Document downloaded from:

http://hdl.handle.net/10251/104772

This paper must be cited as:

Mylonas, CC.; Duncan, NJ.; Asturiano Nemesio, JF. (2017). Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture. 472:21-44. doi:10.1016/j.aquaculture.2016.04.021

The final publication is available at

https://doi.org/10.1016/j.aquaculture.2016.04.021

Copyright Elsevier

Additional Information

Document downloaded from:

http://hdl.handle.net/10251/104772

This paper must be cited as:

Mylonas, CC.; Duncan, NJ.; Asturiano Nemesio, JF. (2017). Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture. 472:21-44. doi:10.1016/j.aquaculture.2016.04.021

The final publication is available at

https://doi.org/10.1016/j.aquaculture.2016.04.021

Copyright Elsevier

Additional Information

*Manuscript
Click here to download Manuscript: Mylonas et al 20160325.docx
Click here to view linked References

2

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

4

Constantinos C. Mylonas[1,*], Neil J. Duncan[2], Juan F. Asturiano[3]

6

[1] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Center for Marine
8 Research, P.O. Box 2214, Iraklion, Crete 71003, Greece

[2] IRTA, Sant Carles de la Rapita, 43540 Tarragona, Spain

10 [3] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal,
Universitat Politècnica de València, Edificio 7G, 46022 Valencia, Spain

12

*Corresponding author: +30 2810 337878 tel; +30 2810 337778 fax; mylonas@hcmr.gr

14

16

Table of Contents

1. INTRODUCTION 4

18 **2. SPERMATOGENESIS AND SPERMIATION 5**

2.1. DIFFERENT TYPES OF TESTIS DEVELOPMENT 5

20 2.2. ENDOCRINE CONTROL 9

2.3. SPERM MATURATION 10

22 **3. NEED FOR THE USE OF EXOGENOUS HORMONES IN MALE BREEDERS 12**

3.1. HORMONAL INDUCTION OF SPERMATOGENESIS AND SPERMIATION 12

24 3.2. ENHANCEMENT OF SPERM PRODUCTION IN POORLY SPERMIATING FISHES 13

3.3. SYNCHRONIZATION OF SPERM PRODUCTION TO OVULATION FOR *IN VITRO* FERTILIZATION 15

26 **4. ENVIRONMENTAL FACTORS INFLUENCING SPERMATOGENESIS AND SPERMIATION 16**

5. HORMONAL METHODS TO ENHANCE SPERM PRODUCTION 18

28 5.1. GTH PREPARATIONS OF PISCINE ORIGIN 18

5.2. RECOMBINANT GTHS OF PISCINE ORIGIN 22

30 5.3. GTH PREPARATIONS OF MAMMALIAN OR HUMAN ORIGIN 24

5.4. GNRHA INJECTIONS AND DELIVERY SYSTEMS 26

32 **6. OTHER APPROACHES TO ENHANCE SPERM PRODUCTION 29**

6.1. PHEROMONE APPLICATIONS 29

34 6.2. HORMONES ADDED TO SPERM *IN VITRO* TO INCREASE SPERM QUALITY 31

7. EFFECT OF HORMONAL THERAPIES ON SPERM QUALITY 32

36 7.1. CHANGES IN SPERMATOZOA DENSITY 32

7.2. CHANGES IN MOTILITY PARAMETERS 34

38 7.3. CHANGES IN SPERM COMPOSITION 35

7.4. RELATIONSHIPS BETWEEN MOTILITY PARAMETERS, AND FERTILIZATION AND HATCHING RATES 38

40 **8. MONITORING SPERM QUALITY PARAMETERS 40**

8.1. SPERM DENSITY 40

42 8.2. COMPUTER-ASSISTED SPERM ANALYSIS (CASA) 41

8.3. ASSISTED SPERM MORPHOMETRY ANALYSIS (ASMA) 42

44 8.4. FLUORESCENT STAINING 44

8.5. OTHER TECHNIQUES 45

46 8.6. SPERMATOZOA SUBPOPULATIONS 45

9. REPRESENTATIVE PRACTICAL PROTOCOLS OF THE USE OF HORMONES FOR THE ENHANCEMENT
48 **OF SPERMIATION IN FISHES 47**

10. CONCLUSION 49

50 **11. ACKNOWLEDGEMENTS 50**

12. REFERENCES 52

52

Abstract

54 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
56 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
58 spermatozoa subpopulations. Hormonal manipulations are employed to induce spermatogenesis in species such as the freshwater eels, to synchronize maximal sperm volume
60 to ovulation for *in vitro* fertilization and to enhance sperm production in species with poor spermiation. The hormones that are employed include gonadotropins (GtH) of piscine or
62 mammalian origin, and gonadotropin-releasing hormone agonists (GnRHa) administered by injections or controlled-release delivery systems, with or without dopaminergic inhibitors.
64 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
66 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
68 parameters have been used to evaluate fish sperm quality, including sperm volume and density, spermatozoa motility and morphometry, and seminal plasma composition. The
70 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
72 accurate technique. The development of Assisted Sperm Morphology Analysis (ASMA) software has introduced a new approach for sperm evaluation studies, demonstrating changes
74 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
76 fertilization capacity. The article concludes with a few practical protocols for the enhancement of sperm production in aquaculture species.

78

Keywords: spermiation, broodstock management, sperm enhancement, sperm quality

80

1. Introduction

82 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
84 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
86 to condition the fish and stimulate them to undergo gametogenesis (oogenesis and spermatogenesis), maturation and spawning. However, in many commercially produced
88 species, there are important reproductive dysfunctions that hinder the efficient and reliable production of fertilized eggs (Mylonas et al., 2010). Reproductive dysfunctions are most
90 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
92 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;
94 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
96 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
98 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
100 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
102 knowledge on the reproductive function of male fish in aquaculture, broodstock management and methods to enhance spermatogenesis and sperm production. In addition, the article
104 provides an extensive review of the available sperm quality evaluation methods.

2. Spermatogenesis and spermiation

106 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
108 spermatogonia, which then undergo multiple mitotic divisions –their total number being species-specific and genetically determined- that result in late B spermatogonia. In phase
110 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
112 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
114 & Miura, 2001; Miura et al., 2002; Schulz & Miura, 2002; Schulz et al., 2010; Vizziano et al., 2008).

116 The spermatozoa are released from the spermatocysts into the sperm ducts where maturation -the process that renders them capable of vigorous motility and fertilization
118 (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
120 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
122 "stripping"). Sperm release can be synchronized with female spawning via tactile or pheromonal communications (Stacey, 2003).

124

2.1. Different types of testis development

126 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;
128 Schulz & 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
130 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
132 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
134 development of the germ cells that leave the spermatocysts.

Different species of fish present different seasonality in spermatogenesis and
136 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.
138 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
140 spermatogenesis amongst species results in differences in the presence and abundance of the different developmental stages of germ cells observed amongst species. Guppies (Poecilidae)
142 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).
144 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
146 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.
148 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.
150 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
152 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
154 ordered by phylogeny (Parenti & Grier, 2004) with clearly defined structural criteria, based on the tubule or lobule network and the position of spermatogonia and spermatocysts (Grier et
156 al., 1980; Grier, 1993; Parenti & 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
158 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
160 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
162 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
164 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
166 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
168 at different stages of development throughout the lobules, and spermatocysts may move only slightly towards the lumen prior to the release of the spermatozoa.

170 Important aquaculture species have both tubular and unrestricted lobular testis type. Tubular type testes are found in various fishes (Parenti & Grier, 2004), ranging from the
172 primitive non-teleost order Acipenseriformes (which includes paddlefish and sturgeons) to early teleost orders such as Cypriniformes (carps), Siluriformes (catfishes), Salmoniformes
174 (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
176 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

178 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,
180 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
182 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
184 (*Lutjanus guttatus*, a Perciformes species with unrestricted lobular type testes) (Ibarra-Castro & Duncan, 2007) is difficult and the amount of sperm collected is usually not sufficient for
186 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
188 (Viveiros et al., 2002).

Testicular development can also be classified as cystic or semicystic. In cystic
190 development, when the spermatocysts rupture they release spermatozoa, while in semicystic development they release spermatids, which then differentiate into spermatozoa and complete
192 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
194 to have semicystic development, including Perciformes–Bleniidae (Lahnsteiner & Patzner, 1990), Ophiliiformes-Ophiliidae (Mattei et al., 1993), Scorpaeniformes–Scorpaenidae
196 (Muñoz et al., 2002) Pleuronectiformes–Soleidae (Garcia-Lopez et al., 2005), Siluriformes–Callichthyidae (Spadella et al., 2007), Syngnathiformes–Syngnathidae (Biagi et al., 2010) and
198 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
200 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
202 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
204 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
206 sperm volume requirements for successful reproduction due to the paired spawning
behaviour, where the male and female spawn with genital ducts held closely together so that
208 the released sperm is immediately next to the released eggs (Morais et al., 2014).

210 **2.2. Endocrine control**

Hormonal regulation of fish spermatogenesis and spermiation has been described previously
212 (Billard et al., 1990; Miura & Miura, 2001; Miura et al., 2002; Peñaranda et al., 2010a; Schulz
& Miura, 2002; Schulz et al., 2010; Vizziano et al., 2008; Watanabe & Onitake, 2008).
214 Spermatogenesis and spermiation are regulated by the two pituitary gonadotropins (GtH),
namely Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), whose synthesis
216 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
218 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
220 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
222 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
224 LH/FSH production, though both stimulatory and inhibitory effects have been documented
(Biran et al., 2014; Moussavi et al., 2013; Tsutsui & Ubuka, 2014; Wang et al., 2015). The
226 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

228 (Miura & 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

230 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

232 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

234 Sertoli cells (Miura & Miura, 2003). The androgen 11 ketotestosterone (11-KT) is the major regulator of spermatogenesis and spermiogenesis, while 17,20β-dihydroxy-4-pregnen-3-one

236 (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

238 maturation (Miura & Miura, 2003), at which stage the spermatozoa acquire the capacity for forward motility and fertilization upon spawning. Regarding the function of the two GtHs,

240 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

242 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

244 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

246 differentiation, and germ cell development (Miura & Miura, 2001; Schulz & Miura, 2002; Schulz et al., 2010).

248

2.3. Sperm maturation

250 Sperm maturation (*i.e.* spermiation) is the phase of male gamete development during which the spermatozoa change from nonfunctional, flagellated gametes to fully matured

252 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
254 sperm duct (Miura et al., 1992; Morisawa & Morisawa, 1988), and the progestin 17,20β–P
together with changes in pH and ionic composition of seminal plasma are involved in this
256 process (see section 6.2.)

In salmonids and Japanese eel (*Anguilla japonica*) the sperm is not capable of motility
258 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 & Morisawa,
260 1988; Ohta et al., 1997a,b). 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
262 increase of pH of seminal plasma, which induces an increase of sperm cAMP, allowing its
motility (Miura et al., 1992). Miura & Miura (2003) proposed a model in the Japanese eel in
264 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.

266 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
268 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
270 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
272 the increase of sperm volume (and decrease of sperm density) during spermiation. Cyclical
shifts in gonadal steroidogenesis have been described in group-synchronous spawning
274 species, causing waves of progestins in the plasma, related with each spawning event or with
peaks of sperm production (Asturiano et al., 2002).

276

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

278 Although spawning induction therapies are more often targeted towards females in aquaculture situations, hormonal therapies in male broodstock may be needed for the 280 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, 282 for example in order to facilitate sperm collection or increase availability during *in vitro* fertilization protocols.

284

3.1. Hormonal induction of spermatogenesis and spermiation

286 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,c, 2002; 288 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 290 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 292 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 294 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 296 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 298 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 300 the exogenous GtH hormonal treatment, spermatogenesis proceeds and spermiation is achieved.

302 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
304 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
306 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)
308 were used to induce complete maturation of these fish (Komatsu et al., 2006a). The treatment promoted spermatogenesis, inducing the development and maturation of spermatogenic germ
310 cells, and leading to spermiation in under-yearling male testes.

In Siluridae *spp*, various hormonal therapies –though producing variable results- have
312 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
314 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
316 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
318 collecting sperm by stripping in catfishes could not be solved by the administration of exogenous hormones.

320

3.2. Enhancement of sperm production in poorly spermiating fishes

322 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
324 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, excessive
326 handling during the spawning season, etc.) or as a result of selective breeding for fast growth

(Mylonas & Zohar, 2001b; Zohar, 1996; Zohar & Mylonas, 2001). In these situations, males
328 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
330 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
332 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
334 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
336 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
338 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 & Zohar, 2001b). In fact, treatment of
340 fish with GnRHa or GtH preparations has a marked effect on seminal fluid production
(Clemens & Grant, 1964; 1965; LaFleur & Thomas, 1991; Takashima et al., 1984).
342 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
344 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
346 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
348 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
350 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

352 increased seminal fluid production, which is necessary to allow the spermatozoa to be "washed" out of the testes (Fig. 1).

354

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

356 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
358 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
360 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
362 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
364 overripe after ovulation and lose their fertilization potential (Bromage & Roberts, 1995; Duncan et al., 2013; Mylonas et al., 2010; Urbanyi et al., 2009). Therefore, most research –
366 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
368 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
370 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
372 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
374 and method of hormone used, the species and temperature (these aspects will be considered in section 5). In males, the latency time from hormone application to spermiation is generally
376 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 & 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, rains, etc.), 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 & 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 >

402 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
404 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
406 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
408 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,
410 Eicosapentaenoic, EPA; Docosahexaenoic, DHA and Arachidonic acid, ARA) and anti-oxidants (vitamin E and selenium) (Asturiano et al., 2001; Beirão et al., 2015). Temperature
412 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
414 al., 2014; Breckels & Neff, 2013; Gallego et al., 2012b; Williot et al., 2000). Consequentially, temperature has been used in some fishes to predict the spawning period
416 (Baynes et al., 1993; Rothbard & Yaron, 1995) and to manipulate an advance, delay or elongation of the spawning period (Brauhn, 1971; Hall et al., 2002; Lang & Tiersch, 2007).

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

426 production and once this has been achieved then the need for further measures (*i.e.* exogenous hormones) to improve sperm production can be assessed.

428

5. Hormonal methods to enhance sperm production

430 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

432 exact nature of the endocrine control of spermatogenesis and spermiation was known (see reviews by Mylonas & Zohar, 2001a; Zohar & Mylonas, 2001). Later on, evidence had

434 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,b, 1998b; Mylonas &

436 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

438 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 --

440 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

442 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

444 spermiogenesis and spermiation, but this effect may be through stimulation of other hormones (Schulz et al., 2010), especially in the case of using GnRHas.

446

5.1. GtH preparations of piscine origin

448 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

450 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 & Mylonas, 2001).

452 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.

454 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

456 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

458 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.

460 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

462 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

464 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

466 needing long-term hormonal treatments with repeated administration of these extracts. Finally, PEs can transmit diseases to very expensive and genetically selected broodstock.

468 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

470 (Cyprinidae family) GtHs have been commercialized, although species-specificity of fish LH can limit their use only to phylogenetically-related fishes.

472 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

474 sperm production 1 d after treatment in common tench (*Tinca tinca*) (Linhart et al., 1995) and

higher sperm volume and motility was obtained from common bream (*Abramis brama*)
476 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
478 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,
480 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
482 (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),
484 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).
486 Spermatozoa motility and fertilization success of sperm from hormone treated males were not
statistically different compared to controls (Miranda et al., 2005).

488 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 & Godinho,
490 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
492 was induced in pirapitinga (*Piaractus brachypomus*) after a single intramuscular injection
(Nascimento et al., 2010). Finally in the tambaqui (*Colossoma macropomum*), intramuscular
494 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 & Godinho, 2009). Also, various
496 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 more
498 than 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

500 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

502 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

504 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}

506 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

508 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

510 highest numbers of spermatozoa per individual were collected 15-42 h post CPE treatment; the sperm motility ranged between 26-100% depending on the stripping sequences; higher

512 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

514 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.,

516 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).

518 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

520 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

522 beluga may be closely related to the genus *Acipenser*, even though it has been assigned to the genus *Huso*.

524 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 526 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, 528 and manuals or books written in local languages.

530 **5.2. Recombinant GtHs of piscine origin**

Recently, studies have examined the production and use of recombinant (re) LH and FSH of 532 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, 534 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 536 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 538 popular for producing recombinant mammalian glycosylated proteins (Demain & Vaishnav, 2009). Homologous reFSH and reLH have been produced for zebrafish (So et al., 2005), 540 channel catfish (*Ictalurus punctatus*) (Zmora et al., 2007), goldfish (*Carassius auratus*) (Hayakawa et al., 2008), Japanese eel (Kobayashi et al., 2010), European seabass (Molés et 542 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). 544 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) 546 stimulated only the FSH receptors, whilst 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* 548 *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
550 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
552 stages of spermatogenesis in immature Japanese eel (Kamei et al., 2006) and immature European seabass (Mazón et al., 2014).

554 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
556 injection of 20 µl g^{-1} of superworm hemolymph containing either reFSH or reLH increased sperm volume >4X after 24 h compared to control fish. Full spermatogenesis and the
558 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
560 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
562 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
564 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
566 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.,
568 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
570 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
572 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 x 10^9 cells ml^{-1}

574 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

576 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

578 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

580 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

582 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

584 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.

586

5.3. GtH preparations of mammalian or human origin

588 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

590 (PMSG) and human Chorionic Gonadotropin (hCG) (Donaldson & Hunter, 1983). However, only hCG –which is purified from the urine of pregnant women- has been used routinely in

592 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 &

594 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

596 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

598 circulation compared to pituitary GtHs (Zohar & Mylonas, 2001). A drawback of hCG that is

often quoted by aquaculturists is that the treatment becomes less effective with repeated use

600 (*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

602 (Zohar & Mylonas, 2001). For example, hCG antibodies were observed in circulation in

striped bass after a single treatment of hCG (Zohar & Mylonas, 2001), but no antibodies

604 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).

606 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 &

608 Donaldson, 1968). This early success, in addition to the long half-life of hCG in circulation

(Ohta & Tanaka, 1997; Zohar & Mylonas, 2001) has resulted in the use of hCG to enhance

610 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

612 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

614 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*

616 *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

618 & Poursaeid, 2014) and 312 IU kg^{-1} in pejerrey (Miranda et al., 2005) to 10,000 IU kg^{-1} in

goldfish (Yamazaki & Donaldson, 1968) and 50,000 IU kg^{-1} in silver perch (*Leiopotherapon*

620 *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}.

622 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

624 more effective in increasing sperm volume, density, motility and kinetic features, throughout most weeks of the treatment (Gallego et al., 2012b).

626

5.4. GnRHa injections and delivery systems

628 The commercial synthesis and widespread use of GnRHa for human medicine (Ulloa-Aguirre & Timossi, 2000), made a number of different GnRHas available for spawning induction 630 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 632 structural similarity of native GnRHs among fishes (Lethimonier et al., 2004). Being of synthetic nature, a GnRHa offers absolute biosecurity against disease transmission threats 634 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 636 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 & Eales, 1996; Le Gac et al., 1993; 638 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 640 inhibition of GtH release, GnRHa treatments are usually combined with DA (*e.g.* domperidone, pimozide, reserpine or metoclopramide). Another advantage of GnRHa is that 642 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 & Zohar, 2001a). Various 644 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 6 646 and/or 10 of the peptide (Zohar & Mylonas, 2001), which makes them more active and resistant to enzymatic degradation (Zohar et al., 1990).

648 The potential of long-term administration of GnRHa via delivery systems was recognized from the early beginnings of commercial aquaculture (Fontenele, 1955). This is
650 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
652 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
654 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
656 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
658 maturation in females, but also for enhancing spermiation in males (Mañanos et al., 2009; Mylonas & Zohar, 2001a; Mylonas et al., 2010).

660 The available GnRHa delivery systems may be in the form of solid implantable pellets of cholesterol (Weil & Crim, 1983), Ethylene-Vinyl Acetate (EVAc) (Mylonas et al., 2007;
662 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
664 microspheric/injectable systems have been used effectively in controlling reproductive function, each method having specific advantages. For example, the injectable systems can
666 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,
668 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
670 water temperature (Crim et al., 1988; Mañanos et al., 2002; Mylonas et al., 1998a; Mylonas & Zohar, 2001a; Vermeirssen et al., 2004; Zohar, 1996).

672 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

674 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).

676 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

678 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-

680 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

682 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

684 (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

686 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

688 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

690 microspheres induced sustained elevations in sperm production for >29 days (Clearwater & Crim, 1998). Similarly in the European seabass, GnRHa implants and microspheres induced

692 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

694 treatment (Sorbera et al., 1996). In fact, the amount of sperm produced after 35 days was almost 2X higher than the amount produced prior to the GnRHa treatment, even after repeated

696 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 >4X 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 of pheromones and the mechanisms of production/reception and biological function have been described in just a few species, such as the goldfish (DeFraipont & 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, whilst 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

722 flow through aquarium with a concentration of 10^{-10} M declining to 10^{-11} M of 17,20β-P increased sperm volume >3X (from 22 μg prior to exposure to 77 μg after exposure)

724 compared to 21 μg of sperm in control fish exposed to the ethanol carrier (DeFraipont & Sorensen, 1993). It has been shown that female goldfish produce and release 17,20β-P (also

726 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

728 substances (Sorensen et al., 1999; Sorensen & Stacey, 2004). During ovulation, females produce and release prostaglandin $F_{2\alpha}$ (PGF), which stimulates male spawning behavior and

730 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

732 both with *in vivo* competition between two males for an ovulated female and with *in vitro* fertilization using the stripped sperm.

734 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

736 common carp increased significantly their sperm volume (4X) 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

738 concentration of 17,20β-P increased significantly their sperm volume (2X) and motility duration compared to controls and pre-exposure values (Pinheiro et al., 2003). In salmonids,

740 PGF have been identified as probable pheromones that stimulate sperm production. Both Atlantic salmon (*Salmo salar*) (Moore & Waring, 1996) and brown trout (*Salmo trutta*)

742 (Moore et al., 2002) mature parr were shown to increase significantly (2X) sperm volume when exposed to 10^{-8} M concentration of either $PGF_{2\alpha}$ or $PGF_{1\alpha}$. These studies demonstrate

744 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

746 wild, since behavior and pheromones may be the mechanism that increases sperm volume in the moment that sperm is required for spawning.

748

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

750 After spermiation, spermatozoa undergo maturation (or capacitation), a process that renders them capable of forward motility upon ejaculation and competent to fertilize an oocyte

752 (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

754 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),

756 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

758 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

760 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

762 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

764 spermatozoa motility (Tubbs & Thomas, 2009). The *in vitro* progestin stimulation of sperm motility has been described in different sciaenid species (Tubbs & Thomas, 2008), such as the

766 spotted sea trout (*Cynoscion nebulosus*), Atlantic croaker (*Micropogonias undulatus*) and red drum (*Sciaenops ocellatus*), and in the southern flounder (*Paralichthys lethostigma*) (Tan et

768 al., 2014).

Sperm motility has been improved by incubating sperm samples with 20β-S (10-200

770 nM) for 1-5 min in the spotted sea trout (Tubbs & 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 & Grant, 1965), which is effected by the MIS (Scott et al., 2010). The increased fluid content of the testes

796 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"
798 more available spermatozoa from the testes, and over time the spermatozoa density of the collected sperm decreases significantly (Table 4). In Atlantic halibut (*Hippoglossus*
800 *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
802 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.,
804 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
806 spermatozoa density in various fishes range between 20-300%, but coupled with the significant increases in expressible sperm, the end result is that there is an increase in total
808 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
810 time through repeated treatments, sperm concentration may be reduced by up to 400X compared to normal values, as observed in meagre (*Argyrosomus regius*) after 17 weeks of
812 spermiation induction with the multiple use of GnRHa implants (Fig. 3), and participation in two spawning events each week (unpublished data).

814 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.
816 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
818 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
820 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 intra-testicular 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 intra-testicular 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 & Cosson, 2005; Genz et al., 2014; Woolsey & 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 2X 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 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

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
872 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
874 study also showed significant correlations between different fatty acids and sperm quality parameters, especially between spermatozoa velocity and the consumption of ARA present in
876 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
878 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,
880 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.,
882 2015). After the hormonal treatment, the sperm showed more than 60% of motile spermatozoa, with PUFA being the fatty acid class with the highest concentration in sperm,
884 and DHA having the highest concentration among PUFA.

886 ***Seminal plasma proteins***

During the last few years, the importance of seminal plasma proteins in relation to sperm
888 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
890 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
892 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,
894 whereas spermatozoal immunoglobulin concentrations were higher in samples with low motility (Lahnsteiner & Radner, 2010). Both results probably are related to antibacterial

896 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 &
898 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
900 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
902 plasma of common carp was related to sperm motility parameters (Wojtczak et al., 2007b).

904 ***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,
906 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
908 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
910 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
912 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
914 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
916 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
918 hormonal treatment.

As mentioned earlier (Section 2.3) sperm maturation (*i.e.* capacitation) occurs in the
920 sperm duct (Miura et al., 1992; Morisawa & 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 & 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 & 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

946 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
948 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*)
950 (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
952 PM and fertilization success (Bozkurt & 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 -
954 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
956 range of sperm motility values, to avoid masking the real correlations between the motility values, and fertilization and hatching success.

958 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
960 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
962 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
964 (*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
966 (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
968 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,
970 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 & 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
996 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
998 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
1000 "measurements made with flow cytometer had the strongest relationship to haemocytometer
counts, demonstrating the usefulness of this technique". Computer-Assisted Sperm Analysis
1002 also allows the quantification of sperm density (Ehlers et al., 2011) and, finally, some
researchers have applied also spectrophotometric techniques (Fauvel et al., 1999).

1004

8.2. Computer-Assisted Sperm Analysis (CASA)

1006 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
1008 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,
1010 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
1012 technique. These systems evolved from the multiple photo-micrographic exposure and video-
micrographic techniques for spermatozoa tracks, using a computer equipped with imaging
1014 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
1016 (Kime et al., 1996; Van Look & Kime, 2003; Wilson-Leedy & Ingermann, 2007).

To use CASA, the spermatozoa movement is video-recorded after activation, and then
1018 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

1020 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

1022 sperm sample. However, for some authors the most useful parameters are the specific spermatozoa velocities (Gallego et al., 2013a; Kime & Tveiten, 2002; Rurangwa et al., 2004).

1024 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

1026 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

1028 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

1030 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

1032 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

1034 systems must be calibrated to each species of interest, trying to follow previously standardized methods, and details of the set-up must be reported together with the obtained

1036 results to guarantee repetitiveness of the process.

1038 **8.3. Assisted Sperm Morphometry Analysis (ASMA)**

The development of ASMA software for the study of spermatozoa morphometry has

1040 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

1042 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

1044 increasing number of aquaculture studies (Table 7).

Spermatozoa ultrastructure and morphology were studied previously by transmission
1046 electron microscopy, scanning electron microscopy or laser light-scattering spectroscopy and stroboscopic illumination (reviewed by Pérez et al., 2009). However, these techniques are
1048 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
1050 (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
1052 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
1054 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
1056 (Kime et al., 2001) and the ASMA methodology used for livestock production animals needs set-up adjustments to be applicable to fish (Van Look & Kime, 2003). One of the first
1058 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-
1060 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
1062 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;
1064 Peñaranda et al., 2010c).

Several studies have evaluated the relationships between morphology, motility and
1066 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
1068 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

1070 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 et al., 2011b).

1072 During the last years, ASMA techniques are becoming relatively common in studies of fish reproductive strategies or fish comparative physiology (Table 7).

1074

8.4. Fluorescent staining

1076 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

1078 sperm quality, using simple and rapid protocols based on the use of fluorescent staining (He & Woods III, 2004; Ogier de Baulny et al., 1999; Rurangwa et al., 2004; Segovia et al.,

1080 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

1082 membranes. An intact plasma membrane will prevent these products from entering the spermatozoa and staining the nucleus. Phenanthridines, such as propidium iodide (PI, Garner

1084 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

1086 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

1088 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

1090 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

1092 highly functional mitochondria (Asturiano et al., 2006; Graham, 2001).

1094 ### 8.5. Other techniques

The cryopreservation of the sperm can cause different damages to the spermatozoa. For

1096 example, it can induce DNA fragmentation and changes in proteins profile (Zilli & Vilella,

2012), as well as increases on the reactive oxygen species (ROS) inducing alterations at the

1098 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-

1100 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.,

1102 2011). The TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end-

labeling) and SCSA (sperm chromatin structure assay) assays are flow cytometry-based

1104 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

1106 information about damages in specific genes have been developed using qPCR approaches.

For example, after gilthead seabream sperm cryopreservation, the lesions generated in nuclear

1108 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

1110 techniques will be used more widely in the field of sperm quality in the future.

1112 ### 8.6. Spermatozoa subpopulations

Computer-Assisted Sperm Analysis systems are able to track a very large number of

1114 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

1116 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

1118 spermatozoa subpopulations coexist in the same sperm sample (Gallego, 2013). Thus, the

spermatozoa can be classified into different subpopulations within each sample according to
1120 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
1122 kinetic parameters, the samples are characterized considering not only the mean values of
CASA parameters, but also the relative proportions of each subpopulation (Holt & Harrison,
1124 2002; Martinez-Pastor et al., 2005).

Sperm subpopulation analysis has been studied mostly in mammals (Dorado et al.,
1126 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
1128 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
1130 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.,
1132 2015).

The spermatozoa subpopulations found in the different species have several
1134 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;
1136 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
1138 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 (Gallego et
1140 al., 2012b), or even being germ cells forced out during the stripping process (Marco-Jiménez
et al., 2006a).

1142 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 20ºC 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 1,000 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 1,500 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 x 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 & 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 & Billard, 1987), gonadotropin-calibrated CPE (Rothbard & Yaron, 1995) or GnRHa in combination with or without DA (Billard et al., 1987; Roelants et al., 2000; Saad & 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 > 4X 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

1194 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

1196 by 3-4X 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-

1198 day period after hormonal treatment (Linhart et al., 1995) and production was observed to peak at 72 h post treatment (Caille et al., 2005).

1200 Finally, controlled-release delivery systems loaded with GnRHa have been used successfully in a variety of marine fishes including European seabass, striped bass, Atlantic

1202 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

1204 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

1206 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

1208 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

1210 percentage or duration. Sperm collection in European seabass in response to hormonal therapy may range between 1-4 ml kg^{-1} at every sampling, which can be done on a weekly

1212 basis.

1214 10. Conclusion

The review has demonstrated the wide range of successful hormone manipulations that have

1216 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

1218 more recently identified hormones may become important in future applications. Recent

studies have demonstrated the potential of reGtHs to both induce spermatogenesis and
1220 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
1222 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
1224 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
1226 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
1228 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,
1230 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
1232 between studies.

1234 **11. Acknowledgements**

We would like to thank the COST Action FA1205 (AQUAGAMETE) titled “Assessing and
1236 improving the quality of aquatic animal gametes to enhance aquatic resources - The need to harmonize and standardize evolving methodologies, and improve transfer from academia to
1238 industry” for initiating the production of this special issue and funding partially this article. JFA has been partly funded by the Spanish Ministry of Economy and Competitiveness
1240 (REPRO-TEMP; AGL2013-41646-R) and the European Training Network IMPRESS (Marie Sklodowska-Curie Actions; Grant agreement nº: 642893). ND has been funded partly by the
1242 INIA-FEDER project RTA2011-00050, and CCM and ND have been supported also by the EU project DIVERSIFY (7FP-KBBE-2013-GA 602131).

12. References

1246 Adriaenssens, B., van Damme, R., Seebacher, F., Wilson, R.S., 2012. Sex cells in changing environments: can organisms adjust the physiological function of gametes to different
1248 temperatures? Global Change Biology 18, 1797-1803.

Agulleiro, M.J., Scott, A.P., Dunca, N.J., Mylonas, C.C., Cerdá, J., 2007. Treatment of GnRHa-
1250 implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comparative Biochemistry and Physiology
1252 A147, 885-892.

Aizen, J., Meiri, I., Tzchori, I., Levavi-Sivan, B., Rosenfeld, H., 2005. Enhancing spawning in the
1254 grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. General and Comparative Endocrinology 142, 212-221.

1256 Alavi, H.S.M., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biology International 29, 101-110.

1258 Alavi, H.S.M., Butts, I.A.E., Hatef, A., Babiak, I., Mommens, M., Trippel, E.A., Litvak, M.K., 2011. Sperm morphology, seminal plasma composition, ATP content and analysis of sperm motility
1260 and flagellar wave parameters in Atlantic halibut (*Hippoglossus hippoglossus* L.). Canadian Journal of Zoology 89, 219-228.

1262 Alavi, S.M.H., Hatef, A., Mylonas, C.C., Gela, D., Papadaki, M., Rodina, M., Kašpar, V., Pšenička, M., Podhorec, P., Linhart, O., 2012. Sperm characteristics and androgens in *Acipenser*
1264 *ruthenus* after induction of spermiation by carp pituitary extract or GnRHa implants. Fish Physiology and Biochemistry 38, 1655-1666.

1266 Arantes, F.P., Santos, H.B., Rizzo, E., Sato, Y., Bazzoli, N., 2011. Influence of water temperature on induced reproduction by hypophysation, sex steroids concentrations and final oocyte
1268 maturation of the "curimatã-pacu" *Prochilodus argenteus* (Pisces: Prochilodontidae). General and Comparative Endocrinology 172, 400-408.

1270 Asturiano, J.F., Sorbera, L.A., Ramos, J., Kime, D.E., Carrilo, M., Zanuy, S., 2000. Hormonal regulation of the European sea bass reproductive cycle: an individualized female approach.
1272 Journal of Fish Biology 56, 1155-1172.

Asturiano, J.F., Sorbera, L.A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, L., Bromage, N., 2001.
1274 Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194,
1276 173-190.

Asturiano, J.F., Sorbera, L.A., Ramos, J., Kime, D.E., Carrillo, M., Zanuy, S., 2002. Group-
1278 synchronous ovarian development, ovulation and spermiation in the European sea bass (*Dicentrarchus labrax*) could be regulated by shifts in gonadal steroidogenesis. Scientia
1280 Marina 66, 273-282.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M.,
1282 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiology and Biochemistry
1284 30, 283-293.

Asturiano, J.F., Pérez, L., Garzón, D.L., Peñaranda, D.S., Marco-Jiménez, F., Martínez-Llorens, S.,
1286 Tomás, A., Jover, M., 2005. Effect of different methods for the induction of spermiation on semen quality in European eel. Aquaculture Research 36, 1480-1487.

1288 Asturiano, J.F., Marco-Jiménez, F., Pérez, L., Balasch, S., Garzon, D.L., Peñaranda, D.S., Vicente, J.S., Viudes-de-Castro, M.P., Jover, M., 2006. Effects of hCG as spermiation inducer on
1290 European eel semen quality. Theriogenology 66, 1012-1020.

Asturiano, J.F., Marco-Jiménez, F., Peñaranda, D.S., Garzón, D.L., Pérez, L., Vicente, J.S., Jover, M.,
1292 2007. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reproduction in Domestic Animals 42, 162-166.

1294 Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. II: Effect of spermiation advancement, catheterization
1296 of semen, and production-scale application. Theriogenology 66, 2036-2046.

Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2014.
1298 Effect of thermal regime on fatty acid dynamics in male European eels (*Anguilla anguilla*) during hormonally-induced spermatogenesis. Aquaculture 430, 86-97.
1300 Baeza, R., 2015. Roles of lipids and fatty acids through the spermatogenesis of European eel (*Anguilla anguilla*), Universitat Politècnica de València, Ph.D. Thesis, 185 pp.
1302 Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2015a. Relationship between sperm quality parameters and the fatty acid composition of the muscle,
1304 liver and testis of European eel. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 181, 79-86.
1306 Baeza, R., Peñaranda, D.S., Vílchez, M.C., Tveiten, H., Pérez, L., Asturiano, J.F., 2015b. Exploring correlations between sex steroids and fatty acids and their potential roles in the induced
1308 maturation of the male European eel. Aquaculture 435, 328-335.
Baudiffier, D., Hinfray, N., Vosges, M., Creusot, N., Chadili, E., Porcher, J.M., Schulz, R.W., Brion,
1310 F., 2012. A critical role of follicle-stimulating hormone (Fsh) in mediating the effect of clotrimazole on testicular steroidogenesis in adult zebrafish. Toxicology 298, 30-39.
1312 Baynes, S., Howell, B.R., Beard, T.W., 1993. A review of egg production by captive sole, *Solea solea* (L.). Aquaculture Research 24, 171-180.
1314 Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72, 1251-1261.
1316 Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2011a. Changes in *Solea senegalensis* sperm quality throughout the year. Animal Reproduction Science 126, 122-129.
1318 Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., Herráez, M.P., 2011b. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 62, 22-31.
1320 Beirão, J., Soares, F., Pousão-Ferreira, P., Diogo, P., Dias, J., Dinis, M.T., Herráez, M.P., Cabrita, E., 2015. The effect of enriched diets on *Solea senegalensis* sperm quality. Aquaculture 435, 187-
1322 194.
Bernáth, G., Bokor, Z., Kása, E., Várkonyi, L., Hegyi, Á., Kollár, T., Urbányi, B., Żarski, D., Radóczi
1324 Ifj, J., Horváth, Á., 2015. Comparison of two different methods in the cryopreservation of Eurasian perch (*Perca fluviatilis*) sperm. Cryobiology 70, 76-78.
1326 Biagi, F., Addis, A., Corso, G., Carpupino, M., 2010. Spermatogenesis and sperm structure in the black-striped pipefish *Syngnathus abaster* (Teleostei, Synghathidae). Biologia Marina
1328 Mediterranea 17, 382-383.
Billard, R., 1977. Effect of various hormones on sperm release in the hypophysectomized goldfish.
1330 IRCS Med. Sci. Libr. Compend. 5, 188.
Billard, R., Marcel, J., 1980. Stimulation of spermiation and induction of ovulation in pike (*Esox*
1332 *lucius*). Aquaculture 21, 181-195.
Billard, R., 1986. Spermatogenesis and spermatology of some teleost fish species. Reproduction
1334 Nutrition Development 26, 877-920.
Billard, R., 1992. Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis,
1336 biology and preservation of gametes. Aquaculture 100, 263-298.
Billard, R., Bieniarz, K., Popek, W., Epler, P., Breton, B., Alagarswami, K., 1987. Stimulation of
1338 gonadotropin secretion and spermiation in carp by pimozide-LRH-A treatment: Effects of dose and time of day. Aquaculture 62, 161-170.
1340 Billard, R., Le Gac, F., Loir, M., 1990. Hormonal control of sperm production in teleost fish. In: Eppler, A., Scanes, C.G., Stetson, M.H. (Eds.), Progress in Comparative Endocrinology.
1342 Wiley-Liss, New York, pp. 329-335.
Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995. Sperm physiology and quality. In: Bromage,
1344 N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science, Oxford, pp. 25-52.
1346 Biran, J., Golan, M., Mizrahi, N., Ogawa, S., Parhar, I.S., Levavi-Sivan, B., 2014. LPXRFa, the piscine ortholog of GnIH, and LPXRF receptor positively regulate gonadotropin secretion in
1348 tilapia (*Oreochromis niloticus*). Endocrinology 155, 4391-4401.
Black, B.J., Pankhurst, N.W., 2009. Effect of gonadotropin releasing-hormone analogue and human
1350 chorionic gonadotropin on milt characteristics and gonadal steroids in yellowfin bream,

1351 *Acanthopagrus australis* (Sparidae). New Zealand Journal of Marine and Freshwater Research
1352 43, 1069-1077.
1353 Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. General and Comparative Endocrinology
1354 165, 535-548.
1355 Bondarenko, O., Dzyuba, B., Cosson, J., Yamaner, G., Prokopchuk, G., Psenicka, M., Linhart, O.,
1356 2013. Volume changes during the motility period of fish spermatozoa: Interspecies
1357 differences. Theriogenology 79, 872-881.
1358 Boryshpolets, S., Kowalski, R.K., Dietrich, G.J., Dzyuba, B., Ciereszko, A., 2013. Different computer-
1359 assisted sperm analysis (CASA) systems highly influence sperm motility parameters.
1360 Theriogenology 80, 758-765.
1361 Bozkurt, Y., Secer, S., 2006. Relationship between spermatozoa motility, egg size, fecundity and
1362 fertilization success in brown trout (*Salmo trutta fario*). International Journal of Biological
1363 Sciences 9, 2141-2144.
1364 Brauhn, L., 1971. Fall spawning of channel catfish. The Progressive Fish-Culturist 33, 150–152.
1365 Breckels, R., Garner, S., Neff, B., 2014. Rapid evolution in response to increased temperature
1366 maintains population viability despite genetic erosion in a tropical ectotherm. Evolutionary
1367 Ecology 28, 141-155.
1368 Breckels, R.D., Neff, B.D., 2013. The effects of elevated temperature on the sexual traits, immunology
1369 and survivorship of a tropical ectotherm. The Journal of Experimental Biology 216, 2658-
1370 2664.
1371 Bromage, N.R., Roberts, R.J., 1995. Broodstock Management and Egg and Larval Quality. Blackwell
1372 Science, Oxford, 424 pp.
1373 Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed
1374 finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-
1375 98.
1376 Brzuska, E., 2001. Artificial spawning of European catfish *Silurus glanis* L.: differences between
1377 propagation results after stimulation of ovulation with carp pituitary and Ovopel. Aquaculture
1378 International 32, 11-19.
1379 Bungum, M., Bungum, L., Giwercman, A., 2011. Sperm chromatin structure assay (SCSA): A tool in
1380 diagnosis and treatment of infertility. Asian Journal of Andrology 13, 69-75.
1381 Butts, I.A.E., Litvak, M.K., Kaspar, V., Trippel, E.A., 2010a. Cryopreservation of Atlantic cod *Gadus*
1382 *morhua* L. spermatozoa: Effects of extender composition and freezing rate on sperm motility,
1383 velocity and morphology. Cryobiology 61, 174-181.
1384 Butts, I.A.E., Rideout, R.M., Burt, K., Samuelson, S., Lush, L., Litvak, M.K., Trippel, E.A.,
1385 Hamoutene, D., 2010b. Quantitative semen parameters of Atlantic cod (*Gadus morhua*) and
1386 their physiological relationships with sperm activity and morphology. Journal of Applied
1387 Ichthyology 26, 756-762.
1388 Butts, I.A.E., Litvak, M.K., Trippel, E.A., 2010c. Seasonal variations in seminal plasma and sperm
1389 characteristics of wild-caught and cultivated Atlantic cod, *Gadus morhua*. Theriogenology 73,
1390 873-885.
1391 Butts, I.A.E., Babiak, I., Ciereszko, A., Litvak, M.K., Słowińska, M., Soler, C., Trippel, E.A., 2011a.
1392 Semen characteristics and their ability to predict sperm cryopreservation potential of Atlantic
1393 cod, *Gadus morhua* L. Theriogenology 75, 1290-1300.
1394 Butts, I.A.E., Ward, M.A.R., Litvak, M.K., Pitcher, T.E., Alavi, S.M.H., Trippel, E.A., Rideout, R.M.,
1395 2011b. Automated sperm head morphology analyzer for open-source software.
1396 Theriogenology 76, 1756-1761.
1397 Butts, I.A.E., Trippel, E.A., Ciereszko, A., Soler, C., Słowińska, M., Alavi, S.M.H., Litvak, M.K.,
1398 Babiak, I., 2011c. Seminal plasma biochemistry and spermatozoa characteristics of Atlantic
1399 cod (*Gadus morhua* L.) of wild and cultivated origin. Comparative Biochemistry and
1400 Physiology Part A: Molecular & Integrative Physiology 159, 16-24.
1401 Butts, I.A.E., Sørensen, S.R., Politis, S.N., Pitcher, T.E., Tomkiewicz, J., 2014. Standardization of
1402 fertilization protocols for the European eel, *Anguilla anguilla*. Aquaculture 426–427, 9-13.
1403 Butts, I.A.E., Baeza, R., Støttrup, J.G., Krüger-Johnsen, M., Jacobsen, C., Pérez, L., Asturiano, J.F.,
1404 Tomkiewicz, J., 2015. Impact of dietary fatty acids on muscle composition, liver lipids, milt

composition and sperm performance in European eel. Comparative Biochemistry and
1406 Physiology Part A: Molecular & Integrative Physiology 183, 87-96.
Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herráez, M.P., 2005. Evaluation of DNA
1408 damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*)
cryopreserved sperm. Cryobiology 50, 144-153.
1410 Cabrita, E., Robles, V., Herraez, P., 2009. Sperm quality assessment. In: Cabrita, E., Robles, V.,
Herraez, P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species.
1412 CRC Press Taylor and Francis Group, Boca Raton, pp. 93-148.
Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez,
1414 M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied
Ichthyology 26, 623-635.
1416 Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., Dinis, M.T., 2011. The influence
of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA
1418 fragmentation. Animal Reproduction Science 125, 189-195.
Cacot, P., Eeckhoutte, P., Muon, D.T., Trieu, N.V., Legendre, M., Mariojouls, C., Lazard, J., 2003.
1420 Induced spermiation and milt management in *Pangasius bocourti* (Sauvage, 1880).
Aquaculture 215, 67-77.
1422 Caille, N., Rodina, M., Kocour, M., Gela, D., Flajšhans, M., Linhart, O., 2005. Quantity, motility and
fertility of tench *Tinca tinca* (L.) sperm in relation to LHRH analogue and carp pituitary
1424 treatments. Aquaculture International 14, 75-87.
Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1992. Stress reduces the quality of gametes produced
1426 by rainbow trout. Biology of Reproduction 47, 1140-1150.
Candolin, U., Reynolds, J.D., 2011. Adjustments of ejaculation rates in response to risk of sperm
1428 competition in a fish, the bitterling (*Rhodeus sericeus*). Proceedings of the Royal Society B:
Biological Sciences 269, 1549-1553.
1430 Cartón-García, F., Riesco, M.F., Cabrita, E., Herráez, M.P., Robles, V., 2013. Quantification of
lesions in nuclear and mitochondrial genes of *Sparus aurata* cryopreserved sperm.
1432 Aquaculture 402-403, 106-112.
Cejko, B.I., Targońska, K., Kowalski, R.K., Żarski, D., Sarosiek, B., Kucharczyk, D., Glogowoski, J.,
1434 2012. The effectiveness of hormonal preparations (Ovopel, Ovaprim, LHRHa, hCG and CPE)
in stimulating spermiation in dace *Leuciscus leuciscus* (L.). Journal of Applied Ichthyology
1436 28, 873-877.
Cejko, B.I., Zarski, D., Krejszeff, S., Kucharczyk, D., Kowalski, R.K., 2013. Effect of hormonal
1438 stimulation on milt volume, number of sperm, and sperm motility in the crucian carp,
Carassius carassius (L.). Israeli Journal of Aquaculture - Bamidgeh 65.2013.912, 7.
1440 Cejko, B.I., Zarski, D., Judycka, S., Kucharczyk, D., Sarosiek, B., Kowalski, R.K., 2014. Effect of two
commercial preparations containing different GnRH analogues with dopamine antagonists on
1442 barbel *Barbus barbus* (L.) sperm quantity and quality. Aquaculture International 22, 97-109.
Celino, F.T., Yamaguchi, S., Miura, C., Ohta, T., Tozawa, Y., Iwai, T., Miura, T., 2011. Tolerance of
1444 spermatogonia to oxidative stress is due to high levels of Zn and Cu/Zn superoxide dismutase.
PLoS ONE 6, e16938.
1446 Chauvigné, F., Verdura, S., Mazón, M.J., Duncan, N., Zanuy, S., Gómez, A., Cerdà, J., 2012. Follicle-
stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells
1448 of an evolutionary advanced teleost. Biology of Reproduction 87(2):35, 1-11.
Chohan, K.R., Griffin, J.T., Lafromboise, M., De Jonge, C.J., Carrell, D.T., 2006. Comparison of
1450 chromatin assays for DNA fragmentation evaluation in human sperm. Journal of Andrology
27, 53-59.
1452 Chu, L., Li, J., Liu, Y., Cheng, C.H.K., 2015. Gonadotropin signaling in zebrafish ovary and testis
development: insights from gene knockout study. Molecular Endocrinology 29, 1743-1758.
1454 Ciereszko, A., Piros, B., Dabrowski, K., Kucharczyk, D., ŁUczyński, M.J., Dobosz, S., Glogowski, J.,
1998. Serine proteinase inhibitors of seminal plasma of teleost fish: distribution of activity,
1456 electrophoretic profiles and relation to proteinase inhibitors of blood. Journal of Fish Biology
53, 1292-1305.

1458 Clearwater, S.J., Crim, L.W., 1998. Gonadotropin releasing hormone-analogue treatment increases sperm motility, seminal plasma pH and sperm production in yellowtail flounder *Pleuronectes*
1460 *ferrugineus*. Fish Physiology and Biochemistry 19, 349-357.
Clemens, H.P., Grant, F.B., 1964. Gonadal hydration of carp (*Cyprinus carpio*) and goldfish
1462 (*Carrasius auratus*) after injections of pituitary extracts. Zoologica 49, 193-210.
Clemens, H.P., Grant, F.B., 1965. The seminal thinning response in carp (*Cyprinus carpio*) and
1464 rainbow trout (*Salmo gairdneri*) after injections of pituitary extracts. Copeia 1965, 174-177.
Cordelli, E., Eleuteri, P., Leter, G., Rescia, M., Spanò, M., 2005. Flow cytometry applications in the
1466 evaluation of sperm quality: semen analysis, sperm function and DNA integrity. Contraception 72, 273-279.
1468 Corriero, A., Medina, A., Mylonas, C.C., Bridges, C.R., Santamaria, N., Deflorio, M., Losurdo, M., Zupa, R., Gordin, H., de la Gándara, F., Belmonte Ríos, A., Pousis, C., De Metrio, G., 2009.
1470 Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna (*Thunnus thynnus L.*) treated with gonadotroping releasing hormone agonist (GnRHa). Animal
1472 Reproduction Science 116, 346-357.
Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Studying sperm
1474 motility in marine fish: an overview on the state of the art. Journal of Applied Ichthyology 24, 460-486.
1476 Crim, L.W., Sherwood, N.M., Wilson, C.E., 1988. Sustained hormone release. II. Effectiveness of LHRH analog (LHRHa) administration by either single time injection or cholesterol pellet
1478 implantation on plasma gonadotropin levels in a bioassay model fish, the juvenile rainbow trout. Aquaculture 74, 87-95.
1480 Cyr, D.G., Eales, J.G., 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish. Reviews in Fish Biology and Fisheries 6, 165-200.
1482 Dabrowski, K., Ciereszko, A., 1994. Proteinase inhibitor(s) in seminal plasma of teleost fish. Journal of Fish Biology 45, 801-809.
1484 Dabrowski, K., Ciereszko, A., Ramseyer, L., Culver, D., Kestemont, P., 1994. Effects of hormonal treatment on induced spermiation and ovulation in the yellow perch *(Perca flavescens)*.
1486 Aquaculture 120, 171-180.
De Leeuw, A.M., Den Daas, J.H., Woelders, H., 1991. The fix vital stain method. Simultaneous
1488 determination of viability and acrosomal status of bovine spermatozoa. Journal of Andrology 12, 112-118.
1490 DeFraipont, M., Sorensen, P.W., 1993. Exposure to the pheromone 17α,20β-dihydroxy-4-pregnen-3-one enhances the behavioural spawning success, sperm production and sperm motility of male
1492 goldfish. Animal Behaviour 46, 245-256.
Demain, A.L., Vaishnav, P., 2009. Production of recombinant proteins by microbes and higher
1494 organisms. Biotechnology Advances 27, 297-306.
Denusta, P.J.T., de Jesus-Ayson, E.G.T., Laron, M.A., Garcia, L.M.B., 2014. Effects of human
1496 chorionic gonadotropin (hCG) and handling stress on spermiation of silver perch *Leiopotherapon plumbeus* (Kner, 1864). Journal of Applied Ichthyology 30, 448-453.
1498 Donaldson, E.M., Hunter, G.A., 1983. Induced final maturation, ovulation and spermiation in cultured fishes. In: Hoar, W.S., Randall, D.J., Donaldson, E.M. (Eds.), Fish Physiology. Academic
1500 Press, Orlando, Florida, pp. 351-403.
Dorado, J., Molina, I., Muñoz-Serrano, A., Hidalgo, M., 2010. Identification of sperm subpopulations
1502 with defined motility characteristics in ejaculates from Florida goats. Theriogenology 74, 795-804.
1504 Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., Hidalgo, M., 2011. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and
1506 centrifugation on PureSperm® gradient. Animal Reproduction Science 125, 211-218.
Duncan, N., Sonesson, A., Chavanne, H., 2013. Principles of finfish broodstock management in
1508 aquaculture: control of reproduction and genetic improvement. In: Allan, G., Burnell, G. (Eds.), Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited,
1510 Cambridge, UK, pp. 23-75.

Dziewulska, K., Rzemieniecki, A., Czerniawski, R., Domagała, J., 2011. Post-thawed motility and fertility from Atlantic salmon (*Salmo salar* L.) sperm frozen with four cryodiluents in straws or pellets. Theriogenology 76, 300-311.

Dziewulska, K., Pilecka-Rapacz, M., Domagała, J., 2015. Spermatozoa motility traits influenced by activation media in the European whitefish (*Coregonus lavaretus* L. 1758). Journal of Applied Ichthyology 31 (Suppl. 1), 22-27.

Dzyuba, B., Boryshpolets, S., Shaliutina, A., Rodina, M., Yamaner, G., Gela, D., Dzyuba, V., Linhart, O., 2012. Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet *Acipenser ruthenus* during sequential stripping. Aquaculture 356–357, 272-278.

Ehlers, J., Behr, M., Bollwein, H., Beyerbach, M., Waberski, D., 2011. Standardization of computer-assisted semen analysis using an e-learning application. Theriogenology 76, 448-454.

Falahatkar, B., Poursaeid, S., 2014. Effects of hormonal manipulation on stress responses in male and female broodstocks of pikeperch *Sander lucioperca*. Aquaculture International 22, 235-244.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., Suquet, M., 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. Journal of Fish Biology 54, 356-369.

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. Journal of Applied Ichthyology 26, 636-643.

Fauvel, C., Boryshpolets, S., Cosson, J., Wilson Leedy, J.G., Labbé, C., Haffray, P., Suquet, M., 2012. Improvement of chilled seabass sperm conservation using a cell culture medium. Journal of Applied Ichthyology 28, 961-966.

Felip, A., Zanuy, S., Carrillo, M., 2006. Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (*Dicentrarcus labrax*). Aquaculture 256, 570-578.

Fernández-Palacios, H., Norberg, B., Izquierdo, M., Hamre, K., 2011. Effects of broodstock diet on eggs and larvae. In: Holt, J. (Ed.), Larval Fish Nutrition. Wiley-Blackwell, Oxford, UK, pp. 153–181.

Figueroa, E., Valdebenito, I., Farias, J.G., 2014. Technologies used in the study of sperm function in cryopreserved fish spermatozoa. Aquaculture Research 45, 1-11.

Flannery, E.W., Butts, I.A.E., Słowińska, M., Ciereszko, A., Pitcher, T.E., 2013. Reproductive investment patterns, sperm characteristics, and seminal plasma physiology in alternative reproductive tactics of Chinook salmon (*Oncorhynchus tshawytscha*). Biological Journal of the Linnean Society 108, 99-108.

Fontenele, O., 1955. Injecting pituitary (hypophyseal) hormones into fish to induce spawning. The Progressive Fish-Culturist 18, 71-75.

Formicki, K., Szulc, J., Korzelecka-Orkisz, A., Tanski, A., Kurzydłowski, J.K., Grzonka, J., Kwiatkowski, P., 2015. The effect of a magnetic field on trout (*Salmo trutta* Linnaeus, 1758) sperm motility parameters and fertilization rate. Journal of Applied Ichthyology 31 (Suppl. 1), 136-146.

Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B., Parker, G.A., 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Current Biology 14, 44-47.

Gallego, V., 2013. Sperm physiology and quality in two marine teleosts: *Anguilla anguilla* & *Takifugu niphobles*, Universitat Politecnica de Valencia, Ph.D. Thesis, 162 pp.

Gallego, V., Peñaranda, D.S., Marco-Jiménez, F., Mazzeo, I., Pérez, L., Asturiano, J.F., 2012a. Comparison of two techniques for the morphometry study on gilthead seabream (*Sparus aurata*) spermatozoa and evaluation of changes induced by cryopreservation. Theriogenology 77, 1078-1087.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012b. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354-355, 7-16.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2013a. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture 416–417, 238-243.

Gallego, V., Carneiro, P.C.F., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Soler, C., Pérez, L.,
1566 Asturiano, J.F., 2013b. Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA software. Theriogenology 79, 1034-1040.
1568 Gallego, V., Martínez-Pastor, F., Mazzeo, I., Peñaranda, D.S., Herráez, M.P., Asturiano, J.F., Pérez, L., 2014a. Intracellular changes in Ca^{2+}, K^{+} and pH after sperm motility activation in the
1570 European eel (*Anguilla anguilla*): preliminary results. Aquaculture 418–419, 155-158.
Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2014b. Sperm motility parameters and
1572 spermatozoa morphometric characterization in marine species: A study of swimmer and sessile species. Theriogenology 82, 668-676.
1574 Gallego, V., Vílchez, M.C., Peñaranda, D.S., Pérez, L., Herráez, M.P., Asturiano, J.F., Martínez-Pastor, F., 2015. Subpopulation pattern of eel spermatozoa is affected by post-activation time,
1576 hormonal treatment and the thermal regimen. Reproduction, Fertility and Development 27, 529-543.
1578 Galvano, P.M., Johnson, K., Wilson, C.C., Pitcher, T.E., Butts, I.A.E., 2013. Ovarian fluid influences sperm performance in lake trout, *Salvelinus namaycush*. Reproductive Biology 13, 172-175.
1580 Garber, A.F., Fordham, S.E., Symonds, J.E., Trippel, E.A., Berlinsky, D.L., 2009. Hormonal induction of ovulation and spermiation in Atlantic cod (*Gadus morhua*). Aquaculture 296, 179-183.
1582 Garcia, L.M.B., 1991. Spermiation response of mature rabbitfish, *Siganus guttatus* Bloch, to luteinizing hormone-releasing hormone analogue (LHRHa) injection. Aquaculture 97, 291-
1584 299.
Garcia, L.M.B., 1993. Sustained production of milt in rabbitfish, *Siganus guttatus* Bloch, by weekly
1586 injection of luteinizing hormone-releasing hormone analogue (LHRHa). Aquaculture 113, 261-267.
1588 Garcia-Lopez, A., Fau., M.-R.G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole *Solea senegalensis* (Kaup): anatomy, histology and histochemistry. Histology and
1590 Histopathology 20, 1179-1189.
Garner, D.L., Johnson, L.A., Yue, S.T., Roth, B.L., Haugland, R.P., 1994. Dual DNA staining
1592 sssessment of bovine sperm viability using SYBR-14 and propidium iodide. Journal of Andrology 15, 620-629.
1594 Garzón, D.L., Peñaranda, D.S., Pérez, L., Marco-Jiménez, F., Espert, X., Müller, T., Jover, M., Asturiano, J.F., 2008. Effects of pH, sodium bicarbonate, cryoprotectants and foetal bovine
1596 serum on the cryopreservation of European eel sperm. Reproduction in Domestic Animals 43, 99-105.
1598 Gasparini, C., Simmons, L.W., Beveridge, M., Evans, J.P., 2010. Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. PLoS ONE 5,
1600 e12146.
Genz, J., McDougall, C.A., Burnett, D., Arcinas, L., Khetoo, S., Anderson, W.G., 2014. Induced
1602 spawning of wild-caught adult lake sturgeon: assessment of hormonal and stress responses, gamete quality, and survival. Journal of Applied Ichthyology 30, 1565-1577.
1604 Graham, J.K., 2001. Assessment of sperm quality: a flow cytometric approach. Animal Reproduction Science 68, 239-247.
1606 Grier, H., Linton, J.R., Leatherland, J.F., DeVlaming, V.L., 1980. Structural evidence for two different testicular types in teleost fishes. The American Journal of Anatomy 159, 331-345.
1608 Grier, H., 1993. Comparative organization of Sertoli cells including the Sertoli cell barrier. In: Russell, L.D., Griswold, M.D. (Eds.), The Sertoli Cell. Cache River Press, Clearwater, Florida, pp.
1610 704-730.
Grier, H.J., Taylor, R.G., 1998. Testicular maturation and regression in the common snook. Journal of
1612 Fish Biology 53, 521-542.
Guzmán, J.M., Cal, R., García-López, Á., Chereguini, O., Kight, K.E., Olmedo, M., Sarasquete, C.,
1614 Mylonas, C.C., Peleteiro, J.B., Zohar, Y., Mañanós, E., 2011a. Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa)
1616 on the reproductive axis of Senegalese sole (*Solea senegalensis*). Comparative Biochemistry and Physiology 158, 235-245.
1618 Guzmán, J.M., Ramos, A.G., Mylonas, C.C., Mananos, E., 2011b. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa)

1620 treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction. Aquaculture 316, 121-128.
1622 Hall, S.G., Finney, J., Lang, R.P., Tiersch, T.R., 2002. Design and development of a geothermal temperature control system for broodstock management of channel catfish *Ictalurus*
1624 *punctatus*. Aquacultural Engineering 26, 277-289.
Harmin, S.A., Crim, L.W., 1993. Influence of gonadotropin hormone-releasing hormone analog
1626 (GnRH-A) on plasma sex steroid profiles and milt production in male winter flounder, *Pseudopleuronectes americanus* (Walbaum). Fish Physiology and Biochemistry 10, 399-407.
1628 Hayakawa, Y., Morita, T., Kitamura, W., Kanda, S., Banba, A., Nagaya, H., Hotta, K., Sohn, Y.C., Yoshizaki, G., Kobayashi, M., 2008. Biological activities of single-chain goldfish follicle-
1630 stimulating hormone and luteinizing hormone. Aquaculture 274, 408-415.
Hayakawa, Y., Nagaya, H., Kaki, H., Hotta, K., Kobayashi, M., 2009. Induction of spermatogenesis in
1632 Japanese eel by recombinant goldfish gonadotropins. Fisheries Science 75, 137-144.
He, S., Woods III, L.C., 2004. Effects of dimethyl sulfoxide and glycine on cryopreservation induced
1634 damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) sperm. Cryobiology 48, 254-262.
1636 Heyrati, F.P., Amiri, B.M., Dorafshan, S., 2010. Effect of GnRHa injection on milt volume in recently stripped rainbow trout *Oncorhynchus mykiss*. Aquaculture Research 41, 487-492.
1638 Hilomen-Garcia, G.V., Baldevarona, R.B., Lacanillao, F., 2002. Milt production of sea bass *Lates calcarifer* Bloch administered an analogue of luteinizing hormone-releasing hormone and 17-
1640 methyltestosterone. Life Marine Sciences 54, 173-182.
Holt, W.V., Harrison, R.A.P., 2002. Bicarbonate stimulation of boar sperm motility via a protein
1642 kinase A—dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. Journal of Andrology 23, 557-565.
1644 Holt, W.V., O'Brien, J., Abaigar, T., 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research.
1646 Reproduction, Fertility and Development 19, 709-718.
Horváth, Á., Wayman, W.R., Urbányi, B., Ware, K.M., Dean, J.C., Tiersch, T.R., 2005. The
1648 relationship of the cryoprotectants methanol and dimethyl sulfoxide and hyperosmotic extenders on sperm cryopreservation of two North-American sturgeon species. Aquaculture
1650 247, 243-251.
Horváth, Á., Bokor, Z., Bernáth, G., Csenki, Z., Gorjan, A., Herráez, M.P., Urbányi, B., Jesenšek, D.,
1652 2015. Very low sperm–egg ratios result in successful fertilization using cryopreserved sperm in the Adriatic grayling (*Thymallus thymallus*). Aquaculture 435, 75-77.
1654 Horvath, L., Tamas, G., Coche, A.G., 1985. Common carp, pt. 2: Mass production of advanced fry and fingerlings in ponds, FAO Training Series (FAO), no. 9. FAO, Rome (Italy), pp. 85.
1656 Horváth, L., Szabó, T., Burke, J., 1997. Hatchery testing of GnRH analogue-containing pellets on ovulation in four cyprinid species. Polskie Archiwum Hydrobiologii 44, 221-226.
1658 Ibarra-Castro, L., Duncan, N.J., 2007. GnRHa-induced spawning of wild-caught spotted rose snapper *Lutjanus guttatus*. Aquaculture 272, 737-746.
1660 Izquierdo, M.S., Fernández-Palacios , H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197, 25-42.
1662 Juario, J.V., Duray, M.N., Duray, V.M., Nacario, J.F., Almendras, J.M.E., 1985. Breeding and larval rearing of the rabbitfish, *Siganus guttatus* (Bloch). Aquaculture 44, 91-101.
1664 Judycka, S., Szczepkowski, M., Ciereszko, A., Słowinska, M., Bodek, G., Dietrich, G.J., 2015. Characterization of Siberian sturgeon (*Acipenser baerii*, Brandt 1869) sperm obtained out of
1666 season. Journal of Applied Ichthyology 31 (Suppl. 1), 34-40.
Kagawa, H., Kasuga, Y., Adachi, J., Nishi, A., Hashimoto, H., Imaizumi, H., Kaji, S., 2009. Effects of
1668 continuous administration of human chorionic gonadotropinm salmon pituitary extract and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in
1670 male Japanese eel, *Anguilla japonica*. Aquaculture 296, 117-122.
Kamei, H., Kaneko, T., Aida, K., 2006. In vivo gonadotropic effects of recombinant Japanese eel
1672 follicle-stimulating hormone. Aquaculture 261, 771-775.

Kanuga, M.K., Drew, R.E., Wilson-Leedy, J.G., Ingermann, R.L., 2012. Subpopulation distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology 77, 916-925.

Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by Drosophila S2 cells and their differential actions on the reproductive biology. Biology of Reproduction 79, 938-946.

Keller-Costa, T., Hubbard, Peter C., Paetz, C., Nakamura, Y., da Silva, José P., Rato, A., Barata, Eduardo N., Schneider, B., Canario, Adelino V.M., 2014. Identity of a tilapia pheromone released by dominant males that primes females for reproduction. Current Biology 24, 2130-2135.

Kim, N.N., Habibi, H.R., Lee, J., Choi, C.Y., 2012. Effects of recombinant gonadotropin hormones on the expression of vitellogenin, gonadotropin subunits and gonadotropin receptors in cinnamon clownfish, *Amphiprion melanopus*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 162, 73-80.

Kime, D.E., Ebrahimi, M., Nysten, K., Roelants, I., Rurangwa, E., Moore, H.D.M., Ollevier, F., 1996. Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; Application to the effects of heavy metals. Aquatic Toxicology 36, 223-237.

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comparative Biochemistry and Physiology C130, 425-433.

Kime, D.E., Tveiten, H., 2002. Unusual motility characteristics of sperm of the spotted wolffish. Journal of Fish Biology 61, 1549-1559.

King, H.R., Young, G., 2001. Milt production by non-spermiating male Atlantic salmon (*Salmo salar*) after injection of a commercial gonadotropin releasing hormone analog preparation, 17α-hydroxyprogesterone or $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one, alone or in combination. Aquaculture 193, 179-195.

Kobayashi, M., Morita, T., Ikeguchi, K., Yoshizaki, G., Suzuki, T., Watabe, S., 2006. In vivo biological activity of recombinant goldfish gonadotropins produced by baculovirus in silkworm larvae. Aquaculture 256, 433-442.

Kobayashi, M., Hayakawa, Y., Park, W., Banba, A., Yoshizaki, G., Kumamaru, K., Kagawa, H., Kaki, H., Nagaya, H., Sohn, Y.C., 2010. Production of recombinant Japanese eel gonadotropins by baculovirus in silkworm larvae. General and Comparative Endocrinology 167, 379-386.

Komatsu, T., Bhandari, R.K., Kobayashi, Y., Nakamura, S., Nakamura, M., 2006a. GnRHa accelerated spermatogenesis in the testes of underyearling golden rabbitfish, *Siganus guttatus* (Bloch). Aquaculture 257, 558-565.

Komatsu, T., Nakamura, S., Nakamura, M., 2006b. A sex cord-like structure and some remarkable features in early gonadal sex differentiation in the marine teleost *Siganus guttatus* (Bloch). Journal of Fish Biology 68, 236-250.

Kowalski, R., Glogowski, J., Kucharczyk, D., Goryczko, K., Dobosz, S., Ciereszko, A., 2003. Proteolytic activity and electrophoretic profiles of proteases from seminal plasma of teleosts. Journal of Fish Biology 63, 1008-1019.

Kowalski, R.K., Hliwa, P., Cejko, B.I., Krol, J., Stabinski, R., Ciereszko, A., 2012. Quality and quantity of smelt (*Osmerus eperlanus* L.) sperm in relation to time after hormonal stimulation. Reproductive Biology 12, 231-246.

Krol, J., Kowalski, R.K., Hliwa, P., Dietrich, G.J., Stabinski, R., Ciereszko, A., 2009. The effects of commercial preparations containing two different GnRH analogues and dopamine antagonists on spermiation and sperm characteristics in the European smelt *Osmerus eperlanus* (L.). Aquaculture 286, 328-331.

Kucharczyk, D., Kujawa, R., Luczynski, M., Glogowski, J., Babiak, I., Wyszomirska, E., 1997. Induced spawning in bream, *Abramis brama* (L.), using carp and bream pituitary extract and hCG. Aquaculture Research 28, 139-144.

1726 Kustan, J.M., Maruska, K.P., Fernald, R.D., 2012. Subordinate male cichlids retain reproductive competence during social suppression. Proceedings of the Royal Society B: Biological
1728 Sciences 279, 434-443.
LaFleur, G.J., Thomas, P., 1991. Evidence for a role of Na^+,K^+-ATPase in the hydration of Atlantic
1730 croaker and spotted seatrout oocytes during final maturation. The Journal of Experimental Zoology 258, 126-136.
1732 Lahnsteiner, F., 2007. Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*). Animal Reproduction Science 97, 151-164.
1734 Lahnsteiner, F., 2011. Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the ability to swim for more than two hours in saline solutions. Aquaculture 314, 221-224.
1736 Lahnsteiner, F., Patzner, R.A., 1990. Functions of the testicular gland of blenniid fish: structural and histochemical investigations. Experientia 46, 1005-1007.
1738 Lahnsteiner, F., Mansour, N., Berger, B., 2004. Seminal plasma proteins prolong the viability of rainbow trout (*Oncorynchus mykiss*) spermatozoa. Theriogenology 62, 801-808.
1740 Lahnsteiner, F., Radner, M., 2010. Lysozyme activities and immunoglobulin concentrations in seminal plasma and spermatozoa of different teleost species and indications on its significance for
1742 sperm function. Theriogenology 74, 246-254.
Lang, R.P., Tiersch, T.R., 2007. Early out-of-season induced spawning of channel catfish *Ictalurus*
1744 *punctatus* (Rafinesque) conditioned in heated earthen ponds. Aquaculture Research 38, 498-507.
1746 Le Comber, S.C., Faulkes, C.G., Van Look, K.J.W., Holt, W.V., Smith, C., 2004. Recovery of sperm activity after osmotic shock in the three-spined stickleback: implications for pre-oviposition
1748 ejaculation. Behaviour 141, 1555-1569.
Le Gac, F., Blaise, O., Fostier, A., Le Bail, P.Y., Loir, M., Mourot, B., Weil, C., 1993. Growth
1750 hormone (GH) and reproduction: a review. Fish Physiology and Biochemistry 11, 219-232.
Leach, B., Montgomerie, R., 2000. Sperm characteristics associated with different male reproductive
1752 tactics in bluegills (*Lepomis macrochirus*). Behavioral Ecology and Sociobiology 49, 31-37.
Lehnert, S.J., Heath, D.D., Pitcher, T.E., 2012. Sperm trait differences between wild and farmed
1754 Chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 344–349, 242-247.
Lethimonier, C., Madigou, T., Muñoz-Cueto, J.-A., Lareyre, J.-J., Kah, O., 2004. Evolutionary aspects
1756 of GnRHa, GnRH neuronal systems and GnRH receptors in teleost fish. General and Comparative Endocrinology 135, 1-16.
1758 Li, P., Li, Z.-H., Hulak, M., Rodina, M., Linhart, O., 2012. Regulation of spermatozoa motility in response to cations in Russian sturgeon *Acipenser gueldenstaedtii*. Theriogenology 78, 102-
1760 109.
Li, P., Hulak, M., Li, Z.H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., Linhart, O., 2013.
1762 Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology 80, 84-89.
1764 Lim, H.K., Pankhurst, N.W., Fitzgibbon, Q.P., 2004. Effects of slow release gonadotropin releasing hormone analog on milt characteristics and plasma levels of gonadal steroids in greenback
1766 flounder *Rhombosolea tapirina*. Aquaculture 240, 505-516.
Lin, F., Dabrowski, K., 1996. Characteristics of muskellunge spermatozoa II: effects of ions and
1768 osmolality on sperm motility. Transactions of the American Fisheries Society 125, 195-202.
Linhart, O., Billard, R., 1994. Spermiation and sperm quality of European catfish (*Silurus glanis* L.)
1770 after implantation of GnRH analogues and injection of carp pituitary extract. Journal of Applied Ichthyology 10, 182-188.
1772 Linhart, O., Peter, R.E., Rothbard, S., Zohar, Y., Kvasnika, P., 1995. Spermiation of common tench (*Tinca tinca* L.) stimulated with injection or implantation of GnRH analogues and injection of
1774 carp pituitary extract. Aquaculture 129, 119-121.
Linhart, O., Gela, D., Rodina, M., Kocour, M., 2004. Optimization of artificial propagation in
1776 European catfish, *Silurus glanis* L. Aquaculture 235, 619-632.
Linhartova, Z., Rodina, M., Nebesarova, J., Cosson, J., Psenicka, M., 2013. Morphology and
1778 ultrastructure of beluga (*Huso huso*) spermatozoa and a comparison with related sturgeons. Animal Reproduction Science 137, 220-229.

1780 Liu, Q.H., Li, J., Xiao, Z.Z., Ding, F.H., Yu, D.D., Xu, X.Z., 2007. Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus*
1782 *major*). Aquaculture 263, 20-25.
López, D.I., Leal, M.C., Viveiros, A.T.M., 2015. Extender composition and osmolality affects post-
1784 thaw motility and velocities of piracanjuba *Brycon orbignyanus* (Valenciennes, 1850) (Characiformes) sperm. Journal of Applied Ichthyology 31 (Suppl. 1), 114-118.
1786 Mañanos, E., Carrillo, M., Sorbera, L.S., Mylonas, C.C., Asturiano, J.F., Bayarri, M.J., Zohar, Y., Zanuy, S., 2002. Luteinizing hormone and sexual steroid plasma levels after treatment of
1788 European sea bass with sustained-release delivery systems for gonadotropin-releasing hormone analogue. Journal of Fish Biology 60, 328-339.
1790 Mañanos, E., Duncan, N., Mylonas, C.C., 2009. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in
1792 Reproductive Aquaculture. CRC Press Taylor and Francis Group, Boca Raton, pp. 3-80.
Mansour, N., Lahnsteiner, F., Berger, B., 2004. Characterization of the testicular semen of the African
1794 catfish, *Claria gariepinus* (Burchell, 1822), and its short-term storage. Aquaculture Research 35, 232-244.
1796 Marco-Jiménez, F., Pérez, L., Castro, M.P.V.d., Garzón, D.L., Peñaranda, D.S., Vicente, J.S., Jover, M., Asturiano, J.F., 2006a. Morphometry characterisation of European eel spermatozoa with
1798 computer-assisted spermatozoa analysis and scanning electron microscopy. Theriogenology 65, 1302-1310.
1800 Marco-Jiménez, F., Garzón, D.L., Peñaranda, D.S., Pérez, L., Viudes-de-Castro, M.P., Vicente, J.S., Jover, M., Asturiano, J.F., 2006b. Cryopreservation of European eel (*Anguilla anguilla*)
1802 spermatozoa: Effect of dilution ratio, foetal bovine serum supplementation, and cryoprotectants. Cryobiology 53, 51-57.
1804 Marco-Jiménez, F., Peñaranda, D.S., Pérez, L., Viudes-de-Castro, M.P., Mylonas, C.C., Jover, M., Asturiano, J.F., 2008. Morphometric characterization of sharpsnout sea bream (*Diplodus*
1806 *puntazzo*) and gilthead sea bream (*Sparus aurata*) spermatozoa using computer-assisted spermatozoa analysis (ASMA). Journal of Applied Ichthyology 24, 382-385.
1808 Maria, A.N., Viveiros, A.T.M., Freitas, R.T.F., Oliveira, A.V., 2006. Extenders and cryoprotectants for cooling and freezing of piracanjuba (*Brycon orbignyanus*) semen, an endangered Brazilian
1810 teleost fish. Aquaculture 260, 298-306.
Maria, A.N., Carvalho, A.C.M., Araújo, R.V., Santos, J.P., Carneiro, P.C.F., Azevedo, H.C., 2015.
1812 Use of cryotubes for the cryopreservation of tambaqui fish semen (*Colossoma macropomum*). Cryobiology 70, 109-114.
1814 Martínez-Páramo, S., Diogo, P., Beirão, J., Dinis, M.T., Cabrita, E., 2012a. Sperm lipid peroxidation is correlated with differences in sperm quality during the reproductive season in precocious
1816 European sea bass (*Dicentrarchus labrax*) males. Aquaculture 358-359, 246-252.
Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E., 2012b. Sea
1818 bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Animal Reproduction Science 131, 211-
1820 218.
Martinez-Pastor, F., Diaz-Corujo, A.R., Anel, E., Herraez, P., Anel, L., de Paz, P., 2005. Post mortem
1822 time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. Theriogenology 64, 958-974.
1824 Martínez-Pastor, F., Cabrita, E., Soares, F., Anel, L., Dinis, M.T., 2008. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts.
1826 Reproduction 135, 449-459.
Martínez-Pastor, F., Tizado, E.J., Garde, J.J., Anel, L., de Paz, P., 2011. Statistical series:
1828 opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783-795.
1830 Mattei, X., Siau, Y., Thiaw, O.T., Thiam, D., 1993. Peculiarities in the organization of testis of *Ophidian* sp. (Pisces Teleostei). Evidence for two types of spermatogenesis in teleost fish.
1832 Journal of Fish Biology 43, 931-937.

Mazón, M.J., Zanuy, S., Muñoz, I., Carrillo, M., Gómez, A., 2013. Luteinizing hormone plasmid
1834 therapy results in long-lasting high circulating Lh and increased sperm production in European sea bass (*Dicentrarchus labrax*). Biology of Reproduction 88, 32, 31-37.
1836 Mazón, M.J., Gómez, A., Yilmaz, O., Carrillo, M., Zanuy, S., 2014. Administration of follicle-stimulating hormone in vivo triggers testicular recrudescence of juvenile European sea bass
1838 (*Dicentrarchus labrax*). Biology of Reproduction 90, 6, 1-10.
Miranda, L.A., Cassará, M.C., Somoza, G.M., 2005. Increase in milt production by hormonal
1840 treatment in the pejerrey fish *Odontesthes bonariensis* (Valenciennes 1835). Aquaculture Research 36, 1473-1479.
1842 Miura, A., Nomura, K., Imaizumi, H., Jinbo, T., Masuda, Y., Tanaka, H., Ohta, H., 2013. Administration of 17α-hydroxyprogesterone into mature male Japanese eel reduces sperm
1844 motility by decreasing potassium ion concentrations in the seminal plasma. Aquaculture 414–415, 217-223.
1846 Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991a. Involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*).
1848 Biomedical Research 12, 241-248.
Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991b. Hormonal induction of all stages of
1850 spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proceedings of the National Academy of Sciences 88, 5774-5778.
1852 Miura, T., Yamauchi, K., Nagahama, Y., Takahashi, H., 1991c. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin.
1854 Zoological Science 8, 63-73.
Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1992. The role of hormones in the acquisition
1856 of sperm motility in salmonid fish. The Journal of Experimental Zoology 261, 359-363.
Miura, T., Miura, C., 2001. Japanese eel: a model for analysis of spermatogenesis. Zoological Science
1858 18, 1055-1063.
Miura, T., Ando, N., Miura, C., Yamauchi, K., 2002. Comparative studies between in vivo and in vitro
1860 spermatogenesis of Japanese eel (*Anguilla japonica*). Zoological Science 19, 321-329.
Miura, T., Miura, C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiology
1862 and Biochemistry 28, 181-186.
Miura, T., Higuchi, M., Ozaki, Y., Ohta, T., Miura, C., 2006. Progestin is an essential factor for the
1864 initiation of the meiosis in spermatogenetic cells of the eel. Proceedings of the National Academy of Sciences 103, 7333-7338.
1866 Molés, G., Zanuy, S., Muñoz, I., Crespo, B., Martínez, I., Mañanós, E., Gómez, A., 2011. Receptor specificity and functional comparison of recombinant sea bass (*Dicentrarchus labrax*)
1868 gonadotropins (Fsh and Lh) produced in different host systems. Biology of Reproduction 84, 1171-1181.
1870 Moon, S.H., Lim, H.K., Kwon, J.Y., Lee, J.K., Chang, Y.J., 2003. Increased plasma 17-hydroxyprogesterone and milt production in response to gonadotropin-releasing hormone
1872 analogue in captive male starry flounder, *Platichthys stellatus*. Aquaculture 218, 703-716.
Moore, A., Waring, C.P., 1996. Electrophysiological and endocrinological evidence that F-series
1874 prostaglandins function as priming pheromones in mature male Atlantic salmon (*Salmo salar*) parr. The Journal of Experimental Biology 199, 2307-2316.
1876 Moore, A., Olsén, K.H., Lower, N., Kindahl, H., 2002. The role of F-series prostaglandins as reproductive priming pheromones in the brown trout. Journal of Fish Biology 60, 613-624.
1878 Morais, S., Mendes, A.C., Castanheira, M.F., Coutinho, J., Bandarra, N., Dias, J., Conceição, L.E.C., Pousão-Ferreira, P., 2014. New formulated diets for *Solea senegalensis* broodstock: Effects of
1880 parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. Aquaculture 432, 374-382.
1882 Morini, M., Peñaranda, D.S., Vilchez, M.C., Gallego, V., Nourizadeh-Lillabadi, R., Asturiano, J.F., Weltzien, F.-A., Perez, L., 2014. Progestin receptors expression in European eel testis through
1884 spermatogenesis, 10th International Symposiun on Reproductive Physiology of Fish, Olhao (Portugal), pp. 107.
1886 Morisawa, M., 2008. Adaptation and strategy for fertilization in the sperm of teleost fish. Journal of Applied Ichthyology 24, 362-370.

1888 Morisawa, S., Morisawa, M., 1988. Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. Journal of Experimental Biology 136, 13-22.
1890 Mousavi, S.E., Yousefian, M., Masoundria, A., Fahimi, S., 2011. Effects of three different GnRH analogues in combination with two different dopamine antagonists on milt production of
1892 endangered non-spermiating male Caspian brown trout, *Salmo trutta caspius* Advances in Environmental Biology 5, 2761-2768.
1894 Moussavi, M., Wlasichuk, M., Chang, J.P., Habibi, H.R., 2013. Seasonal effect of gonadotrophin inhibitory hormone on gonadotrophin-releasing hormone-induced gonadotroph functions in
1896 the goldfish pituitary. Journal of Neuroendocrinology 25, 506-516.
Müller, T., Urbányi, B., Váradi, B., Binder, T., Horn, P., Bercsényi, M., Horváth, Á., 2004.
1898 Cryopreservation of sperm of farmed European eel *Anguilla anguilla*. Journal of the World Aquaculture Society 35, 225-231.
1900 Muñoz, M., Casadevall, M., Bonet, S., 2002. Testicular structure and semicystic spermatogenesis in a specialized ovuliparous species: *Scorpaena notata* (Pisces, Scorpaenidae). Acta Zoologica 83,
1902 213-219.
Mylonas, C.C., Tabata, Y., Langer, R., Zohar, Y., 1995. Preparation and evaluation of polyanhydride
1904 microspheres containing gonadotropin-releasing hormone (GnRH), for inducing ovulation and spermiation in fish. Journal of Controlled Release 35, 23-34.
1906 Mylonas, C.C., Scott, A.P., Zohar, Y., 1997a. Plasma gonadotropin II, sex steroids, and thyroid hormones in wild striped bass (*Morone saxatilis*) during spermiation and final oocyte
1908 maturation. General and Comparative Endocrinology 108, 223-236.
Mylonas, C.C., Scott, A.P., Vermeirssen, E.L., Zohar, Y., 1997b. Changes in plasma gonadotropin II
1910 and sex steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. Biology of
1912 Reproduction 57, 669-675.
Mylonas, C.C., Gissis, A., Magnus, Y., Zohar, Y., 1997c. Hormonal changes in male white bass
1914 (*Morone chrysops*) and evaluation of milt quality after treatment with a sustained-release GnRHa delivery system. Aquaculture 153, 301-313.
1916 Mylonas, C.C., Woods, L.C., III, Thomas, P., Zohar, Y., 1998a. Endocrine profiles of female striped bass (*Morone saxatilis*) in captivity, during post-vitellogenesis and induction of final oocyte
1918 maturation via controlled-release GnRHa-delivery systems. General and Comparative Endocrinology 110, 276-289.
1920 Mylonas, C.C., Zohar, Y., Woods, L.C., Thomas, P., Schulz, R.W., 1998b. Hormone profiles of captive striped bass Morone saxatilis during spermiation, and long-term enhancement of milt
1922 production. Journal of the World Aquaculture Society 29, 379-392.
Mylonas, C.C., Zohar, Y., 2001a. Use of GnRHa-delivery systems for the control of reproduction in
1924 fish. Reviews in Fish Biology and Fisheries 10, 463-491.
Mylonas, C.C., Zohar, Y., 2001b. Endocrine regulation and artificial induction of oocyte maturation
1926 and spermiation in basses of the genus *Morone*. Aquaculture 202, 205-220.
Mylonas, C.C., Sigelaki, I., Divanach, P., Mananos, E., Carrillo, M., Afonso-Polyviou, A., 2003.
1928 Multiple spawning and egg quality of individual European sea bass (*Dicentrarchus labrax*) females after repeated injections of GnRHa. Aquaculture 221, 605-620.
1930 Mylonas, C.C., Bridges, C., Gordin, H., Rios, A.B., Garcia, A., de la Gandara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G., De Metrio, G., Corriero, A., Vassallo-Agius, R.,
1932 Guzman, J.M., Mananos, E., Zohar, Y., 2007. Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive
1934 maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*). Reviews in Fisheries Science 15, 183-210.
1936 Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. General and Comparative Endocrinology 165, 516-534.
1938 Nascimento, A.F., Maria, A.N., Pessoa, N.O., Carvalho, M.A.M., Viveiros, A.T.M., 2010. Out-of-season sperm cryopreserved in different media of the Amazonian freshwater fish pirapitinga
1940 (*Piaractus brachypomus*). Animal Reproduction Science 118, 324-329.

Negatu, Z., Hsiao, S.M., Wallace, R.A., 1998. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. Fish Physiology and Biochemistry 19, 13-21.
1942
Norambuena, F., Mackenzie, S., Bell, J.G., Callol, A., Estévez, A., Duncan, N., 2012. Prostaglandin (F and E, 2- and 3-series) production and cyclooxygenase (COX-2) gene expression of wild and cultured broodstock of senegalese sole (*Solea senegalensis*). General and Comparative Endocrinology 177, 256-262.
1944
1946
Oda, S., Morisawa, M., 1993. Rises of intracellular Ca^{2+} and pH mediate the initiation of sperm motility by hyperosmolality in marine teleosts. Cell Motility Cytoskeleton 25, 171-178.
1948
Ogier de Baulny, B., Labbé, C., Maisse, G., 1999. Membrane integrity, mitochondrial activity, ATP content, and motility of the European catfish (*Silurus glanis*) testicular spermatozoa after freezing with different cryoprotectants. Cryobiology 39, 177-184.
1950
1952
Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Milt production in the Japanese eel *Anguilla japonica* induced by repeated injections of human chorionic gonadotropin. Fisheries Science 62, 44-49.
1954
Ohta, H., Tanaka, H., 1997. Relationship between serum levels of human chorionic gonadotropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. Aquaculture 153, 123-134.
1956
1958
Ohta, H., Ikeda, K., Izawa, T., 1997a. Increases in concentration of potassium and bicarbonate ions promote acquisition of motility *in vitro* by Japanese eel spermatozoa. The Journal of Experimental Zoology 277, 171-180.
1960
Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iimura, N., Hirose, K., 1997b. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiology and Biochemistry 17, 163-169.
1962
1964
Ohta, H., Miyake, H., Miura, C.I., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. Biology of Reproduction 77, 970-977.
1966
Orfão, L.H., Nascimento, A.F., Corrêa, F.M., Cosson, J., Viveiros, A.T.M., 2011. Extender composition, osmolality and cryoprotectant effects on the motility of sperm in the Brazilian endangered species *Brycon opalinus* (Characiformes). Aquaculture 311, 241-247.
1968
1970
Ottesen, O.H., Babiak, I., Dahle, G., 2009. Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 286, 240-245.
1972
Pankhurst, N.W., 1994. Effects of gonadotropin releasing hormone analogue, human chorionic gonadotropin and gonadal steroids on milt volume in the New Zealand snapper, *Pagrus auratus* (Sparidae). Aquaculture 125, 185-197.
1974
Parenti, L.R., Grier, H.J., 2004. Evolution and phylogeny of gonad morphology in bony fishes. Integrative and Comparative Biology 44, 333-348.
1976
Park, I.S., Choi, G.C., Nam, Y.K., Kim, D.S., 2002. The effect of exogenous hormone treatment on spermiation in *Rhynchocypris oxycephalus* (Sauvage and Dabry). Journal of the World Aquaculture Society 33, 494-500.
1978
1980
Peñaranda, D.S., Pérez, L., Fakriadis, G., Mylonas, C.C., Asturiano, J.F., 2008. Effects of extenders and cryoprotectant combinations on motility and morphometry of sea bass (*Dicentrarchus labrax*) spermatozoa. Journal of Applied Ichthyology 24, 450-455.
1982
Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F., 2010a. Molecular and physiological study of the artificial maturation process in European eel males: From brain to testis. General and Comparative Endocrinology 166, 160-171.
1984
1986
Peñaranda, D.S., Marco-Jiménez, F., Pérez, L., Gallego, V., Mazzeo, I., Jover, M., Asturiano, J.F., 2010b. Protein profile study in European eel (*Anguilla anguilla*) seminal plasma and its correlation with sperm quality. Journal of Applied Ichthyology 26, 746-752.
1988
Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010c. European eel sperm diluent for short-term storage. Reproduction in Domestic Animals 45, 407-415.
1990
Peñaranda, D.S., Gallego, V., Caccia, R., Vílchez, M.C., Pérez, L., Gómez, A., Giménez, I., Asturiano, J.F., 2015. First full spermatogenesis and spermiation induced with specific recombinant gonadotropins in a teleost fish, the European eel, 5th International Workshop on the Biology of Fish Gametes. , Ancona (Italy), pp. 28-30.
1992
1994

Pérez, L., Asturiano, J.F., Tomás, A., Zegrari, S., Barrera, R., Espinós, F.J., Navarro, J.C., Jover, M., 2000. Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. Journal of Fish Biology 57, 1488-1504.

Pérez, L., Peñaranda, D.S., Gallego, V., Asturiano, J.F., 2009. Testis development, sperm quality evaluation and cryopreservation in the European eel. In: van den Thillart, G., Dufour, S., Rankin, C. (Eds.), Spawning Migration of the European Eel. Springer The Netherlands, pp. 333-362.

Pérez, L., Vílchez, M.C., Gallego, V., Morini, M., Peñaranda, D.S., Asturiano, J.F., 2016. Role of calcium on the initiation of sperm motility in the European eel. Comparative Biochemistry and Physiology A 191, 98-106.

Peter, R.E., Lin, H.R., Van Der Kraak, G., 1988. Induced ovulation and spawning of culture freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. Aquaculture 74, 1-10.

Peter, R.E., Lin, H.R., van der Kraak, G., Little, M., 1993. Releasing hormones, dopamine antagonists and induced spawning. In: Muir, J.F., Roberts, R.J. (Eds.), Recent Advances in Aquaculture. Blackwell Scientific Publications, Oxford, pp. 25-30.

Pilastro, A., Scaggiante, M., Rasotto, M.B., 2002. Individual adjustment of sperm expenditure accords with sperm competition theory. Proceedings of the National Academy of Sciences 99, 9913-9915.

Pinheiro, M.F.M., Souza, S.M.G., Barcellos, L.J.G., 2003. Exposure to $17\alpha,20\beta$,-dihydroxy-4-pregnen-3-one changes seminal characteristics in Nile tilapia, *Oreochromis niloticus*. Aquaculture Research 34, 1047-1052.

Podhorec, P., Socha, M., Sokolowska-Mikolajczyk, M., Policar, T., Svinger, V., Drozd, B., Kouril, J., 2011. Dopamine control of LH release in the tench (*Tinca tinca*). General and Comparative Endocrinology 175, 34-38.

Psenicka, M., Dietrich, G.J., Wojtczak, M., Nynca, J., Rodina, M., Linhart, O., Cosson, J., Ciereszko, A., 2008. Acrosome staining and motility characteristics of sterlet spermatozoa after cryopreservation with use of methanol and DMSO. Cryobiology 56, 251-253.

Quintero-Moreno, A., Miró, J., Teresa Rigau, A., Rodríguez-Gil, J.E., 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59, 1973-1990.

Rahman, M.S., Morita, M., Takemura, A., Takano, K., 2003. Hormonal changes in relation to lunar periodicity in the testis of the forktail rabbitfish, *Siganus argenteus*. General and Comparative Endocrinology 131, 302-309.

Rainis, S., Mylonas, C.C., Kyriakou, Y., Divanach, P., 2003. Enhancement of spermiation in European sea bass (*Dicentrarchus labrax*) at the end of the reproductive season using GnRHa implants. Aquaculture 219, 873-890.

Riesco, M.F., Martínez-Pastor, F., Chereguini, O., Robles, V., 2011. Evaluation of zebrafish (*Danio rerio*) PGCs viability and DNA damage using different cryopreservation protocols. Theriogenology 77, 122-130.

Roelants, I., Mikolajczyk, T., Epler, P., Ollevier, F., Chyb, J., Breton, B., 2000. Induction of spermiation in common carp after enhanced intestinal uptake of sGnRH-A and pimozide. Journal of Fish Biology 56, 1398-1407.

Rosenthal, H., Asturiano, J.F., Linhart, O., Horvath, A., 2010. On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. Journal of Applied Ichthyology 26, 621-622.

Rothbard, S., Yaron, Z., 1995. Carps (Cyprinidae). In: Bromage, N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science Ltd, Oxford, pp. 321-352.

Rudolfsen, G., Müller, R., Urbach, D., Wedekind, C., 2008a. Predicting the mating system from phenotypic correlations between life-history and sperm quality traits in the Alpine whitefish *Coregonus zugensis*. Behavioral Ecology and Sociobiology 62, 561-567.

Rudolfsen, G., Figenschou, L., Folstad, I., Kleven, O., 2008b. Sperm velocity influences paternity in the Atlantic cod (*Gadus morhua* L.). Aquaculture Research 39, 212-216.

2050 Rurangwa, E., Volckaert, F.A.M., Huyskens, G., Kime, D.E., Ollevier, F., 2001. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable
2052 staining and standardized fertilization in African catfish (*Clarias gariepinus*). Theriogenology 55, 751-769.
2054 Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1-28.
2056 Rzemieniecki, A., Domagałła, J., Glogowski, J., Ciereszko, A., Trzebiatowski, R., Kouřil, J., Hamáčkova, J., Babiak, I., 2004. Induced spermiation in 3-year-old sterlet, *Acipenser ruthenus*
2058 L. Aquaculture Research 35, 144-151.
Saad, A., Billard, R., 1987. Spermatozoa production and volume of semen collected after hormonal
2060 stimulation in the carp, *Cyprinus carpio*. Aquaculture 65, 67-77.
Sanches, E.A., Okawara, R.Y., Caneppele, D., Toledo, C.P.R., Bombardelli, R.A., Romagosa, E.,
2062 2015. Sperm characteristics of *Steindachneridion parahybae* (Steindachner, 1877) throughout 112 h of storage at four temperatures. Journal of Applied Ichthyology 31 (Suppl. 1), 79-88.
2064 Schiavone, R., Zilli, L., Vilella, S., Fauvel, C., 2006. Human chorionic gonadotropin induces spermatogenesis and spermiation in 1-year-old European sea bass (*Dicentrarchus labrax*):
2066 assessment of sperm quality. Aquaculture 255, 522-531.
Schreck, C.B., 2010. Stress and fish reproduction: The roles of allostasis and hormesis. General and
2068 Comparative Endocrinology 165, 549-556.
Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiology and
2070 Biochemistry 26, 43-56.
Schulz, R.W., de França, L.R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura,
2072 T., 2010. Spermatogenesis in fish. General and Comparative Endocrinology 165, 390-411.
Scott, A.P., Sumpter, J.P., Stacey, N., 2010. The role of the maturation-inducing steroid in male fishes:
2074 a review. Journal of Fish Biology 76, 183-224.
Segovia, M., Jenkins, J.A., Paniagua-Chavez, C., Tiersch, T.R., 2000. Flow cytometric evaluation of
2076 antibiotic effects on viability and mitochondrial function of refrigerated spermatozoa of nile tilapia. Theriogenology 53, 1489-1499.
2078 Shaliutina, A., Hulak, M., Dzuyba, B., Linhart, O., 2012. Spermatozoa motility and variation in the seminal plasma proteome of Eurasian perch (*Perca fluviatilis*) during the reproductive season.
2080 Molecular Reproduction and Development 79, 879-887.
Shangguan, B., Crim, L.W., 1999. Seasonal variations in sperm production and sperm quality in male
2082 winter flounder, *Pleuronectes americanus*: the effects of hypophysectomy, pituitary replacement therapy, and GnRHa treatment. Marine Biology 134, 19-27.
2084 Sieczynski, P., Cejko, B.I., Grygoruk, C., Glogowski, J., 2015. Cryopreservation of Siberian sturgeon (*Acipenser baerii*, Brandt, 1869) and sterlet (*Acipenser ruthenus*, Linnaeus, 1758) semen and
2086 its influence on sperm motility parameters assessed using a computer-assisted sperm analysis (CASA) system. Journal of Applied Ichthyology 31 (Suppl. 1), 99-103.
2088 Slater, C.H., Schreck, C.B., Amend, D.F., 1995. GnRHa injection accelerates final maturation and ovulation/spermiation of sockeye salmon (*Oncorhynchus nerka*) in both fresh and salt water.
2090 Aquaculture 130, 279-285.
So, W.-K., Kwok, H.-F., Ge, W., 2005. Zebrafish gonadotropins and their receptors: II. Cloning and
2092 characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits—their spatial-temporal expression patterns and receptor specificity. Biology of Reproduction
2094 72, 1382-1396.
Soler, C., De Monserrat, J.J., Gutiérrez, R., Nuñez, J., Nuñez, M., Sancho, M., Pérez-Sánchez, F.,
2096 Cooper, T.G., 2003. Use of the Sperm-Class Analyser® for objective assessment of human sperm morphology. International Journal of Andrology 26, 262-270.
2098 Sorbera, L.A., Mylonas, C.C., Zanuy, S., Carillo, M., Zohar, Y., 1996. Sustained administration of GnRHa increases milt volume without altering sperm counts in the sea bass. The Journal of
2100 Experimental Zoology 276, 361-368.
Sorensen, P.W., Scott, A.P., Kihslinger, R.L., 1999. How common hormonal metabolites function as
2102 relatively specific pheromonal signals in the goldfish. In: Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., Stefansson, S.O. (Eds.), 6th International Symposium on Reproductive

Physiology of Fish. University of Bergen, Bergen, Norway: Department of Fisheries and Marine Biology pp. 125-128.

Sorensen, P.W., Stacey, N.E., 2004. Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. New Zealand Journal of Marine and Freshwater Research 38, 399-417.

Sørensen, S.R., Gallego, V., Pérez, L., Butts, I.A.E., Tomkiewicz, J., Asturiano, J.F., 2013. Evaluation of methods to determine sperm density for the European eel, *Anguilla anguilla*. Reproduction in Domestic Animals 48, 936-944.

Spadella, M.A., Oliveira, C., Quagio-Grassiotto, I., 2007. Comparative analyses of spermiogenesis and sperm ultrastructure in Callichthyidae (Teleostei: Ostariophysi: Siluriformes). Neotropical Ichthyology 5, 337–350.

Stacey, N., 2003. Hormones, pheromones and reproductive behavior. Fish Physiology and Biochemistry 28, 229-235.

Stacey, N., Zheng, W.-b., Cardwell, J., 1994. Milt production in common carp (*Cyprinus carpio*): stimulation by a goldfish steroid pheromone. Aquaculture 127, 265-276.

Stacey, N.E., Sorensen, P.W., Van Der Kraak, G.J., Dulka, J.G., 1989. Direct evidence that 17α,20ß-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone: preovulatory release is closely associated with male endocrine responses. General and Comparative Endocrinology 75, 62-70.

Stoltz, J.A., Neff, B.D., 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. Journal of Evolutionary Biology 19, 1873-1881.

Suquet, M., Omnes, M.H., Normant, Y., Fauvel, C., 1992. Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture 101, 177-185.

Takashima, F., Weil, C., Billard, R., Crim, L.W., Fostier, A., 1984. Stimulation of spermiation by LHRH analogue in carp. Bulletin of the Japanese Society of Scientific Fisheries 50, 1323-1329.

Tan, W., Aizen, J., Thomas, P., 2014. Membrane progestin receptor alpha mediates progestin-induced sperm hypermotility and increased fertilization success in southern flounder (*Paralichthys lethostigma*). General and Comparative Endocrinology 200, 18-26.

Thomas, P., Pang, Y., Zhu, Y., Detweiler, C., Doughty, K., 2004. Multiple rapid progestin actions and progestin membrane receptor subtypes in fish. Steroids 69, 567-573.

Thomas, P., Tubbs, C., Detweiler, C., Das, S., Ford, L., Breckenridge-Miller, D., 2005. Binding characteristics, hormonal regulation and identity of the sperm membrane progestin receptor in Atlantic croaker. Steroids 70, 427-433.

Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., Clark, A.M., 2009. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Human Reproduction 24, 2061-2070.

Tsutsui, K., Ubuka, T., 2014. Breakthrough in neuroendocrinology by discovering novel neuropeptides and neurosteroids: 1. Discovery of gonadotropin-inhibitory hormone (GnIH) across vertebrates. General and Comparative Endocrinology 205, 4-10.

Tubbs, C., Thomas, P., 2008. Functional characteristics of membrane progestin receptor alpha (mPRα) subtypes: A review with new data showing mPRα expression in seatrout sperm and its association with sperm motility. Steroids 73, 935-941.

Tubbs, C., Thomas, P., 2009. Progestin signaling through an olfactory G protein and membrane progestin receptor in Atlantic croaker sperm: potential role in induction of sperm hypermotility. Endocrinology 150, 473-484.

Tubbs, C., Tan, W., Shi, B., Thomas, P., 2011. Identification of 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) receptor binding and membrane progestin receptor alpha on southern flounder sperm (*Paralichthys lethostigma*) and their likely role in 20β-S stimulation of sperm hypermotility. General and Comparative Endocrinology 170, 629-639.

Tuset, V.M., Dietrich, G.J., Wojtczak, M., Slowinska, M., De Monserrat, J., Ciereszko, A., 2008a. Relationships between morphology, motility and fertilization capacity in rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Journal of Applied Ichthyology 24, 393-397.

Tuset, V.M., Trippel, E.A., De Monserrat, J., 2008b. Sperm morphology and its influence on swimming speed in Atlantic cod. Journal of Applied Ichthyology 24, 398-405.

Tuset, V.M., Dietrich, G.J., Wojtczak, M., Słowińska, M., de Monserrat, J., Ciereszko, A., 2008c. Comparison of three staining techniques for the morphometric study of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology 69, 1033-1038.

Ulloa-Aguirre, A., Timossi, C., 2000. Biochemical and functional aspects of gonadotrophin-releasing hormone and gonadotrpophins. Reproductive Biomedicine Online 1, 48-62.

Urbanyi, B., Horvath, A., Bokor, Z., 2009. Artificial fertilization in aquaculture species: from normal practice to chromosome manipulations. CRC Press Taylor and Francis Group, Boca Raton, USA, pp. 183–216.

Valdebenito, I.I., Gallegos, P.C., Effer, B.R., 2015. Gamete quality in fish: evaluation parameters and determining factors. Zygote 23, 177-197.

Van Der Kraak, G., Pankhurst, N.W., Peter, R.E., Lin, H.R., 1989. Lack of antigenicity of human chorionic gonadotropin in silver carp (*Hypophthalmichthys molitrix*) and goldfish (*Carassius auratus*). Aquaculture 78, 81-86.

Van Look, K.J.W., Kime, D.E., 2003. Automated sperm morphology analysis in fishes: the effect of mercury on goldfish sperm. Journal of Fish Biology 63, 1020-1033.

Velasco-Santamaría, Y.M., Medina-Robles, V.M., Cruz-Casallas, P.E., 2006. Cryopreservation of yamú (*Brycon amazonicus*) sperm for large scale fertilization. Aquaculture 256, 264-271.

Vergílio, C.d.S., Moreira, R.V., de Carvalho, C.E.V., de Melo, E.J.T., 2013. Characterization of mature testis and sperm morphology of *Gymnotus carapo* (Gymnotidae, Teleostei) from the southeast of Brazil. Acta Zoologica 94, 364-370.

Vermeirssen, E.L.M., Scott, A.P., Mylonas, C.C., Zohar, Y., 1998. Gonadotrophin-releasing hormone agonist stimulates milt fluidity and plasma concentrations of 17,20ß-dihydroxylated and 5ß-reduced, 3a-hydroxylated C21 steroids in male plaice (*Pleuronectes platessa*). General and Comparative Endocrinology 112, 163-177.

Vermeirssen, E.L.M., Shields, R.J., Mazorra de Quero, C., Scott, A.P., 2000. Gonadotrophin-releasing hormone agonist raises plasma concentrations of progestogens and enhances milt fluidity in male Atlantic halibut (*Hippoglossus hippoglossus*). Fish Physiology and Biochemistry 22, 77-87.

Vermeirssen, E.L.M., Mazorra de Quero, C., Shields, R.J., Norberg, B., Kime, D.E., Scott, A.P., 2004. Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotropin-releasing hormone agonist implant. Aquaculture 230, 547-567.

Viveiros, A.T.M., Fessehaye, Y., ter Veld, M., Schulz, R.W., Komen, J., 2002. Hand-stripping of semen and semen quality after maturational hormone treatments, in African catfish *Clarias gariepinus*. Aquaculture 213, 373-386.

Viveiros, A.T.M., Godinho, H.P., 2009. Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. Fish Physiology and Biochemistry 35, 137-150.

Viveiros, A.T.M., Orfão, L.H., Maria, A.N., Allaman, I.B., 2009. A simple, inexpensive and successful freezing method for curimba *Prochilodus lineatus* (Characiformes) semen. Animal Reproduction Science 112, 293-300.

Viveiros, A.T.M., Nascimento, A.F., Orfão, L.H., Isaú, Z.A., 2010. Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. Theriogenology 74, 551-556.

Viveiros, A.T.M., Orfão, L.H., Nascimento, A.F., Corrêa, F.M., Caneppele, D., 2012a. Effects of extenders, cryoprotectants and freezing methods on sperm quality of the threatened Brazilian freshwater fish pirapitinga-do-sul *Brycon opalinus* (Characiformes). Theriogenology 78, 361-368.

Viveiros, A.T.M., Isaú, Z.A., Caneppele, D., Leal, M.C., 2012b. Sperm cryopreservation affects postthaw motility, but not embryogenesis or larval growth in the Brazilian fish *Brycon insignis* (Characiformes). Theriogenology 78, 803-810.

Vizziano, D., Fostier, A., Loir, M., Le Gac, F., 2008. Testis development, its hormonal regulation and spermiation induction in teleost fish. In: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Intl, Oxford (UK), pp. 103-139.

Vladić, T.V., 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proceedings of the Royal Society of London B: Biological Sciences 268, 2375-2381.

Von Ihering, R., 1937. A method for inducing spawning in fish. The Progressive Fish-Culturist 34, 15-16.

Wade, M.G., Van der Kraak, G., Gerrits, M.F., Ballantyne, J.S., 1994. Release and steroidogenic actions of polyunsaturated fatty acids in the goldfish testis. Biology of Reproduction 51, 131-139.

Wang, Q., Qi, X., Guo, Y., Li, S., Zhang, Y., Liu, X., Lin, H., 2015. Molecular identification of GnIH/GnIHR signal and its reproductive function in protogynous hermaphroditic orange-spotted grouper (*Epinephelus coioides*). General and Comparative Endocrinology 216, 9-23.

Warnecke, D., Pluta, H.-J., 2003. Motility and fertilizing capacity of frozen/thawed common carp (*Cyprinus carpio* L.) sperm using dimethyl-acetamide as the main cryoprotectant. Aquaculture 215, 167-185.

Watanabe, A., Onitake, K., 2008. The regulation of spermatogenesis in fish: recent cellular and molecular approaches. In: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Intl, Oxford (UK), pp. 141-160.

Wathes, D.C., Abayasekara, D.R.E., Aitken, R.J., 2007. Polyunsaturated fatty acids in male and female reproduction. Biology of Reproduction 77, 190-201.

Weber, G.M., Borski, R.J., Powell, J.F.F., Sherwood, N.M., Grau, E.G., 1995. *In vivo* and *in vitro* effects of gonadotropin-releasing hormone on prolactin in the tilapia *Oreochromis mossambicus*. American Zoologist (Abstract) 34, 121A.

Weil, C., Crim, L.W., 1983. Administration of LHRH analogues in various ways: effect on the advancement of spermiation in prespawning landlocked salmon, *Salmo salar*. Aquaculture 35, 103-115.

WHO, U.N., 1999. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Cambridge University Press, Cambridge, UK, 286 pp.

Williot, P., Kopeika, E.F., Goncharov, B.F., 2000. Influence of testis state, temperature and delay in semen collection on spermatozoa motility in the cultured Siberian sturgeon (*Acipenser baeri* Brandt). Aquaculture 189, 53-61.

Williot, P., Gulyas, T., Ceapa, C., 2002. An analogue of GnRH is effective for induction of ovulation and spermiation in farmed Siberian sturgeon *Acipenser baerii* Brandt. Aquaculture Research 33, 735-737.

Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67, 661 - 672.

Wilson-Leedy, J.G., Kanuga, M.K., Ingermann, R.L., 2009. Influence of osmolality and ions on the activation and characteristics of zebrafish sperm motility. Theriogenology 71, 1054-1062.

Wojtczak, M., Kuzminski, H., Dobosz, S., Mikolajczyk, T., Dietrich, G.J., Kowalski, R., Kotlowska, M., Enright, W.J., Ciereszko, A., 2005. Milt characteristics in European whitefish (*Coregonus lavaretus*) in relation to season and hormonal stimulation with a gonadotropin-releasing hormone analogue, azagly-nafarelin. Advances in Limnology 60, 171-185.

Wojtczak, M., Dietrich, G.J., Słowińska, M., Dobosz, S., Kuźmiński, H., Ciereszko, A., 2007a. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture 270, 259-264.

Wojtczak, M., Dietrich, G.J., Irnazarow, I., Jurecka, P., Słowińska, M., Ciereszko, A., 2007b. Polymorphism of transferrin of carp seminal plasma: Relationship to blood transferrin and sperm motility characteristics. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 148, 426-431.

Woolsey, J., Ingermann, R.L., 2003. Acquisition of the potential for sperm motility in steelhead (*Oncorhynchus mykiss*): effect of pH on dynein ATPase. Fish Physiology and Biochemistry 29, 47-56.

Yamazaki, F., Donaldson, E.M., 1968. The spermiation of goldfish (*Carassius auratus*) as a bioassay for salmon (*Oncorhynchus tshawytscha*) gonadotropin. General and Comparative Endocrinology 10, 383-391.

Yambe, H., Kitamura, S., Kamio, M., Yamada, M., Matsunaga, S., Fusetani, N., Yamazaki, F., 2006.
2268 L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. Proceedings of the National Academy of Sciences 103, 15370-15374.
2270 Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49-73.
2272 Yaron, Z., Bogomolnaya, A., Drori, S., Biton, I., Aizen, J., Kulikovsky, Z., Levani-Sivan, B., 2009. Spawning induction in the carp: past experience and future prospects - a review. The Israeli
2274 Journal of Aquaculture 61, 5-26.
Zarski, D., Cejko, B.I., Krejszeff, S., Palinska-Zarska, K., Horváth, Á., Sarosiek, B., Judycka, S.R.,
2276 Kowalski, K., Łazczynska, B., Kucharczyk, D., 2015. The effect of osmolality on egg fertilization in common carp, *Cyprinus carpio* Linnaeus, 1758. Journal of Applied Ichthyology
2278 31 (Suppl. 1), 159-163.
Zhang, Z., Lau, S.-W., Zhang, L., Ge, W., 2015. Disruption of zebrafish follicle-stimulating hormone
2280 receptor (fshr) but not luteinizing hormone receptor (lhr) gene by TALEN leads to failed Follicle activation in females followed by sexual reversal to males. Endocrinology 156, 3747-
2282 3762.
Zheng, W., Strobeck, C., Stacey, N., 1997. The steroid pheromone 4-pregnen-17,20ß-diol-3-one
2284 increases fertility and paternity in goldfish. The Journal of Experimental Biology 200, 2833-2840.
2286 Zilli, L., Vilella, S., 2012. Effect of cryopreservation on bio-chemical parameters, DNA Integrity, protein profile and phosphorylation state of proteins of seawater fish spermatozoa. In: Katkov,
2288 I.I. (Ed.), Current Frontiers in Cryobiology. InTech, InTechOpen. pp. 391-414.
Zmora, N., Kazeto, Y., Kumar, R.S., Schulz, R.W., Trant, J.M., 2007. Production of recombinant
2290 channel catfish (*Ictalurus punctatus*) FSH and LH in S2 Drosophila cell line and an indication of their different actions. Journal of Endocrinology 194, 407-416.
2292 Zmora, N., Stubblefield, J.D., Wong, T.-T., Levavi-Sivan, B., Millar, R.P., Zohar, Y., 2015. Kisspeptin antagonists reveal Kisspeptin 1 and Kisspeptin 2 differential regulation of
2294 reproduction in the teleost, *Morone saxatilis*. Biology of Reproduction 93, 76, 71-12.
Zohar, Y., Goren, A., Fridkin, M., Elhanati, E., Koch, Y., 1990. Degradation of gonadotropin-
2296 releasing hormones in the gilthead seabream *Sparus aurata* II. Cleavage of native salmon GnRH, mammalian LHRH and their analogs in the pituitary, kidney and liver. General and
2298 Comparative Endocrinology 79, 306-319.
Zohar, Y., 1996. New approaches for the manipulation of ovulation and spawning in farmed fish.
2300 Bulletin of the National Research Institute for Aquaculture, Supplement 2, 43-48.
Zohar, Y., Mylonas, C.C., 2001. Endocrine manipulations of spawning in cultured fish: from
2302 hormones to genes. Aquaculture 197, 99-136.

2304

2306 **Figure legends**

2308 **Figure 1.** Schematic representation of the dysfunction in the reproductive axis of cultured male breeders, and the exogenous hormonal interventions for the enhancement of
2310 spermiation. DA = dopamine antagonist, FSH =follicle stimulating hormone, GnRH = gonadotropin-releasing hormone, GnRHa = GnRH agonist, GtH = gonadotropin, LH =
2312 luteinizing hormone, 11-KT = 11 ketotestosterone, 17,20β-P = 17,20β-dihydroxy-4-pregnen-3-one.

2314 **Figure 2.** Mean (+SEM) volume of expressible sperm and spermatozoa density over a 27-day period, from European seabass (*Dicentrarchus labrax,* n = 6) administered different
2316 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
2318 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
2320 were no significant differences among treatments in spermatozoa density. (From the experiments reported by Rainis et al., 2003).

2322 **Figure 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
2324 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).
2326 Statistically different means are indicated by different letter superscripts (one-way ANOVA, $P = 0.012$; DNMR, $P \leq 0.05$).

2328 **Figure 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

2330 spermatozoa, but also a large number of spermatocysts with spermatids and spermatocytes at various stages, while the central part (B) contained mostly spermatozoa.

2332 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)

2334 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

2336 gamete cells at earlier developmental stages. In addition, the somatic cells lining the tubules became hypertrophied. Somatic cells (som), spermatocytes (spc), spermatids

2338 (spt) and spermatozoa (spz). The bar in all sections is 100 μm (fFrom the experiments reported by Rainis et al., 2003).

2340

2342 **Table 1.** Representative applications of hormonal manipulations for the enhancement of spermiation using various gonadotropin (GtH) preparations of piscine origin.

Species	Common name	Treatment [1]	Enhancement [2] period (h)	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 [3]	(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)

2356	*Cyprinus carpio*	common carp	CPE	24	(Li et al., 2013)
	Huso huso	beluga	CPE	48	(Linhartova et al., 2013)
2358	*Odontesthes bonariensis*	pejerrey	CPE, salmon PE	24	(Miranda et al., 2005)
	Piaractus brachypomus	cirapitinga	CPE	12	(Nascimento et al., 2010)
2360	*Prochilodus argenteus*	curimatã-pacu	CPE	-	(Arantes et al., 2011)
	Prochilodus lineatus	curimba	CPE	8	(Viveiros et al., 2009, 2010)
2362	*Silurus glanis*	European catfish	CPE	24-48	(Linhart et al., 2004)
	Tinca tinca	common tench	CPE	24	(Linhart et al., 1995)

2364 [1] Ground pituitaries or pituitary extracts, mainly Carp Pituitary Extract (CPE), or pituitary extracts (PE) from other fishes.

[2] The enhancement period is in days (d) after treatment. The absence of a number indicates that this parameter was not reported in the article.

2366 [3] Through the use of an controlled-release osmotic pump loaded with salmon PE.

2368

Table 2. Representative applications of hormonal manipulations for the enhancement of spermiation using gonadotropin (GtH) preparations of
2370 mammalian origin.

	Species	Common name	Treatment [1]	Enhancement [2] period (d)	Reference
2372					
	Abramis brama	common bream	hCG	-	(Kucharczyk et al., 1997)
2374	*Acipenser ruthenus*	sterlet	hCG	1	(Rzemieniecki et al., 2004)
	Anguilla anguilla	European eel	hCG [3]	35	(Asturiano et al., 2006)
2376	*Anguilla japonica*	Japanese eel	hCG [3]	35	(Ohta & Tanaka, 1997)
	Carassius auratus	goldfish	hCG	1	(Yamazaki & Donaldson, 1968)
2378	*Dicentrarchus labrax*	European seabass	hCG	7	(Schiavone et al., 2006)
	Leiopotherapon plumbeus	silver perch	hCG	1	(Denusta et al., 2014)
2380	*Leuciscus leuciscus*	dace	hCG	2	(Cejko et al., 2012)
	Odontesthes bonariensis	pejerrey	hCG	2	(Miranda et al., 2005)
2382	*Pagrus auratus*	New Zealand snapper	hCG	1	(Pankhurst, 1994)
	Pangasius bocourti	Mekong catfish	hCG	1	(Cacot et al., 2003)
2384	*Rhynchocypris oxycephalus*	Chinese minnow	hCG	1	(Park et al., 2002)
	Sander lucioperca	pikeperch	hCG	1	(Falahatkar & Poursaeid, 2014)
2386	*Siganus argenteus*	forktail rabbitfish	hCG	2	(Rahman et al., 2003)

[1] Human chorionic gonadotropin (hCG).

2388 [2] The enhancement period is in days (d) after treatment. The absence of a number indicates that this parameter was not reported in the article.

[3] Treatment applied a minimum of 5 times at weekly intervals.

2390

2392

Table 3. Representative applications of hormonal manipulations for the enhancement of spermiation using injections of agonists of

2394 gonadotropin-releasing hormone (GnRHa) and sustained-release GnRHa-delivery systems (implants or microspheres).

Species	Common name	Method[1]	GnRHa[2] type	Enhancement[3] period (d)	Reference
Acanthopagrus australis	yellowfin bream	Inj	Trp	2	(Black & 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[4]	1	(Cejko et al., 2014)
		OP	Ala[4]	0.5	(Cejko et al., 2014)
Carassius carassius	crucial carp	Inj	Arg[4]	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[4]	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[4]	2	(Hilomen-Garcia et al., 2002)
Leuciscus leuciscus	dace	Inj	Arg[4]	2	(Cejko et al., 2012)

2412			OP	Ala[4]	2	(Cejko et al., 2012)
	Morone chrysops	white bass	FA	Ala	7	(Mylonas et al., 1997c)
2414	*Morone saxatilis*	striped bass	EVAc, FA	Ala	14	(Mylonas et al., 1997b)
			FA	Ala	14	(Mylonas et al., 1995)
2416	*Oncorhynchus mykiss*	rainbow trout	Inj	Ala	7	(Heyrati et al., 2010)
	Osmerus eperlanus	smelt	Inj	Ala[4]	3	(Kowalski et al., 2012)
2418			Inj	Ala[4]	1	(Krol et al., 2009)
	Pagrus auratus	New Zealand snapper	Inj	Ala	1	(Pankhurst, 1994)
2420	*Pangasius bocourti*	Mekong catfish	Inj	Arg	2	(Cacot et al., 2003)
	Perca flavescences	yellow perch	Inj	Ala	4	(Dabrowski et al., 1994)
2422	*Platichthys stellatus*	starry flounder	Chol	Ala	35	(Moon et al., 2003)
	Pleuronectes americanus	winter flounder	Inj	Ala	12	(Harmin & Crim, 1993)
2424			Chol	Ala	35	(Shangguan & Crim, 1999)
	Pleuronectes ferrugineus	yellowtail flounder	Chol, FA	Ala	29	(Clearwater & Crim, 1998)
2426	*Rhombosolea tapirina*	greenback flounder	Chol	Ala	35	(Lim et al., 2004)
	Rhynchocypris oxycephalus	Chinese minnow	Inj	Ala	2	(Park et al., 2002)
2428	*Salmo salar*	Atlantic salmon	FA	Ala	10	(Mylonas et al., 1995)
			EVAc	Ala	10	(Zohar, 1996)
2430			Inj	Arg[4]	8	(King & Young, 2001)
	Siganus guttatus	golden rabbitfish	Inj	Ala	1	(Garcia, 1991)

2432				7	(Garcia, 1993)
Solea senegalensis	Senegalese sole	EVAc	Ala[4]	25	(Guzmán et al., 2011a, b)
2434 *Tinca tinca*	common tench	Inj	Arg	5	(Linhart et al., 1995)
		EVAc	Ala	5	(Linhart et al., 1995)

2436 [1] 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

2438 [2] 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

2440 [3] The enhancement period is in days (d) after treatment.

[4] Combination treatment with a dopamine antagonist (DA) such as pimozide, domperidone, reserpine or metoclopramide, or steroids.

2442

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

Species	**Common name**	**Hormone [1] treatment**	**Motility [2] %**	**Motility [2] duration**	**Sperm [2] density**	**Sperm [2] velocity**	**Reference**
Abramis brama	common bream	GtH	(+)				(Kucharczyk et al., 1997)
		hCG	(+)				(Kucharczyk et al., 1997)
Acanthopagrus australis	yellowfin bream	GnRHa		(=)	(+)		(Black & 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 & Dabrowski, 1996)
Esox lucius	pike	GtH			(-)		(Billard & 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)
2464	*Leuciscus leuciscus*	dace	GnRHa	(=)		(-)	(=)	(Cejko et al., 2012)
			GtH	(=)		(-)	(=)	(Cejko et al., 2012)
2466	*Morone chrysops*	white bass	GnRHa	(=)		(=)		(Mylonas et al., 1997c)
	Morone saxatilis	striped bass	GnRHa			(+/-)		(Mylonas et al., 1997b)
2468			GnRHa			(+/=)		(Mylonas et al., 1995)
	Oncorhynchus mykiss	rainbow trout	GnRHa		(=)	(=)		(Heyrati et al., 2010)
2470	*Osmerus eperlanus*	smelt	GnRHa	(+)		(+/=)	(+)	(Kowalski et al., 2012)
			GnRHa	(=)	(=)	(-)	(=)	(Krol et al., 2009)
2472	*Pangasius bocourti*	Mekong catfish	GnRHa			(=)		(Cacot et al., 2003)
			hCG			(=)		(Cacot et al., 2003)
2474	*Platichthys stellatus*	starry flounder	GnRHa	(=)		(+/=)	(=)	(Moon et al., 2003)
	Pleuronectes americanus	winter flounder	GnRHa			(=)		(Harmin & Crim, 1993)
2476			GnRHa	(+/-)		(-)		(Shangguan & Crim, 1999)
			GtH	(=)	(=)	(-)		(Shangguan & Crim, 1999)
2478	*Pleuronectes ferrugineus*	yellowtail flounder	GnRHa	(+)	(+)	(=)		(Clearwater & Crim, 1998)
	Rhombosolea tapirina	greenback flounder	GnRHa		(=)	(-)	(=)	(Lim et al., 2004)
2480	*Rhynchocypris oxycephalus*	Chinese minnow	GnRHa			(-)		(Park et al., 2002)
			hCG			(-)		(Park et al., 2002)
2482	*Salmo trutta*	brown trout	GnRHa	(=)	(=)	(-)		(Mousavi et al., 2011)

Siganus guttatus	golden rabbitfish	GnRHa	(-)	(Garcia, 1991; Garcia, 1993)
Silurus glanis	European catfish	GtH	(=)	(Linhart & Billard, 1994)
Solea senegalensis	Senegalese sole	GnRHa	(-)	(Guzmán et al., 2011b)

[1] GnRHa = gonadotropin-releasing hormone agonists (w/without DA); human Chorionic Gonadotropin = hCG; Pituitary extracts or piscine GtHs (mainly LH) = GtH

[2] (+) = increase; (-) = decrease; (=) = not affected. The absence of any symbol indicates that this parameter was not reported in the article.

2490

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

Quantification method	Requirements	Advantage	Disadvantages
Neubauer improved haemocytometer	Microscope, haemocytometer and trained personnel	Inexpensive, precise, and well described in the literature	Time consuming
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 a	Trained personnel required, need to extrapolate by equation, and expensive if fluorospheres are required

2492 2494 2496 2498 2500 2502 2504 2506 2508

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

2510

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 & 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)

2512 2514 2516 2518 2520 2522 2524 2526 2528

		Oncorhynchus mykiss	rainbow trout	(Lahnsteiner et al., 2004)
2530		*Salmo trutta*	brown trout	(Formicki et al., 2015)
		Steindachneridion parahybae	surubim-do-Paraíba	(Sanches et al., 2015)
2532	Activation media tests	*Coregonus lavaretus*	European whitefish	(Dziewulska et al., 2015)
		Cyprinus carpio	common carp	(Zarski et al., 2015)
2534		*Oncorhynchus mykiss*	steelhead	(Kanuga et al., 2012)
		Perca fluviatilis	Eurasian perch	(Lahnsteiner, 2011)
2536	Broodstock management	*Dicentrarchus labrax*	European seabass	(Felip et al., 2006)
		Solea senegalensis	Senegalese sole	(Beirão et al., 2011a)
2538	Spermiation induction	*Acipenser ruthenus*	sterlet	(Alavi et al., 2012)
		Anguilla anguilla	European eel	(Asturiano et al., 2005; Gallego et al., 2015)
2540		*Hippoglossus hippoglossus*	Atlantic halibut	(Vermeirssen et al., 2004)
		Osmerus eperlanus	smelt	(Kowalski et al., 2012)
2542	*In vitro* fertilization trials	*Anguilla anguilla*	European eel	(Butts et al., 2014)
		Hippoglossus hippoglossus	Atlantic halibut	(Ottesen et al., 2009)
2544		*Oncorhynchus mykiss*	rainbow trout	(Tuset et al., 2008c)
		Pagrus major	red seabream	(Liu et al., 2007)
2546	Diet evaluation	*Anguilla anguilla*	European eel	(Baeza et al., 2015a; Butts et al., 2015)
		Dicentrarchus labrax	European seabass	(Martínez-Páramo et al., 2012a, b)
2548		*Gadus morhua*	Atlantic cod	(Butts et al., 2011c)

		Solea senegalensis	Senegalese sole	(Beirão et al., 2015)
2550	Comparative physiology	*Acipenser gueldenstaedtii*	Russian sturgeon	(Li et al., 2012)
		Gadus morhua	Atlantic cod	(Butts et al., 2010c)
2552		*Hippoglossus hippoglossus*	Atlantic halibut	(Alavi et al., 2011)
		Oncorhynchus mykiss	rainbow trout	(Wojtczak et al., 2007a)
2554		*Oncorhynchus tshawytscha*	chinook salmon	(Lehnert et al., 2012)
		Salvelinus namaycush	Lake trout	(Galvano et al., 2013)

2556

2558 **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)

2560 2562 2564 2566 2568 2570 2572 2574 2576

2578

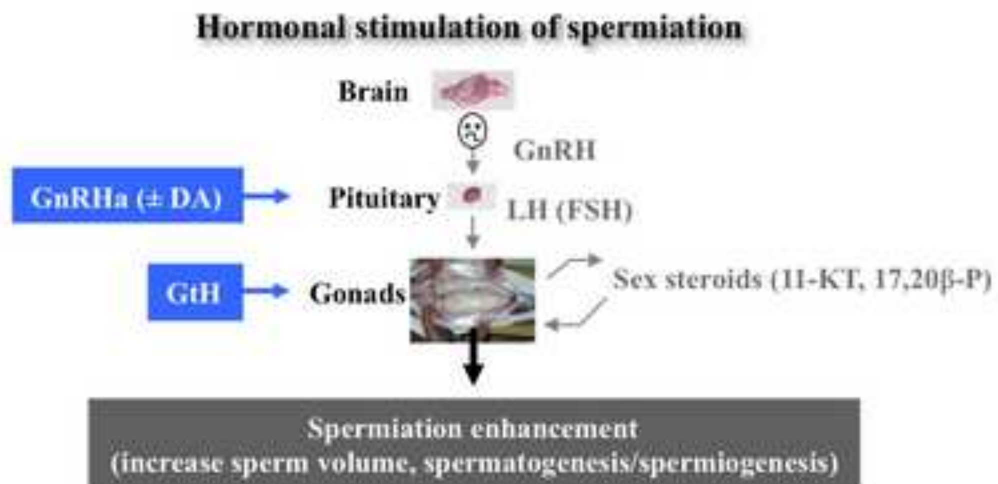
Sparus aurata	gilthead seabream	(Marco-Jiménez et al., 2008)
Takifugu niphobles	pufferfish	(Gallego et al., 2014b)

2580

2582

Figure 1 BPG axis

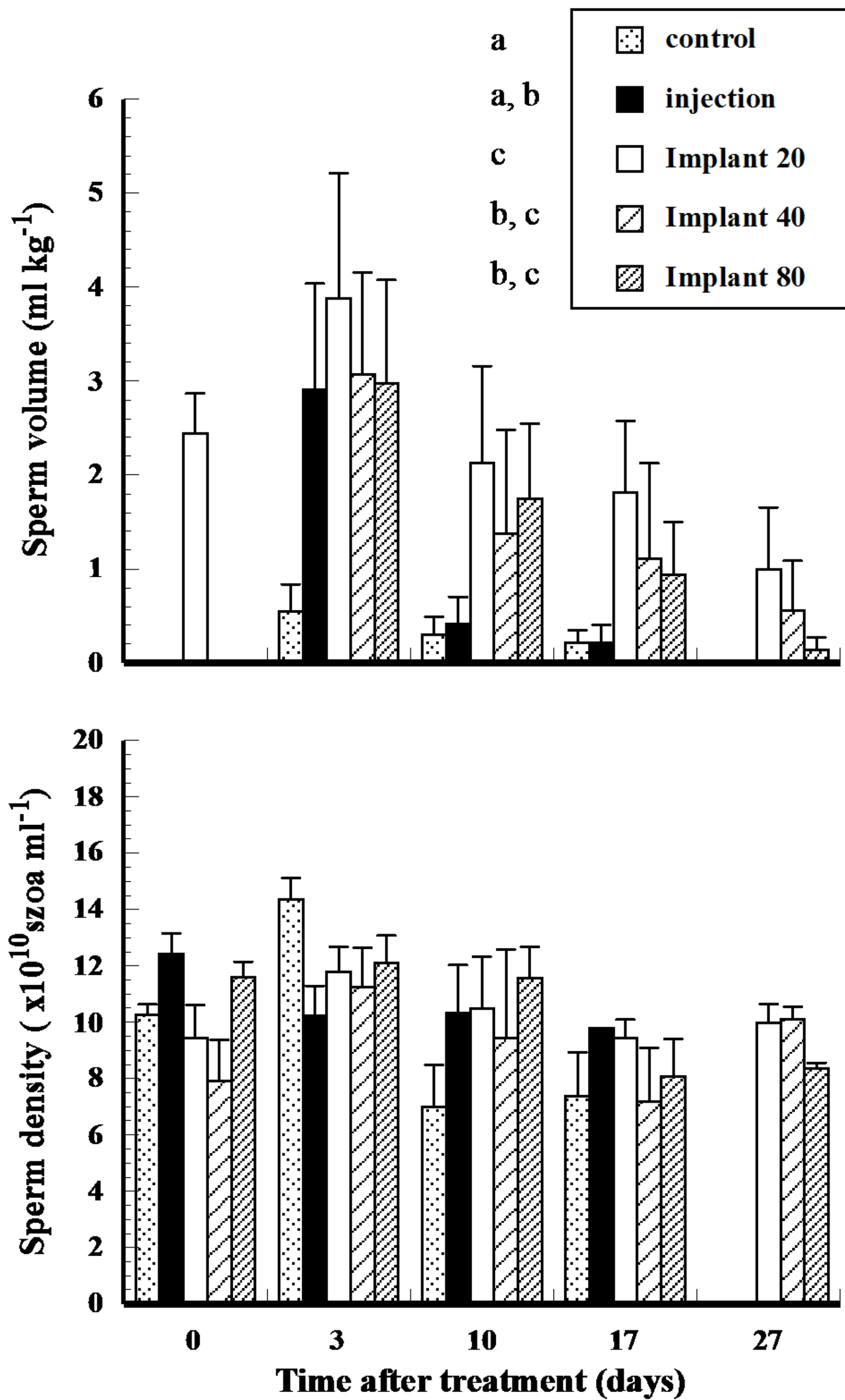
Click here to download high resolution image



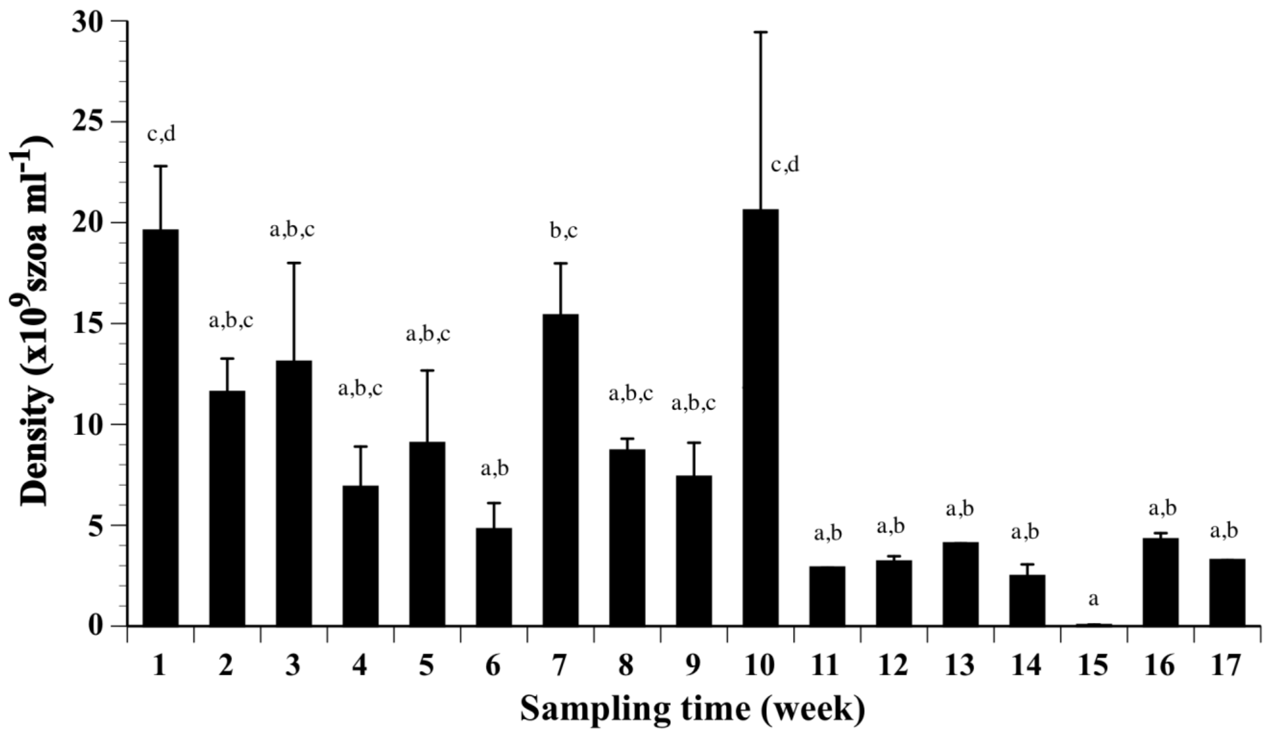
Mylonas et al.

Fig. 1

Figure(s)



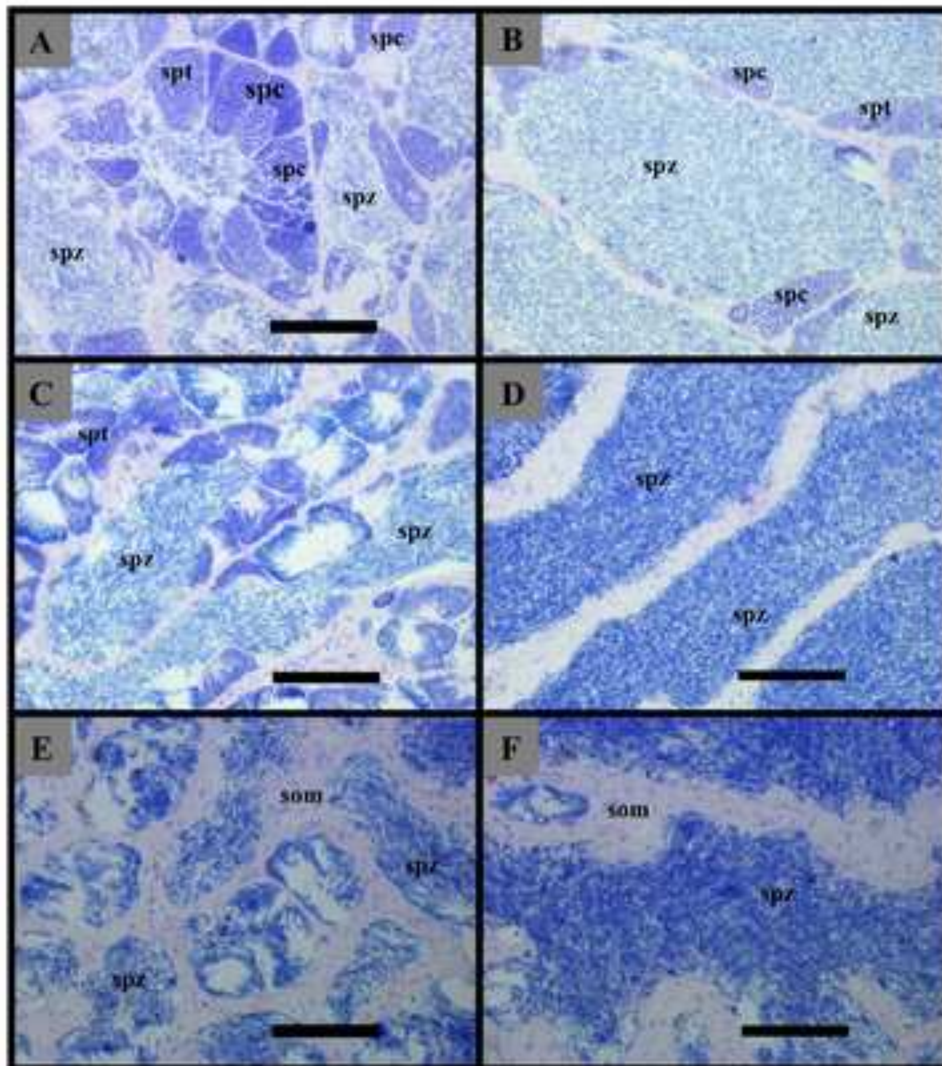
Mylonas et al.
Fig. 2



Mylonas et al.
Fig. 3

Figure 4 histology

Click here to download high resolution image



Mylonas et al.
Fig. 4