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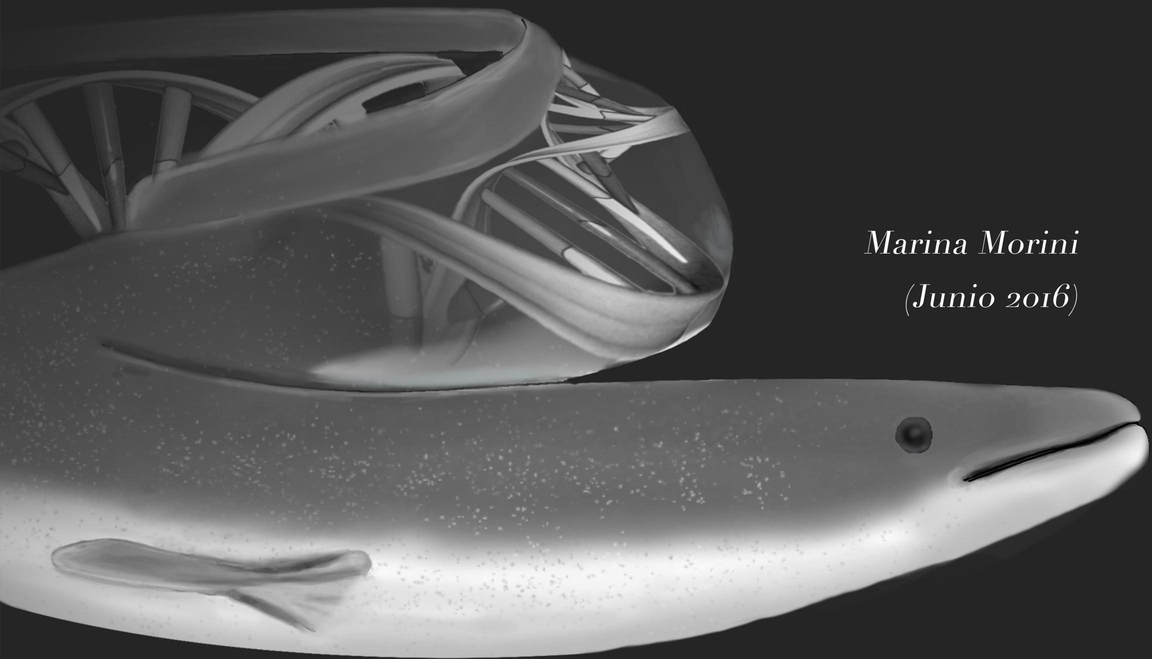
Molecular approaches related to the European eel (*Anguilla anguilla*) reproductive process



Marina Morini

(Junio 2016)

Directores:
Juan F. Asturiano Nemesio
David Sánchez Peñaranda

MOLECULAR APPROACHES RELATED TO THE EUROPEAN EEL (*Anguilla anguilla*) REPRODUCTIVE PROCESS

Marina Morini

This Thesis has been submitted in accordance with the requirements for the degree of Doctor at the Universitat Politècnica de València.

Esta tesis ha sido presentada para optar al grado de Doctor con Mención Europea por la Universitat Politècnica de València.

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ICTA

THESIS SUPERVISORS

Dr. Juan F. Asturiano Nemesio
Dr. David Sánchez Peñaranda

Grupo de Acuicultura y Biodiversidad
Instituto de Ciencia y Tecnología Animal
Universitat Politècnica de València

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TABLE OF CONTENTS

SUMMARY 1

RESUMEN 3

RESUM 5

GENERAL INTRODUCTION 8

1. Biological overview of the European eel 10

   1.1 European eel life cycle 10

   1.2 European eel phylogenetical position 12

   1.3 Eel status 13

   1.4 Eel reproduction in captivity 15

2. Gonadotropic axis 16

   2.1 Brain 17

   2.2 Pituitary 18

   2.3 Gonads 19

   2.4 Gametogenesis 22

3. Fertilization 23

4. Projects and grants involved in this Thesis 24

OBJECTIVES 26

CHAPTER 1 **Expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis** 30

CHAPTER 2	**Nuclear and membrane progestin receptors in the European eel: characterization and expression *in vivo* through spermatogenesis**	54
CHAPTER 3	**Temperature modulates testis steroidogenesis in European eel**	102
CHAPTER 4	**Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel**	130
	GENERAL DISCUSSION	154
	1. Brain, pituitary and gonadal control of European eel reproduction	156
	2. Evolutionary history of nuclear steroid receptors in vertebrates	159
	3. Interactions between steroid receptors	162
	4. The effect of temperature on European eel maturation and gamete quality	164
	FUTURE PERSPECTIVES	166
	CONCLUSIONS	168
	REFERENCES	174

SUMMARY

The European eel (*Anguilla anguilla,* L., 1758) population is in dramatic decline, so much so that this species has been listed as "Critically Endangered" on the Red List of Threatened Species, by the International Union for Conservation of Nature (IUCN). The European eel has a complex life cycle, with sexual maturation blocked in the absence of the reproductive oceanic migration, and an inability to mature in captivity without the administration of hormonal treatments. Even though experimental maturation induces gamete production of both sexes, the fertilization results in infertile eggs, unviable embryos and larvae, which die within a few days of hatching. Therefore, understanding the eel reproductive physiology during maturation is very important if we want to recover the wild eel population. Furthermore, due to its phylogenetic position, representative of a basal group of teleosts, the Elopomorphs, the *Anguilla* species may provide insights into ancestral regulatory physiology processes of reproduction in teleosts, the largest group of vertebrates.

In this thesis, characterization, phylogeny and synteny analyses have given us new insight into the evolutionary history of the reproductive process in vertebrates. The European eel possesses five membrane (mPRs) and two nuclear (nPR or pgrs) progestin receptors. Eel mPRs clustered in two major monophyletic groups. Phylogeny analysis of vertebrate nPRs and PLCζ1 (sperm specific protein) places both eel PLCζ1 and nPR sequences at the base of the teleost clade, which is consistent with the basal position of elopomorphs in the phylogeny of teleosts. To further resolve the origin of the duplicated eel nPRs, synteny analyses of the nPR neighboring genes in several vertebrate genomes were performed. Phylogeny and synteny analyses allowed us to propose the hypothesis that eel duplicated nPRs originated from the 3R.

In order to gain a better understanding of the role of the genes implicated in eel reproduction, analyses of their regulation during experimental maturation were carried out. The change in salinity induced parallel increases in E_2 plasma and nuclear estrogen receptor expression levels, revealing a stimulatory effect of salinity on the E_2 signalling pathway along the BPG axis, leading to a control of

spermatogonial stem cell renewal. Brain and pituitary estrogen receptors may then mediate the stimulation of androgens and steroidogenic enzymes linked to androgen synthesis. Androgen synthesis is not dependent on temperature, but further maturation requires higher temperatures to induce a change in the steroidogenic pathway towards estrogen and progestin synthesis. This is consistent with our studies on estrogen and progestin receptors. In the testis, progestin seems to regulate meiosis through membrane and nuclear progestin receptors, and final sperm maturation seems to be controlled by both estrogen and progestin through the estrogen and progestin membrane receptors. Finally, eel sperm-specific PLCζ1 seems to have an important function in spermatozoa by inducing egg activation and temperature may play a role in its regulation, especially during the process of spermiogenesis.

This thesis attempts to evaluate the physiological function of the genes involved in eel reproduction during spermatogenesis, and demonstrates that salinity and temperature play crucial roles in the sexual maturation of the male European eel.

RESUMEN

La anguila europea (*Anguilla anguilla,* L., 1758) está sufriendo un declive dramático y ha sido incluida en la categoría de especies "En peligro crítico" en la Lista Roja de Especies Amenazadas, por la *International Union for Conservation of Nature* (IUCN). La anguila europea tiene un ciclo de vida complejo, con un bloqueo de la maduración sexual que se mantiene hasta que se produce la migración reproductiva, y no madura en cautividad sin la aplicación de tratamientos hormonales. Pero incluso cuando la inducción de la maduración sexual conlleva la producción de gametos de ambos sexos, los resultados de la fertilización son huevos no fértiles, embriones no viables, o larvas que mueren pocos días después de la eclosión. Por tanto, la comprensión de la fisiología reproductiva de la anguila durante la maduración es imprescindible para recuperar sus poblaciones naturales. Además, dada su posición filogenética, como representantes de un grupo basal de los teleósteos, los elopomorfos, las especies del género *Anguilla* podrían proporcionar nuevas perspectivas sobre los procesos ancestrales de regulación de la fisiología de la reproducción de los teleósteos, el mayor grupo de vertebrados.

En esta tesis, los resultados de caracterización, análisis de filogenia y sintenia ofrecen nuevas perspectivas de la historia evolutiva del proceso reproductivo de los vertebrados. La anguila europea posee cinco receptores de progestágenos de membrana (mPRs) y dos nucleares (nPR o pgrs). Los mPRs de la anguila se engloban en dos grandes grupos monofiléticos. Las filogenias de los nPRs y de la PLCζ1 (una proteína específica del esperma) sitúan a las secuencias de la anguila de PLCζ1 y de nPRs en la base del grupo de los teleósteos, lo que coincide con la posición basal de los elopomorfos en la filogenia de los teleósteos. Para resolver el origen de la duplicidad de los nPRs de anguila, se realizaron análisis de sintenia de los genes próximos a los nPRs, en los genomas de varios vertebrados. Los análisis de filogenia y sintenia nos permitieron formular la hipótesis de que los nPRs duplicados de la anguila se originaron en la 3° duplicación del genoma que se produjo en teleósteos.

Para entender mejor el papel de los genes implicados en la reproducción de la anguila, se hicieron análisis de su regulación durante la maduración experimental. El cambio de salinidad indujo aumentos paralelos del nivel plasmático de E_2 y de la expresión de los receptores nucleares de estrógenos, que refleja un efecto estimulador de la salinidad sobre la ruta de señalización del E_2 dentro del eje cerebro-hipófisis-gónada, que conlleva el control de la renovación de las espermatogonias indiferenciadas. Los receptores de estrógeno en el eje cerebro-hipófisis-gónada podrían así mediar la estimulación de la síntesis de andrógenos y de los enzimas esteroidogénicos unidos a ella. Esa síntesis de andrógenos no depende de la temperatura, pero la continuación del proceso de maduración requiere de temperaturas más altas para inducir un cambio en las rutas esteroidogénicas hacia la síntesis de estrógenos y progestágenos. Esto coincide con nuestros estudios sobre receptores de estrógenos y de progestágenos. En el testículo, los progestágenos parecen regular la meiosis mediante la participación de los receptores de progestágenos de membrana y nucleares, y la maduración final del esperma parece estar controlada tanto por estrógenos como por progestágenos mediante los receptores de estrógenos y de progestágenos de membrana. Finalmente, la PLCζ1 específica del esperma de anguila podría tener una importante función en la activación del huevo inducida por el espermatozoide, y la temperatura podría jugar un papel en su regulación, especialmente durante el proceso de espermiogénesis.

Esta tesis intentó evaluar la función fisiológica de los genes implicados en la reproducción de la anguila durante la espermatogénesis, y demuestra que la salinidad y la temperatura juegan papeles cruciales en la maduración sexual de los machos de anguila europea.

RESUM

La població d'anguila europea (*Anguilla anguilla*, L., 1758) està sofrint un declivi dramàtic, i aquesta espècie ha estat inclosa en la categoria d'espècies "En perill crític" en la Llista Roja d'Espècies Amenaçades per la *International Union for Conservation of Nature* (IUCN). L'anguila europea té un cicle de vida complex, amb un bloqueig de la maduració sexual que es manté fins que es produeix la migració reproductiva, i no madura en captivitat sense l'aplicació de tractaments hormonals. Però, fins i tot quan la inducció de la maduració sexual comporta la producció de gàmetes d'ambdós sexes, els resultats de la fertilització son ous no fèrtils, embrions no viables o larves que moren pocs dies després de l'eclosió. Per això, la comprensió de la fisiologia reproductiva de l'anguila durant la maduració és imprescindible per aconseguir la recuperació de les poblacions naturals d'anguila. A més, donada la seua posició filogenètica com a representant d'un grup basal de teleostis, els elopomorfos, les espècies del gènere *Anguilla* podrien proporcionar noves perspectives al voltant dels processos ancestrals de regulació de la fisiologia de la reproducció dels teleostis, el grup més nombrós dels vertebrats.

En aquesta tesi, els resultats de caracterització i l'anàlisi de la filogènia i la sintènia ofereixen noves perspectives de la història evolutiva del procés reproductiu dels vertebrats. L'anguila europea posseeix cinc receptors de progestàgens de membrana (mPRs) i dos nuclears (nPR o pgrs). Els mPRs de l'anguila s'engloben en dos grans grups monofilètics. L'anàlisi filogenètic dels nPRs i de la PLCζ1 (una proteïna específica de l'esperma) de l'anguila respecte a les de la resta de vertebrats situa a les seqüències d'aquestes proteïnes en la base dels grups dels teleostis, la qual cosa coincideix amb la posició basals dels elopomorfos en la filogènia dels teleostis.

Per tal de resoldre l'origen de la duplicitat dels nPRs de l'anguila, es realitzaren anàlisis de sintènia dels gens pròxims als dels nPRs en els genomes de diversos vertebrats. Aquests anàlisis ens permeteren formular la hipòtesi de que els nPRS duplicats de l'anguila es van originar en la tercera duplicació del genoma que es va produir en teleostis.

Per arribar a entendre millor el paper dels gens implicats en la reproducció de l'anguila, s'analitzà la seua regulació durant la maduració experimental. Els canvis en la salinitat induïren augments en paral·lel del nivell plasmàtic d'E_2 i de l'expressió dels receptors nuclears d'estrògens, reflectint un efecte estimulador de la salinitat sobre la ruta de senyalització d'E_2 en l'eix cervell-hipòfisi-gònada, que comportaria el control de la renovació dels espermatogonis indiferenciats. Els receptors d'estrògens en l'eix cervell-hipòfisi-gònada podrien, d'aquesta forma, intervindre en l'estimulació de la síntesi d'andrògens i dels enzims esteroidogènics units a la síntesi d'andrògens. Aquesta síntesi d'andrògens no depén de la temperatura, però la continuació del procés de maduració requereix de temperatures més altes per induir un canvi en les rutes esteroidogènics cap a la síntesi d'estrògens i progestàgens. En els testicles, els progestàgens pareixen regular la meiosi mitjançant la participació dels receptors de progestàgens de membrana i nuclears, i la maduració final de l'esperma sembla estar controlada tant pels estrògens com per progestàgens de membrana. Finalment, la PLCζ1 específica de l'esperma de l'anguila podria tindre una funció de rellevància en l'activació dels ous induïda pels espermatozoides, i la temperatura podria tindre el seu paper en la regulació d'aquesta, especialment durant el procés de l'espermiogènesi.

Aquesta tesi ha avaluat la funció fisiològica dels gens implicats en al reproducció de l'anguila durant l'espermatogènesi, i ha demostrat que la salinitat i la temperatura tenen papers clau en la maduració sexual dels mascles d'anguila europea.

GENERAL INTRODUCTION

1. Biological overview of the European eel

1.1 European eel life cycle

Due to its unique life cycle, the European eel is a particularly interesting model for the investigation of the regulatory mechanisms of reproductive physiology. The European eel (*Anguilla anguilla* L., 1758*)* is a catadromous species, with a complex, atypical and poorly understood life cycle (Fig. 1).

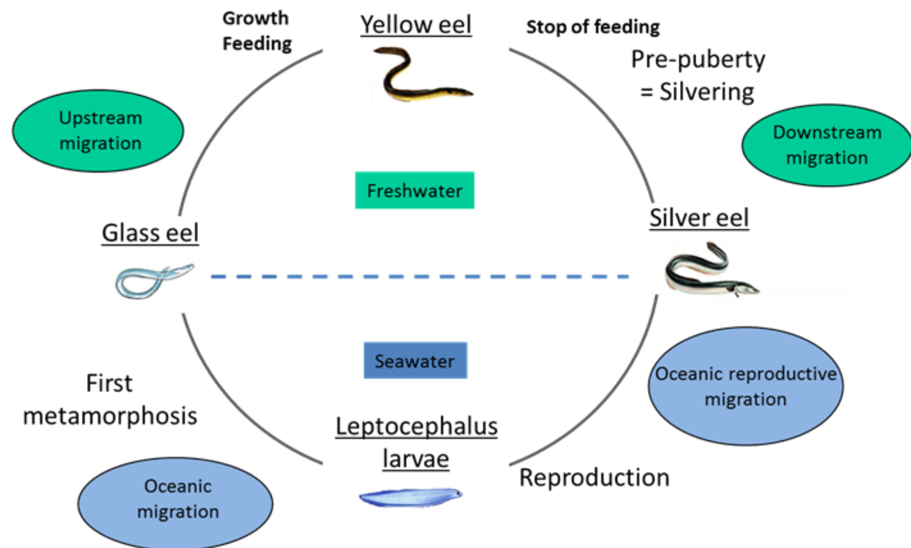

Figure 1. European eel life cycle

Neither European eel eggs nor spawning adults have ever been collected; indeed, the smallest larvae ever caught were from the Sargasso Sea. The larvae, called leptocephalus, has a laterally compressed body and looks like a leaf with a small head. They are planktonic, and are transported by the Gulf Stream to the coastal waters of Europe and Northern Africa, where they metamorphose into small, thin and unpigmented glass eels (Tesch, 2003). At this stage, the glass eels display the anguilliform shape. They migrate into coastal waters and estuaries mostly between October and March/April, and turn into the pigmented elver stage eels. The elvers migrate into continental waters between May and September. As they grow larger

they become known as yellow eels. The yellow eels undergo a sedentary and feeding phase in freshwater prior to metamorphosis into the silver eel stage (called silvering). Yellow eels can stay in freshwater from two to twenty-five years (Asturiano et al., 2011), in some cases even exceeding 50 years depending on the habitat and growth conditions.

Silvering is a puberty related event which marks the beginning of sexual maturation, migration and the reproductive phase (Dufour et al., 2003; Aroua et al., 2005). Silvering is marked by a change in skin colour, with the eels becoming similar to pelagic fish. The belly, initially yellow, turns silvery white, and the back and the sides, initially dark brown or green, become black. Other changes mark the pelagic life of silver eels: enlarged eyes (to improve vision at great depths), black elongated pectoral fins, an increase in skin thickness and a more visible lateral line. Finally, because silver eel stop feeding during their migration, the digestive tract degenerates. Silver eels are still sexually immature when they start their reproductive migration, with sexual maturation occuring during the supposedly 6-7 month migration period (Tesch, 2003; van Ginneken and Maes, 2005). Female silver eels are twice as large and may also be twice as old as the males (Tesch, 2003; van den Thillart and Dufour, 2009).

The eel migration across the Atlantic ocean to reach what is believed to be the Sargasso Sea (5.000-6.000 km) is influenced by various factors, most importantly the decrease in temperature of the autumn, but also by the moon-phase, atmospheric conditions, the decrease in hours of daylight and an increase in water discharges (Tesch, 2003; Bruijs and Durif, 2009). After the migration, the matured eel spawn and probably die after spawning.

Sexual maturation and gonadal development of wild eel probably happen at low temperatures during the oceanic migration, as eels migrate at depths of between 200-600 m and temperatures between 10-12 °C (Aarestrup et al., 2009). However, the spawning probably takes place at high temperatures, as it is known that the temperature of the supposed spawning area in the Sargasso Sea is about 20 °C (Boëtius and Boëtius, 1967, 1980). The impact of the temperature on the European eel maturation process has been demonstrated both in

females (Pérez et al., 2011; Mazzeo et al., 2014) and in males (Gallego et al., 2012, 2014; Baeza et al., 2014), with a clear effect on ovary development observed.

Little is known about the eel´s reproductive migration. As a consequence, it is very difficult to replicate the environmental factors which occur during this migration, such as temperature, photoperiod or hydrostatic pressure. After the silvering stage, dopaminergic inhibitions in addition to a deficient stimulation of gonadotropin-releasing hormone (GnRH) block the eel sexual maturation as long as the reproductive oceanic migration is not performed (Dufour et al., 1988, 2005; Pasqualini et al., 2004; Vidal et al., 2004). So, in captivity, eels are blocked in a pre-pubertal stage and do not spontaneously mature (Dufour et al., 2003; Montero et al., 1996). To induce sexual maturation and gonadogenesis it is necessary to use chronic hormonal treatments, usually weekly injections of carp/salmon pituitary extract for females (Asturiano et al., 2002, Fontaine et al., 1964; Pedersen, 2003) and weekly injections of human chorionic gonadotropin (hCG) for male eels (Asturiano et al., 2005; Boëtius and Boëtius, 1967; Gallego et al., 2012; Huang et al., 2009; Ohta et al., 1996, 1997a; Pérez et al., 2000).

1.2 European eel phylogenetical position

The European eel *Anguilla anguilla* is a member of the Elopomorpha superorder (Greenwood et al., 1966), a diverse group of predominantly marine teleost fishes comprising about 1.000 species, placed in 25 families (Chen et al., 2014; Nelson, 2006). The European eel form part of the Anguilliforme order, and the family Anguillidae. The Anguillidae contains a single genus, *Anguilla*, which comprises about 18 species distributed in tropical, subtropical and temperate areas from all over the world, except the western coasts of North America and South America and the South Atlantic. Although the phylogenetic relationship between the representants of the genus is still uncertain, the genus *Anguilla* has a monophyletic origin (Minegishi et al., 2005) estimated at 20–50 million years ago.

From an evolutive point of view, the eels, including the European eel,

branch at the base of the teleosts. Due its phylogenetical position (Fig. 2), studies on this species may provide insights into ancestral regulatory functions in teleosts, the largest group of vertebrates (Henkel et al., 2012a). Thus, the European eel is a relevant model which may have conserved characteristics that are less derived than those of most other teleost groups, providing information on ancestral vertebrate physiological regulations.

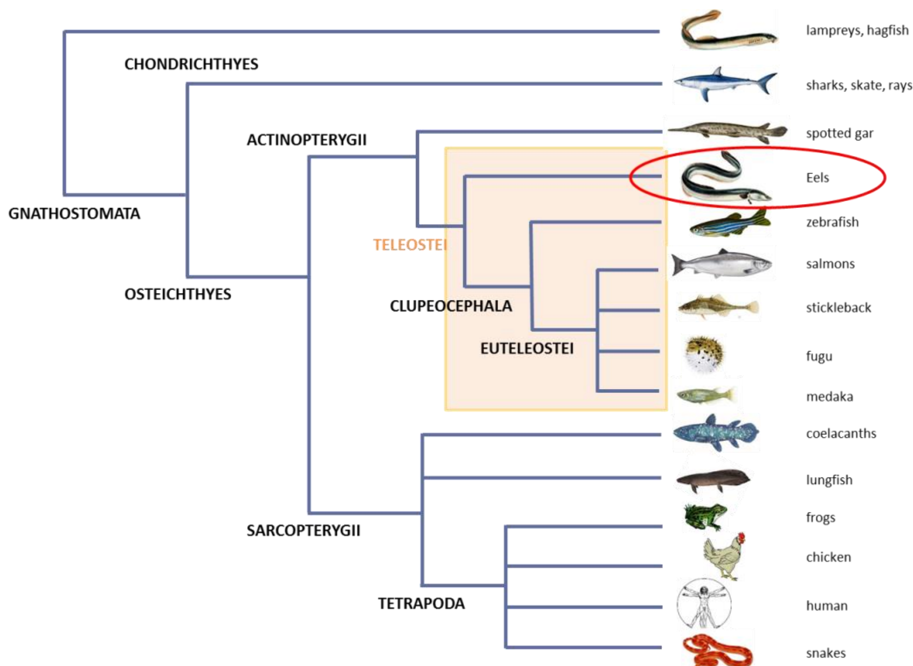

Figure 2. Vertebrate evolutionary tree

1.3 Eel status

The European eel is an important species for European aquaculture, with a production of 6.500 Tm/year (FAO, 2013).

In 2006, the ICES (International Council for the Exploration of the Sea) advised that the stock was outside of safe biological limits and that current fisheries were not sustainable. In 2009, ICES advised that the level of the eel stock for all stages including glass eel, yellow eel and silver eel was at a historical minimum. The reasons for this decline are

uncertain but may include overexploitation, pollution, non-native parasites and other diseases, migratory barriers and other habitat loss, mortality during passage through turbines or pumps, and/or oceanic-factors affecting migrations. The management plan proposed had to take into account the diversity of causal factors implicated to ensure the protection and sustainable use of the population of the European eel.

The European eel stock has been suffering a gradual decline for at least half a century and from 1980 to 2010 recruitment declined sharply. The European eel stock has decreased by 95-99%, compared to its levels in 1960-80 (ICES 2013), leading to the listing of the species as "Critically Endangered" on the Red List of Threatened Species, by the International Union for Conservation of Nature (IUCN). The European eel has also received attention from the European Union, which in 2007 published a regulation (Reglament 1100/2007, 18th September 2007) establishing measures for the recovery of the stock of European eel. This regulation was mandatory in all aspects for all the state members of the EU. Since then, every state has elaborated their Management Plan for the European eel. For instance, the Spanish plan, elaborated by the different autonomic communities, was approved by the EU in 2010, and included measures for habitat restoration, reintroductions, and fishery restrictions.

Also, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed the European eel in 2007 as species "not necessarily threatened with extinction, but in which trade must be controlled to avoid utilization incompatible with their survival'' in Appendix II (CITES 2007). European eel can not be exported to non EU countries.

Although the recruitment indices have increased in the recent three years, they are still only between 4 and 12% of the average levels recorded between 1960–1979. Due to the eel´s long lifespan, the impact of management actions on mortality indicators is visible immediately, but at least 5–10 years are necessary before being able to notice any effect of management measures on the glass eel or yellow eel stocks (Joint EIFAAC/ICES/GFCM WGEEL REPORT 2014).

1.4 Eel reproduction in captivity

Eel aquaculture started in 1879 in Japan (Matsui, 1952) followed by Italy and France (Gousset, 1990; Ciccotti and Fontenelle, 2001). Total world eel aquaculture production is about 250.000 Tm/year, but it mainly involves Japanese eel, with European eel production only accounting for about 5.000 Tm/year (FAO statistics). Nevertheless, eel farming still depends on the fishing of juvenile specimens, such as glass eels or elvers from the wild. Glass eels or elvers are stocked in recirculation systems at 25-28 ºC, and over a period of 18-21 months grow to reach commercial size; 120 grams in Spain (Pérez et al., 2004), and higher in the northern Europe.

The first artificial maturation of Japanese eel (*Anguilla japonica*) occurred in Japan in the 1960s (Tanaka et al., 2003); and the first fertilised eggs and larvae from Japanese eel were successfully obtained by Yamamoto and Yamauchi (1974) using hormonal treatments. However, the larvae did not feed, and the transition into leptocephalus larvae did not occur. In 2001, Tanaka et al. reared feeding larvae and succeeded in the production of leptocephali. The production of glass eel stage specimens and even further to the yellow eel stage was first obtained in 2003 (Tanaka et al., 2003). In 2010, these Japanese researchers reported that they had successfully closed the Japanese eel life cycle in captivity, by producing glass eels from farmed eels. Nevertheless, the egg quality is unstable, and the survival rates of the larvae are usually extremely low.

Concerning the European eel, many efforts have been made to reproduce this species in captivity. In contrast to the Japanese eel, European eels show great individual variability and much slower response to hormonal stimulation (Palstra et al., 2005). In the 1930s, artificial maturation in male eels was achieved for the first time by Maurice Fontaine the (Fontaine, 1936). Spermatogenesis and spermiation of the European eel were obtained by intraperitoneal injections of urine extract from pregnant women. In 1980, Boëtius and Boëtius were the first to obtain fertilized European eel eggs, and Bezdenezhnykh et al. (1983) to obtain the first larvae. Nevertheless, the experimental maturation resulted in infertile eggs, and unviable

embryos and larvae dying within a few days of hatching. Fertilized eggs were further obtained by artificial breeding in 1997 (Amin et al., 1997). In 2001, Asturiano et al. (2002) were the first to achieve ovulation and spawning of the European eel by the "Japanese method" (Ohta et al., 1997a). In Denmark, several experiments have resulted in the fertilisation of eggs and the development of larval stages, but death has ensued before or within a few days of hatching (Palstra et al., 2005; Pedersen et al., 2003). Later, from 2010 to 2014, the European project PRO-EEL (Reproduction of European eel: towards a self-sustained aquaculture) was performed in order to expand the knowledge base on European eel reproduction and to develop standardised protocols for the controlled production of viable eggs and the culture of larvae. Within the framework of this European project, the Towkiewicz group from Denmark was able to produce larvae, which remained alive for up to 22 days (Butts et al., 2014). Also, Pérez et al. from the Aquaculture and Biodiversity of Valencia group (GAB) obtained the first fertilization, hatching and larvae in Spain (Pérez et al., 2012).

So, at the moment, it is impossible to reproduce the European eel in captivity. Considering the dramatic decline in the wild eel populations (ICES 2013), understanding the mechanisms that control eel reproduction is very important in order to improve egg and sperm quality and to succeed in closing the eel life cycle in captivity. Achieving a commercial production of glass eels is imperative if we are to reduce the pressure on the wild population and to preserve and enhance the wild stock.

2. Gonadotropic axis

In European eel, as in all teleosts, reproduction is controlled by the gonadotropic axis or BPG (Brain-Pituitary-Gonad) axis, in which stimulatory or inhibitory effects are regulated by three connected constituents: the brain, the pituitary and the gonads (Fig. 3).

2.1 Brain

In the brain, in response to environmental cues, different factors are produced to exert stimulatory or inhibitory effects on reproduction (Zohar et al., 2010). The neuropeptide gonadotropin-releasing hormone (GnRH), involved in regulating vertebrate reproduction, is released and triggers the release of gonadotropins by the pituitary gland. The protein Kisspeptin seems to play an important role in the onset of puberty, by activating the release of GnRH in vertebrates, including matured eels (Pasquier et al., 2012). Kisspeptin may also act directly on the pituitary through an inhibitory effect on LHβ expression (Pasquier, 2011).

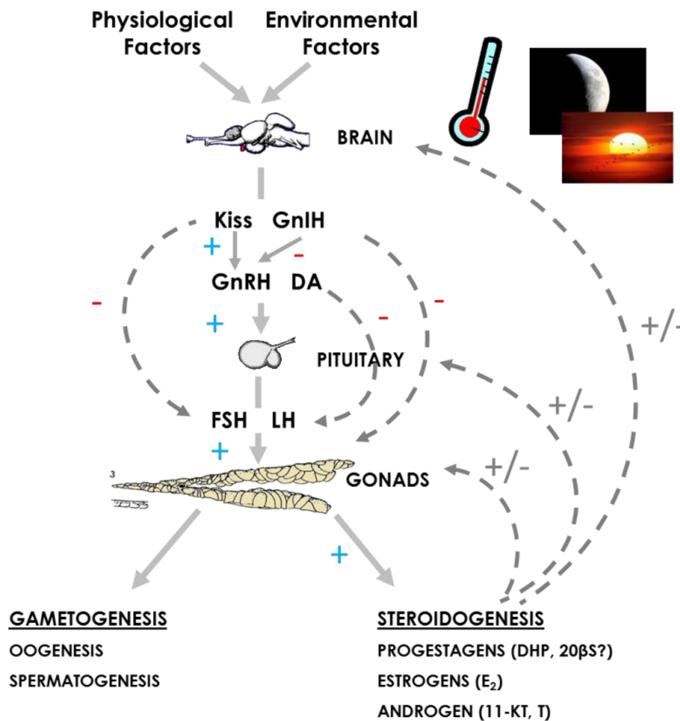

Figure 3. The Brain-Pituitary-Gonad (BPG) axis

The gonadotropin-inhibitory hormone (GnIH) acts on the pituitary and GnRH neurons to inhibit reproductive functions by decreasing the release and synthesis of gonadotropin (reviewed by Tsutsui et al., 2007). Finally, the neurotransmetter dopamine (DA) is known to have an important inhibitory effect on both LH synthesis and secretion (Dufour et al., 1988; Vidal et al., 2004). Although brain factors (neuropeptides, neurotransmitters) have been shown to stimulate the release of gonadotropin, studies indicate that the relative effects on gene transcription of the FSH and LH subunits depend upon the species, sex and reproductive status of the fish.

2.2 Pituitary

The pituitary secretes two gonadotropins (GTHs), the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), which act through their specific membrane receptors, FSHR and LHR, in the gonads. In vertebrates, the GTHs induce steroidogenesis and gametogenesis (reviewed by Schulz et al., 2001).

Together with the thyroid stimulating hormone (TSH), FSH and LH are members of the pituitary glycoprotein family. They are composed of a common subunit α and a specific subunit β (Quérat et al., 2000). Their specific receptors are members of the superfamily of G-protein-coupled receptors, which contain seven transmembrane domains (TMD) (reviewed by Oba et al., 2001). In mammals, both LH and FSH are expressed by the same gonadotropic cells but act on different cell types and have different functions. In contrast to mammals, teleost LH and FSH are expressed in separate gonadotropic cells (Schmitz et al., 2005).

LH and FSH have different functions and expression patterns at different stages of the reproductive cycle, with FSH involved in the control of puberty and gametogenesis, whereas LH mainly regulates final gonadal maturation and spawning (Schulz and Miura, 2002). Regarding spermatogenesis, it is generally accepted that the LH regulates sex steroid production in the Leydig cells and FSH regulates Sertoli cell activities, such as supporting germ cell survival and development (Schulz and Miura, 2002). However an important

variation in the LH and FSH expression patterns among teleosts has been observed. For example, in rainbow trout (*Oncorhynchus mykiss*), the expression levels of FSH-β were much higher than those of LH-β in the pre-gametogenesis and early gametogenetic stages, whereas the expression levels of LH-β mRNA were higher at the end of maturation (Gómez et al., 1999). However, in European sea bass (*Dicentrarchus labrax*), glycoprotein-α, FSH-β, LH-β mRNA increased simultaneously with the gonadosomatic index (GSI) during spermatogenesis (Mateos et al., 2003). In eel, FSH seems to mediate gonadotropin stimulation in the early stages of the gametogenesis, while LH seems to be involved in the end of gametogenesis (European eel: Aroua et al., 2005; Schmitz et al., 2005; Japanese eel: Jeng et al., 2007; Yoshiura et al., 1999).

2.3 Gonads

In the steroidogenic pathways (Fig. 4), the first and limiting step is the conversion of cholesterol into pregnenolone by the P450scc enzyme (cyp11a1), a cholesterol side-chain cleavage enzyme.

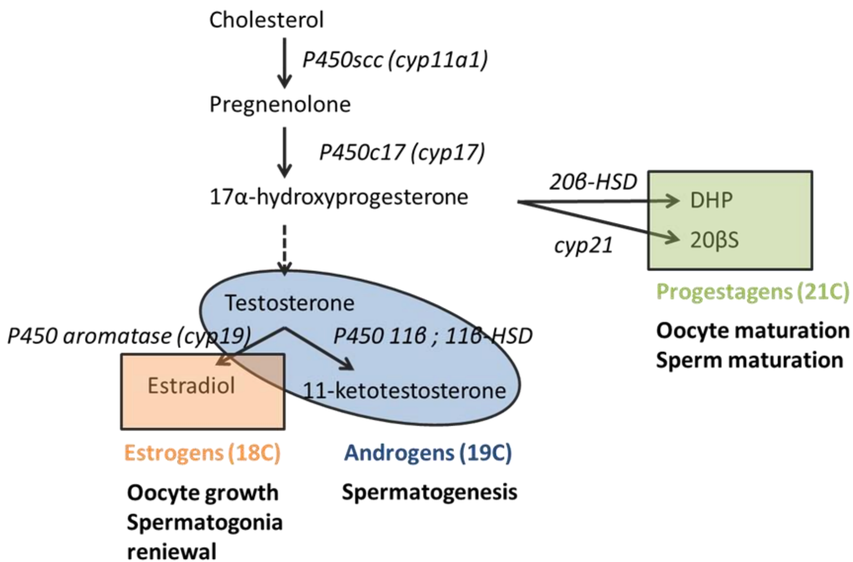

Figure 4. Principal steps of the steroidogenic enzymatic pathways

The cytochrome P450c17 (cyp17) is an enzyme which exhibits two different activities, hydroxylase and lyase. The P450c17 enzyme is responsible for the hydroxylation of pregnenolone and progesterone (hydroxylase activity), but also acts upon 17-hydroxyprogesterone and 17-hydroxypregnenolone (lyase activity) (Reviewed by Diotel et al., 2011). In teleosts, there are two types of P450c17, P450c17-I which possesses 17α-hydroxylase and 17,20-lyase activities, and P450c17-II which only possesses 17α-hydroxylase activity (*Oryzias latipes*: Zhou et al., 2007; *Verasper moseri*; Jin et al., 2012).

Testosterone (T), the aromatizable androgen, can be converted into 17β-estradiol by the cytochrome P450 aromatase (cyp19). In mammals, aside for pigs (Graddy et al., 2000), only a single copy of the aromatase gene, *CYP19A1*, has been characterized. In contrast to vertebrates, in most teleosts, two paralogs of the aromatase gene, known as *cyp19a1a* and *cyp19a1b*, have been identified, and are mainly expressed in the ovary and brain, respectively. These two genes have been identified in many fish species, including the Nile tilapia *Oreochromis niloticus* (Chang et al., 2005), the zebrafish *Danio rerio* (Trant et al., 2001), the goldfish *Carassius auratus* (Callard et al., 1997); the Chinese rare minnow *Gobiocypris rarus* (Wang et al., 2010); the atlantic halibut, *Hippoglossus hippoglossus* (van Nes et al., 2005), the rainbow trout *Oncorhynchus mykiss* (Tanaka et al., 1992; Valle et al., 2002); the European sea bass *Dicentrarchus labrax* (Blazquez and Piferrer, 2004), the orange-spotted grouper, *Epinephelus coioides* (Zhang et al., 2004)and the ricefield eel, *Monopterus albus* (Zhang et al, 2008). Nevertheless, only a single copy of P450 aromatase has been identified in the eel (called cyp19a1) and it is expressed in the ovary, brain and pituitary (Ijiri et al., 2003; Jeng et al., 2012b; Peñaranda et al., 2014).

Estradiol (E_2) is derived from the aromatization of T into 17b-estradiol (fig 4) or from androstenedione into estrone and further into estradiol. Estrogens are known to be involved in the regulation of oogenesis, spermatogenesis, vitellogenesis, gonadotro-pin regulation, and other aspects of reproduction, in addition to the pleiotropic effects they have on many target organs such as the gonads, the cardiovascular system, the liver, the skeleton, and the nervous system (Bazer et al.,

2010; Heldring et al., 2007; Hess, 2003; Horner, 2009; Matthews and Gustafsson, 2003; Nilsson et al., 2001; Nagler et al., 2012; Pang and Thomas, 2009; Shi et al., 2013; for review see Nelson and Habibi, 2013). In Japanese eel males, estradiol has been shown to stimulate a spermatogonial stem cell renewal factor (Miura et al., 1999).

Testosterone can also be converted into 11-ketotestosterone (11-KT; a non aromatizable androgen), considered the most active steroid hormone in male teleosts (Miura and Miura, 2003a). 11-KT is necessary for the initiation of spermatogenesis and sperm production, regulating spermatogonial proliferation toward meiosis in fish (Miura et al., 1999; Fig. 5). The convertion of T into 11-KT can be brought about by the actions of two enzymes, 11β-hydroxylase (cytochrome P450-11β) and 11β-hydroxysteroid dehydrogenase (11β-HSD; Jiang et al., 2003). The enzyme P450-11β metabolizes T into 11β-hydroxytestosterone, the substrate for the production of 11-KT, and the enzyme 11β-HSD metabolizes the 11β-hydroxytestosterone into 11-KT, and cortisol into cortisone. Teleost 11β-HSD sequences (Kusakabe et al., 2003: *Oncorhynchus mykiss*; Jiang et al., 2003: Japanese eel) are similar to mammalian 11β-HSD type 2 (Albiston et al., 1994). In eel, two homologous of mammalian 11β-HSD type 2 are present in the testis: 11β-HSD and 11β-HSD short form (11β-HSDsf) (Albiston et al., 1994; Jiang et al., 2003; Kusakabe et al., 2003; Ozaki et al., 2006).11β-HSDsf seems to be the major/main enzyme in the conversion of 11β-hydroxytestosterone (11β-OHT) into 11-KT (Ozaki et al., 2006), while 11β-HSD mainly converts cortisol into cortisone (Jiang et al., 2003).

In male fish, the progestins: 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) are the maturation-inducing steroids (MIS), and mediate the process of sperm maturation and spermiation (Scott et al., 2010). Nevertheless, DHP has also been proposed to be an essential factor for meiosis initiation, at the beginning of spermatogenesis (Miura et al., 2006). Two enzymes, 20β-hydroxysteroid dehydrogenase (20β-HSD) and 21-hydroxylase (cyp21) mediate the synthesis of progestin in fish. 20β-HSD is considered the main enzyme producing DHP (Lubzens et al., 2010), while the cyp21 enzyme seems to synthesize 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), identified as the MIS in the perciform family

Sciaenidae (Trant and Thomas, 1989). In eels, both DHP and 20βS appear to be involved in the regulation of spermatogenesis (Asturiano et al., 2000; Ohta et al., 2002).

The products of steroidogenesis, such as estrogens (E_2) and androgens (T, 11-KT), can exert negative or positive feedback effects on the brain and pituitary but also on the testis itself (Fig. 3). Other metabolites produced out of the reproductive axis may also be involved in the maturation process, such as the insulin-like growth factor-I (IGF-I) (Legac et al., 1996; reviewed by Schulz et al., 2010) or leptin (Morini et al., 2015b) produced by the liver.

2.4 Gametogenesis

Gametogenesis is a gonadal process in which primordial germ cells undergo cell division and differentiation to form mature haploid gametes. In vertebrates, the gametes, ovum (oogenesis) or spermatozoa (spermatogenesis), are produced by the gonads, testes or ovaries. Spermatogenesis is a very well organized process which can be divided into the following stages: proliferation of spermatogonia, meiosis, spermiogenesis, and sperm maturation (reviewed by Schulz et al., 2010, Miura and Miura, 2011) (Fig. 5).

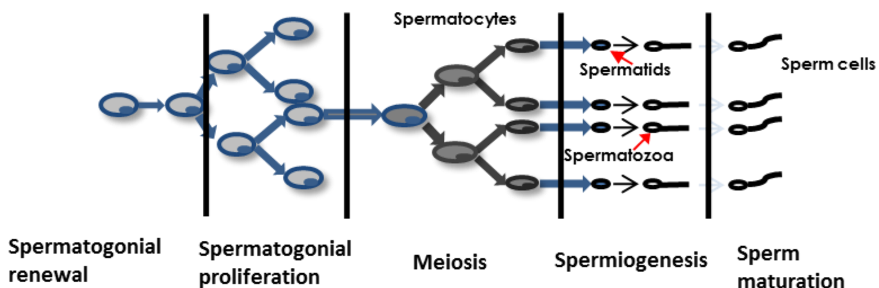

Figure 5. The spermatogenesis process

Firstly, the spermatogonial stem cells, called type A spermatogonia, undergo mitotic proliferation through a specific number of mitotic cycles. Some of the type A spermatogonia cells renew the stock of

type A spermatogonia, others become type B spermatogonia. After the proliferation of spermatogonia, type B spermatogonia differentiate into primary spermatocytes. Primary spermatocytes enter into the first meiotic division to produce secondary spermatocytes, followed by a second meiotic division to produce haploid spermatids. Spermatids have small, round and heterogeneous nuclei. The round spermatids suffer remarkable morphological changes and transform into spermatozoa, with the formation of the cell head and its condensed nucleus, the midpiece, and the flagellum. Finally, the spermatozoa are released from the seminal cysts into the lobular lumen or efferent duct (spermiation) and later they acquire the ability to become motile during their passage through the sperm duct. During all these phases, up until spermiation, the germ cells are in close contact with the Sertoli cells, which provide them withphysical support and the factors needed for survival, proliferation and differentiation (reviewed by Miura and Miura, 2011).

3. Fertilization

Almost all fish species reproduce sexually, permitting the mixing of the genes of the two sexes. Female eels, like the majority of marine fish, spawn pelagic eggs which are fertilized by males shortly after their release into the sea water.

Once released, the egg and spermatozoa are destined to die within minutes or hours unless they find each other and fuse in the process of fertilization. Teleost spermatozoa penetrate into the egg through the micropyle, a funnel shape opening(s) in the zona pellucida through which one spermatozoa can enter (Hart, 1990). After fertilization, the egg is activated and initiates its developmental program, and the haploid nuclei of the two gametes fuse to form the genome of a new diploid organism. A centrally important factor in initiating egg activation at fertilization is a rise in free Ca^{2+} in echinoderms, ascidians, and vertebrate eggs (reviewed by Runft et al., 2002).

Studies show that the initiation of the fertilization calcium wave in verterbrates can generally be best explained by a diffusion of a

sperm-specific activating substance released into the oocyte after gamete fusion (Swann et al., 2006; Parrington et al., 2007; Saunders et al., 2007; Whitaker, 2006). This sperm factor corresponds to a sperm-specific phospholipase C (PLC) called PLCζ (Swann and Lai, 2013; Ito et al., 2011) (Fig 6).

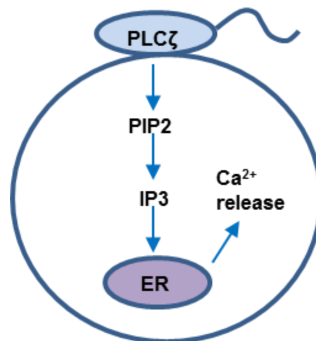

Figure 6. Schematic reaction chain of the PLCζ during fertilization

After fertilization, PLCζ induces a reaction chain by cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Igarashi et al., 2007; Miao and Williams, 2012). These two metabolites, in turn, cause the release of IP3-mediated Ca^{2+} from the endoplasmic reticulum, and the activation of targets such as DAG-sensitive protein kinase Cs (PKCs) (Miyazaki et al., 1993; Saunders et al., 2002; Swann and Yu, 2008; Yu et al., 2008).

4. Projects and grants involved in this Thesis

All the experiments carried out in this thesis were funded by the projects PRO-EEL (Reproduction of European eel towards self-sustained aquaculture), from the European Community's 7th Framework Programme under the Theme 2 ''Food, Agriculture and Fisheries, and Biotechnology'', Grant Agreement No. 245257; http://www.pro-eel.eu/; SPERMOT funded by the Spanish Ministry of Economy and Competitiveness (REPRO-TEMP; AGL2013-41646-R); COST Office (COST Action FA1205: AQUAGAMETE, http://aquagamete.webs.upv.es/); and IMPRESS (Marie Sklodowska Curie Actions; Grant agreement nº: 642893).

OBJECTIVES

The general objective of the present PhD Thesis was to increase the current knowledge on the eel reproductive physiology, in order to improve the control of the production of high quality gametes and viable eggs of European eel (*Anguilla anguilla*) in captivity. The specific objectives were:

- To characterize different genes implicated in the reproduction of the European eel: 2 nuclear progestin receptors *pgr1* and *pgr2*; 5 membrane progestin receptors: *mPRα, mPRAL1 (alpha like 1), mPRAL2 (alpha like 2),mPRγ, mPRδ; and plcz*

- To relate the expression of gene involved in eel reproduction and the steroid hormones synthesis with the stage of European eel maturation:
    - Steroidogenic enzymes: *P450scc, P450c17-I, 11βHSD, P450 a1, cyp21*
    - *Steroid hormone: Testosterone (T),* 11-ketotestosterone (11KT), estradiol (E_2), 17α,20β-dihydroxy-4-pregnen-3-one (DHP)
    - Plcz, three nuclear and two membrane estrogen receptors, two nuclear and five membrane progestin receptors

- To study the effect of the temperature on the European eel spermatogenesis, measuring the expression of:
    - Steroidogenic enzymes: *P450scc, P450c17-I, 11βHSD, P450 a1, cyp21*
    - *Steroid hormone: Testosterone (T),* 11-ketotestosterone (11-KT), estradiol (E_2), 17α,20β-dihydroxy-4-pregnen-3-one (DHP)
    - Plcz

CHAPTER 1

The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis

Marina Morini[1], David S. Peñaranda[1], M. Carmen Vílchez[1], Helge Tveiten[2], Anne-Gaëlle Lafont[3], Luz Pérez[1], Sylvie Dufour[3], Juan F. Asturiano[1,*]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València, Camino de Vera s/n. 46022, Valencia, Spain.

[2]Norwegian Institute of Fisheries and Aquaculture, Muninbakken 9-13, Breivika, P.O. Box 6122 NO-9291 Tromsø, Norway.

[3]Muséum National d'Histoire Naturelle, Sorbonne Universités, Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, CNRS 7208- IRD207- UPMC-UCBN, Paris, France.

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Abstract

Estradiol (E_2) can bind to nuclear estrogen receptors (ESR) or membrane estrogen receptors (GPER). While mammals possess two nuclear ESRs and one membrane GPER, the European eel, like most other teleosts, has three nuclear ESRs and two membrane GPERs, as the result of a teleost specific genome duplication. In the current study, the expression of the three nuclear ESRs (ESR1, ESR2a and ESR2b) and the two membrane GPERs (GPERa and GPERb) in the brain-pituitary-gonad (BPG) axis of the European eel was measured, throughout spermatogenesis.

The eels were first transferred from freshwater (FW) to seawater (SW), inducing parallel increases in E_2 plasma levels and the expression of ESRs. This indicates that salinity has a stimulatory effect on the E_2 signalling pathway along the BPG axis.

Stimulation of sexual maturation by weekly injections of Human chorionic gonadotropin (hCG) induced a progressive decrease in E_2 plasma levels, and different patterns of expression of ESRs and GPERs in the BPG axis. The expression of nuclear ESRs increased in some parts of the brain, suggesting a possible upregulation due to a local production of E_2. In the testis, the highest expression levels of the nuclear ESRs were observed at the beginning of spermatogenesis, possibly mediating the role of E_2 as spermatogonia renewal factor, followed by a sharply decrease in the expression of ESRs. Conversely, there was a marked increase observed in the expression of both membrane GPERs throughout spermatogenesis, suggesting they play a major role in the final stages of spermatogenesis.

1. Introduction

In male vertebrates, sex steroids, androgens, estrogens, and progestins, play significant roles in the control of spermatogenesis (Schultz and Miura, 2002). They are small lipophilic hormones, which can diffuse through the cell membrane (Oren et al., 2004). Estradiol (E_2) binds to intracellular nuclear estrogen receptors (ESRs) and modulates gene transcription (Mangelsdorf et al., 1995), which corresponds to the classic genomic mechanism of steroid action. Two

nuclear ESRs, ESR1 and ESR2 (also named ERα or NR3A1, and ERβ or NR3A2, respectively), are present in mammals. They belong to the nuclear steroid receptor superfamily, as well as androgen, progestin, gluco- and mineralocorticoid receptors (Carson-Jurica et al., 1990; Laudet et al., 1992). Teleost species have at least three distinct ESR subtypes, including ESR1, ESR2a and ESR2b (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002), with ESR2a (also named ERβ2) and ESR2b (also named ERβ1) resulting from the third whole genome duplication (3R) event that occurred in teleost lineage (Hawkins et al., 2000; Lafont et al., in press).

In addition to the classic genomic functions, E_2 can bind itself to membrane receptors, which activates intracellular signalling pathways through a fast, non-genomic action (for review see: Thomas et al., 2012, or Nelson and Habibi, 2013). In mammals, the former orphan receptor GPR30 was characterized as an E_2 membrane receptor, and is also called G-protein coupled estrogen receptor GPER (Filardo and Thomas, 2005; Filardo et al., 2007; for review see Prossnitz and Maggiolini, 2009). Two membrane GPERs have recently been observed in most teleosts including in the eel, likely resulting from teleost 3R (Lafont et al., in press).

The European eel (*Anguilla anguilla*) has a complex catadromous life cycle which includes a 5000-6000 km oceanic reproductive migration to reach its spawning site in an unknown area of the Sargasso sea. Eels are euryhaline fish which are subjected to high variations in salinity during their life cycle (Daverat et al., 2006). After their juvenile growth period in continental waters, eels change from yellow eels to prepubertal silver eels, future genitors that will undergo the transoceanic reproductive migration In captivity, the reproductive cycle is still not closed, and long-term hormonal treatments (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are required to induce sexual maturation in silver eels (Boëtius and Boëtius, 1967; Pérez et al., 2000; Asturiano et al., 2006; Gallego et al., 2012). This, together with the dramatic reduction in the wild European eel population (ICES, 2012) have increased interest in deciphering the basic mechanisms controlling the reproduction of this species. Furthermore, the phylogenetical position of the European eel,

branching at the base of teleosts, may provide insights into ancestral regulatory functions in teleosts, the largest group of vertebrates (Henkel et al., 2012a, b). As far as we know, this is the first study on male teleosts to looks at the expression of the three nuclear (ESR1, ESR2a and ESR2b) and two membrane (GPERa, GPERb) estrogen receptors in the BPG axis throughout the spermatogenetic process.

2. Materials and methods

2.1 Fish maintenance, hormonal treatments and sampling

One hundred male European eels (mean body weight 100±6 g) were purchased from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia, Spain) and transferred to the Aquaculture Laboratory in the Polytechnic University of Valencia. The 100 males were randomly distributed and kept at 20 °C in two freshwater 200-L aquaria equipped with separated recirculation systems, thermostats/coolers, and covered to maintain constant darkness.

One group of 8 eels were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation in freshwater (FW). The rest of the fish were gradually acclimatized over the course of one week to seawater (37±0.3‰ of salinity). Groups of 8 eels were anaesthetized and sacrificed by decapitation in seawater conditions (SW). Once a week for 8 weeks the rest of the fish were anesthetized, weighed and injected with hCG (1.5 IU g^{-1} fish; Profasi, Serono, Italy), to induce the spermatogenesis as previously described by Pérez et al. (2000). Groups of 8 eels were anaesthetized and sacrificed by decapitation each week (W1-8) through the hormonal treatment. For the analysis of ESR expression through the spermatogenesis, the 8 latter groups have been redistributed to 4 groups based on their spermatogenic stage.

Total body weight and testis weight were recorded to calculate the gonadosomatic index [GSI = (gonad weight/total body weight)*100]. Blood samples were collected, centrifugated and stored at -20 °C until E_2 plasma level analysis. Testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological analysis. Samples of anterior brain (dissected into three parts: olfactory bulbs, telencephalon, mes-/di-encephalon), pituitary and testis were stored

in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 °C until extraction of total RNA.

Because eels stop feeding at the silver stage and throughout sexual maturation thee fish were not fed throughout the experiment. They were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Gonadal histology

The formalin-fixed testis samples were dehydrated in ethanol, embedded in paraffin, sectioned to 5-10 µm thickness with a Shandom Hypercut manual microtome (Shandon, Southern Products Ltd., England), and stained using the current haematoxylin and eosin method. The slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope (Nikon, Tokyo, Japan). The stages of spermatogenesis were determined according to the germ cell types present in the testis (Miura and Miura, 2001; Leal et al., 2009) their relative abundance, the degree of development of the seminal tubules and the sperm production of the male at the time of sacrifice (Morini et al., submitted). The stages considered were: Stage SPGA: dominance of A spermatogonia, B spermatogonia present in low numbers; Stage SPGB/SPC: dominance of B spermatogonia and spermatocytes, in some cases low numbers of spermatids; Stage SD : dominance of spermatids, in some cases a small number of spermatozoa; Stage SZ : dominance of spermatozoa (Fig 1).

2.3 Extraction and Reverse-Transcription

Total RNA of the testis, anterior brain parts and pituitary were isolated using a Trizol reagent (Life Technologies, Inc, Carlsbad, CA) as described by Peñaranda et al. (2013). RNA concentration was evaluated using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The testis RNA was treated using a DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). 20 µl cDNA was synthesized from 500 ng of testis total RNA, using a qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA). The brain parts and

pituitary RNAs were treated using a DNase (gDNA Wipeout Buffer, Qiagen, Hilden, Germany). Using a Quantiscript Reverse Transcriptase (Qiagen, Hilden, Germany), 20 µl cDNA was synthesized from 500 ng of total RNA in the case of the olfactory bulb and pituitary, and from 1 µg in the case of the telencephalon and the mes-/diencephalon.

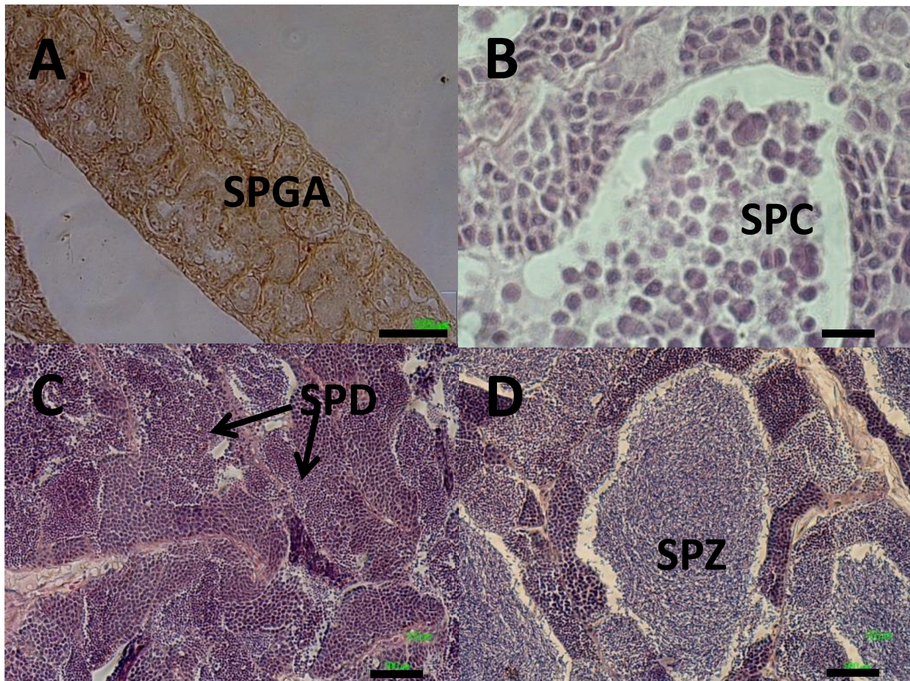


Figure 1. Histological sections of European eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment. A: SPGA (spermatogonia A); B: SPGB/SPC (spermatogonia B/spermatocyte); C: SD (spermatid), D: SZ (spermiation). Scale bar: A=100 µm; B= 10 µm, C, D= 50 µm; Cell types: SPG= spermatogonia; SPC: spermatocytes; SD: spermatids; SZ: spermatozoa

2.4 Gene expression analyses by quantitative real-time PCR

The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using specific qPCR primers for each European eel estrogen nuclear and membrane receptor (Lafont et al., in press) and

the Acidic ribosomal phosphoprotein P0 (ARP) (Weltzien et al., 2005) was used as the reference gene (Table I).

Table I. Quantitative PCR primer sequences for nuclear estrogen receptors (*ESR1*, *ESR2a* and *ESR2b*) and membrane progestin receptors (*GPERa* and *GPERa*).

Name	Sequence (5'- 3')	Orientation	Tm	Reference
ARP	GTGCCAGCTCAGAACACTG	Forward	56.36	Weltzien et al., 2005
	ACATCGCTCAAGACTTCAATGG	Reverse	60.09	
ESR1	GCCATCATACTGCTCAACTCC	Forward	58.20	Lafont et al., in press
	CCGTAAAGCTGTCGTTCAGG	Reverse	59.32	
ESRb1	TGTGTGCCTCAAAGCCATTA	Forward	58.71	Lafont et al., in press
	AGACTGCTGCTGAAAGGTCA	Reverse	57.16	
ESRb2	TGCTGGAATGCTGCTGGT	Forward	59.93	Lafont et al., in press
	CCACACAGTTGCCCTCATC	Reverse	58.44	
GPERa	CAACTTCAACCACCGGGAGA	Forward	61.81	Lafont et al., in press
	TGACCTGGAGGAAGAGGGACA	Reverse	62.86	
GPERb	CAACCTGAACCACACGGAAA	Forward	60.36	Lafont et al., in press
	TGACCTGGAAGAAGAGGGACA	Reverse	60.59	

2.4.1 Reference gene

The stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1. The BestKeeper calculated that variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. In the testis: SD= 0.83; $p<0.05$ with a Cq geometric mean of 24.21±1.77; in the brain and pituitary, olfactory bulb: SD= 0.81; telencephalon: SD= 0.48; mes-/diencephalon: SD= 0.58, pituitary: SD= 0.63; $p<0.05$ and the Cq geometric mean of the olfactory bulb: 23.39±1.76; telencephalon: 21.76±1.40; mes-/diencephalon: 21.89±1.49; pituitary: 22.34±1.55.

2.4.2 SYBR Green assay

To determine the expression of each ESR and GPER gene, qPCR assays were performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas GMBH). The qPCR program used for all was an initial step of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 1 s and 60 °C for 10 s and 72 °C for 7 s. To evaluate assay specificity, the machine performed a melting curve analysis directly after PCR by slowly (0.1 °C/s) increasing the temperature from 68 to 95 °C, with a continuous registration of any changes in fluorescent emission intensity.

The total volume for qPCR reaction was 20 µl, with 5 µl of diluted cDNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl). The transcript levels were determined by the efficiency-adjusted relative quantification method described by Weltzien et al. (2005). Serial dilutions of the cDNA pool of the gonad tissues were run in duplicate and used for the standard curve fto measure all ESRs and GPERs in the testis. Serial dilutions of the cDNA pool of the brain and pituitary tissues were used for the standard curve from which to measure all the ESRs and GPERs in the different parts of the brain and pituitary. A 1/32 dilution of the standard curve was included in each run of the corresponding gene

as a calibrator. Target and reference genes in unknown samples were run in duplicate PCR reactions. A non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates. All ESR and GPER data were normalised to eel reference gene ARP. qPCR calculations were performed according to the Roche Applied Science protocole, Technical Note No. LC 13/2001, part 4 "Calibrator normalized relative quantification".

2.5 Immunoassays for E_2

Plasma concentrations of 17β-estradiol (E_2) were measured by [means of] radioimmunoassays. The assay characteristics and cross-reactivities of the E_2 antiserum were previously examined by Frantzen et al. (2004) and further validated for eel plasma by Mazzeo et al. (2014).

2.6 Statistics

Statistical analyses were performed to study the expression of ESRs and GPERs in the different tissues, as well as the E_2 plasma levels in FW, SW and throughout spermatogenesis (SPGA, SPGB/SPC, SD or SZ).

Two non-parametric tests were performed. Means between FW and SW conditions were compared by Mann-Whitney *U* test. Means between the stages of spermatogenesis were compared by Kruskal-Wallis ANOVA. Differences were considered significant when $p<0.05$.

All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

3. Results

Male eel ESRs (ESR1, ESR2a and ESR2b) and GPERs (GPERa and GPERb) expressions were studied in the BPG axis after the transfer from FW to SW (before hCG treatment) and throughout hormonally induced spermatogenesis (SPGA, SPGB/SPC, SD and SZ stages). E_2 plasma levels were also measured.

3.1 Histological observation

The different spermatogenic stages were determined based on histological analyses of European eel testis during hCG hormonal treatment. Mean GSI were calculated for each spermatogenic stage: SPGA: GSI = 0.07±0.02; SPGB/SPC: GSI = 0.74±0.1; SD: GSI = 3.65±0.4; SZ: GSI = 7.89±0.4. Spermiating males were observed from the fifth week of hCG treatment (W5).

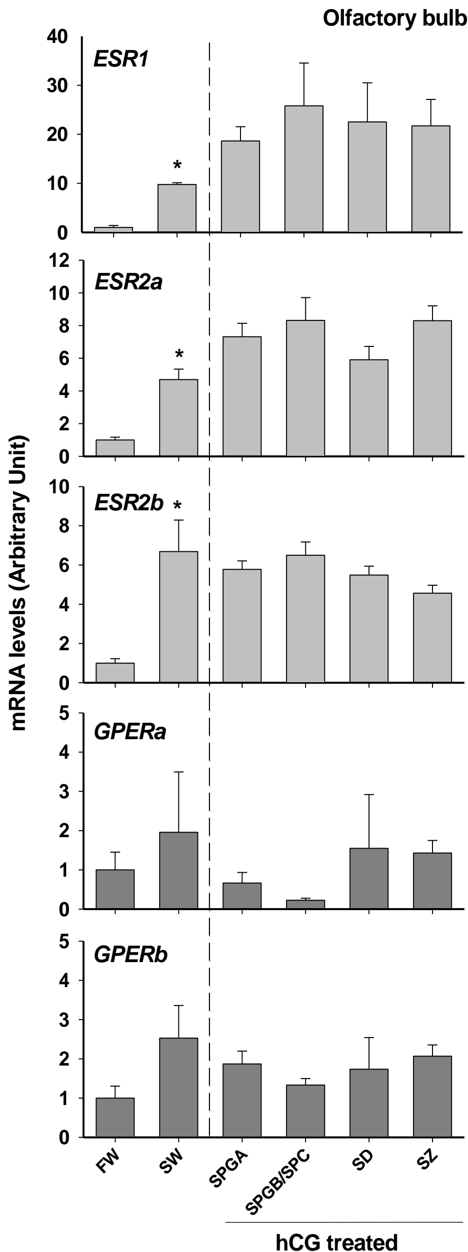
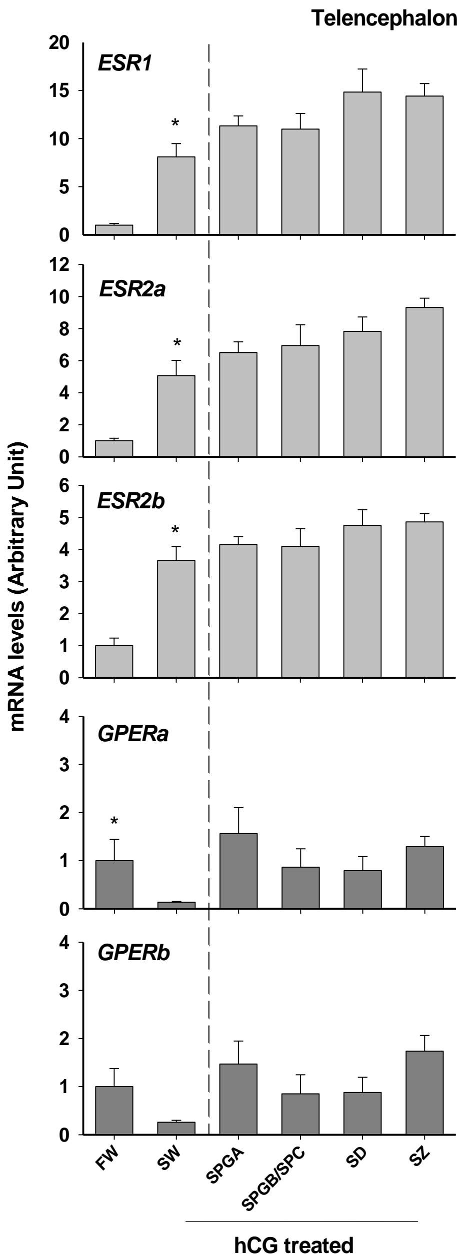


Figure 2. Expression in the olfactory bulb of the male European eel nuclear ERs (ESR1, ESR2a and ESRb2) and GPERs (GPERa and GPERb) in freshwater (FW, n=6) and sea water conditions (SW, n=5), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p<0.05$). Asterisks indicate significant differences between FW and SW condition. SPGA= Spermatogonia A stage (n=8), SPG/SPC= Spermatogonia B/Spermatocyte stage (n=11), SD= Spermatid stage (n=9), SZ= Spermatozoa stage (n=18). See main text for description of development stages.

3.2 Brain estrogen receptor expressions

In all the brain parts, the expressions of the three ESRs (ESR1, ESR2a, ESR2b) increased from FW to SW (Figs.

2, 3 and 4) (9.75, 4.7 and 6.7-fold higher in SW in the olfactory bulb, respectively; 8, 5, and 3.7-fold higher in SW in the telencephalon, respecti-vely; and 7.8, 3.8 and 3.5-fold higher in SW in the mes-/diencephalon, respectively) ($p<0.05$).



During spermatogenesis, ESRs mRNA in the brain remained stable until the end of the experiment, except in the mes-/diencephalon, where the expression of the three ESRs increased ($p<0.05$), with them being 1.9, 2 and 1.7-fold higher respectively at SZ than at SPGA.

Figure 3. Expression in the telencephalon of the male European eel nuclear ERs (*ESR1, ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n=7) and sea water conditions (SW, n=6), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p<0.05$. n=5-19. Asterisks indicate significant differences between FW and SW condition. SPGA= Spermatogonia A stage (n=9), SPGB/SPC= Spermatogonia B/Spermatocyte stage (n=11), SD= Spermatid stage (n=10), SZ= Spermatozoa stage (n=21). See main text for description of development stages.

Concerning the GPERs, the expression levels of GPERa in the telencephalon and GPERb in the mes-/diencephalon decreased with the change

from FW to SW ($p<0.05$), with them being 12.5 and 4-fold higher respectively. GPERa and GPERb expression levels then remained stable until the end of spermatogenesis.

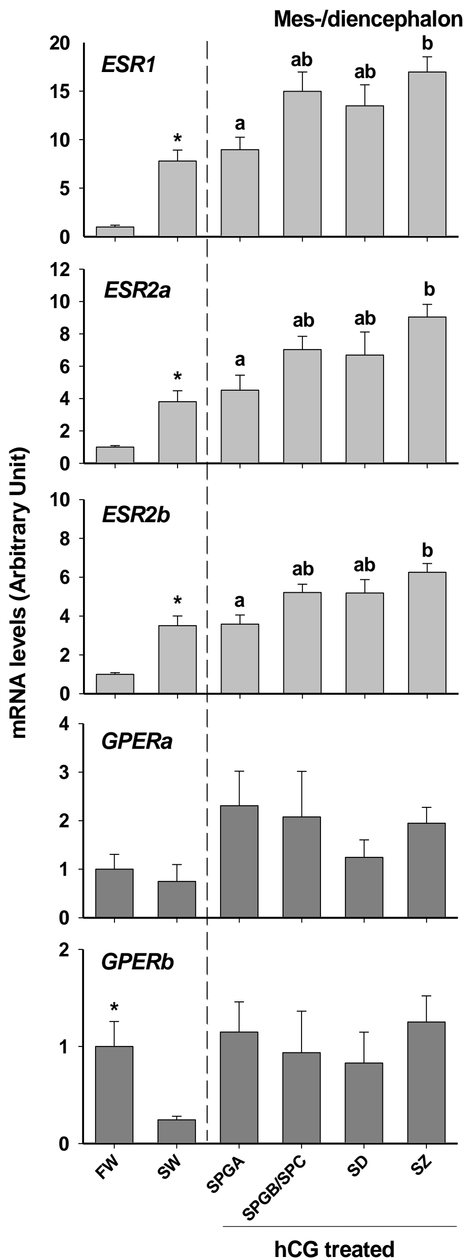


Figure 4. Expression in the mes/-diencephalon of the male European eel nuclear ERs (*ESR1, ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n=7) and sea water conditions (SW, n=6), through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p<0.05$. n=5-19. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA= Spermatogonia A stage (n=9), SPGB/SPC= Spermatogonia B/Spermatocyte stage (n=12), SD= Spermatid stage (n=10), SZ= Spermatozoa stage (n=23). See main text for description of development stages.

3.3 Pituitary estrogen receptor expressions

In the pituitary, the expression of the three ESRs increased with the change from FW to SW ($p<0.05$) (Fig. 5). The expression of ESR1, ESR2a and ESR2b was 7, 4 and 3-fold higher respectively, in SW than in FW. During spermatogenesis, ESR1 and ESR2b showed significant changes in the pituitary throughout testis development, while ESR2a remained stable.

ESR1 expression levels increased from stage SPGA to stage SPGB/SPC ($p<0.05$), and then decreased from SPGB/SPC until the final maturation stage SZ ($p<0.05$).

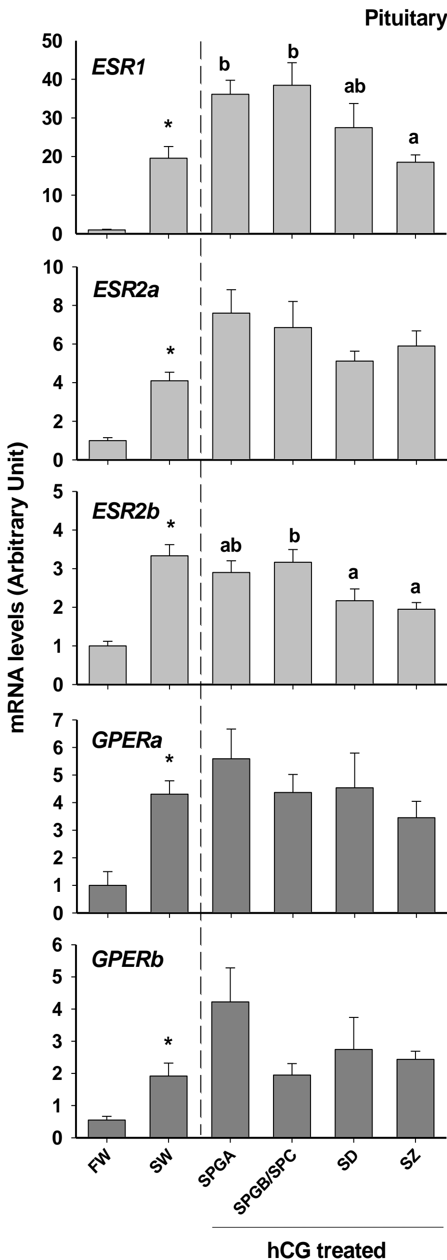


Figure 5. Expression in the pituitary of the male European eel nuclear ERs (ESR1, ESR2a and ESRb2) and GPERs (GPERa and GPERb) in freshwater (FW, n=5) and sea water conditions (SW, n=6), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p<0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA= Spermatogonia A stage (n=7), SPGB/SPC= Spermatogonia B/Spermatocyte stage (n=12), SD= Spermatid stage (n=8), SZ= Spermatozoa stage (n=19). See main text for description of development stages.

ESR2b remained stable from stage SPGA to stage SPGB/SPC, and then progressively decreased until the final maturation stage SZ.

Concerning the GPERs, the expression levels of both GPERa and GPERb increased with the change from FW to SW ($p<0.05$), with them being 4 and 2-fold higher respectively. GPERa and GPERb expression levels then remained stable until the end of spermatogenesis.

3.4 Testis estrogen receptor expressions

In the testis, the expression levels of ESR1 and ESR2a increased with the change from FW to SW ($p<0.05$), with them being 3 and 2-fold higher respectively (Fig. 6). In contrast, the expression of ESR2b remained stable with the change of salinity. During spermatogenesis, the expressions of the three ESRs decreased sharply from stage SPGA to SPGB/SPC: the expressions of ESR1, ESR2a and ESR2b were 7, 9.5 and 9-fold lower respectively, at stage SPGB/SPC compared to stage SPGA, then remained low until stage SZ ($p<0.05$).

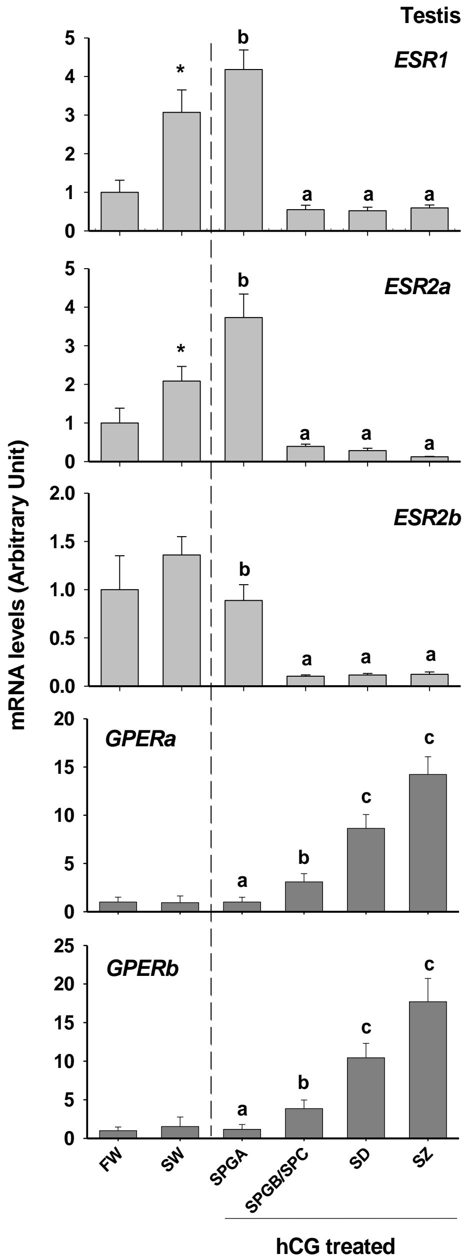


Figure 6. Expression in the testis of the male European eel nuclear ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW) and sea water conditions (SW), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p<0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocyte stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for description of development stages

Neither GPERa nor GPERb expression levels altered

significantly between FW and SW, but they did increase progressively and markedly throughout spermatogenesis ($p<0.05$), with levels being 14.2 and 15.2-fold higher at stage SZ than at stage SPGA.

3.5. Estradiol plasma levels

E_2 plasma levels increased significantly with the change from FW to SW (Fig. 7), with them being 4-fold higher in SW than in FW ($p<0.05$). In hCG treated males, E_2 plasma level decreased progressively until stage SZ ($p<0.05$), and were 2-fold lower at stage SZ than at stage SPGA. By the end of spermatogenesis, E_2 plasma thus returned to very similar levels to those recorded in the initial FW stage.

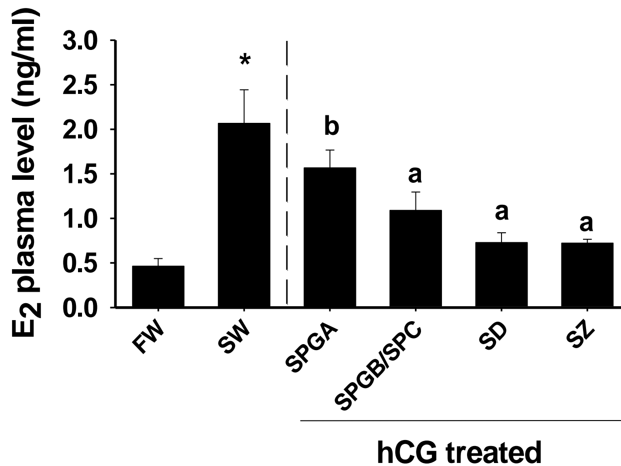


Figure 7. Estradiol (E_2) plasma level of male European eel in freshwater (FW, n=6) and sea water conditions (SW, n=5), and through testis of development stage. Means are given ± SEM. Differences were considered significant when $p<0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA= Spermatogonia A stage (n=10), SPGB/SPC= Spermatogonia B/Spermatocyte stage (n=15), SD= Spermatid stage (n=10), SZ= Spermatozoa stage (n=25). See main text for description of development stages.

4. Discussion

4.1 Effect of salinity on estradiol plasma levels

Before any hormonal treatment, the E_2 plasma levels of immature male European eels increase sharply with the change from FW to SW (37g/L), suggesting that salinity plays a role in sex steroidogenesis. These results match those from previous studies, where the increase in salinity to SW conditions (37g/L) augmented E_2 plasma levels in both male (Peñaranda et al., 2016) and female eels (Quérat et al., 1987). According to Quérat et al. (1987), the E_2 plasma level was higher in SW than in FW, in both hypophysectomized as well as in intact female silver European eels, which suggests that an extra-pituitary mechanism is at work modulating levels of E_2 with the transfer to SW. The increase of E_2 in SW may be related to the osmoregulation, as relation between osmoregulation and reproduction has been demonstrated. For instance in salmon, an anadromous species, there is evidence of a negative relationship between sexual maturation and SW adaptability (For review, see McCormick and Naiman, 1985; Lundqvist et al., 1989; Staurnes et al., 1994; Madsen et al, 1997). The catadromous European eel may respond in the opposite way to its oceanic salinity changes, attending to its reproductive migration in SW.

4.2 Effect of salinity on estrogen receptor expression

The change from FW to SW induced variation of E_2 receptor expression through the BPG axis. ESR1, ESR2a and ESR2b expression levels increased in the anterior brain and in the pituitary, as well as GPERa and GPERb in the pituitary, and ESR1 and ESR2a in the testis. In contrary, GPERa increased in the telencephalon and GPERb increased in the mes-/diencephalon.

The parallel increases in E_2 plasma levels and E_2 receptors could reflect a positive autoregulation by E_2 of the expression of its receptors. In the Japanese female eel, E_2 treatment induces an up regulation of ESR1 but not of ESR2 (Jeng et al., 2012b).). The ESRs are also differentially regulated by E_2, according to tissue, stage of maturation, gender and species, in teleosts. In the European eel, while ESR and E_2 showed the same expression pattern with the change of

salinity, ESR expression levels in brain and pituitary remained high whereas E_2 plasma levels sharply decreased through the spermatogenesis. In the fathead minnow (*Pimephales promelas*), E_2 treatment induces an up-regulation of ESR1 in the testis and a down-regulation in the ovaries, a downregulation of ESR2b in both male and female gonads, while ESR2a does not appear to be affected. In the pituitary, the three ESRs are up-regulated in females but show no significant differences (ESR1, ESR2b) nor decreases (ESR2a) in males undergoing E_2 treatment. In the brain no significant change was observed for any ESRs (Filby et al., 2006). In goldfish, the response of the ESRs to E_2 seems to depend on the stage of maturation (for review see Nelson and Habibi, 2013).

The increase in the E_2 receptor expression levels in SW observed in the male eels in this experiment could also be the result of other hormones involved in SW tolerance. For instance, the growth hormone (GH) is a pleiotropic hormone which regulates various functions in teleosts, including SW acclimation (McCormick, 2001; Eckert et al., 2001). In the European eel, GH was shown to increase the number of nuclear E_2 receptors, as seen in the liver (Messaouri et al., 1991; Peyon et al., 1996). Future studies could investigate the potential effect of GH or other osmoregulatory hormones on E_2 receptors in the BPG axis.

4.3 Brain and pituitary estrogen receptor expression levels during induced spermatogenesis

Several studies have demonstrated that E_2 is an important regulatory factor in the brain, due to the role it plays in the neuroendocrine system controlling reproductive functions (for review see Beyer, 1999), but also because of its neurotrophic, neuroprotective and organizational properties (Behl, 2002). In this study we observed a progressive decrease in male European eel E_2 plasma levels during induced spermatogenesis, contrary to the increase observed in experimentally matured female eels (Pérez et al., 2011). Nevertheless, in both sexes, androgen (testosterone and 11-ketotestosterone) plasma levels increase during induced maturation (Aroua et al., 2005; Peñaranda et al., 2010).

A local production of E_2 in the brain/pituitary could exert autocrine and/or paracrine actions in these organs, throughout spermatogenesis. Although most teleosts possess duplicated aromatase *cyp19a1* genes (enzyme responsible of the conversion of androgens to estrogens), eels have a single *cyp19a1* expressed in the brain, pituitary and gonads (Ijiri et al., 2003; Jeng et al., 2012a; Peñaranda et al., 2014). Jeng et al. (2012a), showed that hCG in males strongly up-regulates aromatase immunoreactivity in the brain and pituitary, leading to a local production of E_2. In rainbow trout (*Oncorhynchus mykiss*), aromatase expression has been localized to the neuroendocrine regions which also express ESRs, indicating that locally produced estrogens can affect neuroendocrine functions through genomic effects in a paracrine way (Menuet et al., 2003, Diotel et al., 2010). According to Pellegrini et al. (2005), in fish, E_2 may be also involved in the regulation of neuro-glial communications in the hypothalamus and in the neurohypophysis.

In this study, ESR2a expression levels in the pituitary remained stable throughout spermatogenesis, whereas ESR1 and ESR2b expression levels decreased, until testis development was complete. According to Lafont et al. (in press), ESR1 expression levels in the pituitary of female European eels increased as the eels matured, showing a difference in the expression of ESR1 between the sexes. ESR2a and ESR2b expression levels remained unchanged in both sexes (Lafont et al., in press; this study).

In the brain, the expression levels of the three ESRs progressively increased in the mes-/diencephalon until the spermatozoa stage. In the female European eel, only ESR1levels increased in the forebrain with maturation, with ESR2a and ESR2b levels remaining stable (Lafont et al., in press). These results again suggest a differential regulation of the nuclear estrogen receptors during the maturation of male and female eels. According to Jeng et al. (2012b), E_2 plasma levels may up-regulate the expression of ESR1 in the brain of female Japanese eel. As E_2 plasma levels decreased throughout spermatogenesis in male eels, the expression of ESRs does not seem to respond to a peripheral E_2 production but rather to a local production in the brain. Nevertheless, other factors may be involved in the increase in the

expression of ESR1, ESR2a and ESR2b in the brain. An interaction between androgen and estrogen in endocrine tissues has already been demonstrated (Panet-Raymond et al., 2000), and a cross talk between androgens and estrogens and their receptors has been highlighted in mice bone (Kousteni et al., 2001). Finally, according to Larsson et al., 2002, both androgens and estrogens, are involved in the physiological regulation of brain androgen receptors in the Atlantic croaker (*Micropogonias undulatus*), another teleost species, during the reproductive cycle. In the male European eel, we cannot rule out an interaction between androgens and the regulation ESR1, ESR2a and ESR2b expressions in the brain.

Concerning GPERs, they are both expressed in the brain and in the pituitary of male European eel, just as previously seen in the female (Lafont et al., in press). These results suggest GPERs in the eel brain are likely to play a role in the reproductive system of both sexes. These two studies on eel are so far the only ones to look at duplicated GPERs. Previous studies on zebrafish and humans have discovered GPER expressed in different brain regions, including regions that control reproduction and sex behavior (Liu et al., 2009; reviewed by Olde and Leeb-Lundberg, 2009). We did not observe any major change in the expression of GPERs in the brain and pituitary during spermatogenesis.

ESRs and GPERs are co-expressed in all the brain parts and in the pituitary of male as well as female European eels. Further analyses are required but these results may suggest potential interactions between nuclear and membrane E_2 receptors.

4.4 Testis estrogen receptor expression during spermatogenesis

The three ESRs were shown to be expressed in the eel testis, and with the same expression pattern throughout spermatogenesis. The highest expression levels were measured at stage SPGA. All three ESR expression levels then sharply decreased at stage SPGB/SPC, and remained low until the end of spermatogenesis.

When studying Japanese eel, Miura et al. (1999) discovered that E_2 plays an important role in spermatogonial renewal. They

demonstrated that low concentrations of E_2 act in the primary stages of spermatogonia through receptors present in Sertoli cells, stimulating and maintaining spermatogonia proliferation prior to the progression of further stages of spermatogenesis. The high expression levels of all ESRs at stage SPGA that we observed corroborates the proposed role of estrogens as a spermatogonial renewal factor. In the European eel, the parallel regulation of the three ESRs suggests that the role of E_2 as a spermatogonial renewal factor is mediated by ESR1, ESR2a and ESR2b.

In the testis, the expression pattern of GPERs during spermatogenesis was notably different from that of ESRs. The expression levels of both GPERs were low at the beginning of the spermatogenesis (SPGA stage), and increased sharply until the end of spermatogenesis. These results suggest that both GPERa and GPERb may play a role in the final sperm maturation process. The high expression levels of both GPERs at the spermatozoa stage suggests that these receptors may are mainly localized in the germ cells in the eel. Nevertheless, as GPER was shown to be localized in the Leydig and Sertoli cells, spermatogonia, spermatocytes and spermatozoa of humans, in the spermatogonia cell line of mice (*Mus musculus*), and in the Sertoli cells, pachytene spermatocytes and round spermatids of rats (*Rattus norvegicus*) (Luconi et al., 2004; reviewed by Correia et al., 2015), it can not be excluded that this may be due to Sertoli cell proliferation in the final sperm maturation process.

It is well known that in female fish GPER mediates the E_2-induced meiotic arrest of oocytes (Pang et al., 2008; Pang & Thomas, 2009). Nevertheless, its role in male fish has still yet to be explored. In adult male zebrafish (*Danio rerio*), a RT-PCR analysis of gene expression in the isolated purified early and late germ cells revealed that GPER is mainly expressed in early germ cells of the testis, including the spermatogonia and spermatocytes, suggesting GPER may play a part in mediating estrogen action early on in spermatogenesis (Liu et al., 2009). In contrast, according to their expression profile during spermatogenesis, European eel GPERs (GPERa and GPERb) may have a major role in the final sperm maturation process. This suggests that GPER have differential functions during spermatogenesis depending

on the teleost species. In humans, although the role of estrogens in spermatogenesis is still unclear, decreased spermatozoa numbers and motility in men who were genetically deficient in aromatase were observed, suggesting aromatase/estrogens play a role not only during the development and maintenance of spermatogenesis, but also in the final maturation of spermatozoa (Carreau et al., 2010; reviewed by Correia et al., 2015). Accordingly, studies have demonstrated the presence of sex steroid membrane receptors in human spermatozoa and have shown that the effects of estrogens and progesterone on human ejaculated spermatozoa may be mediated by GPERs and membrane progestin receptors (mPRs) respectively (Revelli et al., 1998; Luconi et al., 2004; Carreau et al., 2010). Similarly, in the European eel, sex steroid membrane receptors may be involved in final sperm maturation. We recently found high expression levels of mPRs in the final stage of spermatogenesis in male eel testis (Morini et al., submitted). This study suggests that, both in the eel as well as in humans, progestin and estrogen membrane receptors have are implicated in the completion of spermatogenesis.

In conclusion, this is the first study to describe the expression levels of five estrogen receptors (three nuclear ESRs and two membrane GPERs) along the BPG axis of a male teleost. The presence of all these estrogen receptors in theBPG axis suggests an implication in the control of male eel reproduction. Our results support the evidence that the regulation of eel ESRs and GPERs expressions are tissue and stage-specific, as shown by the different expression profiles found in the different BPG tissues throughout spermatogenesis. It appears likely that testes ESRs play a role in spermatogonia renewal, while testes GPERs are mainly involved in the end of spermatogenesis. The three ESRs and both GPERs in the brain and pituitary may control neuroendocrine functions, mediating the autocrine or paracrine actions of locally-produced estrogen, during the entire process of eel spermatogenesis. Finally, the expression of both ESRs and GPERs in the BPG axis indicates a possible cooperation between genomic and non-genomic estrogen actions in the control of reproduction.

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CHAPTER 2

Nuclear and membrane progestin receptors in the European eel: characterization and expression in vivo though spermatogenesis

Marina Morini1, David S. Peñaranda[1], María C. Vílchez[1], Rasoul Nourizadeh-Lillabadi[2], Anne-Gaëlle Lafont[3], Sylvie Dufour[3], Juan F. Asturiano[1], Finn-Arne Weltzien[2], Luz Pérez[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València, 46022, Valencia, Spain.

[2]Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences - Campus Adamstuen, Post Office Box 8146, 0033 Oslo, Norway.

[3]Muséum National d'Histoire Naturelle, Sorbonne Universités, Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, CNRS 7208- IRD207- UPMC-UCBN, Paris, France.

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Abstract

A complete characterization of all progestin receptor genes (PRs) in the European eel, five membrane PRs (mPRs): mPRα (alpha), mPRAL1 (alpha-like1), mPRAL2 (alpha-like2), mPRγ (gamma), mPRδ (delta) and two nuclear PRs (nPRs or PGRs): pgr1 and pgr2, has been performed for the first time in a teleost species. In silico studies showed that the Pgr DNA-binding domain and ligand-binding domain are well conserved among vertebrates, whereas the A/B domain it is not; mPR phylogeny placed three of the eel mPRs together with the vertebrate mPRα, being termed mPRα, mPRAL1 and mPRAL2, while the other two mPRs clustered, respectively, with vertebrate mPRγ and mPRδ.

Our in vivo study showed differential expression patterns along the brain-pituitary-gonad axis. An increase in nPR transcripts was observed in brain (pgr1) and pituitary (pgr1 and pgr2) during the spermatogenesis, suggesting that they are good candidates to mediate the reception of DHP signal, in order to regulate the spermatogenesis from proliferating cell stage to final sperm maturation. In the testis, the higher level of mPRγ, mPRδ and pgr2 transcripts at spermatogonia stage suggest their involvement on early spermatogenesis; and the higher mPRα, mPRAL1 and mPRAL2 transcripts at the spermatozoa stage, suggest them as good candidates to regulate final sperm maturation in European eel. Further studies should be done to confirm the role of both nuclear and membrane receptors on eel spermatogenesis.

1. Introduction

The European eel (*Anguilla anguilla*) have a complex catadromous life cycle. This includes a long reproductive migration across the Atlantic Ocean, for supposedly 6-7 month, to reach their spawning site in unknown areas of the Sargasso Sea. Before leaving the European coast, the silver eel reproductive development is blocked in a pre-pubertal stage until the 5000-6000 km oceanic reproductive migration can occur (Dufour et al., 1988). Because the pre-pubertal silver eels are the most advanced stage of the wild eels caught in river or coastal areas, it is difficult to simulate the variable environmental

factors which would occur during the migration, and a long-term hormonal treatment (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are currently necessary to mature eels in captivity (Asturiano et al., 2006; Huang et al., 2009; Pérez et al., 2011; Gallego et al., 2012). Besides its complex life cycle, the phylogenetical position of the European eel, branching at the basis of the teleosts, which are the largest group of vertebrates (Henkel et al, 2012a, b), makes this species a perfect model to study the ancestral regulatory functions which are controlling reproduction.

In all vertebrates, progestins have a crucial function in gametogenesis. It is known that in male fish two progestins: 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) are the maturation-inducing steroids (MIS), mediating the process of sperm maturation and spermiation (Scott et al., 2010). In Japanese and European eels, DHP has been proposed to be an essential factor for the meiosis initiation at the beginning of spermatogenesis (Miura et al., 2006; Peñaranda et al., 2010), but also to regulate final sperm maturation through the increase of pH in the seminal plasma, which induces an increase in intracellular cAMP, making the sperm cells capable of motility and fertilization (Miura et al., 1995). Furthermore, it is known that progestins regulate spermiation in Salmonidae and Cyprinidae (Ueda et al., 1985), increase milt production in Moronidae (Asturiando et al., 2002) and Salmonidae (Baynes et al., 1985), and stimulate sperm motility in Anguillidae (Miura et al., 1995) and Sciaenidae (Tubbs et al., 2008).

Progestins, as small lipophilic steroid hormones, can diffuse through the cell membranes (Oren et al., 2004) and bind to nuclear progestin receptors (nPRs or Pgrs) belonging to the nuclear steroid receptor family. Receptor activation leads to modulation of gene transcription and translation activity (Mangelsdorf et al., 1995), resulting in a relatively slow biological response. However, many progestin actions are non-genomic, and involve rapid activation of intracellular signal transduction pathways mediated by membrane progestin receptors (mPRs). The mPRs are 7-transmembrane receptors coupled to G-proteins, but they do not belong to the G protein coupled receptor (GPCR) superfamily. Instead, they are members of the progestin and

adipoQ receptor (PAQR) family (Tang et al., 2005; Thomas et al., 2007). Evidence has been obtained that steroid hormones, thyroid hormones, and vitamin D, similarly to water soluble signalling molecules, exert this rapid cell surface-initiated hormone action through binding to membrane receptors, which lead to the activation of intracellular second messenger pathways (Revelli et al., 1998; Watson et al., 1999; Falkenstein et al., 2000; Norman et al., 2004). The mPRs were first discovered and characterized in fish ovaries (Zhu et al., 2003a), and five isoforms (mPRα, mPRβ, mPRγ, mPRδ, mPRε) were subsequently identified in humans and other vertebrates (Zhu et al., 2003b; Thomas et al., 2012; Peterson et al., 2013). Both nPRs and mPRs are highly expressed in testis (Ikeuchi et al., 2002; Hanna et al., 2009) and brain (Thomas et al., 2012; Peterson et al., 2013), but the functions mediated by them are still unclear. In Japanese eel, progestin receptor 1 (pgr1) is expressed in testis germ cells, Sertoli cells, and testis interstitial cells, whereas progestin receptor 2 (pgr2) mRNA has been detected only in testis germ cells (Miura et al., 2006). According to Chen et al. (2012) the only nPR present in Atlantic cod (*Gadus morhua*) testis is involved both in the beginning of spermatogenesis, mediating the mitotic proliferation of spermatogonia, and in the final spermatogenesis, in processes associated with the spermiation/spawning period. In Atlantic croaker (*Micropogonias undulatus*), the expression of the membrane PRα in all early to mid-spermatogenic cell types suggest its involvement in the regulation of early stages of spermatogenesis (Tubbs et al., 2010) but it has also been related with the induction of sperm hypermotility (Thomas et al., 2004).

The objective of this study was to characterize all the progestin receptor genes (from nuclear and membrane receptors) in the European eel, as well as to study their gene expression profiles during the spermatogenesis process in the BPG axis, in order to have a first approach to understand the role of the progestin signaling on European eel spermatogenesis.

2. Materials and methods

2.1 Fish maintenance, hormonal treatments and sampling

One hundred European eel males (mean body weight 100±6 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were transferred to the Aquaculture Laboratory in the Polytechnic University of Valencia. They were randomly distributed and kept in fresh water, in two 200-L aquaria (approximately 50 males per aquarium), equipped with separated recirculation systems, thermostats/coolers, and covered to maintain constant shadow.

The fish were gradually acclimatized for one week to sea water (37±0.3‰ of salinity) and kept at 20 °C during the whole experimental period. Then, to induce the sex maturation, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG, Profasi, Serono, Italy; 1.5 IU g^{-1} fish) during 8 weeks, as previously described by Gallego et al. (2012).

Groups of 5-8 eels were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation before the start of the hormonal treatment in freshwater conditions (after arrival to the laboratory), and in sea water conditions (one week after sea water acclimation), and later each week of the hormonal treatment. Morphometric parameters such as total body weight and testis weight were recorded to calculate individual gonadosomatic indices [GSI = (gonad weight/total body weight)*100] (Pankhurst et al., 1982). For histological analysis, testicular tissue samples were fixed in 10% formalin buffered to pH 7.4 with phosphate buffer.

All samples of brain, pituitary, testis, liver, gill, muscle, spleen, pectoral fin, heart, posterior kidney and head kidney were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 °C until extraction of total RNA (Peñaranda et al., 2010). The brain was dissected into five parts: olfactory bulbs, telencephalon, mes-/diencephalon, cerebellum, and medulla oblongata.

2.2 Ethics amendment

As the eels stop feeding at the silver stage and throughout sexual maturation, they were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (UPV) (Permit Number: 2014/VSC/PEA/00147). All efforts were made to minimize animal suffering and stress.

2.3 Gonadal histology

To determine the maturational stage of the testis, formalin-fixed samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope. Stages of spermatogenesis were determined according to the germ cell types present in the testis (Leal et al., 2009; Miura et al., 2011) and their relative abundance, the degree of development of the seminal tubules, the GSI and the sperm production by the male in the week of the sacrifice. The stages considered were: Stage 1: A Spermatogonia (SPGA): dominance of A spermatogonia, B spermatogonia present in low number, presence/absence of lumen; mean GSI = 0.08 (0.0-0.36); Stage 2 Spermatogonia B/Spermatocytes (SPGB/SPC): dominance of B spermatogonia, spermatocytes present, in some cases low number of spermatids appeared, mean GSI = 0.80 (0.29-1.52); Stage 3 spermatids (SD): spermatids very abundant, some sperm cells could appear, mean GSI = 4.02 (1.79-5.93); Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell, mean GSI =7.35 (3.41-12.8) (S1 Fig.).

2.4 Identification of progestin receptor sequences

2.4.1 European and Japanese eel genome database analyses

All the genomic sequences of nPRs and mPRs were retrieved from European and Japanese eel genomes by performing TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) (Henkel et al., 2012a, b). The exons and splicing junctions were predicted using the empirical nucleotidic splicing signatures, i.e.: intron begins with ''GT'' and ends with ''AG''. The following peptide sequences were used as queries: *Danio rerio* mPRα (acc. number AY149121.1), *Carassius auratus* mPRα (AB122987.1), *C. auratus* mPRγ (AB284132.1), *C. auratus* mPRδ (AB284133.1), *Oreochromis niloticus* mPRγ (XM_003456742), *Anguilla japonica* ePR1 (AB032075.1), A. japonica ePR2 (AB028024.1). The percentage of European eel PR identity was calculated with Sequences Identities And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html).

2.4.2 Phylogenetic analysis of nuclear and membrane progestin receptors

With the aim to obtain a better understanding of nPR and mPR family evolution, phylogenetic analyses were performed on osteichthyans of key-phylogenetical positions: mammalians; sauropsids (birds and reptiles); a representative of an early sarcopterygian, the coelacanth (*Latimeria chalumnae*); the non-teleost actinopterygian spotted gar (*Lepisosteus oculatus*); the European and Japanese eels, as members of an early group of teleosts (elopomorphs), and other teleosts.

Two unrooted phylogenetic trees were constructed with amino acid sequences of known or predicted sequences of nPRs and progestin and adipoQ receptor (PAQR) family (For accession/ID number, see Supplemental Table S1). The sequences were retrieved from NCBI or ENSEMBL, first aligned using Clustal Omega (Sievers et al., 2011) with seaview 4.5.4 software (http://doua.prabi.fr/software/seaview), and later manually adjusted. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the resulting alignment was determined using ProTest software (Abascal et al., 2005). Both phylogenetic trees of nPRs and Progestin and adipoQ receptor were constructed based on

the sequence alignments, using the maximum likelihood method (PhyML software (Stamatakis et al., 2008) with 1000 bootstrap replicates, and subsequently visualized using treedyn (http://phylogeny.lirmm.fr/phylo_cgi/).

2.4.3 Synteny analysis of nuclear progestin receptor genes

Neighboring genomic regions of the duplicated eel nuclear progestin receptors, pgr1 and pgr2, were characterized manually on the European and Japanese eel genomic databases, using CLC DNA Workbench 6 software and the GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html). BLAST analyses were performed in the European and Japanese eel genomes to identify potential additional paralogs of the pgr neighboring genes. Homologs of eel pgr neighboring genes were then identified, using PhyloView of Genomicus v82.01, in other vertebrate genomes, i.e. human, zebra finch (*Taeniopygia guttata*), spotted gar, zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*), tilapia (*Oreochromis niloticus*) and fugu (*Takifugu rubripes*). BLAST analyses were also performed to search potential pgr paralogs in the genomes of these species. For each pgr neighboring gene family, when only one gene was annotated in all the above-mentioned genomes, BLAST analyses were performed to search for potential additional paralogs.

2.5 Gene expression analyses by quantitative real-time PCR

2.5.1 Primers and reference gene

The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using the Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC ACT G; ARPrv: ACA TCG CTC AAG ACT TCA ATG G (Morini et al., 2015a) as reference gene because its mRNA expression has been shown to be stable during experimental maturation (Weltzien et al., 2005). The qPCR expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD

value higher than 1 are defined as unstable. In the testis: SD= 0.82; $p<0.05$ with a Cq geometric mean of 24.14±1.76; in the brain and pituitary, olfactory bulb: SD= 0.81; telencephalon: SD= 0.35; mes-/diencephalon: SD= 0.46, pituitary: SD= 0.62; $p<0.05$ and a Cq geometric mean of olfactory bulb: 23.51±1.76; telencephalon: 21.95±1.28; mes-/diencephalon: 22.02±1.37; pituitary: 22.39±1.54.

European eel progestin receptor specific qPCR primers (Table 1) were designed based on in situ full-length European eel coding sequences. All the primers were designed on two different exons, in order to avoid amplification of potential genomic contamination, and all the primers were tested on genomic DNA and RNA to confirm that potentially contaminant was not amplified. All primers were designed using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA) and were purchased from Integrate DNA Technology, Inc. (IDT, Coralville, IA).

2.5.2 SYBR Green assay

To determine the expression of each progestin receptor gene, qPCR assays were conducted as previously described by Peñaranda et al. (2013) using a model 7500 unit (Applied Biosystems; Foster City, CA, USA). After an initial activation of Taq polymerase at 95 °C for 10 min, 40 PCR cycles were performed at the following cycling conditions: 95 °C for 1 s, 60 °C for 30 s.

The total volume for PCR reaction was 20 µl, performed from 5 µl of diluted (1:20 for the nPRs; 1:40 for the mPRs) DNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Peñaranda et al. (2014). Serial dilutions of cDNA pool of gonad tissues were run in duplicate and used as standard curve for both nPRs and for three mPRs (mPRγ, mPRAL1 and mPRAL2). Serial dilutions of cDNA pool of brain tissues were used as standard curve for mPRα and mPRδ. One of these dilutions was included in each run of the corresponding gene as a calibrator.

Table 1. Quantitative PCR primer sequences for nuclear progestin receptors (pgr1 and pgr2) and membrane progestin receptors (mPRα, mPRγ, mPRδ, mPRAL1 and mPRAL2).

Name	Sequence (5'- 3')	Orientation	Length / Efficiency
pgr1	AGTTTGCCAATCTCCAGGTG	Forward	107bp Eff 2,04
	ATCAAACTGTGGCTGGCTCT	Reverse	
pgr2	GCCTCTGGATGTCACTACGG	Forward	95bp Eff 1,95
	CCGGCACAAAGGTAGTTCTG	Reverse	
mPRα	CTGTCGGAGACGGTGGACTT	Forward	151bp Eff 1,91
	CCAGGAAGAAGAAGGTGTAGTG	Reverse	
mPRγ	AAACAGCACCTTCCACCTGT	Forward	102bp Eff 2,02
	TGCAGAAACGGTAAGCCAAG	Reverse	
mPRδ	GCAGCTTCCAGATGACCAAT	Forward	147bp Eff 1,99
	GCAGCATGTAGACCAGCAGA	Reverse	
mPRAL1	CTGGCCTACATGAGCTTCAG	Forward	92bp Eff 2,01
	CCCACGTAGTCCAGGAAGAA	Reverse	
mPRAL2	CCTGGCGCTACTACTTCCTG	Forward	70bp Eff 2,07
	AGCAGGTGTGTCCAGACGTT	Reverse	

Target and reference genes in unknown samples were run in duplicate PCR reactions. Non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates.

2.5.3 Eel progestin receptors tissue distribution

In order to investigate the tissue distribution of each PR mRNA expression in male and female European eels; gonads (testes and ovaries) and somatic tissues (liver, heart, gill, muscle, spleen, pectoral fin, posterior kidney, head kidney, brain, pituitary) were collected from three immature male eels (mean body weight 118±14 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) and three wild female eels (mean body weight 608±35 g) captured by local fishermen in the Albufera lagoon (Valencia, Spain). Total RNA was extracted following the method used by Peñaranda et al. (2014), treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min, and reverse-transcripted using superscript III (Invitrogen) and random hexamer primers on 1 µL total RNA, according to the manufacturer's protocol. All tissues were analyzed by qPCR.

2.5.4 Progestin receptor gene expression profiles during artificial maturation

To study the regulation of the nPRs and mPRs during European eel artificial maturation, total RNA of testis, olfactory bulbs, telencephalon, mes-/diencephalon and pituitary from hCG treated male silver eels was isolated using Trizol reagent (Life Technologies) as described by Peñaranda et al. (2013). Testis RNA was treated and purified with DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). Twenty µl of cDNA were synthesized from 500 ng of testis total RNA, using qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA).

Olfactory bulbs, telencephalon, mes-/diencephalon and pituitary RNA were treated with deoxyribonuclease (gDNA Wipeout Buffer, Qiagen), from 500 ng of total RNA for the olfactory bulb and pituitary, or from 1 µg for the telencephalon and the mes-/diencephalon. First-strand cDNA was synthesized in 20 µl reactions using Quantiscript

Reverse Transcriptase (Qiagen). RNA concentration was evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain).

2.6 Statistics

Normality of each variable was first checked. Variables that did not have a normal distribution were log-transformed and their normality was checked again. Then, data were analyzed by analysis of variance (one-way ANOVA), using the Student–Newman–Keuls test to compare means. Variance homogeneity was checked with the Bartlett test. Differences were considered significant when $p<0.01$. All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

3. Results

3.1 Characterization of progestin receptor genes

The complete CDS sequences (Coding Sequence) of two nPRs were retrieved from the European eel genome. Furthermore, the complete CDS sequences of four mPR genes (mPRα, mPRAL2, mPRγ, mPRδ) were retrieved from both European and Japanese eel genomes, and the complete mPRAL1 CDS sequence was retrieved from the Japanese eel genome while the partial corresponding sequence was retrieved from the European eel genome. To characterize the nPRs and mPRs of European and Japanese eels in the eel genomes, two phylogenetic trees were constructed, one for the nPRs family and another with part of the PAQR family (PAQR3-9). Concerning nPRs phylogenetic analyses (Fig. 1A), the early sarcopterygian coelacanth clustered at the basis of the monophyletic sarcopterygians nPR group, and eel Pgr1 and Pgr2 branched with the spotted gar at the basis of the monophyletic actinopterygian nPR group, constituting an actinopterygian nPR clade as sister clade of the sarcopterygian nPR.

The complete European eel *pgr1* CDS was a 2133bp sequence, the resulting predicted amino acid sequence consisted of 711 aa

(GenBank accession number AFV13730.1), and complete European eel *pgr2* CDS was a 2028bp sequence, the resulting predicted amino acid sequence consisted of 676 aa (GenBank accession number AFV13731.1).

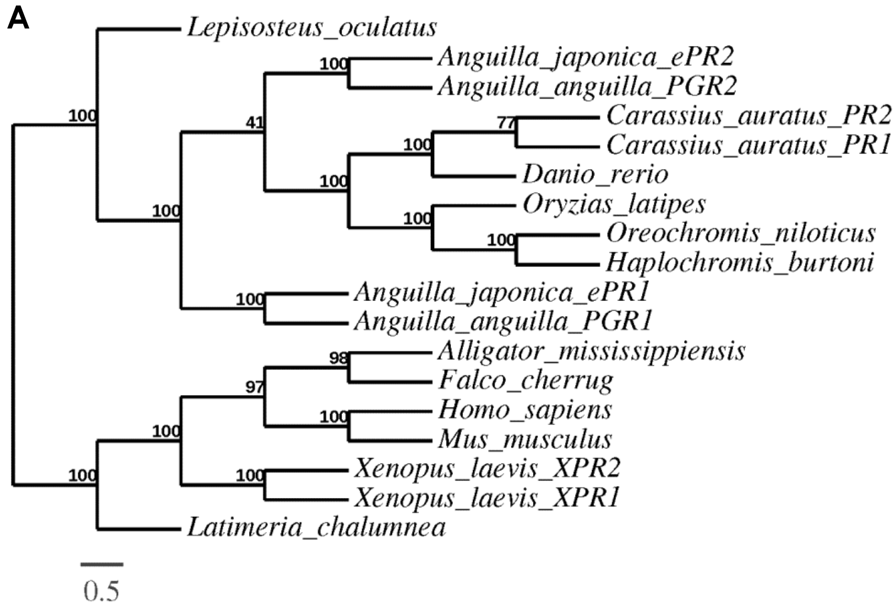

Figure 1a. Consensus phylogenetic tree of nuclear progestin receptors. This phylogenetic tree was constructed based on the amino acid sequences of nuclear progestin receptor (for the references of each sequence see S1 Table) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%).

Although both nPRs were composed of 8 exons, they shared only 25.38% sequence identity at the amino acid level (S2 Fig.). The European and Japanese eel Pgr amino acid sequence differed by 8 and 26 amino acids, for Pgr1 and Pgr2 respectively. The eel *pgr* gene sequence could be subdivided into four or five domains (Fig. 2) as described by Krust (1986). The putative DNA-binding domain (DBD) was from residues 338 to 415 and ligand-binding domain (LBD) was from residues 463 to 710 for Pgr1, and for Pgr2 the DBD was from residues 305 to 382, and the LBD was from residues 428 to 675.

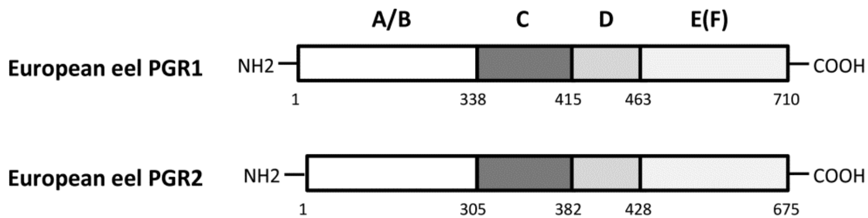

Figure 2. Comparison of nuclear European eel PGR1 and PGR2. The functional domains A/B, C (DNA binding domain), D, E/F (Ligand binding domain) are schematically represented, with the numbers of amino acid residues indicated below.

The PAQR phylogeny is composed of vertebrate PAQR3 to PAQR9 protein sequences (Fig. 1b), and was divided into two major groups: the first group comprised the mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5) and mPRδ (PAQR6) from the mPR subfamily. Within this clade, the mPRs subfamily clustered in two major groups: the mPRγ and δ group; and the mPRα and β group. Three eel mPRs were placed together with vertebrate mPRα, and were called mPRα, mPRAL1 (alpha-like1), mPRAL2 (alpha-like2). The two other eel mPRs (mPRγ, mPRδ) clustered together with their respective mPR types amongst vertebrate representatives.

The second major group is more divergent, and comprises three other members of the PAQR family (PAQR9, PAQR3, PAQR4). The complete CDS of the mPRs (mPRα, mPRAL1, mPRAL2, mPRγ and mPRδ,) were 1059, 1077, 1055, 1071 and 1005 bp, respectively, giving open reading frames (ORF) of respectively 353, 359, 351, 357 and 335 aa sequences. The mPRα, mPRAL1 and mPRAL2 forms were devoid of introns, while the mPRγ and mPRδ forms comprised 7 and 5 introns, respectively.

The predicted European eel mPRs showed a similar structure with the same exon number as the corresponding predicted Japanese eel amino acid sequences. Only differed from 2, 6, 2 and 17 aa for mPRα, mPRγ, mPRδ and mPRAL2; and differed from 5 aa for mPRAL1 when compared with the corresponding Japanese eel partial sequence (S3 Fig.).

B

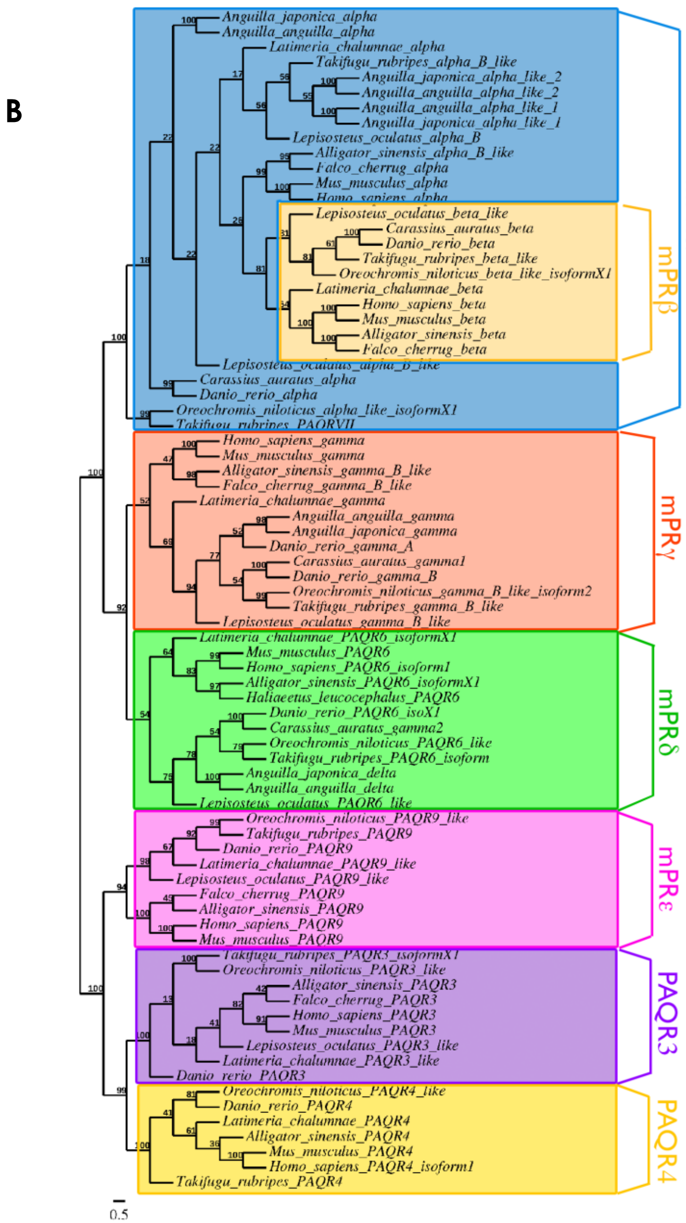

Figure 1b. Consensus phylogenetic tree of vertebrate PAQR family. These phylogenetic trees were constructed based on the amino acid sequences of members of PAQR family, including PAQR3 to PAQR9 (for the references of each sequence see S1 Table) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%).

However, eel mPRs showed very low sequence identity. Higher percentages of identity were found between the mPRα, mPRAL1 and mPRAL2, with 55 to 62% percentage of identity. Both mPRγ and mPRδ showed very low sequence identity (about 30%) with other mPRs. A seven transmembrane structure was predicted for eel mPRα, mPRγ, mPRAL1 and mPRAL2 subtypes with TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Although the mPRδ protein was predicted as possessing five transmembrane domains with this software, INTERPROSCAN 5 (www.ebi.ac.uk/Tools/pfa/iprscan/) predicted seven transmembrane domains (Fig. 3).

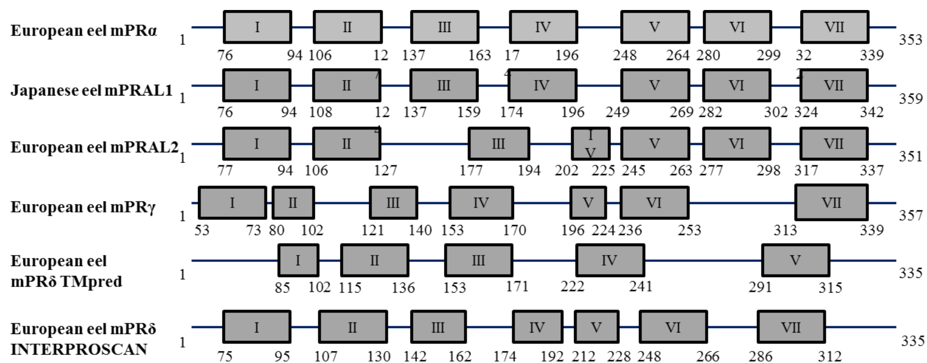

Figure 3. Comparison of membrane European and Japanese eel PRs. The 7 transmembrane domains are schematically represented for each eel mPR; with the two different eel mPRδ representation possible according to the predict program used.

3.2 Syntenic analyses of nuclear progestin receptor genes

To better understand the evolutionary history of the PGRs, we compared the genomic regions that encompass eel *pgr1* and *pgr2* with homologous regions in other vertebrate genomes such as sarcopterygians (human, zebra finch), teleosts (zebrafish, stickleback, tilapia and fugu) and a non-teleost actinopterygian (*spotted gar*) (Fig. 4).

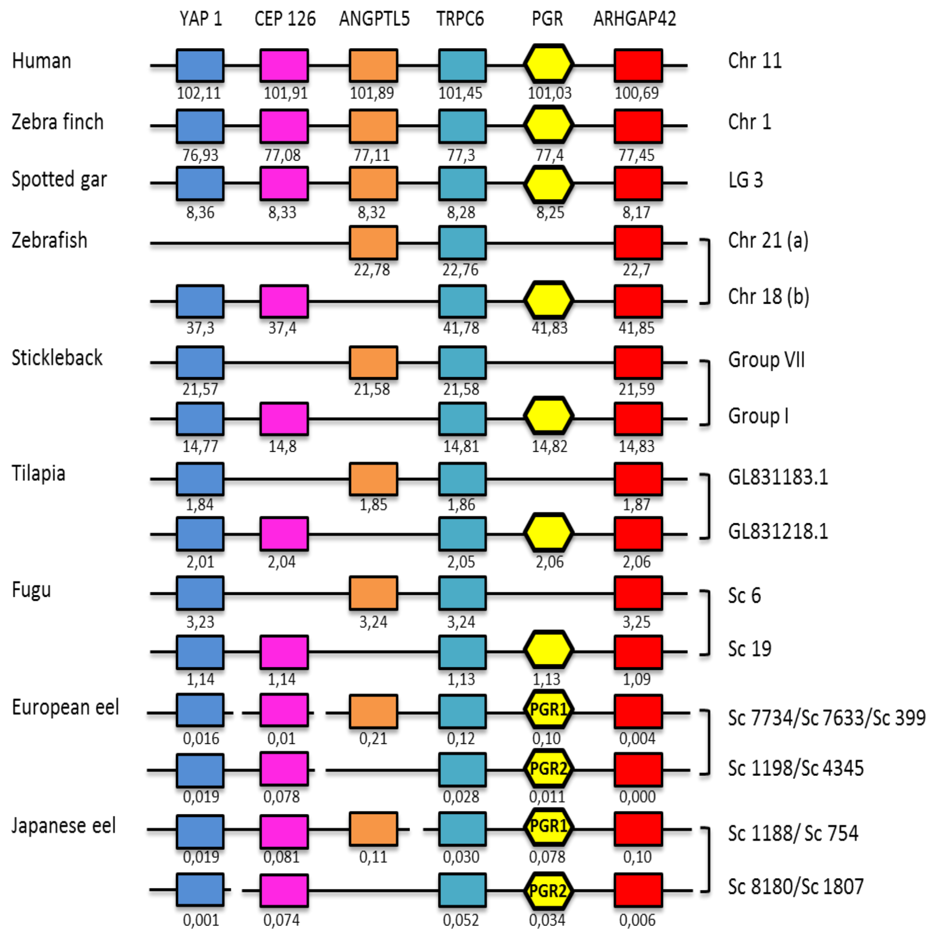

Figure 4. Conserved genomic synteny of vertebrate *pgr*. Genomic synteny maps comparing *pgr* and neighboring genes from human, non-teleost actinopterygian (spotted gar), and teleost species including the two eel *pgr* (*pgr1* and *pgr2*) genomic regions, are represented. The *pgr* genomic region has been duplicated in teleost species, likely as a result of the teleost specific third round of genome duplication. The duplicated *pgr1* paralog has been conserved in the eels but lost in the other teleosts studied. Genes are named after their human orthologs according to the Human Genome Naming Consortium (HGNC). Orthologs of each gene are represented in the same color and displayed in the same column. The genes reproduced in this figure are not necessarily presented in the same order as they appear on the chromosomes and scaffolds, except for human, and their positions are indicated in 10^6 base pairs. The detailed genomic locations of the genes are given in Supporting S2 Table.

Comparative analyses of the small scaffolds of European and Japanese eel genomes allowed us to retrieve five *pgr* neighboring gene families: *yap1, cep126, angptl5, trpc6* and *arhgap42*. As for *pgr* genes, these neighboring genes are duplicated in the European and Japanese eels with the exception of *angptl5*. The single paralog of *angptl5* is located on the genomic region of eel *pgr1*. The other *angptl5* paralog has been lost on the genomic region of eel *pgr2*. The eel *pgr* neighboring genes are also located in the *pgr* genomic regions of all vertebrate species investigated in this study, which supports the orthology of the vertebrate *pgr* genes. The synteny analysis shows that the *pgr* genomic region has been duplicated in teleosts, likely as a result of the teleost-specific third whole genome duplication (3R). As eels, the other teleosts have conserved duplicated *yap1, trpc6* and *arhgap42* genes, and only a single *angptl5* gene. In contrast to the eels, the other teleosts investigated have lost the *pgr1* gene, located in the eels on the same paralogon as the single *angptl5* gene and also lost one *cep126* gene on this paralogon. Zebrafish has further lost one *yap1* gene on this paralogon. The single *pgr* gene conserved by zebrafish, stickleback, tilapia and fugu, is orthologous to eel *pgr2*. In zebrafish, this *pgr2* gene is located on the 3R-paralogon "b" according to the Official Zebrafish Nomenclature Guidelines (http://zfin.org).

3.3 Tissue distribution of progestin receptors mRNA in the European eel

Tissue distribution of all nPR and mPR transcripts revealed a differential expression in male and female European eel (Table 2, S4 Fig.).

Concerning nPRs (S4 Fig. 1A-B and S4 Fig. 2A-B), *pgr2* was highly expressed in the pituitary and both *pgr1 and pgr2* were highly expressed in the brain of female eel, while in male eel they were highly expressed in the testis and other tissues outside the brain-pituitary-gonad (BPG) axis (*pgr1* in kidney and muscle, *pgr2* in the gill).

Concerning mPRs (S4 Fig. 1C-G and S4 Fig. 2C-G), both *mPRAL1* and *mPRAL2* were detected in all tissues studied, and the highest expression was in the cerebellum, both in male and female eels.

Table 2. Tissue distribution summary of progestin receptors in the European eel. Progestin receptor mRNA expression in immature male (A), and in immature female (B) (*n* = 3). Data are normalised to eel ARP.

A

Male	*mPRα*	*mPRAL1*	*mPRAL2*	*mPRγ*	*mPRδ*	*pgr1*	*pgr2*
liver	-	+	-	-	-	-	-
heart	-	+	+	-	-	-	-
gill	-	+	+	+	-	-	++
muscle	-	+	+	-	+++	+++	-
spleen	-	+	+	-	-	-	-
fins	-	+	+	+	+	-	+
post. kidney	-	+	+	+++	-	+++	-
head kidney	+	+	++	+	-	-	-
gonad	-	+	+	+	-	+++	+++
olfactory bulb	-	+	+	-	+	+	+
telencephalon	-	+	+	-	+	+	+
mes/diencephalon	-	+	+	+	+	+	+
cerebellum	+++	+++	+++	-	++	+	-
medulla oblongata	-	+	+	-	+	+	+
pituitary	+	+	+	+	-	-	+

B

Female	*mPRα*	*mPRAL1*	*mPRAL2*	*mPRγ*	*mPRδ*	*pgr1*	*pgr2*
liver	+	++	++	-	-	-	+
heart	+	++	++	-	+	-	+
gill	+	+	+	+	+	-	+
muscle	-	+	+	-	-	-	-
spleen	+	++	++	-	-	-	-
fins	+	++	++	+	+	-	+
post. kidney	+	+	+	+	-	-	+
head kidney	+++	+	+++	-	-	-	+
gonad	+	++	+	+++	-	-	+
olfactory bulb	+	++	+++	-	+	+	+
telencephalon	+	++	++	-	+	+	+++
mes-/diencephalon	+	++	++	-	++	+	+
cerebellum	+	+++	+++	-	+	+++	+
medulla oblongata	+	++	++	-	+++	+	++
pituitary	+	++	++	+	+	+	+++

For instance, in male eel, cerebellum *mPRAL2* was 5-fold more expressed than in the pituitary, and 8,000-fold more expressed than male *mPRγ*. In contrast, the receptors mPRα and mPRδ were lowly expressed in the tissues of male and female eel. mPRα was mainly expressed in the cerebellum of male eel and in the head kidney of females. mPRδ expression was detected in the muscle and in different brain parts of male eels, while in females it was detected in the gill, fins, and highly in the brain parts. Finally, the receptor mPRγ was mostly expressed in the gonads and in peripheral tissues (gill, fins, posterior-/head kidney) both in male and female eels.

3.4 Brain and pituitary progestin receptors mRNA expression during spermatogenesis

Significant variations were found in nPR gene expression levels through the BPG axis during the course of spermatogenesis (Fig. 5). The *pgr1* mRNA transcripts increased along spermatogenesis ($p<0.01$), being significantly higher (2-4 fold) than in SPGA stage: at SPGB/SPC stage in mes-diencephalon (MD) (Fig. 5A), at SD stage in telencephalon (T) (Fig. 5B) and at SZ stage in the olfactory bulbs (OB) (Fig. 5C). In contrast, no variation in *pgr2* mRNA expression was observed in the forebrain (Fig. 5E-G). In the pituitary, both nPRs increased throughout the maturation for (by 3.5-fold for *pgr1*, and by 4-fold for *pgr2* from SPGA to SPGB/SPC stage) (Fig. 5D,H), and then were maintained at high levels until the SZ stage.

Regarding to mPRs, none of them showed significant differences in brain and pituitary throughout the spermatogenesis (Fig. 6).

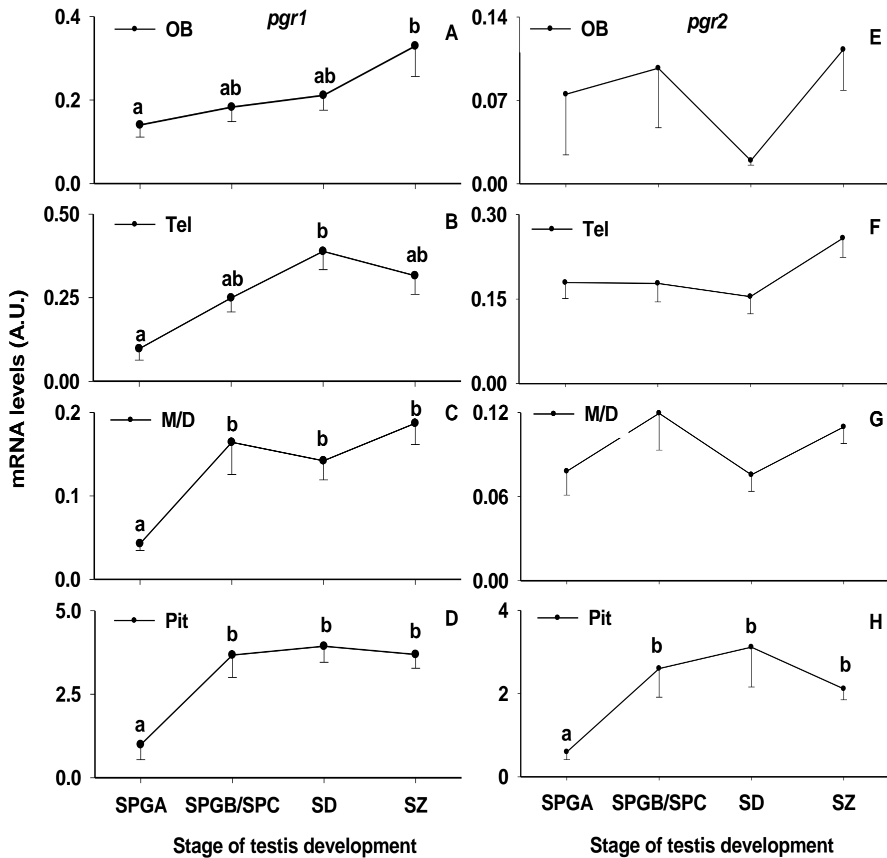

Figure 5. Expression of European eel nuclear progestin receptors. mRNA expression of *pgr1* (A-D) and *pgr2* (E-H) in different brain parts and pituitary of male eel kept at 20 °C during experimental maturation. Data are normalised to eel ARP. Means are given ± SEM. Significant differences ($p<0.01$ n=6-12) between tissues. SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages, OB: olfactory bulb, T: Telencephalon, M/D: Mes-/Diencephalon, PIT: pituitary.

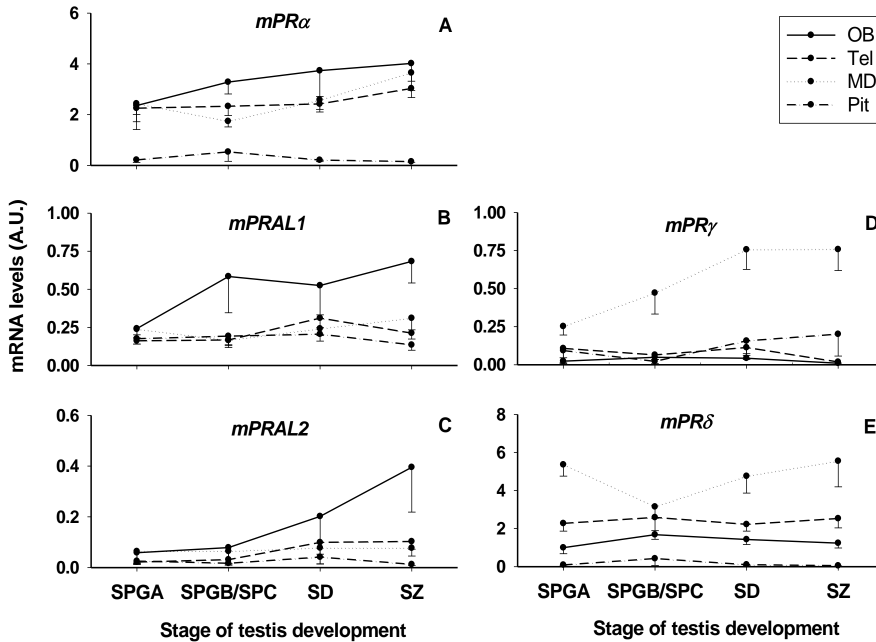

Figure 6. Expression of the eel membrane progestin receptors in different brain parts and pituitary. mRNA expression of *mPRα* (A), *mPRAL1* (B), *mPRAL2* (C), *mPRγ* (D), *mPRδ* (E) during experimental maturation in fish testis kept at 20 °C. Data are normalised to eel ARP. Means are given ± SEM (n=6-17). SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.

3.5 Testis progestin receptor mRNA expression during spermatogenesis

In the testis (Fig. 7), different PR expression profiles were observed during the course of spermatogenesis.

Regarding the nPRs (Fig. 7F-G), *pgr1* mRNA expression was stable during testis development (Fig. 7F), while *pgr2* mRNA expression decreased quickly at the beginning of spermatogenesis (from SPGA to SPGB/SPC stage), and then kept stable from SPGB/SPC to SZ

stages, being 4-fold higher at SPGA compared to later stages (Fig. 7G). *pgr2* mRNA levels thus showed an opposite profile compared to the pituitary.

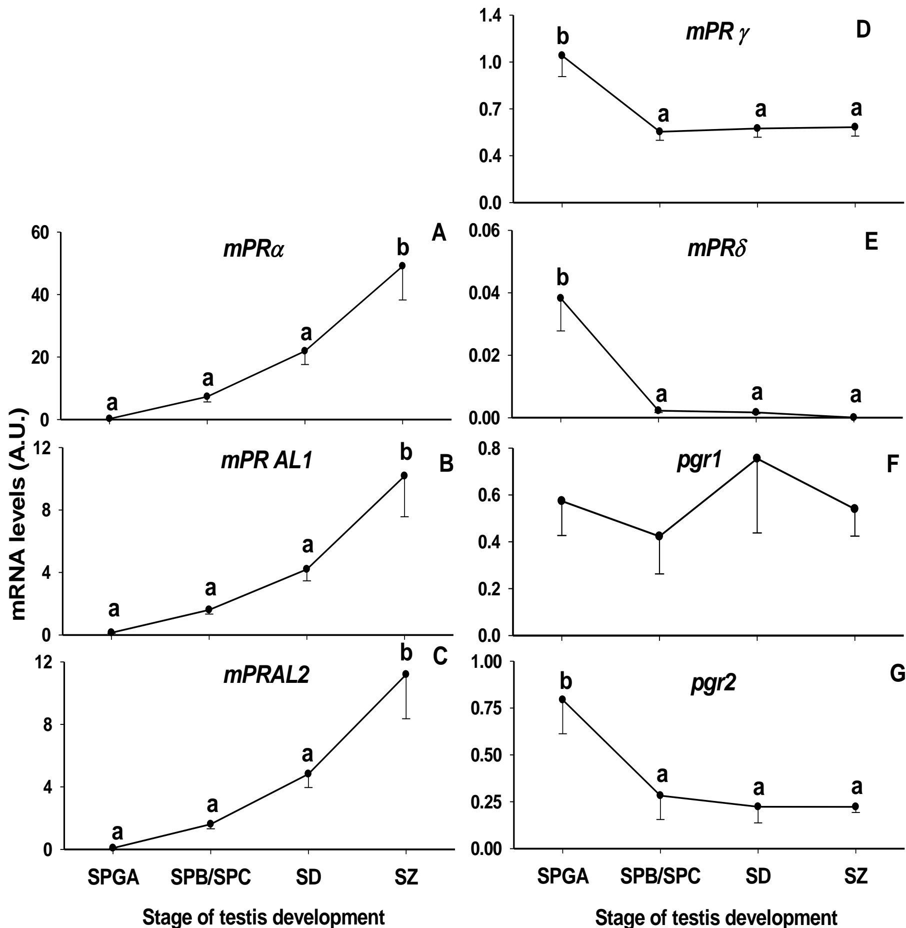

Figure. 7. Expression of the European eel membrane and nuclear progestin receptors. mRNA expression of *mPRα, mPRAL1, mPRAL2, mPRγ, mPRδ* (A-E) and *pgr1* and *pgr2* (F-G) and during experimental maturation in fish testis kept at 20 degrees. Data are normalised to eel ARP. Means are given ± SEM. Significant differences ($p<0.01$; n=6-17). SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.

Different patterns were observed among mPRs (Fig. 7A-E). *mPRα*, *mPRAL1* and *mPRAL2* showed similar expression profiles, with a progressive increase through spermatogenesis, being significant at SZ stage (12-fold higher for *mPRAL1* and *mPRAL2*, 50-fold higher for *mPRα*). *mPRγ* and *mPRδ*, on the other hand, showed an opposite expression pattern, decreasing from the SPGA to SPGB/SPC stage, similar to what was observed for *pgr2* mRNA expression, and maintained at low expression levels in the following stages ($p<0.01$).

4. Discussion

4.1 Duplicated nuclear progestin receptors in the eel

In this study, we identified duplicated nuclear progestin receptor genes (*pgr1* and *pgr2*) in the genomes of the European and Japanese eels, in agreement with the previous cloning of two progestin receptors from Japanese eel testis (ePR1 and ePR2 Todo et al., 2000; Ikeuchi et al., 2002). In contrast, a single PGR has been reported in pufferfish (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*), zebrafish, medaka (*Oryzias latipes*), and stickleback (Hanna et al., 2010). Our BLAST analyses confirmed the presence of a single *pgr* paralogon in these species, as well as in other teleosts such as tilapia.

Phylogeny and synteny analyses allowed us to infer the origin of the duplicated eel *pgr*. Phylogeny analysis clustered teleost PGR in two clades, one encompassing Japanese and European eel PGR1, and the second one encompassing all other teleost PGR, with Japanese and European eel PGR2 at the basis of this second clade.

Synteny analysis showed that *pgr* genomic region has been duplicated in the eel as well as in the other teleosts investigated, likely as a result of the teleost specific 3R. This allows us to raise the hypothesis that eel duplicated *pgr* (*pgr1* and *pgr2*) originated from the 3R. Synteny analysis showed that the other teleosts investigated (zebrafish, stickleback, tilapia and fugu) have conserved a single *pgr*, orthologous to eel *pgr2*. This is in agreement with the phylogeny analysis clustering eel PGR2 with the other teleost PGR. These results suggest that *pgr1* paralog would have been lost in the teleost

lineage, after the emergence of the basal teleost group of elopomorphs. All teleosts would have conserved *pgr2*. Considering that *pgr2* is located on the 3R-paralogon “b” in zebrafish, it could be named *pgrb,* according to the official zebrafish nomenclature. Eel *pgr1* and *pgr2* could be named *pgra* and *pgrb*, respectively. Other teleost species would have conserved *pgrb*, following Zebrafish Nomenclature.

Previous studies from our groups have shown that eels have conserved more 3R-duplicated paralogs than other teleosts species. This is the case as well for *hox* genes (Henkel et al., 2012a, b), as for hormone and receptor genes such as leptin and its receptors (Morini et al., 2015b) and estradiol receptors (Lafont et al., in press).

A previous study showed the presence of two PGRs in goldfish (Li et al., unpublished data; BAO48148, BAO48149, NCBI). Our phylogeny analysis indicated that the two goldfish PGRs clustered together and inside the PGR2 (PGRb) clade. This suggests that the two goldfish *pgr* may result from the tetraploidisation of this species and not from the teleost 3R. As a consequence, the goldfish *pgr* paralogs could be named *pgrb1* and *pgrb2*, instead of *pgr1* and *pgr2*, respectively. Similarly, the two PGR reported in *Xenopus laevis* (Liu et al., 2005), which cluster together and inside the sarcopterygian PGR clade in our phylogeny analysis, may result from tetraploidisation of this species.

The conservation of duplicated PGR may reflect evolutionary processes such as neo- or sub-functionalisation. Regarding the vertebrate PGR amino acid sequences, alignment clearly showed that DBD and LBD domains were well conserved among vertebrates, whereas A/B domain had lower identity. Almost all PGR residues critical for progestin binding (Williams et al., 1998) were conserved in the LBD of both Japanese eel (Todo et al., 2000; Ikeuchi et al., 2002; Hanna et al., 2010) and European eel PGR1 and PGR2, except for a leucine which was replaced by a valine residue in the PGR2 amino acid sequences.

4.1 Multiple membrane progestin receptors in the eel

We identified five membrane progestin receptors in the genomes of

the European and Japanese eels. The mPRs belong to the progestin and adipoQ receptor (PAQR) family. This family includes five mPR subtypes: mPRα (PAQR7) first identified and characterized in spotted seatrout (*Cynoscion nebulosus* (Zhu et al., 2003b), mPRβ (PAQR8) and mPRγ (PAQR5) identified and characterized in humans and other vertebrates (Zhu et al., 2003a), mPRδ (PAQR6) and mPRε (PAQR9), which respond to progestins in yeast recombinant expression systems (Thomas et al., 2012; Pang et al., 2013; Smith et al., 2008), together with PAQR3, PAQR4, two adiponectin receptors (ADR1 and ADR2) and two monocyte to macrophage differentiation proteins (MMD, MMD2) (Thomas et al., 2007). In the present study, phylogenetic analyses including PAQR3 to PAQR9 were performed in order to determine the relationship of eel mPRs characterized with other PAQR forms. The resulting tree clustered the five eel mPRs in 3 groups: mPRα, mPRAL1 (alpha-like1), mPRAL2 (alpha-like2) clustered with the vertebrate mPRα/mPRβ clade, and eel mPRγ and mPRδ clustered with mPRγ and mPRδ clade, respectively. The second part of the phylogenetic tree showed that mPRε, PAQR3, PAQR4 evolutionally diversified from other groups of mPRs, with PAQR3 clearly closer to PAQR4. It can be noticed that the mPRα clade formed a paraphyletic group supported by low bootstrap values, which included the mPRβ clade. According to our in silico and phylogenetic analyses, the European and Japanese eel genomes seem devoid of mPRβ and mPRε. Both mPRAL1 and mPRAL2 could possibly be derived from eel mPRα as a result of a local eel specific duplication; however, the low phylogenetic resolution does not allow to conclude on this. Nevertheless, all eel mPRα or alpha-like are devoid of introns in their coding region, similar to catfish mPRα and β, as described Kazeto et al. (2005). In our study, as mPRδ and mPRγ lack any particular sequence signature, the nomenclature was only based on phylogenetic analyses.

The eel is not the only teleost with mPR derived forms. In the pufferfish genome database, Kazeto et al. (2005) found three uncharacterized forms (FmPRLP 1–3) with FmPRLP 1 and 2 closely related to mPRα, whereas FmPRLP 3 shared high identity with the β form. Thus, these derived mPR forms seem to be expressed only in teleost species, which confer them broad progestin physiological mechanisms.

The presence of both nPRs and mPRs in actinopterygian and sarcopterygian members suggests that they both arose around the same time, early in the vertebrate evolution, coinciding with the appearance of critical steroidogenic enzymes (Thomas et al., 2007). The emergence of both mPRs and nPRs in early vertebrates might suggest a complimentary relationship between the two receptor systems, leading to a wide range of progestin mechanism of action and multiple possible responses of progestin target cells.

4.2 Differential tissue distribution of progestin receptors

In the present study, different expression patterns were found for all the receptors in male and female European eel. Furthermore, male and female eel progestin receptors showed differential expression patterns when compared with the corresponding genes in other species. In female catfish, IpmPRa transcript is expressed in all the tissues (Kazeto et al., 2005) which is similar to the eel tissue distribution of *mPRAL1* or *mPRAL2*, but is different to the eel *mPRa*, which is mainly expressed in the cerebellum of male eels and in the head kidney of female eels. In the seatrout, *mPRa* gene is expressed in the brain, pituitary and gonads (Zhu et al., 2003a), in the zebrafish in testis, ovary and head kidney (Kazeto et al., 2005), and in humans in gonads and kidney (Zhu et al., 2003b). Thus, the different mPRa tissues distribution among the different species investigated until now may indicate species-specific differences of the mPRa function.

The eel mPR subtypes showed a wide distribution, and even mRNA co-expression of some subtypes was observed in a few tissues, such as kidney, similar to what is found in human (Zhu et al., 2003b), and the brain. Finally, we found that tissue distributions of both mPRALs were ubiquitous in male and female eels.

Concerning the nPRs, both *pgr1* and *pgr2* subtypes showed tissue-specific and sex-related expression. Both were mainly expressed in the pituitary and in the brain in female eel, while in males they were mainly expressed in the gonads and in other tissues outside the BPG axis (*pgr1* in kidney and muscle, *pgr2* in the gill). The tissue distribution of nPRs in the European eel is similar to what was found in the

Japanese eel (Ikeuchi et al., 2002). Nevertheless, the different nPR tissue distribution found among teleosts may indicate species-specific differences of the nPR function.

Thus, eel nuclear and membrane PRs were expressed in the neuroendocrine and non-reproductive tissues. Further analyses are required to determine the function of both nuclear and membrane PRs and their potential interactions in some tissues from the BPG axis were they were highly co expressed. However, our results suggest that these receptors could be involved not only in reproduction, but also in other non-reproductive functions.

4.3 Expression of progestin receptors through spermatogenesis

Neuroendocrine mechanisms regulated by progestins influence a wide variety of brain functions. These mechanisms have been shown to be mediated by specific nPRs (Mani et al., 2008; Hanna et al., 2010), or by mPRs (Sleiter et al., 2009).

Although nPRs and membrane mPRα and mPRβ are quite well studied in mammals, information is lacking on the function of mPRγ, mPRδ and mPRε. The present study is the first to report mRNA expression of five membrane and two nuclear PRs through spermatogenesis in fish. In the brain and pituitary, mRNAs for all five mPR subtypes were constantly expressed during spermatogenesis. *mPRγ*, *mPRAL1* and *mPRAL2* showed low expression in all the brain parts and the pituitary, whereas mPRα was highly expressed; and mPRδ showed the greatest brain expression, like in human brain (Thomas et al., 2012; Pang et al., 2013). In human, the mPRs seem to be involved in the negative feedback of progesterone on the gonadotropin-releasing hormone secretion (Sleiter et al., 2009; Thomas et al., 2012), while further research is required to elucidate the specific signalling roles of mPRs in the eel brain and pituitary.

Concerning the nPRs, our study showed very low expression of both *pgr* in different brain parts, but high expression in the pituitary, which is similar to the nPR mRNA pattern found by Pang et al. (2013) in humans. Furthermore, both nPRs in the pituitary were up-regulated throughout the induced-hCG maturation, showing higher expression

from spermatogonia B /spermatocyte stage to spermatozoa stage, which correspond to stages proliferating cell to full spermiation. Only *pgr1* mRNA expression increased in all the brain parts through spermatogenesis, corresponding with the plasma levels of DHP found in the European eel, which significantly increased during the spermatogenesis (S5 Fig.). While further experimental studies will be necessary to determine their physiological role, it seems that the nPRs are involved in the spermatogenesis process; from our data we suggest that PGR1 is the main progestin receptor in the brain, while both PGR1 and PGR2 could mediate DHP signaling in the pituitary through spermatogenesis.

In cyprinids, DHP has a well-known role as pheromone released by females during final oocyte maturation, which induces in males courtship behavior, increased LH, steroid and milt production (Scott et al., 2010). As in cyprinids, in female eels an increase of DHP levels is observed before ovulation (Huertas et al., 2006), which can be possibly released to the environment to act as a pheromone. In this sense, the increase of *pgr1* in the olfactory bulb at spermiating stage suggests a pheromone action of progestins which should be further investigated in this species.

Nuclear and membrane PRs are highly expressed in fish testis (Ikeuchi et al., 2002; Hanna et al., 2009). However, while the reproductive functions of the PRs have been well studied in mammalian models, less information is available in teleost fish. In Japanese eel, progestins induce early spermatogonia to enter in the meiotic prophase (Miura et al., 2006), further regulating sperm maturation (Miura et al., 1995; Schultz et al., 2010). Both Pgr and mPRa were suggested to be involved at the beginning and/or at the final sperm maturation (Thomas et al., 2004; Tubbs et al., 2008; Chen et al., 2012; Tan et al., 2014). Tubbs et al. (2010) showed that mPRa was expressed in all testicular germ cell stages in Atlantic croaker, with an up-regulation of mPRa in both ovaries and testes under gonadotropin control, most likely mediated by increases in LH secretion at the end of the reproductive cycle. In European eel testis, the three mPRalpha/alpha-like (*mPRa, mPRAL1* and *mPRAL2*) showed the same expression pattern as in the Atlantic croaker, increasing until the spermatozoa

stage. This expression pattern concords with the increase of eel DHP plasma levels from proliferating spermatogonia to spermatozoa stage (S5 Fig.), which may suggest an implication of these receptors on the regulation of the final spermatogenesis in the testis, mediated by DHP. However, *mPRγ, mPRδ* and *pgr2* showed an opposite profile, with high expression in the testis during the early spermatogenic stage, and showing a fast decrease onwards, until spermatozoa stage.

The zebrafish showed the same Pgr expression profile as eel mPRγ, mPRδ and pgr2, with strong expression observed in spermatogonia and early spermatocyte stages (Hanna et al., 2010). Nevertheless, in cod, the expression of the sole *pgr* mRNA varies in an opposite way, reaching peak levels in spawning testes (Chen et al., 2012). Our current limited understanding of the role of these progestin receptors in the testicular function in male fish provides few indications of what testicular functions can be regulated by each receptor. According to Miura et al. (2006), DHP plays an important role on the initiation of the meiosis and on further spermatogenesis. Although one recent work mention that *pgr* knockout male zebrafish was fertile (Zhu et al., 2015), this surprising fact was not clearly explained, thus we still consider that progestins and then progestin receptors are involved in fish spermatogenesis as previous authors have demonstrated (Hanna et al., 2010; Chen et al., 2010, 2011, 2012; Miura et al., 2006).

From the pattern of *in vivo* expression of the different receptors through spermatogenesis in the eel, we can hypothesize that testis mPRα, mPRAL1 and mPRAL2 could be involved on the final sperm maturation, while testis mPRγ, mPRδ and pgr2 could be involved on mediating DHP effects on early spermatogenic stages. Further studies of the progestin receptors will be necessary to provide a better knowledge of their specific biological functions in teleost fish.

In conclusion, we have performed a complete description of progestin receptors genes present in the European eel. Two nPR and five mPR genes were identified in the genome of European and Japanese eel. The two nPRs showed the DNA-binding domain (DBD) and the ligand-binding domain (LBD) well conserved among vertebrates, whereas the A/B domain showed lower degree of conservation. Phylogenetic analysis of mPRs placed three eel mPRs

together with the vertebrate mPRα, called mPRα, mPRAL1 (alpha-like1) and mPRAL2 (alpha-like2), while the other two eel mPRs clustered respectively with vertebrate mPRγ and mPRδ. The present study of PR transcript expression suggest that nuclear PR receptors are the most important in brain and pituitary, while in the testis the membrane PR receptors are the most important ones through spermatogenesis: *mPRγ* and *mPRδ* during early spermatogenesis, and *mPRα, mPRAL1* and *mPRAL2* during sperm maturation. Further studies should be performed to elucidate the specific role of these receptors on the spermatogenesis process.

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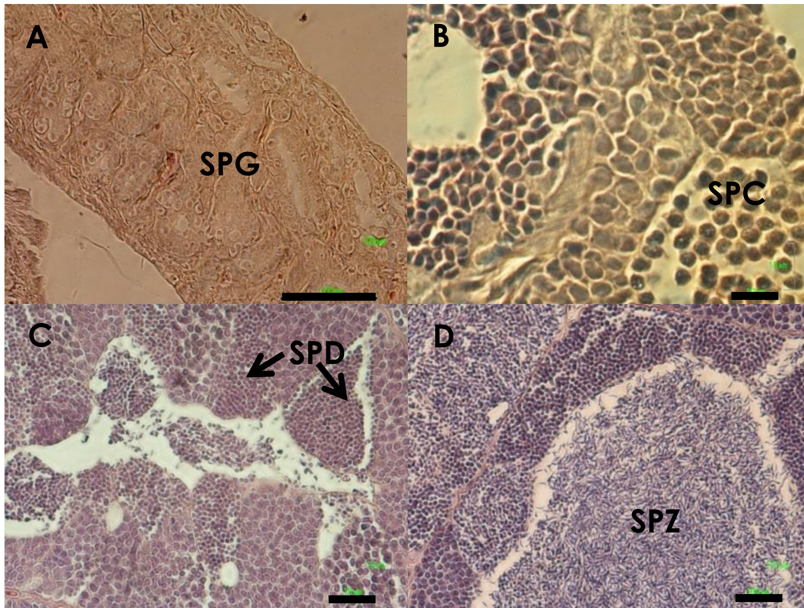
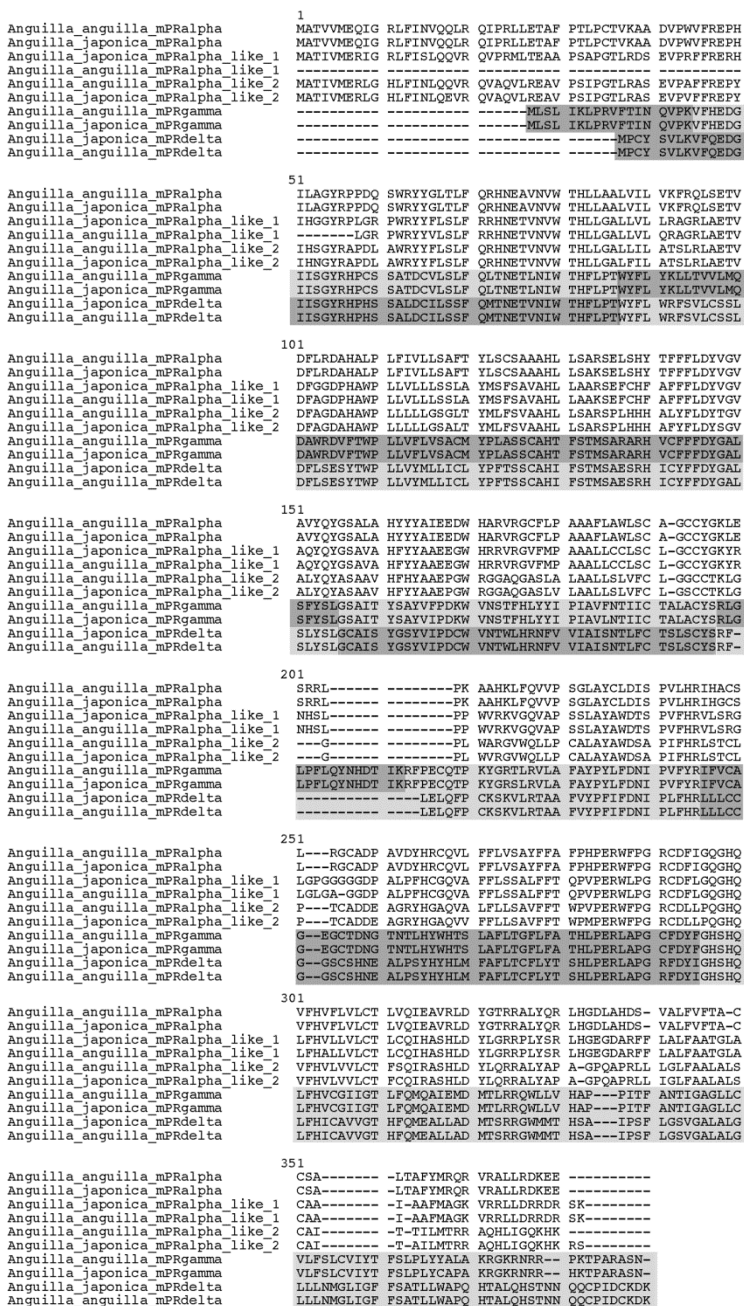
Supporting information captions



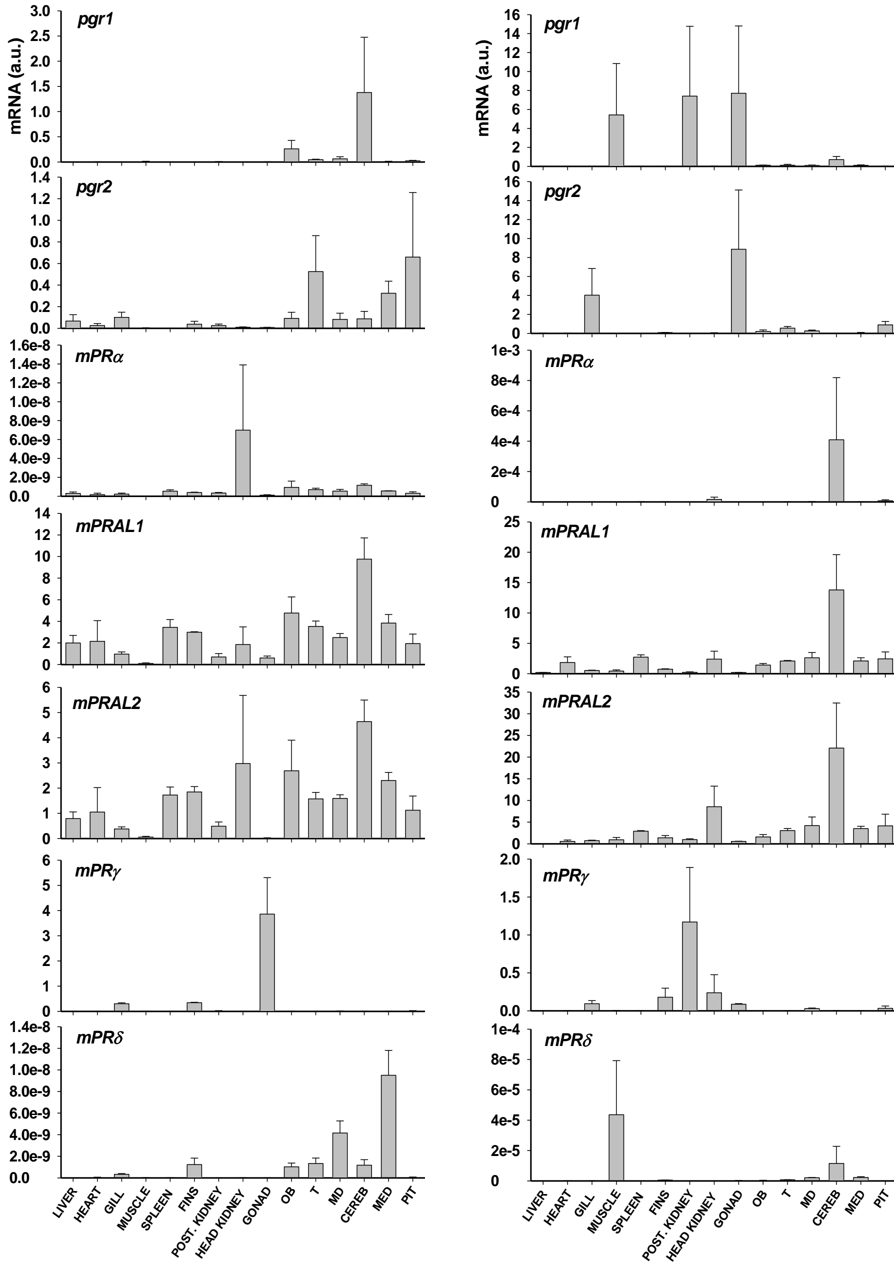
S1 Fig. Histological sections of eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment. A: SPGA (spermatogonia A); B: SPGB/SPC (spermatogonia B/spermatocyte); C: SPD (spermatid), D: SPZ (spermiation). Scale bar: A=100 µm; B= 10 µm, C, D= 25 µm; Cell types: SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: spermatozoa. See main text for definition of gonad developmental stages.

```
                           1
Anguilla_anguilla_PGR1   MDNNHQDKME SLYTPARASP TPDAESIKRA RNLIKTYSES FGSYVEGIVR DDSNNIQSLS
Anguilla_japonica_PGR1   MDNNHQDKME SLYTPARASP TPDAESIKRA RNLIKTYSES FGSYVEEIVR DDSNNIQSLS
Anguilla_anguilla_PGR2   MDSVRKDKSG AT-SP--TAS RPRDTFMKTD NDLTEGFSDS TSNYMAGFCS T-ANSIYSLS
Anguilla_japonica_PGR2   MDSVRKDKSG AT-SP--TAS RPRDTFMKTD NDLTEGFSDS TSNYMAGSCS T-ANSMYSLS

                        61
Anguilla_anguilla_PGR1   ---------- ----SVPLLM RNFGNMDTVT CAPGSGSDSE IWKDFVVPGN SVGSKDTCGH
Anguilla_japonica_PGR1   ---------- ----SVPLLM RNFGNMDTLT CAPGSGSDSE IWKDFVVPGN SVVSKDTCGH
Anguilla_anguilla_PGR2   GVSPT----- ---------M RNSGNVDTTC HGANSTND-- TTESVAVAEN TARYNDSREA
Anguilla_japonica_PGR2   GVSSTMRNSG NALSGVSSTM RNSGNVDTTR HGANSTND-- TTESVAVAEN AARYNDSREA

                       121
Anguilla_anguilla_PGR1   VEISTKAENL SWAAAPLSRE ETLAKGTVTV PATVPKESFT ATSNNSSASG ISIKDEQQSL
Anguilla_japonica_PGR1   VEISTKAENL SWAAAPLSRE ETLAKGTVTV PATVPKESFT ATSNTSSASG ISIKDEQQSL
Anguilla_anguilla_PGR2   GRTESKANNS SWTTSLADNE -----GLALP PASGSKVNLS GVSSSSVGIC KFIKDEQDSS
Anguilla_japonica_PGR2   GRTESKANNS PWTTSLADNE -----GLALP PASGSKASLS GVSSSSVGNC KFIKDEQDSS

                       181
Anguilla_anguilla_PGR1   LKMEPQSSDF CPYTANIPKL NPSYLTNTAS TKQLGYGEQP DTSAHSSPPA QKIVLDTARY
Anguilla_japonica_PGR1   LKMEPQSSDF CPYTANIPKL NPSYLTNTAS TKQLGYGEQP DTSAHSSPPA QKIVLDTARY
Anguilla_anguilla_PGR2   SSMEPQSPYF HPSG-NITTS ---------- --NSSYGTCD EDSATHHPPH M---------
Anguilla_japonica_PGR2   SSMEPQSPYF HPSG-NITTS ---------- --NSSYGTCE EDSATHHPPH M---------

                       241
Anguilla_anguilla_PGR1   SADFGSDNPL PQATNIKTDP CSSFSSFVGE GILTRASMGY SQQALQTLPV HKSEPFRLSA
Anguilla_japonica_PGR1   SADLCSDNPL PQATNIKTDP CSSFSSFVGE GILTRASMGY SQQAIQTLPV HKSEPFRLSA
Anguilla_anguilla_PGR2   FTDYNRTTAL PLIPKITED- QFSFPYPVGE VVANSCLTGY GQRSPQNSLM FKSELSKLSL
Anguilla_japonica_PGR2   FTDYNRTTAL PLIPEITED- QFSFPYPVGE VVANSCLTGY GQRSPQNSLR FKSELCKLSL

                       301
Anguilla_anguilla_PGR1   SSAPADSPFW CQSTGPSEDH HLQIDYLSPA GLHNTCKYS- STNAYSSYLG VLPQRVCVIC
Anguilla_japonica_PGR1   SSAPADSPFW CQSTGPSEDH HLQIDYLSPA GLHSTCKYS- STNAYSSYLG VLPQRVCVIC
Anguilla_anguilla_PGR2   PTTSPESQSW CQSTGLSEDQ HFETGYLPPG EIRNTYVTHN SLKSHSLYMG MLSQKFCLIC
Anguilla_japonica_PGR2   PTSSPESQSW CQSTGLSEDQ HFETGYLPPG EIRNICETHN SLKSHSVYMG MLSQKFCLIC

                       361
Anguilla_anguilla_PGR1   GDEASGCHYG VLTCGSCKVF FKRAVEGHHN YLCAGRNDCI VDKIRRKNCP ACRLRKCYQA
Anguilla_japonica_PGR1   GDEASGCHYG VLTCGSCKVF FKRAVEGHHN YLCAGRNDCI VDKIRRKNCP ACRLRKCYQA
Anguilla_anguilla_PGR2   GDEASGCHYG VLTCGSCKVF YKRAVEGHQN YLCAGRNDCI VDKIRRKNCP ACRLRKCYQA
Anguilla_japonica_PGR2   GDEASGCHYG VLTCGSCKVF YKRAVEGHQN YLCAGRNDCI VDKIRRKNCP ACRLRKCYQA

                       421
Anguilla_anguilla_PGR1   GMILGGRKLK KLGALKAAGL TQALVAHSLT PRRLSGDSQA LMPLGCLPGV RELHLSPQII
Anguilla_japonica_PGR1   GMILGGRKLK KLGALKAAGL TQALVAHSLT PRRLSGDSQA LMPLGCLPGV RELHLSPQII
Anguilla_anguilla_PGR2   GMTLGGRKMK KLSALKVLGL TQSLAVRSPL G--ASYEGQA LATLPSMPMV RELQFTPQML
Anguilla_japonica_PGR2   GMTLGGRKMK KLSALKVLGL TQSLAVRSPL G--ASYEGQA LATLPSMPMV RELQFTPQIL

                       481
Anguilla_anguilla_PGR1   SVLESIEPEV VYSGYDNSQP DMPNMLLNSL NRLCERQLLR IVKWSKSLPG FRSLHINDQM
Anguilla_japonica_PGR1   SVLESIEPEV VYSGYDNSQP DMPNMLLNSL NRLCERQLLR IVKWSKSLPG FRSLHINDQM
Anguilla_anguilla_PGR2   SVLESIEPET VYSGYDGTQP ETPNLLLNSL NRLCERQLLW IVRWSKSLPG FRSLHINDQM
Anguilla_japonica_PGR2   SILENIEPET VYSGYDATQP ETPHLLLNSL NGLCERQLLW IVRWSKSLPG FRSLHINDQM

                       541
Anguilla_anguilla_PGR1   ALIQYSWMSL MVFSLGWRSF QNVTSEYLYF APDLILNEEY MRRSPIFDLC MAMQFIPQEF
Anguilla_japonica_PGR1   ALIQYSWMSL MVFSLGWRSF QNVTSDYLYF APDLILNEEY MRRSPIFDLC MAMQFIPQEF
Anguilla_anguilla_PGR2   TLIQYSWMSM MVFSLGWRSF QNVTREFLYF APDLILSEEK MRNSPISDLC MAMQIIPQAF
Anguilla_japonica_PGR2   TLIQYSWMSL MVFSLGWRSF QNVTREFLYF APDLILGEEK MRNSPISDLC MAMQIIPQAF

                       601
Anguilla_anguilla_PGR1   ANLQVTKEEF LCMKVLLLLN TVPLEGLKSQ PQFDEMRQNY IHELTKAIHL RENGVVACSQ
Anguilla_japonica_PGR1   ANLQVTKEEF LCMKVLLLLN TVPLEGLKSQ PQFDEMRQNY IHELTKAIHL RENGVVACSQ
Anguilla_anguilla_PGR2   DNLHVTKEEF LCMKVLLLLN TVPLEGLRSQ AQFDEMRHGY IRELTKAIQL TERGVVASSQ
Anguilla_japonica_PGR2   DNLQVTKEEF LCMKVLLLLN TVPLEGLRSQ AQFDEMRHGY IRELTKAIQL TERGVMASSQ

                       661
Anguilla_anguilla_PGR1   RFYHLTKLMD HMHDIVKKLH LYCLSTFIQA DAMRVEFPEM MSEVIASQLP RVLAGMVKPL
Anguilla_japonica_PGR1   RFYHLTKLMD HMHDIVKKLH LYCLSTFIQA DAMRVEFPEM MSEVIASQLP RVLAGMVKPL
Anguilla_anguilla_PGR2   RFYHLTKLMD AMHEIVRKVN LYCLSTFIQA EAMQVEFPEM MSEVITSQLP KVLAGMVRPL
Anguilla_japonica_PGR2   RFYHLTKLMD AMHEIVRKVN LYCLSTFIQA EAMQVEFPEM MSEVITSQLP KVLAGMVRPL

                       721
Anguilla_anguilla_PGR1   LFHTK
Anguilla_japonica_PGR1   LFHTK
Anguilla_anguilla_PGR2   LFHKK
Anguilla_japonica_PGR2   LFHKK
```

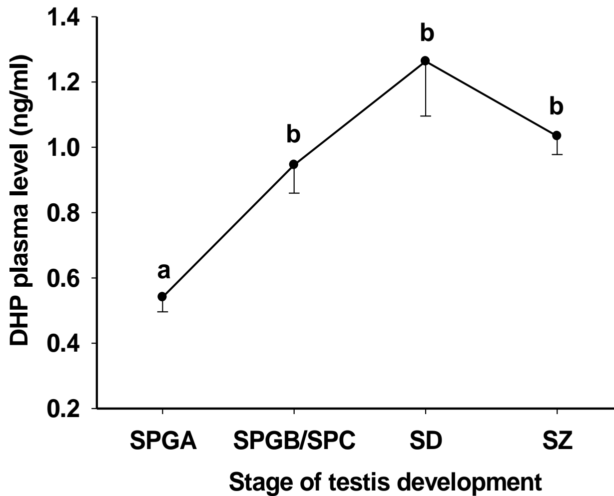
S2 Fig. Multiple sequence alignment of the European eel nPRs at amino acid level.



S3 Fig. Multiple sequence alignment of the European eel mPRs at amino acid level. Exons are indicated in dark grey or light grey.



S4 Fig. Tissue distribution of progestin receptors in the European eel. Progestin receptor mRNA expression in immature female (1), and in immature male (2) of *pgr1* (A), *pgr2* (B), *mPRa* (C), *mPRγ* (D), *mPRδ* (E), *mPRAL1* (F), *mPRAL2* (G) mRNA expression. Data are normalised to eel ARP. Values are presented as means ± SEM (n = 3). OB: olfactory bulb, T: Telencephalon, M/D: Mes-/Diencephalon, CEREB: cerebellum, MED: medulla oblongata, PIT, pituitary.



S5 Fig. 17α,20β-dihydroxy-4-pregnen-3-one (DHP) plasma level through spermatogenesis.

S1 Table. Accession number of sequences used for phylogenetic analyses.

Sequence name	Species name	Accession
membrane progestin receptor beta	*Alligator sinensis*	XP_006033874.1
membrane progestin receptor	*Alligator sinensis*	XP_006032505.1
membrane progestin receptor alpha-	*Alligator sinensis*	XP_006019324.1
progestin and adipoQ receptor family member 6 isoform X1	*Alligator sinensis*	XP_006038577
progestin and adipoQ receptor	*Alligator sinensis*	XP_006037621
progestin and adipoQ receptor	*Alligator sinensis*	XP_006027387
progestin and adipoQ receptor family member 9 partial	*Alligator sinensis*	XP_006027327
membrane progestin receptor alpha	*Falco cherrug*	XP_005433117.1
membrane progestin receptor beta	*Falco cherrug*	XP_005441640.1
membrane progestin receptor	*Falco cherrug*	XP_005443489.1
progestin and	*Falco cherrug*	XP_005435421
progestin and adipoQ receptor	*Falco cherrug*	XP_005443775
progestin and adipoQ receptor	*Haliaeetus*	XP_010562388

membrane progestin receptor alpha (weltzien lab)	*Anguilla anguilla*	scaffold5681.1
membrane progestin receptor gamma 1 (weltzien lab)	*Anguilla anguilla*	scaffold2805.1
membrane progestin receptor gamma 2 (weltzien lab)	*Anguilla anguilla*	scaffold952.1
membrane progestin receptor alpha	*Anguilla japonica*	scaffold5127.1
membrane progestin receptor gamma 1	*Anguilla japonica*	scaffold2302.1
membrane progestin receptor gamma2	*Anguilla japonica*	scaffold53.1
Membrane progestin receptor alpha like 1	*Anguilla japonica*	Scaffold 4651.1
Membrane progestin receptor alpha like 1	*Anguilla anguilla*	Contig 552991, contig 347951, contig 30968, contig 1128323
Membrane progestin receptor alpha like 2	*Anguilla anguilla*	Contig 1426891, contig 998808, contig 330155, contig 873764,
membrane progestin receptor alpha like 2	*Anguilla japonica*	scaffold 1135.1
membrane progestin receptor alpha	*Carassius auratus*	BAD06917.1
membrane progestin receptor gamma-1	*Carassius auratus*	BAF37035.1
membrane progestin receptor gamma-2	*Carassius auratus*	BAF37036.1
membrane progestin receptor beta	*Carassius auratus*	BAF37034.1
membrane progestin receptor alpha	*Danio rerio*	AAN78115.1
membrane progestin receptor gamma	*Danio rerio*	NP_956481.1
membrane progestin receptor	*Danio rerio*	NP_001003573.1
membrane progestin receptor beta	*Danio rerio*	AAN78114.1
progestin and adipoQ receptor family member 3	*Danio rerio*	NP_957004

progestin and adipoQ receptor family member 9	*Danio rerio*	XP_005166589
progestin and adipoQ receptor family member 6 isoformX1	*Danio rerio*	XP_005158451
progestin and adipoQ receptor family	*Danio rerio*	XP_693997
membrane progestin receptor alpha-B-like	*Lepisosteus oculatus*	XP_006631325.1
membrane progestin receptor gamma-B-like	*Lepisosteus oculatus*	XP_006628883.1
membrane progestin receptor beta-like	*Lepisosteus oculatus*	XP_006625758.1
membrane progestin receptor alpha-B	*Lepisosteus oculatus*	XP_006631465
progestin and adipoQ receptor family member 6-like	*Lepisosteus oculatus*	XP_006641879
progestin and adipoQ receptor family member 9-like	*Lepisosteus oculatus*	XP_006627442
progestin and adipoQ receptor family member 3-like	*Lepisosteus oculatus*	XP_006626814
membrane progestin receptor alpha-like isoform X1	*Oreochromis niloticus*	XP_005456756.1
membrane progestin receptor gamma-B-like isoform 2	*Oreochromis niloticus*	XP_003456791.1
membrane progestin receptor beta-like isoform X1	*Oreochromis niloticus*	XP_003437799.1
progestin and adipoQ receptor family member 9-like	*Oreochromis niloticus*	XP_005465345
progestin and adipoQ receptor family member 3-like	*Oreochromis niloticus*	XP_003444582
progestin and adipoQ receptor family member 6-like isoform X1	*Oreochromis niloticus*	XP_003443093
progestin and adipoQ receptor family member 4-like	*Oreochromis niloticus*	XP_003442580
membrane progestin receptor gamma	*Takifugu rubripes*	XP_003969864.1
membrane progestin receptor beta-like	*Takifugu rubripes*	XP_003971417.1
progestin and adipoQ receptor family member 3 isoform X1	*Takifugu rubripes*	XP_003974709
progestin and adipoQ receptor family member 6 isoform X1	*Takifugu rubripes*	XP_003965729
progestin and	*Takifugu rubripes*	XP_003966777

adipoQ receptor family member 9		
progestin and adipoQ receptor family member 4	*Takifugu rubripes*	XP_003961438
progestin and adipoQ receptor family member VII	*Takifugu rubripes*	NP_001035912
membrane progestin receptor alpha-B-like	*Takifugu rubripes*	XP_011607479
membrane progestin receptor alpha	*Homo sapiens*	NP_848509.1
membrane progestin receptor beta	*Homo sapiens*	NP_588608.1
membrane progestin receptor gamma	*Homo sapiens*	NP_001098024.1
progestin and adipoQ receptor family member 6 isoform 1	*Homo sapiens*	NP_079173
progestin and adipoQ receptor family member 4 isoform 1	*Homo sapiens*	NP_689554
progestin and adipoQ receptor family member 3	*Homo sapiens*	NP_001035292
progestin and adipoQ receptor family member 9	*Homo sapiens*	NP_940906 XP_293613
membrane progestin receptor alpha	*Mus musculus*	NP_001272775.1
membrane progestin receptor beta	*Mus musculus*	NP_083105.3
membrane progestin receptor gamma	*Mus musculus*	NP_083024.1
progestin and adipoQ receptor family member 3	*Mus musculus*	NP_940814 XP_132242
progestin and adipoQ receptor family member 6	*Mus musculus*	NP_940802 XP_355445
progestin and adipoQ receptor family member 9	*Mus musculus*	NP_940806 XP_147043
progestin and adipoQ receptor family member 4	*Mus musculus*	NP_076313
Membrane progestin receptor gamma	*Latimeria chalumnae*	XP_005997123.1
Membrane progestin receptor beta	*Latimeria chalumnae*	XP_006009425.1
Membrane progestin receptor alpha	*Latimeria chalumnae*	XP_005993988.1
progestin and adipoQ receptor family member 6 isoform X1	*Latimeria chalumnae*	XP_006005171
progestin and adipoQ receptor family member 4	*Latimeria chalumnae*	XP_006004865
progestin and adipoQ receptor family member 9 like	*Latimeria chalumnae*	XP_005997958
progestin and adipoQ receptor family member 3 like	*Latimeria chalumnae*	XP_005992487
nuclear progestin receptor	*Oryzias latipes*	BAN84406.1
nuclear progesterone receptor Pgr	*Danio rerio*	ABO61201.1

progesterone receptor	*Alligator mississippiensis*	XP_006274202.1
progesterone receptor	*Falco cherrug*	XP_005432129.1
progesterone receptor isoform X1	*Oreochromis niloticus*	XP_003450208.1
progesterone receptor	*Haplochromis burtoni*	ACM51148.1
progesterone receptor	*Homo sapiens*	ABB72139.1
progesterone receptor	*Mus musculus*	NP_032855.2
progesterone receptor 2	*Carassius auratus*	BAO48149.1
progesterone receptor 1	*Carassius auratus*	BAO48148.1
pgr-201	*Latimeria chalumnae*	ENSLACT00000025487
Pgr-201	*Lepisosteus oculatus*	ENSLOCT00000006693
progesterone receptor type 1	*Anguilla anguilla*	AFV13730.1
progesterone receptor type 2	*Anguilla anguilla*	AFV13731.1
progestogen receptor type 2	*Anguilla japonica*	BAB85993.1
progesterone receptor	*Anguilla japonica*	BAA89539.1

S2 Table. Names, references and locations of the genes used in the PGR synteny analysis (Fig. 4)

GENE	SPECIES	LOCATION	START	STRAND	LOCATION	END	STRAND
YAP1	HUMAN	chr 11	102,110,461	F	chr 11	102,233,423	F
	ZEBRA FINCH	chr 1	76,931,691	R	chr 1	77,014,180	R
	SPOTTED GAR	LG3	8,360,941	F	LG3	8,405,073	F
	TILAPIA	GL831183.1	1,836,000	R	GL831183.1	1,844,015	R
	TILAPIA	GL831218.1	2,012,449	R	GL831218.1	2,033,276	R
	STICKLEBACK	group VII	21,566,379	R	group VII	21,571,454	R
	STICKLEBACK	group I	14,774,869	R	group I	14,796,093	R
	FUGU	sc 6	3,230,449	R	sc 6	3,234,945	R
	FUGU	sc 19	1,142,732	F	sc 19	1,159,371	F
	ZEBRAFISH	chr 21	-	-	chr 21	-	-
	ZEBRAFISH	chr 18	37,302,757	R	chr18	37,374,658	R
	EUROPEAN EEL	sc 7734	15,663	R	cont 115697	118	R
	EUROPEAN EEL	sc 1198	19,287	R	sc 1198	64,971	R
	JAPANESE EEL	sc 1188	18,987	R	sc 1188	65,769	R
	JAPANESE	sc 8180	8,251	F	sc 5725	8,75	F

	EEL					5	
CEP126	HUMAN	chr 11	101,915,015	F	chr 11	102,001,058	F
	ZEBRA FINCH	chr 1	77,075,755	R	chr 1	77,104,833	R
	SPOTTED GAR	LG3	8,328,532	F	LG3	8,346,660	F
	TILAPIA	GL831183.1	-	-	GL831183.1	-	-
	TILAPIA	GL831218.1	20,396,70	R	GL831218.1	20.43452	R
	STICKLEBACK	group VII	-	-	group VII	-	-
	STICKLEBACK	group I	14,805,098	R	group I	14,807,616	R
	FUGU	sc 6	-	-	sc 6	-	-
	FUGU	sc 19	1,137,660	F	sc 19	1,138,553	F
	ZEBRAFISH	chr 21	-	-	chr 21	-	-
	ZEBRAFISH	chr 18	37,399,117	R	chr 18	37,426,780	R
	EUROPEAN EEL	sc 7633	9,885	F	sc 7632	16,139	F
	EUROPEAN EEL	sc 1198	77,913	R	sc 1198	9,351	R
	JAPANESE EEL	sc 1188	80,551	R	sc 1188	96,278	R
	JAPANESE EEL	sc 1807	73,881	F	sc 1807	81,264	F
ANGPTL5	HUMAN	chr 11	101,890,674	R	chr 11	101,916,522	R
	ZEBRA FINCH	chr 1	77,109,694	F	chr 1	77,121,394	F

	SPOTTED GAR	LG3	8,319,777	R	LG3	8,327,394	R
ANGPTL 5	TILAPIA	GL831183.1	1,851,478	F	GL831183.1	1,857,985	F
	TILAPIA	GL831218.1	-	-	GL831218.1	-	-
	STICKLEBACK	group VII	21,575,116	F	group VII	21,578,135	F
	STICKLEBACK	group I	-	-	group I	-	-
	FUGU	sc 6	3,239,000	F	sc 6	3,240,783	F
	FUGU	sc 19	-	-	sc 19	-	-
	ZEBRAFISH	chr 21	22,784,214	R	chr 21	22,794,783	R
	ZEBRAFISH	chr 18	-	-	chr 18	-	-
	EUROPEAN EEL	sc 399	207,176	R	sc 399	215,944	R
	EUROPEAN EEL	-	-	-	-	-	-
	JAPANESE EEL	sc 1188	106,445	F	sc 1188	115,142	F
	JAPANESE EEL	-	-	-	-	-	-
TRPC6	HUMAN	chr 11	101,451,564	R	chr 11	101,872,562	R
	ZEBRA FINCH	chr 1	77,295,400	F	chr 1	77,324,263	F
	SPOTTED GAR	LG3	8,275,513	R	LG3	8,307,177	R
	TILAPIA	GL831183.1	1,860,644	F	GL831183.1	1,874,50	F

						9	
	TILAPIA	GL831218.1	2,045,612	F	GL831218.1	2,051,990	F
	STICKLEBACK	group VII	21,581,377	F	group VII	21,588,762	F
	STICKLEBACK	group I	14,810,368	F	group I	14,815,632	F
	FUGU	sc 6	3,243,057	F	sc 6	3,247,974	F
	FUGU	sc 19	1,129,216	R	sc 19	1,135,443	R
	ZEBRAFISH	chr 21	22,764,652	R	chr 21	22,775,844	R
	ZEBRAFISH	chr 18	41,782,282	F	chr 18	41,818,549	F
	EUROPEAN EEL	sc 399	125,048	R	sc 399	167,365	R
	EUROPEAN EEL	sc 4345	27,721	R	sc 4345	32,724	R
	JAPANESE EEL	sc 754	29,678	F	sc 754	69,304	F
	JAPANESE EEL	sc 1807	51,651	R	sc 1807	63,801	R
PGR	HUMAN	chr 11	101,029,624	R	chr 11	101,130,524	R
	ZEBRA FINCH	chr 1	77,400,054	F	chr 1	77,430,281	F
	SPOTTED GAR	LG3	8,249,265	R	LG3	8,264,108	R
	TILAPIA	GL831183.1	-	-	GL831183.1	-	-
	TILAPIA	GL8312	2,055	F	GL8312	2,05	F

		18.1	,322		18.1	8,180	
	STICKLEBACK	group VII	-	-	group VII	-	-
	STICKLEBACK	group I	14,817,967	F	group I	14,822,383	F
	FUGU	sc 6	-	-	sc 6	-	-
	FUGU	sc 19	1,125,205	R	sc 19	1,127,892	R
	ZEBRAFISH	chr 21	-	-	chr 21	-	-
	ZEBRAFISH	chr 18	41,829,847	F	chr 18	41,848,227	F
	EUROPEAN EEL	sc 399	105,092	R	sc 399	115,989	R
	EUROPEAN EEL	sc 4345	10,842	R	sc 4345	18,294	R
	JAPANESE EEL	sc 754	78,396	F	sc 754	89,282	F
	JAPANESE EEL	sc 1807	34,029	R	sc 1807	41,968	R
ARHGAP42	HUMAN	chr 11	100,687,653	F	chr 11	100,991,937	F
	ZEBRA FINCH	chr 1	77,454,971	R	chr 1	77,557,821	R
	SPOTTED GAR	LG3	8,174,507	F	LG3	8,247,740	F
	TILAPIA	GL831183.1	1,874,553	R	GL831183.1	1,892,720	R
	TILAPIA	GL831218.1	2,060,334	R	GL831218.1	2,130,732	R
	STICKLEBACK	group VII	21,590,222	R	group VII	21,596,520	R
	STICKLEBA	group I	14,82	R	group I	14,8	R

	CK		6,603			68,822	
	FUGU	sc 6	3,248,745	R	sc 6	3,258,437	R
	FUGU	sc 19	1,090,791	F	sc 19	1,122,492	F
	ZEBRAFISH	chr 21	22,702,337	F	chr 21	22,759,459	F
	ZEBRAFISH	chr 18	41,853,404	R	chr 18	41,893,801	R
	EUROPEAN EEL	sc 399	4,281	F	sc 399	92,304	F
	EUROPEAN EEL	sc 4345	63	F	sc 4345	8,226	F
	JAPANESE EEL	sc 754	101,070	R	sc 754	190,565	R
	JAPANESE EEL	sc 1807	5,664	F	sc 1807	31,392	F

CHAPTER 3

Temperature modulates testis steroidogenesis in European eel

David S. Peñaranda[1], Marina Morini[1], Helge Tveiten[2], M. Carmen Vílchez[1], Victor Gallego[1], Ron P. Dirks[3], Guido E.E.J.M. van den Thillart[3], Luz Pérez[1], Juan F. Asturiano[1,*]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. 46022, Valencia, Spain.

[2]Norwegian Institute of Fisheries and Aquaculture, Muninbakken 9-13, Breivika, P.O. Box 6122 NO-9291 Tromsø, Norway.

[3]Leiden University, Institute of Biology, Leiden, Gorlaeus Laboratories, POB 9505, 2300RA Leiden, The Netherlands

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Abstract

This study evaluates the effects of temperature on hCG-induced spermatogenesis in European eel (*Anguilla anguilla*), subjected to three thermal regimes: T10: 10 °C (first 4 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15: 15 °C (first 4 weeks) and 20 °C (last 9 weeks); and T20: constant 20 °C for the duration of the experiment. At 10 °C, maturation stopped in the A spermatogonial stage (SPG1), and no further maturation was observed until the temperature was ≥15 °C. With the aim of explaining these results, the influence of temperature on steroidogenic enzyme gene expression and steroid synthesis was tested. The initial synthesis of androgens (T and 11-KT) increased at SPG1, and was not influenced by temperature. Likewise, the gene expression of the steroidogenic enzymes linked to androgen synthesis (*aacyp11a1, aacyp17-I* and *aa11βHSD*) also increased at SPG1. In contrast, no correlation was seen between the increase in E2 and the *aacyp19a1* gene expression peak in the testes, with E2 increasing as a consequence of the seawater acclimation carried out before hormonal treatment, and peaking the aacyp19a1 gene expression at B spermatogonial stage (SPG2). *Aacyp21* gene expression was also higher at SPG2, and this stage was only reached when the rearing temperature was ≥15 °C.

In conclusion, androgen synthesis is not dependent on temperature, but further maturation requires higher temperatures in order to induce a change in the steroidogenic pathway towards estrogen and progestin synthesis. This study demonstrates that temperature plays a crucial role in European eel maturation, even perhaps controlling gonad development during the reproductive migration.

1. Introduction

The European eel (*Anguilla anguilla*) is a teleost fish with a peculiar life cycle in which pubertal individuals undertake, apparently in 6-7 months, a transatlantic migration to the spawning areas in the Sargasso Sea (Tesch, 1978). The precise route that they take and the depth they swim at are not well known. However, satellite tags, used to document the oceanic migratory route up to 1300 km off the

European coasts, have shown that they make daily vertical migrations between depths of 200 and 1000 m. It appears that they swim in shallower and warmer waters through the night (means of 282 m and 11.7 °C), while at dawn they descend to deeper and colder waters (means of 564 m and 7-10 °C) (Aarestrup et al., 2009). However, several authors have expressed doubts regarding the validity of the data gathered by satellite tags as they may have a negative effect on the swimming performance and energetics of the fish (Methling et al., 2011).

Other telemetry studies indicate that the eels in the Mediterranean swim under the thermocline during the day, at 13 °C, and during the night ascend to shallower waters, of around 18 °C (Tesch, 1989). Tesch (1978) discovered that in the coastal waters off the North-East coast of Spain, the eels prefer depths of approximately 400 m during the day and 50-215 m at night.

Since the 1960s, the natural stocks of European eel have declined dramatically due to several factors including overfishing, habitat reduction and pollution (Feunteun, 2002) and at the same time it being a highly valued species particularly in demand in Europe and Asia. Reproduction in captivity is a possible alternative able to reduce the pressure on natural populations and supply glass eels to eel farms. The availability of good quality sperm is necessary to reach this objective.

In some fish species, reproduction in captivity can be controlled using environmental factors exclusively (photoperiod, temperature, salinity), but often the use of exogenous hormones is the only effective way of inducing sexual maturation and spermiation. The eel (*Anguilla* spp.) does not mature spontaneously in captivity and must receive long-term hormonal treatment (Boëtius and Boëtius, 1967; Gallego et al., 2012; Tanaka et al., 2001).

Among the environmental factors, water temperature plays a key role in the sexual development of many fish species (Van Der Kraak and Pankhurst, 1996). In the case of the European eel, the temperature of the hypothetical spawning area is around 20 °C (Boëtius and Boëtius, 1967), and that is the reason why the maturation of males and

females of this species has traditionally been performed in water of that temperature (Peñaranda et al., 2010; Pérez et al., 2009). However, the influence of temperature on the maturation process of the European eel has recently been noted both in females (Mazzeo et al., 2014; Pérez et al., 2011) and in males (Baeza et al., 2015; Tanaka et al., 2001).

Sexual maturation requires steroids (androgens, estrogens and progestins) which are derived from cholesterol and depend on the species, sex, and reproductive stage (Young et al., 2005). The present study has attempted to evaluate the influence of temperature (using 3 thermic regimes) on the dynamics of steroidogenic enzyme gene expression and steroid synthesis in European eel testis.

To date, most of the steroidogenic enzymes have been cloned and their expression has been analysed by both PCR approaches and *in situ* hybridisation (Tokarz et al., 2013). The P450scc enzyme (cyp11a1) acts as the catalyst for the first and rate-limiting step in steroidogenesis, and is responsible for the conversion of cholesterol into pregnenolone. In teleosts (Tokarz et al., 2013), including the Japanese eel (*Anguilla japonica*; Ijiri et al., 2006), its gene expression and immunolocalization are located in the Leydig cells. One of the enzymes responsible for metabolising pregnenolone is the cytochrome P450c17 (cyp17) enzyme. Two forms of P450c17 (I and II) were discovered in medaka (*Oryzias latipes*; Zhou et al., 2007). P450c17-I was identified as being responsible for 17β-estradiol (E_2) production while P450c17-II played a key role in the production of 17a,20β-dihydroxy-4-pregnen-3-one (DHP) (Zhou et al., 2007). P450c17 (*cyp17-I*) was cloned and characterised in Japanese eel by Kazeto et al. (2000a), who reported a significant increase in its gene expression after salmon pituitary extract injections in female eels.

Regarding androgens, 11-ketotestosterone (11-KT) is considered to be the most important in teleosts (Miura and Miura, 2003a) and is biosynthesised from testosterone (T) by two enzymes, 11β-hydroxylase (cytochrome P450-11β) and 11β-hydroxysteroid dehydrogenase (11β-HSD; Jiang et al., 2003). In teleosts, 11β-HSD sequence is similar to mammalian 11β-HSD type 2 (Albiston et al., 1994). Some examples can be found in the rainbow trout (*Oncorhynchus mykiss*, Kusakabe et

al., 2003), tilapia (*Oreochromis niloticus*) and Japanese eel (Jiang et al., 2003). In eel, two homologous genes of mammalian 11β-HSD type 2 are present in the testis: 11β-HSD (Albiston et al., 1994; Jiang et al., 2003; Kusakabe et al., 2003) and 11β-HSD short form (11β-HSDsf) (Ozaki et al., 2006), both enzymes with 11β-dehydrogenase activity.

Cytochrome P450 aromatase (cyp19) acts as a catalyst for the synthesis of estrogens, which regulate important processes throughout spermatogenesis (Miura et al., 2003b). In contrast to the two paralogous genes of P450 aromatase found in other teleosts (Blázquez and Piferrer, 2004), in eels, only one aromatase cDNA has been identified (termed *cyp19a1*) and is expressed in the ovary, brain and pituitary (Ijiri et al., 2003; Peñaranda et al., 2014). Although E2 has traditionally been considered a female hormone, in Japanese eel it has been seen to stimulate spermatogonial stem cell renewal (eSRS34, Miura et al., 2003b).

20β-hydroxysteroid dehydrogenase (20β-HSD) and 21-hydroxylase (Cyp21) are the main enzymes responsible for progestin synthesis in fish. Teleostean 20β-HSD is the candidate enzyme to produce DHP (Lubzens et al., 2010), the maturation inducing steroid (MIS) in eel (Kazeto et al., 2011; Peñaranda et al., 2010). Two types of carbonyl reductase-like 20β-hydroxysteroid dehydrogenase (CR-20β-HSD) cDNAs were cloned from female rainbow trout ovary, both with 20β-HSD and carbonyl reductase-like 20β-HSD (CR-20β-HSD) activity in trout ovary (Guan et al., 1999). In female Japanese eel, 20β-HSD enzymatic activity was increased by hormonal treatment, mainly in the mid-vitellogenic stage (Kazeto et al., 2011). In addition, it has been reported that CR-20β-HSD plays a role in testicular recrudescence in male catfish, leading to sperm maturation (Sreenivasulu et al., 2012). The cyp21 enzyme is responsible for synthesising 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), which was identified as the MIS in the perciform family Sciaenidae (Trant and Thomas, 1989). In some species, both steroids appear to participate in regulating oocyte maturation (Asturiano et al., 2000; Ohta et al., 2002), but until now with eels, the cyp21 gene has been linked to cortisol production through the conversion of progesterone into 11-deoxycorticosterone (Li et al., 2003) in head kidney.

If we consider the limited knowledge available to us on the reproductive migration of this species, it seems probable that gonadal development, which takes several months, happens at low temperatures, while the spawning and the spermiation happen at higher temperatures. Therefore, our hypothesis is that temperature could play a crucial role in regulating the progress of maturation during reproductive migration, inhibiting or inducing the gene expression of steroidogenic enzymes through androgen synthesis at low temperatures and estrogen and progestin at higher temperatures.

2. Material and Methods

2.1 Fish maintenance, hormonal treatment and sampling

A total of 317 adult male eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture Laboratory at the Universitat Politècnica de València, Spain. Growth at the fish farm is carried out in freshwater conditions and at 27 °C, thus once the fish arrived at our facilities, they were acclimated at 20 °C and freshwater conditions over the period of a week. They were then distributed in aquaria equipped with separated recirculation systems, coolers and covered to maintain constant darkness. The fish were gradually acclimatized to seawater (salinity 37±0.3%; 20 °C) over the course of another week and randomly distributed in six 200-L aquaria (approximately 100 males per treatment). Finally, the animals were underwent three thermal regimes: T10: 10 °C (first 4 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15: 15 °C (first 4 weeks) and 20 °C (last 9 weeks); and T20: 20 °C during the whole experimental period.

As previously described by Gallego et al. (2012), after being anaesthetized with benzocaine dissolved in seawater (60 ppm) the males were administered weekly intraperitoneal injections of recombinant human chorionic gonadotropin (rechCG; 1.5 IU g^{-1} fish; Ovitrelle®, Merck Serono Europe Limited, UK) in order to induce maturation and spermiation.

Each week groups of 5-8 eels per thermal regime were anaesthetized

with benzocaine dissolved in seawater (>60 ppm) and sacrificed by decapitation (total amount: ~273 fish). Total weights and gonad weights were recorded to calculate the gonadosomatic index (GSI = 100 gonad weight x total body weight^{-1}). In addition, samples from the testis were collected and stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 °C until the extraction of total RNA (Peñaranda et al., 2010).

Furthermore, testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status.

Additionally, before starting the hormonal treatment, two groups of male eels (n=6) were sacrificed in freshwater (FW) and seawater (SW) conditions with the aim of evaluating the possible influence of salinity.

2.2 Human and Animal Rights

This study was carried out in strict accordance with the recommendations laid out in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (Permit Number: 2014/VSC/PEA/00147). The fish were sacrificed under anaesthesia with benzocaine (>60 ppm), and all efforts were made to minimize suffering. The fish were not fed throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.3 Histology processing

The testis samples were processed and analysed as described by Baeza et al. (2015). The maturation stages (Figure 1) were determined using the following criteria: dominance of germ cell types, degree of development of the seminal tubules, GSI and sperm production by the male sampled in the same week of the sacrifice.

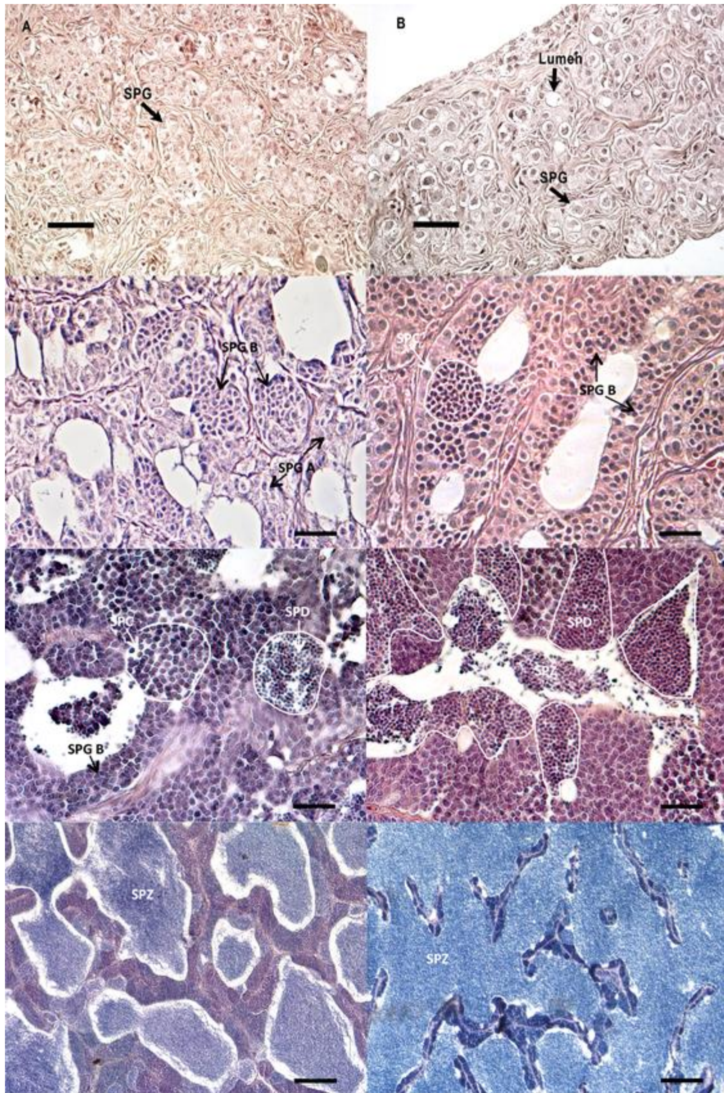

Figure 1. Histological sections of eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment. A) Testis at Freshwater and Seawater conditions (SPG0). B) Testis at SGP1. C) Testis at SGP2. D) Testis at SPC1. E) Testis at SPC2. F) Testis at SD. G) Testis at SZ1. H) Testis at SZ2. See main text for definition of gonad developmental stages. SPG= Spermatogonia; SPC: Spermatocyte; SD: Spermatid; SZ: Spermatozoa. Scale bars, 10 µm.

In summary, Stage SPG0: non-injected fish. Dominance of A spermatogonia. Absence of tubule lumen. Mean GSI=0.016. Stage SPG1: injected fish. Dominance of A spermatogonia. In general, absence of tubule lumen. Gonad in non-proliferating stage. GSI=0.055. Stage SPG2: Dominance of A spermatogonia, with some B spermatogonia. Dominant process: development of tubule lumen and spermatogonial differentiation. Mean GSI= 0.175. Stage SPC1: Dominance of B spermatogonia and presence of spermatocytes. Dominant processes are: spermatogonial mitosis and differentiation. Mean GSI= 0.43. Stage SPC2: Dominance of spermatocytes and B spermatogonia; some spermatids may be present in a low number. Mean GSI=0.72 (0.25-2.00). The dominant process in this stage is germ cell meiosis. Stage SD: is characterized by the abundance of spermatids. Some spermatozoa may appear in the testis, but in scarce numbers. Males in non-spermiating stage; if some milt was produced, it is of low volume (<0.5 ml) and low motility (<10%). Mean GSI= 3.4 (1.4-6.3). The dominant process in this stage is spermiogenesis (spermatid maturation). Stage SZ1: early spermiating stage; abundant sperm cells present inside the tubule lumen. Tubule lumen delimited by a multiple germ cell layer. Males in spermiating stage (volume >0.5 ml; sperm motility >10%). Mean GSI= 6.09 (3.5-9.5). Early spermiation stage. Stage SZ2 is characterized by a dominance of spermatozoa and a reduction in the percentage of other germ cells in comparison to SZ1. The cell walls delimiting the tubule lumen merging between them, and showing reduced width compared to SZ1. Mean GSI=7.61 (3-13). Males showing high sperm motility, high sperm volume. Stage of maximum spermiation.

2.4 Measurement of gene expression by real-time quantitative PCR (qPCR)

2.4.1 Primer design

Eel acidic ribosomal phosphoprotein P0, aa-arp (Table 1) was used as a reference gene in the qPCR analysis because its mRNA expression is stable throughout hormonal treatment (Weltzien et al., 2005). The expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1 (0.94; $p<0.05$) and a Cq arithmetic

mean of 24.7±1.93 in the testis. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. The Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers for aacyp11a1, aacyp17-I, aa11βHSD, aaCR20β-HSD and aacyp21 (Table 1).

Table 1. Primer sequences used in quantitative PCR.

Name	**Sequence (5'- 3')**	**Orientation**	**Usage**	**GenBank Accession number.**	**Reference**
aacyp19a1	TTC AAG GGA ACG AAC ATC ATC AGA AAC GGT TGG GCA CAG T	Forward Reverse	qPCR (115 bp)(E=102%)	KF990052	Peñaranda et al. 2014
aacyp11a1	GGA GTC CTT CTG AAG GAT GGG CCT GAA CCT GTG GAG CGA TT	Forward Reverse	qPCR (82 bp (E=88.3%))	AZBK00000000	confirmed in this work
aacyp17-I	TGT CGC CCC TCC TCA TAC C ACT CTG GCC CCT TTT CCA ACT	Forward Reverse	qPCR (79 bp) (E=93%)	AZBK00000000	confirmed in this work
aa11β-hsd	CAA GGG AGA CTC GTG ACC AT CCC AGG GTT CCA GTT CGT	Forward Reverse	qPCR (104 bp)(E=101%)	AZBK00000000	confirmed in this work
aaCR20β-hsd	CAG CTC GAT ATC TCC GAT CC TCC TCT GTG ATG TCA TTG CTG	Forward Reverse	qPCR (317 bp)(E=90.8%)	AZBK00000000	confirmed in this work
aacyp21	CTG TAT CCC AAA GGA CAC CA ATG GCA GAA CAC GAG T	Forward Reverse	qPCR (115 bp)(E=99.4%)	AZBK00000000	confirmed in this work
aaArp	GTG CCA GCT CAG AAC ACG ACA TCG CTC AAG ACT TCA ATG G	Forward Reverse	qPCR (107 bp)(E=109)	AY763793	Weltzien et al. 2005

aacyp19a1= Anguilla anguilla aromatase P450 a1; aacyp11a1= Anguilla anguilla P450scc; aacyp17-I = Anguilla anguilla P450c17-I; aa11β-hsd= Anguilla anguilla 11βHSD; aaCR20β-hsd= Anguilla anguilla CR20β-hsd; aacyp21= Anguilla anguilla cyp21; aaArp: Anguilla anguilla acidic ribosomal phosphoprotein P0. qPCR amplicon length is given in parenthesis following

the forward qPCR primers. qPCR efficiency for each primer pair is given in parenthesis following the reverse qPCR primer.

To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon-exon boundary. All the primers were tested on gDNA and RNA to confirm that potentially contaminant gDNA was not amplified. The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the qPCR products.

2.4.2 SYBR Green assay (qPCR)

Total RNA was isolated from the RNAlater preserved tissues as described by Pérez et al. (2011), and then purified and treated with DNase I using NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) following the guide instructions. RNA concentration, quality, and integrity were evaluated using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). First-strand cDNA was synthesized from 500 ng of testis total RNA, using a qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA) with a mix of random hexamer and oligo(dT) primers.

In order to quantify the gene expression, qPCR assays were developed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas GMBH). The PCR protocol included an initial step of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 1 s and 60 °C for 30 s. To evaluate assay specificity, the machine performed a melting curve analysis directly following PCR by slowly (0.1 °C/s) increasing the temperature from 68 to 95 °C, with a continuous registration of changes in fluorescent emission intensity.

The total volume for every PCR reaction was 20 µl, performed from diluted (1:10) DNA template (5 µl), forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Weltzien et al. (2005). Target and reference genes in unknown samples were run in duplicate PCR reactions, and a cDNA pool from various samples was included in each run as a

calibrator. Non-template control (cDNA was replaced by water) for each primer pair was run in duplicate on all plates.

2.5 Steroids

Plasma concentrations of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) were measured by mean of radioimmunoassays, as described previously (Frantzen et al., 2004; Schulz, 1985). Assay characteristics and cross-reactivities of the E2 and T antisera have previously been examined by Frantzen et al. (2004) and further validated for eel plasma by Mazzeo et al. (2014). The characteristics and cross-reactivities of the DHP assay have previously been described by Tveiten et al. (2010) and validated for eel plasma by Peñaranda et al. (2010). The cross-reactivities of a new 11-KT antiserum used in this work have previously been described by Johnsen et al. (2013). To validate the recovery of 11-KT from plasma in the eel assay, a plasma pool was spiked with 45 ng 11-KT/ml of plasma and then underwent ether extraction as described below. The resulting product was then assayed by the 11-KT RIA at three different dilutions. The dilutions were found to be parallel to the standard assay curve. Steroid recovery after ether extraction was 71.9±2.8%. The 11-KT values were corrected for recovery losses. The inter- and intra-assay coefficients of variation (CV) for the 11-KT assay were 15.4% (n=7) and 5.3% (n=10), respectively.

2.6 Statistical analysis

Differences in the data were analysed by analysis of variance of least square (General Lineal Model, GLM), including fixed temperature, weeks of treatment and stages of development. A Student-Newman-Keuls test was used to compare means, and the differences were considered significant when $P<0.05$. The results are presented as means ± standard error of the mean (SEM). All statistical procedures were analysed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

In order to understand the role of the steroids and the steroidogenic

enzymes responsible of their synthesis throughout spermatogenesis, the results will be shown with regard to the stages of the spermatogenesis determined histologically. A comparison of least square means (LSM) was performed considering the three thermal regimes as a unique group at each stage. Additionally, the least square mean of T10, T15 and T20 groups was compared in each gonadal stage.

3. Results

3.1 Testis development

All males responded to the hormonal treatment and showed testis development, but the different thermal regimes resulted in notable variations in the dynamics of the process (Figure 2).

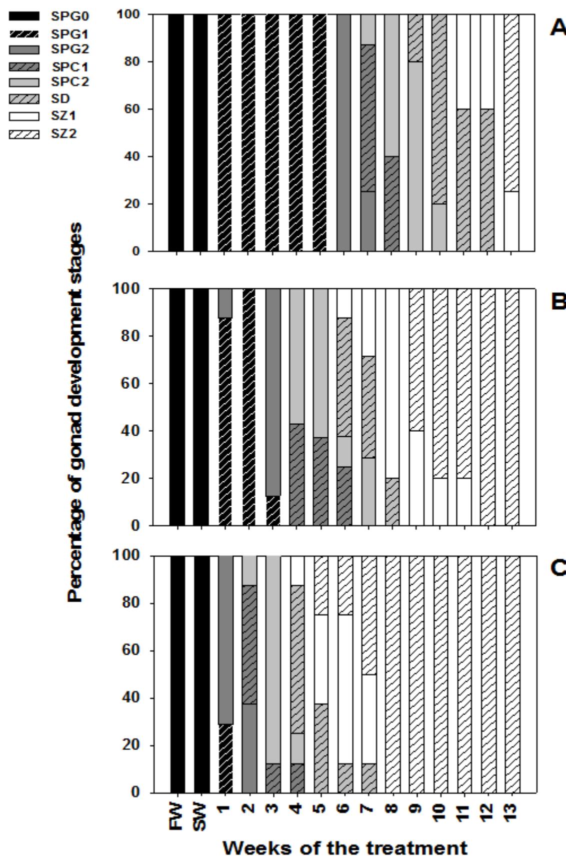

Figure 2. Relative percentages of testis develop-mental stages (SPG0-SZ2) prior to hormonal treatment in freshwater (FW) and seawater conditions (SW), and along hCG treatment at different thermal regimes: A) T20 group; B) T15 group and C) T10 group in male eels.

Seawater acclimation did not induce any gonad development (SPG 0). At 20 °C and 15 °C, after the third week of treatment no fish were found in SPG2 (Figures 2B and 2C). Moreover, it was necessary to increase the temperature to 15 °C in the T10 group for the fish to reach SPG2 (6th week, Figure 2A).

The fish reared at 20 °C only needed four weeks to complete spermatogenesis (presence of spermatozoa in the testis, SZ1 stage) and five weeks to reach the most advanced stage of gonad development (SZ2). However, at lower temperatures nine weeks (in the case of T15) and 13 weeks (T10) were needed before fish reached SZ2.

3.2 Expression of testis steroidogenic enzyme genes and steroid production during the treatment

3.2.1 Androgen synthesis

Temperature modulated the expression of testis steroidogenic enzyme genes. The cyp11a1 enzyme is responsible for the synthesis of pregnenolone from cholesterol and cyp17-I mediates the synthesis of 17a-OH-pregnenolone from pregnenolone, opening the androgen synthetic pathway. The increased expression of both genes was not linked to temperature, with the highest values being recorded in the first few weeks of treatment and after seawater acclimation (up to 37‰; Figure 3). 11β-OHT is converted into 11-KT by the 11β-HSD enzyme. A parallel increase was observed between the expression of aa11βHSD and 11-KT plasma levels (Figure 4). In T20 and T15, one hCG injection was enough to increase aa11βHSD expression and 11-KT plasma levels (Figures. 4A-B and 4D-E). Although the increase of both the gene expression and 11-KT plasma levels happened regardless of the temperature, the peak in the expression of aa11βHSD was delayed by one week at lower temperatures, with it being recorded in the 2nd week of hormonal treatment in T15 and T10, and in the 1st week in T20. The subsequent decrease in aa11βHSD expression and 11-KT plasma levels was less pronounced in T10 (Figures. 4C and 4F) than in the other two groups.

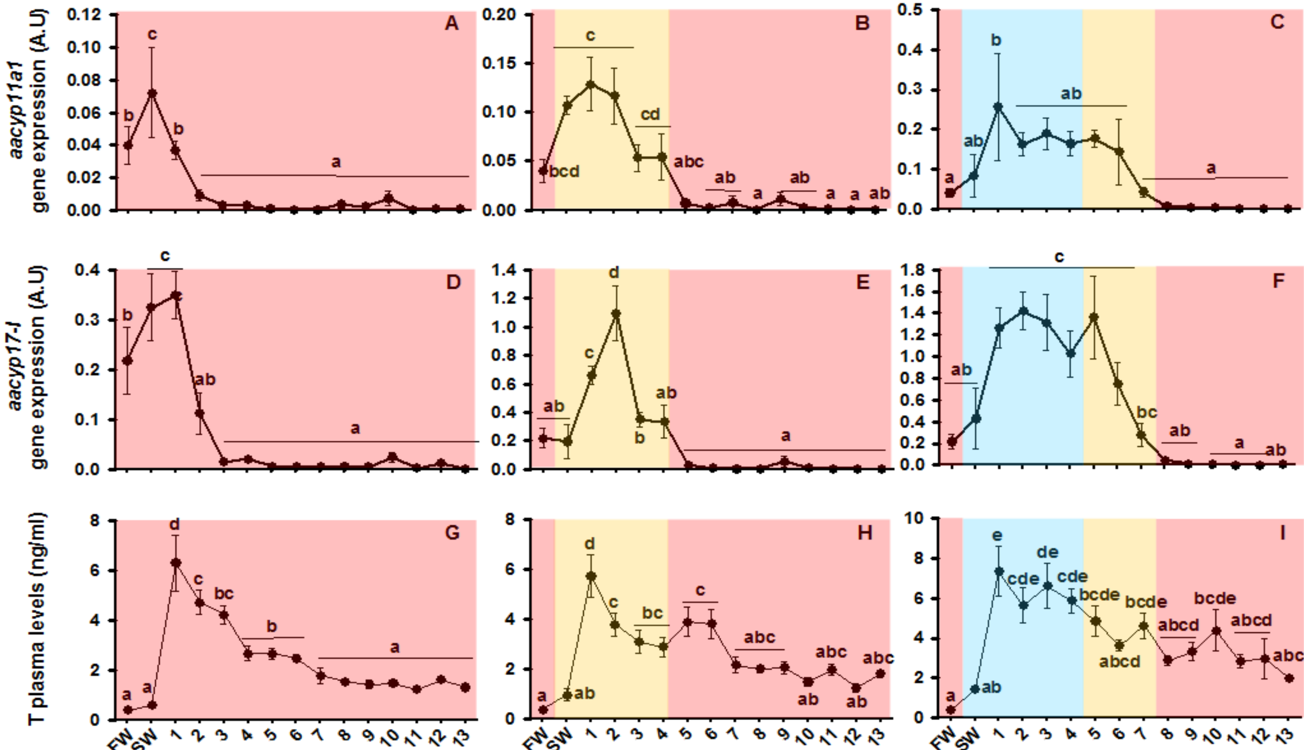

Figure 3. Gene expression of *Anguilla anguilla* P450scc (aacyp11a1; A, B and C), *Anguilla anguilla* P450c17-I (aacypc17-I; D, E and F) and testosterone (T) plasma levels (G, H and I) during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented as following, red colour: 20 °C, orange colour: 15 °C and blue colour: 10 °C.

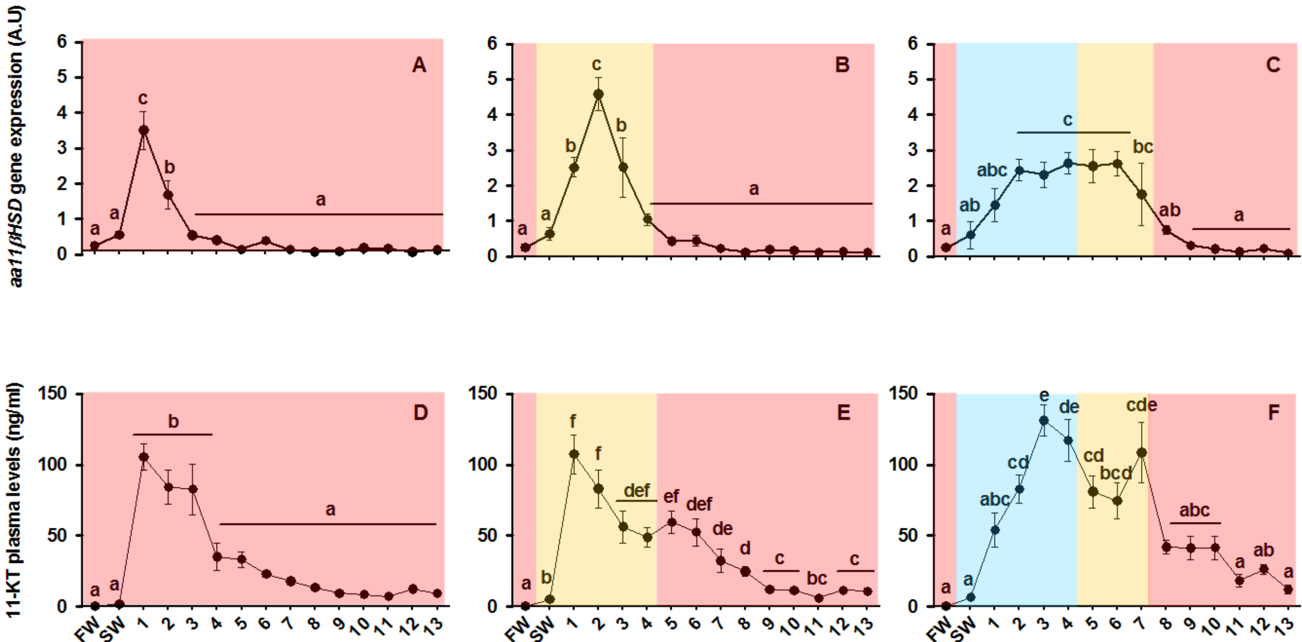

Figure 4. Gene expression of *Anguilla anguilla 11βHSD* (*aa11HSD*; A, B and C) and 11-ketotestosterone (11KT; D, E and F) plasma levels during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented as following, red colour: 20 °C, orange colour: 15 °C and blue colour: 10 °C

3.2.2 Estrogen and progestin synthesis

cyp19a1 is the limiting enzyme for E_2 synthesis. However, an increase in E_2 plasma levels took place prior to the peak in aacyp19a1expression in the testis in all the thermal regimes (Figure 5). The E_2 peak was observed after seawater acclimation, regardless of the temperature, and decreased throughout the course of spermatogenesis.

In contrast, the expression of aacyp19a1 in the testis was modulated by temperature. Lower temperatures delayed the peak in aacyp19a1, recorded in the 2nd, 3rd and 6th weeks in T20, T15 and T10, respectively (Figures 5A-C). It is remarkable that the increase in aacyp19a1 expression took place when the temperature was over 10 °C (Figure 5C).

Cyp21 is the enzyme which mediates the pathway for the production of 20βS, and 20βHSD is responsible for DHP synthesis. No significant differences in the expression of aaCR20β-HSD in any of the thermal regimes were recorded at any point in the hormonal treatment, compared to freshwater conditions (data not shown). Again, no significant differences in aacyp21 gene expression were observed in T20 (Figure 6A) throughout the treatment, but in T15 a progressive increase was registered up until the 4th week of treatment. The levels then decreased when the water temperature was increased from 15 to 20 °C (Figure 6B). In T10, low values of aacyp21 mRNA transcript were observed until the temperature was increased to 15 °C (from week 4), the levels then decreased after the 7th week (Figure 6C), when the water temperature was increased again to 20 °C.

Fish with significantly higher DHP plasma values than untreated fish were observed in the 3rd week of treatment in T20 (Figure 6D). Lower temperatures delayed this increase to the 5th and 6th weeks in T15 and T10, respectively (Figures 6E and 6F). Also, the highest DHP levels were delayed at lower temperatures, with them being reached in week 4 in T20 in contrast to week 10 in the T15 and T10.

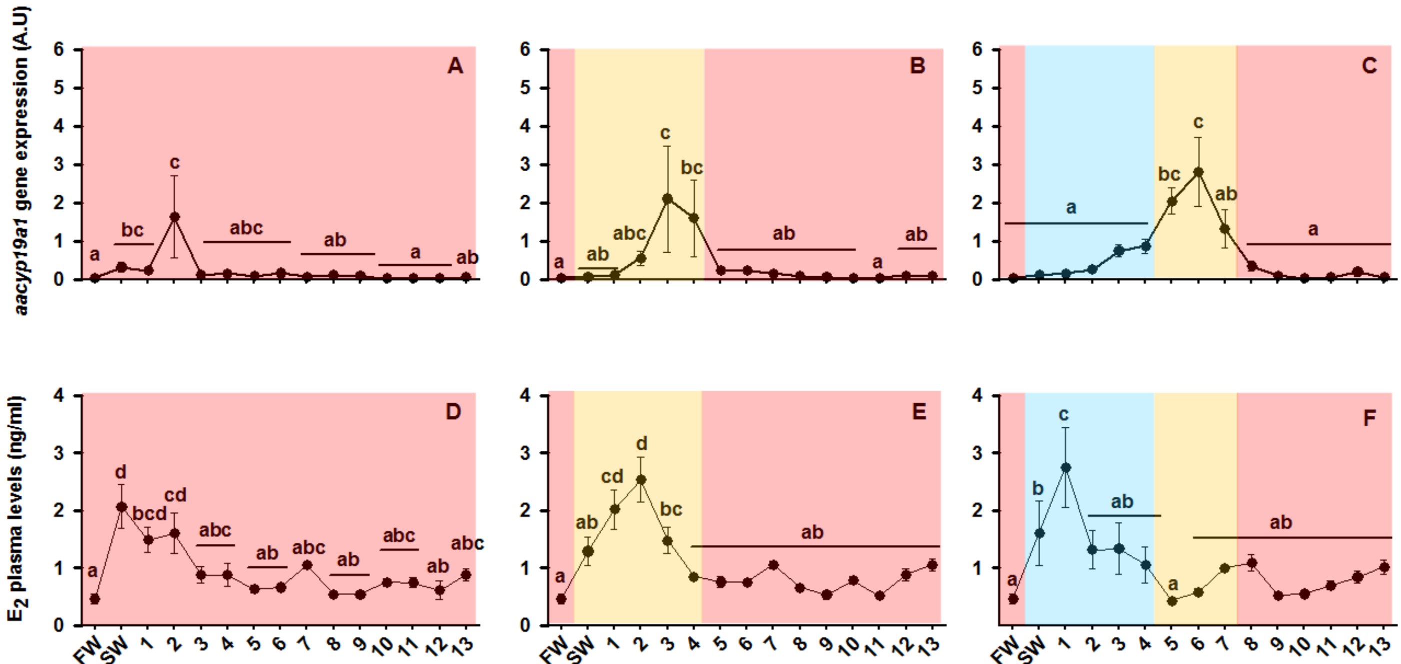

Figure 5. Gene expression of *Anguilla anguilla* aromatase P450 a1 (aacyp19a1; A, B and C) and estradiol (E2; D, E and F) plasma levels during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented as following, red colour: 20 °C, orange colour: 15 °C and blue colour: 10 °C.

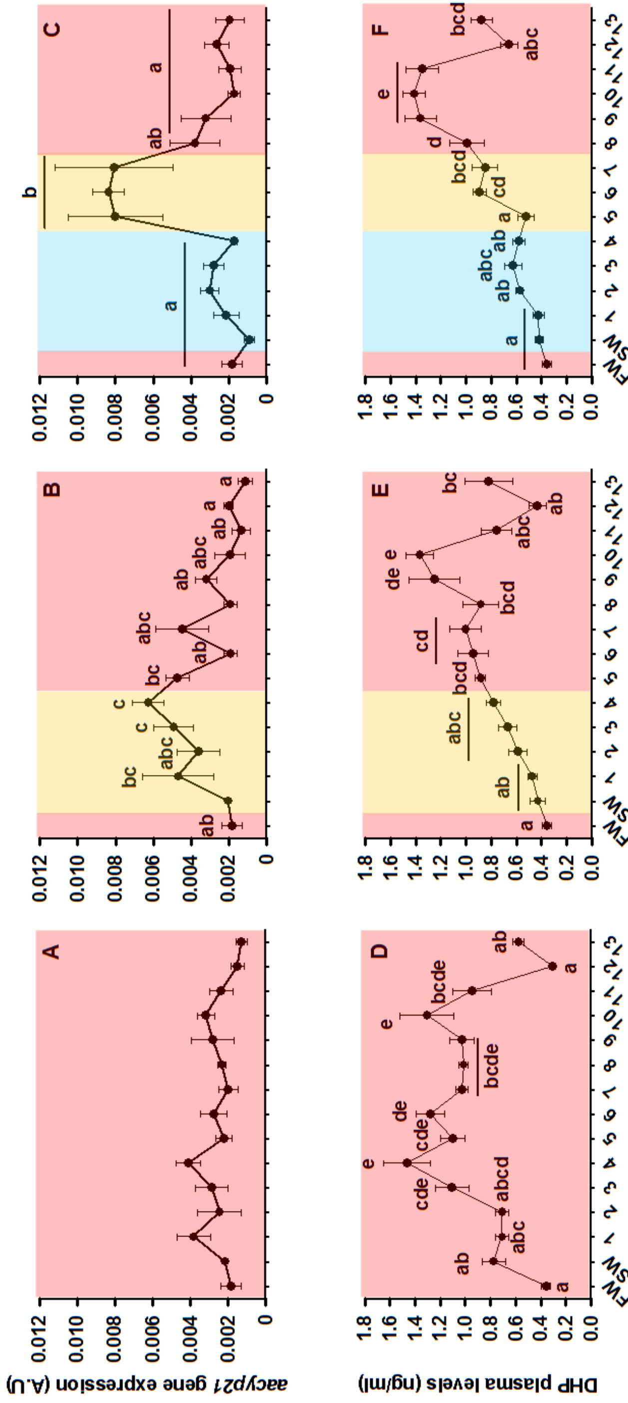

Figure 6. Gene expression of *Anguilla anguilla cyp21* (*aacyp21*; A, B and C) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP; D, E and F) plasma levels during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented as following, red colour: 20 °C, orange colour: 15 °C and blue colour: 10 °C.

3.2.3 Steroid and gene expression throughout spermatogenesis

Firstly, the seawater acclimation was enough to increase the E_2 plasma levels (Figure 7A), which then decreased in the following stages.

This decrease was fastest in T10. Thereafter, the T and 11KT plasma levels increased at SPG1 (Figures 7B and 7C). This increase was higher in T20, but the levels of both androgens decreased in the subsequent stages in all three thermal regimes. There were two registered increases in progestin production, at SPG2 (compared to freshwater conditions) and at SD stage (compared to the previous stages), with no significant difference recorded between the groups (Figure 7D).

Regarding gene expression, the highest levels of cyp11a1 and cyp17-I were registered at SPG1, with a progressive decrease in the subsequent stages (Figures 8A and 8B). This increase, in both genes, was greater at lower temperatures (T20 and T15). Similar results were observed for aa11βHSD gene expression (Figure 8C), but with higher values at high temperatures (T20 and T15).

4. Discussion

Temperature exercised a clear effect on eel maturation, delaying spermatogenesis in thermal regimes with lower temperatures. The gonad development of the fish reared at 20 °C throughout the whole treatment showed similar results to those observed in previous studies (Peñaranda et al., 2010). Nevertheless, the fish reared at 10 °C arrested their maturation at SPG1, and no further maturation was observed until the temperature was increased (15 °C). In hormonally-treated female eels, a delay in gonad maturation caused by low rearing temperatures was also observed (Mazzeo et al., 2014; Pérez et al., 2011; Sudo et al., 2011). In the European female eel, both high (20 °C) and low (10 °C) temperatures induced steroidogenesis in previtellogenic eel ovaries, but high temperatures during hormonal treatment caused an acceleration of ovarian growth from the mid to late vitellogenic stage (Mazzeo et al., 2014; Pérez et al., 2011).

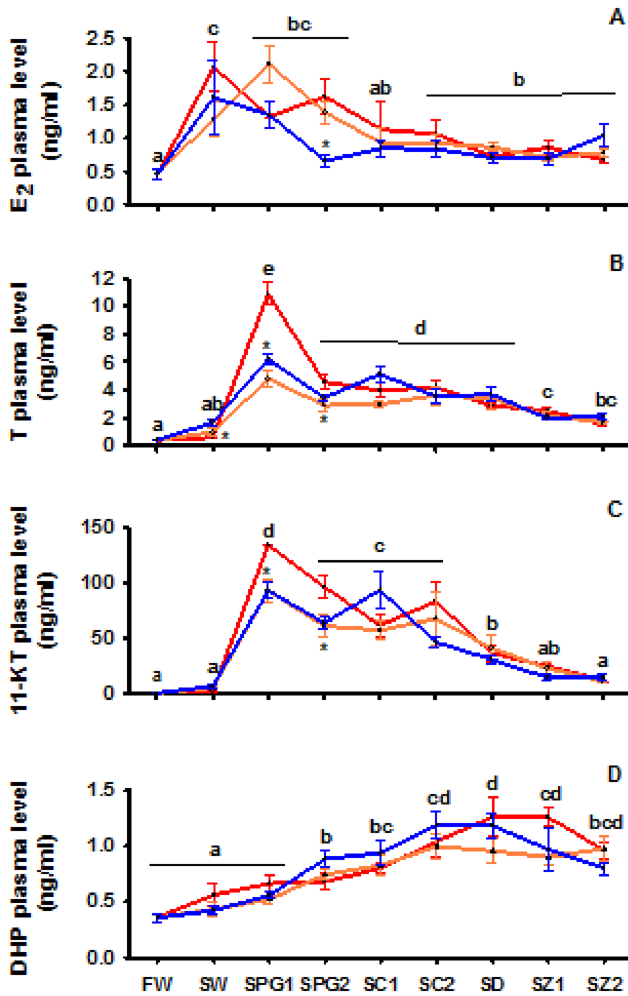

Figure 7. A) Testosterone (T), B) 11-ketotestosterone (11KT), C) estradiol (E_2) and D) 17α,20β-dihydroxy-4-pregnen-3-one (DHP) plasma at the different stages of gonad development. Different superscripts mean significant differences ($p<0.05$; n=6-15) through spermatogenesis considering the three thermal regimes as a unique group in each stage and asterisk reports significant differences ($p<0.05$; n=5-8) between thermal regimes (red line: T20 group; orange line: T15 group; blue line: T10 group). See main text for definition of gonad developmental stages. FW= Freshwater, SW= Seawater, SPG1= Spermatogonia 1 stage, SPG2= Spermatogonia 2 stage, SPC1= Spermatocyte 1 stage, SPC2= Spermatocyte 2 stage, SD= Spermatid stage, SZ1= Spermatozoa 1 stage and SZ2= Spermatozoa 2 stage.

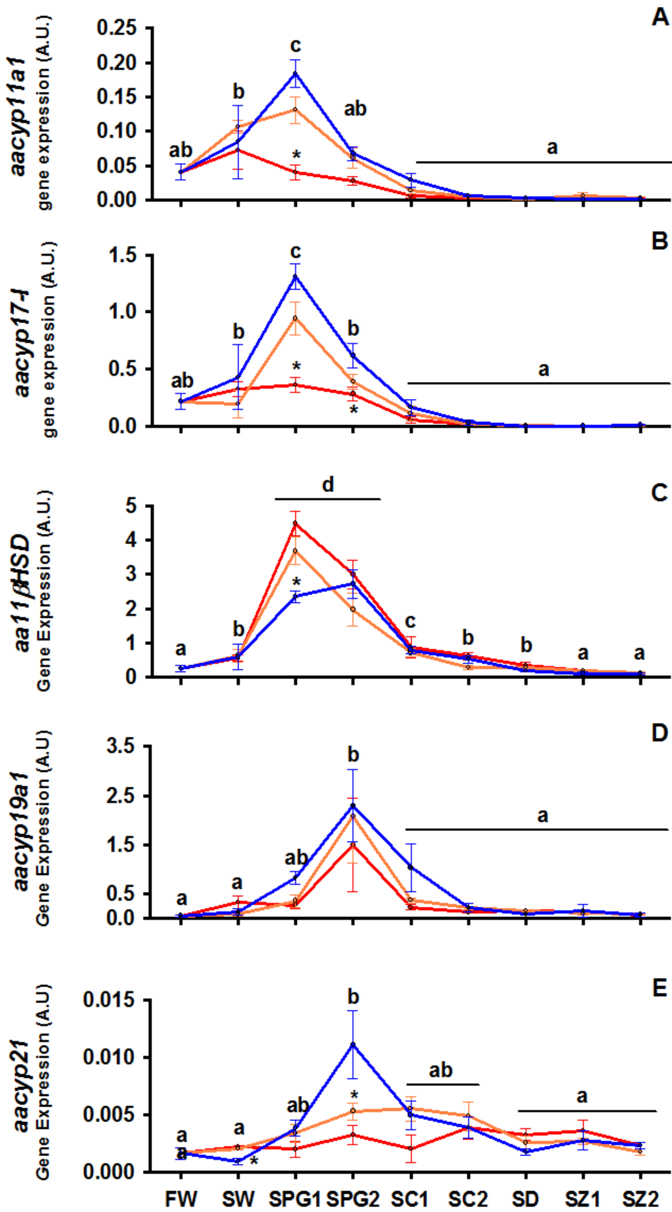

Figure 8. A) Gene expression of *Anguilla anguilla P450c17-I* (*aacypc17-I*), B) *Anguilla anguilla P450scc* (*aacyp11a1*), C) *Anguilla anguilla 11βHSD* (*aa11HSD*), D) *Anguilla anguilla* aromatase *P450 a1* (*aacyp19a1*) and E) *Anguilla anguilla cyp21* (*aacyp21*) at the different stages of gonad development. Different superscripts mean significant differences ($p<0.05$; n=6-15) through spermatogenesis considering the three thermal regimes as a unique group in each stage and asterisk reports significant differences

(p<0.05; n=5-8) between thermal regimes (red line: T20 group; orange line: T15 group; blue line: T10 group). See main text for definition of gonad developmental stages. FW= Freshwater, SW= Seawater, SPG1= Spermatogonia 1 stage, SPG2= Spermatogonia 2 stage, SPC1= Spermatocyte 1 stage, SPC2= Spermatocyte 2 stage, SD= Spermatid stage, SZ1= Spermatozoa 1 stage and SZ2= Spermatozoa 2 stage.

In Japanese female eel, fish maintained at 10 °C did not reach ovulation; however when the water temperature was increased from 10 to 20 °C, the fish ovulated in the subsequent weeks (Sato et al. 2006). Sudo et al. (2011) observed lower gonadotropins expression in fish reared with a gradual temperature decrease from 25 to 15 °C compared to fish reared at 25 °C. These results from female eels appear to corroborate our study, since the first stages of maturation are possible independently of the temperature, even when this temperature is low (10 °C), but higher temperatures being necessary for further maturation.

4.1 Androgen synthesis

An increase in the expression of *aacyp11a1* and *aacyp17-I* in testis was recorded irrespective of the thermal regime. Both enzymes are required for the synthesis of T (Hinfray et al., 2013), thus the increase in these two cytochromes at the onset of spermatogenesis is consistent with the increase in T in the plasma at SPG1.

In Japanese eel testis, after a single hCG injection at 20 °C the expression of *cyp11a1* was stimulated. Unlike the data collected in this experiment, levels of *cyp11a1* mRNA and protein dropped 3 days after hormonal stimulation, but the reason could be that in Japanese eel the hormonal treatment consisted of a single hCG injection, and in our experiment the fish were treated with weekly hCG injections. Similar results have been reported in rainbow trout, with an increase in the expression levels of both genes at the beginning of spermatogenesis (Kusakabe et al., 2006).

As was observed for *aacyp11a1* and *aacyp17-I* gene expression, the increase of *aa11βHSD* gene expression was not temperature dependent. The *aa11βHSD* gene expression reported in the current study is the sum of both 11β-HSD type 2 subtypes (11β-HSD and 11β-

HSDsf) present in eels. In the Japanese eel, *11β-HSD* mRNA transcripts were not found in immature eel testis, but appeared at day 1, and peaked at day 3 after the administering of hCG injections. Thereafter, the levels decreased rapidly from day 6, and fell very low after day 12 (Jiang et al., 2003). As aforementioned, the difference could be that maturation of the Japanese eel was induced using a single injection, while we applied weekly hCG injections. Since the 11-KT plasma levels increased in tandem with the *aa11βHSD* gene expression, it is likely that both 11β-HSD type 2 subtypes play an important role in 11-KT synthesis. The increase in *aa11βHSD* transcripts and 11-KT plasma levels were observed at SGP1 when A spermatogonia cells were predominant in the testes, and decreased with the spermatogenesis progression. These results corroborate the role of 11-KT during spermatogenesis as proposed by Schulz et al. (2010).

4.2 Estrogens and progestin synthesis

As reported in previous studies (Quérat et al., 1987), the increase of salinity to seawater conditions (37%) increased E_2 plasma levels. Nevertheless, the E_2 increase did not correlate with the *aacyp19a1* gene expression in the testes. In fact, the peak in E_2 in plasma was observed prior to the *aacyp19a1* gene expression. As a consequence, E_2 plasma might come from an extra-gonadal source, but also from the gonad. In the Japanese eel it has been demonstrated that *17β-HSD-I* is responsible for the testicular conversion of androstenedione into T and oestrone into E_2, and vice versa (Kazeto et al., 2000b). In eels, the 17β-HSD activity opens up an alternative pathway for E_2 production, which could be the reason why we observed an increase in E_2 in plasma but no *aacyp19a1* gene expression in the testes. *aacyp19a1* mRNA transcripts were also affected by the temperature, with the peak in *aacyp19a1* expression being delayed by lower temperature thermal regimes. The peak in *aacyp19a1* was not observed until the temperature was ≥15 °C. In teleosts, the influence of temperature on the *cyp19a* transcription by DNA methylation of the gonadal aromatase promoter has been demonstrated (Martínez et al., 2014), and recently a down-regulation of ovarian *aacyp19a1* in female European eels caused by high

temperatures in CPE-treated eels was reported (Carp Pituitary Extract, CPE; Mazzeo et al., 2014).

The peak in the *aacyp19a1* expression was reached at SPG2, after an increase in 11-KT and T (stage SPG1). SPG2 is characterised by development of tubule lumen, and the presence of mainly type A spermatogonia. In European sea bass (*Dicentrarchus labrax*), the highest levels of *cyp19a* and estrogen receptor gene expression were found in spermatogonia and spermatocytes (Viñas and Piferrer, 2008), suggesting that estrogens may play a role in the proliferation and differentiation of spermatogonia cells, in addition to being a spermatogonial stem cell renewal factor, as proposed by Miura et al. (2003b) in Japanese eel. In other teleosts the exposure to E2 had no effect on the testis cyp19a1 protein and expression (Filby et al., 2006; Zhang et al., 2008), but it did have an effect on the neural cyp19b1 gene expression (Diotel et al., 2010).

Recently, a novel type of 20α-hydroxysteroid dehydrogenase (omhsd17b3like) was identified in masu salmon (Su et al., 2015). In the Japanese eel, it was demonstrated that CR20βHSD did not have a 20βHSD activity, and this novel 20βHSD and not CR20βHSD is the 20βHSD responsible for DHP production (Su et al., 2015). In the present study, a continual increase in DHP was observed as the weeks of treatment and stages of development progressed, but the levels of *aaCR20βHSD* gene expression did not vary significantly over the same time period. These results confirm that the CR20βHSD enzyme is not responsible for DHP production in eels.

The expression of the *aacyp21* gene seems to be regulated by temperature, since the values were low at 10 °C, and it was necessary to increase the temperature to 15 °C in order to observe a significant increase. In fact, in T10 and T15, the increase took place at 15°C, but in both groups a decrease was reported at 20 °C. Traditionally, eel maturation has been induced at a temperature of 18-20 °C (Mazzeo et al., 2014; Pérez et al., 2011; Tanaka et al., 2001), and this could be the reason why Adachi et al. (2003) reported that 20βS levels at 20 °C in the Japanese eel were much lower than DHP levels.

The *aacyp21* gene expression increased at SPG2, when A

spermatogonia were predominant and some germ cells started to differentiate in B spermatogonia. Also, at this stage (SPG2), higher DHP plasma levels were found, suggesting that progestins can be involved in spermatogonial differenciation or in the testis morphogenesis, since tubule lumen formation was observed at this stage (SPG2). These new possible roles of progestins need to be further studied in the future.

A second increase in DHP plasma levels was reported at SPC2, when meiosis is the dominating process, indicating that the progestins play a role in the meiosis process, as it was proposed by Miura et al. (2006) in Japanese eel. High DHP plasma levels were maintained in the following stages, suggesting that the DHP could also have a role in sperm maturation, as reported by Miura and Miura (2003a) or Kazeto et al. (2011) in Japanese eel (Kazeto et al., 2011).

5. Conclusions

In conclusion, temperature modulates steroidogenesis, the gonad maturation and the spermiation process in eels. Irrespective of the temperature, the onset of spermatogenesis was characterised by an increase in estrogen and androgen plasma levels, which stimulated the proliferation of spermatogonia. Nevertheless, at 10 °C maturation was arrested at SPG1, and for spermatogenesis to advance further, it was necessary to increase the temperature to 15 °C. In fact, this temperature (15 °C) was required to induce a shift in steroidogenesis from androgen synthesis to estrogen and progestin synthesis, with *aacyp19a1* and *aacyp21* gene expression increasing at 15 °C. It could be hypothesized that the testis of male eels migrating at low temperatures develop up to spermatogonia proliferation, but no further maturation occurs until the fish reach higher temperatures, in the Sargasso Sea, and then spermatogenesis can be completed.

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CHAPTER 4

Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel

Marina Morini[1], David S. Peñaranda[1], M. Carmen Vílchez[1], Víctor Gallego[1]., Rasoul Nourizadeh-Lillabadi[2],

Juan F. Asturiano[1], Finn-Arne Weltzien[2], Luz Pérez*[1]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València, Camino de Vera s/n. 46022, Valencia, Spain.

[2]Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences - Campus Adamstuen, P.O. Box 8146 Dep, 0033 Oslo, Norway.

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Abstract

Activation at fertilization of the vertebrate egg is triggered by Ca^{2+} waves. Recent studies suggest the phospholipase C zeta (PLCζ), a sperm-specific protein triggers egg activation by an IP3-mediated Ca^{2+} release and allow Ca^{2+} waves at fertilization.

In the present study we cloned, characterized, and phylogenetically positioned the European eel PLCζ (PLCζ1). It is 1521bp long, with 10 exons encoding an open reading frame of 506 amino acids. The amino acid sequence contains an EF-hand domain, X and Y catalytic domains, and a carboxy-terminal C2 domain, all typical of other PLCζ orthologous. The sequence is truncated not only at the N-terminus of the EF-hand domain, as in all teleost PLCζ, but also in the C-terminal region of the X-domain and in a large part of the N-terminal X/Y linker region.

The tissue distribution was studied, and the gene expression was determined in testis during induced sexual maturation at three different thermal regimes. Also, brain and pituitary expression were studied through sex maturation at constant temperature. *plcζ1* was expressed in brain of male and female, in testis but not in ovaries. By first time in vertebrates, it is reported *plcζ1* expression in the pituitary gland. Testis *plcζ1* expression increased through spermatogenesis under all the thermal regimes, but being significantly elevated at lower temperatures. It was very low when testis contained only spermatogonia or spermatocytes, while maximum expression was found during spermiogenesis. These results support the hypothesis for an eel sperm-specific PLCζ1 inducing egg activation, similarly to mammals and some teleosts, but different from some other teleost species, which express this protein in ovaries, but not in testes.

1. Introduction

Sperm fusion with the egg induces egg activation in all animals studied so far through a rise in intracellular Ca^{2+} (Stricker, 1999; Tarin, 2000; Kashir et al., 2010; Horner and Wolfner, 2008). Three models have been proposed for mechanisms by which fertilization-induced Ca^{2+}

waves are initiated: a) Ca^{2+} bolus/conduit (Jaffe, 1983, 1991), where the sperm trigger the entering of extracellular Ca^{2+} into the oocyte; b) membrane receptor (Jaffe, 1990; Evans and Kopf, 1998), with an intracellular Ca^{2+} release provoked by the binding of an oocyte surface receptor with a sperm ligand; or c) a soluble sperm factor (Swann et al., 2006; Parrington et al., 2007; Saunders et al., 2007) released into the oocyte after gamete fusion, triggering egg activation. This sperm factor corresponds to a sperm-specific phospholipase C (PLC) called PLCζ (Swann and Lai, 2013; Ito et al., 2011). After fertilization, PLCζ induces a reaction chain by cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Igarashi et al., 2007; Miao and Williams, 2012). These two metabolites, in turn, cause IP3-mediated Ca^{2+} release from the endoplasmic reticulum, and the activation of such targets as DAG-sensitive protein kinase Cs (PKCs) (Miyazaki et al., 1993; Saunders et al., 2002; Swann and Yu, 2008; Yu et al., 2008).

During the last ten years, several studies have demonstrated the importance of the soluble sperm factor to allow Ca^{2+} waves at fertilization. Injection of recombinant PLCζ cRNA (Saunders et al., 2002) or protein (Kouchi et al., 2004) into mouse eggs leads to Ca^{2+} oscillations at fertilization. Saunders et al. (2002) showed that when endogenous PLCζ was removed by immunodepletion, mouse sperm protein extracts lost their ability to release Ca^{2+}. Moreover, *in vitro* fertilization of mouse eggs with sperm from transgenic mice expressing lower amounts of PLCζ (due to a short hairpin RNAs targeting PLCζ) induced Ca^{2+} oscillations that ended prematurely, negatively affecting egg activation and embryonic development (Knott et al., 2005). Furthermore, infertile men whose sperm failed in egg activation showed abnormal expression and localization of PLCζ in the sperm (Yoon et al., 2008; Heytens et al., 2009). Until now, mammalian PLCζ orthologues have been reported in mice, monkeys, humans, boars, hamsters, and bulls (Cox et al., 2002; Saunders et al., 2002; Yoneda et al., 2006; Young et al., 2009; Cooney et al., 2010). In non-mammals, PLCζ orthologues were reported in the chicken (Coward et al., 2005), medaka (Ito et al., 2008), quail (Mizushima et al., 2009) and in two pufferfish species *Takifugu rubripes* (Fugu) and *Tetraodon nigroviridis*

(Tetraodon) (Coward et al., 2011). In these non-mammalian species, like chicken or medaka, PLCζ mRNA is expressed in the testis, in line with the situation in mammals. In contrast, in two pufferfish species, *plcζ1* is expressed in the ovary, but not in the testis (Coward et al., 2011).

Due to its unique life cycle and its phylogenetical position, the European eel (*Anguilla anguilla*) is a particularly interesting model to investigate the regulatory mechanisms of reproductive physiology and for providing insights into ancestral regulatory functions in teleosts. Prepubertal silver eels migrate across the Atlantic Ocean to reach their probable spawning area in the Sargasso Sea (Tesch, 1978). Gonadal development and maturation probably takes place during the supposedly 6-7 month migration period, at low temperature, whereas the spawning takes place at high temperatures, considered to be around 20 °C (Boëtius and Boëtius, 1967, 1980). However, as detailed information from the field is still lacking, it is difficult to simulate the variable environmental factors which would occur during the migration (temperature, photoperiod, pressure, etc). That is why, in captivity, silver eels are blocked in a pre-pubertal stage (Dufour et al., 2003; Pasqualini et al., 2004; Vidal et al., 2004) and must receive a long-term hormonal treatment to induce sexual maturation and spermiation (Boëtius and Boëtius, 1967; Ohta et al., 1996, 1997a; Asturiano et al., 2005; Huang et al., 2009; Pérez et al., 2000; Gallego et al., 2012).

In this study, we characterized and cloned the *Anguilla anguilla plcζ1* mRNA, analysed the structure and investigated the position of this protein among vertebrates by phylogenetic analyses, studied the tissue distribution of this gene and finally, for the first time in teleost, we studied the expression profile of *plcζ1* in the brain and gonad through spermatogenesis. The impact of water temperature on the maturation process of European eel has been highlighted in females (Pérez et al., 2011; Mazzeo et al., 2014) and males (Gallego et al., 2012, 2014; Baeza et al., 2014), and in order to simulate the natural conditions during the reproductive migration and testing its potential effect on *plcζ1* expression, three different thermal regimes were tested for the gene expression profile experiments, two variable regimes (changing

gradually from 10 to 20 °C or from 15 to 20 °C), and one constant regime (20 °C).

2. Materials and methods

2.1 Fish maintenance, hormonal and thermic treatments, and sampling

Three hundred and seventeen male European eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were hormonally matured at the Aquaculture Laboratory at the Polytechnic University of Valencia. They were randomly distributed and kept in six 200-L fiberglass tanks (approximately 50 males per aquaria, 2 aquaria per treatment) equipped with separate recirculation systems, thermostats/coolers, and covered to maintain constant shadow.

The fish were gradually acclimatized for one week to seawater (37±0.3‰ of salinity) and the water temperature was kept at 20 °C or changed to 15 °C in one week or to 10 °C in two weeks, depending on thermal groups. Starting three weeks after arrival to the Aquaculture Laboratory, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG, Profasi®, Serono, Italy); 1.5 IU g^{-1} fish; during 13 weeks to induce maturation and spermiation, as previously described by Pérez et al. (2000).

During the experiment, the animals were maintained in three thermal regimes (2 aquaria per treatment): T10-T20: 10 °C (first 5 weeks, with one week of temperature acclimation), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15-T20: 15 °C (first 6 weeks, with two weeks of temperature acclimation) and 20 °C (last 9 weeks); and T20: 20 °C during the whole experimental period. These thermal regimes were previously described by Gallego et al. (2012).

Groups of 5-8 eels per treatment were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation each week along the hormonal treatment. Morphometric parameters such as total body, gonad weights were recorded to calculate the gonadosomatic index

(GSI = (gonad weight/total body weight)*100) for each fish (Pankhurst, 1982). Furthermore, testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status. Samples of pituitary, testis, liver, heart, gill, muscle, spleen, fins, and kidney were collected for analyses of gene expression levels by qPCR. Brains were dissected into five parts: olfactory bulbs, telencephalon, mes-/di-encephalon, cerebellum, and medulla oblongata. All the samples were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 °C until extraction of total RNA (Peñaranda et al., 2010).

Because eels stop feeding at the silver stage and throughout sexual maturation, the fish were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Gonadal histology

Fixed testis samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope. Stages of spermatogenesis were determined according to the most advanced germ cell type present and their relative abundance, degree of development of the seminal tubules, GSI and sperm production by the male in the same week of the sacrifice. Stage 1 Spermatogonia (SPG): dominance of spermatogonia, in some cases, a few spermatocytes were present in low number, mean GSI = 0.08 (0.0-0.36); Stage 2 Spermatocytes (SPC): spermatocytes were present in proportion ≥50% with spermatogonia, in some cases appeared low number of spermatids, mean GSI = 0.72 (0.27-1.54); Stage 3 spermatids (SD): spermatids were the dominant germ cell, some sperm cells can appear, mean GSI = 3.28; and Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell, mean GSI =7.35 (3.41-12.8) (Fig. 1).

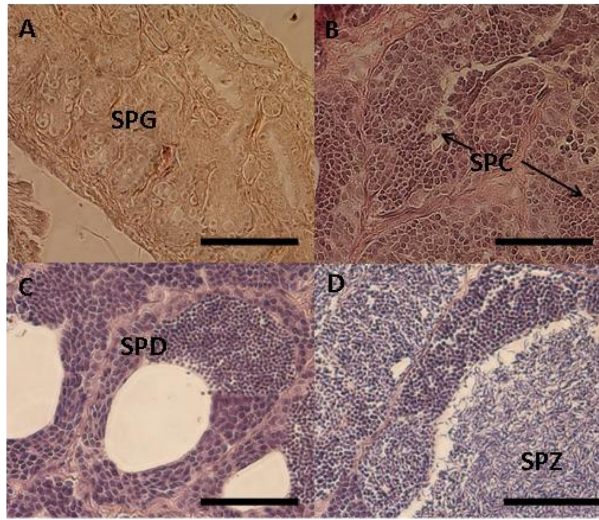

Figure 1. Histological sections of eel testis at different developmental stages during chorionic gonadotropin (hCG) hormonal treatment. A: spermatogonia; B: spermatocyte; C: spermatids, D: spermiation. SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: spermatozoa, Scale bar: A=100µm; B, C, D= 50µm

2.3 Isolation of PLCζ sequence

2.3.1 European eel genome database analysis

The TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) was used to retrieve the genomic sequence of the PLCζ from the European and Japanese eel genomes (Henkel et al., 2012a, b)

Exons and splice junctions were predicted using the empirical nucleotidic splicing signatures, i.e.: introns begin with ''GT'' and end with ''AG''. The peptidic sequences of *Tetraodon nigroviridis* PLCζ1 sequence (Accession: HQ185299. GI: 322510422. 1,889 bp mRNA) were used as query.

Percentage of European eel PLCζ1 identity with other osteichtian PLCζ sequences was calculated with Secuences Identites And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html)

2.3.2 Partial cloning of the PLCZ1 gene

cDNA was generated using 1 µg of total RNA. A mixture of cDNA from different tissues of female silver eels were used as template for amplification of PLCζ. Partial PLCζ cDNA was amplified by PCR using specific primers which were designed based on the predicted PLCζ sequence of European eel using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA):
PLCζ1fw1:GGCTTCCTCCGGTACATGGA;
PLCζ1rv1:TGTAGTTGGAGGACAGCGTGC;
PLCζ1fw2:AGATTCATCAGCAGGATCTATCC;
PLCζ1rv2:TACTGGCCCATGAAGTCGTT.
PCR amplification was run in a Hybaid PCR express, using 25 µl of reaction mixture containing 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µl of cDNA template. PCR products were visualized in 2% agarose gel stained with SYBR Safe DNA gel Stain (Invitrogen) and bands of expected size were purified using Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent E. coli JM109 cells (Promega). Positive colonies were isolated and plasmids extracted by Qiagen Plasmid Mini Kit (Qiagen). Plasmids with insert were sent to Eurofins Genomics (Germany) for sequencing.

2.3.3 Phylogenetic analysis

Amino-acid sequences of known or predicted sequences of gene coding for the PLCζ from 14 species retrieved from NCBI or ENSEMBL were first aligned using ClustalW (Thompson et al., 1994), then manually adjusted. Human, *Homo sapiens*, and mouse, *Mus musculus* PLCβ1 were used as outgroup. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the resulting alignment was determined using ProTest software (Abascal et al., 2005). Phylogenetic analysis of the PLCζ sequence alignment was performed using the maximum likelihood method (PhyML software, Stamatakis and Ott, 2008), with 1.000 bootstrap replicates.

2.4 Gene expression analyses by quantitative real-time PCR

2.4.1 Primers and reference gene

Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC ACT G; ARPrv: ACA TCG CTC AAG ACT TCA ATG G (Aroua et al., 2007; Weltzien et al., 2006) was used as reference gene in the quantitative real-time Reverse Transcriptase-Polymerase chain reaction (qPCR) because its mRNA expression has been shown to be stable during experimental maturation (Weltzien et al., 2005). The qPCR expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD [±Cq]) lower than 1. In the testis, T10-T20: SD= 0.79 ; T15-T20: SD= 0.97; T20: SD= 0.79; $p<0.05$ with a Cq geometric mean of T10-T20: 24.3±1.73 ; T15-T20: 24.37±1.96; T20: 25.17±1. 73; in the brain and pituitary olfactory bulb: SD= 0.85; telencephalon: SD= 0.56; mes-/di-encephalon: SD= 0.53, pituitary: SD= 0.77; $p<0.05$ and a Cq geometric mean of olfactory bulb: 23.74±1. 8; telencephalon: 22.43±1.48; mes-/di-encephalon: 22.17±1.44; pituitary: 22.77±1.71. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. European eel PLCζ specific qPCR primers qPLCζ1fw: GAA GAG CCA CCT GTT TGC AT; qPLCζ1rv: CAG CAG TCG ATC TCC AGA CA; were designed based on the full-length European eel CDS sequences. All the primers were designed on two different exons, in order to avoid amplification of potential genomic contamination, using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA). All primers were purchased from Integrate DNA Technology, Inc. (IDT, Coralville, IA).

2.4.2 SYBR Green assay

To quantify gene expression, qPCR assays were performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA), with PCR protocol previously described by Peñaranda et al. (2013).

The total volume for every PCR reaction was 20 µl, performed from diluted (1:20) DNA template (5 µl), forward and reverse primers (250

nM each), and SYBR Green/ROX Master Mix (12 µl) (Fermentas GMBH). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Weltzien et al. (2005). Serial dilutions of cDNA pool of gonad tissues were run in duplicate and used as a common standard curve. One of these dilutions was also included in each run as a calibrator. Target and reference genes in unknown samples were run in duplicate PCR reactions. Non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates.

2.4.3 PLCζ tissue distribution

In order to investigate the tissue distribution of PLCζ mRNA expression, gonads (testes and ovaries) and somatic tissues (liver, heart, gill, muscle, spleen, fins, kidney, brain, pituitary) were collected from three immature male eels (mean body weight 118 ± 14 g; mean GSI <0.1) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) and three female eels (mean body weight 608 ± 35 g; mean GSI 0.9 ± 0.3) from the Albufera lagoon (Valencia, Spain). Samples were stored in RNAlater (Ambion, Austin, Texas, USA) immediately after decapitation and stored at 20 °C until RNA extraction. The brain was dissected into five parts: olfactory bulbs, telencephalon, mes-/di-encephalon, cerebellum and medulla oblongata as previously reported by Weltzien et al. (2005).

Total RNA was extracted following the method used by Hildahl et al. (2011). Total RNA was treated with DNase I (Turbo DNA-free; Ambion) at 37°C for 30 min. First-strand cDNA was prepared from 1µL total RNA using superscript III (Invitrogen) according to the manufacturer's protocol. All tissues were analysed by qPCR.

2.4.4 PLCζ expression through spermatogenesis

To study PLCζ expression during spermatogenesis, total RNA of gonads, olfactory bulb, telencephalon, mes-/di-encephalon and pituitary was isolated from the RNAlater preserved tissues as described by Peñaranda et al. (2013). Testis RNA of males from thermal groups T10-T20, T15-T20 and T20 was treated and purified with DNase I of

NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized from 500 ng of testis total RNA, using qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA) with 15 µl RNA used as template.

Total RNA extracted from the olfactory bulb, telencephalon, mes-/di-encephalon and the pituitary of males from the thermal group T20 was treated with deoxyribonuclease (gDNA Wipeout Buffer, Qiagen), using a total volume of 14 µl for 500 ng of total RNA for the olfactory bulb ant pituitary, or 1 µg for the telencephalon and the mes-/di-encephalon. First-strand cDNA was synthesized in 20 µl reactions using Quantiscript Reverse Transcriptase (Qiagen) with 14 µl used as template, which were obtained in the previous step. RNA concentration and quality were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain).

2.5 Statistics

Each variable was first checked for normality. If the variables did not have a normal distribution, they were log-transformed and their normality was checked again. Then, data were analyzed by analysis of variance (One-way ANOVA), using the Student-Newman-Keuls test to compare means. Variance homogeneity was checked with the Bartlett test. Differences were considered significant when $p<0.05$.

Statistical analyses (One-way ANOVA) were also performed to study the evolution of PLCζ expression in one tissue throughout sex development, and to study the differences in expression between thermal regimes in the same developmental stage (SPG, SPC, SD or SZ).

T-test analyses were performed to compare differences between males and females in a same tissue from the data obtained in the study of PLCζ tissue distribution.

All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

3. Results

3.1 Characterization of European eel PLCζ1

The *plcζ1* gene was identified *in silico* in both the European and Japanese eel genomes. The European eel *plcζ1* predicted sequence differed from the Japanese eel predicted sequence by 30 nucleotides and 13 amino acids. From the *Anguilla anguilla plcζ1* predicted sequence, specific primers were designed to clone and confirm this sequence. Two overlapping fragments covering 1167 bp were cloned and sequenced. The European eel *plcζ1* cDNA sequence (Fig. 2) differed from the corresponding partial sequence characterized in the European eel genome by only 2 nucleotides and a gap of 3 nucleotides in position 700 of the European eel *plcζ1* cDNA sequence. This gap led to a lack of 1 amino acid, which did not affect the reading frame. The complete *plcζ1* CDS was 1521 bp long, composed by 10 exons giving an open reading frame (ORF) of 506 amino acids (GenBank accession number AFV13732.1) (Fig. 2).

European eel *plcζ1* showed a high identity when compared with *plcζ* from other teleosts: from 76.69% for the Fugu *Takifugu rubripes* to 79.32% for the Atlantic cod *Gadus morhua*, with the highest identity. The European eel *plcζ1* share 78% of identity with the non-teleost actinopterygian spotted gar *Lepisosteus oculatus*. When compared with sarcopterygian Plcζ amino acid sequences, European eel Plcζ1 presented 70.44% of sequence identity with the human, 66.82% with the mouse and the lizard, and 69.13% with the chicken. The highest identity with a sarcopterygian Plcζ was found with the coelacanth, with 72.22% of identity. Classical domains of European eel Plcζ1 protein were predicted using Interproscan software (http://www.ebi.ac.uk/interpro/) and revealed a typical Plcζ domain structure with the following conserved domains: EF hand-like domains from position 16 to 98, X domain in position 101-240, Y domain in position 243-360, and C2 domain in position 383-467.

Figure 2. Multiple sequence alignment of European eel, Mouse and Fugu PLCζ at amino acid level. "*" Conserved residues, ":" conservation between groups of strongly similar properties, "." conservation between groups of weakly similar properties. Residues in red: AVFPMILW, small and hydrophobic. Residues in blue: DE, acidic. Residues in pink : RK, Basic – H. Residues in green: STYHCNGQ, Hydroxyl + sulfhydryl + amine + G. EF-hand like domain, X-domain, Y-domain and C2-domain are shown above the alignment.

3.2 Phylogeny

We performed phylogenetic analyses on five actinopterygian Plcζ1 amino acid sequences (four Plcζ1 from teleost species and one from a non-teleost species, the spotted gar), and five sarcopterygian PLCζ1 amino acid sequences, with the PLCβ1 (phospholipase C, beta1) from two mammalian species as outgroup (Fig. 3).

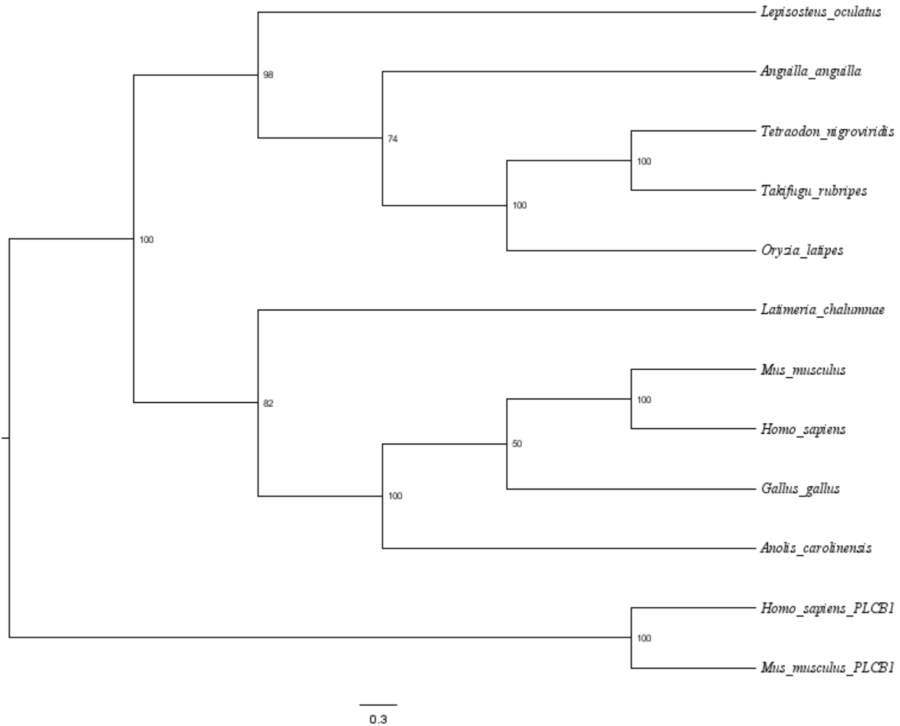

Figure 3. Consensus phylogenetic tree of the vertebrate Phospholipase C zeta. This phylogenetic tree was constructed based on the amino-acid sequences of PLCζ (for the references of each sequence see Table I) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The tree was rooted using the two sequences of the mouse and human phospholipase beta1.

In this phylogenetic analysis, the actinopterygian and sarcopterygian Plcζ1 clustered in two monophyletic groups. In the actinopterygian group, the European eel Plcζ1 clustered with the spotted gar at the basis of the teleost clade, constituting an actinopterygian Plcζ1 clade

as sister clade of the sarcopterygian Plcζ1. This phylogeny confirmed that European eel Plcζ1 is orthologous with actinopterygian Plcζ1 and sarcopterygian Plcζ1.

Table I. Accession number of the sequences used for phylogenetic analyses

Sequence name	Species name	Accession number
PLCZ1-001	*Mus musculus*	ENSMUSP00000032356
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1-like, partial	*Anolis carolinensis*	XP_008108585
PLCZ1-201	*Gallus gallus*	ENSGALP00000021386
PLCZ1-201	*Oryzias latipes*	ENSORLT00000005752
PLCZ1-201	*Latimeria chalumnae*	ENSLACT00000000957
PLCZ1-201	*Takifugu rubripes*	ENSTRUP00000043591
PLCZ1-201	*Homo sapiens*	ENSP00000402358
PLCZ1-201	*Lepisosteus oculatus*	ENSLOCP00000018988
PLCZ1-201	*Gasterosteus aculeatus*	ENSGACP00000013217
PLCZ1-201	*Tetraodon nigroviridis*	ENSTNIP00000003915
PLCB1-005	*Homo sapiens*	ENSP00000367908
PLCB1-005	*Mus musculus*	ENSMUSP00000105743

3.3 *plcζ1* tissue distribution in the European eel

plcζ1 mRNA expression was compared in various tissues of female and male European eels (Fig. 4). The *plcζ1* showed a differential expression in male and female European eel. In female eels, very low

expression of *plcζ1* was detected in peripherical tissues such as liver, heart, gill, muscle, spleen, fins, kidney, ovary or pituitary whereas there was high expression in the different brain parts.

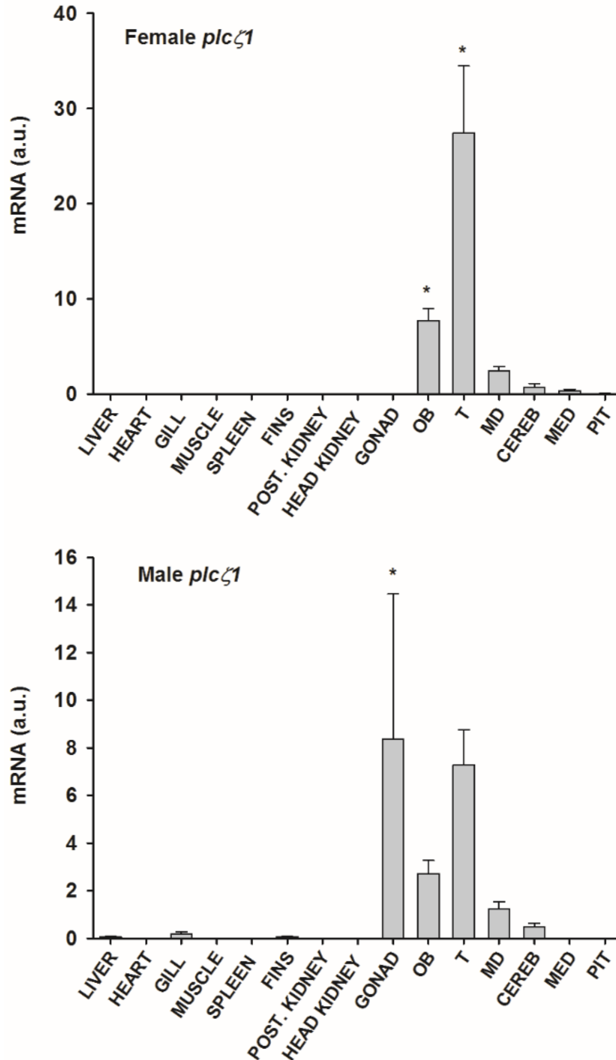

Figure 4. Tissue distribution of (A) *plcζ1 in female*, (B) *plcζ1* in immature *male* European eel. Data are normalised to eel *arp*. Asterisk indicates significant differences between males and females in a same tissue ($p<0.05$; n=3). Values are presented as means ± SEM (n = 3). OB : olfactory bulb, T : Telencephalon, M/D : mes-/di-encephalon, CEREB : cerebellum, MED : medulla oblongata, PIT, pituitary.

In male eels, no expression was found in the muscle and in the kidney. Low expression of male *plcζ1* mRNA was detected in the liver, gill, heart, spleen, fins, medulla oblongata, cerebellum and pituitary. However there was high expression in the testis, olfactory bulb, telencephalon and mes-/di-encephalon. *plcζ1* in the olfactory bulb, telencephalon and gonads was expressed at higher levels in females compared to the males (p<0.05).

3.4 *plcζ1* expression during spermatogenesis

Once demonstrated the expression of male eel *plcζ1* in the brain, pituitary and testis, we studied the testis *plcζ1* mRNA expression of the males from all the thermal regimes through spermatogenesis; and the brain (olfactory bulb, telencephalon, mes-/di-encephalon) and pituitary *plcζ1* mRNA expression in group T20 (kept at 20 °C) through spermatogenesis (Fig. 5).

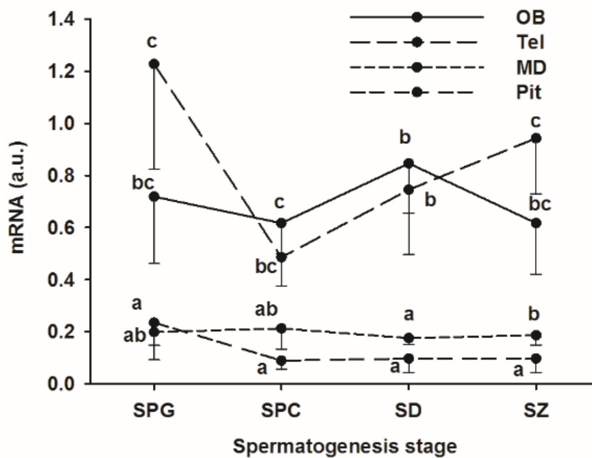

Figure 5. European eel *plcζ1* expressions during experimental maturation in 3 brain parts and in the pituitary in fishs kept at 20 degrees. Data are normalised to eel *arp*. Small letters indicate significant differences between the olfactory bulb, the telencephalon, the mes-/di-encephalon and the pituitary, in the same gonad development stage (p<0.05; n=6-12). Results are given as mean ± SEM. SPG= Spermatogonia stage, SC= Spermatocyte stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages, OB: olfactory bulb, Tel: telencephalon, MD: mes-/di-encephalon, Pit: pituitary

In the brain and pituitary of the males kept at 20 °C, *plcζ1* expression was stable from spermatogonia to spermatozoa and did not show significant differences throughout maturation. The higher *plcζ1* expression was observed in the telencephalon and in the olfactory bulb. The mes-/di-encephalon and the pituitary showed the lower *plcζ1* expression levels ($p<0.05$).

Testis *plcζ1* expression (Fig. 6) increased through spermatogenesis in all the thermal regimes.

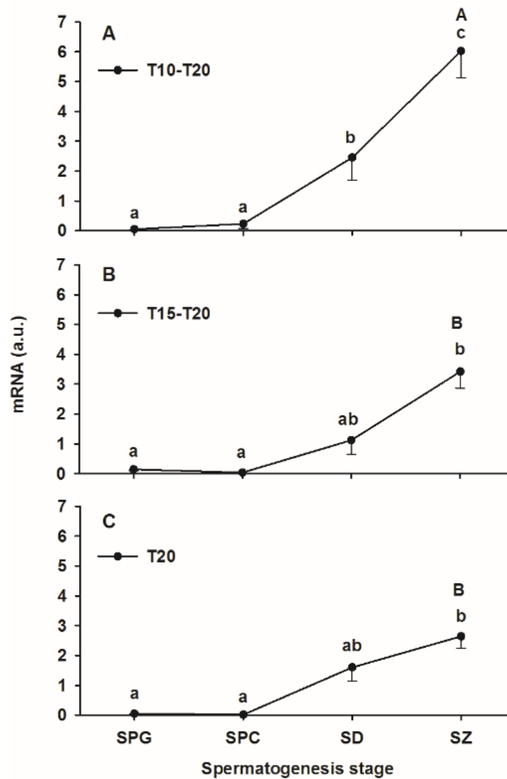

Figure 6. European eel *plcζ1* expressions during experimental maturation in fish testis kept in different thermal regimes. Data are normalised to eel *arp*. Capital letters indicate significant differences between the thermal treatments in the same gonad development stage ($p<0.05$; n=8-17). Small letters indicate significant differences through spermotogenesis in the same thermal treatment ($p<0.05$; n=6-17). Results are given as mean ± SEM. SPG= Spermatogonia stage, SC= Spermatocyte stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.

The *plcζ1* expression was very low when testis showed only spermatogonia (S1) or spermatocytes (S2) (Fig. 6). Maximum *plcζ1* expression was found between S3 and S4 (spermiogenesis) when it was 75-fold higher than at S1 ($p<0.05$). Furthermore, when comparing thermal regimes for a same stage of development, testis *plcζ1* was significantly highly expressed at stage spermatozoa in the lower thermal regime (T10-T20) than in the higher thermal regimes (T15-T20 and T20, $p<0.05$).

4. Discussion

European eel *plcζ1* sequence showed a Plcζ typical domain structure but its sequence is shorter when compared with other vertebrate Plcζ, suggesting that eel Plcζ1 could have conserved its activity, but maybe at a lower level. The expression of eel testis *plcζ1* mRNA increase through spermatogenesis reaching maximum levels during spermiogenesis, and its expression is significantly higher at lower temperature compared to higher temperatures, suggesting that temperature may play a role in the regulation for *plcζ1* transcription in the testis, when *plcζ1* seems to acquire its function.

4.1 Molecular structure and function

The European eel *plcζ1* contains an EF-hand domain located in the amino-terminal region of the molecule, X and Y catalytic domains, and a carboxy-terminal C2 domain, all typical of other PLCζ orthologues (for review see Kashir et al, 2013; Ito et al, 2011, Parrington et al, 2007). Similar to Medaka (Ito et al, 2008), and Fugu and Tetraodon *plcζ* (Coward et al, 2011), the European eel *plcζ1* sequence is shorter when compared with mammalian *PLCζ*, showing an EF-hand domain truncated at the N-terminus, like in all the teleosts *plcζ* studied so far (Fig. 2). EF-hands are involved in binding Ca^{2+} and are thought to be important for the oscillatory Ca^{2+} activity of the enzyme (Ito et al, 2011). In teleost species, studies showed that a deletion of a part of the EF-hand domain reduces the Ca^{2+} oscillatory activity of Plcζ (Kouchi et al, 2005; Kuroda et al, 2006). It remains possible that eel Plcζ1 does not trigger Ca^{2+} oscillation, however medaka Plcζ, which is similarly truncated at its N-terminus, can induce

Ca^{2+} oscillation in mouse oocytes but at a lower activity than for full-length mammalian PLCζ (Coward et al, 2011; Kuroda et al, 2006; Ito et al, 2008). These results suggest that these domains are involved in the Ca^{2+} signal but are not obligatory to induce Ca^{2+} oscillation. This means eel Plcζ1 could have conserved its activity, but maybe at a lower level. Furthermore, EF-hand domains seem also to play a role in nuclear translocation (Kouchi et al, 2004, 2005; Yoda et al 2004). According to Kuroda et al. (2006), Trp13, Phe14, and Val18, which may be necessary for appropriate conformation for nuclear translocation, may also be necessary to keep normal Ca^{2+} oscillation-inducing activity as well. Nevertheless, despite the lack of a part of the N-terminal of all teleosts studied so far, at least some of these Plcζs still can trigger Ca^{2+} oscillations.

The XY domain, known to form together the active site responsible for PIP2 cleavage (Parrington et al, 2007), is highly conserved. On the contrary, the X/Y linker region, between the two catalytic domains, is a poorly conserved domain with a high diversity of amino acid residues among vertebrate. In the C terminus of the X domain and in the X/Y linker region, PLCζ possesses a cluster of basic amino acid residues (lysine and arginine), which is found in many nuclear proteins (Kuroda et al, 2006, Jones and Nixon, 2000). Eel PLCζ1 is truncated in the C-terminal region of the X-domain and in a large part of the N-terminal X/Y linker region, on approximately 85 amino acids, when compared with the other osteichthyan PLCζ sequences. This loss of protein part leads to a change in the protein conformation (data not shown) which may affect the protein function. Furthermore, due to its loss, eel PLCζ1 misses these two nuclear targeting regions localised in the lost part, which may affect the nuclear translocation of the protein. According to Kuroda et al. (2006), in the mouse, nuclear targeting was absent for point mutation of Lys299 and/or Lys301 in the C terminus of X domain, and nuclear translocation was lost when the residues from the NLS were replaced by glutamate. Nevertheless, these substitutions did not affect PLCζ ability to induce the Ca^{2+} oscillation. Furthermore, European eel PLCζ1 still possesses region for enzymatic catalisis and substrate/Ca^{2+} binding, which are very well conserved residues among osteichthyans, so European eel PLCζ1 catalytic function could be preserved. Further studies to confirm the

PLCζ1 function for initiating the Ca^{2+} oscillation after fertilization in eel are necessary.

To better understand the evolutionary history for the PLCζ family, we performed phylogenetic analyses on osteichthyans of key-phylogenetical positions: the human and the mouse, representative of mammalians; the anole lizard and the chicken, representative of sauropsids; the coelacanth, a representative of early sarcopterygians; the spotted gar, a non-teleost actinopterygian; the European eel, a member of an early group of teleosts (elopomorphs), and three members of teleosts (*Medaka*, *Takifugu* and *Tetraodon*). The *Anguilla anguilla* PLCζ1 branch with the spotted gar at the basis of the teleost PLCζ group. Each species exhibits only one PLCζ, which seem to suggest that this protein has not been affected by the teleost-specific third whole-genome duplication. The duplicated gene must have been lost during evolution.

4.2 Sex-specific and species-specific tissue distribution of *plcζ1*

PLCζ is known to be sperm-specific, but eel *plcζ1* mRNA was highly expressed in different brain parts, also showing low expression in the pituitary and peripherical tissues of male and female eels. Tissue distribution of eel *plcζ1* mRNA revealed a differential expression in male and female European eel, with high *plcζ1* expression in testis, and very low in ovary, like in every *plcζ* orthologous from mammals (Cox et al, 2002; Saunders et al, 2002; Yoneda et al, 2006; Young et al, 2009), birds (Coward et al, 2005; Mizushima et al, 2009), and some teleosts like medaka (Ito et al, 2008) and eel, but different to other fish like in the two pufferfish species *Takifugu rubripes* and *Tetraodon nigroviridis* (Coward et al, 2011). While *plcζ* mRNA is thought to be only expressed in male gametes, eel *plcζ1* also expressed in the brain and pituitary of male and female. Yoshida et al. (2007) found expression of *Plcζ* mRNA in brains of both male and female mice, and Coward et al. (2011) found expression of *plcζ* in *Tetraodon* brain, but its function in the brain is unknown. Nevertheless, it is the first evidence of pituitary expression of PLCζ in vertebrates. These results showed different tissue specific patterns of expression in *plcζ* mRNA, which is not only expressed in fish testis, but also in the brain or in the ovary.

PLCζ function is well documented in sperm vertebrates, nevertheless further studies of PLCζ expression and functions in somatic tissues are necessary.

4.3 *plcζ1* expression is stable in brain but increase in testis through spermatogenesis

This is the first study of the effect of the eel sexual maturation on the expression of brain and pituitary *plcζ1* mRNA. In the European eel, *plcζ1* mRNA expression is stable in the pituitary and in the brain through the spermatogenesis. The significance of *plcζ1* mRNA expression in the brain and in the pituitary is unknown, further studies of Plcζ1 protein synthesis in the brain-pituitary-gonad axis should be performed to clarify the role of this protein in the reproductive function.

According to our results, *plcζ1* mRNA expression increases in the testis through spermatogenesis regardless of thermal regime, reaching maximum levels during spermiogenesis. Mizushima et al. (2009) searched the *PLCζ* mRNA expression in quail sperm cells and found expression in elongate spermatids but not in spermatocytes or in round spermatids. Furthermore, they demonstrated that injection of chicken or quail elongated spermatids lead to successful fertilization and development of mouse and quail eggs, but none of the round spermatids alone induced blastodermal development. These results of *PLCζ* mRNA expression and spermatogenic cell injection support the evidence that the egg activation potency of PLCζ during spermatogenesis is acquired in elongated spermatids in quail. This is in accordance with our results showing a European eel *plcζ1* mRNA expression 75-fold higher at the spermatozoa stage than at the spermatogonia stage, suggesting that eel Plcζ1 function is acquired during the stage of spermiogenesis.

PLCζ function in the process of fertilization is known, but it seems to play further roles in spermatogenesis. For instance, Ito et al. (2010) observed that PLCζ knock-out mice was unable to complete spermatogenesis with spermatocytes failing to proceed beyond elongation, underlying the involvement of PLCζ in spermatogenesis.

The observed increase in eel *plcζ1* mRNA expression during spermiogenesis regardless of thermal regimes clearly indicates that this increase is independent of temperature. However, at the final step of spermatogenesis (stage spermatozoa) European eel *plcζ1* mRNA expression in the testis was significantly higher for the males subjected to the temperature T10-T20 compared to T15-T20 and T20, suggesting that temperature may play a role in the regulation for *plcζ1* transcription in the testis, especially during the process of spermiogenesis, precisely when *plcζ1* seems to acquire its function.

The present study shows that *plcζ1* mRNA synthesis in the eel testis starts after the onset of spermatogenesis. Our results support the hypothesis of a sperm-specific Plcζ1 egg activation in the European eel, similar to many other vertebrates. However, expression of *plcζ1* mRNA showed different tissue specific patterns, expressing in the brain or in the ovary like the two pufferfish species *Takifugu rubripes* and *Tetraodon nigroviridis*. Further studies of the function of PLCζ in the Brain-Pituitary-Gonad axis are necessary to clarify the physiologic processes which control sexual maturation and fertilization. Due to its phylogenetical position and its complex life cycle, the European eel may be a very useful model to explore the evolutionary origins of PLCζ and its functional role in the egg activation.

In conclusion, the Plcζ1 expression pattern found in the European eel suggests an important function of this protein in the spermatozoa of this species.

Acknowledgements

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GENERAL DISCUSSION

1. Brain, pituitary and gonadal control of European eel reproduction

European eels do not reach gonadal maturation in captivity, and hormonal treatment is necessary to induce a complete spermatogenesis in male eel. So, understanding the real molecular control mechanisms that control European eel spermatogenesis in natural conditions is a complicated task. The gene expression profiles obtained by the repeated hCG injections can be considered abnormal. Nevertheless, our studies equip us with a better understanding of the physiological mechanisms which control eel spermatogenesis in captivity, and these results provide us with new information on eel maturation. So, following our results, a possible physiological model is proposed (Fig 1).

The change from freshwater to sea water may activate the eel maturation process. Indeed, the expression of sex steroid receptors, i.e. the three nuclear ERs in the anterior brain, nuclear ERs and membrane GPERs in the pituitary, and ESR1 and ESR2a in the testis, seem to respond to the increase of E_2 plasma levels which occurs with the increase in salinity (Chapters 1 and 3; Fig 1). We suggest that the increase in these ERs and plasma E2 may be a physiological response to this specific environmental change. Furthermore, in the testis, the high expression levels of all the nuclear ERs at the SPGA stage supports the role proposed by Miura et al. (1999) of estrogens being a spermatogonial renewal factor in the Japanese eel. So, in the European eel, this proposed role would be mediated by ESR1, ESR2a and ESR2b.

All nuclear and membrane ER and PR are expressed in all the brain parts studied (olfactory bulb, telencephalon, mes-/diencephalon) (Chapter 1 and 2), and this suggests that PRs and ERs may modulate a wide variety of neural functions. Furthermore, they were found in regions known to be involved in the neural control of gonadotropin release. Indeed, mGnRH, considered to be the principal GnRH responsible for the release of gonadotropin in the European eel, is also expressed in the pituitary, olfactory bulbs, telencephalon and mes-/diencephalon (Dufour et al., 1993).

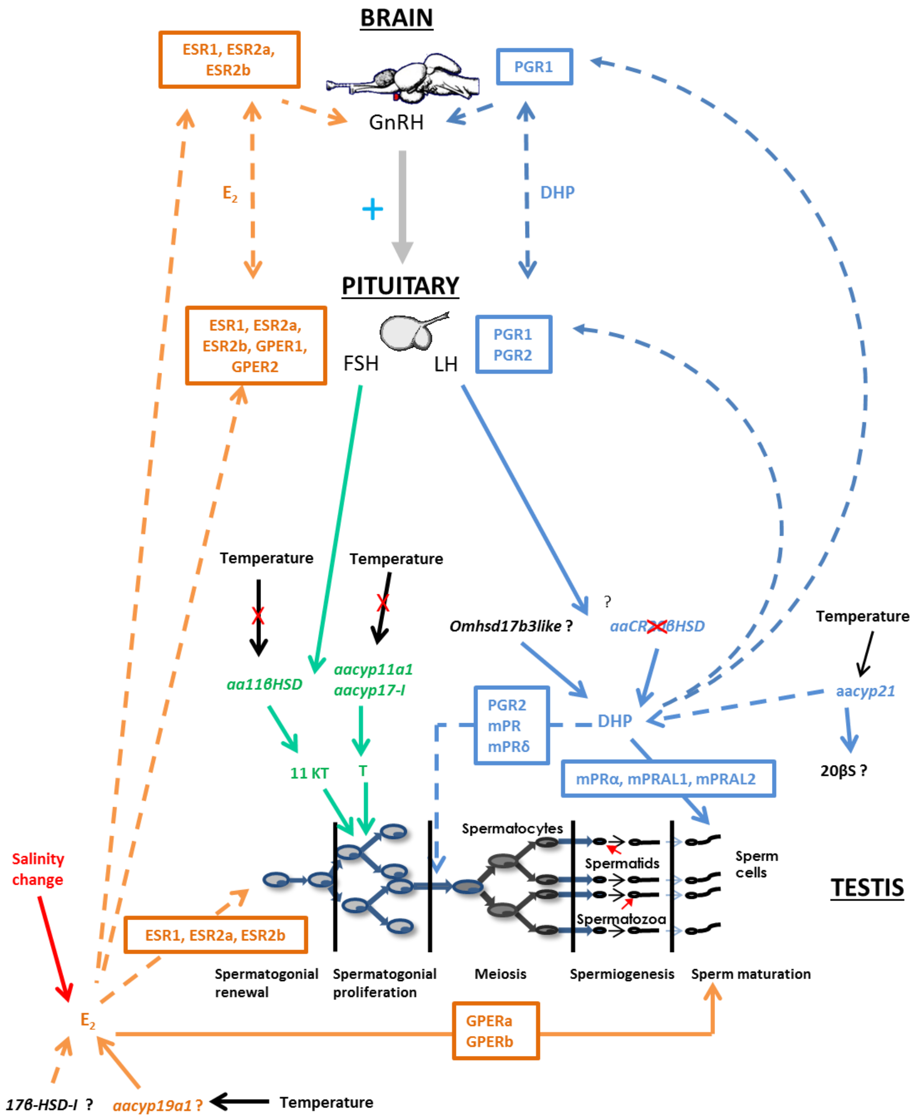

Figure 1. Schematic summary of spermatogenesis mecanisms in European eel testes. T: temperature; cyp: cytochrome P450; HSD : hydroxysteroid dehydrogenase; KT : ketotestoterone ;T: testosterone; DHP: 17α,20β-dihydroxy-4-pregnen-3-one; 20βS: 17α,20β,21-trihydroxy-4-pregnen-3-one; E_2: estradiol; ESR: estrogen receptor; GPER: G protein coupled estrogen receptor; PGR: progestin receptor; mPR: membrane progestin receptor; mPRAL: membrane progestin receptor alpha like.

So, in the brain, these receptors may be involved in the control of reproduction and the eel maturation process, through the regulation of mGnRH expression. The increase in E_2 levels and nuclear ERs in the brain in line with the increase in water salinity may lead to an increase in GnRH in the brain (Fig 1).This increase in brain GnRH may stimulate the synthesis and secretion of FSH by the pituitary. According to Miura et al. (1991a,b), the release of gonadotropin by the pituitary leads to a switch from spermatogonial stem cell renewal to proliferation towards meiosis, through the gonadal biosynthesis of androgen, especially 11-KT. In the European eel, the increase in 11-KT plasma levels was observed at the beginning of spermatogenesis, at SPG1, when A spermatogonia cells were predominant in the testes. So, FSH release may in turn stimulate the production of 11KT in the testis, stimulating the spermatogenesis process (Fig 2). *In vitro* studies demonstrated that in immature testis of Japanese eel, FSH stimulated the secretion of T and 11-KT, essential hormones for the onset of spermatogenesis (Kamei et al., 2003, Khan et al., 1987; Miura et al., 1991a; Ohta and Tanaka, 1997b). The increase in 11KT expression levels may exert a positive feedback on pituitary LH expression, as androgens were shown to stimulate LH synthesis *in vivo* in the European eel (Dufour et al., 1983, Peñaranda et al., 2010).

Therefore, the release of LH may induce the production of DHP by the gonad, as DHP is produced in response to gonadotropin stimulation (Schulz and Miura, 2002; Fig 1). According to Miura et al. (1991b, 1992, 1995, 2006) DHP is an essential hormone involved in the initiation of meiosis and in the regulation of final maturation. These results correspond to the DHP level measured in ours studies (Chapters 2 and 3) where an increase in eel DHP plasma levels has been observed between the proliferating spermatogonia and the spermatozoa stage. In the European eel, DHP seems to induce early spermatogonia to enter meiosis, through the membrane progestin receptors mPRγ and mPRδ, and the nuclear progestin receptor pgr2 in the testis (Fig 1). Concerning final sperm maturation, it seems that DHP act directly throught a membrane receptor localized in the spermatozoa to stimulate the carbonic anhydrase activity which causes an increase in the seminal plasma pH, which in turn increases the sperm content of cAMP, thereby allowing the acquisition of sperm motility (Miura et al.

1991b, 1992, 1995, 2003a). According to our results, this effect of DHP on final sperm maturation may be mediated by the membrane progestin receptors mPRa, mPRAL1 and mPRAL2 (Fig 1). DHP plasma levels from meiosis to final spermation may be modulated by a feedback effect of DHP on the brain and the pituitary. Indeed, we have demonstrated that brain Pgr1, and both pituitary Pgr1 and Pgr2 may receive the DHP signal in order to regulate spermatogenesis from meiosis to final sperm maturation (Chapter 2).

According to our results, final sperm maturation seems to be controlled not only by DHP, but also by E2, through the two membrane receptors GPERa and GPERb (Chapter 1; Fig 1). These results support the proposed role of estrogen related to final spermatogenesis in mammals (Carreau et al., 2010), but further analyses are needed in order to understand the physiological mecanisms of the E2 action at the end of spermatogenesis in the European eel.

2. Evolutionary history of nuclear steroid receptors in vertebrates

The steroid receptors (SRs): estrogen receptor (ER), androgen receptor (AR), progestin receptor (PR), glucocorticoid receptor (GR), and mineralcorticoid receptor (MR), are members of the nuclear receptor family. They arose from one ancestral steroid receptor (ancSR) through a series of gene duplications which occurred early in vertebrate evolution (Dehal and Boor, 2005; for review see Eick and Thornton, 2011; Thornton, 2001) (Fig 2).

The first duplication event produced an estrogen receptor and a 3-ketosteroid receptor, which correspond to AncSR2, the last common ancestor of AR, PR, GR, and MR. AncSR2 underwent a second duplication to yield a corticoid receptor, the ancestor of GR/MR, and a 3-ketogonadal steroid receptor, the ancestor of AR/PR (for review see Eick and Thornton, 2011). This duplication may be the result of the first of two whole-genome duplication events which occurred early in the vertebrate lineage (Van de Peer et al., 2009).

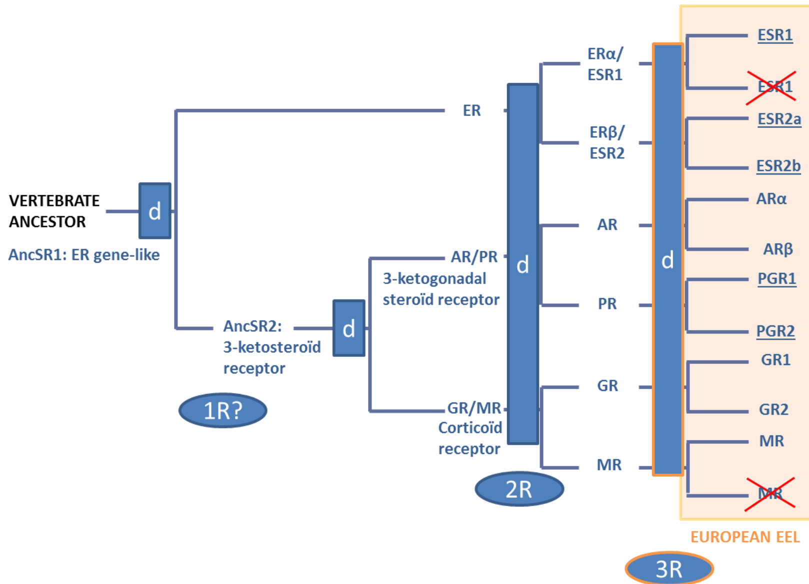

Figure 2. Evolutionary history of the sex steroid receptors in teleosts. R: Round of whole genome duplication; d: duplication event

Finally, the three receptors (i.e. ER, the corticoid receptor, and the 3-ketogonadal steroids receptor) produced the six steroid receptors currently found in jawed vertebrates, in a final round of duplication possibly due to the second whole-genome duplication event which took place during early vertebrate evolution (Thornton, 2001).

Teleost genomes differ from the rest of the vertebrate genomes because of an additional whole-genome duplication event, the teleost genome duplication (TGD) or third whole-genome duplication (Amores et al., 1998, 2011). Thus, compared to other vertebrates, teleosts possess a higher number of genes, and as a consequence, an increased number of steroid receptors. Indeed, studies on eels have demonstrated the presence of 3 ERs (ESR1, accession number CUH82767, ESR2a, accession number CUH82768; ESR2b, accession number CUH82769; Lafont et al., in press), 2 PRs (PGR1, accession number AFV13730; PGR2, accession number AFV13731) (Chapter 2) and 2 ARs (ARα, accession number: CBV44425; ARβ, accession number CBV44424; Lafont et al., unpublished; Ikeuchi et al., 1999; Todo et al., 1999). Only a single GR has been cloned in the Japanese eel (accession number AB506765), while at least two types seem to be

present in the European eel (Marsigliante et al., 2000) and in other teleost species (Prunet et al., 2006; reviewed by Stolte et al., 2006). Finally, only a single MR has been isolated in teleosts (Greenwood et al., 2003; Prunet et al., 2006).

Due to its phylogenetical position, belonging to an early emerging group of teleosts (elopomorphs), the European eel share the inferred whole-genome duplication at the base of the teleost lineage (Henkel et al., 2012a). Furthermore, as it did not undergo additional genome duplication events (4R) specific to some teleost, as well as Salmonid species (Danzmann et al., 2008), the *Anguilla* species may provide insights into ancestral regulatory functions in teleosts.

Phylogeny and synteny analyses of ER and PR in European eel show that this larger number of nuclear steroid receptors results from the teleost whole genome duplication (3R). Indeed, synteny analysis has shown that the *pgr* genomic region has been duplicated in the eel as well as in the other teleosts investigated. Furthermore, according to the phylogenetic analysis, each nuclear steroid sequence is clustered at the base of the teleost clades (Chapter 2; Lafont et al, in press). This is consistent with the basal position of elopomorphs in the phylogeny of teleosts, which was also observed in our previous phylogeny analyses of various gene families (Morini et al., 2015a; Pasquier et al., 2011, 2012). The presence of two ESR2 and PR in the eel, compared to a single ESR2 and PR in non-teleost actinopterygian and sarcopterygian species, strengthens the current hypothesis that these nuclear receptors are likely to be the result of the teleost 3R (Bardet et al., 2002; Hawkins et al., 2000). The presence of two AR (Ogino et al., 2009) and GR (Greenwood et al., 2003) paralogs is consistent with the previous hypotheses that larger number of nuclear steroid receptors is the result of additional gene duplications specific to teleosts. Nevertheless, the presence of one ESR1 in most teleost species including the eel (Lafont et al., in press), and the presence of duplicated PR in eels but not in most teleosts studied (Morini et al., submitted, Chapter 2) suggests that one of 3R-duplicated ESR1 has been lost shortly after the emergence of the teleost species, while the *pgr1* paralog was lost in the teleost lineage, after the emergence of the basal teleost group of elopomorphs. Further studies of eel MR are

necessary to confirm the presence of a single eel *MR* paralogon and, consequently, to gain a greater understanding of the evolutive history of this nuclear steroid receptor.

Gene duplication through evolution is an important mechanism to improve the diversification of genes and gene functions, leading to phenotypic complexity, diversity and innovation. As teleosts underwent three whole-genome duplications, fish species exhibit more diversified genes compared to sarcopterygians, and this resulted in the enrichment of their development, signaling, and behavioral genes, through processes of sub- and/or neofunctionalization. The genomic complexity of teleosts might be the reason behind their evolutionary success and biological diversification.

3. Interactions between steroid receptors

The binding of sex steroids to their respective receptors is commonly accepted, nevertheless, every hormone can affect the expression of the other receptors, leading to a complex combined effect of such interaction.

Concerning the nuclear steroid receptors, physiological interactions between androgens and estrogens through physical interaction between ESR1 and AR has already been demonstrated (Panet-Raymond et al., 2000). These interactions can be triggered by different mecanisms. A steroid-receptor coactivator may confer the androgen receptor the capacity to bind estrogen, resulting in an estrogen effect on androgen targets (Yeh et al., 1998); or a direct estrogen dependent interaction between AR and ESR1 steroid receptors, may modulate the AR transcriptional activity (Panet Raymond et al., 2000). Furthermore, cross talk between androgens and estrogens and their receptors has been noted in mice bone (Kousteni et al., 2001) and interactions between androgen and estrogen receptors has been investigated in humans (Kreitmann and Bayard, 1979). In the male European eel, as E_2 plasma levels decrease and brain ESR1, ESR2a and ESR2b remain stable throughout spermatogenesis, we can hypothesise that other factors, such as

androgens, may interact with brain ESR1, ESR2a and ESR2b. Although there is no evidence of a direct interaction between progestin and nuclear estrogen receptors, a brain progestin effect mediated by nuclear ESR cannot be excluded.

Furthermore, several rapid nongenomic effects of progestin and estrogens have been attributed to membrane receptors. Some of these effects have been demonstrated in human spermatozoa (Baldi et al., 2000; Luconi et al., 2001, 2004). While the isolation of both mPR and GPER in human spermatozoa remains unclear, the evidence points towards the existence and functional activity of both a progestin and an estrogen membrane receptor in human spermatozoa (Luconi et al., 2004). Indeed, in humans, progestin has been demonstrated to stimulate calcium influxes and to induce the activation of spermatozoa through the induction of capacitation, hyperactivated motility and acrosome reaction, whereas E_2 seems to negatively modulate sperm responsiveness to progestin (Luconi et al., 2004). Both progestin and E_2 effects seem to be mediated by specific receptors on the plasma membranes of human spermatozoa. Similarly, in the European eel, the high expression levels of mPRs (mPRa, mPRAL1, mPRAL2) and GPERs (GPERa, GPERb) at the end of spermatogenesis (SPZ stage) suggest that these receptors are localized in the membrane of the germ cells, as has been found to be the case in humans. Although further studies are necessary in order to be able to draw conclusions on the effect of both E2 and progestin on final spermatogenesis, they may interact with eachother in order to regulate the maturation of sperm cells in the male European eel.

Sex steroids may interact with both nuclear and membrane steroid receptors. Furthermore, complex interactions between the two receptor systems with their specific sex steroid may occur in order to activate nonclassical signaling pathways. Direct interactions between the receptors lead to an additional level of control and add to the increasing complexity of steroid signaling pathways.

4. The effect of temperature on European eel maturation and gamete quality

The mechanism by which temperature controls maturation and spawning in fishes is unclear, however, water temperature is an important environmental factor involved in the sexual development of many fish species (Van Der Kraak and Pankhurst, 1996). In female eels, studies have demonstrated that temperature can modulate oogenesis (Pérez et al., 2011; Mazzeo et al., 2014), and that low temperatures facilitate early ovarian development. In male eels, studies have shown that high temperatures are required for the development of final spermatogenesis and to guarantee sperm quality (Chapter 3; Baeza et al., 2014; Gallego et al., 2012). During their oceanic migration, eels would appear to to be subjected to increasing temperatures, from the cold water of the Eastern coasts of the Atlantic Ocean to the warmer water of the Sargasso Sea. As the eel migration takes several months, it seems probable that gonadal development occurs at low temperatures, whereas spawning takes place at higher temperatures. Beside this change in temperature during the oceanic reproductive migration, the eels seem to be subjected to a daily change in temperature due to circadian vertical migrations. According to Aarestrup et al. (2009), during the night, eels try to find warm water (300 m depth and 12 °C approx.), and during the day, eels are able to cope with cooler temperatures (600 m depth and 9 °C approx.).

In this thesis we have used 3 thermic regimes to demonstrate (Chapter 3), a clear influence of temperature on steroidogenic enzyme gene expression and steroid synthesis in male European eels. Temperature modulates steroidogenesis, testis maturation and the spermiation process. Furthermore, we have shown (Chapter 4) that temperature may play a key role in eel egg fertilization.

Thanks to our *in vivo* results, we can hypothethize that the onset of spermatogenesis is characterised by an increase in estrogen plasma levels, stimulated by a change in salinity and independent of the temperature. This first step is followed by an increase in androgen plasma levels, resulting from aa11βHSD, aacyp11a1 and aacyp17-1 activity (Fig 1). The concentrations of these enzymes and steroids

increase even at low temperatures, suggesting that the change in salinity itself is a signal for the beginning of eel maturation and for the stimulation of spermatogonia renewal/proliferation.

However, at 10 °C the maturation is blocked and eels cannot reach the spermiation phase. Increasing the temperature to 15 °C is necessary in order to induce a shift in testis steroidogenesis, from androgen to estrogen and progestin synthesis (through an increase in *aacyp19a1, aacyp21* and maybe *omhsd17b3like* gene expression; Su et al., 2015).

So, to complete the spermatogenesis process; during the migration of eel males at low temperatures the testis development seems to reach the spermatogonia proliferation stage, whereas final spermatogenesis can be achieved when the fish reache higher temperatures. We have shown that higher temperatures (T10 thermal regime) lead to higher PLCζ expression levels (Chapter 4). Our *in vivo* results suggest that a thermal regime which begins with low temperatures and is then followed by increasing temperatures, is more akin to that experienced by this species in the wild and, therefore, more physiologically effective, and should result in improved experimental male eel maturation and embryo quality. Furthermore, other studies have found that, without hCG treatment, 11KT and T reached higher levels at 10 °C than at 20 °C (Peñaranda et al., unpublished), suggesting a major proliferation of spermatogonia at low temperatures, at the beginning of spermatogenesis. These results support previous studies on female eels, which have demonstrated that low temperatures facilitate early ovarian development (Pérez et al., 2011; Mazzeo et al., 2014). However, a constant temperature of 20 °C throughout hormonal treatment seems to be more effective for inducing fast eel spermiation (Gallego et al., 2012), which is interesting from an aquacultural perspective. Future epigenetic analyses should provide further information on the effects of temperature.

Future perspectives

In this thesis, we have performed phylogenetic analyses of all the receptors studied (membrane and nuclear), and syntenic analyses of the duplicated nuclear *pgr* genes were added to complete the evolutionary history of the PGRs. To gain a greater understanding of the origin of these sex steroid receptors, it would be interesting to carry out syntenic analyses of the membrane progestin receptors.

As we previously proposed, interactions may occur between sex steroid receptors and androgens, or between progestin and estrogen. Further studies of ligand binding specificity measures may provide us with helpful information on the complex interaction mechanisms involved in the control of gamete maturation.

Temperature variations strongly influenced the spermatogenesis process and eel maturation. Nevertheless, further experiments on temperature effects with different timings for the temperature changes, or even combined with other environmental factors (such as pressure or salinity), would allow us to get closer to the environmental stimuli at work in the wild and, as a consequence, may result in improved spermatogenesis.

Finally, our gene expression and steroid level results suggest that sex steroid receptors/sex steroids are phase-specific throughout European eel spermatogenesis. However, the study of the corresponding protein could provide us with useful information on eel maturation. Furthermore, a spatial mRNA expression study through the gonadotropic axis would provide us with a complete overview of the steroid function in eel reproductive physiology.

CONCLUSIONS

- Due to its phylogenetical position, belonging to an early emerging group among teleosts (elopomorphs), the European eel underwent three whole-genome duplications, and exhibits more diversified genes compared to sarcopterygians.

- European eel spermatogenesis seems to be affected by two major environmental factors: the change of salinity which seems to control the onset of the whole process, and temperature which appears to regulate final testis maturation.

- E_2 and DHP effects are tissue-specific, with different expression patterns of the respective receptors along the brain-pituitary-gonad axis.

- In the brain, estrogen and progestin receptors may be implicated in the neuroendocrine control of reproduction through E_2 and DHP signals.

- In the pituitary, nuclear progestin receptors (pgr1 and pgr2) receive the DHP plasma signals, while nERs and GPERs mediate the autocrine or paracrine actions of locally-produced E_2.

- In the testis, E_2 is involved in spermatogonial stem cell renewal through the nuclear estrogen receptors (ESR1, ESR2a and ESR2b), 11-KT promotes spermatogonial proliferation towards meiosis, DHP regulates meiosis through mPRγ, mPRδ and pgr2, and sperm maturation seems to be controlled by both E_2 and DHP through GPERa and GPERb; mPRα, mPRAL1 and mPRAL2.

- Spermatogenesis is regulated by complex interactions between sex steroids and steroid receptors, and further analyses are required in order to fully understand the function of these interactions during eel maturation. The level of complexity increases with the interactions between nuclear and membrane receptors, along the entire gonadotropic axis (i.e. in all the brain parts, the pituitary and the gonads).

- The eel sperm-specific PLCζ1 which induces egg activation is significantly elevated in the T10 thermal regime, suggesting that temperature may play a role in the regulation of *plcζ1* transcription in the testis, and therefore in the physiological processes which control sexual maturation and fertilization.

- Debido a su posición filogenética, dentro de uno de los grupos más antiguos de los teleósteos (Elopomorfos), el genoma completo de la anguila europea se ha duplicado en tres ocasiones, y presenta una mayor diversidad de genes que el grupo de los sarcopterigios.

- La espermatogénesis en la anguila europea parece estar influida por dos importantes factores ambientales: el cambio de salinidad que parece controlar el inicio de la espermatogénesis, y la temperatura que parece regular la maduración final del testículo.

- Los efectos del E_2 y DHP son específicos de tejido, mostrando diferentes patrones de expresión de sus respectivos receptores a lo largo del eje cerebro-hipófisis-gónada.

- En el cerebro, los receptores de estrógenos y de progestágenos podrían estar implicados en el control neuroendocrino de la reproduccion a traves de señales de E_2 y de DHP.

- En la hipófisis, los receptores nucleares de progestágenos (pgr1 and pgr2) reciben la señal del DHP plasmático, mientras los receptores de estrógenos nucleares y de membrana median las acciones autocrina o paracrina del E_2 producido en la misma hipófisis.

- En el testículo, el E_2 está implicado en el control de la renovación de las espermatogonias indiferenciadas, mediante los receptores nucleares de estrógenos (ESR1, ESR2a y ESR2b), la 11-KT induce la proliferación de las espermatogonias, la DHP regula la meiosis mediante los receptores mPRγ, mPRδ y pgr2, y la maduración final del esperma parece estar controlada por el E_2 y el DHP a través de los receptores GPERa y GPERb; mPRa, mPRAL1 y mPRAL2.

- La espermatogénesis está regulada por interacciones complejas entre los esteroides sexuales y los receptores de esteroides, y serán necesarios más análisis para entender completamente la función de estas interacciones durante la maduración de la anguila. El nivel de complejidad aumenta con las interacciones entre los receptores nucleares y los de

membrana, presentes en todo el eje gonadotrópico (todas las partes del cerebro, la hipófisis y las gónadas).

- La PLCζ1, una proteína específica del esperma y que induce la activación del huevo, en anguila ve su nivel aumentado significativamente en el régimen térmico T10, lo que sugiere que la temperatura podría tener un papel en la regulación de la transcripción de la *plcζ1* en el testículo, y por lo tanto en los procesos fisiológicos que controlan la maduración sexual y la fertilización.

REFERENCES

Aarestrup, K., Ökland, F., Hansen, M.M., Righton, D., Gargan, P., Castonguay, M., Bernatchez, L., Howey, P., Sparholt, H., Pedersen, M.I., McKinley, R.S., 2009. Oceanic Spawning Migration of the European Eel (*Anguilla anguilla*), Science 325, 1660.

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21, 2104-2105.

Adachi, S., Ijiri, S., Kazeto, Y., Yamauchi, K., 2003. Oogenesis in the Japanese Eel, *Anguilla japonica*. Eel biology. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), . Eel biology , vol. 5., Springer, Tokyo, pp. 301-317.

Albiston, A.L., Obeyesekere, V.R., Smith, R.E., Krozowski, Z.S., 1994. Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme. Mol. Cell Endocrinol. 105, R11-17.

Amin, E. M., 1997. Observations on reproduction techniques applicable to the European eel (*Anguilla anguilla* L.). En: Genetics and breeding of Mediterranean aquaculture species. D. Bartley y B. Basurco (eds.). Cahiers Options Mé- diterranéennes 34: 223-234.

Amores, A., Force, A., Yan, Y.L, Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M., Postlethwait, J.H., 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282, 1711-4.

Amores, A., Catchen, J., Ferrara, A., Fontenot, Q., Postlethwait, J.H., 2011. Genome evolution and meiotic maps by massively parallel DNA sequencing : spotted gar,an outgroup for the teleost genome duplication. *Genetics* 188, 799-808.

Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S., 2005. Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. Neuroendocrinology 82, 221–232.

Aroua, S., Weltzien, F.A., Le Belle, N., Dufour, S., 2007. Development of real-time RT-PCR assay for eel gonadotropins and their application to the comparison of in vivo and in vitro effects of sex steroids. Gen. Comp. Endocrinol. 153, 333-343.

Asturiano, J.F., Sorbera, L.A., Ramos, J., Kime, D.E., Carrillo, M., Zanuy, S., 2000. Hormonal regulation of the European sea bass

(*Dicentrarchus labrax*, L.) reproductive cycle: an individualized female approach. J. Fish Biol. 56, 1155-1172.

Asturiano, J.F., Pérez, L., Tomás, A., Zegrari, S., Espinós, F.J., Jover, M., 2002. Inducción hormonal de la maduración gonadal y puesta en hembras de anguila europea (*Anguilla anguilla*). Cambios morfológicos y desarrollo oocitario. Boletín del Instituto Español de Oceanografía 18, 1-10.

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

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

Asturiano, J.F., Mazzeo, I., Gallego, V., Ciccotti, E., Pérez, L., Capoccioni, F., 2011. Estimación de la edad en que las hembras de anguila (*A. anguilla*) de la Albufera de Valencia realizan su migración reproductiva Poster XIII Congreso Nacional de Acuicultura Libro de Resúmenes, pp.: 267-268.

Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2014. Effect of thermal regime on fatty acid dynamics in male European eels (*Anguilla anguilla*) during hormonally-induced spermatogenesis. Aquaculture 430, 86-97.

Baeza, R., Peñaranda, D.S., Vílchez, M.C., Tveiten, H., Pérez, L., Asturiano, J.F., 2015. Exploring correlations between sex steroids and fatty acids and their potential roles in induced maturation of male European eel. Aquaculture 435, 328-335.

Baldi, E., Luconi, M., Muratori, M., Forti, G., 2000. A novel functional estrogen receptor on human sperm membrane interferes with progesterone effects. Mol. Cell. Endocrinol. 161,31-5.

Bardet, P.L., Horard, B., Robinson-Rechavi, M., Laudet, V., Vanacker, J.M., 2002. Characterization of oestrogen receptors in zebrafish (*Danio rerio*). J. Mol. Endocrinol. 28, 153-163.

Baynes, S.M., Scott, A.P., 1985. Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 57, 150-160.

Bazer, F.W., Wu, G., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Bayless, K., 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Mol. Hum. Reprod. 16, 135-152.

Behl, C., 2002. Oestrogen as a neuroprotective hormone. Nat. Rev. Neurosci. 3, 433-442.

Beyer, C., 1999. Estrogen and the developing mammalian brain. Anat Embryol 199, 379-390.

Bezdenezhnykh, V.A., Prokhorchik, G.A., Petrikov, A.M., Petukov, V.B., Plyuta, M.V., 1983. Obtaining the larvae of European eel *Anguilla anguilla* L. (Pisces, Anguillidae) under experimental conditions. Dokl. Akad. Nauk SSSR, 268, 1264-1266 (In Russian).

Blazquez, M., Piferrer, F. 2004. Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*), Mol. Cell. Endocrinol. 219, 83–94.

Boëtius, I., Boëtius, J., 1967. Studies in the European eel, *Anguilla anguilla* (L.). Experimental induction of the male sexual cycle, its relation to temperature and other factors. Meddelser fra Danmarks Fiskeri- og Havunderogelser 4, 339-405.

Boëtius, I., Boëtius, J., 1980. Experimental maturation of female silver eels, *Anguilla anguilla*. Estimates of fecundity and energy reserves for migration and spawning, Dana 1, 1-28.

Bruijs, M.C.M., Durif, C.M.F., 2009. Silver Eel Migration and Behaviour, in G. van den Thillart et al. (Eds.), Spawning Migration of the European Eel, Springer Science + Business Media B.V. 65-95.

Boletín Oficial del Estado, 2013. Real Decreto 53/2013 sobre protección de animales utilizados en experimentación y otros fines científicos. BOE 34, 11370-11371.

Butts, I.A.E., Sørensen ,S.R., Politis, S.N., Pitcher, T.E., Tomkiewicz, J., 2014. Standardization of fertilization protocols for the European eel, *Anguilla anguilla*. Aquaculture 426-427, 9-13.

Callard, G.V., Tchoudakova, A. 1997. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish, J. Steroid Biochem. Mol. Biol. 61, 387-392.

Carreau, S., Wolczynski, S., Galeraud-Denis, I., 2010. Aromatase, oestrogens and human male reproduction. Phil. Trans. R. Soc. B. 365, 1571-1579.

Carson-Jurica, M.A., Schrader, W.T., O'malley. B.W., 1990. Steroid Receptor Family: Structure and Functions. Endocr. Soc. 11, 201-220.

Chang, X., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajura, H., Sudhakumari, C.C., Nagahama, Y. 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 141, 101-115.

Chen, J.N., López, J.A., Lavoué, S., Miya, M., Chen, W.J., 2014. Phylogeny of the Elopomorpha (Teleostei): evidence from six nuclear and mitochondrial markers. Mol Phylogenet Evol 70, 152-161.

Chen, S.X., Bogerd, J., García-López, A., de Jonge, H., de Waal, P.P., Hong, W.S., Schulz, R.W., 2010. Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor, Biol. Reprod. 82, 171-181.

Chen, S.X., Bogerd, J., Andersson, E., Almeida, F.F.L., Taranger, G.L., Schulz, R.W., 2011. Cloning, pharmacological characterization, and expression analysis of Atlantic salmon (*Salmo salar* L.) nuclear progesterone receptor. Reproduction 141, 491-500.

Chen, S.X., Almeida, F.F.L., Andersson, E., Taranger, G.L., Schmidt, R., Schulz, R.W., Bogerd, J., 2012. Cloning, pharmacological characterization and expression analysis of Atlantic cod (*Gadus morhua,* L.) nuclear progesterone receptor. Gen. Comp. Endocrinol. 179, 71-77.

Ciccotti, E., Fontenelle, G., 2001. A review of eel, *Anguilla anguilla* aquaculture in Europe: perspectives for its sustainability. J. Taiwan Fish Res 9, 27-43.

CITES Proposal 2007: Inclusion of *Anguilla anguilla* (L). in Appendix II in accordance with Article II §2(a). In: Convention on International Trade in Endangered Species ofwild flora and fauna. Fourteenth meeting of the Conference of the Parties, The Hague, Netherlands, 3–15 June 2007. CoP14, Prop. 18. http://www.cites.org/common/cop/14/raw_props/EDE04 Anguilla%20anguilla.pdf. Accessed 8 Aug 2007

Cooney, M.A., Malcuit, C., Cheon, B., Holland, M.K., Fissore, R.A., D'Cruz, N.T., 2010. Species-specific differences in the activity and nuclear localization of murine and bovine phospholipase C zeta 1. Biol. Reprod. 83, 92-101.

Correia, S., Cardoso, H.J., Cavaco J.E., Socorro, S., 2015. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev. Mol Med. 17: e2.

Coward, K., Ponting, C.P., Chang, H.Y., Hibbitt, O., Savolainen, P., Jones, K.T., Parrington, J., 2005. Phospholipase Cζ, the trigger of egg activation in mammals, is present in a non-mammalian species. Reproduction 130, 157-163.

Coward, K., Ponting, C.P., Zhang, N., Young, C., Huang, C.J., Chou, C.M., Kashir, J., Fissore, R.A., Parrington, J., 2011. Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCζ) in pufferfish. Mol. Reprod. Dev. 78, 48-56.

Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K., Lai, F.A., 2002. Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca2+ oscillations, activation and development of mouse oocytes. Reproduction 124, 611-623.

Danzmann, R.G., Davidson, E.A., Ferguson, M.M., Gharbi, K., Koop, B.F., Hoyheim, B., Lien, S., Lubieniecki, K.P., Moghadam, H.K., Park, J., Phillips, R.B., Davidson, W.S., 2008. Distribution of ancestral proto-Actinopterygian chromosome arms within the genomes of 4R-

derivative salmonid fishes (Rainbow trout and Atlantic salmon). BMC Genomics 9, 557.

Daverat, F., Limburg, K.E., Thibault, I., Shiao, J.C., Dodson, J.J., Caron, F., Tzeng, W.N., Iizuka, Y., Wickström, H., 2006. Phenotypic plasticity of habitat use by three temperate eel species, *Anguilla anguilla, A. japonica* and *A. rostrata*. Mar. Ecol. Prog. Ser. 308, 231-241.

Dehal, P., Boore, J.L., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol 3(10): e314.

Diotel, N., Page, Y.L., Mouriec, K., Tong, S.K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B.C., Kah, O., 2010. Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front. Neuroendocrinol. 31, 172-192.

Diotel, N.,Do Rego, J.L., Anglade1, I., Vaillant, C., Pellegrini, E., Vaudry, H.,Kah, O., 2011. The brain of teleost fish, a source, and a target of sexual steroids. Front. Neurosci. 5, 1-15.

Dufour, S., Delerue-Le Belle, N., Fontaine, Y.A., 1983. Effects of steroid hormones on pituitary immunoreactive gonadotropin in European freshwater eel, *Anguilla anguilla* L. Gen. Comp. Endocrinol. 52, 190–197.

Dufour, S., Lopez, E., Le Menn, F., Le Belle, N., Baloche, S., Fontaine, Y.A., 1988. Stimulation of gonadotropin release and ovarian development, by the administration of a gonadoliberin agonist and of dopAmine antagonists, in female silver eel pretreated with estradiol. Gen. Comp. Endocrinol. 70, 20-30.

Dufour, S., Montero, M., Le Belle, N., Bassompierre, M., King, J.A., Millar, R.P., Peter, R.E., Fontaine, Y.A., 1993. Differential distribution and response to experimental sexual maturation of two forms of brain gonadotropin-releasing hormone (GnRH) in the European eel, *Anguilla anguilla*. Fish Physiol Biochem. 1-6, 99-106.

Dufour, S., Burzawa-Gerard, E., Le Belle, N., Sbaihi, M., Vidal, B., 2003. Reproductive endocrinology of the European eel, *Anguilla anguilla*. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology, Springer, Tokyo, pp. 373-383.

Dufour, S., Weltzien, F.A., Sébert, M.E., Le Belle, N., Vidal, B., Vernier, P., Pasqualini, C., 2005. DopAminergic inhibition of reproduction in

teleost fishes: ecophysiological and evolutionary implications, Ann. NY Acad. Sci. 1040, 9-21.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M.H., Hirano, T., Grau, E.G., 2001. Hormonal Control of Osmoregulation in the Channel Catfish Ictalurus punctatus. Gen. Comp. Endocrinol. 122, 270-286.

Eick, G.N., Thornton, J.W., 2011. Evolution of steroid receptors from an estrogen sensitive ancestral receptor. Mol. Cell. Endocrinol. 334, 31-38.

Evans, J.P., Kopf, G.S., 1998. Molecular mechanisms of sperm-egg interactions and egg activation. Andrologia 30, 297-307.

Falkenstein, E., Tillmann, H.C., Christ, M., Feuring, M., Wehling, M., 2000. Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. Pharmacol. Rev. 52, 513-556.

FAO, 2013. Food and Agriculture Organization. Departamento de pesca y acuicultura. Estadísticas. http://www.fao.org/fishery/statistics/es.

Feunteun, E., 2002. Management and restoration of European eel population (*Anguilla anguilla*): An impossible bargain. Ecol. Eng. 18, 575-591.

Filardo, E., Thomas, P., 2005. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol. Metab. 16, 362-367.

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., Thomas, P., 2007. Activation of the Novel Estrogen Receptor G Protein-Coupled Receptor 30 (GPR30) at the Plasma Membrane. Endocrinology 148, 3236-3245.

Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. J. Mol. Endocrinol. 37, 121-134.

Frantzen, M., Arnesen, A.M., Damsgård, B., Tveiten, H., Johnsen, H.K., 2004. Effects of photoperiod on sex steroids and gonad maturation in Artic charr. Aquaculture 240, 561-576.

Fontaine, M., 1936. Sur la maturation complète des organes génitaux de l'anguille mâle et l'émission spontanée de ses produits sexuels. C. R. Acad. Sci. Paris 202, 1312-1315.

Fontaine, M., Bertrand, E., Lopez, E., Callamand, O. 1964. Sur la maturation des organes génitaux de l'anguille femelle (*Anguilla anguilla* L.) et l'émission spontanée des oeufs en aquarium, C.R. Acad. Sci. Paris 259, 2907-2910.

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

Gallego, V., Vílchez, M.C., Peñaranda, D.S., Pérez, L., Herráez, M.P., Asturiano, J.F., Martínez-Pastor, F., 2014. The subpopulation pattern of eel sperm is affected by post-activation time, hormonal treatment and thermal regimen. Reprod. Fertil. Dev. 27, 529-543.

Gómez, J.M., Weil, C., Ollitrault, M., Le Bail, P.Y., Breton, B., Le Gac, F.L., 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 113, 413-428.

Gousset, B., 1990. European eel (*Anguilla anguilla* L.) farming technologies in Europe and in Japan: application of a comparative analysis. Aquaculture 87, 209-235.

Graddy, L.G., Kowalski, A.A., Simmen, F.A., Davis, S.L., Baumgartner, W.W., Simmen, R.C., 2000. Multiple isoforms of porcine aromatase are encoded by three distinct genes, J. Steroid Biochem. Mol. Biol. 73, 49-57.

Greenwood, P.H., Rosen, D.E., Weitsman, S.H., Myers, G.S., 1966. Phyletic studies of teleostean fishes, with a provisional classification of living forms. Bull Am Mus Nat Hist 131, 339-456.

Greenwood, A.K., Butler, P.C., White, R.B., Demarco, U., Pearce, D., Fernald, R.D., 2003. Multiple Corticosteroid Receptors in a Teleost Fish: Distinct Sequences, Expression Patterns, and Transcriptional Activities. Endocrinology 144, 4226-4236.

Guan, G., Tanaka, M., Todo, T., Young, G., Yoshikuni, M., Nagahama, Y., 1999. Cloning and expression of two carbonyl reductase-like 20β-hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). Biochem. Biophys. Res. Commun. 255, 123-128.

Hanna, R.N., Zhu, Y., 2009. Expression of membrane progestin receptors in zebrafish (*Danio rerio*) oocytes, testis and pituitary. Gen. Comp. Endocrinol. 161, 153-157.

Hanna, R.N., Daly, S.C., Pang, Y., Anglade, I., Kah, O., Thomas, P., Zhu, Y., 2010. Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. Biol. Reprod. 82, 112-122.

Hart, N.F., 1990. Fertilization in teleost fishes: mechanisms of sperm–egg interactions. Int. Rev. Cytol. 121, 1–66. Heinsbroek LTN (1991) A review of eel culture in Japan and Europe. Aquacult Fish Manag 22, 57-72.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. U.S.A. 97, 10751-10756.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., Gustafsson, J.A., 2007. Estrogen receptors: how do they signal and what are their targets. Physiol. Rev. 87, 905-931.

Henkel, C.V., Burgerhout, E., de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J., Spaink, H.P., Dufour, S., Weltzien, F.A., Tsukamoto, K., van den Thillart, G.E., 2012a. Primitive duplicate Hox clusters in the European eel's genome. PLoS One 7:e32231.

Henkel, C.V., Dirks, R.P., de Wijze, D.L., Minegishi, Y., Aoyama, J., Jansen, H.J., Turner, B., Knudsen, B., Bundgaard, M., Hvam, K.L., Boetzer, M., Pirovano, W., Weltzien, F.A., Dufour, S., Tsukamoto, K., Spaink, H.P., van den Thillart, G.E., 2012b. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene 511, 195-201.

Hess, R.A., 2003. Estrogen in the adult male reproductive tract: a review. Reprod. Biol. Endocrinol. 1, 52.

Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S.Y., Fissore, R.A., Hamer, R., Deane, C.M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C.A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L., De Sutter, P., 2009. Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. Hum. Reprod. 24, 2417-2428.

Hildahl, J., Sandvik, G.K., Edvardsen, R.B., Fagernes, C., Norberg, B., Haug, T.M., Weltzien, F.A., 2011. Identification and gene expression analysis of three GnRH genes in female Atlantic cod during puberty provides insight into GnRH variant gene loss in fish. Gen. Comp. Endocrinol. 172, 458-467.

Hinfray, N., Nobrega, R.H., Caulier, M., Baudiffier, D., Maillot-Marechal, E., Chadili, E., Palluel, O., Porcher, J.M., Schulz, R., Brion, F. 2013. Cyp17a1 and Cyp19a1 in the zebrafish testis are differentially affected by oestradiol. J. Endocrinol. 216, 375-388.

Horner, V.L., Wolfner, M.F., 2008. Transitioning from egg to embryo: Triggers and mechanisms of egg activation. Dev. Dyn. 237, 527-544.

Horner, K.C., 2009. The effect of sex hormones on bone metabolism of the otic capsule-an overview. Hear. Res. 252, 56-60.

Huang, H., Zhang, Y., Huang, W.R., Li, S.S., Zhu, P., Liu, Y., Yin, S.W., Liu, X.C., Lin, H.R., 2009. Molecular characterization of marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles during artificially induced gonadal development. Gen. Comp. Endocrinol. 162, 192-202.

Huertas, M., Scott, A.P., Hubbard, P.C., Canário, A.V.M., Cerdà, J., 2006. Sexually mature European eels (*Anguilla anguilla* L.) stimulate gonadal development of neighbouring males: Possible involvement of chemical communication. Gen. Comp. Endocrinol. 147, 304-313.

ICES. 2012. Report of the Joint EIFAAC/ICES Working Group on Eels (WGEEL), 3–9 September 2012, Copenhagen, Denmark. ICES CM 2012/ACOM:18. 824 pp.

ICES. 2013. ICES Working Group on Eels. Report of the ICES Advisory Committee on Fisheries Management 18, 253 pp.

Igarashi, H., Knott, J.G., Schultz, R.M., Williams, C.J., 2007. Alterations of PLCβ1 in mouse eggs change calcium oscillatory behaviour following fertilization. Dev. Biol. 312, 321-330.

Ijiri, S., Kazeto, Y., Lokman, P.M., Adachi, S., 2003. Yamauchi K. Characterization of a cDNA encoding P-450 aromatase (CYP19) from Japanese eel ovary and its expression in ovarían follicles during induced ovarian development. Gen. Comp. Endocrinol. 130, 193-203.

Ijiri, S., Takei, N., Kazeto, Y., Todo, T., Adachi, S., 2006. Changes in localization of cytochrome P450 cholesterol side-chain cleavage (P450scc) in Japanese eel testis and ovary during gonadal development. Gen. Comp. Endocrinol. 145, 75-83.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 1999. cDNA Cloning of a Novel Androgen Receptor Subtype. Journal of Biological Chemistry, 274, 25205-25209.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 2002. A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*. FEBS Lett. 510, 77-82.

Ito, M., Shikano, T., Oda, S., Horiguchi, T., Tanimoto, S., Awaji, T., Mitani, H., Miyazaki, S., 2008. Difference in Ca2+ oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg activating sperm factor candidate, between mouse, rat, human, and medaka fish. Biol. Reprod. 78, 1081-1090.

Ito, M., Nagaoka, K., Kuroda, K., Kawano, N., Yoshida, K., Harada, Y., Shikano, T., Miyado, M., Oda, S., Toshimori, K., Mizukami, Y., Murata, T., Umezawa, A., Miyazaki, S., Miyado, K., 2010. Arrest of spermatogenesis at round spermatids in PLCZ1-deficient mice. 11th International symposium on Spermatology (abstract). Japan.

Ito, J., Parrington, J., Fissore, RA., 2011. PLCζ and its role as a trigger of development in vertebrates. Mol. Reprod. Dev. 78, 846-853.

Jaffe, L.F., 1983. Sources of calcium in egg activation: A review and hypothesis. Dev. Biol. 99, 265-276.

Jaffe, L.A., 1990. First messengers at fertilization. J. Reprod. Fertil. Suppl. 42,107-116.

Jaffe, L.F., 1991. The path of calcium in cytosolic calcium oscillations: A unifying hypothesis. Proc. Natl. Acad. Sci. USA 88, 9883-9887.

Jaillon, O., Aury, J.M., Brunet, F., Petit, J.L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S., Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biémont, C., Skalli, Z., Cattolico, L., Poulain, J., de Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau, J.P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S., Kellis, M., Volff, J.N., Guigó, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birre, B., Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quétier, F., Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J., Roest Crollius, H., 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. Nature 431, 946-957.

Jiang, J.Q., Wang, D.S., Senthilkumaran, B., Kobayashi, T., Kobayashi, H.K., Yamaguchi, A., 2003. Isolation, characterization and expression of 11β-hydroxysteroid dehydrogenase type 2 cDNAs from the testes of Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*). J. Mol. Endocrinol. 31, 305-315.

Jeng, S.R., Yueh, W.S., Chen, G.R., Lee, Y.H., Dufour, S., Chang, C.F., 2007. Differential expression and regulation of gonadotropins and their receptors in the Japanese eel, *Anguilla japonica*. Gen. Comp. Endocrinol. 154,161-173.

Jeng, S.R., Yueh, W.S., Pen, Y.T., Gueguen, M.M., Pasquier, J., Dufour, S., Chang, C.F., Kah, O., 2012a. Expression of Aromatase in Radial Glial Cells in the Brain of the Japanese Eel Provides Insight into the Evolution of the cyp191a Gene in Actinopterygians. PLoS One 7, e44750.

Jeng, S.R., Pasquier, J., Yueh, W.S., Chen, G.R., Lee, Y.H., Dufour, S., Chang, C.F., 2012b. Differential regulation of the expression of cytochrome P450 aromatase, estrogen and androgen receptor subtypes in the brain-pituitary-ovarian axis of the Japanes eel

(*Anguilla japonica*) reveals steroid dependent and independent mechanisms. Gen. Comp. Endocrinol. 175, 163-172.

Jin, G.X.,Wen, H.S., He, F., Li, J.F., Chen, C.F., Zhang, J.R., Chen, X.Y., Shi, B., Shi, D., Yang, Y.P., Qi, B.X., Li, N., 2012. Molecular cloning, characterization expression of P450c17-I and P450c17-II and their functions analysis during the reproductive cycle in males of barfin flounder (*Verasper moseri*). Fish. Physiol. Biochem. 38, 807-817.

Johnsen, H., Tveiten, H., Torgersen, J.S., Andersen, O., 2013. Divergent and sex-dimorphic expression of the paralogs of the Sox9-Amh-Cyp19a1 regulatory cascade in developing and adult Atlantic cod (*Gadus morhua* L.). Mol. Reprod. Dev. 80, 358-370.

Jones, K.T., Nixon, V. L., 2000. Sperm-induced Ca2+ oscillations in mouse oocytes and eggs can be mimicked by photolysis of caged inositol 1,4,5-trisphosphate: evidence to support a continuous low level production of inositol 1,4,5-trisphosphate during mammalian fertilization. Dev. Biol. 225, 1-12.

Kamei, H., Oshira, T., Yoshiura, Y., Uchida, N., Aida, K., 2003. Androgen secretion activity of recombinant follicle-stimulating hormone of Japanese eel, *Anguilla japonica* in immature and maturing eel testes. Fish Physiol. Biochem. 28, 97-98.

Kazeto, Y., Ijiri, S., Todo, T., Adachi, S., Yamauchi, K,. 2000a. Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. Gen. Comp. Endocrinol. 118, 123-133.

Kazeto, Y., Ijiri, S., Matsubara, H., Adachi, S., Yamauchi, K., 2000b. Cloning of 17b-hydroxysteroid dehydrogenase-I cDNAs from Japanese eel ovary. Biochem. Biophys. Res. Commun. 279, 451-456.

Kazeto, Y., Goto-Kazeto, R., Thomas, P., Trant, J.M., 2005. Molecular characterization of three forms of putative membrane-bound progestin receptors and their tissue-distribution in channel catfish, Ictalurus punctatus. J. Mol. Endocrinol. 34, 781-791.

Kazeto. Y., Tosaka, R., Matsubara, H., Ijiri, S., Adachi, S. 2011. Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation of the Japanese eel. J. Steroid Biochem. Mol. Biol. 127, 149-154.

Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. Hum. Reprod. Update 16, 690-703.

Kashir, J., Deguchi, R., Jones, C., Coward, K., Stricker, SA., 2013. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. Mol. Reprod. Dev. 80, 787-815.

Khan, I.A., Lopez, E., Leloup-Hâtey, J., 1987. Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature European eel (*Anguilla anguilla* L.). Gen Comp. Endocrinol. 68 91-103.

Knott, J.G., Kurokawa, M., Fissore, R.A., Schultz, R.M., Williams, C.J., 2005. Transgenic RNA interference reveals role for mouse sperm phospholipase Cζ in triggering Ca2+ oscillations during fertilization. Biol. Reprod. 72, 992-996.

Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T., Miyazaki, S., 2004. Recombinant phospholipase Cζ has high Ca2+ sensitivity and induces Ca2+ oscillations in mouse eggs. J. Biol. Chem. 279, 10408-10412.

Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K., Miyazaki, S., 2005. The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Cζ. J. Biol. Chem. 280, 21015-21021.

Kousteni, S., Bellido, T., Plotkin, L.I., O'Brien, C.A., Bodenner, D.L., Han, L., Han, K., DiGregorio, G.B., Katzenellenbogen, J.A., Katzenellenbogen, B.S., Roberson, P.K., Weinstein, R.S., Jilka, R.L., Manolagas, S.C.,2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.Cell 104, 719-730.

Kreitmann, B., Bayard, F., 1979. Androgen interaction with the oestrogen receptor in human tissues. J. Steroid Biochem. 11, 1589-1595.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M., Chambon, P., 1986. The chicken oestrogen receptor sequence:

homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J. 5, 891-897.

Kuroda, K., Ito, M., Shikano, T., Awaji, T., Yoda, A., Takeuchi, H., Kinoshita, K., Miyazaki, S., 2006. The role of X/Y linker region and N-terminal EF-hand domain in nuclear translocation and Ca2+ oscillation inducing activities of phospholipase Cζ, a mammalian egg activating factor. J. Biol. Chem. 281, 27794-27805.

Kusakabe, M., Nakamura, I., Young, G., 2003. 11β-Hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in Rainbow trout: cloning, sites of expression, and seasonal changes in gonads. Endocrinology 144, 2534-2545.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins. J. Endocrinol. 189, 541-554.

Lafont, A.G., Rousseau, K., Tomkiewicz, J., Dufour, S. 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla* sp: identification, evolutionary history and differential expression regulation Mol. Cell. Endocrinol., in press.

Laudet, V., Hänni, C., Coll, J., Catzeflis, C., Stéhelin, D., 1992. Evolution of the nuclear receptor gene family. EMBO J. 11, 1003-1013.

Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177-187.

LeGac, F., Loir, M., LeBail, P.Y., Ollitrault, M. 1996. Insulinlike growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. Mol. Reprod. Dev. 44, 23-35.

Li, Y.Y., Inoue, K., Takei, Y., 2003. Interrenal steroid 21-hydroxylase in eels: primary structure, progesterone-specific activity and enhanced expression by ACTH. J. Mol. Endocrinol. 31, 327-340.

Liu, X.S., Ma, C., Hamam, A., Liu, X.J., 2005. Transcription-dependent and transcription-independent functions of the classical progesterone receptor in Xenopus ovaries. Dev. Biol. 283, 180-190.

Liu, X., Zhu, P., Sham, K.W.Y., Yuen, J.M.L., Xie, C., Zhang, Y., Liu, Y., Li, S., Huang, X., Cheng, C.H.K., Lin, H., 2009. Identification of a membrane estrogen receptor in zebrafish with homology to mammalian gper and its high expression in early germ cells of the testis. Biol. Reprod. 80, 1253-1261.

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: How fish eggs are formed. Gen. Comp. Endocrinol. 165, 367-389.

Luconi, M., Bonaccorsi, L., Forti, G-, Baldi, E., 2001 Effects of estrogenic compounds on human spermatozoa: evidence for interaction with a nongenomic receptor for estrogen on human sperm membrane Mol. Cell. Endocrinol. 178, 39-45.

Luconi, M., Francavilla, F., Porazzi, I., Macerola, B., Forti, G., Baldi, E., 2004. Human spermatozoa as a model for studying membrane receptors mediating rapid nongenomic effects of progesterone and estrogens. Steroids 69, 553-559.

Lundqvist, H., Borg, B., Berglund, I., 1989. Androgens impair seawater adaptability in smolting Baltic salmon (*Salmo salar*). Can. J. Zool. 67, 1733-1736.

Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (*Carassius auratus*). Biochim. Biophys. Acta 1490, 145-152.

Madsen, S.S., Mathiesen, A.B., Korsgaard, B., 1997. Effects of 17β-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (Salmo salar). Fish Physiol Biochem 17, 303-312.

Mangelsdorf, D.F., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P, Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835-839.

Mani, S., 2008. Progestin receptor subtypes in the brain: the known and the unknown. Endocrinology 149, 2750-2756.

Marsigliante, S., Barker, S., Jimenez, E., Storelli, C., 2000. Glucocorticoid receptors in the euryhaline teleost *Anguilla anguilla*. Mol. Cel. Endocrinol. 162,193-201.

Martínez, P., Viñas, A.M., Sánchez, L., Díaz, N., Ribas, L., Piferrer, F., 2014. Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. Front. Genet. 5, 340.

Mateos, J., Mananos, E., Martinez-Rodriguez, G., Carrillo, M., Querat, B., Zanuy, S., 2003. Molecular characterization of sea bass gonadotropin subunits (alpha, FSH beta, and LH beta) and their expression during the reproductive cycle. Gen. Comp. Endocrinol. 133, 216-232.

Matsui, I., 1952. Studies on the morphology, ecology and pondculture of the Japanese eel (*Anguilla japonica* Temminck and Schlegel). J Shimonoseki Coll Fish 2, 1-245.

Matthews, J., Gustafsson, J.A., 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol. Interv. 3, 281-292.

Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R., Tveiten, H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature modulates the progression of vitellogenesis in European eel. Aquaculture 434, 38-47.

McCormick, S.D., Naiman, R.J., 1985. Hypoosmoregulation in an anadromous teleost: influence of sex and maturation. J. Exp. Zool. 234, 193-198.

McCormick, 2001. Endocrine Control of Osmoregulation in Teleost Fish. Amer. Zool. 41,781-794.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881-1892.

Menuet, A., Anglade, I., le Guevel, R., Pellegrini, E., Pakdel, F., Kah, O., 2003. Distribution of Aromatase mRNA and protein in the Brain and Pituitary of female Rainbow Trout: Comparison with Estrogen Receptor a. J. Comp. Neurol. 462, 180–193.

Messaouri, H., Baloche, S., Hardy, A., Leloup-Hatey, J., and Burzawa-Gerard, E., 1991. Hepatic 17β-estradiol receptors and vitellogenesis in *Anguilla anguilla* L. after treatment by 17β-estradiol alone or associated with bovine growth hormone. Gen. Comp. Endocrinol. 82, 238.

Methling, C., Tudorache, C., Skov, P.V., Steffensen, J.F. 2011. Pop up satellite tags impair swimming performance and energetics of the European eel (*Anguilla anguilla*). PLoS ONE 6, e20797.

Miao, Y.L., Williams, C.J., 2012. Calcium signaling in mammalian egg activation and embryo development: The influence of subcellular localization. Mol. Reprod. Dev. 79, 742-756.

Minegishi, Y., Aoyama, J., Inoue, J.G., Miya, M., Nishida, M., Tsukamoto, K., 2005. Molecular phylogeny and evolution of the freshwater eels genus *Anguilla* based on the whole mitochondrial genome sequences. Mol Phylogenet Evol 24, 134-146.

Miura, T., Yamauchi, K., Takahashi, H. and Nagahama, Y. 1991a. Involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*). Biomed. Res. 12, 241-248.

Miura, T., Yamauchi, K., Nagahama, Y. and Takahashi, H. 1991b. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci. 8, 63-73.

Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1992. The role of hormones in the acquisition of sperm motility in salmonid fish. J. Exp. Zool. 261, 359-363.

Miura, Y., Kasugai, T., Nagahama, Y., Yamauchi, K., 1995. Acquisition of potential for sperm motility in vitro in Japanese eel *Anguilla japonica*. Fish Sci. 61, 533-534.

Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Com. 264, 230-234.

Miura. T., Miura, C., 2003a Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181-186.

Miura. T., Ohta, T., Miura, C., Yamauchi, K., 2003b. Complementary deoxyribonucleic acid cloning of spermatogonial stem cell renewal factor. Endocrinology 144, 5504-5510.

Miura, T., Higuchi, M., Ozaki, Y., Ohta, T., Miura, C., 2006. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. PNAS 103, 7333-7338.

Miura, C., Miura, T., 2011. Analysis of spermatogenesis using an eel model. Aqua-bioScience Monographs, Vol.4, No.4, pp. 105-129.

Miyazaki, S., Shirakawa, H., Nakada, K., Honda, Y., 1993. Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. Dev. Biol. 158, 62-78.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., Shimada, K., 2009. Phospholipase Cζ mRNA expression and its potency during spermatogenesis for activation of quail oocyte as a sperm factor. Mol. Reprod. Dev. 76, 1200-1207.

Montero, M., Le Belle, N., Vidal, B., Dufour, S., 1996. Primary cultures of dispersed pituitary cells from estradiol-pretreated female silver eels (*Anguilla anguilla* L.): immunocytochemical characterization of gonadotropic cells and stimulation of gonadotropin release. Gen. Comp. Endocrinol. 104, 103-115.

Morini, M., Peñaranda, D.S., Vílchez, M.C., Gallego, V., Nourizadeh-Lillabadi, R., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2015a. Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel. Comp. Bioch. Physiol. A 187, 168-176.

Morini, M., Pasquier, J., Dirks, R., van den Thillart, G., Tomkiewicz, J., Rousseau, K., Dufour, S., Lafont, A.G., 2015b. Duplicated leptin receptors in two species of eel bring new insights into the evolution of the leptin system in vertebrates. PLoS One 10, e0126008.

Morini, M., Peñaranda, D.S., Vílchez, M.C., Nourizadeh-Lillabadi, R., Lafont, A.G., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2016. Nuclear and membrane progestin receptors in the European eel: characterization and expression in vivo through spermatogenesis., submitted.

Nagler, J.J., Cavileer, T.D., Verducci, J.S., Schultz, I.R., Hook, S.E., Hayton, W.L., 2012. Estrogen receptor mRNA expression patterns in

the liver and ovary of female rainbow trout over a complete reproductive cycle. Gen. Comp. Endocrinol. 178, 556-561.

Nelson, J.S., 2006. Fishes of the world, 4th edition. Hoboken: Wiley.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15-24.

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., Gustafsson, J.A., 2001. Mechanisms of estrogen action. Physiol. Rev. 81, 1535-1565.

Norman, A.W., Mizwicki, M.T., Norman, D.P., 2004. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model, Nat. Rev. Drug. Discov. 3, 27-41.

Oba, Y., Hirai, T., Yoshiura, Y., Kobayashi, T., Nagahama, Y., 2001. Fish gonadotropin and thyrotropin receptors: the evolution of glycoprotein hormone receptors in vertebrates. Comp. Bioch. Physiol. B. 129, 441-448.

Ogino, Y., Katoh, H., Kuraku, S., Yamada, G., 2009. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. Endocrinology 150, 5415-5427.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Milt production in the Japanese eel *Anguilla japonica* induced by repeated injections of human chorionic gonadotropin. Fish Sci. 62, 44-49.

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., Hirose, K., 1997. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol. Biochem. 17, 163-169.

Ohta, H., Tanaka, H., 1997b. Relationship between serum levels of human chorionic gonadptropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. Aquaculture 153, 123-134.

Ohta, K., Yamaguchi, S., Yamaguchi. A., Gen, K, Okuzawa, K., Kagawa, H., Matsuyama, M., 2002. Biosynthesis of steroid in ovarian follicles of red sea bream, *Pagrus major* (Sparidae, Teleostei) during final oocyte maturation and the relative effectiveness of steroid

metabolites for germinal vesicle breakdown in vitro. Comp. Biochem. Physiol. B 133, 45-54.

Olde, B., Leeb-Lundberg, L.M.F., 2009. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol. Metab. 20, 409-416.

Oren, I., Fleishma, S.J., Kessel, A., Ben-Tai, N., 2004. Free diffusion of steroid hormones across biomembranes: a simplex search with implicit solvent calculations, Biophys. J. 87, 768-779.

Ozaki, Y., Higuchi, M., Miura, C., Yamaguchi, S., Tozawa, Y., Miura, T., 2006. Roles of 11beta-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology 147, 5139-5146.

Palstra, A., Cohen, E., Niemantsverdriet, P., van Ginneken, V., van den Thillart, G., 2005. Artificial maturation and reproduction of European silver eel: Development of oocytes during final maturation. Gen. Comp. Endocrinol. 249, 533-547.

Panet-Raymond, V., Gottlieb, B., Beitel, L.K., Pinsky, L., Trifiroa, M.A., 2000. Interactions between androgen and estrogen receptors and the effects on their transactivational properties. Mol. Cel. Endocrinol.167, 139-150.

Pang, Y., Dong, J., Thomas, P., 2008. Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. Endocrinology 149, 3410-3426.

Pang, Y., Thomas, P., 2009. Involvement of estradiol-17β and its membrane receptor, G protein coupled receptor 30 (GPR30) in regulation of oocyte maturation in zebrafish, *Danio rario*. Gen. Comp. Endocrinol. 161, 58-61.

Pang, Y., Dong, J., Thomas, P., 2013. Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors δ and ε (mPRδ and mPRε) and mPRδ involvement in neurosteroid inhibition of apoptosis. Endocrinology 154, 283-295.

Pankhurst, N.W., 1982. Relation of visual changes to the onset of sexual maturation in the European eel *Anguilla anguilla* (L.). J. Fish Biol. 21, 127-140.

Parrington, J., Davis, L.C., Galione, A., Wessel, G., 2007. Flipping the switch: How a sperm activates the egg at fertilization. Dev. Dyn. 236, 2027-2038.

Pasqualini, C., Vidal, B., Belle, N.L.E., Sbaihi, M., Weltzien, F.A., Vernier, P., Zohar, Y., Dufour, S., 2004. An antagonist to GnRH in the control of reproduction in teleost fish: dopAminergic inhibition. Ancestral origin and differential conservation within vertebrates. J. Soc. Biol. 198, 61-67.

Pasquier, J., Lafont, A.G., Leprince, J., Vaudry, H., Rousseau, K., Dufour, S., 2011. First evidence for a direct inhibitory effect of kisspeptins on LH expression in the eel, *Anguilla anguilla*. Gen. Comp. Endocrinol. 173, 216-225.

Pasquier, J., Lafont, A.G., Jeng, S.R., Morini, M., Dirks, R., van den Thillart, G., Tomkiewicz, J., Tostivint, H., Chang, C.F., Rousseau, K., Dufour, S., 2012. Multiple kisspeptin receptors in early osteichthyans provide new insights into the evolution of this receptor family. PLoS One 7, e48931.

Pedersen, B.H., 2003. Induced sexual maturation of the European eel *Anguilla anguilla* and fertilisation of the eggs. Aquaculture 224, 323-338.

Pellegrini, E., Menuet, A., Lethimonier, C., Adrio, F., Gueguen, M.M., Tascon, C., Anglade, I., Pakdel, F., Kah, O., 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish, Gen. Comp. Endocrinol. 142, 60-66.

Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F., 2010. Molecular and physiological study of the artificial maturation process in European eel males: from brain to testis. Gen. Comp. Endocrinol. 166, 160-171.

Peñaranda, D.S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J.F., Weltzien, F.A., 2013. Molecular characterization of three GnRH receptors in the European eel, *Anguilla anguilla*: tissue-distribution and changes in transcript abundance during artificially induced sexual development. Mol. Cell. Endocrinol. 369, 1-14.

Peñaranda, D.S., Mazzeo, I., Gallego, V., Hildahl, J., Nourizadeh-Lillabadi, R., Pérez, L., Weltzien, F.A., Asturiano, J.F., 2014. The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel. Reprod. Domest. Anim. 49, 512-521.

Peñaranda, D.S., Morini, M., Tveiten, H., Vílchez, M.C., Gallego, V., Dirks, R.P., van den Thillart, G.E.E.J.M., Pérez, L., Asturiano, J.F., 2016. Temperature modulates the testis steroidogenesis in European eel. Comp. Biochem. Physiol. Part A 197, 58-67.

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

Pérez, L., Barrera, R., Asturiano, J.F., Jover, M., 2004. Producción de anguilas: pasado, presente y futuro. Revista AquaTIC 20, 51-78.

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

Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., van den Thillart, G.E.E.J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol. 174, 51-59.

Pérez, L., Vílchez, M.C., Gallego, V., Mazzeo, I., Peñaranda, D.S., Weltzien, F-A., Dufour, S., Asturiano, J.F., 2012. Trying to reproduce the European eel (*Anguilla anguilla*) under captivity: experiments with females. Domestication in Finfish Aquaculture, Olsztyn (Poland), Octobre 2012. Book of Proceedings, p. 11-15.

Peterson, S.L., Intlekofer, K.A., Moura-Conlon, P.J., Brewer, D.N., DelPinoSans, J., Lopez, J.A., 2013. Novel progesterone receptors: neural localization and possible functions. Front. Neurosci. 7, 164.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially

regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509-515.

Prossnitz, E.R., Maggiolini, M., 2009. Mechanisms of estrogen signaling and gene expression via GPR30. Mol. Cell. Endocrinol. 308, 32-38.

Prunet, P., Sturm, A., Milla, S., 2006. Multiple corticosteroid receptors in Wsh: From old ideas to new concepts. Gen. Comp. Endocrinol. 147, 17-23.

Quérat, B., Nahoul, K., Hardy, A., Fontaine, Y.A., Leloup-Hâtey, J., 1987. Plasma concentrations of ovarian steroids in the freshwater European silver eel (*Anguilla anguilla*): effects of hypophysectomy and transfer to sea water. J. Endocr. 114, 289-294.

Quérat, B., Sellouk, A., Salmon, C., 2000. phylogenetic analysis of the vertebrate glycoprotein hormone family including new sequences of sturgeon (*Acipenser baeri*) b subunits of the two gonadotropins and the thyroid-stimulating hormone. Biol. Reprod. 63, 222-228.

Revelli, A., Massobrio, M., Tesarik, J., 1998. Nongenomic actions of steroid hormones in reproductive tissues. Endocr. Rev. 19, 3-17.

Runft, L.L., Jaffe, L.A., Mehlmann, L.M., 2002. Egg activation at fertilization: where it all begins. Dev. Biol. 245, 237-254.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K., 2006. Effects of temperature on vitellogenesis in Japanese eel *Anguilla japonica*. Fish. Sci. 72, 961-966.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLCζ: A sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. Development 129, 3533-3544.

Saunders, C.M., Swann, K., Lai, F.A., 2007. PLCζ, a sperm-specific PLC and its potential role in fertilization. Biochem. Soc. Symp. 74, 23-36.

Schmitz, M., Aroua, S., Vidal, B., Le Belle, N., Elie, P., Dufour, S., 2005. Differential regulation of luteinizing hormone and follicle-stimulating hormone expression during ovarian development and under sexual steroid feedback in the European eel. Neuroendocrinology 81, 107-119.

Schulz, R.W., 1985. Measurement of five androgens in the blood of immature and maturing male rainbow trout, *Salmo gairdneri* (Richardson). Steroids 46, 717-726.

Schulz, R.W., Vischer, H.F., Cavaco, J.E.B., Santos, E.M. ,Tyler, C.R., Goos, H.J.Th., Bogerd, J. 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Bioch. Physiol B. 129, 407-417.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation Fish Physiol. Bioch. 26, 43-56.

Schulz, R.W., França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390-411.

Scott, A.P., Sumpter, J.P., Stacey, N., 2010. The role of the maturation-inducing steroid, 17,20β-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish Biol. 76, 183-224.

Shi, Y., Liu, X., Zhu, P., Li, J., Sham, K.W.Y., Cheng, S.H., Li, S., Zhang, Y., Cheng, C.H.K., Lin, H., 2013. G-protein-coupled estrogen receptor 1 is involved in brain development during zebrafish (*Danio rerio*) embryogenesis. Biochem. Biophys. Res. Commun. 435, 21-27.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., Mc William, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539. doi: 10.1038/ msb.2011.75.

Sleiter, N., Pang, Y., Park, C., Horton, T.H., Dong, J., Thomas, P., Levine, J.E., 2009. Progesterone receptor A (PRA) and PRB-independent effects of progesterone on gonadotropin-releasing hormone release. Endocrinology 150, 3833-3844.

Smith, J.L., Kupchak, B.R., Garitaonandia, I., Hoang, L.K., Maina, A.S., Regalla, L.M., Lyons, T.J., 2008. Heterologous expression of human mPRα, mPRβ and mPRγ in yeast confirms their ability to function as membrane progesterone receptors. Steroids 73, 1160-1173.

Sreenivasulu, G., Senthilkumaran, B., Sridevi, P., Rajakumar, A., Rasheeda, M.K., 2012. Expression and immunolocalization of 20β-hydroxysteroid dehydrogenase during testicular cycle and after

hCG induction, in vivo in the catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 175, 48-54.

Stamatakis, A., Ott, M., 2008. Efficient computation of the phylogenetic likelihood function on multi-gene alignments and multi-core architectures. Phil. Trans. R. Soc. B 363, 3977-3984.

Staurnes, M., Sigholt, T., Gulseth, O.A., Elliassen, R., 1994. Effects of maturation on seawater tolerance of anadromous Arctic char. Trans. Am. Fish Soc. 123, 402– 407.Stolte, E.H., van Kemenade, B.M.L.V., Savelkoul, H.F.J., Flik, G., 2006. Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. J. Endocrinol. 190, 17-28.

Stolte, E.H., van Kemenade, L.V., Savelkoul, H.F.J., Flik, G., 2006. Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. J. Endocrinol. 190, 1-28

Stricker, S.A., 1999. Comparative biology of calcium signalling during fertilization and egg activation in animals. Dev. Biol. 211, 157-176.

Su, T., Ijiri, S., Kanbara, H., Hagihara, S., Wang, De-S., Adachi, S., 2015. Characterization and expression of cDNAs encoding P450c17-II (*cyp17a2*) in Japanese eel during induced ovarian development. Gen. Comp. Endocrinol. 221, 134-143.

Sudo, R., Tosaka, R., Ijiri, S., Adachi, S., Suetake, H., Suzuki, Y., Horie, N., Tanaka, S., Aoyama, J., Tsukamoto, K., 2011. Effect of temperature decrease on oocyte development, sex steroid, and gonadotropin β-subunit mRNA expression levels in female Japanese eel *Anguilla japonica*. Fish. Sci. 77, 575-582.

Swann, K., Saunders, C.M., Rogers, N.T., Lai, F.A., 2006. PLCζ (zeta): A sperm protein that triggers Ca2+ oscillations and egg activation in mammals. Semin. Cell Dev. Biol. 17, 264-273.

Swann, K., Yu, Y., 2008. The dynamics of calcium oscillations that activate mammalian eggs. Int. J. Dev. Biol. 52, 585-594.

Swann, K., Lai, F.A., 2013. PLCζ and the initiation of Ca2+ oscillations in fertilizing mammalian eggs. Cell Calcium 53, 55-62.

Tan, W., Aizen, J., Thomas, P., 2014. Membrane progestin receptor alpha mediates progestin-induced sperm hypermotility and

increased fertilization success in southern flounder (*Paralichthys lethostigma*). Gen. Com. Endocrinol. 200, 18-26.

Tanaka, M., Telecky, T.M., Fukada, S., Adachi, S., Chen, S., Nagahama, Y. 1992. Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of P450arom mRNA and the production of oestradiol-17 beta in the ovary, J. Mol. Endocrinol. 8, 53-61.

Tanaka, H., Kagawa, H., Ohta, H., 2001. Production of leptocephali of Japanese eel (*Anguilla japonica*) in captivity. Aquaculture 201, 51-60.

Tanaka, H., Kagawa, H., Ohta, H., Unuma, T., Nomura, K., 2003. The first production of glass eel in captivity: fish reproductive physiology facilitates great progress in aquaculture. Fish. Physiol. Biochem. 28, 493-497.

Tang, Y.T., Hu, T., Arterburn, M., Boyle, B., Bright, J.M., Emtage, P.C., Funk, W.D., 2005. PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. J. Mol. Evol. 61, 372-380.

Tarin, J.J., 2000. Fertilization in protozoa and metazoan animals: a comparative overview. In: Tarin, J.J., Cano, A. (Eds.), Biochemistry and Molecular Biology of Fishes, Cellular and Molecular Aspects, Springer, Berlin Heidelberg, pp 277-314.

Tesch, F.W., 1978. Telemetric observations on the spawning migration of the eel (*Anguilla anguilla*) west of the European continental shelf. Environ. Biol. Fishes 3, 203-209.

Tesch, F.W., 1989. Changes in swimming depth and direction of silver eels (*Anguilla anguilla* L.) from the continental shelf to the deep sea. Aquat. Living Resour. 2, 9-20.

Tesch, F., 2003. The Eel. Blackwell, Oxford, pp. 1-408.

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

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., Tubbs, C., 2007. Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology 148, 705-718.

Thomas, P., Pang, Y., 2012. Membrane progesterone receptors (mPRs): evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. Neuroendocrinology 96, 162-171.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Thornton, J.W., 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. PNAS 98, 5671-5676.

Todo, T., Ikeuchi, T., Kobayashi, T., Nagahama, Y., 1999. Fish Androgen Receptor: cDNA Cloning, Steroid Activation of Transcription in Transfected Mammalian Cells, and Tissue mRNA Levels. Biochem. Biophys. Res. Commun. 254, 378-383.

Todo, T., Ikeuchi, T., Kobayashi, T., Kajiura-Kobayashi, H., Suzuki, K., Yoshikuni, M., Yamauchi, K., Nagahama, Y., 2000. Characterization of a testicular 17a,20bdihydroxy- 4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*), FEBS Lett. 465, 12-17.

Tokarz, J., Möller, G., Hrabê de Angelis, M., Adamski, J., 2013. Zebrafish and steroids: What do we know and what do we need to know? J. Steroid Biochem. Mol. Biol. 137, 165-173.

Trant, J.M., Thomas, P., 1989. Isolation of a novel maturation-inducing steroid produced in vitro by ovaries of Atlantic croaker. Gen. Comp. Endocrinol. 75, 397-404.

Trant, J.M., Gavasso, S., Ackers, J., Chung, B.C., Place, A.R. 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J. Exp. Zool. 290, 475-483.

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

Tubbs, C., Pace, M., Thomas, P., 2010. Expression and gonadotropin regulation of membrane progestin receptor alpha in Atlantic croaker (*Micropogonias undulatus*) gonads: Role in gamete maturation. Gen. Comp. Endocrinol. 165, 144-154.

Tsutsui, K., Bentley, G.E., Ubuka, T., Saigoh, E., Yin, H., Osugi, T., Inoue, K., Chowdhury, V.S., Ukena, K., Ciccone, N., Sharp, P.J., Wingfield, J.C., 2007. The general and comparative biology of gonadotropin-inhibitory hormone (GnIH). Gen. Comp. Endocrinol. 153, 365-370.

Tveiten, H., Frantzen, M., Scott, A.M., Scott, A.P., 2010. Synthesis of 17, 20beta, 21-trihydroxypregn-4-en-3-one by ovaries of reproductively mature Atlantic cod *Gadus morhua*. J. Fish Biol. 77, 33-53.

Ueda, H., Kanbegawa, A., Nagahama, Y., 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 59, 24-30.

Valle, L.D., RAmina, A., Vianello, S., Belvedere, P., Colombo, L. 2002. Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum), J. Steroid Biochem. Mol. Biol. 82, 19-32.

Van den Thillart, G., Dufour, S., 2009. How to estimate the reproductive success of European silver eel. In: van den Thillart G., Dufour, S., Rankin, J. C. (Eds.). Spawning migration of the European eel: Reproduction index, a Useful Tool for Conservation Management: Fish & Fisheries Series, pp. 4-9.

Van de Peer, Y., Maere, S., Meyer, A., 2009. The evolutionary significance of ancient genome duplications. Nat. Rev. Genet. 10, 725-732.

Van Der Kraak, G., Pankhurst, N.W., 1996. Temperature effect on the reproductive performance of fish. In: Wood, C.M., McDonald, D.G. (Eds), Global warming: implications for freshwater and Marine Fish, Cambridge University Press, Cambridge, pp. 159-176.

Van Ginneken, G. Maes, 2005. The European eel (*Anguilla anguilla* Linnaeus), its Lifecycle, Evolution and Reproduction: A Literature Review. Rev. Fish. Biol. Fisheries 15, 367-398.

Van Nes, S., Moe, M., Anderse, O. 2005. Molecular characterization and expression of two cyp19 (P450 aromatase) genes in embryos, larvae, and adults of Atlantic halibut (*Hippoglossus hippoglossus*). Mol. Reprod. Dev. 72, 437-449.

Viñas, J., Piferrer, F., 2008. stage-specific gene expression during fish spermatogenesis as determined by laser-capture microdissection and quantitative-pcr in Sea bass (*Dicentrarchus labrax*) gonads Biol. Reprod. 79, 738-747.

Vidal, B., Pasqualini, C., Le Belle, N., Holland, M.C.H., Sbaihi, M., Vernier, P., Zohar, Y., Dufour, S., 2004. DopAmine inhibits luteinizing hormone synthesis and release in the juvenile European eel: neuroendocrine lock for the onset of puberty. Biol. Reprod. 71, 1491-1500.

Wang, J., Liu, X., Wang, H., Wu,T ., Hu, X., Qin, F., Wang, Z., 2010. Expression of two cytochrome P450 aromatase genes is regulated by endocrine disrupting chemicals in rare minnow Gobiocypris rarus juveniles. Comp. Biochem. Physiol. C. 152, 313-320.

Watson, C.S., Gametchu, B., 1999. Membrane-initiated steroid actions and the proteins that mediate them, Proc. Soc. Exp. Biol. Med. 220, 9-19.

Weltzien, F.A., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. Gen. Comp. Endocrinol. 142, 134-142.

Weltzien, F.A., Pasqualini, C., Sébert, M.E., Vidal, B., Le Belle, N., Kah, O., Vernier, P., Dufour, S., 2006. Androgen-dependent stimulation of brain dopAminergic systems in the female European eel (*Anguilla anguilla*), Endocrinology 147, 2964-2973.

Whitaker, M., 2006. Calcium at fertilization and in early development. Physiol. Rev. 86, 25-88.

Williams, S., Sigler, P., 1998. Atomic structure of progesterone complexed with its receptor. Nature 393, 392-396.

Yamamoto, K., Yamauchi, K., 1974. Sexual maturation of Japanese eel and production of eel larvae in the aquarium. Nature 251, 220-222.

Yeh, S., Miyamoto, H., Shima, H., Chang, C., 1998. From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc. Natl. Acad. Sci. USA 95, 5527-5532.

Yoda, A., Oda, S., Shikano, T., Kouchi, Z., Awaji, T., Shirakawa, H., Kinoshita, K., Miyazaki, S., 2004. Ca2+ oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. Dev. Biol. 268, 245-257.

Yoneda, A., Kashima, M., Yoshida, S., Terada, K., Nakagawa, S., Sakamoto, A., Hayakawa, K., Suzuki, K., Ueda, J., Watanabe, T., 2006. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Cζ. Reproduction 132, 393-401.

Yoon, S.Y., Jellerette, T., Salicioni, A.M., Lee, H.C., Yoo, M.S., Coward, K., Parrington, J., Grow, D., Cibelli, J.B., Visconti, P.E., Mager, J., Fissore, R.A., 2008. Human sperm devoid of PLC, zeta 1 fail to induce Ca2+ release and are unable to initiate the first step of embryo development. J. Clin. Invest. 118, 3671-3681.

Yoshida, N., Amanai, M., Fukui, T., Kajikawa, E., Brahmajosyula, M., Iwahori, A., Nakano, Y., Shoji, S., Diebold, J., Hessel, H., Huss, R, Perry, A.C.F., 2007. Broad, ectopic expression of the sperm protein PLCZ1 induces parthenogenesis and ovarian tumours in mice. Development 134, 3941-3952.

Yoshiura, Y., Suetake, H., Aida, K., 1999. Duality of gonadotropin in a primitive teleost, Japanese eel (*Anguilla japonica*). Gen. Comp. Endocrinol. 114, 121-131.

Young, C., Grasa, P., Coward, K., Davis, L.C., Parrington, J., 2009. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. Fertil. Steril. 91, 2230-2242.

Young, G., Lokman, P.M., Kusakabe, M., Nakamura, I., Goetz, F.W., 2005. Hormones and their Receptors in Fish Reproduction. 1st ed. World Scientific Press, Singapore.

Yu, Y., Halet, G., Lai, F.A., Swann, K., 2008. Regulation of diacylglycerol production and protein kinase C stimulation during sperm- and PLCζ-mediated mouse egg activation. Biol. Cell. 100, 633-643.

Zhang, X., Hecker, M., Park, J.W., Tompsett, A.R., Newsted, J., Nakayama, K., Jones, P.D., Au, D., Kong, R., Wu, R.S., Giesy, J.P. 2008. Real-time PCR array to study effects of chemicals on the hypothalamic–pituitary–gonadal axis of the Japanese medaka. Aquat. Toxicol. 88, 173-182.

Zhang, Y., Zhang, W., Zhang, L., Zhu, T., Tian, J., Li, X., Lin, H. 2004. Two distinct cytochrome P450 aromatases in the orange-spotted grouper (*Epinephelus coioides*): cDNA cloning and differential mRNA expression, J. Steroid Biochem. Mol. Biol. 92, 39-50.

Zhang, Y., Zhang, W., Yang, H., Zhou, W., Hu, C., Zhang, L. 2008. Two cytochrome P450 aromatase genes in the hermaphrodite ricefield eel, *Monopterus albus*: mRNA expression during ovarian development and sex change, J. Endocrinol. 199, 317-331.

Zhou, L.Y., Wang, D.S., Shibata, Y., Paul-Prasanth, B., Suzuki, A., Nagahama, Y., 2007. Characterization, expression and transcriptional regulation of P450c17-I and -II in the medaka *Oryzias latipes*. Biochem. Biophys. Res. Commun. 26, 619-625.

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P., 2003a. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. USA. 100, 2231-2236.

Zhu, Y., Bond, J., Thomas, P., 2003b. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. USA. 100, 2237-2242.

Zhu, Y., Liu, D., Shaner, Z.C., Chen, S., Hong, W., Stellwag, E.G., 2015. Nuclear progestin receptor (Pgr) knockouts in zebrafish demonstrate role for Pgr in ovulation but not in rapid non-genomic steroid mediated meiosis resumption. Front. Endocrinol. 6, 37.

Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438-455.