UNIVERSIDAD POLITÉCNICA DE VALENCIA
DEPARTAMENTO DE CIENCIA ANIMAL

STUDY OF MALE REPRODUCTIVE PHYSIOLOGY AND SPERM CONSERVATION IN THE EUROPEAN EEL (*Anguilla anguilla*, L.)

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Informan:

Que la Tesis Doctoral titulada: “Study of male reproductive physiology and sperm conservation in the European eel (A*nguilla anguilla*, Ll.)” ha sido realizada por el Licenciado en Ciencias Biológicas D. David Sánchez Peñaranda en el Departamento de Ciencia Animal bajo su dirección y que, una vez revisado y comprobado el trabajo, consideran que reúne los requisitos necesarios para la obtención del grado de Doctor, por lo que autoriza su presentación.

Y para que así conste firman el presente informe en Valencia a veintiuno de abril de dos mil ocho.

Fdo: Dr. Juan F. Asturiano Nemesio

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RESUMEN

La anguila europea es una especie de gran importancia comercial en la acuicultura europea, pero el dramático descenso de las poblaciones naturales ha provocado la inclusión de esta especie en la lista CITES (convención del comercio internacional de especies en peligro de la fauna y flora salvaje). Considerando esta situación, se hace necesaria más investigación en el control reproductivo.

El presente documento contiene 5 estudios que se pueden dividir en dos partes principales: la primera, una descripción del proceso de la maduración artificial de la anguila, seguida por una segunda parte centrada en el de desarrollo de técnicas para la evaluación de la calidad espermática, y la conservación del esperma.

Una vez fijado el tratamiento hormonal en anteriores estudios, nosotros hemos estudiado la evolución de la calidad espermática a lo largo del periodo de espermiación, tratando de determinar las semanas con mayor porcentaje de muestras con alta calidad espermática. Para la evaluación de la calidad espermática los parámetros analizados fueron los siguientes: porcentaje de células vivas, motilidad, y morfometría de la cabeza del espermatozoide. El porcentaje de células vivas fue medido poniendo a punto técnicas de tinción fluorescentes, mientras que la medida del tamaño de la cabeza fue llevado a cabo por un programa informático (sistema ASMA), pero este análisis presentó problemas debido a la especial forma de la cabeza del espermatozoide de esta especie. Para validar esta técnica fue necesario comparar los resultados obtenidos por el sistema ASMA con los obtenidos por el microscopio electrónico de barrido.

Distintos machos que recibieron el mismo tratamiento hormonal mostraron diferentes respuestas en cuanto al nivel de desarrollo gonadal y calidad espermática. Para entender qué factores podían influir en en estos factores, se realizó por primera vez la descripción de la maduración de machos de anguila europea, analizando la expresión de GnRHs y gonadotropinas, los niveles plasmáticos de 11-KT, los parámetros morfométricos y la calidad espermática en relación con el estado de desarrollo de la gónada.

Para completar el trabajo, se desarrollaron nuevas técnicas para el almacenamiento a corto y largo plazo de esperma de anguila europea. Hemos diseñado un medio que conserva el esperma fresco durante varios días, además de un medio para la crioconservación del esperma inmerso en nitrógeno líquido.

ABSTRACT

European eel is an important commercial fish in the European aquaculture, but the dramatic decrease in natural populations has caused the incorporation of this species in the CITES list (convention on the international trade in endangered species of wild fauna and flora). Considering this situation, more research on the eel reproductive control is necessary.

The present document contains 5 studies that can be divided in two principal parts: first, a description of the artificial eel maturation process, followed by a second part with the development of techniques for the sperm quality evaluation and sperm conservation.

Once the optimal hormonal treatment was fixed in previous studies, we studied the evolution of the sperm quality through the spermiation period, trying to determine the weeks with higher percentage of samples with high quality sperm. To evaluate the sperm quality the following parameters were analyzed: percentage of live cells, motility and spermatozoa head morphometry. The percentage of live cells was measured by fluorescent staining techniques, while the measurement of head size was performed using a computer program (ASMA system), but this analysis presented problems due to the special spermatozoa head shape. To validate this technique it was necessary to compare the results obtained by ASMA system with those obtained by scanning electron microscopy.

Males receiving the same hormonal treatment show different individual responses in terms of gonad development and sperm quality. To understand which factors can influence in the gonad maturation and the sperm quality we made the first description of European eel male maturation process analyzing GnRHs and gonadotropin expression, 11-KT plasma levels, morphometric parameters and sperm quality in relation with the testis stage of development.

To complete this work, new techniques for the short- and long-term storage of European eel sperm were developed. We have designed one medium that preserves the fresh sperm during several days, as well as a new method to cryopreserve the sperm immersed in liquid nitrogen.

RESUM

L'anguila europea és una espècie de gran importància comercial en la acuicultura europea, però el dramàtic descens de les poblacions naturals ha provocat la inclusió d'aquesta espècie en la llista CITES (convenció del comerci internacional d'especies en perill de la fauna i flora). Considernat aquesta situació, es fa necessària més investigació en el control reproductiu.

El present document contén 5 estudis que es poden dividir en dos parts principals: la primera es una descripció del procés de maduració artificial de l'anguila, correguda per una segona part centrada en el desenvolupament de les técniques per a la evaluación de la calitat espermática, i la conservació de l'esperma.

Una volta fixat el tractament hormonal en anteriors estudis, nosaltres vam estudiar l'evolució de la qualitat espermàtica a llarg del periode d'espermiació, tractant de determinar les setmanes amb major porcentage de mostres amb alta qualitat espermàtica. Per a l'evaluació de la qualitat espermàtica els paràmetres analizats foren els següents: percentatge de cèl.lules vives, motilitat i morfometria del cap de l'espermatozoide. El percentatge de cèl.lules vives siguí mesurat ficant a punt tècniques de tinció fluorescent, mentres que la mesura de la grandària del cap va ser feta per un program informàtic (sistema ASMA), però aquest anàlisi va presentar problemes a causa de l'especial forma del cap de l'espermatozoide d'aquesta espècie. Per a validar aquesta tècnica va ser necessari comparar els resultats obtenguts pel sistema ASMA amb els obtenguts per mitja del microscopi electrònic d'escombrat.

Differents mascles que reberen un mateix tractament hormonal mostraren diferents respostes en quant al nivell de desenvolupament gonadal i de qualitat espermàtica. Per entendre quins factors poden influir en la maduració gondal i la qualitat espermática, es va fer la primera descripción de la maduració en mascles de anguila euroepa, analizant l'expressió de GnRHs i gonadotropinas, els nivells plasmàtics de 11-KT, els paràmetres morfomètrics i la qualitat espermàtica en relació amb l'estat de desenvolupament de la gònada.

Per a completar el treball, foren desenvolupades noves tècniques per al emmagatzematge a curt i llarg termini d'esperma d'anguila europea. Nosaltres hem disenyat un medi que conserve l'esperma fresc durant diversos dies, al

mateix temps que un medi per a la conservació de l'esperma inmers en nitròg
líquid.

ABREVIATURAS

[cAMP]	Cyclic adenosine monophosphate concentration
ANOVA	Analysis of variance
AR	Androgen receptor
ASMA	Assisted sperm morphology analysis
ARP	Acidic ribosomal phosphoprotein P_0
BCF	Beating cross frequency
BGP	Brain-pituitary-gonad
BSA	Bovine serum albumin
CA	Carbonic anydrase
DA	Dopamine
CASA	Computer assisted sperm analysis
DHP	17,20β-dihydroxy-4pregnen-3-one
DMSO	Dimethyl sulfoxide
E_2	17β-Estradiol
EI	Eye index
eSRS	Eel spermatogenesis-related substance
FBS	Foetal bovine serum
FSHβ	Follicle-stimulating hormone-β
FSHr	Follicle-stimulating hormone receptor
GLM	General linear model
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic index
sGTH	Salmon gonadotropin
IGF-I	Insulin-like growth factor I
hCG	Human chorionic gonadotropin
HSI	Hepatosomatic index
11-KT	11-ketotestosterone
LHβ	Luteinizing hormone
LHr	Luteinizing hormone receptor
LSM	Least square means
ME_2SO	Dimethyl sulfoxide

PCA	Principal component analysis
PBS	Phosphate buffer solution
PI	Propidium iodide
P.I.T	Passive integrated transponders
PR	Progestin receptor
qrt RT-PCR	Quantitative real time Reverse Transcriptase-Polymerase Chain Reaction
S	Stage
SEM	Scanning electron microscopy
S.E.M	Standard error of the means
TEM	Transmission electron microscopy
TH	Tirosine hydroxylase
TGF-Bs	Transforming growth factor-Bs
VCL	Curvilinear velocity
VSL	Straight line velocity
VAP	Angular path velocity

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1 INTRODUCTION

1.1 EUROPEAN EEL BIOLOGY

The European eel (*Anguilla anguilla*, L. 1758) is classified in the subdivision elopomorpha, a group that appeared in early evolution of teleosts. The genus *Anguilla* is composed by 15 species (Watanabe, 2003). The geographic ranges of some representative species are as follows: *A. marmorata*, which lives in tropical regions of the Indian and Pacific Oceans; *A. mossambica*, in the eastern coast of Africa; *A. japonica*, in the eastern coast of the Eurasian Continent; *A. anguilla*, in the western and eastern coasts of the Eurasian Continent; *A. rostrata*, in the eastern coast of North America; *A. reinhardti*, in the eastern coast of Australia; *A. australis australis*, in the south-eastern Australia; *A. celebesensis*, in the Sulawesi Island region (Fig. 1.1).

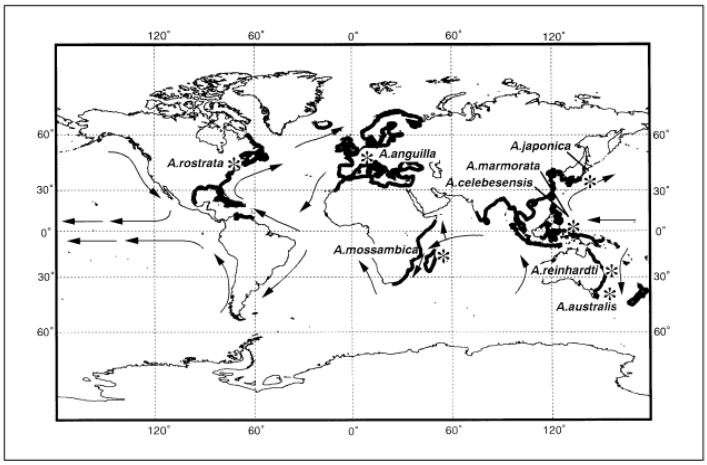

Figure 1.1. Distribution of eel in the world (Aoyama and Tsukamoto, 1997).

Their catadromous life history strategy is special, spending most of their lives in freshwaters, until their long migration to the seawater spawning regions (Fig. 1.2).

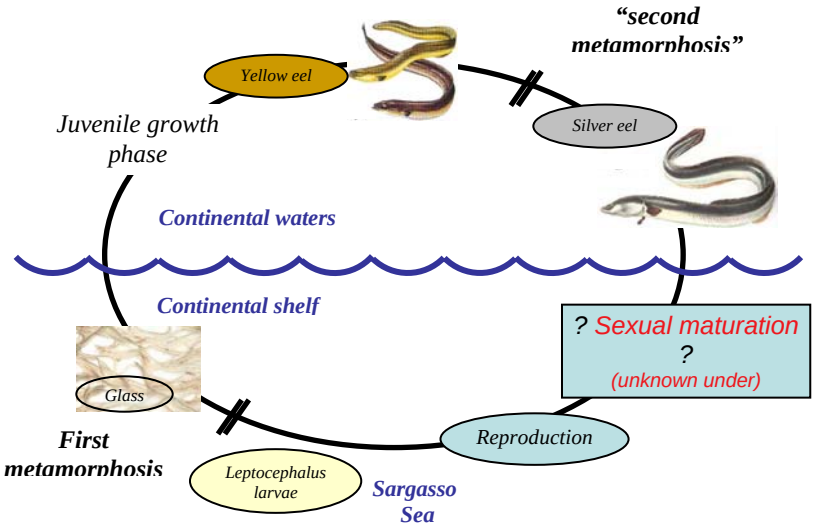

Figure 1.2 Life cycle of *Anguilla anguilla*.

The mystery has been always linked with the eel biological cycle. In 1856 a new fish from the Strait of Messina was discovered, and named *Leptocephalus brevirostris* (Kaup). Forty years later the Italians, Grassi and Calandruccio (Grassi 1986), discovered that this fish was a larvae of the river eel. Taking as basis these discoveries, in the 1960's, Tucker (1959) and d'Ancona (1960) hypothesized that eel spawning areas could be located in the Mediterranean, close to the Strait of Messina. Schmidt (1912a, 1922, 1925) performed large number of explorations from the Mediterranean to south-east of the Bermudas. In the spawning area never parental eels or eggs were observed. However larvae with 5 millimetres in length around Sargasso Sea were captured. Taking as basis these studies the panmixia theory was proposed, in which the eel distributed in Europe belongs to a completely homogeneous population, with a single spawning location. Recent molecular works studies indicated that this theory can be overstatement (Daemen et al., 2001; Wirth and Bernatchez, 2001; Maes and Volckaert, 2002), getting strength the theory that the eel population is a genetic mosaic consisting of several groups (Van Ginneken and Maes, 2005).

The eel larvae, named leptocephali (Fig. 1.3) return to European waters from Sargasso Sea helped by the Gulf Stream and active swimming. The duration of this travel is less than one year (Lecomte-Finiger, 1994). When they arrive near to the coast (continental shelf), before to entry in the rivers the leptocephali suffer metamorphosis into glass eels (Lecomte-Finiger, 1994;

McCleave et al., 1998; Pérez et al., 2004). Once in the feeding habitat, the eels can spend long time in, depending of sex: 5-8 years the males and 8-20 years the females. The sex determination occurs when the size is around 30 centimetres, but it can be influenced by density or environmental factors; for example high densities provide higher proportion of males (Bark et al., 2005). Under unclear environmental conditions, pubertal eels return to the sea to perform the reproductive migration.

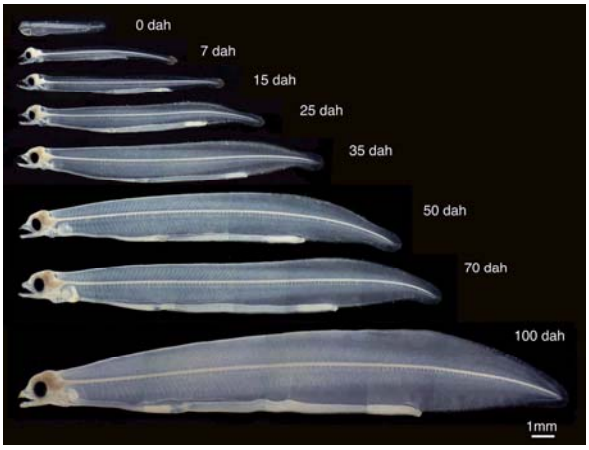

Figure 1.3. Example of leptocephali (Tanaka et al., 2001)

1.2 PHYSIOLOGYCAL DESCRIPTION OF EEL MATURATION

1.2.1 Gonadotropin releasing hormone (GnRH)

In teleosts, the control of reproduction by the brain-pituitary-gonad (BPG) axis is well established (Fig. 1.4).

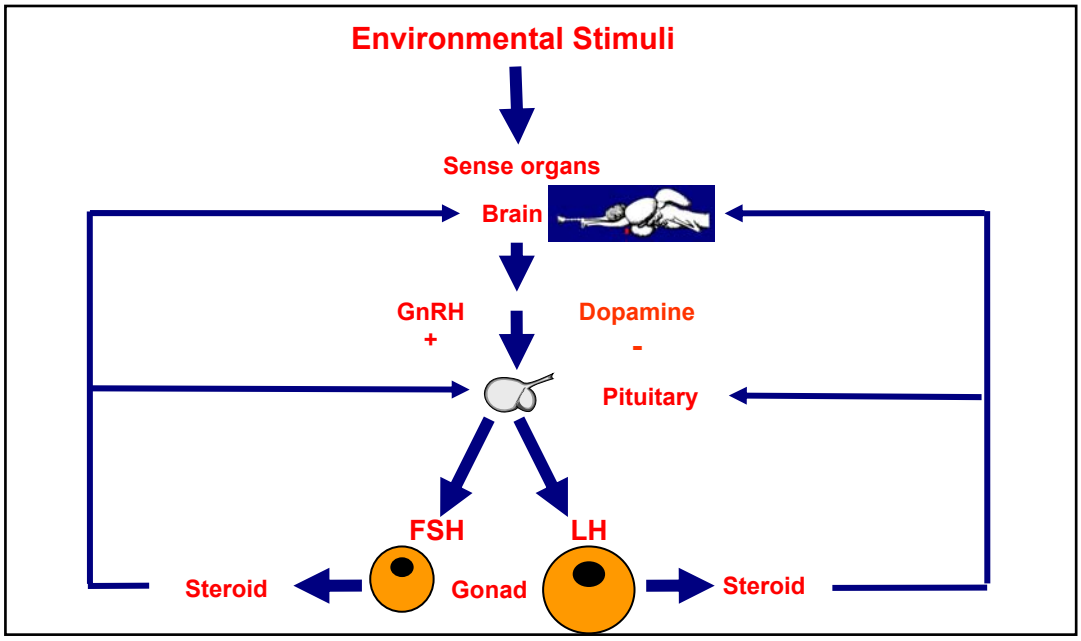

Figure 1.4. Brain–pituitary-gonad axis in teleosts

A hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), is the physiological stimulator of gonadotropin release, acting on the pituitary gland (Amoss et al., 1971; Matsuo et al., 1971). Twelve different GnRH molecular forms have been characterized from vertebrates and two from a protochordate, usually named as the species in which they have been characterized (Powell et al., 1996; Jimenez-Linan et al., 1997). Recently, another clasification was proposed to define the different GnRHs. All the forms that are expressed in the hipothalamus and preoptic area were named GnRH I, chicken GnRH-II was named GnRH II and salmon GnRH changed to GnRH III (Fernald and White, 1999). The structure of all prepro-GnRH genes consists in four exons separated by three introns (Sherwood et al., 1997; Suetake and Aida, 1997; White and Fermald, 1998; White et al., 1998), and the different variants are synthesized by splicing during transcription process. The brain of teleosts contains at least two GnRH variants,

but in more evolved teleosts usually there are synthesized three GnRH variants: sGnRH, sbGnRH and cGnRH-II (Powell et al., 1994; White et al., 1995; Senthilkumaran et al., 1999; Lethimonier et al., 2004). The presence of two GnRH variants (sGnRH; cGnRH-II) in the brain of a single teleost was first demostrated in goldfish (Yu et al., 1988), while gilthead seabream was the first species where were described three GnRH variants in teleosts (Powell et al., 1994). GnRHs distribution is not homogenous in the brain. In Masu salmon (with two variants) sGnRH is found in the anterior ventral brain, and cGnRH-II in the midbrain (Amano et al., 1991). In *Sparus aurata*, with three GnRH variants, sGnRH is synthesized in the olfatory bulbs, sbGnRH in the preoptic area and cGnRH-II in the diencephalon-mesencephalon (Gothilf et al., 1996; White and Fernald, 1998).

The principal function of GnRH is the release of gonadotropic, although this effect may partially depend on specific physiological conditions (Marchant et al., 1989; Le Gac et al., 1993; Lin et al., 1993; Blaise et al., 1995, 1997; Melamed et al., 1995, 1998). For example in immature rainbow trout GnRH can stimulate FSHβ but not LHβ expression, while in pituitaries of mature fish the reverse occurs (Kawauchi et al., 1989).

In other hand, different studies have been performed to know the possible specific function for each GnRH variant. Usually, the levels of sbGnRH are higher in the mature fish, and it is considered that this form can be responsible of the gonadotropin release (Powell et al., 1994; Holland et al., 1998, Rodríguez et al., 2000, 2004; González-Martinez et al., 2002). Both cGnRH and sGnRH seem to be responsibles of the behaviour or in several neuronal functions (Zohar et al., 1995; Fernald and White, 1999). In Masu salmon, it has been reported the sGnRH as the responsible of gonadotropin release (Amano et al., 1997).

In European eel the distribution of GnRHs in the brain has been studied (Dufour et al., 1993; Montero et al., 1994), finding higher cGnRH-II concentration in di-/mesencephalon and met-/myelencephalon, while higher mGnRH concentrations were registered in the pituitary, olfactory bulbs, telencephalon and di- /mesencephalon (Fig. 1.5). The mGnRH localization in the eel brain is related to the sGnRH sytem of the Masu Salmon (Amano et al., 1991), to the mGnRH system of the amphibian brain (Muske, 1993) and to the cGnRH-I system in the

avian brain (Millam et al., 1993). cGnRH-II in eel is found principally in the midbrain, as in the rest of teleosts.

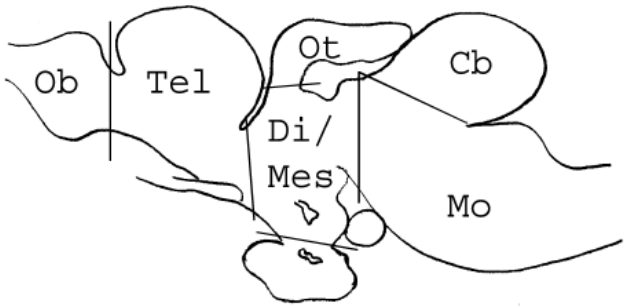

Figure 1.5. Principal parts of the brain in European eel (Weltzien et al., 2005b).

When GnRH expression was analyzed in European eel females, an opposite regulation was observed in mGnRH and cGnRH-II expression, registering a significant mGnRH increase in matured eels, and the opposite for cGnRH-II (Dufour et al., 1993; Montero et al., 1993). mGnRH is considered as the responsible of gonadotropin release in European eel (Dufour et al., 1993; Montero et al., 1994), although it has been reported that it could stimulate the motor activity (previous step to the reproductive migration; Dufour et al., 1991). In Japanese eel, mGnRH was localized in a diversity of body tissues, while cGnRH-II expression was found in olfactory epithelium, brain, pituitary and gonad (Okubo et al., 1999a,b, 2002). The widespread expression of mGnRH could be due to a physiological function in an autocrine or paracrine manner, besides its paper in the synthesis and release of gonadotropins (Okubo et al., 1999a). In contrast, the cGnRH-II is limited to a few tissues, being its function unknown. Its presence in olfactory epithelium suggests that could be involved in pathways correlated with enviromental changes or that it can play a role in the control of sexual behaviour (Muske, 1993; King and Millar, 1995). In other hand, the observation of cGnRH-II fibers in the neurohypophysis of the European eel, indicates that cGnRH-II should have a role in the gonadotropin release.

cGnRH-II is present in all vertebrates (Dellovade et al., 1993). In primitive gnathostomes all the basic functions are ascribed to GnRH, but the evolution have reduced its role, losing its hypophysiotropic function in birds and mammals. In the eel, cGnRH-II represents an intermediate evolutionary situation: it may still

serve for two functions, a neuromodulatory and a hypophysiotropic one (Montero et al., 1994).

It has been studied the influence of GnRH expression on the testicular differentiation in the Japanese eel. Although the olfactory, preoptic and midbrain GnRH neuronal system contribute to pituitary innervation, an association can be assumed between preoptic GnRH expression and testicular differentiation (Chiba et al., 1999).

mGnRH and cGnRH expressions have not been still analyzed during gonad development in European eel males, what could be important to confirm the posible role of the different GnRHs, as well as their influence in the gonad development.

1.2.2 The role of dopamine

The release of gonadotropic hormones in European eel and other teleost fish is subjected to a double neuroendocrine control: a positive effect of GnRH and a negative effect produced by dopamine (DA; Peter and Paulencu, 1980; Kah et al., 1987; Dufour et al., 1988, 2005; Linard et al., 1996; Montero et al., 1996; Vidal et al., 2004). The European eel is considered as a good model to study this neuronal control, by its unique life cycle, in which the gonad maturation is blocked until the reproductive oceanic migration.

Dopamine is a catecholamine, like adrenaline and nonadrenaline, and its synthesis depends on the tyrosine hidroxylase enzyme. Immunocytochemical studies demonstrated the existence of abundant innervations of the pars proximal distalis of the eel pituitary by tyrosine hydroxylase-positive neurons (Boularand et al., 1998). Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the catecholamine biosynthesis (like dopamine). Weltzien et al. (2005a,b) analyzed TH expression in the different parts of the European eel brain, finding the highest expression in the olfactory bulb, followed by the di-/mesencephalic areas and the telencephalon/preoptic area. TH synthesis was affected by some steroids, like testosterone, that provided a positive effect, but not by 17β-estradiol (E_2, Weltzien et al., 2005a, 2006). Considering the high TH expression in the olfactory bulb, Weltzien et al. (2006) and Sébert et al. (2008) proposed that the olfactory function could have a role in eel navigation during its reproductive migration toward the oceanic spawning grounds.

The gonadotropin stimulating effect of the GnRH was demonstrated in European eel treated females with injections of GnRH-analog, but due to dopamine inhibition it was necessary to add a dopamine antagonist (Dufour et al., 1988, 2005; Montero et al., 1996; Pasqualini et al., 2004; Vidal et al., 2004). The dopamine antagonist employed was pimozide, which in mammals is an antagonist of DA-D2 receptors. Two different cDNA sequences of DA-D2 receptors were found in eel brain (pituitary and olfactory bulb), named D2A and D2B (Pasqualini et al., unpublished data). In fact, a triple treatment using steroids, GnRH analogues and dopamine antagonists was necessary to trigger the endogenous production and release of gonadotropins in European eel (Dufour et al., 2003; Vidal et al., 2004). However in E_2 treated males, GnRH injections originated a low effect (Olivereau et al., 1986). This slight effect of GnRH, without DA antagonist, suggests a less effective dopaminergic inhibition in male than in female eel. Until now, the GnRH expression has been never analized during gonad development in male European eel, and its study can help to understand the influence of GnRHs in the male maturation process.

1.2.3 Gonadotropins

In teleost fish, it is well stablished the occurrency of two gonadotropins, FSH and LH, like in the rest of tetrapods. They were first named as GTH I and GTH II. Gonadotropins are glycoproteins consisting of two subunits, α and β. While α subunit is common in both gonadotropins (FSH and LH), β subunit is different in every case. All of them have been sequenced in European eel (Querat et al., 1990a,b; Degani et al., 2003; Schmitz et al., 2005). In most teleosts, FSH regulates the first steps of the gonad maturation, while LH is involved in the final gonad development. In salmonids, vitellogenesis and spermatogenesis are likely to be controlled by FSH, while the processes of final oocyte maturation/ovulation and spermiation are regulated by LH (Swanson, 1991). In female eels, FSHβ was found in high levels in immature previtellogenic eels, while LH could found at the vitellogenic stage and germinal vesicle migration stage (Nagae et al., 1996; Yoshiura et al., 1999; Degani et al., 2003). However, some researchers indicated that both gonadotropins are present in immature previtellogenic eels (Schmitz et al., 2005; Aroua et al., 2005).

Gonadotropins does not act directly on germ cells, but rather through the biosynthesis of 11-ketotestosterone (11-KT; Miura et al., 1991a,b). In salomonids,

FSH and LH are equipotent in stimulating the production of the androgens, 11-KT and testosterone in salmon males, but LH is more potent in stimulating 17α,20β-dihydroxy-4-pregnen-3-one (DHP) at the final maturation and spawning in both males and females (Swanson et al., 1989; Planas and Swanson, 1995; Maugars and Schmitz; 2008). The steroidogenic induction performed by FSH could be through a direct action on the Leydig cells (binding LH receptors, Schulz et al., 2001) or it may trigger the release of growth factors in Sertoli cells, which in turn can stimulate the steroid biosynthesis in Leydig cells (Lejeune et al., 1996).

The gonadotropin expression has been deeply analyzed in European eel females (Huang et al., 1997; Degani et al., 2003; Vidal et al., 2004; Schmitz et al., 2005; Aroua et al., 2007; Sébert et al., 2007), but in male European eel it has never been studied during male gonad development. In any case, most of the studies have been performed in female Japanese eel (Nagae et al., 1997; Suetake et al., 2002; Jeng et al., 2002, 2007; Kamei et al., 2005, 2006; Han et al., 2003; Saito et al., 2003; Ozaki et al., 2007). The repeated injections of exogenous sGTH in the Japanese and European eel female gave as results an abnormal LHβ and FSHβ expression, with an over-expression of LHβ during ovarian maturation and a hard decrease of FSHβ after the first injections (Nagae et al., 1997; Suetake et al., 2002; Schmitz et al., 2005).

In order to know if the GTH profiles observed in artificially matured eels are similar to the natural pattern, Saito et al. (2003) compared the gonadotropin expression in naturally maturing New Zealand longfinned eel with artificially maturing Japanese eel. New Zealand longfinned eel migrates to the spawning grounds at the sea later than other eel species, with the ovaries having reached the mid-vitellogenic stage. With the firsts injections a quick decrease in the FSHβ expression was observed in artificially maturing female eels, whereas in New Zealand longfinned eels in mid-vitellogenic stage a FSHβ increase was observed. Respect to LHβ profile, a LHβ over-expression was found in artificially maturing eels compared to the New Zealand eels.

Gonadotropins exert their function through the binding with membrane receptors located in the gonad. Gonadotropin receptors are membrane-bound receptors belonging to the superfamily of G-protein-coupled receptors, which contain seven transmembrane domains. In testis coho salmon (*Oncorhynchus kisutch*) FSH receptors (FSHR) were localized in the Sertoli cells, but not in

Leydig cells, while LH receptors (LHR) were found in Leydig cells but not in Sertoli cells of testis coho salmon (Miwa et al., 1994). In female coho salmon, FSHR were observed both on thecal and intensively on granulose cells in vitellogenic ovary (but not in preovulatory ovary), whereas LHR were synthesized in granulose cells of preovulatory follicles (Miwa et al., 1994). Schulz et al (2001) studied the gonadotropin-receptor union. A receptor structurally resembling tetrapod FSH-Rs responded specifically to salmon FSH; however the receptor similar to the tetrapod LH-Rs responded to salmon LH, but also to salmon FSH.

The study of eel GTH receptors has been only done in the Japanese eel, being sequenced partially (Jeng et al., 2007). In this species, FSH and LH receptors increased their expression during gonadal maturation, but no effect was observed when treatments with sexual steroids were administrated (Jeng et al., 2007). In contrast with the results observed in coho-salmon, Ohta et al. (2007) reported that FSH receptor in Japanese eel is expressed mainly by Leydig cells, which are the responsible of steroid production, and by Sertoli cells surrounding spermatogonia type A and early type B.

1.2.4 Gonadal steroids and feedbacks

The gonadal steroids are essential for the teleost reproduction, and they are able to affect practically all tissues. Both in testis and ovary, the synthesis of steroids is performed in somatic cells. Thecal and granulose cells are the responsible of the steroid production in the ovary (Fostier et al., 1983; Kagawa et al., 1984; Nagahama, 1994). Taking as basis the salmonid model, the thecal cells produce testosterone during vitellogenic process or 17αHP during the oocyte maturation, while the granulose cells use these steroids to produce E_2 and DHP. Respect to the testis, the steroid production is localized mainly in the Leydig cells. Some evidences indicate that Sertoli cells could be implicated in steroid synthesis, especially progestins as DHP (Nagahama, 1983).

In teleosts, the gonad steroids have an important role in the spermatogenesis process. The target tissue can be the gonad, or it can perfom their function through the liver, pituitary and/or brain, where steroid receptors have been found (Pakdel et al., 1989; Salbert et al., 1991; Valotaire et al., 1993; Ikeuchi et al., 1999; Todo et al., 1999). As in mammals, the hormonal steroids cause a feedback on the brain-pituitary-gonad axis. This feedback can be

positive or negative, causing the synthesis or release of gonadotropins and GnRH (Kobayashi and Stacey, 1990).

The influence of the steroid administration on the teleost reproduction has been analyzed in some species, like in male tilapia hybrids. In cultured pituitary cells from immature male tilapia, FSHβ expression was stimulated by exposure to testosterone (Melamed et al., 1997), while in pituitary cells at the end of the spermiation a FSHβ decrease was observed after administration of T or E_2. In regressed fish, neither T nor 11-KT had any effect on the testis cells. Respect to the steroid influence in LHβ expression, in immature fish, E_2 or testosterone increase the LHβ synthesis in teleosts (Trinh et al., 1986; Querat et al., 1991; Dickey and Swanson, 1995; Gur et al., 1995; Huggard et al., 1996; Schmitz et al., 2005). In pituitary cells from sexually maturing rainbow trout, T and E_2 stimulated the LHβ expression, but no difference was observed in in pituitary cells of spawing fish (Xiong et al., 1994).

The same steroids can have different effects depending on the fish physiological stage, and this makes more difficult the study of the role of sex steroids on eel maturation, because in this species hormonal injections are necessary to obtain the gonadal development. Several studies have been performed in this field in European eel. It has been demostrated that injections of E_2 increase the LHβ synthesis in the eel pituitary, but not their release (Dufour et al., 1983). On the other hand, E_2 *in vitro* did not promote the luteinizant hormone-β (LHβ) expression in pituitary cells, and this difference suggests that the stimulatory effect of E_2 is exerted by indirect ways *in vivo* conditions (Huang et al., 1997; Aroua et al., 2007). *In vitro*, testosterone stimulates the LHβ expression in pituitary cells, while *in vivo* the testosterone decrease the FSHβ expression (Schmitz et al., 2005). A GnRH control could be involved, since a parallel stimulation of LHβ and GnRH levels was observed in female European eel under the E_2-treatment effect (Montero et al., 1995). However, in Japanese eel the sex steroids did not affected brain mGnRH content (Jeng et al., 2002).

In Japanese eel females, during artificial gonad development induced by repeated injection of salmon gonadotropin (sGTH), E_2 was maintained low until the final of vitellogenesis, while the testosterone level was always higher until the E_2 increase (Ijiri et al., 1995). This abnormal steroid profile may result in the inhibition of spontaneous final oocyte maturation and ovulation.

The 11-KT is the androgen responsible of the spermatogonia proliferation to spermiogenesis in the eel (Miura et al. 1991a,b, 1996; Miura and Miura, 2001, 2003), and it is considered the most important androgen in teleosts (Borg, 1994). In other hand, 11-KT plays a role in the eel female maturation, promoting the oocyte growth (Lokman et al., 2007). In some species, like male catfish, the 11-KT has a positive effect in the LHβ synthesis (Rebers et al., 1997). Finally, it has been demonstrated that this androgen has an influence in the secondary morphologic changes observed in all the eel species during the maturation (Rohr et al., 2001).

1.3 SPERMATOGENESIS

The spermatogenesis process in eel male is divided into the following stages: spermatogonial stem-cell renewal, spermatogonial proliferation, spermiogenesis and sperm maturation (Fig. 1.6, Miura et al., 1997, 1998, 2002b, 2006; Miura and Miura, 2001, 2003; Schulz et al., 2002).

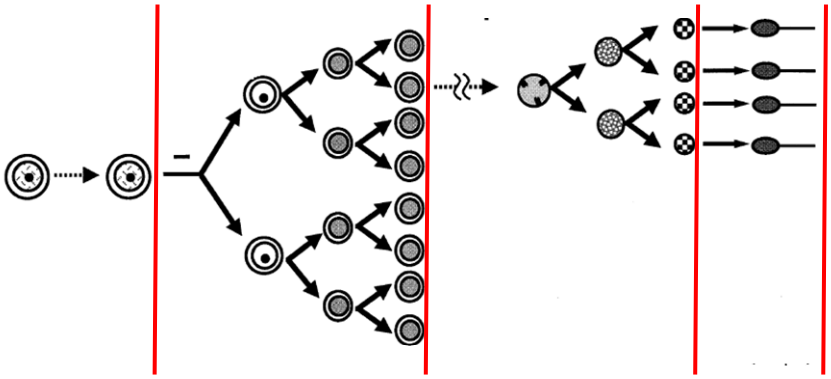

Figure 1.6. Spermatogenesis stages (Miura and Miura, 2003).

Both in Japanese and European eel males maintained under artificial conditions the testis is immature, containing only premitotic spermatogonia, type A and early-type B spermatogonia (Yamamoto et al., 1972; Khan et al., 1987; Miura et al., 1991a,b, 1995a, 1996a, 1997; Ohta and Tanaka, 1997). The development is found inhibited by the expression of different factors, named eSRS3, eSRS4, eSRS21 (Miura et al., 1998, 2002a) but the synthesis of these factors can be suppressed with only one injection of human chorionic gonadotropin (hCG) or 11-KT. In both eel species it has been reported that is necessary to administrate a hormonal treatment with human chorionic gonadotropin to start the spermatogenesis (Colombo et al., 1987; Khan et al., 1987; Miura et al., 1991a; Pérez et al., 2000, 2003; Asturiano et al., 2004, 2005, 2007; Marco-Jiménez et al., 2006; Garzón et al., 2008). As a consequence of this hormonal treatment, the expression of E_2 receptor is stimulated mainly during the first 6 days, but its expression is maintained during the whole spermatogenesis. It

was seen that E_2 receptor was expressed in the Sertoli and intersticial cells. Although E_2 is a female hormone, its existence in vertebrate male gonad has been previously reported (Schlinger and Arnold, 1992; Fasano and Pieratoni, 1993; Betka and Callard, 1998), and its positive effect on the spermatogonial stem cell division in Japanese eel male was demonstrated by Miura et al. (1999). It was reported that E_2 stimulated the eSRS34 synthesis, which is considered as a spermatogonial stem cell renewal factor (Miura et al., 2003).

When gonadotropins are secreted from the pituitary, spermatogonial mitosis switches from stem cell renewal to proliferation toward meiosis. But they have not a direct effect, actually, the 11-KT is the responsible androgen of the spermatogonia proliferation to spermiogenesis (Miura et al. 1991a,b, 1996; Miura and Miura, 2001, 2003). Gonadotropins activate the 11-KT production in the gonad, being synthesized by Leydig cells. This 11-KT production influences in the Sertoli cells (Miura et al., 1996), inducing the production of growth factors n, such as insulin-like growth factor-I (IGF-I) and activin β. IGFs stimulate DNA synthesis in spermatogonia cells (Loir and LeGac, 1994; LeGac et al., 1996), and although it is necessary for the spermatogenesis regulation, the principal IGF-I role is to promote (together with 11-KT) that the mitotic process started by activin β not was interrupted. IGF-I not only is produced by Sertoli cells, its synthesis has been registered in liver and germinal cells. Activin β is also a growth factor which belongs to the transforming growth factor-βs (TGF-βs) family. It is synthesized strictly in the Sertoli cells, and its role is to induce the proliferation of spermatogonia, even though does not induce meiosis (Miura et al., 1995b). Both hormones, IGF-I and activin β, have an influence in the gonadotropin expression, IGFs showing a positive effect on the pituitary LHβ expression, and activin β stimulating FSHβ expression (Aroua et al., 2008).

11-KT is the steroid that occupies a central role in the sexual maturation process in male fish (Kime et al., 1991; Miura et al., 1991a,b; Borg, 1994; Guiguen et al., 1995). Despite the fact that 11-KT is a male-specific androgen, it has been found in elevated levels in some female teleost (Leatherland et al., 1982; Slater et al., 1994). Studies in *Anguilla australis* and *A. dieffenbachii* showed that 11-KT levels were higher in pre-migratory “silver” females than in non-migratory, “yellow” females (Lokman et al., 1998). Since the 11-KT cannot be aromatized, maybe it is directly involved in mediating some of the morphological

changes that occur during silvering in eels. To check it, eel females (*Anguilla australis*) were treated with 11-KT, and these animals presented external changes in the head shape and pectoral fin color (Rohr et al., 2001). 11-KT not only affected the external morphology, also increased the liver and gonad mass (Rohr et al., 2001). Asanuma et al. (2003) showed that treatment in eel females (*Anguilla japonica*) with 11-KT enhanced the vitellogenin production caused by E_2. Some researches suggested that 11-KT can bind to liver estrogen receptors (Peyon et al., 1997), but the discovery of an specific androgen receptor in male eel liver (Ikeuchi et al., 1999) supports the notion that 11-KT could be acting directly in the liver. Moreover, it was observed that the 11-KT increased significantly the diameter of previtellogenic oocytes and oocyte nuclei, although these changes were not accompanied with obvious ultrastructural changes (Lokman et al., 2007).

It is possible to induce the spermatogenesis and spermiation in eel male with only one injection of hCG (Khan et al., 1987; Ohta and Tanaka, 1997; Miura et al., 1991a), which causes a significant increase of 11-KT levels. It has been demonstrated that the 11-KT can promote all the stages of spermatogenesis *in vitro* (Miura et al., 1991a, 1996). Also, *in vitro* conditions, a recombinant Japanese eel follicle-stimulating hormone (rjeFSH) stimulated the 11-KT production, evidencing this gonadotropin as a responsible of the onset of the spermatogenesis (Kamei et al., 2003). 11-KT receptors have been identified in Japanese eel gonad, being sequenced two different subtypes (Androgen receptors, AR1, AR2; Ikeuchi et al., 1999; Todo et al., 1999).

Recently, the progestin has been identified as an essential factor for the initiation of the meiosis in the Japanese eel (Miura et al., 2006). Hormonal treatment with hCG induces an increase in 17α,20β-dihydroxy-4-pregnen-3-one (DHP) level, and it is the unique progestin that stimulates the DNA replication in spermatogonia cells. DHP production is increased in presence of 11-KT, being synthesized only in germinal cells. Two different receptors have been sequenced for this progestin: PR1 and PR2 (Todo et al., 2000; Ikeuchi et al., 2002), finding the progestin receptor 1 (PR1) in Sertoli, germinal and interstitial cells, while PR2 was only observed in germinal cells.

The spermiogenesis process is characterized by morphological changes, through which the spermatids are transformed into spermatozoa, but at the end

of the process these gametes are non-functional. During the next spermatogenesis step, named sperm maturation process, these gametes will acquire the ability of movement. Unlike in spermiogenesis, in this phase there are involved physiological but not morphologic changes. An increase in the pH into the sperm duct (around 8.0 in Japanese eel, and 8.5 in European eel) was observed in this phase, which caused an elevation of intracellular cyclic adenosine monophosphate concentration ([cAMP]; Miura et al., 1995a; Ohta et al., 1997). DHP has been related with the sperm maturation, because it acts directly on spermatozoa, inducing the expression of eel spermatogenesis-related substance 22 (eSRS22), which is a homologue of carbonic anhydrase (CA; Miura and Miura, 2003). This enzyme catalyzes the reversible carbonate buffer reaction, regulating in this way the acid-base balance. This enzymatic activation is the responsible of the seminal plasma pH increase observed at the end of the sperm maturation (Fig. 1.7, Miura et al., 1995a; Miura and Miura, 2003).

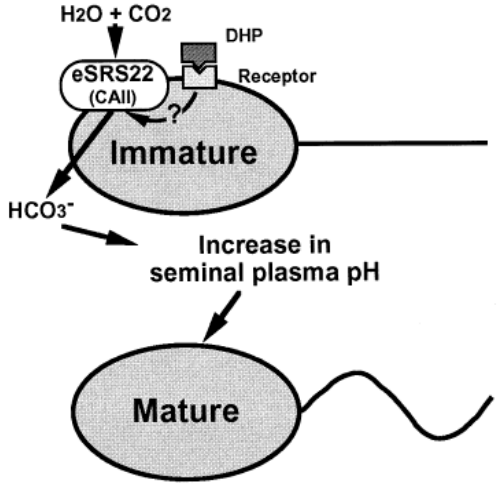

Figure 1.7. DHP effect in the spermatozoa (Miura and Miura, 2003).

1.4 METHODS TO INDUCE THE ARTIFICIAL MATURATION

Previous studies in Japanese eel (*Anguilla japonica*) showed that repeated injections of hCG allow the artificial maturation of immature male eels and induce milt production (Ohta et al., 1996; Ohta and Tanaka, 1997). One injection is enough to promote the spermatogenesis (Miura et al., 1991a), but the number of animals with complete gonad development was higher with repeated injections (Ohta et al., 1996). Also, hCG injections have been used in European eel (Boëtius and Boëtius, 1967; Billard and Ginsburg, 1973, Meske 1973; Bieniarz and Epler, 1977; Dollerup and Graver, 1985; Leloup-Hâtey et al., 1985; Colombo et al., 1987; Khan et al., 1987; Amin 1997; Pérez et al., 2000, 2003; Müller et al., 2004, 2005; Szabó et al., 2005), but only Asturiano et al. (2005) tested five different treatments with hCG looking for the optimization of the hormonal induction. Fish treated with only three weekly injections, or weekly injected only until the onset of spermiation did not show good results in terms of sperm production, but the treatment that provided best results was weekly injections of 1.5 IU hCG/g fish, that caused the highest percentage of spermiating males and the highest number of sperm samples with high quality.

1.5 EVALUATION OF SPERM QUALITY

As a consequence of the hormonal treatment the spermatogenesis takes place and the spermiation begins. The European eel sperm shows a high density (3-6x10^9 spermatozoa/ml; Pérez et al., 2000) and the time of spermatozoa motility is very short after activation, from a few seconds to a few minutes (Gibbons et al., 1983, 1985; Woolley, 1998; Asturiano et al., 2004). Fish sperm quality has traditionally been estimated by a subjective evaluation of sperm motility and concentration, but during recent years an intense advance has occurred in the techniques for the objective evaluation of sperm quality. This evaluation has been achieved by the study of motility parameters by CASA (*computer assisted sperm analysis*) systems (Kime et al., 2001; Asturiano et al., 2004; Rurangwa et al., 2004), spermatozoa morphometry analysis using ASMA (*automated sperm morphometry analysis*; Van Look et al., 2003; Marco-Jiménez et al., 2006) or by using fluorescent staining methods to evaluate membrane functionality, determining on this way the percentage of viable spermatozoa (Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008).

1.5.1 Evaluation of parameters of sperm motility by computer assisted sperm analysis (CASA)

Woolley (1997, 1998) described different aspects of the kinematics of movement, the flagellum structure and its vibratile and rotatory bending movement. However, the first application of CASA systems (Fig. 1.8) on European eel sperm was reported by Asturiano et al. (2005) in an attempt of improving hormonal induction treatments.

This analysis consists in the determination of the exact percentage of motile spermatozoa as well as some motility parameters: curvilinear velocity (VCL, in μm/s), straight line velocity (VSL, in μm/s), angular path velocity (VAP, in μm/s), and beating cross frequency (BCF, in Hz). Rurangwa et al. (2004) reviewed different studies carried out using sperm-tracking systems in African catfish, carp, goldfish, roach, Eurasian perch, trout and lake sturgeon, and concluded that the most useful parameters of velocity are the VCL (the actual velocity along the trajectory) and the VSL (the straight line distance between the start and end points of the track divided by the time of the track). In the case of European eel, data from fast and medium-velocity spermatozoa (VCL >40 μm/s)

have been used to compare motility parameters. The next results were found in samples showing 49.5 ± 2.5% motile spermatozoa: VSL: 26.1 ± 3.3 µm/s, VCL: 125.5 ± 15.6 µm/s, VAP: 44.6 ± 3.7 µm/s, BCF: 17.0 ± 1.2 Hz (Asturiano et al., 2005).

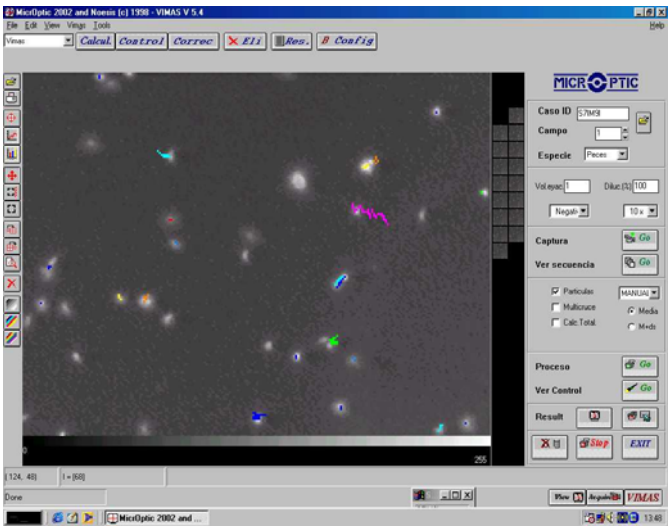

Figure 1.8. CASA system software.

1.5.2 Morphometry characterisation of spermatozoa with assisted sperm morphology analysis (ASMA) and scanning electron microscopy

Previous studies reported that when spermatozoa morphology is analysed by visual methods, the intra- and inter-observer laboratory variations are usually very large (Soler et al., 2003). Spermatozoa ultrastructure and morphology of European and Japanese eels has been studied by transmission electron microscopy (TEM; Ginsburg and Billard, 1972; Çolak and Yamamoto, 1974; Gibbons et al., 1983; Gwo et al., 1992) or scanning electron microscopy (SEM; Gibbons et al., 1983; Okamura et al., 2000). In 2004, an automated system for spermatozoa head morphometry analysis (ASMA) was developed and validated for mammals (Rijsselaere et al., 2004). The ASMA systems require standardisation of methods and variables, but under these conditions their repeatability and validity are much higher than any subjective morphological evaluation (Wang et al., 1991; Coetzee et al., 1998).

Abnormal spermatozoa head morphometry has been associated with reduced fertility in the bull, boar and stallion (Claassens et al., 1996; Van Look

and Kime, 2003). ASMA measurements have shown toxic effects on human spermatozoa head (Davis et al. 1995) and goldfish sperm (Van Look and Kime 2003). This technique has also been used in the field of cryopreservation, in which cryoprotectants or frozen-thawed protocols are known to cause morphological damage to the spermatozoa (Billard 1983; Billard et al. 2000). Kruger et al. (1995) found that spermatozoa head morphometry, determined by ASMA, was predictive of *in vitro* fertilisation rates and its utility has also been reported in detection of fertile and subfertile stallions (Gravance et al., 1997) and rabbits (Marco-Jiménez et al., 2005). Until very recently, ASMA systems had never been used on eel species (Fig. 1.9).

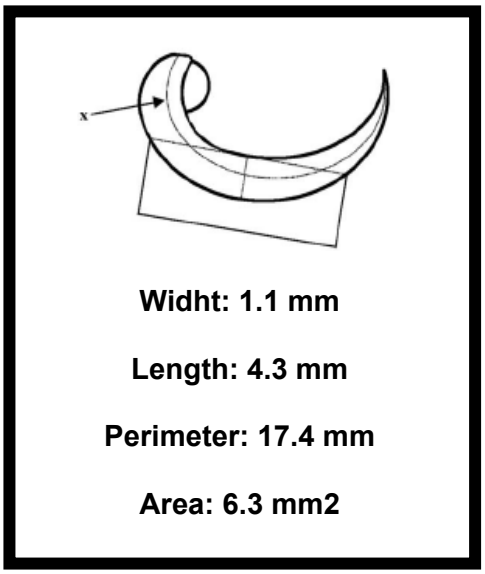

Figure 1.9. Parameter measured by ASMA system (Marco et al., 2005).

The ASMA technique has been used to evaluate the osmotic effects suffered by cells and cryoprotectant effects during the application of cryopreservation protocols (Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008). These are some of the first applications of ASMA methodology in fish and the first one in eel species. They confirm this system as a useful tool, mainly in terms of time-saving and the reduced equipment required in comparison with electron microscopy techniques, with wide applications in

future studies of fish spermatozoa membrane response under the effects of extenders, cryoprotectants, additives, etc.

1.5.3 Fluorescent staining

This technique is based in the use of dyelight fluorophores, which with specific amplitude of the oscillation (λ) emit a light. Depending of its characteristics the fluorophore can inform about cells viability, enzymatic activity, etc. The main benefits of these techniques are the simplicity and the high velocity of evaluation. These methods has been successfully used for assessing spermatozoa in men (Garner and Johnson, 1995), bulls (Garner et al., 1994; Thomas et al.,1998), boars (Garner and Johnson, 1995; Garner et al.,1996), rams (Garner and Johnson, 1995), rabbits (Garner and Johnson, 1995), mice (Garner and Johnson, 1995; Songsasen et al., 1997), poultry and wildfowl (Donoghue et al., 1995; Blanco et al., 2000) and honey bees (Collins and Donoghue, 1999; Collins,2000). Cell viability and mitochondrial function, have also been chosen to evaluate fish sperm quality (Ogier De Baulny et al., 1997, 1999; Segovia et al., 2000; Riley 2002; Flajshans et al., 2004; He and Woods, 2004; Rurangwa et al., 2004). JC-1 or rhodamine 123 are some mitochondrial stains, which are transported into the interior of functioning mitochondria. For example, when the concentration of JC-1 inside the mitochondria increases, the aggregation presents an orange fluorescence (Thomas et al., 1998; Garner and Thomas, 1999). Spermatozoa stained with JC-1 display either green fluorescence for mitochondria with low to medium membrane potential, or yellow-orange for mitochondria with high membrane potential. Previous studies have demonstrated a positive correlation between functional mitochondria and sperm motility (Evenson et al., 1982; Auger et al., 1989).

SYBR 14 and propidium iodide are DNA staining, where spermatozoa are classified as *dead* when nuclei showed red fluorescence over sperm head and *live* when they show green fluorescence (Fig. 1.10).

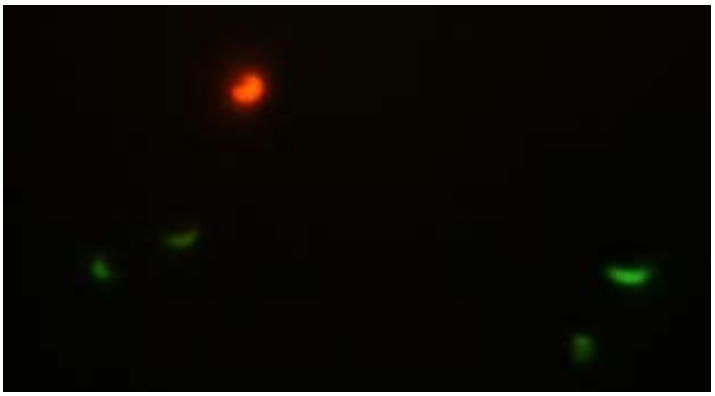

Figure 1.10. Example of SYBR and IP staining in eel sperm.

Another vital staining is the bisbenzimidazole Hoechst 33258, which stains only the dead cells. Spermatozoa are classified as dead when nuclei showed bright blue fluorescence over sperm head, and live when sperm head was not strongly fluorescent (Fig. 1.11, De Leeuw et al., 1991; Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008).

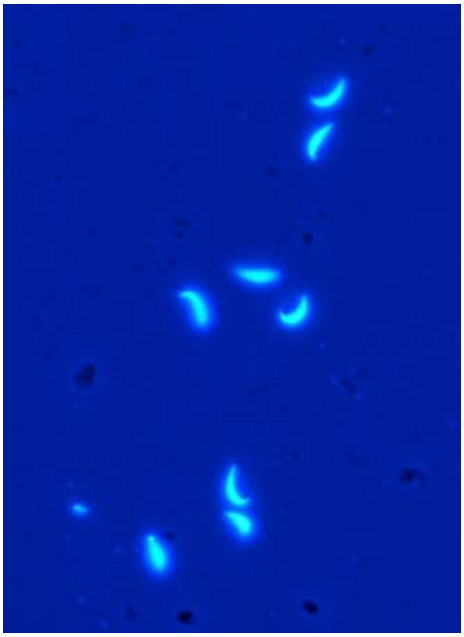

Figure 1.11. Example of Hoechst stanining in eel sperm.

1.6 METHODS FOR EEL SPERM CONSERVATION

The methods of hormonal induction carried out in European eel, both males and females, take several weeks (Pérez et al., 2000; Asturiano et al., 2002, 2005) and unsynchronized maturations can happen, making difficult the egg fertilization. Trying to improve the unresolved control on the reproduction of this species, different methods to preserve the sperm were developed depending on the time of conservation required. For short- time storage the sperm can be diluted in a medium that conserves unchanged the sperm characteristics (Ohta and Izawa 1995, 1996; Aurich et al., 1997; Sansone et al., 2001; Rodina et al., 2004; Peñaranda et al., in press), while for long-time conservation it is necessary to use cryopreservation techniques (Tanaka et al., 2002a; Müller et al., 2004, Szabó et al., 2005; Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008). In European eel, any diluent has been developed to preserve the fresh sperm during several days with high motility, and when the sperm was frozen by cryopreservation techniques the best post-thawing motility achieved by our group was 22.2 ± 1.5%. Further studies are necessaries in this field to improve the European eel sperm conservation.

1.6.1 Diluent media

The use of diluting media or extenders can extend the sperm viability and even increase the spermatozoa motility after an incubation period (Ohta and Izawa, 1996, Ohta et al., 2001; Peñaranda et al., in press). Usually, the diluents have been used to maintain a high motility during short-term storage, by stabilising the physio-chemical conditions (Tan-Fermin et al., 1999), and to improve fertilization rates in some species (Tambasen-Cheong et al., 1995; Ohta and Izawa, 1996). Fish spermatozoa are immotile in seminal plasma, and for this reason several authors have developed diluents with the same ionic composition and osmolality as the seminal plasma (Villani and Catena, 1991; Tan-Fermin et al., 1999; Asturiano et al., 2004). On the other hand, some authors have also tried to prepare inactivation media for fish spermatozoa, with different composition respect to the seminal plasma (Sansone et al. 2001; Tanaka et al. 2002b; Rodina et al. 2004). In the European eel, first studies in this regard were focused on the analysis of the seminal plasma ionic composition and physio-chemical characteristics (Pérez et al. 2003; Asturiano et al. 2004). Changes in

the ionic composition of seminal plasma were correlated with changes in the sperm quality, in terms of spermatozoa motility (Pérez et al. 2003; Asturiano et al. 2004). Asturiano et al. (2004) described that the seminal plasma pH did not vary during the 14 weeks of assay, being constant between 8.4 and 8.6. The seminal plasma osmolality showed a significant decrease during the first spermiation weeks and was maintained around 325-330 mOsm/kg during the rest of the experiment. All these results were used to design a diluting medium isoionic with the seminal plasma, called P1 medium, in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5 (Asturiano et al., 2004). In the case of Japanese eel, similar studies on seminal plasma and diluting media production have been carried out. Ohta and Izawa (1996) managed times of conservation of diluted sperm under refrigeration longer than 3 weeks. Two diluting media (K15 and K30; Ohta and Izawa, 1996; Ohta et al., 2001) previously used in Japanese eel sperm were tested in the European eel, with a significant decrease in the motility 24 hours later (Asturiano et al., 2004).

The development of diluent media for European eel sperm is necessary not only to preserve it, but also to solve the problems in sperm handling and quality evaluation, due to its high density and its short motility time. These problems have been settled in other fish species, like Japanese eel, by diluting the sperm before using it during the fertilization process (Ohta and Izawa, 1996).

1.6.2 Cryopreservation media

Different media and methods have been recently developed for European eel sperm (Müller et al., 2004, Szabó et al., 2005; Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008), which are based in previous studies in the Japanese eel species (Tanaka et al., 2002a). To design the freezing medium, it is necessary to consider the effect of cryoprotectants on the sperm. These must be added to the medium, because the cryopreservation causes lethal damage in spermatozoa and also produces an important loss of membrane function by increasing membrane fragility in live cells (Cabrita et al., 1999). Müller et al. (2004) worked with methanol (MeOH) as cryoprotectant for European eel, obtaining 36 ± 11% of spermatic motility post-freezing, using as extender the modified Kurokura medium (Rodina et al., 2004). Szabó et al. (2005) studied the cryoprotectant role of dimethil sulfoxide (DMSO) and MeOH in different extender media: as TNK (137 mM NaCl, 76.2mM Na HCO_3; Tanaka et al., 2002a),

Kurokura´s modified (350 mg NaCl, 1000 mg KCl, 22 mg $CaCl_2$, 8 mg $MgCl_2$ and 20 mg $NaHCO_3$, for 100 ml; Magyary et al., 1996) and glucose extender (350 mM glucose, 30 mM Tris, pH 8.0; Szabó et al., 2005). The highest post-thawing motility (47 ± 15%) was registered with DMSO and TNK medium. Garzon et al. (2008) tested several cryoprotectants: DMSO, acetamide, ethylene-glycol, propanol, glycerol and MeOH. Their effect was evaluated in terms of percentage of motile cells (activation caused by the cryoprotectants), percentage of live cells (by Hoechst staining), and spermatozoa morphometry pre and post-cryopreservation (by ASMA). The set of results of this experiment showed DMSO, MeOH and glycerol as the best cryoprotectants. These three cryoprotectants were chosen to make a new experiment, using P1 (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5; Asturiano et al., 2004) and P1 modified (125 NaCl, 75 $NaHCO_3$, 2.5 $MgCl_26H_2O$, 1 $CaCl_22H_2O$, 30 KCl, pH 8.5, in mM) as freezing media, and testing the influence of foetal bovine serum (FBS) addition (Garzón et al., 2008). Post-thawing samples in P1 modified medium with DMSO and FBS (25%) showed a significantly higher percentage of motile spermatozoa (22.2±1.5%) that in the rest of conditions. These results coincide with the reported by Tanaka et al. (2002a) and Szabó et al. (2005), who suggested the DMSO as effective cryoprotectant for the eel sperm. P1 modified and TNK medium have been the freezing extenders that have provided the best post-thawing motility, and both contain the same $NaHCO_3$ concentration, suggesting that this component of the medium has an important role in the sperm movement. Studies in this field were carried out by Tanaka et al. (2002b, 2004), observing a $NaHCO_3$ inhibition role on the sperm motility. This inhibition could be important to arrest the sperm activation caused by cryoprotectants in pre-freezing conditions, and in this way to decrease the energy spent by spermatozoa. Foetal bovine serum (FBS) or bovine serum albumin (BSA) have been used in the sperm cryopreservation media due to their effect as buffer of the osmotic shock, because proteins can provide a protection of mechanical type to the cells membrane, diminishing the risks of crystallization, recrystallization or ice melting during the different phases of the process of freezing and thawing (Cabrita et al., 2005; Marco-Jiménez et al., 2006; Garzón et al., 2008).

2 JUSTIFICATIONS AND OBJECTIVES OF THE PRESENT THESIS

2.1.1 Justifications

The male European eel can not achieve the gonad development in artificial conditions, being necessary to inject hormones to obtain the maturation. Once fixed the optimal hormonal treatment (1.5 IU. hCG/g fish; Asturiano et al., 2005), we consider important to study the evolution of sperm quality (motility, percentage of live cells, head spermatozoa morphometry, sperm volume, sperm cocentration) during the spermiation period using the techniques described in previous works (Asturiano et al., 2004; Marco-Jiménez et al., 2006). Thanks to these studies the weeks with the highest sperm quality can be established. The analysis of spermatozoa head morphometry presents problems due to the curved and elongated spermatozoa head. To validate this technique in sperm eel, it will be compared the obtained results by ASMA system with scanning electron microscopy (SEM).

To understand the reproductive physiological mechanisms, and their influence on the sperm quality, every week several males will be sacrificed to study the GnRHs and gonadotropins expression, 11-KT level in plasma and morphometric parameters, being the first time that GnRHs and gonadotropins expression will be described in male European eel. The different studied parameters will be compared with the stage of gonad development and sperm quality, obtaining a complete description of artificial maturation in European eel.

Once described the maturation in European eel and the evolution of sperm quality during its gonad development, the next purpose will be to preserve the eel spermatozoa with high motility for a long time. There are two methods to preserve the sperm, during short-term or long-term storage.

It is considered short-time storage when the fresh sperm can be maintained with high motilities during several days. Different media have been tested in previous works, but they did not provide good results (Asturiano et al., 2004). In order to improve these results, two different methods to preserve the sperm will be tested: one medium that inactivates the sperm motility and another non-activating medium.

The best motility post-thawing achieved by our group was 22.2 ± 1.5%, which we consider too low to obtain a fertilization succes. Taking as basis the sperm physiology, media with different $NaHCO_3$ concentration and pH will be

used to inhibit the sperm activation produced by the cryoprotectants in pre-freezing conditions. This inhibition will permit that the spermatozoa do not spend its energy reserves before the cryopreservation process.

2.1.2 Objectives

1- To assess the applicability of different techniques (ASMA system and vital staining), to evaluate the sperm quality in European eel.

2- To determine the evolution of sperm quality during the whole spermiation period induced by hCG administration.

3- To study the endocrine mechanisms controlling the spermatogenesis and spermiation processes in artificially matured European eel males, and their relationship with the sperm quality.

4- To develop one extender and one appropriate protocol for the short-term storage of European eel sperm under refrigeration.

5- To develop a freezing medium and to improve the cryopreservation techniques for the long-term storage of the European eel sperm.

3 GENERAL MATERIAL AND METHODS

3.1 FISH AND SAMPLING

The fish were obtained from the fish farm Valenciana de Acuicultura, S.A (Puzol, Valencia; East coast of Spain), and they were moved into our facilities, in the Aquaculture Laboratory in the Universidad Politécnica de Valencia. Fishes were gradually acclimatized to sea water (salinity 37 ± 0.3 g/l; 20ºC) for one week and distributed in the corresponding tanks or aquaria (Fig. 3.1).

Figure 3.1. Aquaria and tanks where the eels were distributed.

The male eels were hormonally treated for the induction of maturation and spermation with weekly intraperitoneal injections of hCG (1.5 IU g/fish, Fig. 3.2A), as previously described by Pérez et al. (2000).

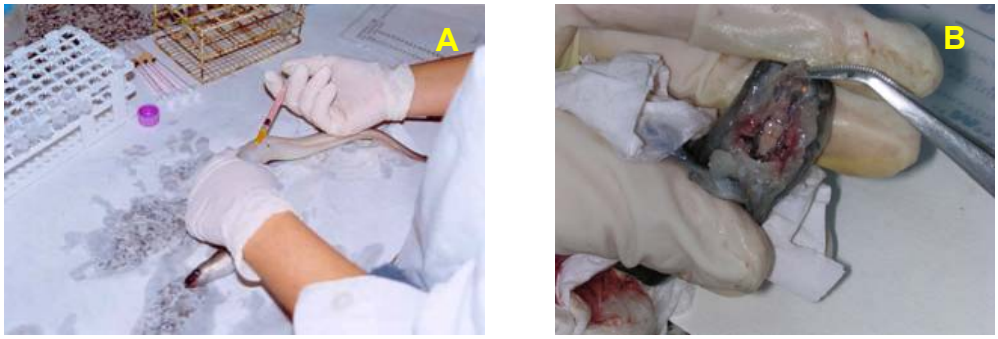

Figure 3.2. (A) Intraperitoneal injection of hCG. (B) Extraction of the different parts of the eel brain.

When it was necessary to sacrifice animals to obtain samples, once a week 10 fish were over-anesthetized, and morphometric parameters as gonadosomatic index (GSI) and eye index (IE; Leloup-Hatey et al., 1985) were recorded, as well as samples of blood, liver, the brain, pituitary were collected, and also sperm samples during the spermiation weeks. The brain was divided in

different parts for its study: olfactory bulb, telencephalon, mesencephalon and diencephalon, cerebellum and medulla oblongata and pituitary (Fig. 3.2B).

Each part of the brain was quickly removed and stored in 0.5 ml RNA later (Ambion Inc, Huntingdon, UK) at -20ºC until its RNA extraction. The blood was centrifuged at 3000 r.p.m during 5 minutes, and the blood plasma was stored at -80ºC until its analysis. The sperm was obtained by applying gentle abdominal pressure to anesthetised males (benzocaine; 60 mg/L), after cleaning the genital area with freshwater and thoroughly drying to avoid contamination of samples with faeces, urine and sea water (Fig. 3.3). During spermiation weeks the motility and percentage of live cells were evaluated every week.

Figure 3.3. Process of sperm extraction: it began with the fish catch, followed by anaesthesic bath, the abdominal cleaning and drying, and finish with the sperm extraction.

3.2 RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from the different parts of the brain (olfactory bulb, telencephalon, mesencephalon-diencephalon) and the pituitary, using traditional phenol/chloroform extraction by the Trizol reagent (Invitrogen, Belgium), followed by a bath water during 20 minutes at 37ºC. Later, one step of deoxyribonuclease treatment (gDNA Wipeout Buffer, Qiagen) was performed, using a total volume of 14 µl for 2 µg of total RNA. First-strand cDNA was synthesized in 20 µl reactions with 14 µl used as template, which were obtained in the previous step. The protocol was carried out according to the manufacturer's instructions.

3.3 QUANTITATIVE REAL TIME RT-PCR

3.3.1 Primers and references genes

Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC ACG; ARPrv: ACA TCG CTC AAG ACT TCA ATG G (Aroua et al., 2007; Weltzien et al., 2006) was used as reference gene in the qrtRT-PCR. It was used as house kepping because its mRNA expression does not vary with experimental treatment. The gene specific primers assigned to evaluate the pituitary gonadotropin expression were: FSHfw: TCT CGC CAA CAT CTC CAT C; FSHrv: TAG CTT GGG TCC TTG GTG ATG and LHfw: TCA CCT CCT TGT TTC TGC TG; LHrv: TAG CTT GGG TCC TTG GTG ATG (Aroua et al., 2007)

The study of the GnRHs expression were localized in olfactory bulbs, telencephalon, diencephalon-mesencephalon for mGnRH, using the next specific primers: mGnRHfw, ACT GGT GTG TCA GGG ATG CT; mGnRHrv, TGC AGC TCC TCT ATA ATA TCT TGC (Sébert et al., submitted), while the cGnRH-II expression was analyzed in diencephalon-mesencephalon (cGnRH-IIfr, CTG ACA TCC ACA CAG CGA CT; cGnRH-IIrv, GGT GTT CAC CAT CAC AGC TAA A ; Sébert et al., submitted).

3.3.2 SYBR Green assay

In order to monitor gene expression of gonadotropins (FSHβ, LHβ) and GnRHs (mGnRH, cGnRH-II), real-time quantitative polymerase chain reaction (PCR) analyses were performed using a Light Cycler system with SYBR Green I sequence-unspecific detection (Fig. 3.4; Roche, Meylan, France).

Figure 3.4. Light Cycler system with SYBR Green I sequence-unspecific detection (Roche).

After an initial *Taq* activation of polymerase at 95ºC for 10 minutes, 41 cycles of PCR were performed using the LightCycler with the following cycling conditions: 95ºC for 15 s, 60ºC for 5 s and 72ºC for 10 s in the study of gonadotropins. In the case of GnRHs, 41 cycles of PCR were made with this cycle: 95ºC for 10 s, 60ºC for 10 s and 72ºC for 13 s. After the PCR, the machine performed a melting curve analysis by slowly (0.1 ºC/s) increasing the temperature from 68 to 95ºC, with a continuous registration of changes in fluorescent emission intensity.

The total volume for every PCR was 10 µl, performed from diluted cDNA template (4 µl), forward and reverse primers (0.5 pm each) and SYBR Green Master Mix (2 µl).

3.4 IMMUNOENZYMATIC ASSAY (ELISA) FOR SEXUAL STEROID (11KT)

The teleost-specific androgen 11-ketotestosterone (11-KT) was measured in blood plasma using the 11-KT EIA kit from Cayman Chemicals (distributed by Scharlab S.L., Barcelona, Spain) according to the instructions of the manufacturer (Fig. 3.5).

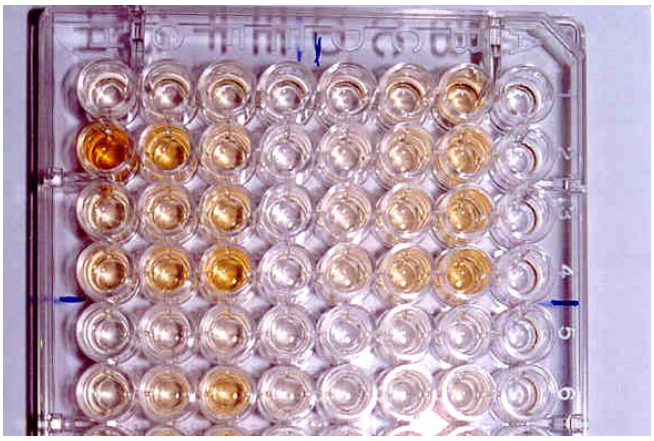

Figure 3.5. Example of ELISA plate.

3.5 GONAD HISTOLOGY

After fixation in 10% buffered formalin (pH 7.4), the gonad samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were made with a manual microtome Shandom Hypercut, and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope.

Stages of spermatogenesis were determinated according to the most advanced germ cell types and their relative abundance (Utoh et al., 2004; Huertas et al., 2006): stage I was determined by the presence of spermatogonia type A and/or B; stage II, by the presence of spermatogonia and spermatocytes; stage III, by the appearance of spermatids; stage IV, appearance of spermatozoa in small lumen; stage V (maturation stage) increase in the number of spermatozoa, as well as in lumen size; and stage VI, characterized by dominance of spermatozoa, with a low proportion of the other germ cells, as well as luminal fusion.

3.6 EVALUATION OF THE MOTILITY

Immediately after collection, the motility of sperm samples was assessed by mixing one drop of sperm with 3 µl of artificial sea water [in Mm: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8; Fig. 3.7] adjusted to 1000 mOsm/kg as activation media. All the sample analyses were

performed by the same trained observer to avoid subjective differences in the motility evaluation.

Figure 3.6. Evaluation of the sperm motility.

3.7 FLUORESCENCE STAIN ANALYSIS

Different fluorescence staining methods can be used to evaluate the cell viability. One of them is Live/Dead Sperm Viability Kit based in SYBR green and Propidium Iodide (PI) dyes (Invitrogen, Barcelona, Spain), that was used in Experiment X to evaluate the viability of spermatozoa pre- and post-freezing.

Sperm diluted in P1 medium (Asturiano et al., 2004; 1:350) was mixed with SYBR Green and PI, and maintained during 20 minutes of incubation at room temperature in the dark. The final SYBR Green concentration was 10^4 times diluted from the original stock (2 µM) and PI 10^3 times from original stock (24 µM). Spermatozoa were classified as *dead* when nuclei showed red fluorescence over sperm head and *live* when they showed green fluorescence.

Another fluorescence stain useful to evaluate the viability is Hoechst 33258, that was used in experiment 1.

Briefly, 4 µL Hoechst 33258 (Sigma, 1 mg/mL in PBS) was mixed with 2 µL eel semen and 998 µL PBS. After 5 min incubation at room temperature in the dark, at least 100 spermatozoa per slide were assessed. Spermatozoa were classified as *dead* when nuclei showed bright blue fluorescence over sperm head, and *live* when they did not showed this strong fluorescence.

The mitochondrial function, studied in Experiment 1, was determinated by JC-1 staining. The stock solution was first prepared as 5 mg/ml of JC-1 (Molecular Probes Inc.) in dimethyl sulphoxide, as staining solution contained 10 µg of JC-1 in 998 ml of PBS solution. Two µl of sperm eel was incubated with staining solution at room temperature in the dark for 15 min. Spermatozoa stained with JC-1 displayed either green fluorescence on mitochondria with low to medium membrane potential, or yellow-orange fluorescence on mitochondria with high membrane potential.

For each fluorescence analysis at least 100 spermatozoa per sample were assessed in a Nikon Eclipse (E-400) epifluorescence microscope, using UV-2A (EX: 330-380 nm, DM: 400, BA: 420) filter.

3.8 SPERMATOZOA MORPHOLOGY ANALYSIS

In all experiments a fraction of sperm samples was diluted 1:50 (v/v) with 2.5% glutaraldehyde in phosphate buffered saline fixative solution (Pursel and Johnson, 1974). Slides were viewed using a 1000X negative phase contrast objective (Nikon Plan Fluor) on an Eclipse E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer®, Morfo Version 1.1, Imagesp, Barcelona, Spain). Approximately 100 spermatozoa were analysed in each sample. The morphological parameters determined were: head perimeter (µm) and head area (μm^2).

3.9 SCANNING ELECTRON MICROSCOPY

In experiment 2, sperm samples were separated from seminal plasma by centrifugation at 1800 g for 10 min at room temperature, pre-washed twice with 0.1 M phosphate buffer pH 7.2 for 1 h. The cells were washed twice with phosphate buffer and post-fixed in 1% osmium tetraoxide for 1 h. After fixation, the samples were washed twice with phosphate buffer, dehydrated through a graded series of ethanol and amylacetate, and finally critical point-dried in liquid CO_2. The dried samples were mounted on aluminium studs by means of conductive silver paint and then coated with gold-palladium. All specimens were examined and photographed in a JEOL JSM 5 410 microscopy operated at 15

kV. Spermatozoa head length, width, perimeter and area were determinated using a public domain ImageJ program (Fig. 3.8; develop at U.S. National Institutes of Health and available at www.rsb.info.nih.gov/ij/).

Figure 3.7. Eel spermatozoa observed by scanning electron microscopy.

4 EXPERIMENT 1

Effects of hCG as spermiation inducer on European eel semen quality

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Abstract

Fish sperm quality has traditionally been estimated by subjective evaluation of motility and sperm concentration. Alternative methods for evaluation of sperm quality have been developed in the last decade and enable estimation of spermatozoa head morphometry, membrane integrity and mitochondrial function.

Weekly injections of human chorionic gonadotropin (hCG) induced spermiation in farmed male European eels. The milt volume increased from the 5th to 12th weeks. Sperm concentration significantly increased from the 5th week, reaching the highest values at the 8th week, while best motility results were registered at the 9th week of treatment. Coinciding with these intervals, the percentage of dead spermatozoa determined with Hoechst staining showed a reduction in the 8th to 11th weeks of treatment, while the percentage of mitochondrial functionality determined by JC-1 staining did not show a similar pattern.

The automatic sperm morphology analysis (ASMA) of the spermatozoa head length, width, area and perimeter showed a significant growth from the 5th to 8th weeks. However, the analysis of isolated descriptive parameters may be difficult to understand because there is variability in these parameters for each week, making knowledge of the growth kinetic complex. The global size of the spermatozoa head was calculated by applying principal component analysis (PCA), because this method establishes new components that make the interpretation of results easier, allowing a whole interpretation of the changes in the cell morphology. PC1 defines the global head size and shows a significant increase between the 5th and 8th weeks of treatment, showing shorter changes until 12th week. PC2 shows a significant increase in the spermatozoa width from the 5th to 7th weeks. Considering the results of the variations in the principal components defining European eel spermatozoa morphometry, it may be concluded that hCG maturative treatment produced thick cells during the first weeks of spermiation, and subsequent samplings showed an increase in cell width and length.

These changes in sperm morphometry coincide with the highest sperm quality assessed as sperm motility and concentration, as well as with the best results obtained in previous studies reporting the best sperm quality between weeks 8 and 10 of hCG treatment. These results support the use of ASMA and Hoechst staining techniques as alternative methods for the evaluation of fish sperm quality.

Introduction

In recent decades, the capture and over-exploitation of eels and elvers have diminished populations, becoming an ecological and economical problem, making the development of techniques for the control of reproduction in captivity necessary. Methods for the hormonal induction of gonad maturation in this species have been developed in previous studies, obtaining significant sperm volumes with a good quality [1-3] as well as ovarian maturation, spawns, egg fertilisation and even hatching [4-5]. Moreover, hybrids between European eel and Japanese eel (*Anguilla japonica*) have been obtained, using the sperm of the European species and Japanese eel oocytes [6]. However, methods for the hormonal induction of gonad maturation in this species usually take several

weeks, and unsynchronised maturations can occur, preventing egg fertilisation. With the aim of resolving the issue of lack of control in the reproduction of this species, the first sperm cryopreservation media and methods have recently been developed [7-10], and different techniques have recently been used to characterise European eel spermatozoa morphometrically [11-12], in an attempt to define a way to evaluate sperm quality.

Human chorionic gonadotropin (hCG) treatment has been used successfully to induce spermiation in both European and Japanese (*A. japonica*) farmed eels [1-3,7,9,13,14]. The hormonal treatment causes morphological changes in males (eye diameter increase, dark dorsolateral and silver ventral colouration, black pectoral fins and well developed cephalic lateral line). The hCG treatment results in the spermiation of most of the males over several weeks [1,3,7,9].

Sperm quality is a measure of the ability of sperm to fertilise an egg successfully, but this capacity may not be reliable, as egg quality may be variable and affect fertilisation success [15]. Currently, any quantifiable physical parameter that directly correlates with the fertilisation capacity of sperm could be used to evaluate sperm quality [15]. The percentage of motile spermatozoa is the most common test used to evaluate fish sperm quality [15-17]. Sperm motility depends on the ATP content and the ability of mitochondria to sustain the high energy demand during motility [18]. Protocols have been developed using fluorescent staining to provide rapid assessment of the mitochondrial functionality and plasma membrane integrity of fish sperm [15,17,19-21]. To assess the non-viable cells, membrane-impermeable nucleic acid stains can be used which positively identify dead spermatozoa by penetrating cells with damaged membranes. An intact plasma membrane will prevent these products from entering the spermatozoa and staining the nucleus. Phenanthridines, such as propidium iodide (PI) [22,23], SYBR14 [21,23] and bisbenzimidazole Hoechst 33258 [24] have commonly been used. Mitochondrial function can be assessed using rhodamine 123 (Rh123) [21] or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), allowing a distinction to be made between spermatozoa with poorly and highly functional mitochondria [25]. Until recently, the morphology of fish and mammalian sperm was examined using manual techniques, but these are subjective, time-consuming and the results

highly variable [26,27] and difficult to apply uniformly [28]. Several reports have described the spermatozoan ultrastructure of European and Japanese eels and examined its morphology based on transmission electron microscopy (TEM) [29-32] or scanning electron microscopy (SEM) [31,33]. New image analysis systems which are easier to use and enable evaluation of a great number of spermatozoa have been developed to measure several morphology parameters. Automatic sperm morphology analysis (ASMA) was previously used in European eel to examine the head spermatozoa morphometry [11,12], showing that it is possible to use this methodology to obtain similar results as from TEM and SEM. This technique has also been used in the field of cryopreservation, in which cryoprotectants or freeze-thawed regimes are known to cause morphological damage to the spermatozoa [34,35], and to study the effect of mercuric chloride on goldfish sperm [27].

The aim of the present study was to examine the effect of weekly hCG treatment on European eel spermatozoa quality, using fluorescent staining techniques and analysing spermatozoa morphometry with the ASMA system.

Material and Methods

Fish and sampling

Thirty-six farmed eel males (137.6 ± 21.43 g of body weight) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into our facilities at the Universidad Politécnica de Valencia. Fish were gradually acclimatised to seawater (salinity 37.0 ± 0.3 g l^{-1}; 20–22 °C) for 10 days. Fish were distributed in four 96 l aquaria and tagged with passive integrated transponders (P.I.T. tags) injected into the epaxial muscle for individual identification. Fish were fasted during the experiment.

Males were hormonally induced to maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU g BW^{-1}), as described previously by Pérez et al. [2]. Once a week during the spermiation period, from week 5–12 of hCG treatment, fish were sampled 24 h after the hormone administration. After cleaning the genital area with fresh water and thoroughly drying to avoid contamination of samples with faeces, urine and seawater, total expressible milt was collected in a graduate tube after applying gentle abdominal pressure to

anaesthetised males (benzocaine; 60 mg l^{-1}). Total expressible milt was expressed as ml 100 g $fish^{-1}$ and samples were maintained at 4 °C.

Determination of spermatozoa concentration was performed immediately after collection from all males and by the same technician to avoid bias in assessment. To avoid sperm aggregation and achieve an appropriate concentration for counting, milt was diluted 1:500–1:1000 with P1 medium containing: 125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 30 mM KCl; pH 8.5 [7]. Sperm concentration was estimated after direct counting on a Thoma haemocytometer and expressed as spermatozoa × 10^9 ml^{-1}.

Sperm quality evaluation

Immediately after collection, the sperm motility was estimated after a final dilution of 1:1000 using artificial seawater (354.7 mM NaCl, 52.4 mM $MgCl_2$, 9.9 mM $CaCl_2$, 28.2 mM Na_2SO_4, 9.4 mM KCl; pH 8) with osmolality adjusted to 1000 mOsm kg^{-1} as an activation media [3]. Immediately after dilution, 2 µl of activated sperm were transferred into one well of a 12-well multi-test slide and covered with a coverslip coated with 1% bovine serum albumin (BSA) in distilled water. All the sample analyses were performed by the same technician to avoid subjective differences in the motility evaluation. The activation process was carried out very quickly, in less than 10 s, as the motility usually drops in a few seconds. Motility was characterised using an arbitrary scale in which 0 represented no motile sperm, I <25%, II = 25–50%, III = 50–75%, IV = 75–90%, and V = 90–100% of the population were vigorously motile.

Sperm viability assessed by Hoechst 33258 staining

Hoechst 33258 was used to evaluate the viability of eel spermatozoa throughout the weeks of spermiation. Briefly, 1 µl of Hoechst 33258 (Sigma–Aldrich, Madrid, Spain; stock solution 1 mg ml^{-1}) was added to 2 µl of eel semen and 997 µl of phosphate buffer solution (PBS). After 5 min incubation at room temperature in the dark, at least 100 spermatozoa per slide were assessed in a Nikon Eclipse (E-400) epifluorescence microscope, using a UV-2A (EX: 330–380 nm, DM: 400, BA: 420) filter and a 1000× lens (Nikon Plan Fluor). Spermatozoa were classified as *dead* when nuclei showed bright blue fluorescence over sperm head, and *live* when sperm head was not strongly fluorescent.

Mitochondrial function determined by JC-1 staining

The JC-1 was obtained from Molecular Probes Inc. (T-3168). The stock solutions were first prepared as 5 mg ml^{-1} of JC-1 in dimethyl sulphoxide (DMSO). The staining solution contained 10 µg of JC-1 in 998 ml of PBS solution. A 2 µl of eel semen was incubated with the staining solution at room temperature in the dark for 15 min. The stained spermatozoa were then examined with B-2A (EX: 450–490 nm, DM: 505, BA: 520) filter and a 1000× lens (Nikon Plan Fluor). At least 100 spermatozoa per sample were assessed in a Nikon Eclipse (E-400) epifluorescence microscope. Spermatozoa stained with JC-1 displayed either green fluorescence for mitochondria with low to medium membrane potential, or yellow–orange fluorescence for mitochondria with high membrane potential.

Spermatozoa morphology analysis

A fraction of collected sperm samples was diluted 1:50 in 2.5% glutaraldehyde in PBS fixative solution [36]. Slides were observed using a 1000× negative phase contrast lens (Nikon Plan Fluor) on Eclipse E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image to one computer. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer®, Morfo Version 1.1, Imagesp, Barcelona, Spain). Approximately 100 spermatozoa were analysed in each sample. The morphological parameters determined were: head length (µm), head width (µm), head perimeter (µm) and head area (μm^2). A total of 194 semen samples were analysed during 8 weeks.
The reference values of morphological characteristics of spermatozoa in the present study were obtained with scanning electron microscopy, according to Marco-Jiménez et al. [11,12].

Statistical analysis

The General Linear Model (GLM, Statgraphics®Plus 5.1; Statistical Graphics Corp., Rockville, MO, USA) was used to analyse the volume, concentration, percentage of dead spermatozoa and mitochondrial function data. The week was included as a fixed effect in the model. Male was a blocking factor which, although having no interest in itself, was included in order to achieve a more accurate comparison between different weeks [37].

The week effect of hCG treatment on morphometry parameters was analysed by a one way analysis of variance (ANOVA) with fixed effects, including

male as a blocking factor. Considering the multidimensional character of morphometry, as well as the evident correlation between the four parameters (length, width, area and perimeter), the previously described ANOVA was applied to the scores obtained after one Principal Component Analysis (PCA, Statgraphics®Plus 5.1; Statistical Graphics Corp., Rockville, MO, USA) developed over the four morphometry parameters. The purpose of PCA is to derive a small number of linear combinations (principal components) from a set of variables that retain as much of the information in the original variables as possible. This allows the summarisation of many variables in a few, jointly uncorrelated, principal components. A good result is obtained when a few principal components account for a high proportion of the total variance. This analysis establishes new concepts (or components) not directly measurable and independent of each other [38] that make the interpretation of results easier. In order to select the number of principal components that should be used in the next step of our analysis, we followed the criterion of selecting only those with an eigenvalue (variance extracted for that particular principal component) higher than 1 (Kaiser criterion).

Results

The means ± standard error of the means (S.E.M.) of milt volume and concentration between weeks 5 and 12 of hCG treatment are shown in Table 1. Milt volume displayed a significant increase from the 5th (0.23 ± 0.08 ml 100 g fish^{-1}) to the 11th weeks (4.18 ± 0.75 ml 100 g fish^{-1}). Sperm concentration (spz × 10^{9} ml^{-1}) significantly increased from week 5th (0.73 ± 0.25) until 8th (2.46 ± 0.23), and then progressively decreased from week 8th to 12th (1.38 ± 0.21), when the values were significantly lower.

Sperm motility increased gradually from the onset of spermiation (week 5) until showing motility categories >III from weeks 8 to 11 of hCG treatment. Best motility results were registered at the 9th week, when 24.24% samples showed motility class II and 24.24% more showed motility classes of ≥III (Table 4.1). Subsequently, sperm motility was reduced until the end of treatment.

4.1A shows the sperm viability results obtained by Hoechst 33258 staining. The percentage of dead spermatozoa showed a significant reduction at the 8th week of treatment (reaching 37.0 ± 4.6%) and maintained similar results until the 11th week of treatment, showing a significant increase (showing 55.8 ± 4.9%) coinciding with the end of the spermiation at the 12th week.

Fig. 4.1B shows the mitochondrial function determined by JC-1 staining. A reduction of cells showing low mitochondrial functionality was observed from the 5th to 8th week of treatment, when the lowest percentage of spermatozoa showed low mitochondrial functionality (78.4 ± 3.3%). From then on, a significant increase occurred and the percentage of cells showing low mitochondrial functionality was maintained until the last sampling.

Table 4. 1

Sperm volume, concentration and frequency of motility categories produced by eel males between 5th and 12th weeks of hCG treatment

	Weeks of hCG treatment							
	5	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Number of samples (*n*)	12	15	34	34	33	30	31	30
Sperm volume (ml 100 g fish^{-1})	0.23 ± 0.08 a	0.99 ± 0.26 ab	1.69 ± 0.29 ab	2.66 ± 0.41 bc	2.67 ± 0.40 bc	3.80 ± 0.52	4.18 ± 0.75 c	3.99 ± 0.76 c
Sperm concentration (spz × 10^9 ml^{-1})	0.73 ± 0.25 a	1.15 ± 0.19 a	1.79 ± 0.15 ab	2.46 ± 0.23 b	2.36 ± 0.26 ab	2.12 ± 0.23 ab	1.66 ± 0.20 ab	1.38 ± 0.21 a
Motility classes (%)								
0	91.67	33.33	14.71	5.88	3.03	13.33	16.13	46.67
I	8.33	66.67	76.47	58.82	48.48	36.67	45.16	50.00
II	–	–	8.82	26.47	24.24	36.67	35.48	3.33
>III	–	–	–	8.82	24.24	13.33	3.23	-

Different letters indicate significant differences in every sampling.

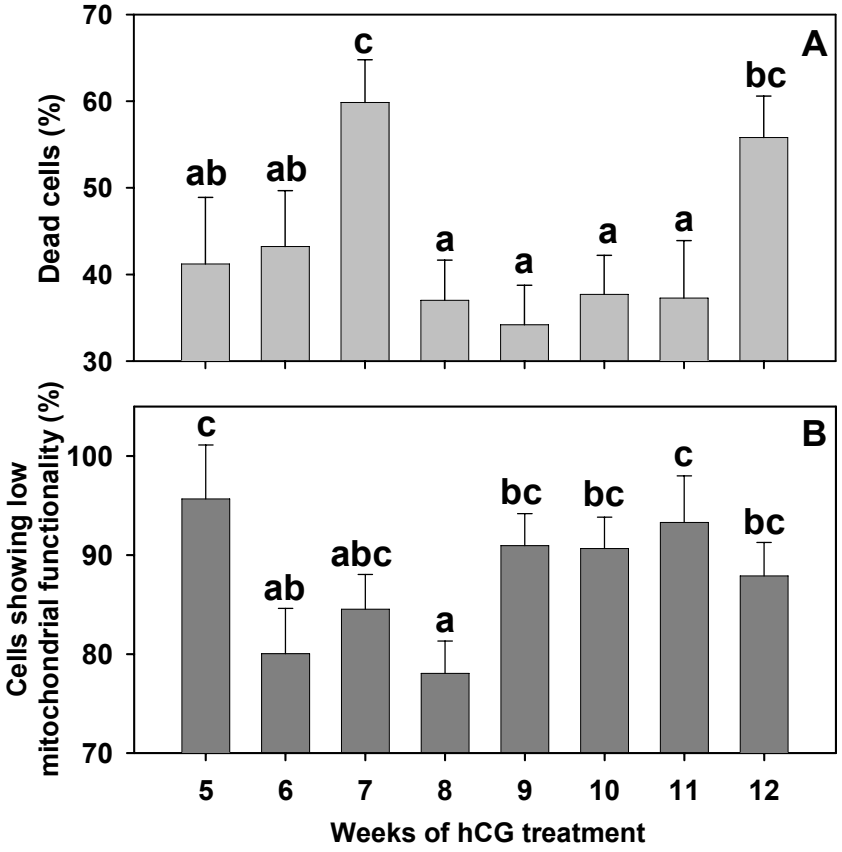

Figure 4.1 (A) Sperm viability obtained by Hoechst 33258 staining, expressed as percentage of dead spermatozoa. (B) Mitochondrial function determined by JC-1 staining, shown as percentage of cells showing low mitochondrial functionality. Different letters indicate significant differences.

Table 4.2 shows the least square means ± S.E.M. for each of the measured parameters (head length, width, perimeter and area) from weeks 5 to 12 of hCG treatment. A higher number of spermatozoa were analysed during the weeks when the highest number of sperm samples were obtained. Generally, the results show a significant growth from weeks 5 to 8, being maintained until the week 11, and decreasing thereafter. However, there is considerable variability within each of the parameters that complicates the determination of the growth kinetic.

Table 4.2. Least square means ± standard error of the means for each of the measured parameters (head length, width, perimeter and area) from 5th to 12th weeks of hCG treatment.

Weeks	*n*	Head length (µm)	Head width (µm)	Area (µm^2)	Perimeter (µm)
5	471	3.99 ± 0.03^{e}	1.07 ± 0.009^{g}	4.90 ± 0.03^{g}	13.63 ± 0.10^{f}
6	1560	4.11 ± 0.01^{d}	1.19 ± 0.004^{b}	5.19 ± 0.02^{f}	14.13 ± 0.05^{d}
7	3007	4.11 ± 0.01^{d}	1.21 ± 0.003^{a}	5.14 ± 0.01^{e}	13.94 ± 0.05^{e}
8	3147	4.31 ± 0.01^{b}	1.13 ± 0.003^{e}	5.44 ± 0.01^{b}	15.32 ± 0.04^{a}
9	2357	4.28 ± 0.01^{b}	1.13 ± 0.003^{e}	5.38 ± 0.01^{c}	15.06 ± 0.05^{b}
10	3060	4.20 ± 0.01^{c}	1.17 ± 0.004^{c}	5.46 ± 0.01^{b}	15.10 ± 0.05^{b}
11	1375	4.38 ± 0.01^{a}	1.10 ± 0.003^{f}	5.51 ± 0.01^{a}	15.09 ± 0.05^{b}
12	1514	4.09 ± 0.01^{d}	1.15 ± 0.004^{d}	5.27 ± 0.02^{d}	14.37 ± 0.06^{c}

Values in the same column with different letters are statistically different ($P < 0.05$). *n*: numbers of spermatozoa analysed in each week.

The global size of head spermatozoa in multivariate analysis was calculated considering four parameters (length, width, area and perimeter). On the basis of an eigenvalue-one criterion, the two most significant components (1 and 2, with eigenvalue of 1.98 and 1.14, respectively) were estimated. They explain 78% of variance (Table 4.3). The component 1 correlates well with length (0.79), area (0.84) and perimeter (0.80) and component 2 with width (0.97) and area (0.42).

Table 4.3. Principal components existing on the head spermatozoa morphology obtained by hCG treatment: influences of the different parameters and its correlations (inside parenthesis) on the two components.

	Components (correlations)	
	1	**2**
Head length	0.56 (0.79)	−0.13 (−0.14)
Head width	−0.11 (−0.15)	0.90 (0.97)
Area	0.60 (0.84)	0.39 (0.42)
Perimeter	0.57 (0.80)	−0.11 (−0.12)
Percentage of variability explained	49.7	28.7

In addition, the percentage of variability explained by each component is shown.

The week effect of hCG treatment on spermatozoa morphometry components obtained by the Principal Component Analysis is shown in Fig. 2, where the comparison of least square means on the levels of the factor *week* is graphically shown as HSD intervals of Tukey. Fig. 2A shows a significant increase in spermatozoa size (component 1) between the weeks 5 and 8 of hCG treatment; from then on, size showed minor but significant changes until week 11, and a significant decrease during week 12. Fig. 4.2B shows a high increase in the spermatozoa width (component 2) from the weeks 5 to 7 of hCG treatment. From the 8th week, changes in width were lower, but significant and without clear tendencies.

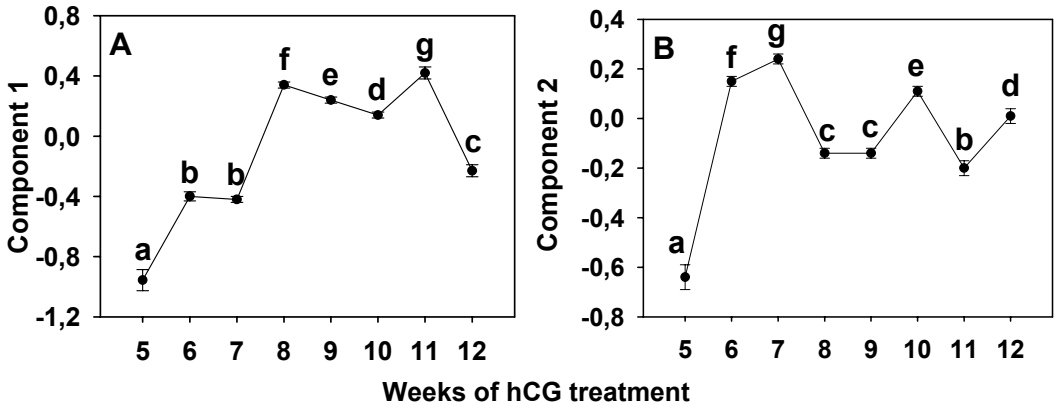

Figure 4.2. Evolution of the different spermatozoa morphometry components, obtained by the Principal Component Analysis, between 5th and 12th weeks of hCG treatment. (A) Spermatozoa size or component 1 and (B) spermatozoa width or component 2. Results are shown as 95% HSD intervals over the means. Different letters indicate significant differences.

Discussion

It has been reported that repeated hCG (1.5 IU g fish^{-1}) administration induced spermiation in farmed European eels [2]. Sperm sample evaluation started at the 5th week of treatment, coinciding with the onset of milt production. The changes in milt volume, concentration and motility from the 5th week until the end of hCG treatment were similar to those observed by other authors [1-3,8,9]. Pérez et al. [2] proved that the most suitable time to obtain sperm with the highest motility is the 9th week, while Müller et al. [9] reported no differences in the motility of sperm samples collected on the 8th, 9th and 10th weeks of treatment. Based on the present study, the 9th week seems the most suitable week of treatment to obtain sperm.

Traditionally, methods of estimating fish sperm quality have been sperm motility and concentration [21]. Other attributes, such as cell viability and mitochondrial function, have also been chosen as indicators to evaluate fish sperm quality [17,19-21]. Fluorescent staining has recently been shown to provide rapid assessment of the integrity of membranes and mitochondrial function in fish sperm [17]. In the present study, the proportion of dead cells

(around 37%) observed after staining with Hoechst 33258 was significantly lower from the weeks 8 to 11 of hCG treatment. These data correspond with the best seminal quality (higher percentage of motile cells and higher concentration), as shown in Table 1.

Previous studies have demonstrated a positive correlation between functional mitochondria and sperm motility [39,40]. However, in the present study, the percentage of mitochondrial functionality determined by JC-1 staining did not show a similar pattern to that obtained with Hoechst 33258. A low variability was observed throughout the weeks of treatment, varying between the best week (8th) and the worst (5th) by <20%. In the monomeric state, JC-1 stains green, and, similarly to rhodamine 123, JC-1 is transported into the interior of functioning mitochondria. However, as the concentration of JC-1 inside the mitochondria increases, the stain forms aggregates which fluoresce orange [41,42]. It is possible that the fluorochrome stained less cells because the conditions were sub-optimal, since the conditions used in the present study were validated in mammal studies [41,42].

In the present study, the effect of hCG treatment on spermatozoa morphometry was analysed with the use of automated sperm morphology analysis (ASMA). This technique has been used previously in eels [11,12]. The eel spermatozoa head is gently curved and elongated with a hook-shaped upper end in a crescent [33]. The head shape is asymmetric along the longitudinal axis. This unusual elongation of the sperm head in eels and other elopomorph fish is a problem for the ASMA system, causing a certain error in the length measurements but not in the rest of morphometry parameters [12]. In fact, results obtained in the present study coincide with the range of measurements of European eel spermatozoa head width (1.0 ± 0.2 μm) reported by Okamura et al. [33], but are relatively lower than head length (5.4 ± 0.4 μm) described by these authors, who measured 20–30 spermatozoa using scanning electron microscopy techniques.

The growth sequence of the spermatozoa head in response to the hormonal maturation treatment showed variation for all parameters analysed (length, width, area and perimeter). All of them showed lowest values on week 5th of hCG treatment and these results agree with the first weeks of spermiation [2] and therefore with immature sperm. From the 5th week, different changes

were observed for each of the parameters. To obtain a better approach to global head size of the spermatozoa, a multivariate analysis method was used. The use of multivariate analysis methods (discriminant analysis, cluster analysis) to define spermatozoa clusters or classes with different morphology has been used in humans [43], monkeys [44] and horses [45]. In fact, descriptive parameters shown in Table 2 suggest some changes in the spermatozoa morphology through the hormonal treatment, but the analysis of isolated parameters is not powerful because it does not consider the correlations between them. However, the ANOVA on the scores, generated by the Principal Component Analysis (Table 3), allows a whole interpretation of the changes in the spermatozoan morphology and subsequently a more informative comparison between different weeks of treatment. In the present study, the Principal Component Analysis established two components (Kaiser criterion) with an accumulated percentage of variability explained of 78.4% (49.7% and 28.7%, for each component, respectively). The first component can be considered as a general size component and the second one distinguishes wide and narrow spermatozoa. Our results showed an increased effect on global head size up to 8th week of treatment, maintaining approximately these values until the 11th week, decreasing later. On the other hand, the head of the spermatozoa from the weeks 5 to 7 is thickened, decreasing somewhat from the weeks 7 to 8, the thickness staying more or less constant during the following treatment weeks.

One interesting aspect revealed by the use of Hoechst staining, and undetected with previous techniques, is the existence of around 37% dead spermatozoa, even in the weeks when best results were registered. This result could indicate a sub-optimal effect of the maturation hormonal treatment. Similarly, Matsubara et al. [46] studying different steroid levels caused by a weekly treatment in artificially matured female Japanese eels, concluded that weekly administrations could be inducing a sub-optimal release of steroids. On the other hand, this could be explained by a group-synchronous spawner character of this species, translated in sperm production in waves as has been previously described in other species such as the European sea bass (*Dicentrarchus labrax*) [47,48]. The forced weekly semen collection could be extracting spermatozoa from different batches or stages of development, which in a natural manner should be released in different waves of sperm production. If

this were the case, some of the extracted spermatozoa could be immature, and so unviable.

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5 EXPERIMENT 2

Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron microscopy

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Abstract

The aim of the present study was to characterise European eel spermatozoa morphometrically, as a basis for future studies on the morphological effects of methods for sperm cryopreservation and sperm quality. This characterisation was carried out measuring several spermatozoa morphology parameters (head length, width, area and perimeter) by scanning electron microscopy (SEM), in comparison with measurements developed in European eel spermatozoa with computer-assisted morphology analysis (ASMA).

Spermatozoa head morphology showed differences in width (1.15 ± 0.01 μm versus 1.12 ± 0.01 μm), perimeter (14.68 ± 0.13 μm versus 13.72 ± 0.19 μm) and area (5.36 ± 0.06 μm^2 versus 1.12 ± 0.01 μm^2) for ASMA and SEM, respectively. When head length was evaluated, significant differences were found, being higher for SEM methodology (5.09 ± 0.04 μm versus 4.29 ± 0.03 μm). The curved and elongated spermatozoa head in eels means a problem for the ASMA system (Sperm Class Analyser®, Morfo Version 1.1, Imagesp, Barcelona, Spain), causing an error in the length measurements. However, similar results were obtained by both techniques when spermatozoa head length was considered as the greater length between two points within the object (4.29 ± 0.03 μm versus 4.31 ± 0.04

μm for ASMA and SEM, respectively). In conclusion, this is one of the first applications of ASMA in fish and the first in this species, and confirms this system as a useful tool with wide applications in future fish spermatozoa studies. Width, perimeter and area could be used as parameters for the spermatozoa morphology evaluation, whereas the length requires a new programming of the Imagesp software.

Introduction

Previous reports have described the spermatozoa ultrastructure of European and Japanese eels and examined its morphology by transmission electron microscopy (TEM) [1-4] or scanning electron microscopy (SEM) [3] and [5]. Other techniques to examine and analyse fish spermatozoa head are laser light-scattering spectroscopy and stroboscopic illumination [6]. Results obtained with these techniques are subjective, time-consuming and highly variable. The search for methods of accurate, objective and repeatable assessment of sperm fertility still remains the aim of many studies. One of these developed computer-assisted applications is an automated system for spermatozoa head morphometry analysis (ASMA), developed and validated for mammals [7] and fish [6].

Abnormal spermatozoa head morphometry has been associated with reduced fertility in the bull, boar and stallion [6,8]. ASMA has increasingly been used with mammalian species, such as man [9], rat [10], rabbit [11], bull [12], dog [13], monkey [14] and alpaca [15]. ASMA measurements have shown toxic effects on human spermatozoa head [16] and the effect of mercuric chloride on goldfish sperm [6]. This technique has also been used in the field of cryopreservation, in which cryoprotectants or frozen-thawed protocols are known to cause morphological damage to the spermatozoa [17,18]. Kruger et al. [19] found that spermatozoa head morphometry, determined by ASMA, was predictive of in vitro fertilisation rates, and it has also been reported to result in detection of fertile and subfertile stallions [20] and rabbit [21]. ASMA systems have never been used in eel species.

ASMA has provided a series of objective parameters, which have facilitated the standardisation of morphological semen evaluation [22]. However, different problems have arisen, such as sample preparation, staining procedure and the

settings of the spermatozoa morphology analyser, which must be optimised for each species [9-11,23-25]. The computer-assisted morphometry analysis requires the standardisation of preparation, staining and sampling methods [9].
The main aim of the present study was to characterise the European eel spermatozoa morphometrically, comparing the results obtained by computer-assisted spermatozoa analysis and by scanning electron microscopy.

Materials and Methods

Fish and samplings

Thirty-six farmed eel males (body weight: 137.6 ± 21.43 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into our facilities. Fish were gradually acclimatised to sea water (salinity 37.0 ± 0.3 g/l; 20–22 C) for 10 days. Fish were distributed in four 96l aquaria and tagged with passive integrated transponders (P.I.T. tags) injected into the epaxial muscle for individual identification. Fish were fasted during the experiment.
Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU/g fish), as previously described by Pérez et al. [26].
Once a week during the spermiation period, from the 5th to 12th weeks of treatment, fish were sampled 24 h after the hormone administration. After cleaning the genital area with freshwater and thoroughly drying to avoid contamination of samples with faeces, urine and sea water, total expressible milt was collected by applying gentle abdominal pressure to anesthetised males (benzocaine; 60 mg/l). A small aquarium air pump was modified to obtain a vacuum breathing force and to collect the sperm. A new tube was used for every male and distilled water was used to clean the collecting pipette between different males. Samples were maintained at 4 C.

Samples evaluation by ASMA

A fraction of collected sperm samples was diluted 1:50 with a 2.5% glutaraldehyde in Dulbecco's phosphate buffered saline fixative solution prepared with DPBS (Sigma) and glutaraldehyde solution (Sigma). Slides were visualised using a 1000× negative phase contrast objective (Nikon Plan Fluor) on Eclipse

E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image. The morphological parameters were analysed using ASMA software (Sperm Class Analyser®, Morfo Version 1.1, Imagesp, Barcelona, Spain). The morphometric parameters determined were: head length (μm), width (μm) and perimeter (μm) and head area (μm^2).

Scanning electron microscopy

Sperm cells were separated from seminal plasma by centrifugation at 1800 g for 10 min at room temperature, pre-washed twice with 0.1 M phosphate buffer pH 7.2 and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer solution pH 7.2 for 1 h. The cells were washed twice with phosphate buffer and post-fixed in 1% osmium tetraoxide for 1 h.

After fixation, the samples were washed twice with phosphate buffer, dehydrated through a graded series of alcohol and amyl acetate, and finally critical point-dried in liquid CO_2. The dried samples were mounted on aluminium studs by means of conductive silver paint and then coated with gold–palladium.

All specimens were examined and photographed in a JEOL JSM 5 410 microscopy operated at 15 kV. Spermatozoa head length, width, perimeter and area were determined using a public domain ImageJ program (developed at U.S. National Institutes of Health and available at www.rsb.info.nih.gov/ij/).

Experimental design

To examine the spermatozoa head morphometry with ASMA system, 203 individual ejaculates, from 36 males, were analysed during 8 weeks. Approximately 75 spermatozoa were analysed in each sample.

To examine the spermatozoa head morphometry from pictures obtained with scanning electron microscopy (SEM), 1 pool from 10 males, to avoid individual differences, was analysed using ImageJ program. A total of 100 spermatozoa head measurements were taken.

Statistical analysis

The mean morphometry measurements for length, width, area and perimeter were compared by analysis of variance (General linea r model, GLM). The results are presented as least square means (LSM) ± standard error of the

means (S.E.M.). Significance level was set at $P < 0.05$. All statistical procedures were run using Statgraphics Plus® 5.1 (Statistical Graphics Corp., Rockville, MO, USA) [27].

Results

A total of 100 spermatozoa were measured by SEM (Fig. 5.1) and 14,898 spermatozoa were measured using ASMA system (Fig. 5.2).

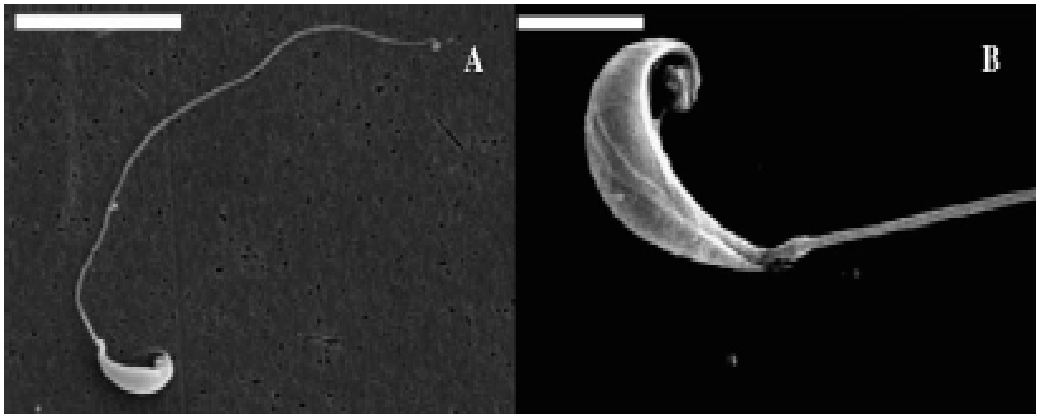

Figure 5.1. Scanning electron microscopy of European eel spermatozoa separated from seminal plasma by centrifugation, fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetraoxide, dehydrated, critical point-dried in liquid CO_2 and coated with gold–palladium. Scale bars: 10 (A) and 2 μm (B).

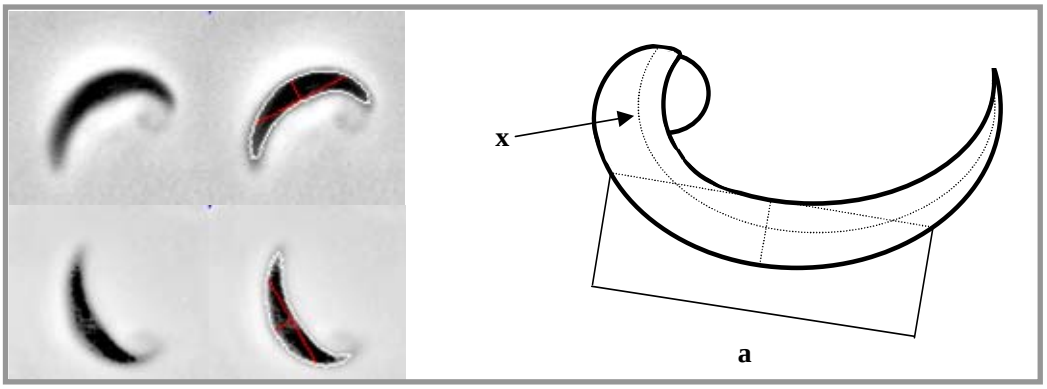

Figure 5.2. Real pictures obtained by phase contrast optic (1000×) and schematic drawing showing how the Sperm Class Analyser®, Morfo Version 1.1 (Imagesp, Barcelona, Spain) measures different morphology parameters of European eel spermatozoa. a= Measured length by ASMA; x = real length of the cell.

Differences were found between spermatozoa head length, width, perimeter and area determined by ASMA and SEM system (Table 5.1, $P < 0.05$).

Table 5.1. Spermatozoa morphological parameters: head length (μm), width (μm), perimeter (μm), and head area (μm^2), considering spermatozoa measured by computer-assisted morphology analysis (ASMA) or scanning electron microscopy (SEM) and analysed by Image J.

	Length (μm)	**Width (μm)**	**Perimeter (μm)**	**Area (μm^2)**
ASMA	4.29 ± 0.03^b	1.15 ± 0.01^a	14.68 ± 0.13^a	5.36 ± 0.06^a
SEM	5.09 ± 0.04^a	1.12 ± 0.01^b	13.72 ± 0.19^b	5.05 ± 0.01^b
P-value	0.000	0.031	0.002	0.000

Different superscript letters (a and b) indicate significant differences between values obtained by ASMA or SEM.

Similar results were obtained for head length for both techniques when length was considered as the greater length between two points within the object (4.29 ± 0.03 μm versus 4.31 ± 0.04 μm for ASMA and SEM, respectively; Fig. 5.2).

Discussion

Previous studies reported that when spermatozoa morphology is analysed by visual methods, the intra- and inter-observer laboratory variations are usually very large [30]. The ASMA systems require standardisation of methods and variables, but under these conditions their repeatability and validity are much higher than any subjective morphological evaluation [31,32].

ASMA system has been used previously in fish, but merely to show the effect of mercuric chloride on goldfish spermatozoa morphology [6]. Eel spermatozoa morphological characteristics have been described to date by transmission electron microscopy (TEM) or SEM observations [1-5]. Currently, image analysis systems have been developed that are easy to use and allow

evaluation of a great number of spermatozoa, for example, in this study 14,898 were measured. In the present study, European eel spermatozoa morphometry was analysed with the use of ASMA software on images captured by phase contrast optic or by SEM, in order to standardise the evaluation of spermatozoa head morphology in this species. The main findings emerging from this study were: (i) that spermatozoa head morphology evaluated by ASMA resulted in a lower length and higher width, perimeter and area than measurements determined by SEM, (ii) software Imagesp cannot detect the curved and elongated spermatozoa head form, causing an error in the length measurements, although not in the other morphological parameters.

The results obtained with SEM are in good agreement with previous studies, in which 5.4 ± 0.4 and 1.0 ± 0.2 μm and 5.6 ± 0.5 and 0.9 ± 0.3 μm were reported for head length and width, respectively [5,33]. Eel spermatozoa head perimeter and area have been measured in the present study for the first time. However, significant differences were observed between SEM and ASMA methodologies. Spermatozoa morphology assessment is influenced by numerous factors, such as semen preparation, magnification level of the objectives, number of evaluated spermatozoa, or the fixation and staining technique [7,20]. Every factor appeared to be species specific [7] and play an important role in the optimal utilisation of computer-aided spermatozoa morphometry systems [25]. Boersma et al. [25] suggested that stained spermatozoa heads produce better contrast in the recognition and digitisation of spermatozoa. However, other works indicate that the stains do not necessarily provide the appropriate grey-level contrast for accurate morphometric analysis [35]. To stain, the samples are placed on a slide and dried in air before fixation, but it may be possible that air dried samples will shrink, flatten or collapse under these conditions [36]. On the other hand, fixation techniques containing aldehydes result in a low coefficient of variation and a high number of acceptable and correctly delineated heads [22]. Thus, in the present study, samples were directly fixed with 2.5% glutaraldehyde solution in Dulbecco's phosphate buffered saline (300 mOsm/kg). The higher measurements reported by ASMA could be explained at least in part because the SEM samples were fixed and dehydrated through a graded ethanol series and finally critical point-dried in liquid CO_2, and perhaps this preparation process provokes a reduction in spermatozoa head size. The different spermatozoa head

length measurement obtained between ASMA and SEM can be explained by the particular aspect of the head of eel spermatozoa, very different to livestock production animals. Fish sperm differs in many aspects from that of mammals [34], and the ASMA methodology used for livestock production animals is not directly applicable to fish [6]. The eel spermatozoa head is gently curved and elongated, with a hook-shaped upper end, which is directed inside in a crescent [5] (Fig. 1). The shape of the head is asymmetric along the longitudinal axis. This elongation of the spermatozoa head in eels and other elopomorph fish means a problem for the Imagesp ASMA system, causing an error in the length measurements (Fig. 2). Once the software captures one spermatozoa, the image is digitised and it is transformed into a variable dummy, 1 or 0 if the pixels are black or white in colour, respectively. The Imagesp software starts the image analysis, determining the maximum length of the pixel with value 1 and drawing a line at this point (*a* in Fig. 2). However, the curvature of this kind of spermatozoa is not recognised and, therefore, the software is not measuring the real length (*x* in Fig. 2), but measures the maximum length determined by the amplitude of curvature of spermatozoa head (*a* in Fig. 2). When this same criterion was applied for measuring the pictures obtained by means of electron microscopy, similar results of length were obtained; 4.31 ± 0.04 μm versus 4.29 ± 0.04 μm, for SEM and ASMA, respectively. Similar spermatozoa curved shapes have been reported in species such as rat [37], making its spermatozoa characterisation also difficult. New developments of Imagesp software are required, and different possibilities are present: (i) to obtain a skeleton of the figure by means of lines parallel to the width within the figure and drawing up to a line between the midpoints (ii) or by means of geodesic ratios, as after the digitalisation of the image, the coordinates are known for each point, and it would be possible to obtain the greatest longitude between two points of the object on the inside passing through the midpoint of the straight line that defines the width (*x* in Fig. 2). Until now, it has not been clear which parameter better defines the head morphometry and, therefore, the fact of not having the use of the length available does not invalidate the use of this software, since the rest of the parameters, width, area and perimeter, can be used.

In conclusion, width, perimeter and area could be used as methods of spermatozoa morphology evaluation, whereas the length requires a new

programming of the Imagesp software. This characterisation of the spermatozoa morphology is one of the first applications of ASMA methodology in fish and the first one in eel species. It confirms this system as a useful tool, mainly in terms of time-saving, higher accuracy and the reduced equipment required in comparison with electron microscopy techniques, with wide applications in future studies of fish spermatozoa membrane physiological response under the compared effects of extenders, cryoprotectants, additives, etc. Additional experiments are required in order to understand the correlation between usual sperm evaluation methods and spermatozoa head morphometry.

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6 EXPERIMENT 3

Molecular and physiological study of the artificial maturation process in the European eel males: from brain to testis

Under preparation

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Abstract

European eel males can be artificially matured (1.5 IU hCG/g fish), but the regulatory mechanisms of their reproductive development are practically unknown. The present study evaluated the expression of GnRHs (mGnRH and cGnRH) and gonadotropin expression (FSHβ and LHβ) during the hormonal treatment. Moreover, 11-ketotestosterone (11KT) levels, testis development, morphology parameters [eye index (EI), gonadosomatic index (GSI), hepatosomatic index (HI)] and sperm quality (motility, viability and head spermatozoa morphometry) were analyzed. Differences in the GnRHs expression were found, considering the mGnRH as responsible of gonadotropin release. One injection was enough to decrease dramatically the FSHβ expression, being close to zero during the rest of the treatment. LHβ expression registered two important peaks, in the 3rd and 7th weeks. These LHβ peaks could be correlated with two important changes in the gonad, first of them coinciding with the late meiosis-spermiogenesis process and the second with the sperm maturation. The 11-KT increased with GSI, and the highest 11-KT values coincided with the later steps of spermartogenesis previous to spermiation. So, it can be considered that this androgen played an important role in the spermatogonia proliferation and in the last phases of spermatogenesis. Furthermore, 11-KT has influence in the changes of morphologic characters, presenting high values when EI and GSI

increased. Sperm production was obtained from the 4th week of treatment, but it was in the 8th week when a significant increase was observed in the sperm quality [viability, high motility (>75%)].

Introduction

The European eel, *Anguilla anguilla*, is a teleost fish with a peculiar life cycle. Prepubertal silver eels migrate 4-5000 km from European coastal waters to their supposed spawning grounds in the Sargasso Sea. If prevented from this oceanic migration, the silver eel will remain physiologically blocked in a prepubertal stage [1-5]. This makes the eel a powerful model organism for investigating the regulatory mechanisms of reproductive development. Furthermore, the European eel belongs to an early branching group of teleosts (Elopomorphs), and the knowledge of the reproductive development in this species may provide information on regulatory mechanisms in other teleost species as well as vertebrates in general.

In mammals, control of reproduction by the brain-pituitary-gonad (BPG) axis is well established. A hypothalamic decapeptide, the gonadotropin-releasing hormone (GnRH), is the physiologic stimulator of gonadotropin release from the pituitary gland. Two different types were described in Japanese eel (*Anguilla japonica*), mGnRH and cGnRH-II, finding mGnRH expression in large number body tissues, while the cGnRH-II expression appeared in olfactory epithelium, brain, pituitary and gonad [6-8]. Their distribution in the brain was studied in European eel [9,10], registering higher cGnRH-II peptide concentration in di/mesencephalon and in the posterior part of the brain (medulla oblongata, corpus cerebellum), while for mGnRH peptide it was in the olfactory bulbs, telecephalon, di/mesencephalon and pituitary. The gonadotropin stimulating effect of this neuropeptid in European eel was demonstrated with injections of GnRH-analog, but due to dopamine inhibition it was necessary to add a dopamine (DA) antagonist for their release [1-5]. In fact, a triple treatment using 17β-estradiol (E_2), GnRH analogues and dopamine antagonists were necessary to trigger endogenous production and release of gonadotropins [5]. However, in males a double treatment (E_2 and GnRH injection) was enough to induce a low gonadotropin release [11]. This slight effect of GnRH, without DA antagonist, suggests a less effective dopaminergic inhibition in male than in female eel.

Unlike eel females, where the gonadotropin expression has been deeply analyzed [1,3,5,12-14], in male European eel the gonadotropin expression has never been studied during the gonadal development. The gonadotropin release induces the gonad development, being the gonad their principal target tissue [15-17]. The gonadal development has been studied in eel males, and consists in the spermatogenesis process, which is divided in the next stages: spermatogonial stem-cell renewal, spermatogonial proliferation, spermiogenesis and sperm maturation [15,16,18-21]. Under culture conditions, Japanese and European eel males have immature testes containing only type A and early-type B spermatogonia [18,22-26]. To continue the spermatogenesis, it has been reported in both species that it is necessary to administrate a hormonal treatment with human chorionic gonadotropin, due to these species do not mature in captivity [22,23,27-38]. This treatment promotes an increase of 11-ketotestosterone (11-KT) levels in the plasma, which is considered as the major androgen in the eel male [22,23,26]. This androgen is produced by the Leydig cells, being its role the induction of the spermatogenesis through of the Sertoli cells [18,20,21]. As a consequence of this stimulation, the Sertoli cells produce growth factors, such as insulin-like growth factor-I (IGF-I) and activin B, which are the responsible factors of spermatogonia mitosis [15,16].

As a result of the hormonal treatment the spermatogenesis takes place and the spermiation begins. The European eel sperm shows a high density (3-6x10^9 spermatozoa/ml [28]) and the time of spermatozoa motility is very short after activation, from a few seconds to a few minutes [39] making difficult the evaluation of sperm motility. Fish sperm quality has traditionally been estimated by subjective evaluation of sperm motility and concentration, but during recent years an intense advance has occurred in the techniques for the objective evaluation of sperm quality. This has been achieved by the study of motility parameters by CASA (*computer assisted sperm analysis*) systems [30,40,41], spermatozoa morphometry analysis using ASMA (*automated sperm morphometry analysis*; [32,37,42,43] or using fluorescent staining methods to evaluate membrane functionality, determining on this way the percentage of viable spermatozoa [33,37,38]. Sperm quality is a measure of the ability of sperm to fertilise an egg successfully, but this capacity may not be reliable in this

species, due to the reduced number of good spawns obtained from artificially matured European eel females.

A physiological description of the male maturation process during the hormonal treatment has not been yet made in the European eel. This is the first study in which GnRH and gonadotropin expression are related with the most important reproductive parameters. Our main purpose has been to carry out a wide description of the European eel male gonad maturation to understand how this process is controlled by hormonal mechanisms, and which are the hormones effects on the sperm quality. This work not only could improve the knowledge of the control mechanisms of male sexual maturation in the European eel, as well as contribute to increase the knowledge in teleost male physiology.

Materials and Methods

Fish and samplings

One hundred and fifty eel males (124.1 ± 12.6 g body weight) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into our facilities. Fish were gradually acclimatized to sea water (salinity 37 ± 0.3 g/l; 20ºC) for one week. Fish were equally distributed in three tanks of 500-L. Males were hormonally treated for the induction of maturation and spermation with weekly intraperitoneal injections of hCG (1.5 IU g/fish; Angelini Farma-Lepori, Barcelona, Spain), as previously was described by Pérez [28].

Once a week during all experiment (13 weeks), 10 males were sacrificed one day after injection obtaining morphometric parameters as gonadosomatic index (GSI), hepatosomatic index (HIS) and eye index (EI, [27]), and samples of blood, brain and pituitary. The brain was divided in different parts for its study: olfactory bulb, telencephalon, mesencephalon and diencephalon, cerebellum and medulla oblongata, but only the first 3 parts were used in this study. Each part was stored in 0.5 ml of RNA later (Ambion Inc, Huntingdon, UK) at -20ºC until extraction. The blood was centrifuged at 3000 r.p.m. during 5 minutes, and the blood plasma was stored at -80ºC until analysis. Moreover, sperm samples were taken during the spermiation weeks, analyzing the spermatozoa motility and the percentage of live cells before sacrificing the fish. The sperm was obtained by applying gentle abdominal pressure to anesthetised males (benzocaine; 60

mg/L), after cleaning the genital area with freshwater and thoroughly drying to avoid contamination of samples with faeces, urine and sea water.

Evaluation of motility

Immediately after collection, the motility of sperm samples was assessed by mixing one drop of sperm with 3 μl of artificial sea water [in Mm: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8] adjusted to 1000 mOsm/kg as activation media. All the sample analyses were performed by the same trained observer to avoid subjective differences in the motility evaluation.

Fluorescence stain analysis

Live/Dead Sperm Viability Kit (SYBR/Propidium Iodide (PI) of Invitrogen (Barcelona, Spain) was used to evaluate the viability of spermatozoa pre- and post-freezing. Sperm diluted in P1 medium ([30]; 1:350) was mixed with SYBR Green and PI, and maintained during 20 minutes in dark incubation at room temperature. The final SYBR Green concentration was 10^4 times diluted from the original stock (2 μM) and PI 10^3 times from original stock (24 μM). At least 100 spermatozoa per sample were assessed in a Nikon Eclipse (E-400) epifluorescence microscope, using UV-2A (EX: 330-380 nm, DM: 400, BA: 420) filter. Spermatozoa were classified as *dead* when nuclei showed red fluorescence over sperm head and *live* when they showed green fluorescence.

Spermatozoa morphology analysis

A fraction of sperm samples was diluted 1:50 (v/v) with 2.5% glutaraldehyde in phosphate buffered saline fixative solution. Slides were viewed using a 1000X negative phase contrast objective (Nikon Plan Fluor) on an Eclipse E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer®, Morfo Version 1.1, Imagesp, Barcelona, Spain). Approximately 100 spermatozoa from each sample were analysed in each sample. The morphological parameters determined were: head perimeter (μm) and head area (μm^2).

RNA extraction and cDNA synthesis

Total RNA was extracted from the different parts (5-50 mg) of the brain (olfactory bulb, telencephalon, mesencephalon-diencephalon) and the pituitary, using traditional phenol/chloroform extraction by the Trizol reagent (Invitrogen, Belgium), followed by a bath water during 20 minutes at 37ºC. Later, one step of deoxyribonuclease treatment (gDNA Wipeout Buffer, Qiagen) was performed, being used a total volume of 14 µl for 2 µg of total RNA as template. This 14 µl were used as template to synthesize the first-strand cDNA in a total volume of 20 µl reactions. The protocol was carried out according to the manufacturer's instructions.

Primers and reference gene

Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC ACG; ARPrv: ACA TCG CTC AAG ACT TCA ATG G [44,45] was used as reference gene in the quantitative real time Reverse Trancriptase- Polymerase chain reaction (qrtRT-PCR). It was used as house kepping because its mRNA expression does not vary with experimental treatment. The gene specific primers assigned to evaluate the pituitary gonadotropin expression were: FSHfw: TCT CGC CAA CAT CTC CAT C; FSHrv: TAG CTT GGG TCC TTG GTG ATG and LHfw: TCA CCT CCT TGT TTC TGC TG; LHrv: TAG CTT GGG TCC TTG GTG ATG [44].

The study of the GnRHs expression were localized in olfactory bulbs, telencephalon, diencephalon-mesencephalon for mGnRH, using the next specific primers: mGnRHfw, ACT GGT GTG TCA GGG ATG CT; mGnRHrv, TGC AGC TCC TCT ATA ATA TCT TGC [46], while the cGnRH-II expression was analyzed in diencephalon-mesencephalon (cGnRH-IIfr, CTG ACA TCC ACA CAG CGA CT; cGnRH-IIrv, GGT GTT CAC CAT CAC AGC TAA A [46]).

SYBR Green assay (qrtRT-PCR)

In order to monitor gene expression of gonadotropins (FSHβ, LHβ) and GnRHs (mGnRH, cGnRH-II), quantitative real-time RT-PCR analyses were performed using a Light Cycler system with SYBR Green I sequence-unspecific

detection (Roche, Meylan, France). After an initial *Taq* activation of polymerase at 95ºC for 10 minutes, 41 cycles of PCR were performed using the LightCycler with the following cycling conditions: 95ºC for 15 s, 60ºC for 5 s and 72ºC for 10 s in the study of gonadotropins. In the case of GnRHs, 41 cycles of PCR were made with this cycle: 95ºC for 10 s, 60ºC for 10 s and 72ºC for 13 s. After the PCR, the machine performed a melting curve analysis by slowly (0.1 ºC/s) increasing the temperature from 68 to 95ºC, with a continuous registration of changes in fluorescent emission intensity.

The total volume for every PCR was 10 µl, performed from diluted cDNA template (4 µl), forward and reverse primers (0.5 pm each) and SYBR Green Master Mix (2 µl).

Gonad histology

After fixation in 10% buffer formalin (pH 7.4), the gonad samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were made with a manual microtome Shandom Hypercut, and stained with haematoxylin and eosin. Slides were observed with Nikon Eclipse E400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope.

Stages of spermatogenesis were determinated according to the most advanced germ cell types and their relative abundance [47,48]: stage I was determined by the presence of spermatogonia type A and/or B; stage II, by the presence of spermatogonia and spermatocytes; stage III, appearance of spermatids; stage IV, by the appearance of spermatozoa in small lumen; stage V (maturation stage) increase in the number of spermatozoa, as well as in lumen size; and stage VI, characterized by dominance of spermatozoa, with a low proportion of the rest of other germ cells, as well as luminal fusion.

Immunoenzymatic assays (ELISA) for sexual steroid (11-KT)

The teleost-specific androgen 11-ketotestosterone (11-KT) was measured in blood plasma using the 11-KT EIA kit from Cayman Chemicals (distributed by Scharlab S.L., Barcelona, Spain) according to the instructions of the manufacture.

Statistical analysis

The variables were subjected to analysis of variance (General Lineal Model, GLM). A Student-Newman-Keuls tests were used for the comparisons between means at a 0.05 significancy level ($p<0.05$). The results are presented as least square means (LSM) ± standard error of the means (SEM). All statistical procedures were run using Statgraphics Plus® 5.1 [49].

Results

GnRHs and gonadotropins variations in eel brain

GnRHs expression was analyzed in different parts of the brain, for the following weeks of the treatment: 0, 2nd, 3rd, 4th, 6th, 7th, 9th and 12th . mGnRH expression was studied in olfactory bulb, telencephalon, and diencephalon-mesencephalon, and cGnRH expression was studied in diencephalon-mesencephalon. At the start of the experiment brain samples (except pituitary) were not taken, and a new batch of males were injected 2 months later in order to have brains from males without treatment (0 week), as well as treated with 1 and 2 hCG injections. This data have not been included in the statistic analyses, but results are showed in the figures to understand better the possible evolution of this hormone during the treatment.

GnRHs expression did not show differences in olfactory bulbs and telencephalon, in contrast of di-/mesencephalon. The maximum mGnRH expression was observed in the third week, followed by significantly lower values in the next weeks (Fig. 6.1B). The expression of cGnRH-II in diencephalon-mesencephalon did not show significant differences in the studied weeks, except during the 4th week when a reduction was observed (Fig. 6.1B).

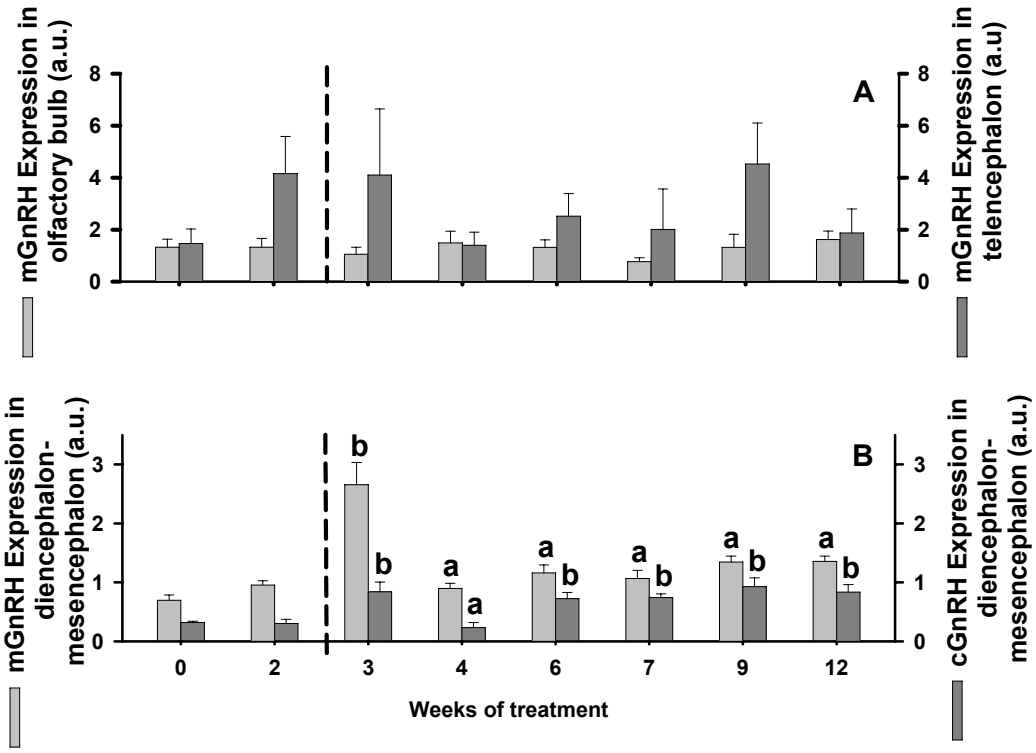

Figure 6.1. GnRHs expression in the different parts of the brain. mGnRH expression in olfactory bulb and telencephalon (A). cGnRH-II and mGnRH expression in mes/di-encephalon (B). Different letters means significant differences ($p<0.05$).

As a consequence of hormone injections, the gonadotropins expression changed through the treatment. The highest level of FSHβ subunit was registered before the first injection of hCG (Fig. 6.2). With only one injection, the expression decreased 50 times, and at the 4th week of treatment it was close to zero. LHβ subunit showed different results: the expression increased progressively during the 7 first weeks, with a special increase at 3rd and resulting significant at 7th week. In the subsequent weeks the LHβ did not show a clear pattern of variation (Fig. 6.2).

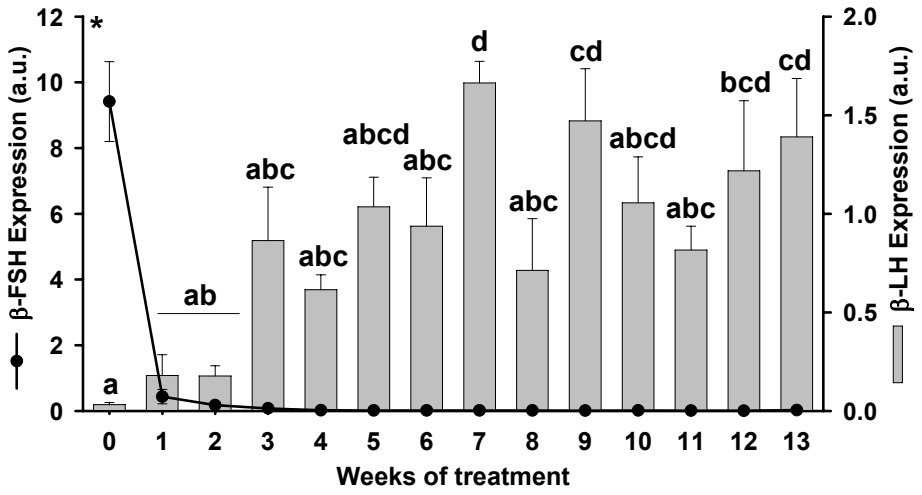

Figure 6.2. Gonadotropin expression in the pituitary (FSHβ and LHβ subunit), during the treatment. Asterisks or different letters means significant differences ($p<0.05$).

Analysis of morphometric parameters and 11-ketotestosterone

Before the hormonal treatment, eels showed a gonadosomatic index of 0.29 ± 0.13%. Four injections were necessary to obtain a significant increase (3.96 ± 0.87%), but the highest value (8.02 ± 1.04%; Fig. 6.3A) was found at the 6th week of treatment. No significant variations were observed during the following weeks (Fig. 6.3A).

A progressive increase of the hepatosomatic index was observed from the 2nd to the 8th weeks, obtaining a significant increase at 7th week (Fig. 6.3B), maintaining until 11th week when showed again a significant increase (Fig. 6.3B).

The eye index had a continuous increase until 6th week, but without significant differences until 5th week (Fig. 6.3C). After this peak, the values decreased until 8th week, being maintained without changes until 12th week, when showed a second peak (Fig. 6.3C).

The basal level of 11-ketotestosterone in blood plasma was 1.14 ± 0.5 ng/ml. After one injection of hCG the values were 4 times higher (4.7 ± 0.37 ng/ml), but the most important increase was found at 3th week of treatment, reaching 50 times the basal level (56.5 ± 7.27 ng/ml). From 4th week, there was a progressive

decrease until 8th week, and then similar lower levels were maintained during the rest of the treatment (Fig. 6.3D).

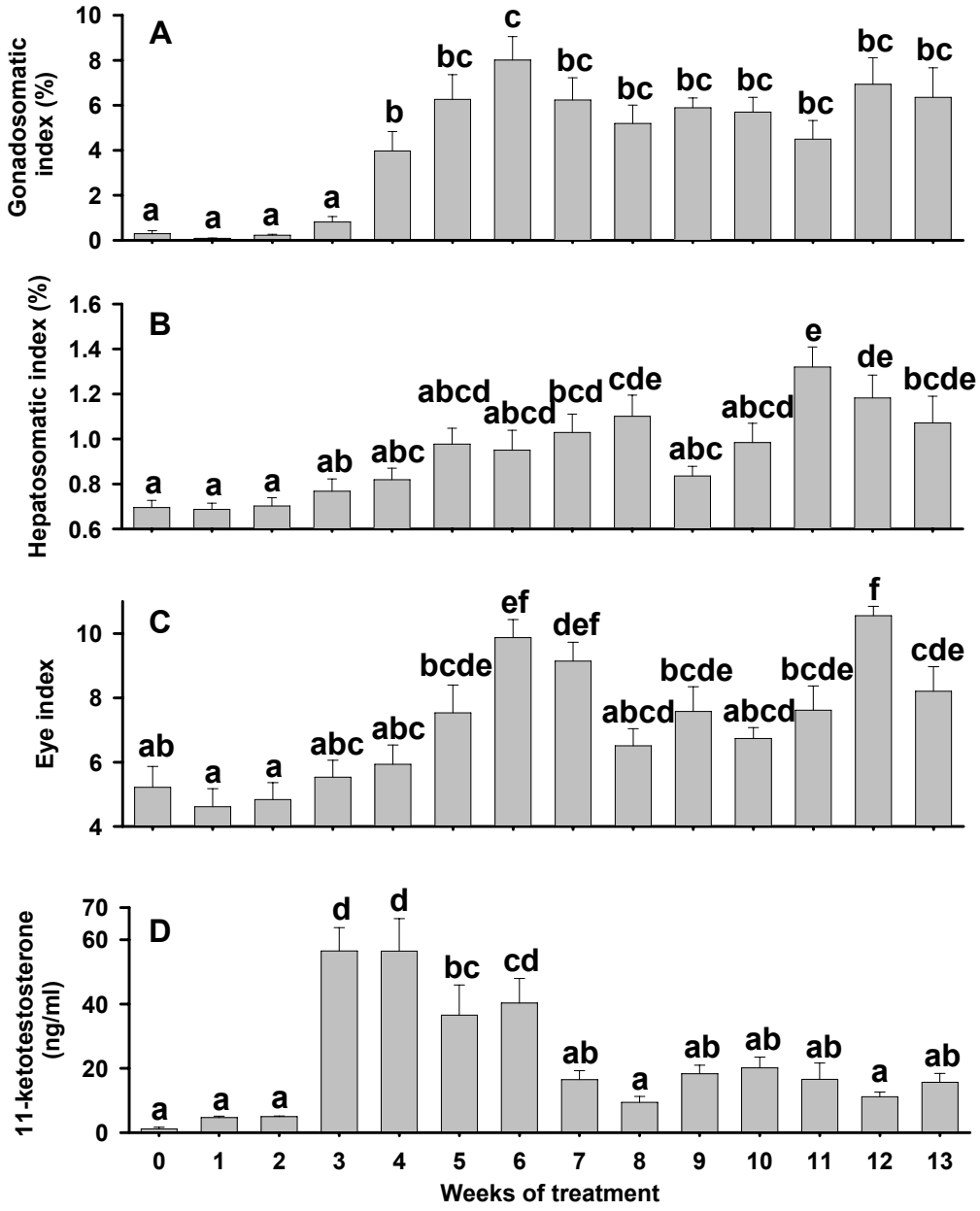

Figure 6.3. Morphometry parameters: gonadosomatic index (GSI, A), hepatosomatic index (HI, B), eye index (EI, C) and plasma 11-ketotestosterone (11KT, D) levels during the treatment.

Evaluation of sperm quality

The milt was obtained from the 4th week to the end of the experiment, but during the 8th week the first samples considered with high quality (≥75% motile cells) were found (Fig. 6.4A), and was reported a significant higher percentage of live cell (Fig. 6.4B).

From this week, the milt production was maintained approximately with the same conditions during the rest of the treatment in both parameters, except in the 11th week when the percentage of live cells showed a significant increase. The first week of spermiation (4th week) registered the lowest head size (data not showed), with a significant increase in the spermatozoa area in the following weeks, finding the highest results in the 10th and 11th. Significant improvements of the perimeter were reported at the 6th, 9th and 11th weeks.

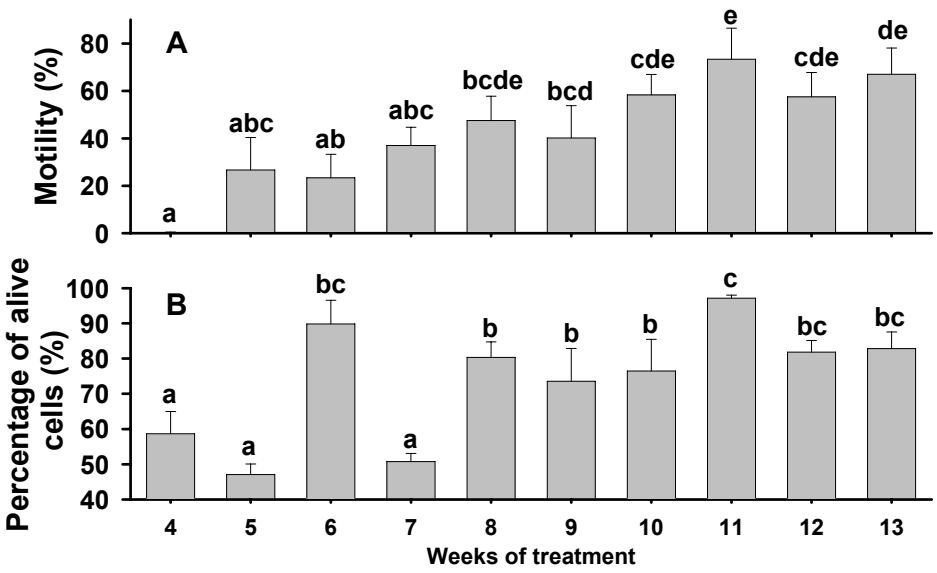

Figure 6.4. Evolution of the (A): motility and (B): percentage of live cells during the treatment.

Gonadal development during the treatment

Testis development in each male was classified in six different stages (S1-S6; Fig. 6.5). Stages S1 to S3 are characterized by the most avanced germinal cell present: spermatogonia (S1), spermatocytes (S2) or spermatids (S3). Stages S4 to S6 are differentiated by the abundance of spermatozoa respect with other germ cells.

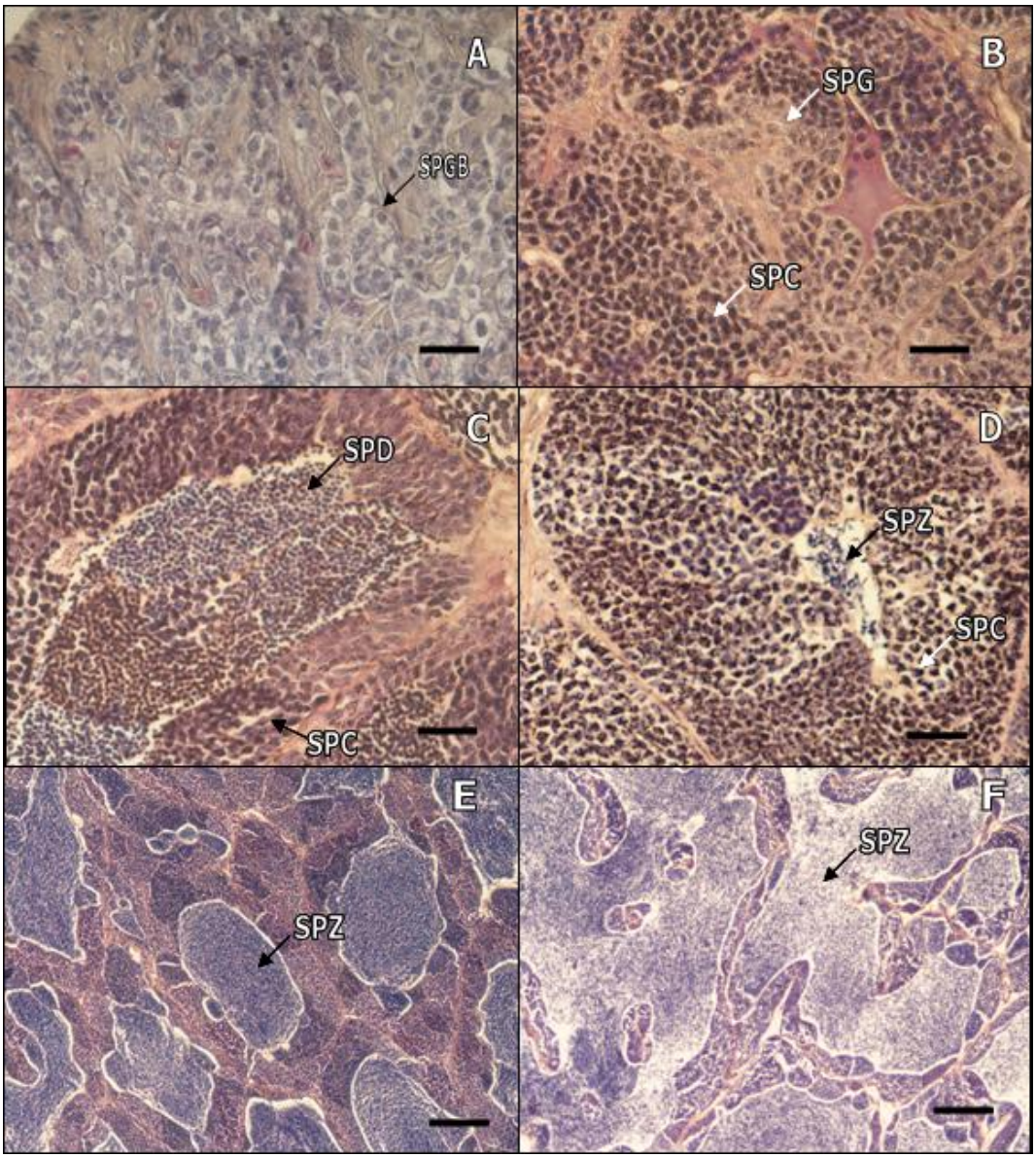

Figure 6.5. Photomicrographs of histological sections for the different stages found during the treatment. (A) Testis at stage 1, SPGA, spermatogonia-A, SPGB, spermatogonia-B; (B) Testis at stage 2, SPC, spermatocyte; (C) Testis at stage 3, SPD, spermatid; (D) Testis at stage 4, SPZ, spermatozoa; (E) Testis at stage 5; (F) Testis at stage 6. Scale bar, 100 μm (A-F).

S1 was present only during the two first weeks of the treatment, while S2 (spermatocyte appearance) was observed in males from 1st to 3th weeks (Fig. 6.6).

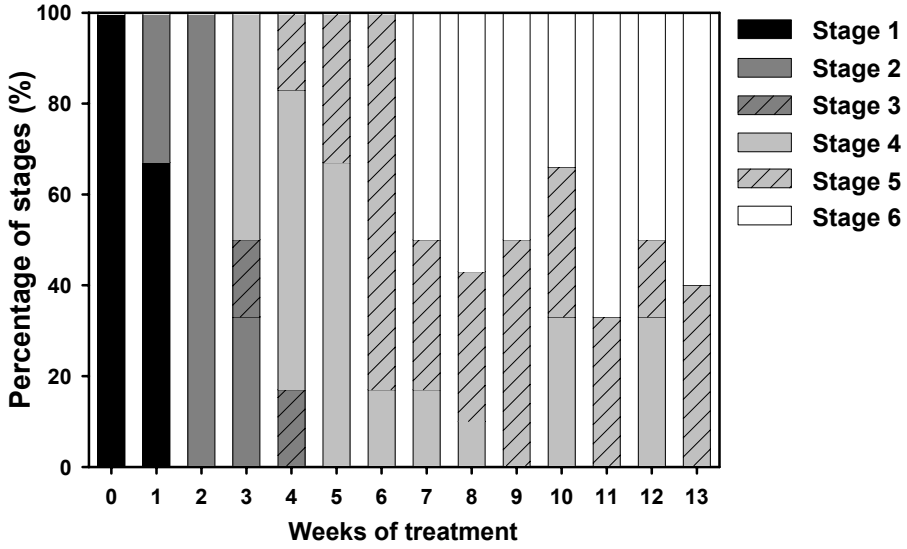

Figure 6.6. Percentage of the different stages of gonad development during the weeks of treatment.

The percentage of males showing stage S3, characterized by the appearance of spermatids without any spermatozoa present, was very low during 3rd and 4th weeks, probably, because this is a short-time stage in the process of cell development. For this reason, the stages 3 and 4 (characterized by the appearance of first spermatozoa) were analyzed together in the figure 6.7. In the 7th week, males in the maximum developmental stage (stage 6) were observed by first time, which continued present during the rest of the treatment.

These categories of gonad development were correlated with the evolution of the most relevant studied parameters. The expression of LHβ subunit (Fig. 6.7A) registered a significant increase at S3/4, coinciding with the late stages of meiosis and spermiogenesis. In the same moment of gonad development, a significant increase was observed for the morphometric parameters (GSI and EI, Fig. 6.7C and 6.7D, respectively). When the gonad was found in the most advances stages (S5, S6), LHβ expression and EI were maintained with high values, while the GSI showed a new statistically significant increase respect to the previous stage. The 11-KT levels registered a progressive increase until S3/4 (spermiogenesis), followed by a significant decrease coinciding with the stages S5 and S6 (spermiation period, Fig. 6.7B). Sperm was obtained from S4 to S6 stages, reporting higher quality sperm (motility and head size) in the stages S5 and S6. The stage 4 showed significantly lower values in

both parameters (Fig. 6.7E and 6.7F), increasing in the following stages (except in the perimeter head spermatozoa in the 6th stage).

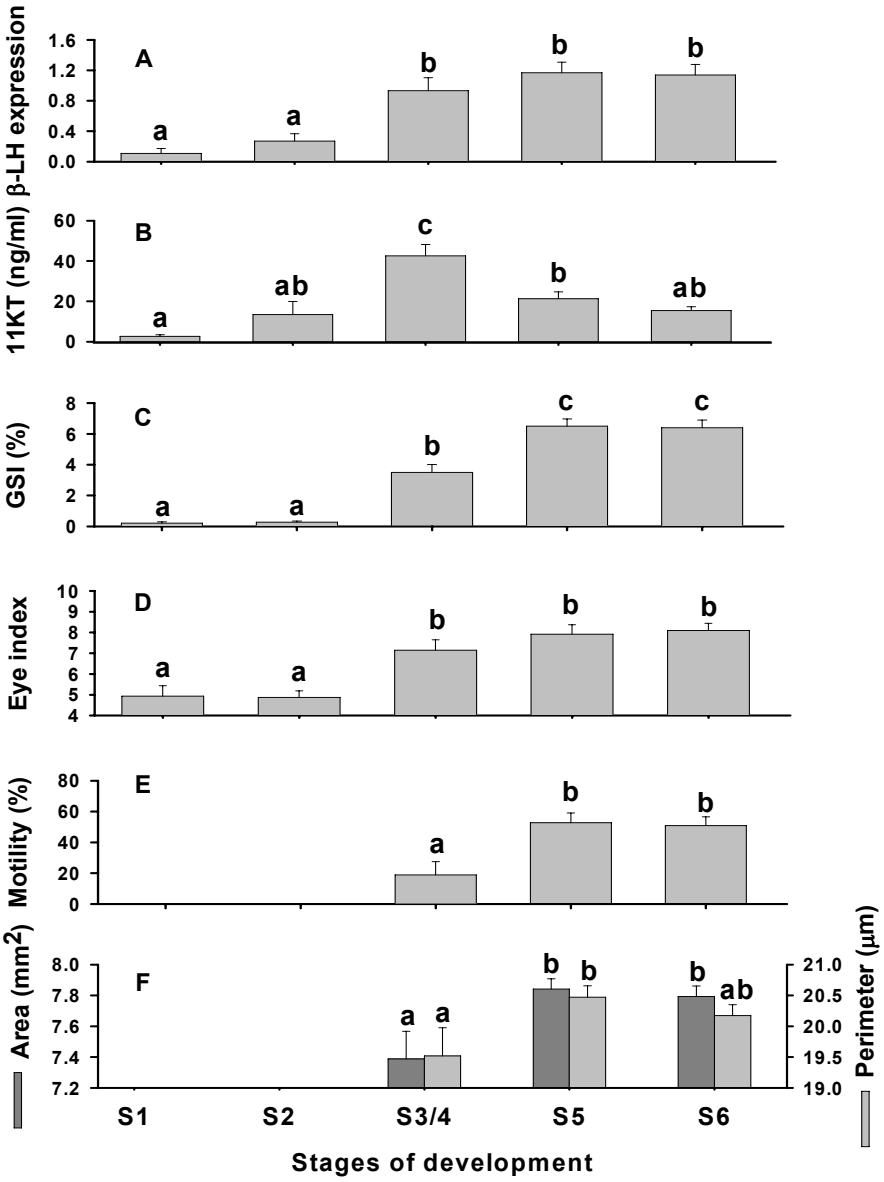

Figure 6.7. Evolution of different parameters studied in relation with the stage of testis development (A) Expression of LHβ subunit; (B) level of 11-ketotestosrone (11KT) in blood plasma; (C) gonadosomatic index (GSI); (D) eye index (EI); (E) motility; (F) spermatozoa head morphometry, area and perimeter.

Discussion

In artificial conditions, the European eel male does not maturate, and it is necessary the weekly administration of hCG (1.5 IU/g fish [29]) to induce the gonad development. A physiological description has been performed in response to this treatment, from the brain to sperm. In the present work, the repeated injection of hCG induced differences on the GnRHs expression in diencephalon-mesencephalon. A significant decrease was observed at 4th week of the treatment for cGnRH-II expression, while a significant higher value of mGnRH expression was found in the third week of the treatment, coinciding with an increase in the LHβ expression and 11-KT levels. In contrast, the high increase observed in LHβ expression at the 7th week was not related with an increase in mGnRH expression. This opposite GnRH regulation was also observed in eel matured females [9,50]. Experimental matured eels showed a significant increase for mGnRH, and a significant cGnRH-II decrease during the treatment.

The cGnRH-II function on gonadotropin synthesis is not clear. The observation of cGnRH-II fibers in the neurohypophysis, indicates a possible role in the gonadotropin release, but some authors proposed that the cGnRH-II can be affected by environmental factors or to play a role in control of sexual behaviour [51,52]. On one hand, the mGnRH is considered as responsible of the direct control of gonadotropin release [9,10] but in the present work a good correlation between both hormones can not be found. One possible explanation for these results could be that at the beginning of the maturation process, the mGnRH could be stimulated by high levels of 11-KT (positive effect of gonadal steroids was demostrated by Dufour et al. [9]), and this mGnRH promoted the LHβ expression. But, coinciding with more advances stages of testis development, the gonadotropin expression could be influenced by direct hormone action in the pituitary increasing its synthesis.

The steroid effect on gonadotropin expression has been evaluated widely in teleosts. For example, Rebers et al. [53] reported that the 11-KT had a positive effect in the LHβ synthesis in the catfish. In other hand, it has been demonstrated that the same steroid can have different effects depending of physiological stage of the fish. For example, in cultured pituitary cells from immature male tilapia, the FSHβ expression was stimulated by exposure to testosterone [54], while *in vitro* conditions, cells of tilapia testis at the end of the spawning season, showed a

FHS-β decrease with the administration of T or E_2, and in regressed fish, neither T nor 11-KT had any effect. This could explain that the 11-KT stimulated at the beginning of the spermatogenesis the mGnRH, and in the final phase acted directly promoting the LHβ expression in the pituitary.

The brain influence seems not to be essential to complete the spermatogenesis process when the fish are matured by hormonal injections. In hypophysectomized European eel males the spermatogenesis can be completed by hCG administration, but the maturing cells were less numerous and spermiation less frequent than in intact eels [22]. These results suggest that hCG worked in cooperation with the pituitary hormones.

The gonadotropic function of FSH in Japanese eel was studied by Kamei et al. [55] using a recombinant Japanese eel FSH (rjeFSH). They demonstrated that rjeFSH stimulated the testosterone and 11-KT secretion in Japanese immature testis, that are essential hormones for the onset of the spermatogenesis [22,23,56]. In the present study, one injection of hCG was enough to decrease dramatically the FSHβ expression. In previous works it has been reported that hCG promotes testosterone secretion in ovaries [57] and testis [22,56,23] from Japanese or European eels. It is known that testosterone inhibits FSHβ expression in European eel (*in vivo* [58]) as well as in goldfish [59] and seabass [60]. Probably the reduction in FSHβ in the present study has been caused by an increase in testosterone induced by hCG injection. This decrease in the FSHβ expression has been also observed in European eel female after salmon pituitary extracts treatment [58], likewise in Japanese eel female [61-63] injected with salmon pituitary extracts or salmon gonadotropin fraction. In order to know if the gonadotropin profiles observed in artificially matured eels are similar to the natural pattern, Saito et al. [63] compared the gonadotropin expression in naturally maturing New Zealand longfinned eel with artificially maturing Japanese eel. With the first injections a quickly decrease in the FSHβ expression was observed in artificially maturing female eels, whereas in New Zealand longfinned eels in mid-vitellogenic stage a FSHβ increase was observed. The artificial induction of maturation, by hormonal injections, can be the responsible of this abnormal gonadotropin profile obtained in the eel.

Despite of inhibitory effect of hCG on FSHβ expression in European eel male, the spermatogenesis was promoted. The hCG is considered as a LH

analogue [64], and in some species, as salmon, it has been reported that the gonadotropins, FSH and LH, are equipotents stimulating (*in vitro*) the production of 11-KT and testosterone at the beginning of the spermatogenesis [65-67]. Probably, hCG acts directly on the gonad, promoting the 11-KT production as has been proposed in the Japanese species [15,68,69]. The 11-KT is the most important androgen in males, and by itself is able to promote the complete spermatogenesis *in vitro* [15,16,23,25,68]. In other works it has been reported that 11-KT induces in the Sertoli cells the production of growth factors, such as activin B [15,16], responsible of the initiation of spermatogenesis [24], and insulin-like growth factor-I (IGF-I) that plays an essential role in the progress of the spermatogenesis [15,16,69].

In the present study just one week of hCG treatment caused a level of 11-KT 4 times higher (4.70 ± 0.37 ng/ml) than in the untreated males (1.14 ± 0.52 ng/ml), with the presence of 33% males in stage 2 of development (early meiotic stage). In the 2nd week all the animals showed this stage of development. Stages 3 (mid meiosis) and 4 (late meiosis and spermiogenesis) were observed in the gonad at the 3rd week of the treatment, coinciding with the highest values of 11-KT. When a fish is classified in the stage 4 of the gonad development, means that the spermatogonies have been developed until spermatids or spermatozoa, therefore the meiosis has been completed. The 11-KT is considered as the responsible of the spermatogonia proliferation [15,16,23,25,68], but not of the meiotic process. In the present study, the highest 11-KT values coincided with the later stages of spermatogenesis (late meiosis and spermiogenesis), so this steroid could have a role in these steps of the spermatogenesis. In a number of teleost species, similar results were observed [70-72], decreasing the 11-KT after the onset of spermiation. At the 4th week, sperm can be obtained by abdominal pressure, beginning the spermiation period. From this week on the 11-KT was decreasing until 8th week, that was the first week with high sperm quality. Some authors have considered that 11-KT could be responsible for the release of mature spermatozoa from testicular cysts into the lobular and sperm duct in male rainbow trout [73], or may have an important role maintaining the viability of salmon spermatozoa [74]. Miura et al. [75] reported that high values of 11-KT causes a negative feedback on its own production in the Japanese eel, and this

can be the mechanism used to control the 11-KT levels during the spermiation period.

hCG promoted the 11-KT production, but also the LHβ synthesis. One injection was enough to obtain 6 times more LHβ expression in the pituitary. At the 3rd week a LHβ increase was observed, although was not significant. This LHβ increase can be induced through an IGF-I effect because has been demonstrated *in vitro* that this growth factor promotes the LHβ synthesis [76]. Another possibility can be the 11-KT influence, since in some species as male catfish, the 11-KT has a positive effect in the LHβ synthesis [53].

The following week, 4th week, there was a significant increase in the GSI, coinciding with 83% of males showing stages 4 and 5 of testis development. The spermatozoa presence requires the hydration of the gonad, providing as a result higher GSI values.

The EI showed a significant increase respect to the untreated males in the 5th and 6th weeks. In *Anguilla australis* females the treatment with 11-KT caused external changes in the secondary morphological parameters (head shape and pectoral fin color [77]), and the levels of 11-KT were higher in silver females than non-migratory females [78]. These studies support that 11-KT has an important role in the development of morphological changes in the eel, and the EI increase registered seems be due to previous 11-KT increase.

The stage 6 of testis development, characterized by the dominance of spermatozoa was observed by first time at 7th week. A significant increase in LHβ expression was produced during the 7th week. This major LHβ expression in the pituitary can be due to the IGF-I effect, produced as consequence of high levels of 11-KT in the previous weeks, or by 11-KT levels itself [53].

A significant increase of HSI respect to the untreated males was registered in the 8th week. The IGF-I production is localized in the liver (furthermore Sertoli cells and germinal cells [69]), and this higher HSI could be consequence of this IGF-I production. 11-KT also could be responsible of this increase, since in *Anguilla australis* high levels of 11-KT increased the liver mass in eel females [77].

The spermiation began at 4th week of treatment. A significant higher percentage of live cells was observed in the 8th week, and this was the first week in which the sperm showed over 75% of motile spermatozoa. Following weeks

registered high quality sperm. In this last period some significant differences were found in EI and HIS. Probably, the reason of these differences is not physiological changes, but could be due to high variability observed between males in the same week.

To avoid these differences between males in the same week, the parameters related with the male maturation (LHβ expression, 11-KT, GSI, EI, sperm motility and spermatozoa head morphometry) were studied in function of the stage of gonad development. LHβ expression registered higher values when the gonad was in the last stages, ratifying that this gonadotropin has influence in the final maturation [58,62,79].

11-KT showed a progressive increase until the late meiosis and spermiogenesis (stages S3/4), and decreased in the spermiation stages S5 and S6. Coinciding with the highest value of 11-KT, a significant increase in the GSI was produced, as consequence of germ cell proliferation. This confirms that one 11-KT role is to promote the spermatogenesis process, and the fact that the high level coincide with the last steps of spermatogenesis indicate the possible function of this androgen in these phases of development. EI was high from S3/4 to S6, supporting the influence of 11-KT in the development of secondary morphologic characters. Obviously, better results of motility and spermatozoa head morphometry were obtained when the gonad was more developed, coinciding with the maximum values in the rest of parameters (LHβ expression, GSI and EI).

A wide study of gonad maturation in European eel male have been performed, being the first time that the gonadal maturation is reported analyzing reproductive parameters from the brain to the testis. The GnRHs and gonadotropin expression were studied, being the first time in which these parameters are described during eel male maturation process. In the future more studies must be carried out, including the analysis of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), to provide important information about the spermatogenesis process and the acquisition of motility by the European eel spermatozoa.

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7 EXPERIMENT 4

European eel sperm diluent for short term storage

In press in Reproduction in Domestical Animals

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Abstract

The sperm of European eel shows a high density and the time of spermatozoa motility is very short after activation with sea water. These characteristics make difficult the sperm handling and its quality assessment. Several diluents were previously described for the Japanese eel obtaining over 3 weeks conservation times under refrigeration, but they rendered bad results in the European species. In the present study several diluents were developed taking as basis the P1 medium, and using different dilutions ratios (1:50,1:100) and two pH (6.5, 8.5). The effect of the addition of bovine serum albumin (BSA, 2% w/v) was also evaluated. At 24 h, undiluted samples already showed significant lower motility and viability than sperm samples diluted in the different media. The results for diluents with pH 6.5 and 8.5 were different. Spermatozoa diluted in media at pH 6.5 can not be activated at 24 h, while samples diluted in the diluents with pH 8.5 and added with BSA did not show significant differences with respect to the fresh sperm motility until 48 h. The viability (percentage of live cells) did not show differences until one week, independently of the dilution ratio. After 1 week, the motility was around 30% in the media containing BSA, which presented no differences for head size of the spermatozoa (perimeter and area) until 72 h and 1 week, respectively. In conclusion, the combination of one medium having similar physic-chemical characteristics to the seminal plasma, including pH 8.5, and supplemented with BSA can be used in different dilution ratios for the sperm short-term storage, preserving its motility capacity.

Introduction

During the last decades, the capture and over-explotation of European eels and elvers have diminished its populations, making necessary the development of techniques for the control of reproduction in captivity. Methods for the hormonal induction of gonad maturation in this species have been developed in previous studies, obtaining significant sperm volumes with a good quality (Boëtius et al. 1967; Billard and Ginsburg 1973; Meske 1973; Bieniarz and Epler 1977; Dollerup and Graver,1985; Leloup-Hâtey et al. 1985; Khan et al. 1987; Amin, 1997; Pérez et al. 2000, 2003; Müller et al. 2004, 2005; Asturiano et al. 2005, 2006; Szabó et al. 2005), as well as ovarian maturation, spawns, egg fertilisation and even hatching (reviewed by Pedersen 2003, 2004; Tomkiewicz 2007).

However, methods for the hormonal induction of gonad maturation in this species usually take several weeks both in males and females, and unsynchronised maturations can occur, avoiding egg fertilisation. Diluting media were developed to prevent these problems and to improve the sperm handling. Usually, the diluents have been used to maintain a high motility after short-term storage of spermatozoa, to stabilise the physic-chemical conditions in the seminal plasma during storage (Tan-Fermin et al. 1999), and to improve fertilization rates in some species (Tambasen-Cheong et al. 1995; Ohta and Izawa 1996). Fish spermatozoa are immotile in seminal plasma, and for this reason several authors have developed diluents with the same ionic composition and osmolality as the seminal plasma (Villani and Catena, 1991; Tan-Fermin et al., 1999; Asturiano et al., 2004). On the other hand, some authors have tried to prepare inactivation media for fish spermatozoa, with different composition respect to the seminal plasma (Sansone et al. 2001; Tanaka et al. 2002b, Rodina et al. 2004).

Several sperm conservation media have been tested in the Japanese eel (*Anguilla japonica*) using the chemical composition of the seminal plasma, and obtaining conservation times under refrigeration of over three weeks (Ohta and Izawa 1996). In a later work Ohta et al. (2001a) reported that the use of diluted sperm could increase the fertilization rate. Moreover, the use of diluting solutions could extend the life of refrigerated sperm and even increase the spermatozoa motility after incubation.

Two diluting media (K15 and K30, Ohta and Izawa 1995; Ohta et al. 2001a) previously used in Japanese eel sperm were tested in the European eel. Twenty-four hours later, the sperm showed an important reduction in the percentage of motile spermatozoa after activation and lower motility parameters (VAP, angular velocity; VCL, curvilinear velocity; VSL, straight line velocity; BCF, beating cross frequency), concluding that these media are not useful to preserve the sperm of the European eel (Asturiano et al. 2004). Looking for one effective medium, the ionic composition and the physic-chemical characteristics of the seminal plasma were studied in the European eel (Pérez et al. 2003; Asturiano et al. 2004). The results were used to design a new diluting medium, named P1, isosmotic, isoionic and with the same pH (8.5) than seminal plasma, which might facilitate sperm dilution for quality analyses and also be used in the first step of dilution of the sperm before the fertilization process.

Tanaka et al. (2002a,b) suggested that $NaHCO_3$ has an inhibitory role of the movement in the Japanese eel sperm, product of its dissociation and its influence in intracellular pH. Two years later, Tanaka et al. (2004) described that $NaHCO_3$ in aqueous solutions is dissociated in: CO_2 + H_2CO_3 (free-CO_2), HCO_3^- and CO_3^{2-}, but the proportion of each component depend of the pH medium. In acid environment the HCO_3^- protonates giving H_2CO_3, which dissociates into H_2O and CO_2. In contrast, in basic medium HCO_3^- loses the H^+, rendering CO_3^{2-}. The inhibitory function corresponds to CO_2 (Tanaka et al., 2002), so in the present study, the P1 medium was tested with a low pH (6.5), to know if the inactivation of the sperm can improve the results obtained with P1 at pH 8.5.

Foetal bovine serum (FBS) or bovine serum albumin (BSA) have been used in the sperm cryopreservation media due to their effect as buffers of the osmotic shock, because proteins can give a protection of mechanical type to the cells membrane, diminishing the risks of crystallization, recrystallization or ice melting during the different phases of the process of freezing and thawing (Cabrita et al. 2005; Peñaranda et al. submitted). In the European eel, the addition of 25% FBS in the sperm freezing medium has shown a positive effect, improving the spermatozoa survival and maintaining its head size (Marco-Jiménez et al. 2006a; Garzón et al. 2008), but in the present study the membrane protector used was BSA (2%), which gave good results in previous works (Peñaranda et al. in press). On the other hand we have checked that the dilution

ratio is an important factor for the eel sperm conservation along time (unpublished results), including cryopreservation (Asturiano et al. 2003, 2004). So, taking as basis these and previous works in Japanese eel (Ohta et al., 1996, 2001a) two different dilution ratios were tested (1:50 and 1:100).

In conclusion, our initial hypotheses were that low pH can inhibit the sperm motility, BSA can protect the cells membranes and can help maintaining the cell viability during short-term storage of European eel sperm. In this regard the present study checked the effect of these three factors: pH (8.5 vs 6.5, adjusted using HCl 1M), BSA addition (2%, w/v) and dilution ratio (1:50 vs 1:100).

Materials and Methods

All the chemicals were reagent grade and purchased from Sigma-Aldrich Química S.A. (Tres Cantos, Madrid, Spain), Angelini Farma-Lepori (Barcelona), Invitrogen (Prat de Llobregat, Barcelona, Spain) and Panreac S.A. (Alcobendas, Madrid, Spain).

Semen collection

One hundred fifty European eel males (body weight: 137.6 ± 21.4 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into our facilities. Fish were distributed in three tanks of 500 L and gradually acclimatised to sea water (salinity 37.0 ± 0.3 g/L, 20 ºC) for 7 days.

Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU/g fish) as previously described by Pérez et al. (2000). To avoid contamination of samples with faeces, urine and sea water the genital area was cleaned with freshwater, total expressible milt was collected by applying gentle abdominal pressure to anesthetised males (benzocaine; 60 mg/L). Once a week during the spermiation period, from the 8^{th} to 13^{th} week of treatment, 10 fish were sampled 24 h after the hormone administration. For the experiments, semen samples from three males having over 50% of total motile spermatozoa were pooled to avoid individual male differences.

Evaluation of motility

Immediately after collection, the motility of sperm samples was assessed by mixing one drop of sperm with 3 μl of artificial sea water [in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8] adjusted to 1000 mOsm/kg as activation media. The motility was calculated evaluating the percentage of the cell are in movement respect to the total cells in the mix. All the sample analyses were performed by the same trained observer to avoid subjective differences in the motility evaluation.

Media composition

Taking as base the P1 medium described by Asturiano et al. (2003, 2004) in mM: 125 NaCl, 20 $NaHCO_3$, 2.5 $MgCl_26H_2O$, 1 $CaCl_22H_2O$, 30 KCl, the effect of pH (8.5 vs 6.5, adjusted using HCl 1M), BSA addition (2%, w/v) and dilution ratio (1:50 vs 1:100) were evaluated in the experiment using the possible combinations (n=10 pools). The sampling times were 24, 48, 72 h and 1 week after sperm dilution, maintaining the diluted samples in Petri plates (10 ml) at 4ºC.

Fluorescence stain analysis

Live/Dead Sperm Viability Kit [SYBR/Propidium Iodide (PI)] of Invitrogen (Barcelona, Spain) was used to evaluate the viability of the spermatozoa at different times (n=10 pools). Sperm diluted in P1 medium (Asturiano et al. 2004; 1:350) was mixed with SYBR Green and PI, and maintained during 20 minutes of incubation at room temperature in the dark. The final SYBR Green concentration was 10^4 times diluted from the original stock (2 μM) and PI 10^3 times from original stock (24 μM). At least 100 spermatozoa per sample were assessed in a Nikon Eclipse (E-400) epifluorescence microscope, using UV-2A (EX: 330-380 nm, DM: 400, BA: 420) filter. Spermatozoa were classified as *dead* when nuclei showed red fluorescence over sperm head and *live* when they show green fluorescence.

Spermatozoa morphometry analysis

A fraction of fresh samples and diluted samples was mixed with 2.5% glutaraldehyde in phosphate buffered saline fixative solution (Pursel and

Johnson, 1974), obtained a final dilution of 1:200 (v/v). Slides were viewed using a 1000X negative phase contrast objective (Nikon Plan Fluor) on Eclipse E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer®, Morfo Version 1.1, Imagesp, Barcelona, Spain). Approximately 100 spermatozoa were analysed in each sample (n=5-10). The morphological parameters determined were: head perimeter (μm) and head area (μm^2).

Statistical analysis

Motility and viability parameters were subjected to analysis of variance (One-Way ANOVA). General Lineal Model (GLM) was performed for spermatozoa head morphometry. A Student-Newman-Keuls procedure was used for the comparisons between means at a 0.05 significant level. The results of motility and viability showed presented as mean ± standard error of the means (SEM), while the results of spermatozoa head morphometry are showed as least square means (LSM) ± standard error of the means (SEM). All statistical procedures were run using Statgraphics®Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

Results

Motility and percentage of live cells

Different media were tested to preserve the sperm along the time, one maintaining (seminal plasma-like) medium, with pH 8.5, and an inactivating medium, with pH 6.5. Every diluent was probed with two dilution ratios: 1:50 and 1:100, with or without BSA.

The sperm quality for undiluted samples decreased with only 24 h of incubation (Fig 7.1a) respect to fresh samples. In contrast, sperm diluted in the pH 8.5 media preserved its motility without differences, except in the case of samples diluted in the media without BSA, dilution ratio 1/100, which showed a reduction of motility (Fig 7.1a). The pH 6.5 media caused a total absence of motility after 24 h (Fig. 7.1a), and as a consequence of this null motility no more dates were obtained from these samples. The pH 8.5 media containing BSA caused motilities without significant differences with the fresh samples until 48

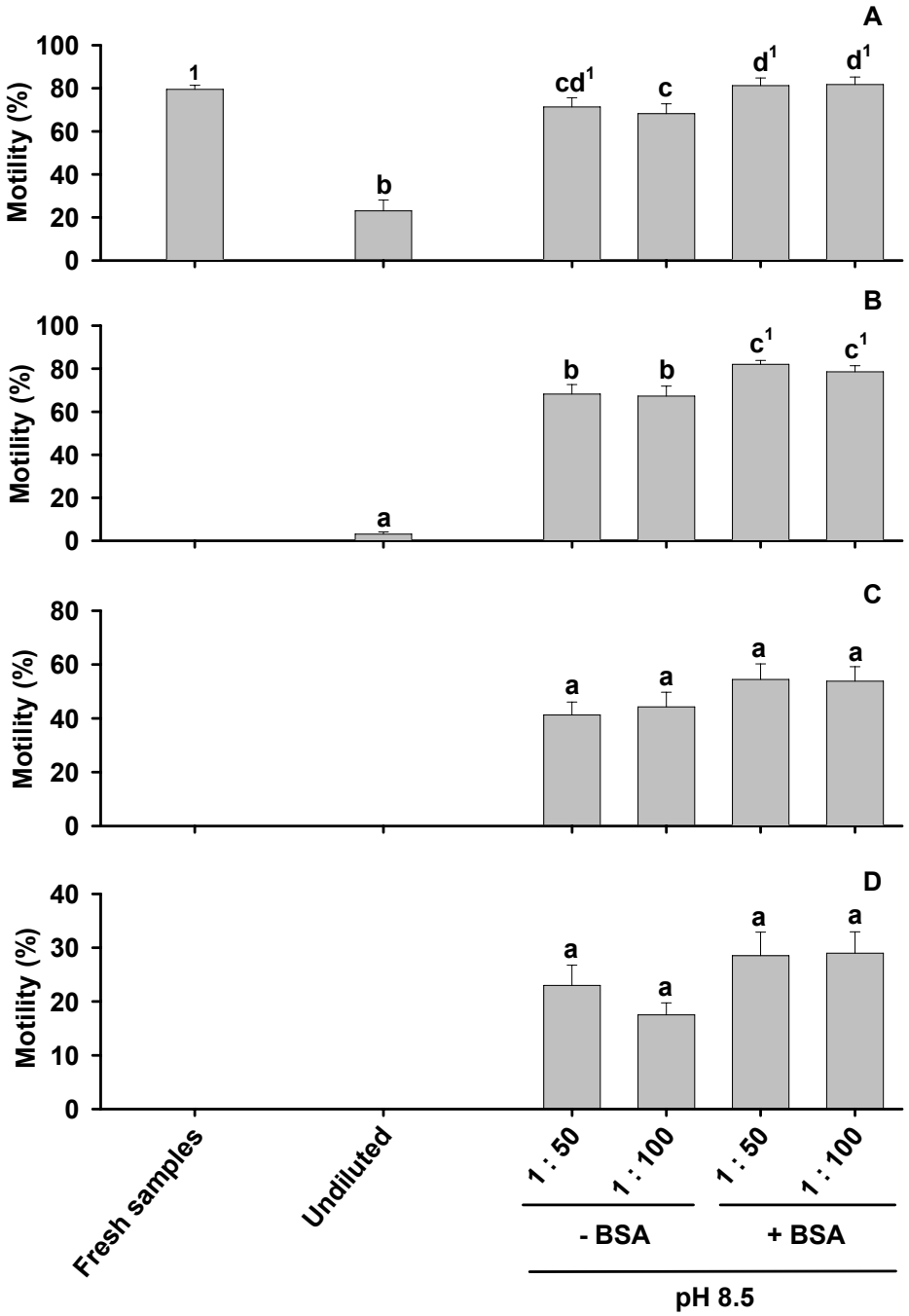

Figure 7.1. Evaluation of the motility of sperm samples in the different tested media compared with fresh and undiluted samples, at different times: (A) 24 h, (B) 48 h, (C) 72 h and (D) 1 week. No motility was observed in the all pH 6.5 media after 24 h of incubation, and no more data were taken in the rest of the experiment. Different letters means significant differences (mean ± sem) between media at the same time of the incubation, and the number (1) indicates no significant difference respect to fresh samples ($p<0.05$).

hours, while the samples diluted in the media without BSA showed significant lower motilities at this time of incubation (Fig. 7.1b). Undiluted sperm samples registered motilities close to zero at 48 h, and for this reason they were not analyzed in the following times of the study. After 72 h and 1 week of incubation, all the samples, independently of the diluting media, showed lower motilities than fresh samples (Fig. 7.1c,d). Between media any difference was registered during this time, being the highest motility around 30% (BSA-1/50: 28.5 ± 13.95; BSA-1/100: 29.0 ± 12.48) after 1 week.

The viability did not show differences after 24 h of incubation (Fig. 7.2a), but at 48 h the undiluted samples registered a significant lower percentage of live cells (Fig. 7.2b). The BSA provided at the sperm significant higher viabilities respect to the media without BSA (Fig. 7.2b,c,d) and did not show any difference with fresh samples during all the experiment.

Spermatozoa head morphometry

Spermatozoa area corresponding to undiluted and diluted samples did not show any difference respect to fresh samples during the first 48 h of incubation (Fig. 7.3a,b). In the next times, no more data of undiluted samples were taken because of their low motility. The spermatozoa head area for diluted samples was not significantly lower than fresh samples until 1 week of incubation (Fig. 7.3c,d), moment in which differences between media were observed. Lower results were registered when the spermatozoa were diluted in BSA media, and this decrease was significantly at dilution ratio 1:50 (Fig. 7.3d).

Similar results were obtained when the spermatozoa perimeter was analyzed. Until 48 h any difference was observed with fresh samples (Fig. 7.4a,b). At 72 h and 1 week the spermatozoa head perimeter in all the media suffered a significant decrease in comparison with fresh samples (Fig. 7.4c,d). The BSA media provided lower results than without BSA media, but only was significantly lower for the media with dilution ratio 1/100 at 72 h.

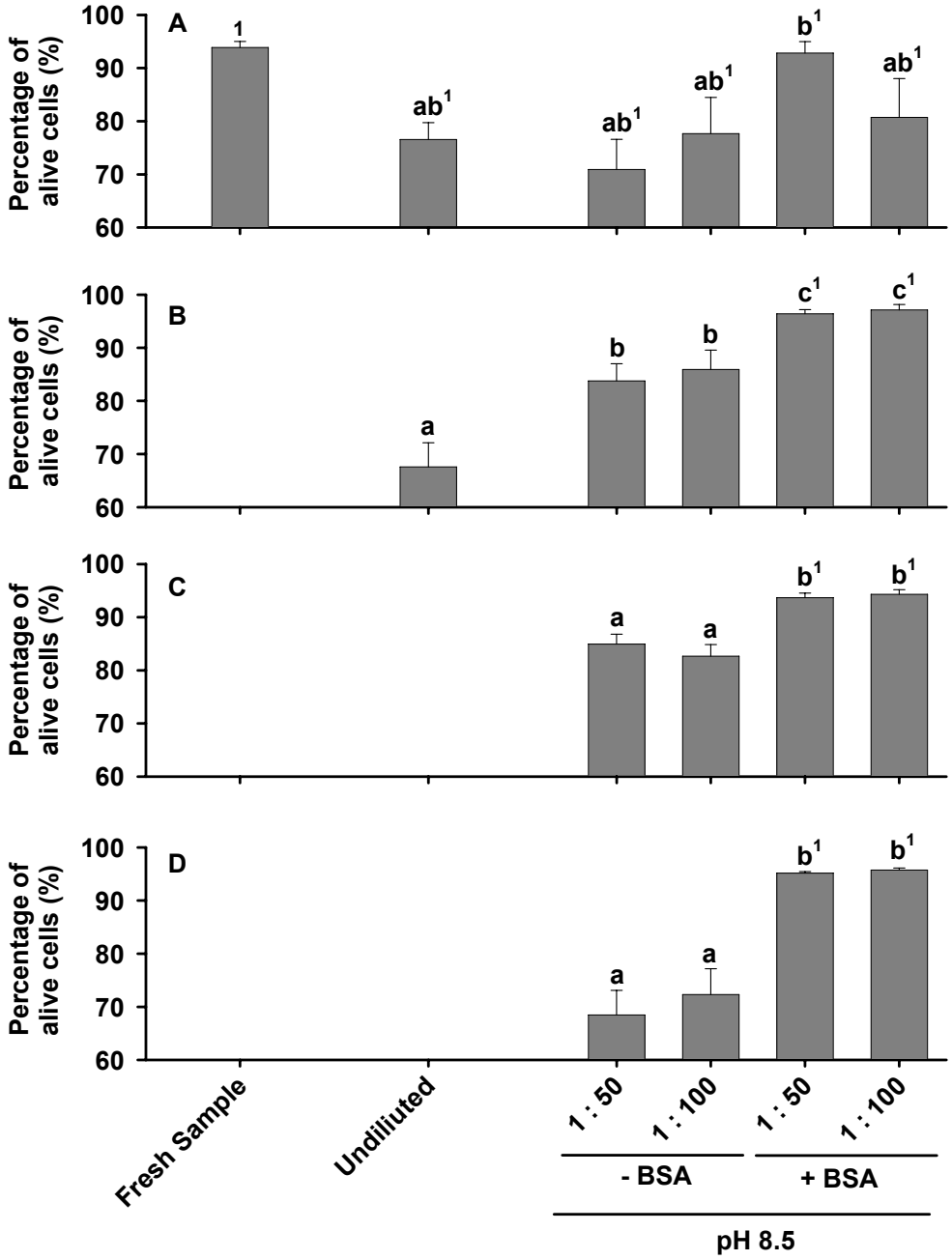

Figure 7.2. Percentage of live cells in the sperm samples diluted with the different tested media compared with fresh and undiluted samples at different times: (A) 24 h, (B) 48 h, (C) 72 h and (D) 1 week. Any difference respect to the fresh samples was registered at 24 h of incubation for pH 6.5 media, and no more data were taken in the rest of the experiment. Different letters means significant differences (mean ± sem) between media at the same time of the incubation, and asterisks indicates no significant difference respect to fresh samples ($p<0.05$).

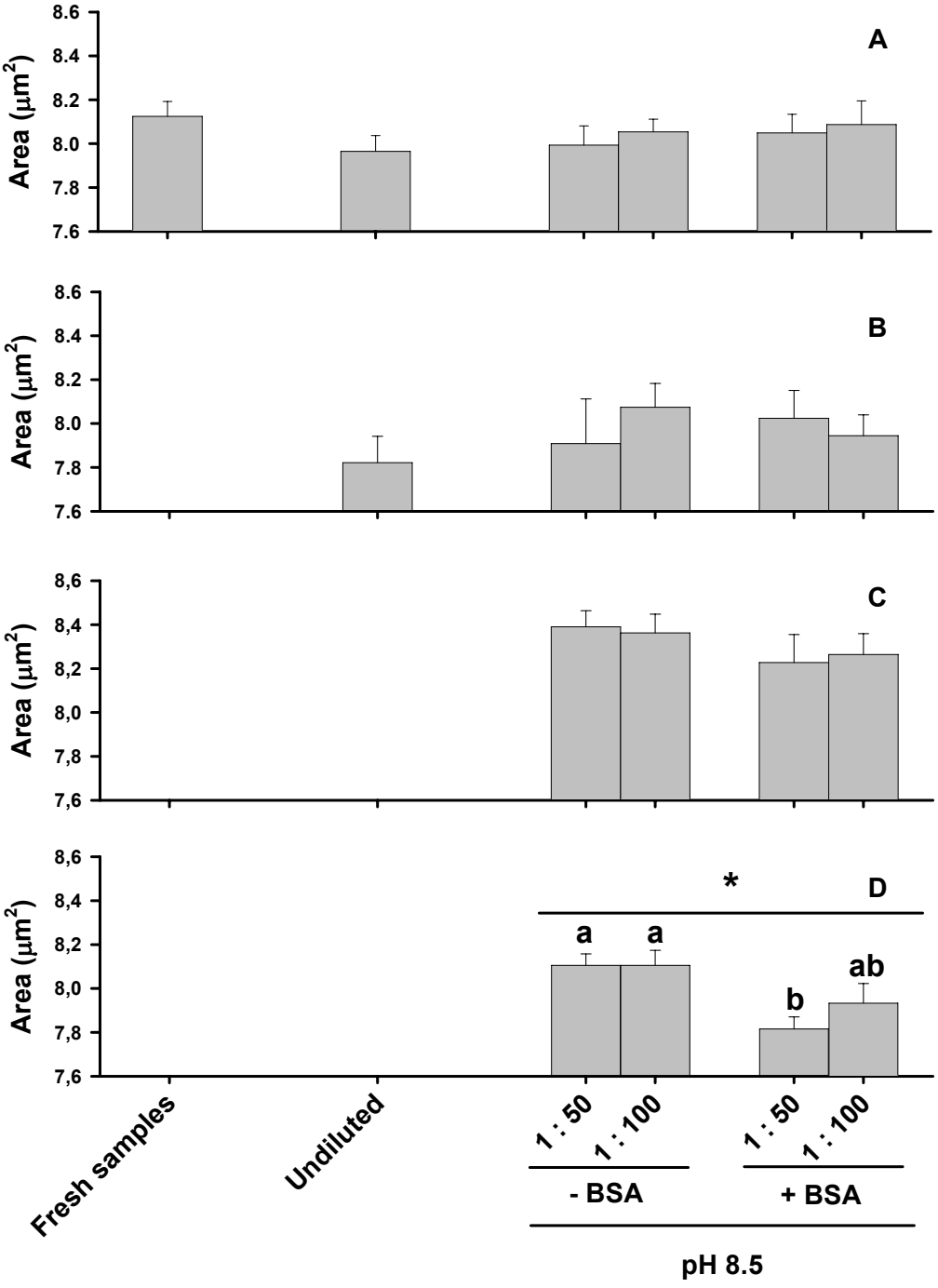

Figure 7.3. Evaluation of spermatozoa area in the sperm samples diluted with the different tested media compared with fresh and undiluted samples, at different times: (A) 24 h, (B) 48 h, (C) 72 h and (D) 1 week. Any difference respect to the fresh samples was registered at 24 h of incubation for pH 6.5 media, and no more data were taken in the rest of the experiment. Different letters means significant differences (lsm ± sem) between media at the same time of the incubation, and asterisk means significant difference respect to fresh samples ($p<0.05$).

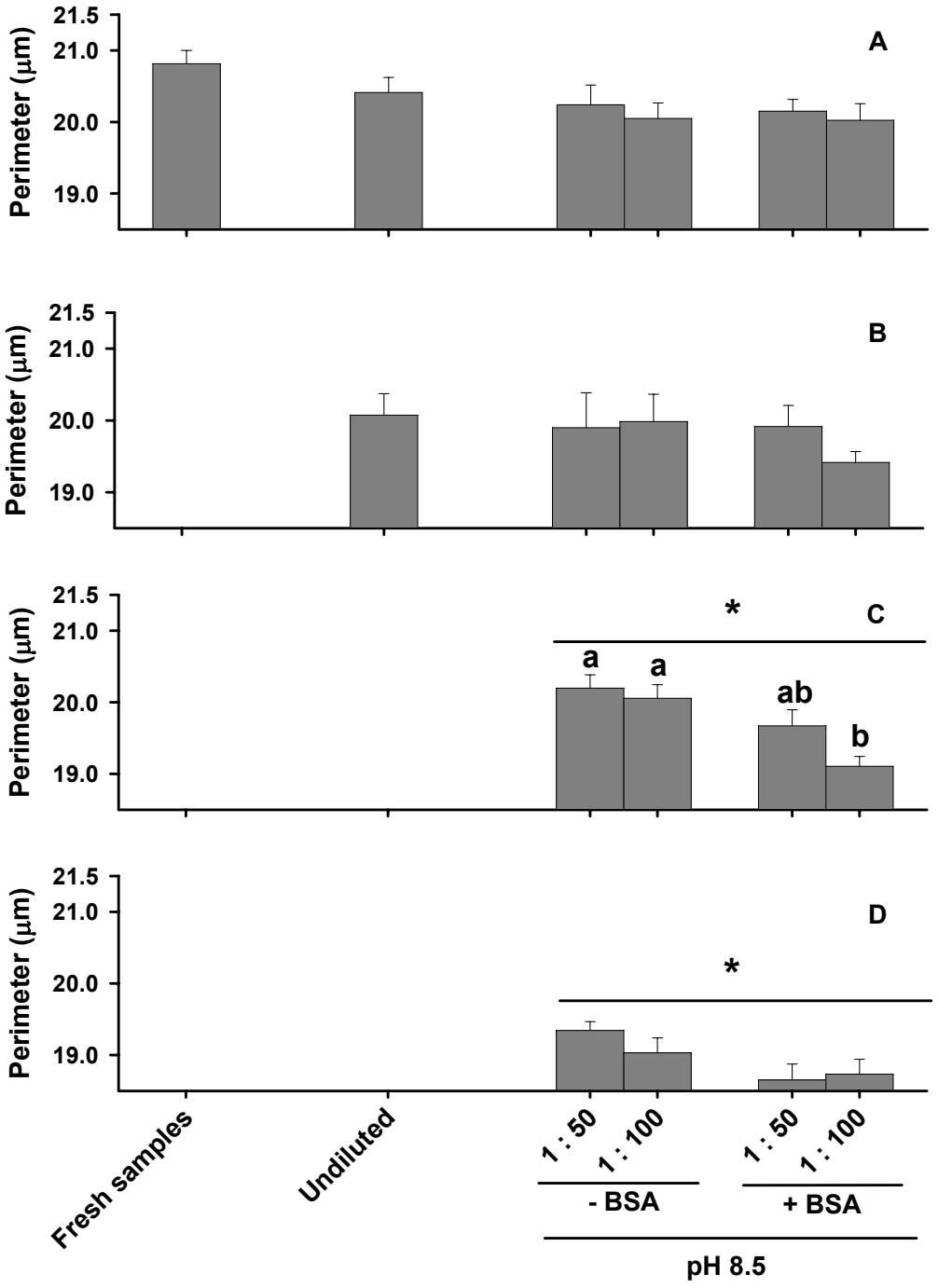

Figure 7.4. Study of spermatozoa area in the sperm samples diluted with the different tested media compared with fresh and undiluted samples, at different times: (A) 24 h, (B) 48 h, (C) 72 h and (D) 1 week. Any difference respect to the fresh samples was registered at 24 h of incubation for pH 6.5 media, and no more data were taken in the rest of the experiment. Different letters means significant differences (lsm ± sem) between media at the same time of the incubation, and asterisk means significant difference respect to fresh samples ($p<0.05$).

Discussion

The fish spermatozoa are immotile in the seminal plasma (Stoss, 1983; Morisawa 1985), and looking for maintaining this stage some diluents have been developed. Several media have been designed in fresh water species, as glucose solutions (Linhart et al. 1995) or potassium solutions (Cosson and Linhart 1996). For eel, which belongs to sea water species, two different strategies have been performed. First of them it is one medium with similar physic-chemical characteristics to the seminal plasma, where in natural conditions the sperm is immotile (Lahnsteiner et al. 1997; Ohta and Izawa 1995, 1996; Ohta et al. 2001a; Peñaranda et al. in press). The second strategy is to develop a medium that inactivates the movement during the incubation (Sansone et al. 2001; Tanaka et al. 2002a,b; Rodina et al. 2004). To design them, it was necessary to consider the following factors: ionic composition, pH and osmotic pressure.

All diluents that were tested had the same osmotic pressure, ionic composition and temperature of incubation. The seminal plasma composition can give additional information about optimal conditions for storage, energy resources and possible peculiarities of metabolism (Lahnsteiner et al. 1997). Consequently, the description of European eel seminal plasma (Asturiano et al. 2004) was used to design the diluent. In Japanese eel, some ions concentration had been increased (K^+ and HCO_3^-) in the medium to improve the motility (Ohta and Izawa 1995, 1996; Ohta et al. 1997, 2001a), but similar probes in European eel did not give good results (unpublished results). Regarding the osmotic pressure, it was fixed in 325 mOsm/Kg because the osmotic pressure of seminal plasma remained constant around this value during the spermiation period (Asturiano et al. 2004). The temperature of incubation was 4 ºC, because high temperatures cause an increase in the spermatozoa metabolism (Cosson et al. 1985).

The variable parameters were the pH and dilution ratio, furthermore the addition of BSA. BSA has been used in other species as gilthead seabream (*Sparus aurata*; Cabrita et al. 2005), European sea bass (*Dicentrarchus labrax*; Zilli et al. 2003; Peñaranda et al. in press) or European eel (Peñaranda et al, submitted). There is direct evidence that BSA adheres rapidly to the spermatozoa membrane at the moment of dilution (Blank et al. 1976), and modifies the sperm lipid composition through lipid exchange or hydrolysis (Davis et al. 1979). In

cultures cells, addition its binding and transport functions (Kragh-Hansen 1981), albumin stimulate sperm cholesterol efflux thanks to its high-density lipoprotein (HDL; Go and Wolf 1985; Thérien et al. 1999). Significant better motilities and viability at 48 hours respect to the media without BSA were registered, what demonstrated its influence in the sperm motility. Similar effect has been observed, in previous works, using it as an antioxidant component in freezing media (Matsuoka et al. 2006; Uysal and Bucak 2007; Uysal et al. 2007), or in diluents to prolong the sperm motility along time (McPartlin et al. 2008; Peñaranda et al. in press). Hossain et al. (2007) reported that the fatty acids can bind to BSA-V, improving the motility and viability. Maybe, some of these interactions could be preserving the eel sperm membrane integrity, and so the motility and viability. Nevertheless, the spermatozoa diluted in BSA media registered lower head size with the time. One possible explanation could be that the lost of cholesterol and the increase of phospholipids in the membrane (Go and Wolf 1985; Thérien et al. 1999) had influence in the cellular osmoregulation. Although the osmotic pressure of the medium was the same than seminal plasma, possibly long incubation with BSA media can promote that the spermatozoa loses part of their osmoregulation capacity. The principal responsible of the osmoregulation is the Na^+/K^+ (Sancho et al. 2003), and the changes in the cellular membrane could finish affecting the functionality of these bombs. Not only the spermatozoa diluted in media containing BSA decreased their head size with the incubation, but the media without BSA also presented lower spermatozoa head morphometry values from 72 h of incubation. The difference respect to BSA media, it was that in the media without BSA the percentage of live cells was significant lower than fresh samples. Marco et al. 2006b reported that in goat sperm the dead cells has lower head size than live cells, so this can be the cause of the significant decreased observed at the end of the experiment. This explanation can not use for the BSA media, because the viability in these media is not different than fresh samples.

In other hand, fertilization probes will be necessary to know if these morphology changes could affect the fertilization capacity, but for the moment the poor quality eggs obtained in European eel females under artificial conditions makes difficult this type of study.

The pH is considered an essential factor in the control of sperm motility (Lahnsteiner et al. 1997; Tan-Fermin et al. 1999; Ohta et al. 2001b; Sansone et al. 2001; Tanaka et al. 2002a,b; Woolsey and Ingermann 2003; Alavi et al. 2004, Tanaka et al. 2004; Alavi and Cosson 2005). At 24 hours the motility was zero for all the samples diluted in pH 6.5 media, although the cells were maintained live and any significant changes in the spermatozoa head morphometry was observed. Possibly, the pH effect had influence in the mechanism of sperm motility, and not in the other parameters. It has been demonstrated that CO_2 inhibits the sperm motility by decreasing the intracellular pH (Morisawa 1994; Woolsey and Ingermann 2003; Cosson 2004; Tanaka et al. 2002a, 2004), but the low values of pH could also affect other factors that have influence in the sperm motility. In sea urchin sperm has been demonstrated that ATPase activity is highly dependent on the pH of the medium (Christen et al. 1983). Other example can be found in rainbow trout, varying the ATP hydrolytic activity of the outer arm of dynein with the pH (Gatti et al. 1989).

The combination of low pH and high $NaHCO_3$ concentration was used by our group (Peñaranda et al., under revision), to arrest the activation of the eel sperm movement produced by DMSO in the freezing medium. To check if the spermatozoa can recover the movement after a few minutes of incubation in the pH 6.5 medium, a fraction was diluted in artificial sea water (pH 8.0-8.2). The spermatozoa showed motilities without difference with fresh samples, so it was demonstrated that the pH affects the spermatozoa movement but not the viability, and this inhibition is reversible. However, it is possible that a long incubation of the sperm at low pH can promote irreversible changes in the cell physiology, that affect the motility spermatic capacity.

When the pH was in physiological values, no differences were observed respect to fresh samples until 48 hours for the motility and until 1 week for the viability when the media contained BSA. Similar studies were performed in Japanese eel (Ohta and Izawa 1995, 1996), maintaining higher percentages of movement after one week of incubation. Nevertheless, the undiluted sperm in European eel was zero at 72 hours, while in Japanese eel at 1 week the sperm motility was around 60%.

The tested ratios of dilution were: 1:50 and 1:100. Both dilutions have given good results in other species (Tan-Fermin et al. 1999; Ohta and Izawa

1995, 1996; Ohta et al. 2001a), and any difference was observed between them in the present study.

These results have demonstrated that high values of pH preserved the eel sperm, with a clear improvement respect to the undiluted samples. The inclusion of BSA in the media provided better results when the motility and the percentage of live cells were analyzed. All the media maintained unchanged the spermatozoa head mophometry until 72 hours for the perimeter and 1 week for the area, what could be an important data for future fertilizations. Any difference was registered between both ratios of dilution, so the lowest dilution seems more useful for sperm storage. Despite the physiological difference between Japanese and European eel, more studies must be performed in this field to approach to the motilities described in the Japanese eel studies.

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8 EXPERIMENT 5

Improvement of European eel sperm cryopreservation method by preventing spermatozoa activation caused by cryoprotectants

Under revision in Cryobiology

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Abstract

Sperm production has been obtained from European and Japanese eels, but its quality and quantity use to be changeable. So, its cryopreservation has been tried in both species. Dimethyl sulfoxide (ME_2SO) is the best cryoprotectant for European eel sperm, but increases the medium osmolality, inducing the activation of spermatozoa motility. To avoid it, different combinations of pH (6.5, 8.5) and $NaHCO_3$ concentrations (20, 40, 80 mM) were tested with two ME_2SO concentrations (5, 10%). Foetal bovine serum (FBS, 25% v/v) was added as a membrane protector to all the freezing media used in the different experiments. The highest ME_2SO and $NaHCO_3$ concentrations at pH 6.5 caused the best post-thawing motility (26.27 ± 3.85%). A second experiment was carried out testing media with ME_2SO 10% with additional $NaHCO_3$ concentrations (100, 120 mM). The highest post-thawing motility (38.26 ± 2.89%) was found in the media containing $NaHCO_3$ 100 mM, but not significant difference was observed with the best in the previous experiment ($NaHCO_3$ 80 mM). In a parallel experiment, and trying to improve the protection ahead of the cryopreservation process, bovine serum albumin (BSA, 5% w/v) was added instead of FBS. Lower motilities were registered with BSA as membrane protector. Spermatozoa activation caused by addition of ME_2SO can be prevented using high $NaHCO_3$ concentrations, improving the cryopreservation process. This effect seems be based on some of

the products dissociated from $NaHCO_3$ in aqueous solution, affecting the intracellular pH, essential in the sperm motility.

Introduction

The European eel cryopreservation [5,16,25] has been justified because the hormonally treatment to induce the eel gonadal maturation is long (9-12 weeks), the sperm quality is variable and is necessary to synchronize the gamete production in both sexes [4].

It is known that cryopreservation causes lethal damage in spermatozoa and also produces an important loss of membrane function by increasing membrane fragility in live cells [8]. The lost of membrane permeability by cryopreservation process has been described as the main damage during freezing and thawing, and changes in membrane stability result in an increase in the permeability of membranes to water and cations [9]. Changes in spermatozoa volume are associated with osmotic imbalance in live cells [19] and volumetric measurement has been shown to be an appropriately precise, accurate and informative method for the detection of functional membrane changes [32].

To achieve the European eel sperm cryopreservation, studies to analyze the ionic composition and physio-chemical characteristics of the seminal plasma were done [3,31]. The results were used to design a diluting medium, named P1, isoionic, isosmotic and with the same pH (8.5) than with the seminal plasma of this species and that might facilitate its dilution for quality analyses, to get a freezing medium or can be used as the first step of dilution of the sperm before the fertilization process.

On the other hand, foetal bovine serum (FBS) or bovine serum albumin (BSA) have been used in the sperm cryopreservation media due to their effect as buffer of the osmotic shock, because proteins can award a protection of mechanical type to the cells membrane, diminishing the risks of crystallization, recrystallization or ice melting during the different phases of the process of freezing and thawing [10,29,36]. In the European eel, the addition of 25% FBS in the sperm freezing medium had shown a positive effect, improving the spermatozoa survival and maintaining its head size, while the use of ME_2SO was showed as the best election respect to others cryoprotectants, like methanol or glycerol [16,25]. ME_2SO increases the medium osmolality [5,21], causing the

activation of the spermatozoa motility (cryoprotectant activation, [26]. To avoid the cryoprotectant activation, the present study will focus in one component of the freezing media: $NaHCO_3$. On one hand, Tanaka et al. (2002) [34] suggested that $NaHCO_3$ has an inhibitory role of the movement in the Japanese eel spermatozoa, product of its dissociation and its influence in intracellular pH. Two years later, Tanaka et al. (2004) [35] described that $NaHCO_3$ in aqueous solutions is dissociated in: CO_2+H_2CO_3 (free-CO_2), HCO_3^- and $CO^3{}_2^-$, and CO_2 has inhibitory effect on the sperm motility.

The aims have been to determine the influence of the pH and the concentration of $NaHCO_3$ in the freezing medium on the sperm cryopreservation, and to find the best percentage of ME_2SO for the freezing of European eel sperm (experiment 1 and 2). Parallel, the effect of BSA addition to the freezing medium was probed trying to improve the cryopreservation process (experiment 3).

Materials and Methods

All the chemicals were reagent grade and purchased from Sigma-Aldrich Química S.A. (Tres Cantos, Madrid, Spain), Angelini Farma-Lepori (Barcelona), Invitrogen (Prat de Llobregat, Barcelona, Spain) and Panreac S.A. (Alcobendas, Madrid, Spain).

Semen collection

One hundred fifty European eel males (body weight: 137.6 ± 21.4 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into our facilities. Fish were gradually acclimatised to sea water (salinity 37.0 ± 0.3 g/L, 20 ºC) for 7 days, and distributed in three tanks of 500 L.

Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of hCG (1.5 IU/g fish) as previously described by Pérez et al. (2000) [30]. After cleaning the genital area with freshwater and thoroughly drying to avoid contamination of samples with faeces, urine and sea water, total expressible milt was collected by applying gentle abdominal pressure to anesthetised males (benzocaine; 60 mg/L). Once a week during the spermiation period, from the 8^{th} to 13^{th} week of treatment, 10 fish were sampled 24 h after the hormone administration. For the experiments,

semen samples from three males having over 50% of total motile spermatozoa were pooled to avoid individual male differences.

Evaluation of motility

After collection, the motility of sperm samples was assessed mixing one drop of sperm with 3 μl of artificial sea water (in Mm: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8) adjusted to 1000 mOsm/kg as activation medium. All the analyzed samples were performed by triplicate and the same trained observer to avoid subjective differences in the motility evaluation.

In the freezing medium the semen is diluted 1:2, and the sperm density was so high to determinate the percentage of motile spermatozoa, causing a problem to evaluate the effect of cryoprotectants in the activation of sperm motility. For instance, a subjective scale was used (category I: 0 - 15%, category II: 16 - 50% and category III: >50%).

Media composition

The effect of pH (8.5 vs 6.5, adjusted using HCl 1M) and different $NaHCO_3$ concentrations were evaluated in the experiment 1 (n = 6 pools). Four freezing media containing 25% FBS (v/v) were tested with ME_2SO 10% or 5% (v/v).

In the second experiment (n = 6 pools), six freezing media (Table 1, media 1-6) were tested, including two new media respect to the previous experiment. In this case, Me_2SO 10% (v/v) and 25% FBS (w/v) were constant in all the media.

Table 8.1. Composition and pH of the freezing media used on the three experiments. In the experiment 1, the media 1-4 were tested with 5 and 10% of ME_2SO, while in the rest of experiments only ME_2SO 10% was used. Foetal bovine serum (FBS; 25% v/v, media 1-6) or bovine serum albumin (BSA; 5% w/v, medium 7) were added as membrane protectors.

	Medium	NaCl (mM)	NaHCO3 (mM)	MgCl2 (mM)	CaCl2 (mM)	KCl (mM)	pH	Membrane protector
Experiment 1	1*	125	20	2.5	1	30	8.5	FBS
	2	125	20	2.5	1	30	6.5	FBS
	3	100	40	2.5	1	30	6.5	FBS
	4	75	80	2.5	1	30	6.5	FBS
Experiment 2	5	50	100	2.5	1	30	6.5	FBS
	6	25	120	2.5	1	30	6.5	FBS
Experiment 3	7	50	100	2.5	1	30	6.5	BSA

* P1 medium described by Asturiano et al. (2004)

In the third and last experiment (n = 6 pools), 25% of FBS was substituted by 5% of BSA in the medium 5 (containing $NaHCO_3$ 100 mM pH 6.5), which showed the best in the previous experiment becoming medium 7.

Freezing and thawing

All the freezing media were maintained at 4 ºC until the sperm dilution. Once diluted (1:2 sperm: freezing medium, v/v [25]), the samples were immediately packaged in 0.25 mL straws (IMV® Technologies, Láigle, Cedex, France), sealed with modelling paste and frozen in liquid nitrogen vapour, 1.6 cm above the liquid nitrogen level for 5 min, before being plunged into the liquid nitrogen for storage. Thawing took place in a 20 °C water bath for 15 s. Three straws were thawed and analysed for each pool.

Fluorescence stain analysis

Live/Dead Sperm Viability Kit (SYBR Green/Propidium Iodide (PI), Invitrogen) was used to evaluate the viability of spermatozoa pre- and post-cryopreservation. Briefly, 80 μL of SYBR Green (stock solution 2 μM) and 40 μL of PI (stock solution 24 μM) were mixed with 15 μL of fresh sample pools. After 10 min of incubation at room temperature in the dark, 150 μL of glutaraldehyde (0.5%) diluted in Dulbecco's phosphate buffered saline (DPBS) was added, acting as fixation solution.

At least 100 spermatozoa per sample were assessed in a Nikon Eclipse (E-400) epifluorescence microscope, using UV-2A (EX: 330-380 nm, DM: 400, BA: 420) filter. Spermatozoa were classified as dead when nuclei showed red fluorescence over sperm head, and live when they showed green fluorescence.

Spermatozoa morphometry analysis

Pre- and post-cryopreservation, a fraction of sperm samples was diluted 1:50 (v/v) with 2.5% glutaraldehyde in phosphate buffered saline fixative solution [33]. Slides were viewed using a 1000X negative phase contrast objective (Nikon Plan Fluor) on Eclipse E400 Nikon microscope. A Sony CCD-IRIS video camera transferred the image. Sperm morphology was analysed using ASMA software (Sperm Class Analyzer®, Morfo Version 1.1, Imagesp, Barcelona, Spain). Approximately 100 spermatozoa were analysed in each sample. The morphological parameters determined were: head perimeter (μm) and head area (μm^2).

Experimental design

The main aim of the present study was to inhibit the spermatozoa motility activation induced by osmolality changes caused by cryoprotectants (cryoprotectant activation) to preserve the energy spent before the cryopreservation, and in this way improving the post-thawing motility.

The study was divided in three experiments. In the first of them, two different percentages of ME_2SO (5, 10%), were probed at several $NaHCO_3$ concentrations and two different pHs: 8.5 and 6.5. In the second experiment, using the concentration of ME_2SO that showed the best results, higher $NaHCO_3$ concentrations were tested. Parallel, using the same samples of the second experiment, a third experiment was done. Taking as bases these previous results, it was decided to use $NaHCO_3$ 100 mM freezing medium to check the BSA effect in the sperm cryopreservation. The choice of this medium was because it was reported as the best freezing medium for European eel in preliminary unpublished experiments. Sperm pools were evaluated pre-freezing and post-thawing considering the motility, the percentage of live cells by fluorescent microscopy, and the morphometry of the spermatozoa head by ASMA.

Statistical analysis

Motility, viability and head morphometry parameters were subjected to analysis of variance (One-Way ANOVA, $p<0.05$), including as fixed effect dilution ratio and FBS addition for the first experiment. Cryoprotectant was added as fix element for the second experiment, and $NaHCO_3$ concentration for third one. The results are shown as mean ± standard error of the means (SEM). All statistical procedures were run using Statgraphics®Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

Results

Experiment 1

Pre-freezing

Figure 8.1A shows the inhibitory effect of $NaHCO_3$ on cryoprotectant activation, while the figure 8.1B shows the motility of the same sperm samples once activated with sea water, immediately after dilution in freezing medium containing ME_2SO. The inhibition of activation caused by ME_2SO was highly effective in the medium containing $NaHCO_3$ 80 mM pH 6.5 (medium 4). The motility showed different results depending on the percentage of ME_2SO. The results obtained in samples diluted in media containing 5% of ME_2SO did not registered significant differences with the activated fresh pools, while most of the media containing 10% of ME_2SO provided significant lower motilities (Fig. 8.1B).

Post-thawing

The pools showed significantly higher motilities in comparison with post-thawed samples, and statistical differences were also found between the media tested. At pH 6.5, the motility of samples frozen in media with ME_2SO 10% was higher with respect to 5% ($p<0.05$, Fig. 8.2A). Medium 4 plus ME_2SO 10% registered the highest motility (26.27 ± 3.85%), being significantly higher than medium 1 plus ME_2SO 10% (10.97 ± 2.04%, Fig. 8.2A).

The cryopreservation process reduced the percentage of live spermatozoa, but different freezing media caused no significantly different results (Fig. 8.2B). On one hand, the cryopreservation produced a decrease in the

spermatozoa head size (area and perimeter, Fig. 8.2C), but the use of the different media combinations affected in a similar way the spermatozoa head morphometry.

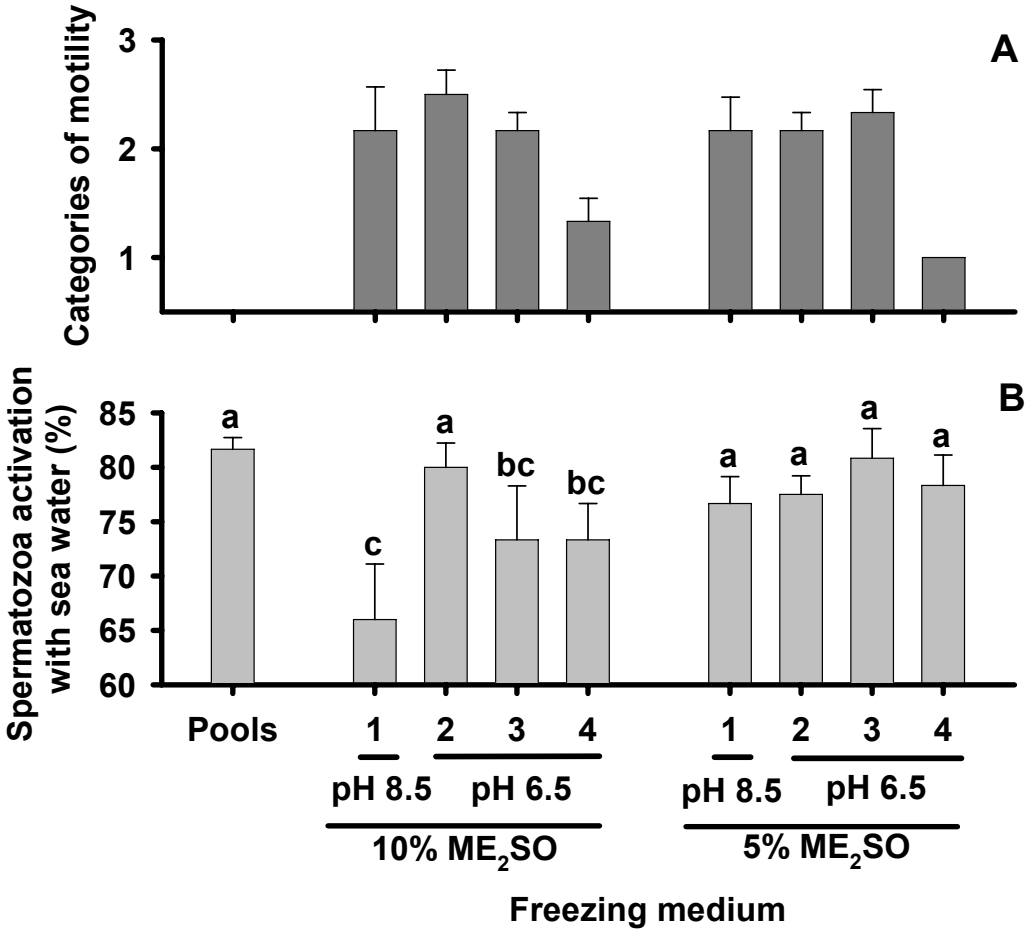

Figure 8.1. Inhibition effect on cryoprotectant activation induced by $NaHCO_3$ (A), and subsequent sperm activation with sea water (B). The effect of different ME_2SO percentages (5 and 10%) and pH (8.5 and 6.5) in the freezing media (1-4) are shown. Different letters means significant differences ($p<0.05$).

Experiment 2

Pre-Freezing

The inhibition of the cryoprotectant activation was $NaHCO_3$ doses-dependent. A total inhibition of cryoprotectant activation was achieved with the medium 6, containing $NaHCO_3$ 120 mM pH 6.5 (Fig. 8.3A), but when later these

samples were activated with sea water, showed a significant lower motility (70.00 ± 5.32%) than fresh samples (81.67 ± 2.79%).

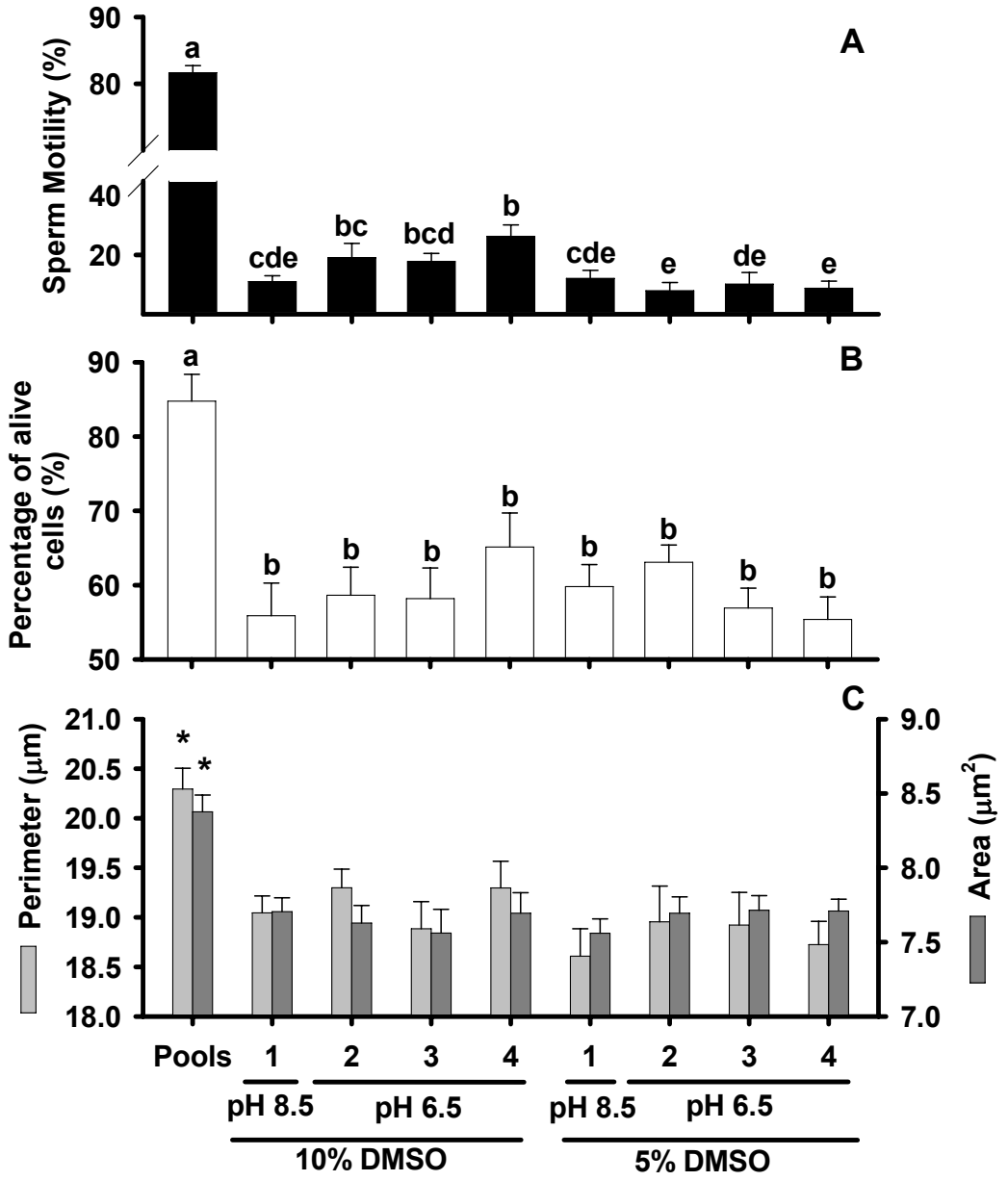

Figure 8.2.Sperm motility (A), percentage of live cells (B) and spermatozoa head morphometry (area and perimeter, C) analyzed post-thawing. The effect of two ME_2SO percentages (5 and 10%) and pH (8.5 and 6.5) in the freezing media (1-4) are compared. Asterisks or different letters means significant differences ($p<0.05$).

The motility of the samples diluted with the rest of the media did not show significant differences with respect the pools (Fig 8.3B).

Post-thawing

Cryopreservation caused a reduction of sperm motility in comparison with fresh samples. Samples frozen in media 4 and 5 showed significantly higher motilities (33.95 ± 3.73% and 38.26 ± 2.89%, respectively) respect the medium 1 (22.36 ± 1.08%), but without significant differences with the rest of the freezing media with pH 6.5 (media 2,3,4,5 and 6; Fig. 8.4A).

The cryopreservation process decreased the percentage of live cells independently of the medium used (Fig 4B). Cryopreservation also caused a reduction of the morphometry parameters (area and perimeter, $p<0.05$), although spermatozoa head area for the medium 2 containing $NaHCO_3$ 20 mM, did not show statistical differences with fresh pools cells, and no differences were found between the media (Fig. 8.4C).

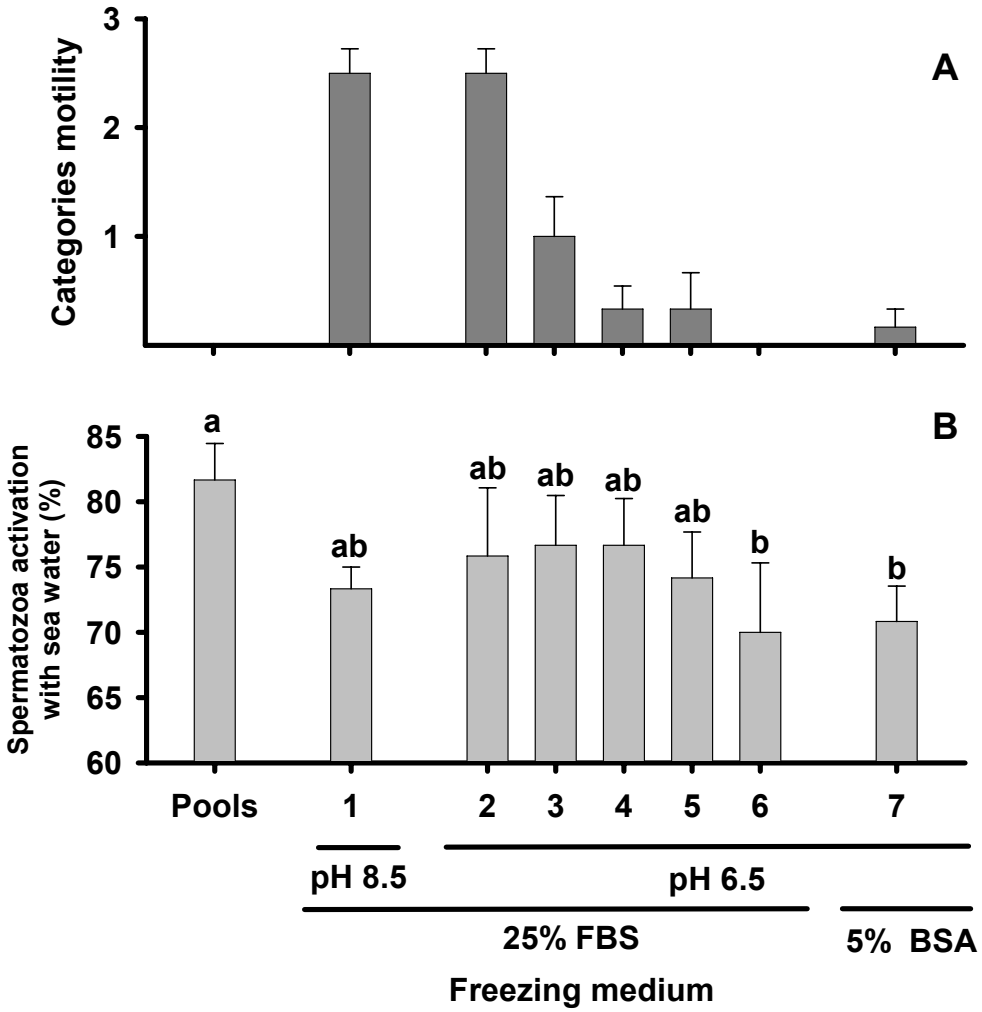

Figure 8.3. Inhibition effect on cryoprotectant activation induced by $NaHCO_3$ (A), and subsequent sperm activation with sea water (B) The effect of higher $NaHCO_3$ concentration in the freezing media (1-7) are shown. Also, the BSA (5%) was adding instead of FBS (25%) in the freezing medium 7. Different letters means significant differences ($p<0.05$).

Experiment 3

Pre-Freezing

Parallel to the second experiment and looking for obtaining better results, instead of 25% of FBS, 5% of BSA was added in the medium 3, containing $NaHCO_3$ 100 mM, pH 6.5, and forming the medium 7. It was not possible to avoid totally the cryoprotectant activation before the freezing, but it was reduced at very low levels (Fig. 8.3A). After activation with sea water the motility was significant lower than fresh samples, but not respect the rest of the media, including the medium 5 having the same composition but added with FBS (Fig. 8.3B).

Post-thawing

Significant worse post-thawing motility (21.66 ± 4.23%) was obtained respect to the same medium with FBS (medium 5), and without difference with some other media, including the medium 1 (Fig. 8.4A). The percentage of live cells and the spermatozoa head morphometry were statistical lower than those of fresh samples (Figs. 8.4B and 8.4C), but any difference was found with the rest of freezing media.

Discussion

To approach the cryopreservation, the presence of cryoprotectant is essential to avoid the injury of cell membranes. ME_2SO has been showed as a good cryoprotectant for European eel sperm [16,25], but it increases the medium osmolality [5,21], and this osmolality increase triggers the spermatozoa motility (called cryoprotectant activation, [26]). The cryoprotectant activation seems to spend partially the cell energy of the spermatozoa, and can be one of the causes of low post-thawing motility obtained until now. This activation can affect the sperm cryopreservation especially in this species because the time of spermatozoa motility is very short once diluted in sea water, from a few seconds to a few minutes [17,18,38]. Sodium bicarbonate was used because it was showed as an inhibitor factor of spermatozoa motility in the Japanese eel sperm and other marine species such as the turbot or flatfish [15,22,34,35]. In aqueous media $NaHCO_3$ is dissociated in several products: CO_2+H_2CO_3 (free-CO_2), HCO_3^- and $CO^2{}_3^-$, and their proportion is affected by pH. If the medium is acid, the most

of HCO_3^- will be converted in free CO_2, and this free CO_2 will act as inhibitor factor of the motility [35]. This effect has been observed in studies on sperm from invertebrates to mammals [11,23,24,39]

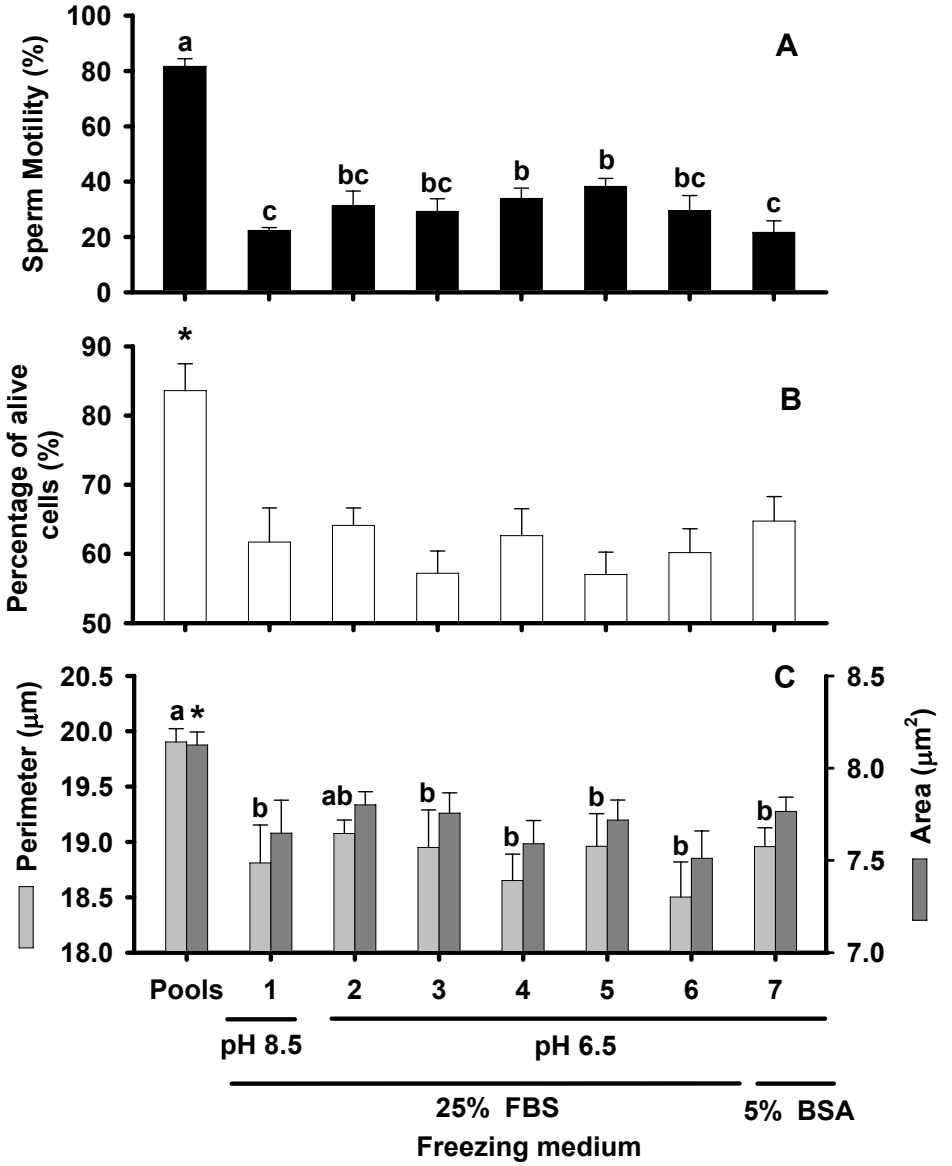

Figure 8.4. Sperm motility (A), percentage of live cells (B) and spermatozoa head morphometry (area and perimeter), C) analyzed post-thawing. The effects of higher NaHCO3 concentration in the freezing media (1-7) are compared. Also, the BSA (5%) was adding instead of FBS (25%) in the freezing medium 7. Asterisks or different letters means significant differences ($p<0.05$).

Taking as basis these physiology mechanisms, the pH of P1 medium (pH 8.5, [3]; called medium 1 in the present study) was reduced to 6.5, increasing its $NaHCO_3$ to raise the proportion of free CO_2 in the freezing medium. Low values of pH were not enough to arrest the sperm motility in contact with ME_2SO, and it was necessary to increase $NaHCO_3$ concentration until 80 mM to cause a reduction in the cryoprotectant activation effect. This reduction provided a significant higher post-thawing motility, being the unique freezing medium able to show significant differences respect to medium 1. Considering this, a total suppressing of cryoprotectant activation was tried. To achieve this objective, higher concentration of $NaHCO_3$ with low pH (6.5) were tested. The pre-cryopreservation motility was totally avoided with $NaHCO_3$ 120 mM, but without causing better post-thawing motility. It was achieved using $NaHCO_3$ 100 mM, with statistical difference with medium 1. Only, the media 4 ($NaHCO_3$ 80 mM) and 5 ($NaHCO_3$ 100 mM) registered differences with medium 1, being medium 5 which showed the best post-thawing motility. Higher number of samples could have been necessary to observe differences between them (media 4 and 5, respectively), but a positive increasing trend was observed when the concentration of $NaHCO_3$ was 100 mM (Fig 4A).

To check if the sperm can recover the motility when it was arrested in the freezing medium, before of cryopreservation, the diluted sperm was activated with sea water. The motility was recovered, but it was affected by the percentage of cryoprotectant and $NaHCO_3$ concentration. The motility observed in the media containing ME_2SO 5% was not different than fresh samples; while in the most of samples diluted with ME_2SO 10% media was significantly lower. ME_2SO usually is employed because of its membrane permeability; and for its preservation of the integrity of isolated proteins and phospholipids membranes [2,6,28]. Nevertheless, if it is added to the medium, increases the osmolality causing a dehydration of cells, and this phenomenon can affect the cell survival [14]. A significant morphometry decrease was reported when the European eel sperm was diluted in ME_2SO-containing freezing medium, as a consequence of this osmotic stress [5]. In the European sea bass, prior to freezing, no effect of ME_2SO concentration (2.5, 5 and 10%) was observed on the percentage of live cells, but a different response was observed on mitochondrias. ME_2SO 10% caused lower mitochondrial functionality than minor percentage of ME_2SO (2.5 or

5%), in both pre- and post-freezing samples [20]. On one hand, the cellular ATP content decreased when higher cryoprotectant concentrations in the medium [20]. These results could explain why in the present study the lowest ME_2SO percentage (5%) showed better motilities in pre-freezing conditions.

In the second experiment, the diluted sperm in medium 6 (with the highest NaHCO3 concentration) registered significantly lower motility when the samples were activated with sea water. One possible reason could be that the concentration of free-CO_2 was too high in this medium, and this ion decreased the pH too much to recover the motility. For the eel sperm motility, a high pH is considered a preliminary condition, previous to the activation, since the influx of H^+ in the cell plays an important role in the spermatic movement [34].

Both ME_2SO concentrations were tested to find the best percentage for the cryopreservation. In pre-freezing conditions ME_2SO 5% provided better results, but in post-thawing conditions, significant higher motilities were found in the media containing ME_2SO10%. It seems that 5% of ME_2SO was not enough concentration to protect the spermatozoa during the cryopreservation. One possible explanation can be that, although higher percentages could be toxic for the spermatozoa, a 10% of ME_2SO showed major protection on the cell during freezing and thawing processes.

The motility was affected by the percentage of cryoprotectant, but not other parameters like spermatozoa head morphometry or the percentage of live cells. Nevertheless, these parameters were significantly lower when they were measured in post-thawing conditions. Coinciding with He and Woods (2004) [20], the cryopreservation process reduced the viability ($p<0.05$), but the level of cryoprotectant concentration was not a significant factor.

The spermatozoa head area and perimeter were decreased by cryopreservation, although the head size was not affected by the percentage of ME_2SO. When the sperm is in contact with freezing media (with high osmolality), the cells shrink as water flows out [1], causing that the live spermatozoa shrink to reach the osmotic equilibrium, resulting in a reduction of head size. The dead cells are not be able to respond to stress or to maintain the isotonic volume, so the dead spermatozoa suffers a higher decrease than live cells in these conditions, with high osmolality, [24]. This phenomenon was observed in

European eel sperm when it was diluted in freezing medium containing ME_2SO, showing a significant decrease in head size respect to fresh samples [5].

The cryopreservation process provoked the loss of membrane integrity, causing cellular death, so the proportion of dead cells is higher than in fresh samples or pre-freezing conditions (Fig. 1B, 2B), causing a decrease in the mean morphometry of the sample (Fig. 2A, [5,24]). In the experiment 1 the difference between 5 and 10% of ME_2SO did not produce changes in the head size, but a significant decrease was induced by cryopreservation process. On one hand, when the post-thawing viability was studied, no differences were found between both percentages of ME_2SO. These results confirm that the difference between 5 and 10% of ME_2SO is not enough to affect these parameters (head size, viability), but a significant decrease respect to the post-thawing motility was produced with the lowest percentage of ME_2SO.

In previous studies [16,24], the addition of FBS in the freezing medium caused a positive effect in the European eel sperm cryopreservation. In the present study, BSA was tried by first time as membrane protector, considering the improvement of sperm cryopreservation results caused in other species as gilthead seabream (*Sparus aurata* [10]), European sea bass (*Dicentrarchus labrax* [29,40]). There is direct evidence that BSA adheres rapidly to the spermatozoa membrane at the moment of dilution [7], and modifies the sperm lipid composition through lipid exchange or hydrolysis [13].

In pre-freezing conditions, BSA (media 7) caused lower pre-freezing sea water activation and post-thawing motility when was compared with a similar FBS-containing medium (media 5). So, some proteins or lipids could exist in FBS medium that are not present in the BSA and this difference could be the responsible of improving the cell protection and motility, but further studies in this field are necessary.

In this present study, the results achieved with P1 medium at pH 8.5 ([16], 22.2±1.5%; medium 1), were improved obtaining approximately 40% of post-cryopreservation motilities. It was necessary to avoid the spermatozoa activation produced by the ME_2SO decreasing the pH and adding more concentration of $NaHCO_3$ in the medium ($NaHCO_3$ 100 mM, pH 6.5, ME_2SO 10% and FBS 25%, medium 5). Probably this percentage of motile cells is enough for fertilization thanks to the high density of eel sperm. Nevertheless, in the future the sperm

cryopreservation can be improved including cryoprotectant combinations or sugars in the freezing media [12,37].

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9 GENERAL DISCUSSION

One injection of hCG is enough to promote the spermatogenesis both in Japanese (Miura et al., 1991a) and European eel (Khan et al., 1987), but in both cases the individual response of the fish ahead of the treatment is not always the same. To try to explain which factors can influence in the maturation, different reproductive parameters were studied during the treatment. Due to any fish has been caught in the reproductive migration or in the spawning place, the physiological values during gonad development are unknown, so it was not possible to compare the results obtained with the natural values. Nevertheless, we have proposed a possible physiological model, taking as basis our results and studies performed in eels or other teleosts.

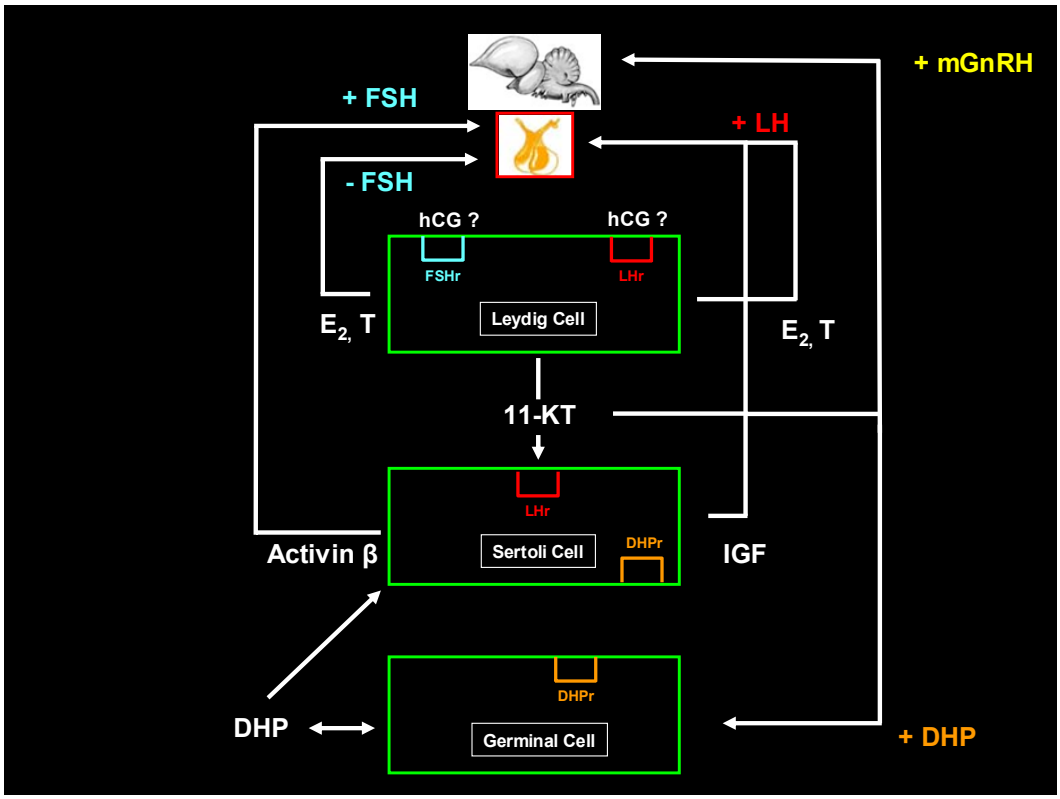

Figure 9.1. Possible physiologycal model in European eel male during gonadal maturation.

European eel does not achieve the gonadal maturation in captivity, being necessary to administrate hGC injections. Although hCG is considered an analogue gonadotropin of LH (Loosfelt et al., 1989), it is able to induce a complete spermatogenesis in male eel. One possible explanation could be that hCG is able of acting over in both gonadotropin receptors, explaining the high

variability in the fish response to the treatment, since hCG could have not the same effectiveness than natural FSH. Furthermore, in European eel female both FSH and LH receptors increased their expression in response to treatment with salmon pituitary extracts (Jeng et al., 2007).

Other possibility is that hCG could bind only on LHr, as it has been seen in African catfish and zebrafish, where hCG specifically activated LHr but not FSHr, while the homologous or recombinant LH recognized both receptors (Bogerd et al., 2001; Vischer and Bogerd, 2003; Kwok et al., 2005). Jeng et al (2007) also suggested that FSH receptor probably could not recognize hCG. In teleost LH can promote the androgen production (11-KT, Testosterone) like FSH does (Swanson et al., 1989; Planas et al., 1995; Maugars and Schmitz, 2008). In higher vertebrates, it is known that LH promotes the androgen synthesis by Leydig cells (Means et al., 1976; Gonzales et al., 1988), and in mammals, FSHr gene knockout mice can complete the spermatogenesis, being fertile animals (Kumar et al., 1997; Dierich et al., 1998; Abel et al., 2000; Krishnamurthy et al., 2000). In Japanese eel, *in vitro*, during the hCG-induced spermatogenesis the FSHr expression increased only slightly, what indicates that in eel males the hCG is not promoting its expression (Ohta et al., 2007). On the other hand, the effect of LH can be different depending on the fish gonadal stage of development. In salmonids, at the beginning of the spermatogenesis, LH can stimulate the androgen production, but at the final maturation and spawning this gonadotropin is more potent in stimulating DHP (Planas et al., 1995; Planas et al., 2000). These results could explain why the same gonadotropin, hCG, is able to induce the whole spermatogenesis in the male eel.

As a consequence of hCG injections, the 11-KT production is stimulated (Khan et al., 1987; Miura et al., 1991a; Ohta and Tanaka, 1997). In the present document one injection of hCG was enough to increase four times the 11-KT level, demonstrating the positive effect of this hormone in the stimulation of 11-KT production. The spermatogenesis can be achieved *in vitro* conditions by administration of 11-KT (Miura et al., 1991b) or in hypophysectimized European eel males by hCG injections (Khan et al., 1987). These results demonstrate that hCG has a direct effect on the gonad, and in artificial conditions the brain is not necessary to get a complete spermatogenesis. In contrast, when the

hypophysectimized European eel males were compared with no hypophysectimized males, the intact fish showed more sensibility to the hCG administration, especially in terms of induction of spermiation, suggesting the participation of positive feedbacks. One possibility could be that in artificial conditions the GnRHs and gonadotropin expression help to achieve a complete spermatogenesis (Ohta et al., 1997; Asturiano et al., 2005), but their presence is not necessary to finish the maturation process. It could be said that in the gonad of treated males are present the necessary factors to obtain the gonad development.

One function of the steroid 11-KT is the induction of the germinal proliferation (Miura et al., 1991a,b 1996; Miura and Miura, 2001, 2003), coinciding with the observed significant increase in the GSI, caused probably as a consequence of highest 11-KT levels. During the weeks where the highest peaks in 11-KT was observed (3rd and 4th), the cells were found in late meiosis and spermiogenesis process, steps in which is assumed that 11-KT plays no role. The European eel is not the unique species that showed this 11-KT level just before the spermiation period (Fostier et al., 1983; Ueda et al., 1983; Mayer et al., 1992; Amiri et al., 1996; Pavlidis et al., 2000; Amer et al., 2001; García-López et al., 2006), being suggested by some authors (Baynes et al., 1985; Malison et al., 1994) that this androgen could have a role in the maintenance of the spermatozoa viability or in the release of mature spermatozoa from testicular cysts into the lobular duct. So, it is possible that this androgen has more than one function in the spermatogenesis, although further research is necessary in this field.

In vitro conditions, the presence of 11-KT in the gonad is enough to induce a full spermatogenesis (Khan et al., 1987; Miura et al., 1991; Ohta and Tanaka, 1997). Also *in vitro* conditions, rjeFSH stimulated the 11-KT production in the gonad (Kamei et al., 2003; Ohta et al., 2007), being assumed that FSH promote the gonad maturation through the 11-KT.

The presence of DHP is necessary to finish the spermatogenesis (Miura et al., 1995a; Ohta et al., 1997), which is the responsible of meiosis process

(Miura et al., 2006) and the acquisition of motility by spermatozoa (Miura and Miura, 2003). *In vitro* conditions, 11-KT sitmulated the DHP synthesis on germinal cells, what could explain that this androgen is able to develop the spermatogenesis by itself. Moreover, these results also can explain why the gonad maturation is achieved in hypophysectimized males (Khan et al., 1987).

In the third experiment, the GnRHs and gonadotropin expression were studied. Despite their natural expression could be different than the results obtained in our experiment, we have tried to hypothesize their function in the maturation process.

The expression of GnRHs showed significantly higher values in diencephalon-mesencephalon during gonad development, but differents results were obtained for mGnRH and cGnRH. Coinciding with Dufour et al. (1993) and Montero et al. (1994), a significant increase was observed for mGnRH, while a decrease was registered for cGnRH. Although the mGnRH expression not always was correlated with peaks of LHβ expression, it can be considered as the principal GnRH responsible of gonadotropin release in the European eel. cGnRH-II suffered a decrease at the 4^{th} week of the treatment, but it is not clear the possible role of this GnRH variant during the gonad development. Maybe, its function in the maturation is supporting the hypophysiotropic role of the mGnRH.

The gonadal steroids can influence in the GnRH expression (Dufour et al., 1993; Amano et al., 1997) but the steroid effect depends on the physiological stage of development. This situation can be a possible explanation of why the 11-KT stimulated the GnRH release at the beginning of the gonadal maturation, but not when the gonad was in the more advanced stages of development.

It is not possible to know the real gonadotropin profile in natural conditions, but when our results are compared with the profile obtained in naturaly matured New Zealand longfinned eel or in other teleost (Mateos et al., 2003; Saito et al., 2003), the gonadotropin profile obtained by hCG injections can be considered abnormal. During the hCG treatment an over expression of LHβ (6 times) and a dramatic decrease of FSHβ expression (50 times; in both cases respect to the untreated males) were registered after only one injection of hCG,

what could cause a premature maturation, and so a low gonad development in some fish. One possible explanation of this gonadotropin profile is the hormone used for the maturation. hCG stimulates in the gonad the expression of E_2 receptor (possibly also the E_2 synthesis; Miura et al., 1999), and the production of the 11-KT and testosterone (Khan et al., 1987; Ohta and Tanaka, 1997; Miura et al., 1991a). Testosterone and E_2 (*in vivo*) have a negative effect in the FSHβ expression (Jeng et al., 2007), while 11-KT, T and E_2 have a positive effect in the LHβ expression (Dufour et al., 1983; Rebers et al., 1997; Schmitz et al., 2005; Jeng et al., 2007). On the other hand, the 11-KT stimulates the IGF-I synthesis (Miura and Miura, 2001) which induces the LH expression in the pituitary (Aroua et al., 2008). By different ways, the hCG has a positive effect in the LH expression and negative in the FSH expression.

The gonad development can be divided in two phases: the seven first weeks of treatment, when the spermatogenesis takes place, and the following weeks that can be classified as spermiation period. During the first period most of the analyzed parameters (LHβ expression, GSI, EI, HIS, quality sperm) were increasing, but in the second phase high variability was observed between fish. One possible explanation can be that in the second phase of the treatment the repeated hCG injections can be inducing an over-maturation in some fish. In this second phase the motility was high, coinciding with high levels of LHβ expression. Although both parameters registered similar evolution during the treatment, the LH is not the direct responsible of the motility, but the DHP progestagen, which production is stimulated by LH effect (Miura and Miura, 2001, 2003). For this reason, further studies about the role of progestagens will provide important information about sperm quality control, being a primordial work in the future.

The induction of reproductive maturation is performed in pubertal fish by hormonal methods. Maybe, some fish are too young (pre-pubertal) to begin the sexual maturation, what could explain the different response (LHβ expression, quality sperm, morphometric parameters) obtained in some cases.

As a consequence of the spermatogenesis process the sperm is produced, and it can be obtained by gentle abdominal pressure. The fertilization is its principal destination, but first it was necessary its study. The motility is considered the principal parameter (appart from fertilization rate) that defines the sperm quality, but in this document two parameters more were applied: percentage of live cells and spermatozoa head morphometry. Our group was the first to apply the ASMA analysis of head size in the European eel, but this species showed problems for its analysis due to the unusual spermatozoa morphology. To verify if this system can be used in this species, the results obtained with this program were compared with those obtained with scanning electron microscopy. The head perimeter and area were the morphometric parameters that we considered more accurate for this type of study. This technique has provided information about the head size during the hormonal induction treatment. The analysis showed higher head size between 8^{th}-11^{th} weeks of hCG treatment, coinciding with the highest motilities and percentages of live cells. When the spermatozoa head morphometry was analyzed in the 3^{rd} experiment, bigger head size and motilities were found coinciding with the most advanced stages of gonad development (S5, S6). Taking as basis these results, we can say that there is a coincidence between sperm quality and spermatozoa head size. On the other hand, the most important application of this technique is the possibility to check the cell response to processes as the cryopreservation or the dilution in a extender medium. We can conclude that this technique will be useful to work with sperm, but in the case of European eel, fertilization probes are necessary to know which is the optimum size to obtain high fertilization rates.

Applying these techniques (motility, size head and viability) we can define which weeks of the treatment showed a good sperm quality. The spermiation began at the 5^{th} week in the 1^{st} experiment and at the 4^{th} week in the 3^{rd} experiment. So we can consider this time as the beginning of sperm production. Nevertheless, the period with high sperm quality was different between experiments (8^{th}-11^{th}, experiment 1; 10^{th}-13^{th}, experiment 3). These changes can be due to the method of maturation, which is performed by hormonal injections, and not all the fish responded in the same way. Fish were obtained from a local fish farm, looking for a higher homogeinity between the groups used in the

different experiments. However, maybe the physiological stage at the beginning of the experiment was different in both experiment, causing dissimilar answer to the treatment.

This study will permit a better synchronisation with the female spawns, since the induction of maturation in eel females is performed with hormonal injections, and the moment of female spawn can be very different between fish. Depending of the hormone used in the treatment, the time of the female spawn can be found between 10^{th}-29^{th} weeks with salmon pituitary extracts (SPE) or 13^{th}-20^{th} weeks with carp pituitary extracts (CPE; Pedersen, 2003, 2004; Palstra et al., 2005; Perez et al 2007). Nevertheless, some problems are presented, as the difference between 1^{st} and 3^{rd} experiment respect to the weeks of the treatment that presented high sperm quality. On the other hand, the fresh sperm only can be maintained with good motility during several hours, since after 24 h it is close to zero. To try to solve these problems, different techniques to preserve the sperm were developed.

To extend the time maintaining good sperm motility, we designed one diluting medium (P1 medium) that can preserve the fresh sperm during several days. To develop this diluent, a medium was designed having the same ionic and osmotic characteristics of the European eel seminal plasma (Asturiano et al., 2004), since the sperm is maintained immotile and with high motility when is diluted in the seminal plasma. No differences in motility were observed respect to fresh samples for the sperm diluted in the P1 medium containing 2% of BSA during the first 48 h of incubation. But perhaps the most significant date, it is that after 1 week of incubation the percentage of live cells was not different than fresh samples, although the motility was around 30%. Unpublished results demonstrated that anaerobic and dark conditions caused better motilties than aerobic environment in eel sperm. These results are undermining the loss of ATP or O_2 as the cause of lower motilities at 1 week in aerobic conditions. One possible explanation could be the presence of free radicals, which are produced in aerobic reactions. The free radicals have a negative effect on the lipids, and could be affecting to some factor important for the sperm motility. These results open the doors to new research in this field, with the design of new media that

preserve a higher motility, since the cell can be maintained live during a long time.

Finally, a new method to cryopreserve the sperm was developed, which will permit to keep sperm with good quality during long time. Different methods have been previously tested by our group to cryopreserve the eel sperm (Marco-Jiménez et al., 2006; Asturiano et al., 2007; Garzón et al., 2008), but the post-thawing motility was not higher than 22.2 ± 1.5%, that we considered low. Garzón et al. (2008) demonstrated that the DMSO was the best cryoprotectant for European eel sperm, but DMSO has a problem. When the DMSO was added in the freezing medium the osmolality increases, causing the activation of the spermatozoa motility, and therefore the energy spend. To avoid this activation was necessary to decrease the pH, and to increase the $NaHCO_3$ concentration in the freezing medium. The medium that caused the highest post-thawing motility (38.26 ± 2.89%) contained $NaHCO_3$ 100 mM and pH 6.5. We consider this result as enough motility for the fertilization due to the high sperm density in this species.

In aqueous media $NaHCO_3$ is dissociated in several products: CO_2+H_2CO_3 (free-CO_2), HCO_3^- and $CO^2{}_3^-$, and their proportion is affected by pH. Depending which product is predominant in aqueous solution, $NaHCO_3$ can have a positive or negative effect on the motility. $NaHCO_3$ is a buffer that can modify the intracellular pH, which it is an essential factor controlling the sperm motility. Low values of pH_i inhibits the spermatozoa motility (Tanaka et al., 2004), since before starting the movement, a exchange K^+/H^+ is produced with the efflux of K^+ and the influx of H^+. If the concentration of H^+ inside the cell is so high (low pH_i), this exchange can be not achieved. When the medium is acid the $NaHCO_3$ is dissociated in high proportions in CO_2, which induces the decrease in the pH_i, and as consequence the motility is arrested. But if the pH medium is high (around 8.5), the principal component is HCO_3^- which increase the intracellular pH, and so induces the spermatozoa motility. Further studies are necessary in this field, since only a small part of the sperm movement physiology is known, especially in teleosts.

Two different methods of sperm preservation have been described, one for short time and another for long time. Each one will permit the application of new techniques inside eel reproduction. For example, the fertilization rate could be improved by the dilution of the sperm as was described in the Japanese eel by Ohta et al., (2001a), and in the case of cryopreservation, this technique would permit the creation of genetic banks.

On the other hand, all these methods of preservation need a last probe: the fertilization. But for the moment the poor quality of eggs obtained from European eel females under artificial conditions makes difficult this type of study.

Right now different groups are working in the European eel reproduction, providing a wide knowledge in this field. But due to the dramatic situation that this species is suffering, it is necessary further research. The study of new methods for eel sperm preservation and its reproductive physiology, not only will be important for eel reproduction but also to understand the reproductive physiology in other teleosts.

10 CONCLUSIONS

1. Cell staining techniques and morphometry ASMA analysis have been validated as useful tools for the evaluation of European eel sperm quality.

2. The hCG treatment (1.5 IU hCG/g body weight, weekly administration), induced the sperm production, showing high quality between 8^{th} and 13^{th} weeks of the treatment.

3. The treatment with hCG provides an abnormal gonadotropin profile, if its is compared with other teleosts.

4. mGnRH seems to be the responsible of gonadotropin release at the beginning of the hormonal treatment.

5. The 11-KT plays a role not only in the spermatogonial proliferation, but also in the late meiosis and spermiogenesis.

6. One medium able to maintain the fresh sperm during one week of incubation (4 ºC) with good motility (30%) was developed.

7. A new cryopreservation technique and one freezing medium able to arrest the sperm activation caused by DMSO, were developed, providing approximately 40% of post-thawing motile spermatozoa.

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