enhancement of spermiation in fishes

Protocols for the enhancement of spermiation have been developed for a variety of commercially important fishes, and below we present some practical information about three representative approaches that have been shown to be effective in enhancing spermatogenesis and sperm production in freshwater eels, freshwater and marine fish.

The most impressive application of a hormonal therapy for the induction of spermatogenesis and spermiation is the protocol used for freshwater eels. Hormonal treatments are based on weekly administration of hCG, normally after a gradual acclimatization to seawater, maintaining the water temperature at 20 °C and fasting the fish throughout the treatment. In the Japanese eel, male maturation has been induced with a single high dose treatment of hCG (Miura et al., 1991c), but better results have been obtained using weekly injections of lower doses of 1000 IU kg^{-1} body weight, allowing the collection of sperm after the 5–6th week of treatment (Ohta et al., 1996; Ohta et al., 1997b). In the European eel, weekly injections of 1500 IU kg^{-1} have been used (Asturiano et al., 2006; Müller et al., 2004; Peñaranda et al., 2010a; Pérez et al., 2000). Sperm samples of high volume and density ($3–6 \times 10^9$ spermatozoa ml^{-1}) are collected between the 6th and 13th weeks of treatment, always 24 h after the administration of the hormone, as studies have demonstrated that this is the time when the highest sperm quality is obtained (Pérez et al., 2000). Different hormonal preparations, such as hCG, rehCG and PMSG - which is less expensive than either hCG or rehCG - were evaluated in a recent trial (Gallego et al., 2012b). The rehCG produced the best results in relation to sperm volume, density, motility and kinetic features throughout most weeks of treatment. Moreover, rehCG was the most cost effective treatment, making it possible to obtain more good quality sperm samples at a lower price than by using the other two hormonal treatments.

For freshwater fishes, a spermiation enhancement protocol should first consider whether the species in question exhibits or not a dopamine inhibition of the action of GnRH, since both cases are common. Carps, in general, exhibit a dopamine inhibition of GnRH stimulated release of LH (Peter et al., 1988). Common carp spawn spontaneously in ponds, and eggs or fry can be collected (Rothbard and Yaron, 1995; Yaron et al., 2009). However, induced spawning is often preferred for intensive hatchery production. The breeders need to be exposed to the appropriate environmental cycling to ensure the progress of gametogenesis and although males often have adequate sperm production, it is common practice to also induce males to enhance sperm production. Sperm production has been enhanced with CPE (Horvath et al., 1985; Saad and Billard, 1987), gonadotropin-calibrated CPE (Rothbard and Yaron, 1995) or GnRHa in combination with or without DA (Billard et al., 1987; Roelants et al., 2000; Saad and Billard, 1987; Takashima et al., 1984). Doses of 10 μg kg^{-1} of GnRHa (Billard et al., 1987; Takashima et al., 1984) or 10 μg kg^{-1} of GnRHa in combination with 10 mg kg^{-1} of the DA pimozide or metoclopramide (Billard et al., 1987; Roelants et al., 2000) provided a >4× increase in sperm volume of similar quality, 24 h after hormone treatment. After treatment with GnRHa/DA, circulating plasma LH levels peaked at 24 h and were maintained elevated until 40–48 h before decreasing (Billard et al., 1987), and plasma levels of LH were directly correlated to volume of sperm collected (Roelants et al., 2000), indicating that the most adequate time point for sperm collection was 24 h after treatment. In the case of another cyprinid, the tench, although studies provided evidence of a dopaminergic inhibition of LH release, both GnRHa alone and GnRHa/DA treatments were equally effective in inducing ovulation (Podhorec et al., 2011). An injection of 20 μg kg^{-1} of salmon GnRHa (Linhart et al., 1995) or mammalian GnRHa (Caille et al., 2005) increased sperm production by 3–4× compared to controls and gave a similar result as an implant of 25 μg kg^{-1} of mammalian GnRHa or an injection of 0.5–2 mg kg^{-1} of CPE. Sperm was collected over a 5-day period after hormonal treatment (Linhart et al., 1995) and production was observed to peak at 72 h post treatment (Caille et al., 2005).

Finally, controlled-release delivery systems loaded with GnRHa have been used successfully in a variety of marine fishes including European seabass, striped bass, Atlantic halibut and meagre, among others. Treatment is usually done at the same time females are treated for spawning, or whenever sperm production appears diminished during the spawning season. Treatments may be repeated after a few weeks, if spermiation declines again, once the release of GnRHa from the delivery system is exhausted. The usual GnRHa treatment dose ranges between 20 and 50 μg GnRHa kg^{-1} body weight and an increase in sperm production can be within 24–48 h. The duration of elevated sperm production lasts for 2–5 weeks (depending on species, GnRHa delivery system and water temperature), and it is usually not associated with changes in sperm quality parameters, such as density, motility percentage or duration. Sperm collection in European seabass in response to hormonal therapy may range between 1 and 4 ml kg^{-1} at every sampling, which can be done on a weekly basis.

10. Conclusion

The review has demonstrated the wide range of successful hormone manipulations that have been employed to date to enhance sperm production in fishes. So far, gonadotropin preparations (PEs and hCG) and GnRHas have been used (with or without DA), though other more recently identified hormones may become important in future applications. Recent studies have demonstrated the potential of reGtHs to both induce spermatogenesis and spermiation, although more work is needed to increase biological activity and establish practical applications. In particular, the use of reFSH to stimulate the early stages of spermatogenesis offers the potential to control the entire process of spermatogenesis, while reLHs also offer great potential to stimulate spermiation and increase sperm volume, while avoiding undesirable effects that can be associated to non-homologous PEs. Pheromones also appear to offer good potential to enhance spermiation in the future. With more research on their precise function, it appears possible that pheromones may induce rapid increases in sperm volume that could be helpful in species with low collectable sperm volumes. Existing methods to evaluate sperm quality have improved during the last years, becoming more accurate and objective, while some new ones are currently under development. However, more efforts are required to standardize the use of all evaluation methods in fish, in order to assure the repeatability of the measurements and to enable easy and meaningful comparisons between studies.

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