Departamento de Ciencia Animal

Effect of thermal regime on the expression of key reproductive genes during hormonally-induced vitellogenesis in female European eels

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RESUMEN

La población de anguilla europea (*Anguilla anguilla* L., 1758) está sufriendo una drástica reducción. A su vez se trata de una especie muy apreciada por el mercado y su ciclo reproductivo no se ha podido cerrar puesto que esta especie no madura en cautividad. Por estas razones, hasta el momento, la producción global de anguila recae sobre las poblaciones naturales. Todos estos factores hacen urgente el cierre del ciclo productivo y para ello es necesario comprender la fisiología reproductiva y las razones de su imposibilidad de madurar en cautividad.

Con la presente tesis, se pretende aportar mayores conocimientos sobre la fisiología reproductiva en hembras de anguila europea maduradas hormonalmente

Para alcanzar este objetivo, se analizó la expresión de los genes: *cyp19a1*, *ara*, *arb*, *gnrhr1a*, *gnrhr1b*, *gnrhr2*, *zpb* and *zpc*, en animales bajo el regimen térmico comunemente utilizado. Por último se quiso evaluar el efecto de la temperatura sobre la vitelogenesis tanto sobre la expresión génica como en el perfil de esteroides (T, 11-KT and E2). De hecho, la anguila europea cuando migra al mar de los Sargazos para reproducirse experimenta un regimen térmico desde temperaturas más bajas a más altas. Tradicionalmente en acuicultura las anguilas se han madurado a temperatura constante y relativamente alta lo que podría afectar a la vitelogenesis y la calidad final de los oocitos.

Para este estudio fue necesario la clonación y caracterización de nuevos genes. Su expresion genica fue estudiada por qPCR tras el diseño de los primers. El perfil esteroidogénico fue analizado por immunoensayos y los estados de desarrollo gonadal se calsificaron histológicamente.

Como primer resultado se obtuvieron la secuencia para 6 genes en anguila europea. El análisis de la expresión génica permitió comprender la función de algunos de los genes estudiados a lo largo de la vitelogénesis (*arb*, *gnrhr1b* and *gnrhr2*) en diferentes regiones del cerebro.

La temperatura se confirmó como un factor crucial durante la vitelogénesis. Por un lado, las anguilas sometidas a bajas temperaturas al inicio de la vitelogenésis mostraron mejores parámetros reproductivos, los cuales podrían tener un efecto en la calidad final de los oocitos. Por otra parte, temperaturas más altas son indispensables para alcanzar los estadios más avanzados de la vitelogenesis.

ABSTRACT

European eel (*Anguilla anguilla,* L., 1758) is suffering a strong population decrease and at the same time it is a very appreciated species and by now it has not been possible closing its cycle life. In fact, this species does not mature in captivity unless hormonally induced. So all the production is up to the natural population. All these factors together make urgent achieving the closing of the productive cycle and for this aim it is important to understand the reproductive physiology and the reasons of this development blockage.

The present thesis wants to be a new contribution to the knowledge of reproductive physiology in female European eel submitted at hormonal treatment. To achieve this goal, expression of genes not previously studied in this species (*cyp19a1*, *ara*, *arb*, *gnrhr1a*, *gnrhr1b*, *gnrhr2*, *zpb* and *zpc)* was analyzed in eels reared under a constant thermal regime, accordingly to the usual rearing conditions. Also, the effect of rearing temperature on gene

expression and steroid profile (T, 11-KT and E2) was studied. In fact, eels migrate to Sargasso Sea to reproduce and during the travel experiment temperature changes, while traditionally they are reared at a constant high temperature which could affect vitellogenesis progression and final oocyte quality.

For the study it was necessary cloning and characterizing some genes which have not still been sequenced in European eel. Gene expression was studied by qPCR after designing primer and optimizing the qPCR race. Steroid profiles were analyzed by immunoassays and the gonadal development stages were established by histology.

The first result obtained at the end of the study were six new genes characterized in European eel.
The analysis of gene expression allowed to understand the involvement of specific genes during vitellogenesis (*arb*, *gnrhr1b* and *gnrhr2*) in different brain regions.
The temperature was conformed as a crucial environmental factor affecting vitellogenesis. On one hand, eels matured at lower starting temperatures showed better reproductive parameters which could have an influence in the final oocyte quality. On the other hand higher temperatures are necessary to achieve further vitellogenetic stages.

RESUM

La població d'anguila europea (*Anguilla anguilla* L, 1758) està sofrint una dràstica reducció: A la vegada que es tracta d'una espècie molt apreciada pel mercat i a més el seu cicle reproductiu no s'ha pogut tancar ja què aquesta espècie que no madura en captivitat. Donat aquestes circumstàncies, fins ara, la producció global d'anguila es depenent de les poblacions naturals. Per tant es

urgent el tancament del seu cicle reproductiu i per a aconseguir-ho es necessari comprendre la fisiologia reproductiva i la causes de la seua impossibilitat de madurar en captivitat.

Amb aquesta tesis, es pretendeix aportar majors coneixements de la fisiologia reproductiva en femelles d'anguila madurades hormonalment.
Per assolir aquest objectiu, es va analitzar l'expressió dels gens: *cyp19a1*, *ara*, *arb*, *gnrhr1a*, *gnrhr1b*, *gnrhr2*, *zpb* and *zpc*, en animals baix el règim tèrmic comunment utilitzat. A més del perfil d'esteroids (T, 11-KT and E2) durant la vitel.logenesis. Per últim es va voler evaluar l'efecte de la temperatura sobre la vitel.logenesis tant en l'expressió génica com el perfil d'esteroids. De fet, l'anguila europea quan migra al mar dels Sargassos per a reproduir-se experimenta un règim tèrmic des de temperatures més baixes a més altes. Tradicionalment en acuicultura les anguiles s'han madurat a temperatura constant i relativament alta, el que podria tindre un efecte sobre la vite.logènesis i la qualitat final dels oocits.

Per a la realització d'aquest estudi va ser necessari la clonació i caracterització de nous gens. La seua expressió gènica va ser estudiada per qPCR tras el diseny dels primers. El perfil esteroidogènic s'analitzà per inmunoassaig i els estadis de desenvolupament es classificaren histològicament.

Com primer resultat es va obtindre la sequència per a 6 gens en anguila europea. L'analisis de l'expressió gènica va permetre comprendre la funció d'alguns dels gens estudiats al llarg de la vitel.logenesis (*arb*, *gnrhr1b* and *gnrhr2*) en les diferents parts del cerbell.
La temperatura es va confirmar com un factor crucial durant la vitel.logenesis. Per una banda, les anguiles sotmeses a temperatures baixes a l'inici de la vitel.logenesis van mostrar millors paràmetres reproductius, els quals podrien tindre un efecte sobre la

qualitat final dels oocits. Per altra banda, temperatures més altes són indispensables per assolir estadis finals de la vitel.logenesis.

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ABBREVIATIONS

11-KT	11-ketotestosterone
17αHP	17α-hydroxyprogesterone
AR	Androgen receptor
CPE	Carp pituitary extract
DA	Dopamine
DHP	17α,20β-dihydroxy-4-pregnen-3-one
E2	17β-estradiol
EI	Eye index
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin release hormone
GnRH-R	GnRH receptor
GSI	Gonado somatic index
GTH	Gonadotropin hormone
hCG	Human chorionic gonadotropin
LH	Luteinizing hormone
P450arom	Aromatase
qPCR	Quantitative Polymerase Chain Reaction
T	Testosterone
TH	Tyrosine hydroxylase
VTG	Vitellogenin
ZP	Zona pellucida

INTRODUCTION

European eel (*Anguilla anguilla* L., 1758) belongs to Elopomorpha superorder, being one of the most ancient teleost. This catadromous and semelparous species displays a complex and not yet totally clarified life cycle. First details on European eel reproduction were provided by Schmidt at the beginning of the 20th century (Schmidt 1912, 1922, 1925), as result of his observations of larvae at the supposed spawning site. European eels spend most of their life in continental water, principally in freshwater, altough nomadic eels can be found also in brackish or saline water (Panfili et al., 2012; Capoccioni et al., 2014) while spawning occurs in the Sargasso Sea. Once hatched, eel larvae, called leptocephali, reach continental waters helped by Gulf Current. However, many details on larvae oceanic life and migration, such as its duration (Bonhommeau et al., 2010), are unknown.

Due to the different morphology that larvae present with respect to adults, it was only at the end of 19th century that Grassi (1896) realized that the fish until the moment known as *Leptocephalus brevirostris* was the eel larval stage. Once reached the continental water, leptocephali suffer a metamorphosis into glass eels, the name they receive as long as they are unpigmented. Once pigmentation has started they are called elvers, and after reaching 30 cm length and pigmentation is complete they are referred to as yellow eels (Tesch, 2003). In continental waters, eels live a stationary and feeding phase whose duration varies according to the habitat and the growth conditions. The variability is higher in females than in males. Males can start migration around 35 cm length (2-15 years) while females, which have to face a higher energy demand considering the cost of vitellogenesis, have to grow more and become mature between 50 and 100 cm, around 4-20 years of age (Tesch, 2003; Durif et al., 2009a). The average age and length of silver eels leaving the Albufera lagoon (Valencia,

Spain) to reach the spawning area was estimated in approximately 8 years and 73 cm (Asturiano et al., 2011).

The end of the stationary phase matches with silvering, a puberty related event which marks the beginning of sexual maturation, migration and reproductive phase (Dufour et al., 2003; Aroua et al., 2005).

Migration starts under the effect of triggering factors, including moon-phase or atmospheric conditions as a decrease in temperature and daylight length after summer or an increase in water discharges (Tesch, 2003; Bruijs and Durif, 2009).

The migration to Sargasso Sea (5,000-6,000 km) lasts approximately 6-7 months and eels fast during whole migration (Tesch, 2003; van Ginneken and Maes, 2005). Their digestive tract is partially reabsorbed and at the end of the maturation the abdominal cavity is totally occupied by the gonads (Fig. 1).

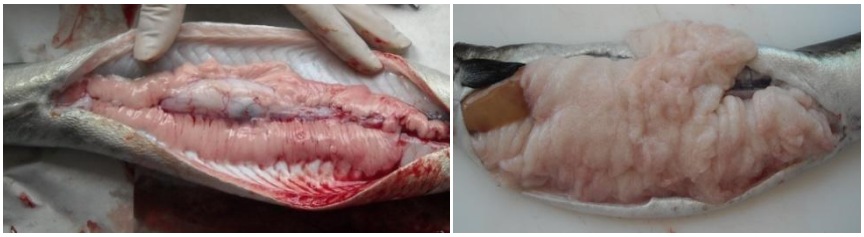

Fig. 1. Undeveloped (A) and developed (B) ovary in European eel before and after the hormonal treatment.

Apparently, eel migration to Sargasso Sea is performed in the upper 500 m of the open ocean, swimming deeper at daytime than at nighttime (Tesch, 1989; Aarestrup et al., 2009). However, to discover more details on eel migration will be necessary further research (van Ginneken and Maes, 2005).

In European eel, as in all the teleosts, the control of reproduction by the hypothalamus-pituitary-gonads axis is well established

(Nagahama, 1994). Vitellogenesis is a complex mechanism which involves environmental stimuli and chemical and hormonal signals which are crucial for the proper egg growth and embryo development (Nagahama, 1994; Brooks et al., 1997; Polzonetti-Magni et al., 2004).

The organs involved in vitellogenesis are brain, pituitary, ovary and liver. The hypothalamus is responsible for the perception of external signals and the release of GnRHs which act on the pituitary gland (Amoss et al., 1971; Matsuo et al., 1971). In European eel, two different types of GnRHs are present (King et al., 1990) and their action is mediated by the binding to G-protein coupled receptors.

The presence of several forms of GnRHs in a single species, acting in multiple tissues, is often associated with the existence of different receptor subtypes. Some species have been shown to encode up to five *gnrhr*s in their genome (Ikemoto and Park, 2005; Moncaut et al., 2005) but the cell/tissue distribution, regulation and function of the various GnRH-Rs are still not clear. Characterization of the spatial and temporal GnRH-R variant gene expression, both at central and peripheral level, would be an important step to improve the knowledge of the physiological consequences of GnRH stimulation.

Although GnRH action is extended also in extra pituitary sites, its principal function is the release of GTHs, even if this effect may partially depend on specific physiological conditions (Marchant et al., 1989; Le Gac et al., 1993; Lin et al., 1993; Blaise et al., 1995, 1997; Melamed et al., 1995, 1998). GTHs are heterodimeric glycoproteins with a common α subunit and different β subunits (FSH and LH specific). Both β subunits and the common α subunits have been cloned in European eel (Quérat et al., 1990a, b; Schmitz et al., 2005).

The release of gonadotropic hormones in European eel and other teleost fish is subjected to a double neuroendocrine control: a

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

DA synthesis depends on TH; Weltzien et al. (2005a,b) analyzed TH expression in the different parts of the European eel brain, finding the highest expression in the olfactory bulb, followed by the di-/mesencephalic area and the telencephalon/preoptic area. TH synthesis was affected by some steroids, like T, that provided a positive effect, but not by E2 (Weltzien et al., 2005a, 2006).

In female eels, FSHβ was found in high levels in immature previtellogenic eels, while LH could be found at the vitellogenic stage and germinal vesicle migration stage (Nagae et al., 1996; Yoshiura et al., 1999; Degani et al., 2003). However, some researchers indicated that both GTHs are present in immature previtellogenic eels (Aroua et al., 2005; Schmitz et al., 2005). Also, the timing in the expression of FSH and LH receptors which mediate the action of GTHs on the gonad show a similar difference, since in previtellogenic eels the expression of the FSH receptor is really much higher than that of LH receptor (Jeng et al., 2007).

The action of GTHs on the ovary brings to the synthesis of steroids involved in vitellogenesis and maturation, in particular the synthesis of E2 by aromatization of androgens. On its hand, E2 reaches the liver and stimulates the synthesis of VTG which from the liver, by blood stream, is transferred to the ovary and enters the oocytes by binding to specific receptors (Lubzens et al., 2010).

Responsible for the androgen aromatization is the enzyme P450 aromatase, a member of the P450 cytochrome superfamily. In teleosts, two paralogous genes of P450arom have been found, *cyp19a1b,* mainly expressed in the brain and *cyp19a1a*, mainly expressed in the gonad (Chang et al., 1997a; Gelinas et al., 1998;

Kishida and Callard, 2001; Kwon et al., 2001; Blázquez and Piferrer, 2004; Choi et al., 2005). In European and Japanese eels, however, only one aromatase cDNA has been identified (termed *cyp19a1*), and is expressed in ovary, brain and pituitary (Ijiri et al., 2003; Tzchori et al., 2004).
Apart from estrogens, it was demonstrated that also steroids traditionally considered more involved in male physiology, play an important role in female eel maturation, as 11-KT which promotes the previtellogenic ovarian growth (Rohr et al., 2001; Matsubara et al., 2003a,b, 2005; Lokman et al., 2007; Kazeto et al., 2011; Setiawan et al., 2012).

The effect of the androgens can be also mediated by binding to ARs. In teleosts, two ARs have been identified and their expression has been demonstrated in a wide range of tissues in different fish species (Todo et al., 1999; Blázquez and Piferrer, 2005). Both AR genes are expressed in Japanese eel (*A. japonica*; Ikeuchi et al., 1999; Todo et al., 1999), but no significant differences in their androgen binding capacity have been detected. In eel, both ARs are activated by 11-KT and by 5α-dihydrotestosterone (Ikeuchi et al., 2001).

The gonadal steroids are essential for the teleost reproduction and they are able to affect practically all tissues. Both in testis and ovary, the synthesis of steroids is performed in somatic cells. Thecal and granulosa cells are the responsible of the steroid production in the ovary (Fostier et al., 1983; Kagawa et al., 1984; Nagahama, 1994). Taking as basis the salmonid model, the thecal cells produce T during vitellogenic process or 17αHP during the oocyte maturation, while the granulosa cells use these steroids to produce E2 and DHP.
In the testis, the steroid production is localized mainly in the Leydig cells. Some evidences indicate that Sertoli cells could be implicated

in steroid synthesis, especially progestins as DHP (Nagahama, 1983).

In eel, the study of the effect of gonadal steroids on reproduction is difficult because hormonal injections are necessary to obtain the gonadal development. In female European eel, it was demonstrated that E2 has a major effect *in vivo* on LH content (Dufour et al., 1983; 2003; Montero et al., 1995). Conversely, *in vitro* treatment with E2 was not effective, suggesting that the positive feedback found *in vivo* is mediated by indirect actions (Huang et al., 1997; Dufour et al., 2003). Unlikely LH, FSH β subunit mRNA decrease during induced sexual maturation suggests a negative feedback on its expression, but further studies are necessary (Dufour et al., 2003).

This physiological cascade of signals has to bring to the production of gametes. While in male eels good quality sperm is obtained (Ohta et al., 1997; Lokman and Young, 2000; Asturiano et al., 2002, 2005; Palstra et al., 2005; Peñaranda et al., 2010), the quality of the eggs and, as consequence, of the embryos, is often very low (Ohta et al., 1996; Lokman and Young, 2000; Pedersen, 2003).

Fish oocytes are surrounded by the chorion, an extracellular protein coating present in all the vertebrates (Spargo and Hope, 2003). Proteins forming the inner layer of the chorion are closely related to mammalian ZP proteins and play a structural role as well as presenting bactericidal properties and supplying a mechanical protection for the oocyte and the developing embryo (Litscher and Wassarman, 2007). Following fertilization, ZP proteins are involved in chorion hardening and polyspermy prevention (Murata, 2003; Modig et al., 2007). For all these reasons, although no direct studies have been realized on ZP proteins absence or mutation in teleosts, a ZP impairment or a no functional ZP can seriously compromise reproductive success.

In the last 20 years, eel populations have suffered a very sharp decrease, as evidenced by a decrease in glass eel captures. In the mid '80s, the number of new glass eels entering the rivers declined until reaching 10% of former levels, and later it has dropped to 1% (Dekker, 2003). It will be translated in the next years in a decline in adult stocks, as there is no way to increase eel juvenile number in European freshwater (ICES, 2006).

Many causes have led to the actual situation, such as climate and oceanic changes, exploitation, habitat loss, migration barriers and pathological agents (Feunteun, 2002; ICES, 2006; Friedland et al., 2007; Bonhommeau et al., 2008). The pathogen which mostly poses a threat to European eel is the nematode *Anguillicoloides crassus.* It was introduced in Europe in the '80s and parasitizes the swimming bladder, affecting swimming performance (Palstra et al., 2007a), inducing an increase in spleen weight (Lefebvre et al., 2004; Neto et al., 2010) and making eels more vulnerable in the presence of stressor agents (Kirk, 2003).

In spite of being listed as a critically endangered species by IUCN in 2010 and being subjected to EU Council Regulation EC No 1100/2007, the natural stock of European eel is exploited by fishery as a high demand for eels exists by both European and Asian markets. However, according to ICES (2006) European eel fishery is not sustainable and for this reason recovery plans are established in European country, including Spain (Informe post-evaluación de los planes de gestión de la anguila europea de España, 2012). By now, fattening glass eels in fish factories is the only way to produce eels in captivity. In fact, alternatives for eel production are impossible because European eel, such as all other Anguillid species, does not mature in captivity. The reason why eels remain at prepubertal stage if oceanic migration is prevented have to be sought in an insufficient production of pituitary GTHs and a strong dopaminergic inhibition (Dufour et al., 2003; Vidal et al., 2004; Weltzien et al., 2009). To bypass the lack of natural maturation in captive eels,

since the ´30s attempts have been made to achieve artificial maturation. First results came from males. Spermatogenesis and spermiation were obtained by Fontaine by intraperitoneal injections of urine extract from pregnant women. Later, it was known that its maturation effect was produced by hCG here contained (Fontaine, 1936; Dufour et al., 2003). In females, ovarian development was obtained in the '60s, again by Fontaine, by administering CPE, rich in LH-like gonadotropin (Fontaine et al., 1964; Dufour et al., 2003). Since then, fish (carp or salmon) pituitary extract and hCG are currently used to obtain maturation in *Anguilla* spp in both sexes, with better results in males than in females (Ohta et al., 1997; Lokman and Young, 2000; Asturiano et al., 2002, 2005; Palstra et al., 2005; Pérez et al., 2008; Peñaranda et al., 2010). Recently, in males, it was demonstrated that stimulation by recombinant hCG produces better results in term of sperm production than traditional treatment with purified hCG (Gallego et al., 2012).

The current protocols for artificial maturation of female eels provide low gamete quality. Possible causes for this could be an inadequate nutritional status prior to maturation, not totally adequate maturation techniques, an incorrect lipid accumulation, egg membrane malformations, a high variability in the progression of oocyte maturation or plasma hormone levels and high contaminant accumulation (Adachi et al., 2003; Seoka et al., 2003; Pedersen, 2004; Kagawa et al., 2005; Palstra et al., 2006; Horie et al., 2008).

In order to improve the gamete quality and to reduce the hormonal treatment, studies have been done to increase the knowledge on the environmental factors which can enhance vitellogenesis. Among them, the ones which have received more attention have been pressure, swimming and temperature.

Effects of pressure on eels have been studied keeping eels in a cage at 450 m depth in the Mediterranean Sea, demonstrating a positive effect on LH content (Dufour and Fontaine, 1985; Fontaine et al.,

1985). Moreover, maintaining female eels at higher water pressure resulted in a positive effect on steroidogenesis and an increase in VTG content (Sébert et al., 2007).

Swimming stimulates fat deposition in oocyte, as it was evidenced by histology and GSI and EI increase (Palstra et al., 2007b; van Ginneken et al., 2007). However, yolk globules have not been observed in these experiments, suggesting that swimming only triggers lipid mobilization which is depressed in yellow eels at prepubertal stage (Palstra et al., 2009). Afterwards, it was found out that swimming stimulates fat deposition in the oocyte and at the same time suppresses vitellogenesis (Palstra et al., 2010). These results suggest that in nature the 2 events - primary oocyte growth (characterized by lipid accumulation in oocytes) and vitellogenesis - are separated. This temporal division implies that final sexual maturation progresses near or at the spawning site. If swimming somehow inhibits vitellogenesis, a different effect was found for spermatogenesis since swimming results in natural full maturation in males (Palstra et al., 2008).

The effect of temperature in eel migration is not totally clear. The range of temperature during which downstream migration occurs is variable, although it takes place in autumn and winter when temperatures decreases (Haro, 1991; Bruijs and Durif, 2009). Temperature, together with salinity, is also supposed to be an orientation cue and to act as landmark to cause the cessation of swimming (Tsukamoto, 2009; Bonhommeau et al., 2010). The exact localization of the spawning site is unknown. In fact, as no adult European eels have been fished in the Sargasso Sea, all data on spawning site position are deduced by the presence of eel larvae and extrapolated by their size (Kleckner and McCleave, 1988; van Ginneken and Maes, 2005).

Spawning on Sargasso Sea occurs at around 23 ºC (Friedland et al., 2007) and experiments carried out with Japanese eel indicate that in that species the optimal temperature for final maturation, ovulation and spawning behavior is 20-22 ºC (Dou et al., 2008; Unuma et al., 2012).

Field studies on eel migration provided evidences that eels, including European eel, performed dial vertical migrations (Tesch, 1989; Aarestrup et al., 2009; Jellyman and Tsukamoto, 2005; 2010). European eel apparently swims in less deep and warmer waters at nighttime (means of 282 m and 11.7 °C), while at dawn it descends to deeper and colder waters (means of 564 m and 7-10 °C) (Aarestrup et al., 2009). The reasons for these vertical displacements in Anguillid spp. are not totally clear, but it is supposed to be aimed to predator avoidance and thermoregulation. Authors suggest that, on one hand, ascending at nighttime helps to meet higher temperature and to maintain swimming activity, while on the other hand, descending to deeper water during the day helps to avoid predation and to maintain temperature below 11 ºC, delaying sexual maturation (Jellyman and Tsukamoto, 2005; Aarestrup et al., 2009). However, several authors have expressed doubts about the full validity of the data collected by satellite tags by their possible negative effect on swimming performance and energetics, hence further studies on eel migration are necessary (Burgerhout et al., 2011; Methling et al., 2011).

Experimental studies have been performed to evaluate the temperature effect on eel maturation and its physiology, in attempt to improve artificial maturation success by modifying rearing conditions. In eel, as in teleost species, development progression depends on temperature. In Japanese eel, vitellogenesis is faster at 20 than at 10 ºC (Sato et al., 2006), while in males no maturation was observed below 10 ºC (Boëtius and Boëtius, 1967). Sudo et al. (2011) studied the effects of temperature decrease (aimed to simulate the temperature descent at fall) on early ovarian

development. They compared eels under decreasing temperatures with eels reared at 20 ºC. In the decreasing temperature group, early ovarian development was stimulated as evidenced by the increase in GSI, oocyte diameter and oil droplet number, and by the higher 11-KT plasma level. In fact, 11-KT plays a role in early ovarian development and lipid accumulation in oocyte and it is probably responsible for the changes recorded in the decreasing temperature group (Rohr et al., 2001; Matsubara et al., 2003b; Lokman et al., 2007; Sudo et al., 2012). On the other hand, in the decreasing temperature group low values of FSHβ and LHβ mRNA were registered, indicating that low temperatures inhibit vitellogenesis (Sudo et al., 2011). As already seen with swimming activity, also these results suggest a temporal separation between primary oocyte growth and vitellogenesis.

Studies on temperature effect during vitellogenesis have been realized in European eel by Pérez et al. (2011). Eels maintained at constant temperature were compared with other under an increasing thermal regime and results suggested that lower temperatures enhance the first steps of ovarian maturation and that a gradual temperature increase brings to GTH profiles more similar to the ones found in wild females of New Zealand longfin eel (*A. dieffenbachii,* Gray, 1842*).* Nevertheless, eels maintained at constant and higher temperatures reached a more advanced developmental stage at the end of the treatment. Hence, further studies are necessary to improve the maturation protocol and define the best condition to combine gamete production and quality and reasonable stimulation duration and cost.

OBJECTIVES

The present thesis wants to be a contribution to the knowledge on reproductive physiology in European eel. Due to the necessity to breed eel in captivity, increasing the knowledge on the physiological mechanism bringing to sexual maturation or blocking it and the influence of environmental factors is considered a first and indispensable step to establish new maturation protocols and setting gamete quality.

Following specific objectives were established:

1. To characterize some of the genes involved in the reproductive process and not yet described in European eel (*aa-cyp19a1*, *aa-gnrhr1a*, *aa-gnrhr1b*, *aa-gnrhr2*, *aa-zpb* and *aa-zpc*) and to develop specific qPCR for their gene expression analysis.
2. To understand the role of the forementioned genes and of *aa-ara* and *aa-arb* by studying their gene expression in male and female eels hormonally induced to maturation maintained at constant temperature.
3. To evaluate the effect of the temperature on vitellogenesis progression and steroidogenesis in female eels maintained under different thermal regimes.

CONTENT AND STRUCTURE OF THE THESIS

The work was realized in collaboration with Dr. Finn-Arne Weltzien laboratory at the Norwegian School of Veterinary Science of Oslo (Norway), where the candidate had the opportunity to realize part of the analysis during a six-month stay.
Androgens and VTG were analyzed in Paris, thanks to the collaboration with Dr. Sylvie Dufour laboratory at the Muséum National d'Histoire Naturelle (Paris, France).
E2 was measured in Dr. Helge Tveiten laboratory at NOFIMA (Tromsø, Norway).
Animals handling, hormonal induction, sample collection, data elaboration and article writing were realized at the Polytechnic University of Valencia, while gene characterization, primer design, primer test and qPCR were carried on in Oslo.
During the whole process, the candidate was involved in the experiment setting, sample collection, check of the animal status and in part of the molecular analysis. She is the first author of 2 papers, while is second author in the articles which were written by Dr. Peñaranda.

The experiment was part of the European project PRO-EEL (Reproduction of European Eel Towards a Self-sustained Aquaculture) which aims at breeding European eel in captivity. The project is funded from the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257.

Results are divided in 4 chapters, matching with 4 different articles and derive from the same animals used in one experiment: results have to be read as an *unicum* in order to have a wider and more complete look into the reproductive process.

Chapter I.

The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel.

D.S. Peñaranda, I. Mazzeo, V. Gallego, J. Hildahl, R. Nourizadeh-Lillabadi, L. Pérez, F.-A. Weltzien, J.F. Asturiano.

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Complete *Anguilla anguilla* aromatase (*aa-cyp19a1*) and partial androgen receptor α and β (*aa-ara* and *aa-arb*) sequences were isolated, and qPCR assays were validated. Expression of these genes in different tissues from immature males and females was determined. Gene expression analysis in brain, pituitary and gonads was performed in both male and female eels during hormonal induced maturation under constant thermal regime (20 and 18 ºC, respectively).

Chapter II.

Molecular characterization of three GnRH receptors in the European eel, *Anguilla anguilla*: Tissue-distribution and changes in transcript abundance during artificially induced sexual development.

D.S. Peñaranda, I. Mazzeo, J. Hildahl, V. Gallego, R Nourizadeh-Lillabadi, L. Pérez, J.F. Asturiano, F.-A. Weltzien.

Molecular and Cellular Endocrinology 369 (2013) 1-14.

Three GnRH-R genes (*aa-gnrhr1a*, *aa-gnrhr1b* and *aa-gnrhr2*) were characterized and specific qPCR assays developed. Expression of these genes in different tissues from immature males and females was determined. Gene expression analysis in brain and pituitary was performed in both male and female eels during hormonal induced maturation under constant thermal regime (20 and 18 ºC, respectively).

Chapter III.

Variations in the gene expression of zona pellucida proteins, *zpb* and *zpc*, in female European eel (*Anguilla anguilla*) during induced sexual maturation.

I. Mazzeo, D.S. Peñaranda, V. Gallego, J. Hildahl, R. Nourizadeh-Lillabadi, J.F. Asturiano, L. Pérez, F.-A. Weltzien.

General and Comparative Endocrinology 178 (2012) 338-346.

Two zona pellucida protein genes (*aa-zpb* and *aa-zpc*) from European eel were characterized and specific qPCR assays developed. Expression of these genes in different tissues from immature males and females was determined. Gene expression analysis in gonad and liver was performed in females during hormonal induced maturation under constant thermal regime (18 ºC).

Chapter IV.

Temperature modulates the vitellogenesis progression in European eel

I. Mazzeo, D.S. Peñaranda, V. Gallego, S. Baloche, R. Nourizadeh-Lillabadi, H. Tveiten, S. Dufour, J.F. Asturiano, F.-A. Weltzien, L. Pérez.

Aquaculture 434 (2014) 38-47.

Wild female eels at silver stage were hormonally induced to sexual maturation under three different thermal regimes. One group was kept at constant temperature (18 ºC) while in the other two groups eels experienced a temperature increase from 10 to 15 ºC and from 15 to 18 ºC. Temperature effect on vitellogenesis progression, steroid and VTG plasmatic levels and P450arom gene expression in ovary was studied.

CHAPTER I

The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel

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Following one referee's request, the supplementary data were included in the text rather than shown in a specific section.

Abstract

This research investigated the regulation of aromatase and androgen receptor gene expression in the brain-pituitary-gonad (BPG) axis of male and female European eels (*Anguilla anguilla*) during induced sexual maturation. Complete *Anguilla anguilla* aromatase (*aa-cyp19a1*) and partial androgen receptor α and β (*aa-ara* and *aa-arb*) sequences were isolated, and qPCR assays were validated and used for quantification of transcript levels for these three genes. Expression levels of the genes varied with sex, tissue, and stage of maturation. *aa-arb* was expressed at higher levels than *aa-ara* in the pituitary and gonad in both sexes, suggesting *aa-arb* is the physiologically most important androgen receptor in these tissues. In the female brain, a decrease in *aa-ara* and an increase in *aa-cyp19a1* were observed at the vitellogenic stage. In contrast, a progressive increase in all three genes was observed in the pituitary and ovaries throughout gonadal development, with *aa-arb* and *aa-cyp19a1* reaching significantly higher levels at the vitellogenic stage. In the male pituitary, a decrease in *aa-arb* and an increase in *aa-cyp19a1* were observed at the beginning of spermatogenesis, and thereafter remained low and high, respectively. In the testis, the transcript levels of androgen receptors and *aa-cyp19a1* were higher during the early stages of spermatogenesis and decreased thereafter. These sex- dependent differences in the regulation of the expression of *aa-ara*, *aa-arb*, and *cyp19a1* are discussed in relation to the role of androgens and their potential aromatization in the European eel during gonadal maturation.

1. Introduction

The lifecycle of the European eel, *Anguilla anguilla* (L.) includes both oceanic and continental phases. European eels reproduce in the Atlantic Ocean, supposedly in the Sargasso Sea, after which the leptocephalus larvae drift towards the European coast, where they

metamorphose into glass eels before entering the long growth phase as yellow eels in fresh or coastal water. At the start of their reproductive migration towards the Sargasso Sea, yellow eels transform into silver eels, which are still immature and remain blocked in the pre-pubertal stage as long as migration is prevented. This makes the eel an interesting model organism in the investigation of the regulatory mechanisms of reproductive development.

The pre-pubertal stagnation is due to insufficient release of pituitary gonadotropins, resulting from both a lack of stimulation by the gonadotropin-releasing hormone (GnRH) and a strong dopaminergic inhibition (Dufour et al., 1988; Vidal et al., 2004). Therefore, in order for eels to mature in captivity, gonadotropin treatment is necessary (e.g. Miura et al., 1991; Pérez et al., 2000; 2011). Upon gonadotropin stimulation, synthesis of gonadal androgens (testosterone (T) and 11-ketotestosterone (11-KT)) and estrogens (17β-estradiol (E2)) increases. These androgens play crucial roles during gonad development, both by stimulating gamete development, and through feedback mechanisms to the brain and pituitary.

Unlike in mammals, plasma levels of androgens are relatively high in both female and male fish (Borg, 1994). The effects of the androgens on their target cells may be direct, through binding to androgen receptors, or indirect, through local aromatization into estrogens by the complex enzyme aromatase. Aromatase is a member of the P450 cytochrome superfamily of enzymes and acts as a catalyst in the creation of estrogens from androgens. In contrast to the single form found in mammals (Harada, 1988), two paralogous genes of P450 aromatase have been found in teleosts, one of which is expressed mainly in the brain (*cyp19a1b*) and the second mainly in the gonad (*cyp19a1a*). This phenomenon of fish possessing two aromatase genes was first reported in goldfish (*Carassius auratus*, Gelinas et al., 1998), zebrafish (*Danio rerio*,

Kishida and Callard, 2001), and tilapia (*Oreochromis niloticus*, Chang et al., 1997a; Kwon et al., 2001), and has since been confirmed in many other teleost species (e.g. Blázquez and Piferrer, 2004; Choi et al., 2005). In eels, however, only one aromatase cDNA has been identified (termed *cyp19a1*), and is expressed in the ovary, brain and pituitary (Ijiri et al., 2003; Tzchori et al., 2004).

In teleosts, two androgen receptor paralogs (ARα and ARβ, encoded by the genes *ara* and *arb*, respectively) have been identified through phylogenetic analyses, and evaluation of tissue distribution and ligand affinity (Sperry and Thomas, 1999a; Olsson et al., 2005; Harbott et al., 2007). The expression of both androgen receptors (AR) has been demonstrated in a wide range of tissues in different fish species (in the brain, pituitary, gonad and peripheral tissues) (Todo et al., 1999; Blázquez and Piferrer, 2005). Although the biochemical characteristics of ARβ are similar to those of mammalian AR (Wilson and French, 1976), the binding characteristics of ARα make this receptor unique to teleosts. While ARα binds only T with high affinity, ARβ is able to bind a wide spectrum of synthetic and natural androgens with approximately the same steroid-binding specificity. In addition, the tissue distribution differs, with ARα found exclusively in the brain, and ARβ in both the central nervous system and the peripheral tissues (Sperry and Thomas, 1999a,b). Both AR genes are expressed in Japanese eel (*A. japonica*; Ikeuchi et al., 1999; Todo et al., 1999), but, unlikely other teleost species, no significant differences in their androgen binding capacity have been detected. Both eel ARs are activated by 11-KT, and also by 5α-dihydrotestosterone which, along with T, is the most important androgen in mammals (Ikeuchi et al., 2001).

The purpose of this study was to measure the levels of *aa-cyp19a1*, *aa-ara,* and *aa-arb* in the brain, pituitary, and gonads during the artificial maturation of European eels of both sexes in order to improve our understanding of their regulation during eel reproductive development.

2. Materials and Methods

2.1. Fish maintenance, hormonal treatment and sampling

2.1.1 Maturation and sampling of female European eel

Forty-four wild female eels at the silver stage (mean body weight 847±28 g; mean body length 71.4±0.8 cm) caught by local fishermen in the Albufera Lagoon (Valencia, Spain) in October and November during their reproductive migration to the sea, were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. They were kept in two 500 L fiberglass tanks equipped with separate recirculation systems. Over the course of two weeks the fish were acclimatized from fresh water to seawater (salinity 37.0±0.3%). Water temperature was maintained at 18 ºC throughout the experiment. The eels were treated weekly with intraperitoneal injections of carp pituitary extract (CPE, Catvis Ltd, Netherlands) for 12 weeks, at a dose of 20 mg/kg body weight (Pérez et al., 2011). The CPE was diluted in NaCl solution (0.9 g/l), centrifuged (1260 *g*, 10 min) and the supernatant was stored at -20 ºC until its use between 1 and 4 weeks later. Groups of 6 eels were anaesthetized and sacrificed by decapitation at weeks 4, 8 and 12. Additionally, before starting the hormonal treatment, two groups of female eels also from Albufera Lagoon (n=6) were sacrificed in fresh (F) and seawater (S) conditions with the aim to evaluate the possible influence of salinity.

Samples from the ovary, pituitary, and brain were collected from the eels at sacrifice and stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until extraction of total RNA (Peñaranda et al., 2010). The brain samples were divided into three sections: the olfactory bulb (OB), telencephalon (Tel), and di- and mesencephalon (DM), by the method described by Weltzien et al. (2005b). In addition, ovarian tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and

measurement of oocyte diameter in order to determine the maturational status (Pérez et al., 2011).

2.1.2 Maturation and sampling of male European eel

One hundred and fifty male eels at the silver stage (mean body weight 124.1±12.6 g; mean body length 39.9±0.21 cm) were purchased from the Valenciana de Acuicultura, S.A fish farm (Puzol, Valencia) and transported to the facilities at the Universitat Politècnica de València. The fish were gradually acclimatized to seawater (salinity 37±0.3%; 20 ºC) over the course of one week and equally distributed into three tanks of 500 L equipped with separate recirculation systems.

In order to induce maturation and spermiation, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g/fish; Angelini Farma-Lepori, Barcelona, Spain) over the course of 13 weeks, as previously described by Pérez et al. (2000). Groups of 10 eels were anaesthetized and sacrificed by decapitation each week along the hormonal treatment. Additionally, before starting the hormonal treatment, 6 male eels also from Valenciana de Acuicultura, S.A fish farm were sacrificed in seawater (S) conditions, being considered as the control group.

Samples from the testis and pituitary were collected from the eels at sacrifice and then stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until the extraction of total RNA (Peñaranda et al., 2010). As the different sections of the brain were not collected during the first few weeks of treatment, the brains from male fish were not included in this study. In addition, testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status.

2.2 Histology processing

The ovarian and testicular samples were processed and analyzed as described by Peñaranda et al. (2013, Chapter II). The diameters of the largest oocytes in the ovarian samples (n ≥ 100/female) were measured using Camera Control Unit software (Nikon, Japan).

Three stages of the ovarian development were distinguished: 1) previtellogenic (PV; mean ovarian diameter 155±6.7 µm) - perinucleolus stage with no or few lipid droplets, or in lipid droplet stage but without yolk vesicles. 2) Early vitelogenic (EV; 211±10.7 µm) - small yolk vesicles restricted to the periphery of the oocyte. 3) Vitellogenic (V; 402±22.6 µm) - mid- and late vitellogenesis are included in this stage. Mid-vitellogenesis is characterized by numerous yolk vesicles in the cytoplasm from the membrane to the nucleus, and a lower proportion of yolk vesicles compared to lipid droplets. Late vitellogenesis is characterized by fewer, enlarged yolk vesicles and a higher proportion of lipid droplets compared with yolk vesicles (Fig. 1A) (Perez et al., 2011; Mazzeo et al., 2012, Chapter III).

Spermatogenesis stages were determined by identifying the most advanced germ cell type present and relative abundance (Peñaranda et al., 2010; 2013 – Chapter II; Fig. 7A). Stage 1 was characterized by the presence of spermatogonia type A and/or B; Stage 2 by the presence of spermatogonia and spermatocytes; Stage 3 by spermatids appearing in the testis tissue, and Stages 4, 5 and 6 by spermatozoa appearing inside the lumen of the lobules. Distinguishing between stages 4-6 was based on the percentage of spermatozoa present in relation to the lobule lumen surface.

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

2.3.1 Primer design

Eel acidic ribosomal phosphoprotein P0, *aa-arp* (Table 1, Weltzien et al., 2005b; Aroua et al., 2007; Peñaranda et al., 2010) was used as a reference gene in the qPCR analysis because its mRNA expression is stable throughout hormonal treatment (Weltzien et al., 2005b). 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 (testis: 0.59; ovary: 0.21; brain and pituitary: 0.53, $p<0.05$) and a Cq arithmetic mean of 19.51±1.11 in the testis, 10.1±0.72 in the ovary, and 19.32±1.67 in the brain. 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 *aa-cyp19a1*, *aa-ara*, and *aa-arb* (Table 1). 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.

2.3.2 SYBR Green assay (qPCR)

Total RNA was isolated from the RNAlater preserved tissues as described by Hildahl et al. (2011a), and then treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min. First-strand cDNA was synthesized from either 1 µg (brain and testis) or 2 µg (ovary) total RNA, using random hexamer primers and superscript III reverse transcriptase (Invitrogen).

qPCR assays were performed as described by Weltzien et al. (2005b) and Peñaranda et al. (2013, Chapter II), using a Light Cycler 480 system with SYBR Green I detection (Roche, Meylan, France). After an initial activation of *Taq* polymerase at 95 ºC for 10 min, 42 PCR cycles were performed at the following cycling conditions: 95 ºC for 10 s, 60 ºC for 10 s, and 72 ºC for 7 s. Each PCR reaction contained a total of 10 µl, comprising 1:10 diluted cDNA template (3 µl), forward and reverse primers (250 nM each), and SYBR Green Master Mix (5 µl). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Weltzien et al. (2005b). 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.

2.3.3 Tissue-Specific Expression of *aa-cyp19a1*

Three silver males from the Valenciana de Acuicultura S.A fish farm, and three silver females from the Albufera lagoon, with average body weights of 118±14.7 g and 632±46.5 g, respectively, were sacrificed. RNA was extracted as described above from nine different tissues: the gonad, liver, pectoral fin, anterior and posterior kidney, heart, gill, pituitary, and brain. The brain was divided into five parts: olfactory bulbs, telencephalon**,** di- and mesencephalon, cerebellum, and medulla oblongata, as previously reported by Weltzien et al. (2005b). Total RNA was treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III reverse transcriptase (Invitrogen) and random hexamer primers according to the standard protocol. The existence of *aa-cyp19a1* transcripts in the various tissues was analyzed by qPCR.

Table 1. Primer sequences used in quantitative PCR and in reverse transcriptase-PCR cloning experiments.

Name	Sequence (5'- 3')	Orientation	Usage	GenBank Accession number.	Reference
aa-cyp19a1	CTC ACA CCA TGA AGC ACC TGG AG	Forward	PCR		Tzchori et al. 2004, confirmed in this work
	GAT GGA AGC TGC CGC TTT ACT GTC	Reverse	PCR[1] (1560bp)		
	TTC AAG GGA ACG AAC ATC ATC	Forward	qPCR[2] (115 bp)		
	AGA AAC GGT TGG GCA CAG T	Reverse	qPCR[3] (BE=2.044)/(GE=2.067)		
aa-ara	CTG TGA AAT GCG TCA GGA GA	Forward	PCR	FR668031	confirmed in this work
	CCG CCA TTT TGT TTA GCA TT	Reverse	PCR[1] (2487 bp)		
	CGG AAG GGA AAC AGA AGT ACC	Forward	qPCR[2] (104 bp)		
	AGC GAA GCA CCT TTT GAG AC	Reverse	qPCR[3] (BE=2.058)/(GE=2.006)		
aa-arb	CCC GTA ACA GAC GGA AGA TA	Forward	PCR	FR668032	confirmed in this work
	GTG CTC GTA CAT GCT GGA GA	Reverse	PCR[1] (1658 bp)		
	CGC TGA AGG AAA ACA GAG GT	Forward	qPCR[2] (115 bp)		
	CAT TCC AGC CTC AAA GCA CT	Reverse	qPCR[3] (BE=2.173)/(GE=2.033)		
aa-arp	GTG CCA GCT CAG AAC ACG	Forward	qPCR[2] (107 bp)	AY763793	Weltzien et al. 2005b
	ACA TCG CTC AAG ACT TCA ATG G	Reverse	qPCR[3] (EB=2.142)/(GE=2.181)		

[1] PCR amplicon length is given in parenthesis following the reverse PCR primers.
[2]qPCR amplicon length is given in parenthesis following the forward qPCR primers.
[3] qPCR efficiency for each primer pair in brain (BE) and gonad (GE) is given in parenthesis following the reverse qPCR primer
aa-cyp19a1= Anguilla anguilla aromatase P450 a1; aa-ara= Anguilla anguilla androgen receptor a; aa-arb= Anguilla anguilla androgen receptor b; aa-arp: Anguilla anguilla acidic ribosomal phosphoprotein P0.

2.4 Molecular cloning of *aa-cyp19a1*, *aa-ara* and *aa-arb* cDNAs

First-strand cDNA was synthesized from 2 µg of total RNA (extracted from the ovary and brain and DNase treated as described in section 2.3.3), using random hexamer primers and superscript III reverse transcriptase (Invitrogen). Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used for the design and theoretical evaluation of PCR primers. Primers for initial fragment cloning were designed from the corresponding Japanese eel sequences (GenBank accession numbers: *ara* AB023960, *arb* AB025361, and *cyp19a1* AY540622).

PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 µl contained 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. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. The PCR products were visualized in 2 % agarose gel stained with ethidium bromide and bands of expected size were purified using a 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 a Qiagen Plasmid Mini Kit (Qiagen). Plasmids with inserts were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, University of Oslo sequencing service, Oslo, Norway).

The partial AR sequences were confirmed by comparing the results with those obtained by Lafont et al. (Unpublished results) (GenBank accession no. FR668031 and FR668032). The *aa-cyp19a1* full-length sequence was confirmed by comparison with the recently sequenced eel genome (Henkel et al., 2012) and the published sequence from

Tzchori et al. (2004). The eel genome (Henkel et al., 2012) was also used to search for potential additional paralogous AR or aromatase genes.

2.5 Statistical analysis

Differences in the data were analyzed by analysis of variance (One-way ANOVA). 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 means (SEM). All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

The gene expression data in the case of both male and female eels are presented according to the stage of gonad development.

3.1 Female eels

3.1.1 Ovarian development

Four hormonal injections were sufficient to stimulate ovarian development to the EV stage in most female eels (83%; Fig. 1B), while eight weeks were necessary to reach the V stage (34%). After 12 weeks of treatment, 83% of females had reached the V stage.

3.1.2 *aa-cyp19a1* and *aa-ARs* expression levels in the brain

In OB and DM, *aa-ara* expression levels were significantly lower in V stage than the previous stage (EV; Fig. 2A). Similar results were also seen in Tel, although this was not significant. *aa-arb* expression levels increased from the PV to the EV stage in OB, while non-significant differences were found in Tel and DM (Fig. 2B). For *aa-cyp19a1,* transcript levels in OB increased continuously from the PV to the EV stage and continued to the V stage (Fig. 4A). In DM, the

increase was visible at the V stage (Fig. 4C). No differences in *aa-cyp19a1* expression levels between the developmental stages were found in Tel (Fig. 4B).

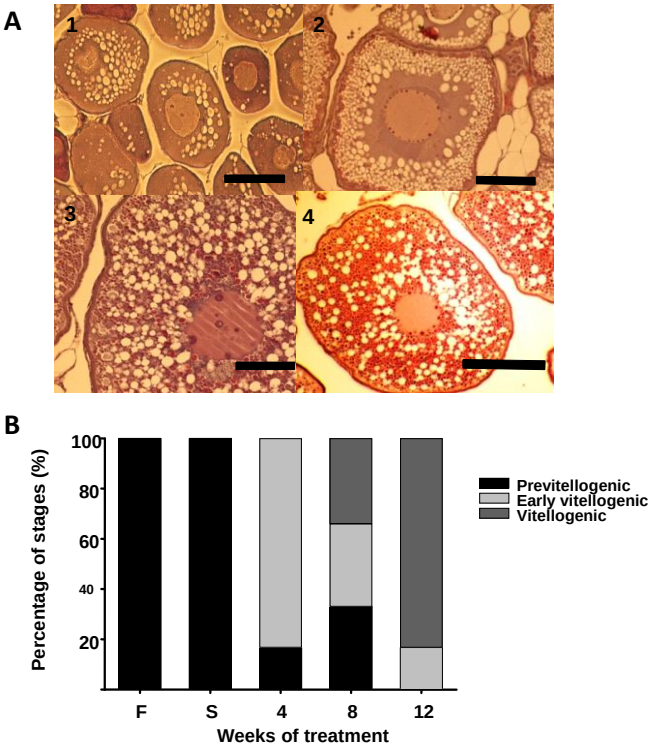

Fig. 1. A) Histological sections of oocytes at different developmental stages during carp pituitary extract (CPE) hormonal treatment. A1) Previtellogenic stage (PV); A2) Early vitellogenic stage (EV); A3) Mid-vitellogenic stage (V); and A4) Late vitellogenic stage (V). lv: lipid vesicle; yg: yolk globules. Scale bars, 100 μm. B) Percentage of the different stages of ovarian development at 4, 8 and 12 weeks of CPE treatment, and fresh (F) and seawater (S) conditions, (n=8, data from Peñaranda et al., 2013, Chapter II).

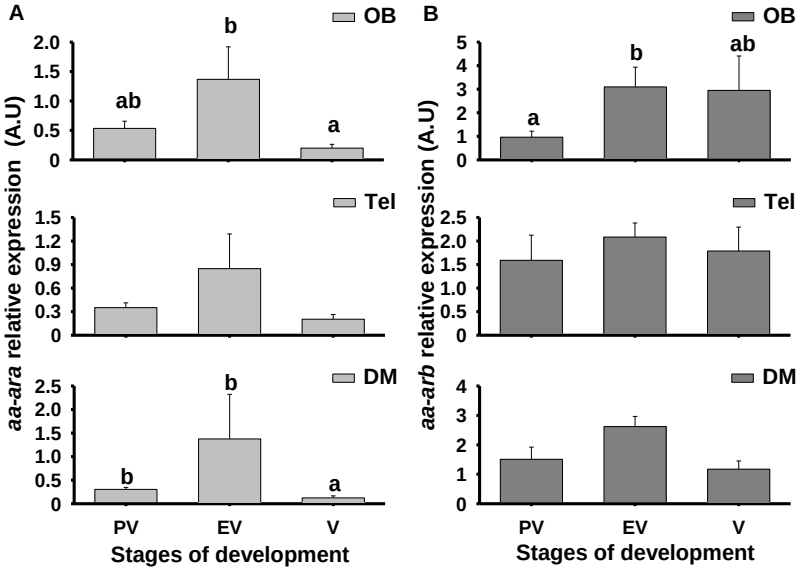

Fig. 2. Gene expression, analyzed by qPCR, of *Anguilla anguilla androgen receptor a* (*aa-ara*) (A) and *Anguilla anguilla androgen receptor b* (*aa-arb*) (B) in female eel brains during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage. OB= Olfactory bulbs, Tel= Telencephalon and DM= Di- and mesencephalon.

3.1.3 *aa-cyp19a1* and *aa-AR*s expression levels in the pituitary

Expression of *aa-ara* and *aa-arb* in the female pituitary differed from each other. While transcript levels of *aa-arb* increased from the PV to the EV stage and further to the V stage, similar but non-significant results were observed for *aa-ara* transcript levels (Fig. 3B). Additionally, the *aa-arb* expression levels were approximately eight times higher than *aa-ara*. *aa-cyp19a1* expression levels increased markedly (about eight-fold) from the PV and EV stages to the V stage (Fig. 4D). The *aa-cyp19a1* expression levels in the pituitary in the V stage were higher than those found at any stage in the different brain regions.

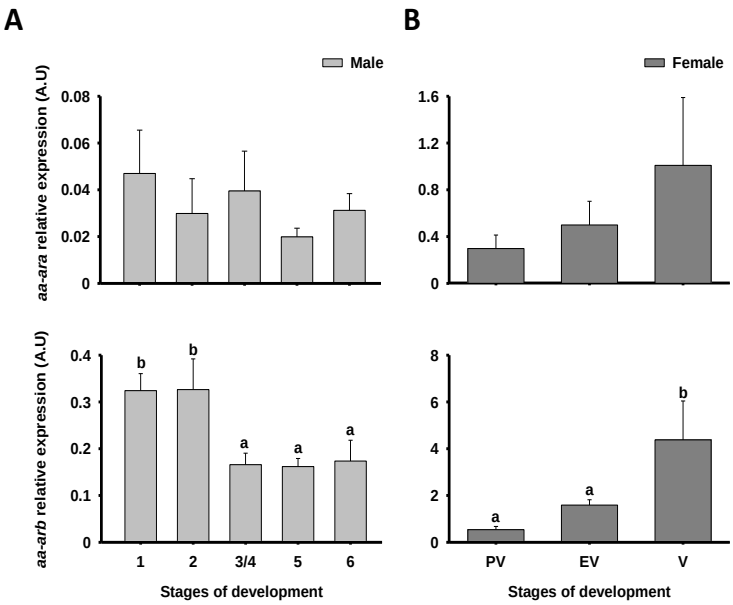

Fig. 3. Gene expression of *Anguilla anguilla androgen receptor a* (*aa-ara*) and *Anguilla anguilla androgen receptor b* (*aa-arb*) analyzed by qPCR in male (A) and female (B) pituitary during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-13). PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

3.1.4 *aa-cyp19a1* and *aa-ARs* expression levels in the ovary

Ovarian *aa-ara* and *aa-arb* expression levels increased during oogenesis (Fig. 5C and 5D). *aa-arb* gene expression levels increased significantly from the PV to the EV stage, and continued into the V stage, whereas a significant increase in *aa-ara* gene expression levels was only seen from the PV to the V stage.

Based on Cq values, *aa-arb* expression levels were about 27-times higher than *aa-ara* levels. Ovarian *aa-cyp19a1* levels varied markedly between samples from the EV and V groups. The average levels were higher than those in the brain, and increased in line with the gonad development, with a significant increase at the V stage (Fig. 6B).

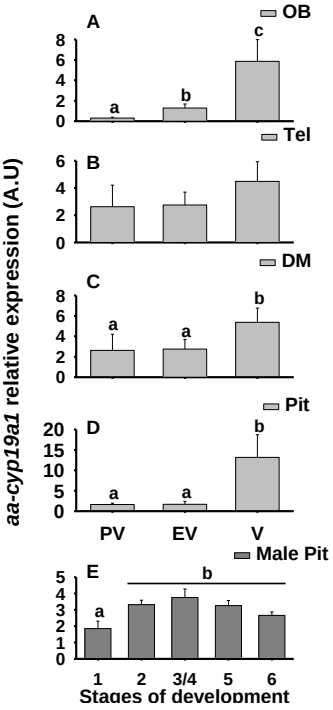

Fig. 4. Gene expression of *Anguilla anguilla aromatase P450 a1 (aa-cyp19a1)* analyzed by qPCR in eel brains (females) and pituitaries (males and females) during artificially induced sexual maturation. A) Female OB, B) Female Tel, C) Female DM, D) Female pituitary, E) Male pituitary. Different superscript letters mean significant differences ($p<0.05$; n=6-15). OB= Olfactory bulbs, Tel= Telencephalon, DM= Di- and mesencephalon, Pit= Pituitary. See main text for definition of gonad developmental stages.

3.2 Male eels

3.2.1 Testicular development

After having been treated with hCG for two weeks, all the males reached spermatogonial stage 2 (Fig. 7B). Stages 3 and 4 were seen after three weeks, whereas stages 5 and 6 were first observed after four and seven weeks, respectively. From seven weeks until the end of the experiments most males were in stage 5 or 6, with some, possibly non-responders, remaining in stage 4.

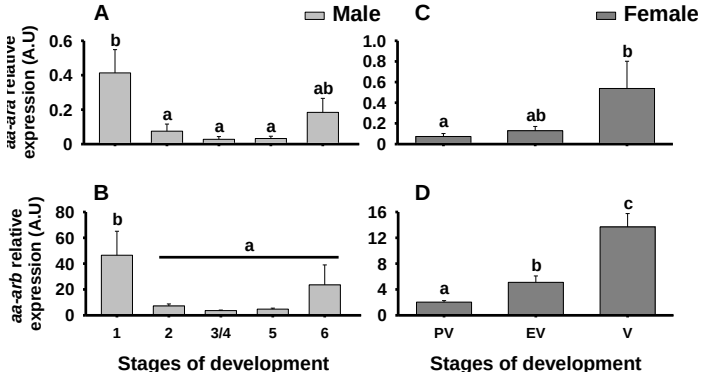

Fig. 5. Gene expression, analyzed by qPCR, of *Anguilla anguilla androgen receptor a* (*aa-ara*) (A), *Anguilla anguilla androgen receptor b* (*aa-arb*) (B) in male and *aa-ara* (C), *aa-arb* (D) in female eel gonads during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

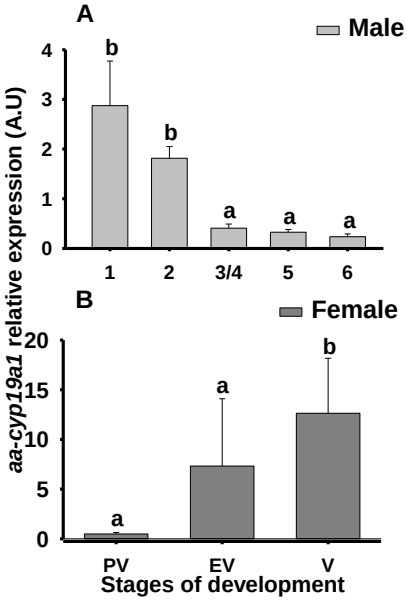

Fig. 6. Gene expression of *Anguilla anguilla aromatase P450 a1 (aa-cyp19a1*) analyzed by qPCR in male (A) and female (B) eel gonads during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

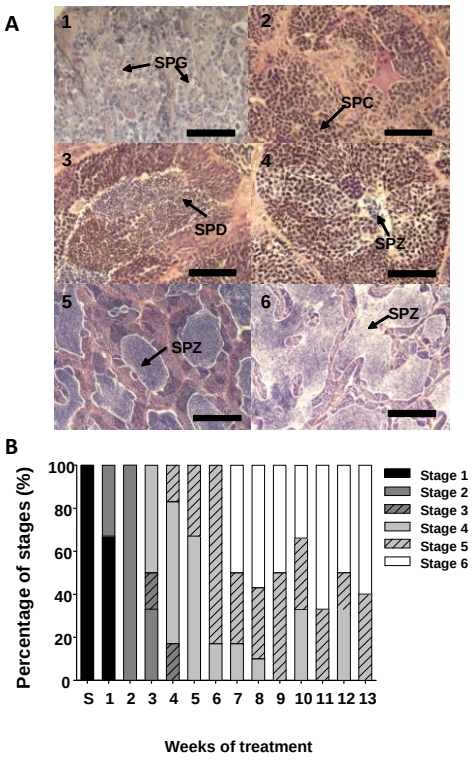

Fig. 7. A) Histological sections of eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment. A1) Testis at stage 1. A2) Testis at stage 2. A3) Testis at stage 3. A4) Testis at stage 4. A5) Testis at stage 5. A6) Testis at stage 6. SPG, spermatogonia; SPC, spermatocytes; SPD, spermatids; SPZ, spermatozoa. B) Relative percentages of testis developmental stages (1-6) along hCG treatment in male eels, and prior hormonal treatment in seawater conditions (S) (n=10, data from Peñaranda et al., 2010).

3.2.2 *aa-cyp19a1* and *aa-ARs* expression levels in the pituitary

Both *aa-ara* and *aa-arb* differed between male and female pituitaries (Fig. 3). While *aa-ara* expression levels increased in the female pituitaries during the gonad development, expression levels in the male pituitaries remained relatively stable with no significant changes between the stages. However, expression levels of *aa-arb*

decreased between stages 2 and 3/4, and thereafter remained stable. Although comparisons between genes/primer pairs should be treated cautiously, the *aa-arb* expression levels were again higher than the *aa-ara* levels (about 7-fold). A significant increase in the *aa-cyp19a1* expression levels in the pituitary coincided with the appearance of spermatocytes in the testis (stage 2), with expression levels remaining elevated in the subsequent developmental stages (Fig. 4E).

3.2.3 *aa-cyp19a1* and *aa-ARs* expression levels in the testis

Both *aa-ara* and *aa-arb* expression levels followed similar results, decreasing at the beginning of spermatogenesis and thereafter remaining at low levels (stage 2; Fig. 5A and 5B). Similar to results from the pituitary, *aa-arb* gene expression levels were higher than *aa-ara* levels (>100 fold). The highest mean expression level of testicular *aa-cyp19a1* gene expression (Fig. 6A) occurred in untreated males (stage 1), and this was followed by a progressive decrease until stages 3 and 4. Low expression levels continued during the more advanced stages of development.

3.3 Detection of *aa-cyp19a1* mRNA transcript in eel tissues

The tissue-specific expression pattern demonstrated different *aa-cyp19a1* transcript levels in the different parts of the brain, although similar levels were observed in both sexes (Fig. 8). Higher *aa-cyp19a1* gene expression levels were observed in Tel and the pituitary than in the cerebellum and medulla oblongata. Lower expression levels were also found in the gonads than Tel and the pituitary, with levels in the testes being no higher than those in the ovaries. A variation in expression levels between the sexes was also observed in the gills; the females did not express *aa-cyp19a1*,

whereas relatively low levels were found in male gills. No transcripts were observed in either sex in the other tissues investigated from prepubertal silver eels.

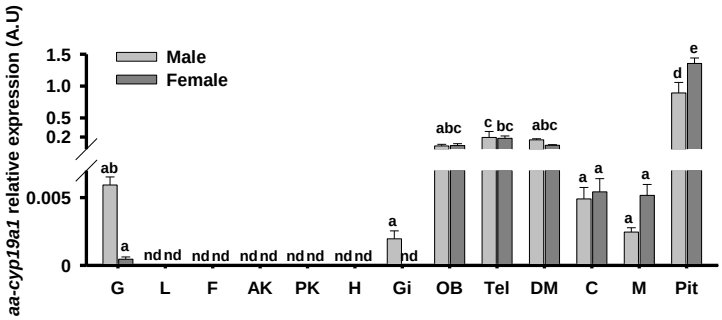

Fig. 8. Quantitative gene expression of *Anguilla anguilla aromatase P450 a1 (aa-cyp19a1)* in various tissues from immature eel, males (gray bars; average body weight 118±14.7 g) and females (dark gray bars; average body weight 632±46.5 g). Different superscript letters mean significant differences ($p<0.05$; $n=3$). Tissues in which the gene was not detected is labeled "nd" (not detectable). Tissue abbreviations are G = gonad (testis or ovary), L = liver, F= pectoral fin, AK= anterior kidney, PK= posterior kidney, H= heart, Gi= gill, OB= olfactory bulbs, Tel= telencephalon, DM= di- and mesencephalon, C= cerebellum, M= medulla oblongata, Pit= pituitary.

3.4 Identification and characterization of the *aa-cyp19a1* gene

One form of aromatase (*aa-cyp19a1*), identical to the one published by Tzchori et al. (2004), was identified from the European eel genome (Henkel et al., 2012), and its sequence confirmed by molecular cloning. A single open reading frame of 1536 bp was identified as sharing a high identity (98%) with Japanese eel *cyp19a1* (Table 2). Additionally, multiple conserved domains were identified, including a transmembrane-spanning domain, an I-helix region, an Ozol's peptide region, an aromatic region, and a heme-binding region (Fig. 9). Alignment of genomic DNA and cDNA sequences revealed nine exons and eight introns, with the following sizes: exon 1 (186 bp, 62 aa), intron 1 (1054 bp), exon 2 (153 bp, 51 aa), intron 2 (313 bp), exon 3(153 bp, 51aa), intron 3(397 bp),

exon 4 (177 bp, 59 aa), intron 4 (839 bp), exon 5 (114 bp, 38 aa), intron 5 (281 bp), exon 6 (114 bp, 38 aa), intron 6 (239 bp), exon 7 (165 bp, 55 aa), intron 7 (620 bp), exon 8 (240 pb, 80 aa), intron 8 (411), exon 9 (234 bp, 78 aa). Sequence alignment confirmed the intermediate nature of the eel *cyp19a1* gene, sharing some sequence features unique to the other teleost *cyp19a1a* gene and other features unique to teleost *cyp19a1b*.

4. Discussion

4.1 aa-cyp19a1 cDNA characterization

The expression levels of *aa-ara*, *aa-arb*, and *aa-cyp19a1* in the brain, pituitary, and gonads of male and female European eels during hormonally induced maturation are measured and described, along with a complete description of the *aa-cyp19a1* sequence. The sequence analysis has demonstrated that classification of the *aa-cyp19a1* gene as a typical teleost ovarian variant or as a brain aromatase variant is not possible, as it appears to contain sequence domains that are specific to both the brain and ovarian variants found in other teleost species (Table 2 and Fig. 9). It has been suggested that the two fish variants arose following the teleost specific round of whole genome duplication (WGD), which was followed by the loss of tissue specific regulatory elements resulting in two independent genes (Diotel et al., 2010). According to this hypothesis, we would expect the eel also to have two variants, as it is assumed that the teleost-specific WGD predates the divergence of the Elopomorphs order, which includes the European eel, as well as other teleosts (Henkel et al., 2012). Elopomorphs, however, diverged early in the evolution of teleost fish and one of the duplicate *cyp19a1* genes was probably lost at an early point in the diversification process. This theory is supported by the observation that, as with tetrapods, *aa-cyp19a1* lacks the additional intron in the 5'UTR.

Table 2. Percent identity between the European eel aromatase (*aa-cyp19a1*) deduced amino acid sequence (http://www.ncbi.nlm.nih.gov/gene/) and representative vertebrate species for the complete open reading frame (ORF) and conserved domains: transmembrane-spanning domain (transmembrane), I-helix region, Ozol's peptide region, aromatic region, and heme-binding region. The alignment was done using CLC Main Workbench 4.0 sequence analysis program (CLC bio, Aarhus, Denmark).

Species	GenBank accession nr	ORF	Transmembrane	I-Helix	% Identity Ozol's peptide	Aromatic	Heme-bidning
Japanese eel	AAS47028	98	93	97	100	100	100
Medaka A	BAA11656	61	36	89	70	83	79
Trout A	1806325[a]	65	71	89	74	83	87
Catfish A	AAB32613	58	50	83	61	58	79
Goldfish A	AAC14013	61	57	89	70	83	83
Medaka B	AAP83449	62	36	89	61	75	87
Trout B	CAC84574	63	57	86	61	92	79
Catfish B	AAL14612	59	36	77	74	83	75
Goldfish B	AAB39408	55	36	80	65	83	79
Xenopus	BAA90529	53	36	77	61	83	75
Chicken	AAA48738	52	36	63	57	92	79
Human	AAA52132	52	43	71	61	83	75

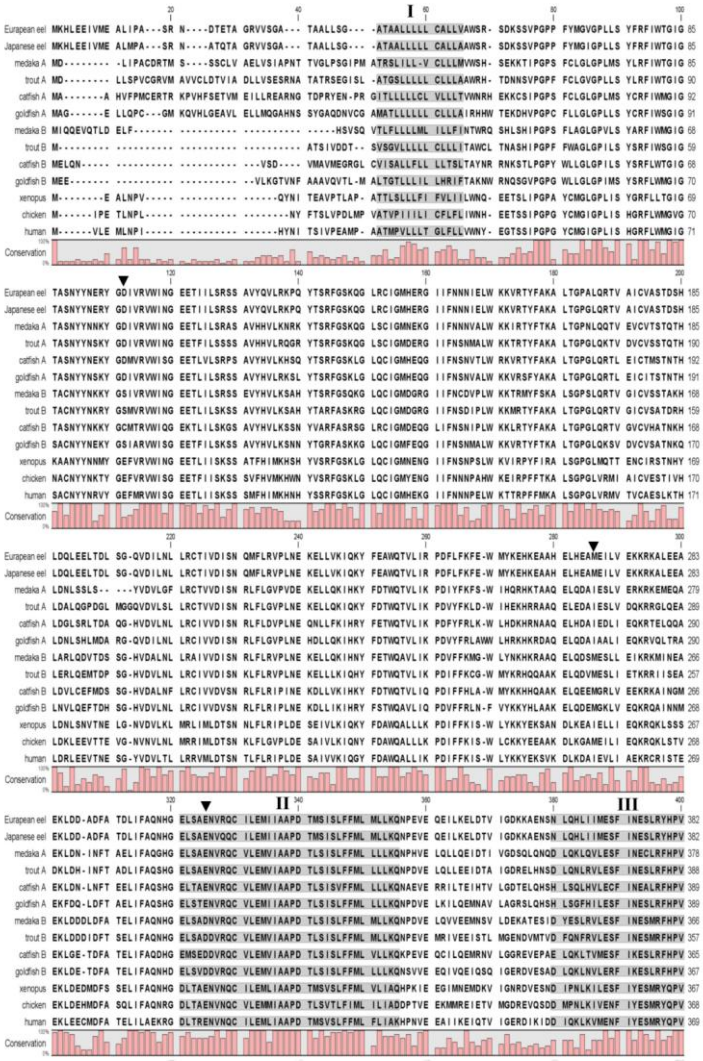

Fig. 9. See next page for figure legend.

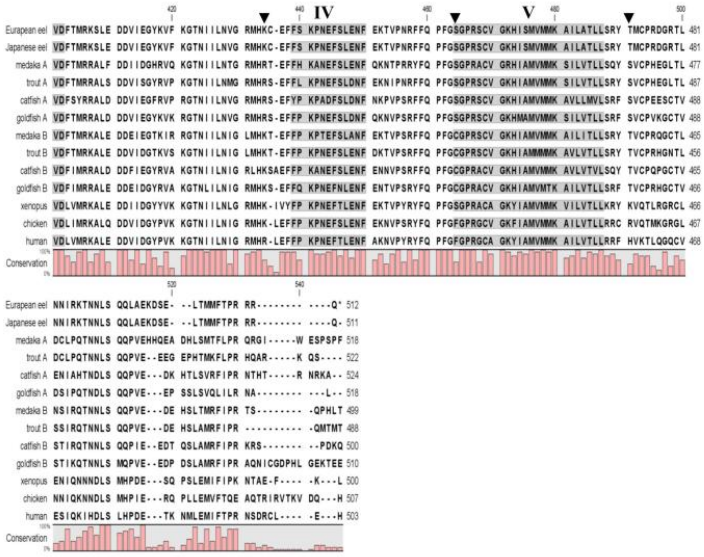

Fig. 9. Alignment of European eel *aa-cyp19a1* deduced amino acid sequence (1536 bp) with one representative of the fish orders Anguilliformes, *Anguilla japonica* (Japanese eel); Perciformes, *Oryzias latipes* (Japanese medaka); Salmoniformes, *Oncorhynchus mykiss* (rainbow trout); Siluriformes, *Ictalurus punctatus* (channel catfish); and Cypriniformes, *Carassius auratus* (goldfish), and representative tetrapods – amphibian, *Xenopus laevis* (African clawed frog); aves, *Gallus gallus* (chicken); and mammalia, *Homo sapiens* (human). Conserved domains are boxed in grey and numbered as follows: I. transmembrane-spanning domain, II. I-helix region, III. Ozol's peptide region, IV. aromatic region, and V. heme-binding region. Arrows identify differentially regulated motifs between fish *cyp19a1a* and *cyp19a1b*. The degree of conservation at each location is shown by the red bar graph. Alignment of the genomic DNA sequence (Henkel et al., 2012) and the cloned transcript sequence revealed nine exons and eight introns.

4.2 Gene expression during female gonadal development

aa-cyp19a1 and *aa-ARs* expression levels in the brain

The significantly higher levels of *aa-ara* and *aa-arb* observed in the EV stage could indicate increased sensitivity of the brain to androgens during this stage of development. The decrease in *aa-ara* gene expression levels in the OB and DM (with similar results in Tel)

of stage V females may be related to the positive effect of T on the expression of tyrosine hydroxylase (Weltzien et al., 2006) and dopamine receptors (D2A and D2B, Pasqualini et al., 2009) in the forebrain; with the lower *aa-ara* levels in stage V indicating less dopaminergic inhibition of gonadotrope activity and thus stimulation of gonad development.

In contrast to the brain *aa-ara* gene expression levels, which decreased from EV to V, the brain *aa-cyp19a1* expression levels increased in V stage females. The opposing nature of this regulation again supports the hypothesis that there is a reduction in inhibition of gonad development by androgens in the later stages, due both to a decrease in AR and to an up-regulation of aromatase expression (Weltzien et al., 2006; Pasqualini et al., 2009). In addition to decreased inhibition by androgens, the increase in expression levels of *aa-cyp19a1* in the forebrain as maturation progresses indicates an increase in the local synthesis of estrogens. Previous studies have shown a decrease in tyrosine hydroxylase and *d2rb* gene expression in the eel forebrain following E2 treatment (Weltzien et al., 2006; Pasqualini et al., 2009; Dufour et al., 2010). Additionally, Montero et al. (1995) observed an up-regulation of GnRH1 (mGnRH) in the forebrain following E2 treatment. Consequently, *aa-cyp19a1* activity may have a dual effect in the eel forebrain during maturation, reducing androgenic stimulation of dopamine inhibition and also stimulating maturation through increased stimulation of GnRH synthesis by E2.

aa-cyp19a1 and *aa-ARs* expression levels in the pituitary

Coinciding with higher expression levels of *aa-ara* and, in particular, *aa-arb* in the pituitary at the V stage, a significant increase was found in *aa-cyp19a1* levels. These significant differences could be

related to the higher T levels found in the plasma of the same fish at the V stage (Fig. 10A).

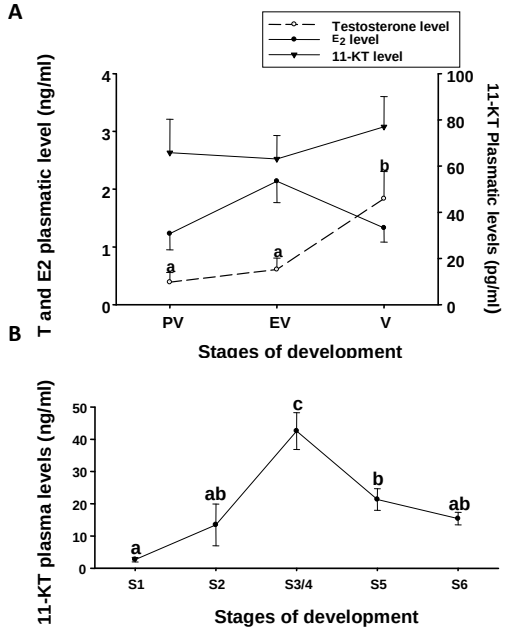

Fig. 10. A) Plasma levels of testosterone, 17β-estradiol (E2), and 11-ketotestosterone (11-KT) in female silver eels along carp pituitary extract hormonal treatment (data from Mazzeo et al. 2014, Chapter IV) (PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage). B) Plasma levels of 11-KT in male silver eels along human chorionic gonadotropin hormonal treatment (data from Peñaranda et al. 2010) (S1-S6, spermatogenic stages 1 to 6). Different superscript letters indicate significant differences in plasma steroid levels between developmental stages ($p<0.05$). To measure T and 11-KT plasma levels, commercial kits, T ELISA KIT (Eurobio Abcys, Les Ulis, France) and 11-KT competitive immunoassay (EIA) Kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), respectively, were used according to the manufacturer's instructions. The assay had a sensitivity of 1-2 pg/ml, and the standard curve was prepared between 0.78 and 100 pg/ml. Both duplicates from each plasma sample were assayed in the same test. Intra-assay variation was 10-15%. E2 plasma levels were measured by means of radioimmunoassay (RIA), according to Schulz (1984). The limit for the assay was 0.2 ng ml^{-1}, with a steroid recovery after ether extraction of 85.6±1.0%. E2 values were corrected for recovery. The inter- and intra-assay coefficients of variation (CV) for the E2 assay were 9.4% (n=4) and 5.2% (n=10), respectively.

In previous studies, E2 did not stimulate gonadotropin synthesis (mRNA or peptide) in the European eel pituitary *in vitro*, but did so

in vivo (Montero et al., 1996; Vidal et al., 2004). T on the other hand, has been shown to strongly induce pituitary *lhb* mRNA levels *in vitro*, but no effect was observed *in vivo* (Aroua et al., 2007). The lack of *in vivo* effect from T can be explained by T having a positive effect on the *d2rb* receptor and tyrosine hydroxylase gene expression in the eel forebrain (Weltzien et al., 2006; Pasqualini et al., 2009). In addition, it should be noted that the female eels used in the *in vivo* experiment by Aroua et al. (2007) were juvenile silver eels, and therefore *aa-ara*, *aa-arb*, and *aa-cyp19a1* transcript levels had probably not yet increased in the pituitary. The stimulation of gonadotropin synthesis *in vivo* by E2 could be explained by E2 having a positive effect on GnRH receptors in the pituitary (Levavi-Sivan et al., 2006; Lin et al., 2010). These data support our results, as both *aa-cyp19a1* and GnRH receptor transcripts increased in the pituitary during the same stage of development (V stage; See Fig. 4D and Peñaranda et al., 2013, Chapter II).

aa-cyp19a1 and *aa-ARs* expression levels in the ovary

As observed in the pituitary, the T increase (Fig. 10A) coincided with increased ovarian *aa-ara*, *aa-arb*, and *aa-cyp19a1* transcript levels at the V stage. This coincidence could indicate that T has a positive feedback on these genes in the pituitary and gonad, but not in the brain. In Japanese eel, *aj-ara* (*Anguilla japonica* ARa) mRNA transcripts were stable during maturation until the migratory nucleus stage, whereas *aj-arb* increased in mid-vitellogenesis (Tosaka et al., 2010). Although comparison of transcript levels between different genes in relative qPCR experiments should be treated with caution, the Japanese results are consistent with our data, since *aa-arb* was expressed in higher levels than *aa-ara* in the pituitary and gonad during vitellogenesis. In fact, Ikeuchi et al. (1999) reported that *aa-arb* has a higher androgen-binding affinity than *aa-ara* in Japanese eel, supporting the predominant presence of the *aa-arb* gene during maturation. No differences were found in

11-KT plasma levels during the treatment (Fig. 10A), but it has been demonstrated that 11-KT plays an important role in controlling pre-vitellogenic oocyte growth in *A. japonica* and *A. australis* (Lokman et al., 2003; Matsubara et al., 2003b; Kazeto et al., 2011), promoting the growth and lipid transfer and/or accumulation of previtellogenic oocytes (Lokman et al., 2007; Endo et al., 2008). No apparent correlation was found between E2 plasma levels and *aa-cyp19a1* expression, possibly because E2 in the ovary has only a local effect. Our gene expression results are consistent with Japanese eel data. Matsubara et al. (2003a) reported increased levels of ovarian *cyp19a1* during vitellogenesis, and a decrease in the subsequent stages. The aromatase activity in hormonally treated Japanese eels remained low when the Gonadosomatic Index (GSI) was below 8% (in the PV and EV stages), but increased in late vitellogenesis (GSI 12-15%) (Jeng et al., 2005).

4.3 Gene expression during male gonadal development

aa-cyp19a1 and *aa-ARs* expression levels in the pituitary

The different expression profiles and transcript levels observed for *aa-ara* and *aa-arb* in the male pituitaries could indicate that they have roles in spermatogenesis, but published information on this is limited to a few tissues and/or a single season/reproductive stage (Harbott et al., 2007; Diotel et al. 2010). In *Spinibarbus denticulatus* (Cyprinidae), significantly higher AR expression was observed in the pituitaries of fully recrudesced fish when compared with early and late recrudesced fish (Liu et al., 2009). Further studies are necessary in order to clarify the roles of these genes during reproductive maturation in male teleosts, but the elevated expression levels of *aa-arb* in stage 1 and 2 male eels could indicate that androgens in the pituitary play a role at the start of spermatogenesis.

As a consequence of the hormonal induction in eels, androgen production is greatly stimulated at the beginning of treatment (Khan et al., 1987; Miura et al., 1991), thus allowing the possibility of T being transformed to E2 by the pituitary aromatase (Miura et al., 1999). This is consistent with the increase in *aa-cyp19a1* observed in our experiment. In fact, it has been observed that locally synthesized E2 can increase the pituitary responsiveness to exogenous GnRH analogs in male goldfish (Trudeau et al., 1991). In addition, directly following the increase in *aa-cyp19a1* (stage 3/4), an increase in *lhb* levels was observed in the male pituitaries of the same fish, with high levels remaining throughout the subsequent stages of development (Peñaranda et al., 2010).

aa-cyp19a1 and *aa-ARs* expression levels in the testis

11-KT is the natural androgen with the highest affinity to both AR in Japanese eel (Ikeuchi et al., 2001). It is possible that the increase in 11-KT could have a negative effect on the expression of *aa-ara* and *aa-arb* in testis. In fact, the quantity of 11-KT in the blood of the same fish increased 16 fold from stage 1 to stage 3/4 (Fig. 10B; Peñaranda et al., 2010). As in the pituitary, *aa-arb* expression levels were much higher (~100 fold) than *aa-ara* levels, thus demonstrating that *aa-arb* is also the physiologically most relevant AR in the testis.

It has been shown that E2 stimulates synthesis of platelet-derived endothelial growth factor in Japanese eel testis, which is a spermatogonial stem cell renewal factor during spermatogenesis (Miura and Miura, 2011). Therefore, high *aa-cyp19a1* expression levels at the start of spermatogenesis are consistent with the role of E2 during male maturation, decreasing over the course of gonad development.

4.4 Conclusion

In summary, *aa-arb* was expressed at higher levels than *aa-ara* in the pituitary and gonad of both sexes of European eels, suggesting that *aa-arb* could be considered the physiologically most relevant AR in these tissues. In the brain, however, it seems that a decrease in *aa-ara* and an increase in *aa-cyp19a1* levels coincide with gonad development, suggesting decreased androgen and increased estrogen levels are necessary for advancement of the maturation process. In the female pituitary and ovary, *aa-ar*, *aa-arb*, and *aa-cyp19a1* expression levels increased in line with gonad maturation. However, in the males the results were different; in the male pituitary, opposing patterns were observed in the expression of *aa-cyp19a1* and *aa-ara*/*aa-arb*, with *aa-cyp19a1* increasing with gonad development, while *aa-ara*/*aa-arb* levels decreased. In the testis, both *aa-cyp19a1* and *aa-ara* gene expression levels decreased during spermatogenesis. A clear sex differentiation related to *aa-cyp19a1* and *aa-ara* and *aa-arb* gene expression along the BPG axis was observed, but it seems that, irrespective of the sex, the balance between androgens and estrogens could be one of the mechanisms by which gonad maturation is controlled.

5. Acknowledgements

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male eels used in the experiments. The English revision was done by Professor Lucy Robertson (Lucy Robertson Writing Services, Norway).

6. Conflict of interest

None of the authors have any conflict of interest to declare.

7. Author contributions

DSP, JFA, FAW and LP participated in the design of the study. VG and IM carried out the experimental work with animal facilities. DSP, JH and RNL performed the molecular biology analyses. DSP analyzed the data and wrote the manuscript, and all co-authors commented on it.

CHAPTER II

Molecular characterization of three GnRH receptor paralogs in the European eel, *Anguilla anguilla*: Tissue-distribution and changes in transcript abundance during artificially induced sexual development.

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Abstract

Gonadotropin-releasing hormone receptor (GnRH-R) activation stimulates synthesis and release of gonadotropins in the vertebrate pituitary and also mediates other processes both in the brain and in peripheral tissues. To better understand the differential function of multiple GnRH-R paralogs, three GnRH-R genes (*gnrhr1a, 1b, and 2*) were isolated and characterized in the European eel. All three *gnrhr* genes were expressed in the brain and pituitary of pre-pubertal eels, and also in several peripheral tissues, notably gills and kidneys. During hormonally induced sexual maturation, pituitary expression of *gnrhr1a* (female) and *gnrhr2* (male and female) was up-regulated in parallel with gonad development. In the brain, a clear regulation during maturation was seen only for *gnrhr2* in the midbrain, with highest levels recorded during early vitellogenesis. These data suggest that GnRH-R2 is the likely hypophysiotropic GnRH-R in male eel, while both GnRH-R1a and GnRH-R2 seems to play this role in female eels.

Keywords: gene expression, gonad maturation, brain, qPCR, screening, sequence

1. Introduction

Gonadotropin-releasing hormone (GnRH) plays a central role in the neuroendocrine control of the reproductive process in vertebrates, notably by stimulating synthesis and release of pituitary gonadotropins (Weltzien et al., 2004). Teleost fish express two or three different forms (GnRH1-3), with only two forms having been detected in early evolved teleosts such as Elopomorphs: GnRH1 and GnRH2 (King et al., 1990; Okubo et al., 1999a). Similar to what has been shown in mammals (Hsueh and Schaeffer, 1985; Chieffi et al., 1991), extra-pituitary sites of GnRH actions have been detected in a number of reproductive and non-reproductive organs of different eel

species such as the ovary, testis, brain, liver, and kidney (Dufour et al., 1993; Okubo et al., 1999a,b).

The actions of GnRH are mediated through binding to membrane receptors (GnRH-R) belonging to the rhodopsin family of G-protein coupled receptors. GnRH-Rs are highly conserved through evolution, as demonstrated by the cloning of a functional *gnrhr* from invertebrates, including the octopus (Kanda et al., 2006) and identification of functional receptors from the tunicate, also including receptor heterodimerization as an additional level of regulation (Kusakabe et al., 2003; Tello et al., 2005; Sakai et al., 2010; 2012). The presence of several forms of GnRH in a single species, acting in multiple tissues, is often associated with the existence of different receptor subtypes. Indeed, there is increasing evidence for the presence of at least two GnRH-R types, Type I and Type II, in most vertebrate classes (Millar et al., 2001; Okubo et al., 2001; Wang et al., 2001; Kah et al., 2007; Hildahl et al 2011b). The first *Gnrhr* was cloned from the mouse αT3 gonadotrope cell line (Reinhart et al., 1992; Tsutsumi et al., 1992) and the first teleost *gnrhr* was cloned from African catfish (*Clarias gariepinus*) pituitary (Tensen et al., 1997). Subsequently, many teleost GnRH-Rs have been identified (Lethimonier et al., 2004; Weltzien et al., 2004; Levavi-Sivan and Avitan, 2005; Kah et al., 2007; Hildahl et al 2011b). Some species have been shown to encode up to five *gnrhr*s in their genome, as is the case for each of two pufferfish (*Fugu rupripes* and *Tetraodon nigroviridis*; Ikemoto and Park, 2005) and the European seabass (*Dicentrarchus labrax*; Moncaut et al., 2005). The cell/tissue distribution, regulation and function of the various GnRH-Rs are still not clear. Characterization of the spatial and temporal GnRH-R variant gene expression is therefore critical to improve our understanding of the physiological consequences of GnRH stimulation. To date, most studies on GnRH-R have focused on the brain and pituitary (Okubo et al., 2001; 2003; Jodo et al.,

2003; Millar et al., 2004; Hapgood et al., 2005; Moncaut et al., 2005). For example, in tilapia (*Astatotilapia burtoni*; Flanagan et al., 2007) and European seabass (González-Martínez et al., 2004), the Type II GnRH-R2a was proposed as the GnRH receptor involved in the control of gonadotropic function. GnRH-Rs also have been identified in peripheral tissues. In human ovary, *Gnrhr* expression was found in luteal granulosa cells (Cheng et al., 2002), and some studies suggest that the GnRH system is involved in the control of follicle atresia in the ovary (Kang et al., 2003). *gnrhr* transcripts in kidney and gill have been reported in several teleost species (Robison et al., 2001; Ikemoto and Park, 2005; Tello et al., 2008; Lin et al., 2010) demonstrating the possible relation between GnRH and osmoregulation. In spotted green pufferfish (*T. nigroviridis*) two of the five isolated GnRH-R forms were not detected in the pituitary (Ikemoto and Park, 2005), while the four functional GnRH-Rs isolated from adult zebrafish showed different tissue distribution (Tello et al., 2008). These data suggest the different receptors to have different functional roles.

In vivo and *in vitro* experiments have shown that gonadal steroids participate in the regulation of GnRH-R transcripts. For instance, estradiol-17β (E2) showed a positive effect on *gnrhr* expression in pre-spawning black porgy (*Acanthopagrus schlegeli*; Lin et al., 2010) and vitellogenic female tilapia (Levavi-Sivan et al., 2006). In tilapia, a GnRH3 analog stimulated pituitary *gnrhr* transcripts *in vivo* and *in vitro* (Levavi-Sivan et al., 2004), whereas in grouper GnRH2 and GnRH3 inhibited while GnRH1 stimulated pituitary *gnrhr* transcript expression (Hsieh et al., 2007). However, more studies are needed to clarify the regulation and functional significance of the individual GnRH-R forms.

European eel is an important aquaculture species, and belonging to the early evolved Elopomorpha, knowledge of its GnRH systems may provide valuable information about the classification and

function of GnRH-Rs in later evolving teleost species. The current work reports for the first time three *gnrhr* genes in an eel species and characterizes their brain and pituitary expression during artificial maturation, hence suggesting the functional significance of each receptor form.

2. Materials and Methods

2.1 Fish maintenance, hormonal treatment and sampling

2.1.1 Maturation of male European eel

One hundred and fifty pre-pubertal male eels (124.1±12.6 g body weight; 39.9±0.21 cm body length) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. For one week, the fish were gradually acclimatized to seawater (salinity 37±0.3 ‰). Simultaneously, the water temperature was changed from environmental conditions to 20 ºC. After one week, fish were distributed in equal numbers in three 500 L fibreglass tanks equipped with separate recirculation systems.

Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of human chorionic gonadotropin (hCG) for 13 weeks (1.5 IU g/fish; Angelini Farma-Lepori, Barcelona, Spain), as previously described by Pérez et al. (2000). From the beginning of the experiment and once per week, 6 males were anaesthetised and sacrificed by decapitation to obtain pituitary and gonad samples that were stored in 0.5 ml of RNAlater (Ambion Inc, Huntingdon, UK) at -20 ºC until RNA extraction.

2.1.2 Maturation of female European eel

Forty-four wild pre-pubertal female eels at the silver stage (body weight 744.7±22.2 g; body length 72.2±0.65 cm) caught by local fishermen in the Albufera Lagoon during their migration to the sea were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. Fish were acclimatised for two weeks from fresh water to sea water (salinity 37.0±0.3‰) after which water temperature was progressively changed from environmental temperature to 18 ºC, a temperature that was maintained during the rest of the experiment. The fish were distributed in equal numbers in two 500 L fibreglass tanks equipped with separate recirculation systems.

Females were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, Catvis Ltd, Netherlands) for 12 weeks (20 mg/kg body weight). The CPE was diluted in 0.9 g/l NaCl solution (0.9 g/l), centrifuged (1260 g, 10 min) and the supernatant stored at -20 ºC until use one to four weeks later. Groups of 6 female eels were anaesthetised and sacrificed by decapitation at 0, 4, 8 and 12 weeks of treatment, in addition to six animals newly obtained from the Albufera Lagoon (fresh water conditions). These animals served to observe a possible seawater acclimation effect. Upon sampling, the brain, pituitary and gonad were removed and stored in 0.5 ml of RNAlater (Ambion) at -20 ºC until extraction. The brain was divided into three parts: olfactory bulb, telencephalon and di-and mesencephalon as previously reported by Weltzien et al. (2005b).

2.2 Histology processing

For both experiments, after fixation in 10% formalin buffered at pH 7.4, ovaries and testes were dehydrated in ethanol and embedded in paraffin for histological staging. Sections of 5 µm thickness were made with a Shandom Hypercut manual microtome and stained with hematoxylin and eosin. Slides were observed with a Nikon

Eclipse E400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope.

Stages of spermatogenesis were determined according to the most advanced germ cell type present and to their relative abundance (Utoh et al., 2004; Huertas et al., 2006; Peñaranda et al., 2010; Fig. 4.1). Stage 1 was characterized by the presence of spermatogonia type A and/or B, stage 2 by the presence of spermatogonia and spermatocytes, at stage 3 spermatids appeared in the testis tissue, and at stages 4, 5 and 6 spermatozoa appeared inside the lumen of lobules. The difference between stages 4-6 was the relative abundance of sperm in the lobule lumen or the different proportions between the volume of lumen filled with sperm and the volume of the whole lobule. Stage 4 was characterized by small lumens, which comprised 10-45% of the whole lobules. Indeed, in some testes parts of the lobules were filled with spermatozoa, while other lobules contained only spermatids. Stage 5 was characterized by an increase in the quantity and proportion of spermatozoa in the gonad, comprising 50-70% of the total volume of the lobule. The lobule wall showed all the germinal cell types: spermatogonia, spermatocytes and spermatids, which did not appear inside the lumen. Stage 6 was characterized by a reduction in the germinal cells other than the spermatozoa with a proportion between spermatozoa and whole lobules between 75-95%. In this stage elongated lumens were observed that were often fused in long wide spaces filled with spermatozoa.

The ovarian development observed during the 12 weeks of treatment was classified in 3 stages, according to previous works (Selman and Wallace, 1989; Kayaba et al., 2001; Pérez et al., 2011; Fig. 4.2): Previtellogenic (PV) - in perinucleolus stage with none or few lipid droplets or in the lipid droplet stage but without yolk vesicles. Early vitellogenic (EV) - small yolk vesicles restricted to the periphery of the oocyte. Vitellogenic (V) - including medium and late vitellogenesis. Medium vitellogenesis is characterized by

abundant yolk vesicles in the cytoplasm from the membrane to the nucleus and a lower proportion of yolk vesicles than lipid droplets. Late vitellogenesis is described by enlarged yolk vesicles and smaller proportion of lipid droplets than yolk vesicles.

2.3 Molecular cloning of eel *gnrhr* cDNAs

In the current work, the nomenclature for GnRH-Rs follows the classification performed by Hildahl et al. (2011b). Total RNA was isolated from RNAlater preserved tissues as described by Hildahl et al. (2011a). First-strand cDNA was synthesized from 2 µg of total RNA using random hexamer primers and superscript III reverse transcriptase (Invitrogen). Design and theoretical evaluation of PCR primers were performed using the Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) and Vector NTI software (Invitrogen; Table 1), respectively. *aa-gnrhr1b* PCR primers for initial fragment cloning were designed from the corresponding Japanese eel sequence (GenBank accession number AB041327). *aa-gnrhr2* primers were selected from a partial sequence of the corresponding receptor from Japanese eel kindly provided by Dr. Kataaki Okubo (University of Tokyo). PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 µl contained 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. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. PCR products were visualized in 2% agarose gel stained with ethidium bromide 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 sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, University of Oslo sequencing service, Oslo, Norway).

The obtained partial sequences were used to design gene specific primers for use with SMART RACE PCR (Clontech, CA, USA) to identify the 5' and 3' end sequences. Following RACE PCR and gel electrophoresis, a single band was isolated, cloned and sequenced as described above. Full-length sequences were confirmed by comparing with the results recently obtained using the massive parallel sequencing of the eel genome (Henkel et al., 2012). This analysis revealed an additional third *gnrhr* gene, which was identified as the putative *aa-gnrhr1a* based on sequence alignment and phylogenetic analysis. A fragment of this gene was then cloned and sequenced as described above. The three *aa-gnrhr* cDNA sequences were submitted to GenBank and subsequently assigned the following accession numbers: JX567769.1 (*aa-gnrhr1a*), JX567770.1 (*aa-gnrhr1b*), JX567771.1 (*aa-gnrhr2*).

2.4 Phylogenetic analysis

The phylogenetic analysis was carried out according to Hildahl et al. (2011b). Briefly, the deduced amino acid sequences from available teleost *gnrhr* genes in addition to at least one representative species from the other vertebrate classes – *Homo sapiens*, *Macaca mulatta, Macaca radiate, Mus musculus* (mammalia); *Gallus gallus* (aves); *Eublepharis macularius* (reptilia); *Rana ridibundus*, *Xenopus laevis* (amphibia) - were aligned, and PHYML analysis was carried out using the Seaview 4.2.12 molecular phylogeny software package (Gouy et al., 2010). The aligned sequences spanned the first transmembrane domain to the end of the seventh transmembrane domain and partial sequences were omitted to ensure good alignment. The model of protein evolution was determined using ProTest v1.2.7 software (Drummond and Strimmer 2001; Guindon and Gascuel 2003; Abascal et al 2005).

Octopus were used as an outgroup, and the robustness of the obtained tree was analyzed by bootstrap analysis using 500 data set replicates. The alignment file was also used to identify conserved residues and variant specific motifs.

2.5 Tissue-Specific Expression of the Eel *gnrhrs*

Three pre-pubertal silver male and three pre-pubertal silver female eels with average body weights of 118.0±6.47 and 710.7±52.61 g, respectively, were sacrificed. RNA was extracted using the methodology explained above from nine different tissues: gonad, liver, pectoral fin, anterior and posterior kidney, heart, gill, pituitary, and brain. The brain was divided into five parts: olfactory bulbs, telencephalon, di- and mesencephalon, cerebellum, and medulla oblongata as previously reported by Weltzien et al. (2005b). Total RNA was treated with DNase I (Turbo DNA-free, Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III reverse transcriptase (Invitrogen) and random hexamer primers according to standard protocol. The expression of *aa-gnrhr* transcripts in the various tissues was analyzed by qPCR.

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

2.6.1 Primer design

Acidic ribosomal phosphoprotein P0, ARP (Table 1; Weltzien et al., 2005b; Aroua et al., 2007; Peñaranda et al., 2010) was used as reference gene in the quantitative real-time reverse trancriptase polymerase chain reaction (qPCR), because its mRNA expression has been shown to be stable also during experimental treatment (Weltzien et al., 2005b). The Primer3 program (http:// frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers to quantify the *aa-gnrhr* gene expression (Table 1).

To avoid detection of genomic DNA (gDNA), primers were designed to span an exon-exon boundary or for one of the primers to anneal to an exon-exon boundary. All primers were tested on gDNA and RNA to confirm that they would not amplify potentially contaminating gDNA. The specificity was confirmed by melting curve analysis, gel electrophoresis, and by sequencing of the qPCR products. Furthermore, the possible cross-reactivity between *aa-gnrhr* assays was tested by qPCR using *aa-gnrhr1a*, *1b*, or *2* clones as template.

2.6.2 SYBR Green assay (qPCR)

In order to measure changes in gene expression of *aa-gnrhr*s, qPCR assays were developed and expression analyses performed using a Light Cycler 480 system with SYBR Green I sequence-unspecific detection (Roche, Meylan, France). After an initial activation of *Taq* polymerase at 95 ºC for 10 min, 42 cycles of PCR were performed using the Light Cycler with the following cycling conditions: 95 ºC for 10 s, 60 ºC for 10 s and 72 ºC for 7 s for *aa-gnrhr1a* and *aa-gnrhr1b*; and 95 ºC for 10 s, 61 ºC for 7 s and 72 ºC for 4 s for *aa-gnrhr2*. To evaluate assay specificity, melting curve analysis was performed directly following PCR by slowly (0.1 ºC/s) increasing the temperature from 68 to 95 ºC with a continuous registration of changes in fluorescent emission intensity. The total volume for every PCR reaction was 10 µl using diluted (1:10) cDNA template (3 µl), forward and reverse primers (250 nM each) and SYBR Green Master Mix (5 µl). Relative standard curves were used to assess PCR efficiency for *aa-gnrhr*s and ARP and to decide which dilutions to use for the unknown samples (Table 1). The efficiency adjusted normalized starting concentration was determined by an efficiency adjusted delta-delta Cq method (Weltzien et al., 2005b). The target gene expression quantification was expressed relative to reference gene expression (ARP; Weltzien et al., 2005b). 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 controls (cDNA was replaced by water) for each primer pair were run on all plates.

2.7 Statistical analysis

The data were subjected to analysis of variance (One-way ANOVA). A Student-Newman-Keuls test was used for the comparison between means at a 0.05 significance level ($p<0.05$). The results are presented as means ± standard error of means (SEM). All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1 Cloning and identification

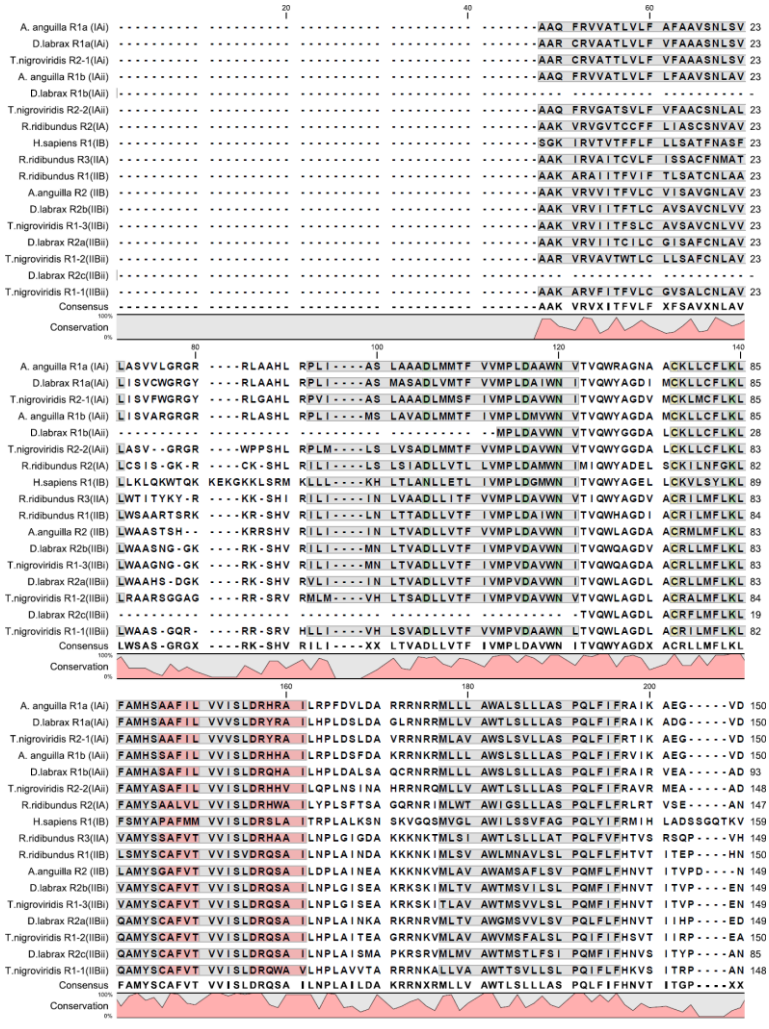
We have isolated three GnRH-R cDNAs in European eel, two Type IA and one Type IIB receptor. These were named according to the nomenclature presented by Hildahl et al. (2011b), whereby the two Type IA receptors were given a putative 1a and 1b designation (*aa-gnrhr1a* and *aa-gnrhr1b*, GenBank accession numbers JX567769.1 and JX567770.1, respectively) based on distinct conserved motifs in the third transmembrane domain. The sub-type of the Type IIB gene (*aa-gnrhr2*, GenBank accession number JX567771.1) could not be determined by conserved sequence or phylogenetic analysis. The full-length sequences were 1158 bp (*r1a*), 1086 bp (*r1b*) and 966 bp (*r2*). All three sequences contain conserved sequences with other vertebrate species (Hildahl et al., 2011b), including Type-specific micro-domains in TM3 and a tri-peptide domain in the third extracellular region. The putative *aa-gnrhr1a* has AAFIL and DRHRAI micro-domains, whereas the putative *aa-gnrhr1b* has SAFIL and DRHHAI. These domains were identified as GAFVT and DRQSAI for *aa-gnrhr2*. In addition both Type IA variants possess the PEY tri-peptide and *aa-gnrhr2* has a SHS sequence similar to other teleost receptors (Fig. 1).

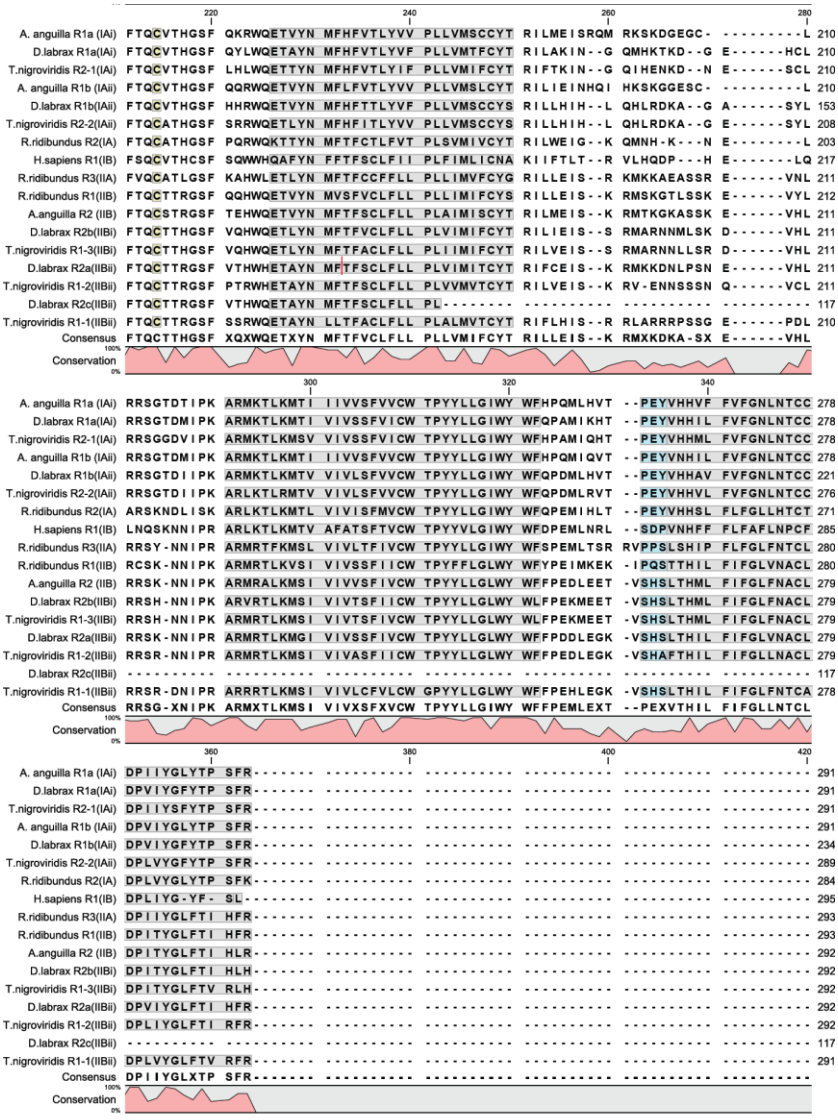
Table 1. Primer sequence for RT-PCR to identify gene fragments, 5′ and 3′ RACE PCR and qPCR.

Name	Sequence (5′-3′)	Orientation	Usage
aa-gnrhr1a	TGG TCA TGA GTT GCT GCT ACA	Forward	qPCR[1] (82 bp)
	AGA CAC CCC TCT CCG TCT TT	Reverse	qPCR[2] (E=1.862)
aa-gnrhr1b	AGA CGG AGG AAT TGG AGG AGG A	Reverse	RACE 5′ PCR
	AAA CCA GCA GCA TGC ACA CCT TCA G	Forward	RACE 3′ PCR
	TCG TCA CGC TCT ACG TTG TC	Forward	qPCR[1] (107 bp)
	AGG CAG GAC TCT CCA CCT TT	Reverse	qPCR[2] (E=2.037)
aa-gnrhr2	GCG CTT GCG CAG GTG GAT GGT GAA C	Reverse	RACE 5′ PCR
	GGA AGA ACC AGT ACC ACA GGC CCA GCA GGT AG	Reverse	Nested RACE 5′ PCR
	CTC CAA ACG CAT GAC CAA AGG GAA GGC AT	Forward	RACE 3′ PCR
	CTT CCA AGG AAG TGC ATC TCC GC	Forward	Nested RACE 3′ PCR
	TCA CCT TCT CCT GCC TCT TC	Forward	qPCR[1] (105 bp)
	TTG GAA GAT GCC TTC CCT TT	Reverse	qPCR[2] (E=1.876)
aa-arp	GTG CCA GCT CAG AAC ACG	Forward	qPCR[1] (107 bp)
	ACA TCG CTC AAG ACT TCA ATG G	Reverse	qPCR[2] (E=2.142)

[1] qPCR amplicon length is given in parenthesis following the forward qPCR primers.
[2] qPCR efficiency for each primer pair is given in parenthesis following the reverse qPCR primer

Fig. 1. (following on next page) Conserved sequences among vertebrate GnRH-R variants. Alignment of representative fish (dl, tn, aa) Type I and Type II GnRH-R variants, non-mammalian tetrapod Type I (rr) and Type II (rr) GnRH-R and human Type I GnRH-R, spanning transmembrane regions 1 – 7. Transmembrane regions are boxed in grey. Additional conserved amino acid residues involved in control of GnRH-R expression and ligand binding (green and yellow), micro-domains in transmembrane region 2 (pink), and a tri-peptide sequence in extracellular domain 3 (blue) are identified. Percent conservation between amino acid sequences is shown at the bottom of the alignment in pink. Receptor variant names as given in GenBank are given following the species name and the Type distinctions according to Hildahl et al., 2011b are given in parenthesis.



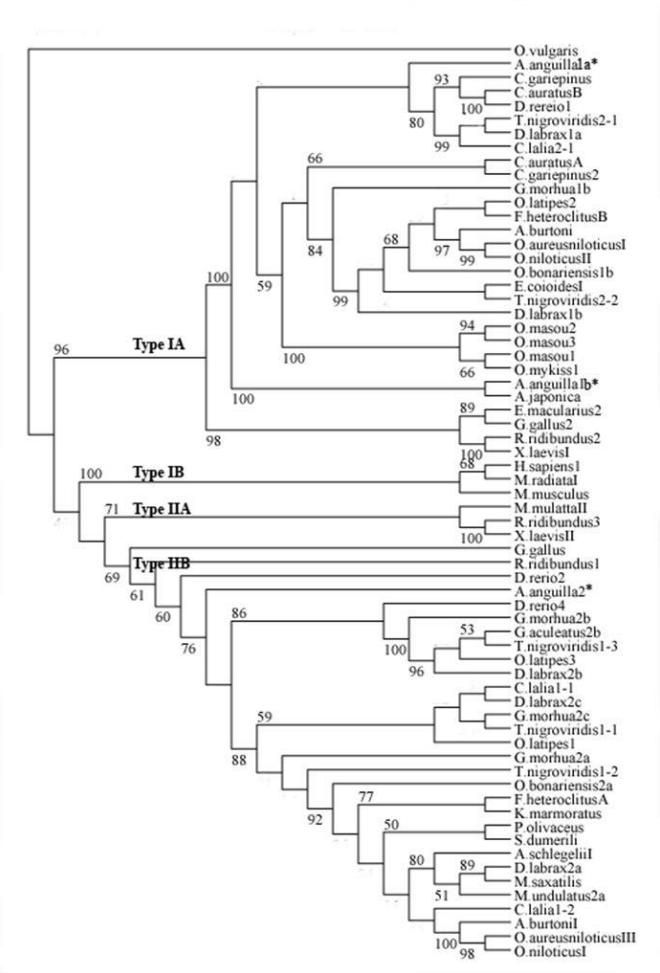


3.2 Phylogenetic analysis

The phylogenetic tree presented in the current study (Fig. 2) supports the division of GnRH-Rs into two Types, I and II, each of which have subforms A and B, giving a total of four clades; IA, IB, IIA and IIB, as presented in Hildahl (2011b). Teleost fish group into

two main clades, IA and IIB, distinct from the mammalian Type IB and tetrapod Type IIA clades. The European eel receptors associated with teleost fish Type I and Type II clades, but sub-types could not be determined from this analysis. *aa-gnrhr1b* grouped with the previously identified Japanese eel *gnrhr*, but these receptors branched from the main teleost Type I clade, prior to the distinction of the *gnrhr1a* and *gnrhr1b* clades. Similarly, *aa-gnrhr2* formed a mono-phylogenetic clade with the teleost Type II *gnrhr*s, but it branched from the teleost sub-type clades prior to their branching. The putative *aa-gnrhr1a* associated with the teleost *gnrhr1a* clade, but the bootstrap analysis did not irrefutably support this distinction.

Fig. 2 (next page). Phylogenetic tree. Phylogenetic tree showing the relationship among vertebrate GnRH-R amino acid sequences spanning transmembrane domain 1 to transmembrane domain 7. The tree was generated by maximum likelihood analysis in Seaview 4 software using an evolutionary replacement model generated by ProtTest. Bootstrap analysis was performed on 500 trees and the values greater than or equal to 50% are provided on the branch nodes. The three European eel GnRH-R variants (accession numbers: JX567769.1, JX567770.1, JX567771.1) are organized in two separate clades with non-mammalian Type IA and Teleost Type IIB receptors. Note the receptor subtypes could not be determined from this analysis and were further determined by conserved sequence analysis. The sequences were as follows with the NCBI accession number and given variant indicator where available in parenthesis (ni = not indicated): A. burtoni (ni, AAK29745; I, AAU89433); A. japonica (ni, BAB11961); A. schlegelii (I, AAV71128); C. auratus.(A, AAD20001; B, AAD20002); C. gariepinus (ni, CAA66128; 2, AAM95605); C. lalia (2–1, BAE87050; 1–2, BAE87049; 1–1, BAE87048); D. labrax (1a, CAE54804; 1b, CAE54806; 2a,CAD11992; 2b partial sequence, CAE54807; 2c, CAE54805); D. rerio (1, NP001138452; 2, NP001138451; 4, NP001091663; 3, NP001170921); E. coioides (I, ABF93210); E. macularius (2, BAD11150); F heteroclitus (A, BAG12379; B, AB471799); G. aculeatus (clone CH213-16009); G. gallus (ni, NP001012627;2, NP989984); G. morhua (1b, ADD92008; 2a, ADD92009; 2b, ADD92010; 2c, ADD92011); H. sapiens (1, NP000397); K marmoratus (ni, ABK88381); M. musculus (ni, NP034453); M. mulatta (II, AAK52745); M. radiate (I, AAK52745); M. saxatilis (ni, AAF28464); M. undulatus (2a, ABB97085); O. bonariensis (1b, ABI75337; 2a, ABI75336); O. latipes (1, BAB70504; 3, BAC97833; 2, BAB70503); O. aureusniloticus (I, AAQ88391; III, AAQ88392); O. mykiss (1, CAB93351); O. niloticus (I, BAC77240; II, BAC77241); O. vulgaris (ni, BAE66647); P. olivaceus (ni, AAY28982); R. ridibundus (1, AAP15162; 2, AAP15163; 3, AAP15164); S. dumerili (ni, CAB65407); T. nigroviridis (2–1, BAE45700; 2–2, BAE45702; 1–1, BAE45694; 1–2, BAE45696; 1–3, BAE45698); X. laevis (I, NP001079176; II, AAK49334). Species Types are identified as defined in described in Hildahl et al (2011b).



3.3 Tissue specific expression of eel *gnrhr* transcripts

qPCR analysis with *SYBR Green* was utilized to characterize the tissue-specific expression pattern of the eel *aa-gnrhr* transcripts in five parts of the dissected brain, in addition to the pituitary, gonad, liver, pectoral fin, anterior and posterior kidney, heart, and gill from pre-pubertal silver eel. Differential expression was found between the sexes (Fig. 3B and 3C). *aa-gnrhr1b* transcripts were detected in all tissues except in the male liver, fin and anterior/posterior kidney

(Fig. 3B). In most of the tissues, females showed higher *aa-gnrhr1b* expression compared to males with the highest levels being observed in the olfactory bulbs, di- and mesencephalon, and cerebellum.

As for *aa-gnrhr1b*, differential expression between sexes was found for *aa-gnrhr2* with no transcripts detected in the female pectoral fin or male cerebellum (Fig. 3C). Gonads, olfactory bulbs, telencephalon, di-and mesencephalon, and medulla oblongata were the tissues that showed highest transcript levels in both sexes. *aa-gnrhr2* transcripts were observed neither in females nor in males in the following tissues: liver, anterior and posterior kidney, heart, and gill. Unlike *aa-gnrhr1b* and *aa-gnrhr2*, *aa-gnrhr1a* did not show differences between sexes (Fig. 3A). The highest transcript levels were found in the gonads and kidney, while no *aa-gnrhr1a* transcripts were detected in the following tissues: liver, fin, heart and gill.

3.4 Gene expression through gonad development

3.4.1 Male gonad development

Changes in gonad development during hormonal treatment in males are shown in Fig. 4-1A to F, and Fig. 5A. Stage 1 was present only during the first 2 weeks of treatment, while stage 2 was observed in males from week 1 to 3. The percentage of males with stage 3 gonads was very low during week 3 and 4, probably because this is a short-time stage in the cell development process. For this reason, stages 3 and 4 were analyzed together (stage 3/4). In week 7, males with maximally developed gonads (stage 6) were observed for the first time, and thereafter during the rest of the experiment.

Gene expression in male pituitary was analyzed using 6 animals every two weeks of treatment. *aa-gnrhr1a* and *aa-gnrhr1b* gene expression was stable during gonad development, showing no

significant differences neither during the weeks of treatment nor at the different stages of development (Fig. 6A-D).

aa-gnrhr2 expression, on the other hand, was elevated compared to fresh water-sampled animals after only two weeks of treatment (Fig. 6E), maintaining this progressive increase along the experiment up to the 10th week of treatment before a non-significant decrease at week 12. When analyzing the data by the stages of gonad development instead of weeks of treatment, *aa-gnrhr2* provided similar results (Fig. 6F), displaying a progressive increase along spermatogenesis and becoming significantly different in the last stages of development (stage 5 and 6).

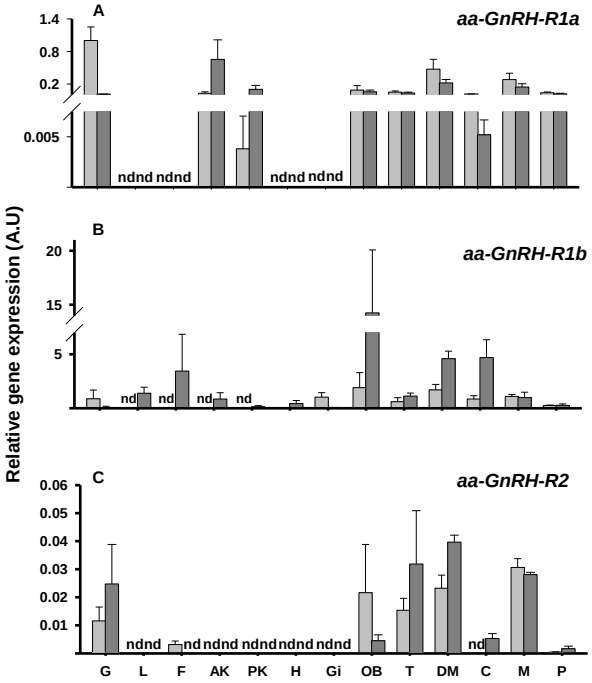


Fig. 3. Quantitative gene expression of three GnRH receptors variants in various tissues from immature eel, males (gray) and females (dark gray). N = 3 for all tissues. Tissues in which genes were not detected are labeled nd (not detectable). Tissue abbreviations are G = gonad, L = liver, F= fin, AK= anterior kidney, PK= posterior kidney, H= heart, Gi= gill, OB= olfactory bulb, T= telencephalon, DM= di-mesencephalon, C= cerebellum, M= medulla oblongata, and P= pituitary.

3.4.2 Female gonad development

The experiment was arrested at 12 weeks of treatment, before ovulation was reached, due to a lack of animals to continue the experiment. Therefore the gonad development was divided in three stages: pre-vitellogenic (PV), early vitellogenic (EV) and vitellogenic (V) stage (Fig. 4-2A to D).

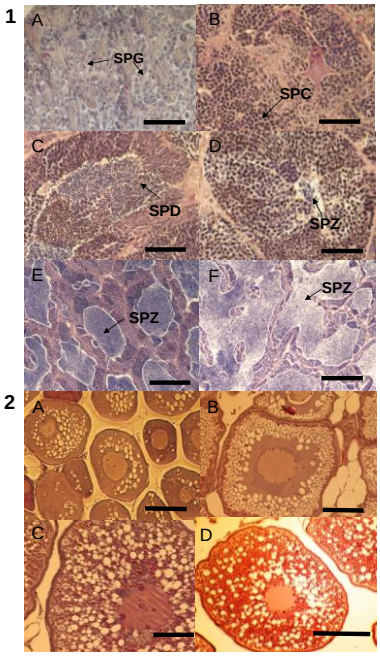


Fig. 4. Photomicrographs of histological sections for the different stages found during treatment. 1) Sections for the different males stages found during treatment: 1.A) Testis at stage 1. 1.B) Testis at stage 2. 1.C) Testis at stage 3. 1.D) Testis at stage 4. 1.E) Testis at stage 5. 1.F) Testis at stage 6. SPG, spermatogonia; SPC, spermatocyte; SPD, spermatid; SPZ, spermatozoa. 2) Sections of oocytes at different times during hormonal treatment. 2.A) Previtellogenic stage. 2.B) Early vitellogenic stage 2C and 2D) Mid and Late Vitellogenic stage. Scale bar, 100 µm.

At 4 weeks of treatment, 83.3% of the females had reached early vitellogenesis (Fig. 5B). After 8 weeks of treatment, similar proportions of the three stages (33 %) were observed, while at 12 weeks 83.3 % of the animals had reached the vitellogenic stage.

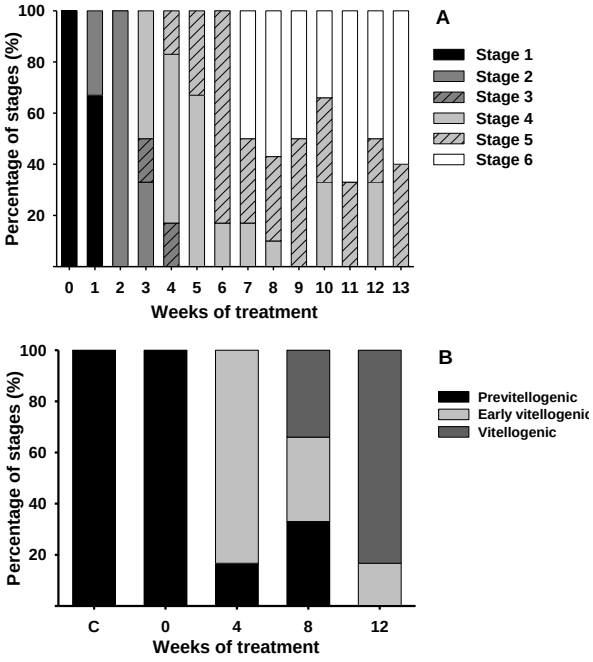


Fig. 5. Percentage of the different stages of gonad development during the weeks of treatment. A) Percentage of stages (1-6) gonad during the treatment in male eels. B) Percentage of the different stages of ovarian development at 0, 4, 8 and 12 weeks of CPE treatment, and fresh water conditions (c). Previtellogenic stage (black); early vitellogenic stage (gray); vitellogenic stage (dark gray).

aa-gnrhr1a expression in the olfactory bulbs decreased from fresh water to seawater samples, remaining low for the remainder of the experiment (Fig. 7A). Similar results were observed in pituitary samples (Fig. 7D). In telencephalon and di-and mesencephalon, no differences were found during the treatment (Fig. 7B and 7C). *aa-gnrhr1b* showed a significant increase at 12 weeks of treatment in the pituitary (Fig. 7H), whereas no differences were observed in the different parts of the brain at any week (Fig. 7E, 7F and 7G).

aa-gnrhr2 did not change in the olfactory bulbs (Fig. 7I), whereas a higher expression was observed at 4 weeks of treatment in the

telencephalon and di-and mesencephalon (Fig. 7J and7K) and a significant increase at 12 weeks in the pituitary (Fig. 7L).

Similar profiles were observed when the gene expression was related to stages of ovarian development. *aa-gnrhr1a* expression levels in the pituitary and different parts of the brain did not change significantly during the different stages of development (Fig. 8A-D). At the vitellogenic stage, a significant increase was shown for *aa-gnrhr1b* in the pituitary (Fig. 8H), but no differences were found in the brain (Fig. 8E-G). Finally, *aa-gnrhr2* showed higher expression in the di- and mesencephalon at the beginning of vitellogenesis (Fig. 8K). Furthermore, its expression increased at the vitellogenic stage in the pituitary (Fig. 8L). No differences were observed in olfactory bulbs and telencephalon for *aa-gnrhr2* transcripts (Fig. 8I and 8J).

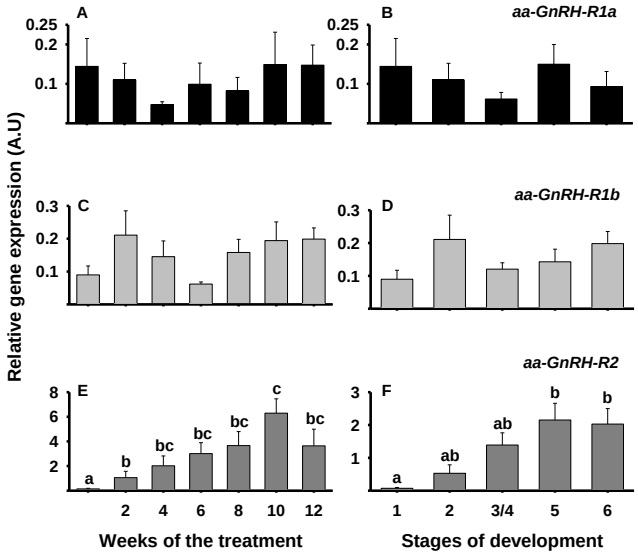


Fig. 6. Effect of treatment on *aa-gnrhr* expression in male eel pituitary. *aa-gnrhr1a* (black), *aa-gnrhr1b* (gray) and *aa-gnrhr2* (dark grey) expression at different weeks of treatment (A,C,E) and in relation to the stage of testis development (B,D,F). Different superscript letters mean significant differences ($p<0.05$), n = 5-6.

4. Discussion

4.1 Phylogenetic analysis

The phylogenetic analysis clearly distinguishes the *aa-gnrhr*s as Type IA (*aa-gnrhr1a* and *aa-gnrhr1b*) and Type IIB (*aa-gnrhr2*) receptors. The branching of the putative *aa-gnrhr1b* and *aa-gnrhr2* prior to the separation of the subtype specific clades reflects the eel position as an early evolved teleost, although distinct subtypes are expected in eel based on the proposed three rounds of whole genome duplication (WGD) in teleost fish (Meyer and Van de Peer, 2005; Kah et al., 2007). Analysis of Hox gene clusters from the recent European eel genome sequencing supports the notion that the teleost specific WGD occurred before the branching of the Anguilliformes (Henkel et al., 2012).

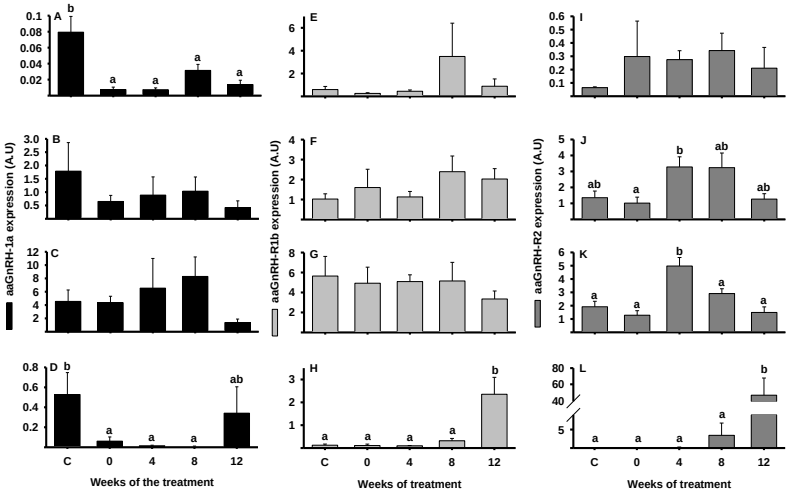


Fig. 7. Effect of treatment on *aa-gnrhr* expression at 0, 4, 8 and 12 weeks of CPE treatment, and fresh water conditions (c) in female eel brains and pituitaries. *aa-gnrhr1a* (gray), *aa-gnrhr1b* (dark grey) and *aa-gnrhr2* (black) expression at different weeks of treatment in olfactory bulb (A,E,I), telencephalon (B,F,J), di-mesencephalon (C,G,K), and pituitary (D,H,L). Different superscript letters means significant differences ($p<0.05$), n = 5-6.

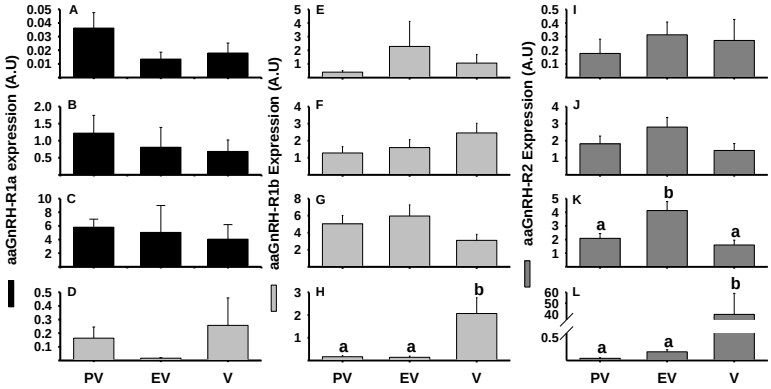


Fig. 8. Effect of treatment on *aa-gnrhr* expression in relation with the stage of ovarian development in eel female brain: previtellogenic stage (PV); early vitellogenic stage (EV); vitellogenic stage (V). *aa-gnrhr1a* (gray), *aa-gnrhr1b* (dark grey) and *aa-gnrhr2* (black) expression at different stages in olfactory bulb (A,E,I), telencephalon (B,F,J), di-mesencephalon (C,G,K) and pituitary (D,H,L). Different superscript letters means significant differences ($p<0.05$), n = 5-6.

The putative distinction of the eel Type I *gnrhr*s based on conserved motifs further supports the branching of these sub-types following a third round of WGD, although lack of a clear phylogenetic distinction of these receptors reflects the low overall conservation of *gnrhr* genes. The teleost Type II *gnrhr*s are more highly conserved with no distinct functional motifs currently identified (Hildahl et al., 2011b), and at present the significance of subtype proliferation is poorly understood. The different teleost Type II *gnrhr* variants are differentially regulated in multiple species (Ikemoto and Park, 2005; Moncaut et al., 2005; Hildahl et al., 2011b) and *gnrhr2a* appears to be the most likely hypophysiotrophic receptor (Flanagan et al., 2007; Lin et al., 2010; Hildahl et al 2011b). Further information concerning the functional regulation of the Type II *gnrhr*s should help clarify their molecular evolution.

4.2 Tissue specific expression of *aa-gnrhr* transcripts

Differential expression profiles were observed for the three *aa-gnrhr* genes in the different sexes and tissues, similar to other teleosts (Yu et al., 1998; Robison et al., 2001). This emphasizes the complex regulation of the different receptor variants, making it difficult to speak about their function in general. The widespread tissue distribution also supports the involvement of GnRH in diverse physiological processes.

All *aa-gnrhr* transcripts were detected in all parts of the brain (except *aa-gnrhr2* in male cerebellum), and also in the pituitary in both sexes. This suggests that these receptors have a functional significance throughout the central nervous system, probably related to neuromodulation, in addition to processes like reproductive behavior (Yamamoto et al., 1997), but possibly other as yet unknown functions. The pituitary showed the lowest mean values for *aa-gnrhr1b* and *aa-gnrhr*2 (*aa-gnrhr1a* did not display significant differences between brain and pituitary). This is in contrast to the Japanese immature eel wherein the highest expression level of *gnrhr1b* was observed in the pituitary (Okubo et al., 2000). Results similar to those obtained in the current work were found in adult Atlantic cod brain (*Gadus morhua*). *gm-gnrhr1b* was classified as receptor IA and *gm-gnrhr2a*, *gm-gnrhr2b* and *gm-gnrhr2c* as receptor IIB, and most of them (not *r2a*) showed higher expression in the brain compared to the pituitary (Hildahl et al., 2011b).

The expression of *gnrhr*s in gonads has been reported in other vertebrate species, including both fish (Lethimonier et al., 2004; Weltzien et al., 2004) and mammals (Hapgood et al., 2005; Ramakrishnappa et al., 2005). GnRH was found to induce apoptosis during the late stages of spermatogenesis in the testis of mature goldfish (*Carassius auratus*; Andreu-Vieyra et al., 2005). Supporting this observation, *gnrhr* expression was detected in ovarian and testicular tissue from recrudescent goldfish (Yu et al., 1998). In

addition, testis from territorial dominant Burton´s mouthbreeder (*Haplochromis burtoni*; Robison et al., 2001) showed much higher *gnrhr* mRNA levels compared to ovarian tissue. The expression of *aa-gnrhr* in ovary and testis of European eel further suggests that these receptors are involved in regulating GnRH functions in the gonads, but it should be emphasized that the fish used for tissue distribution were all immature (silver stage).

Analysis of the tissue distribution of *aa-gnrhr* mRNA showed that the action of GnRH is not limited to the brain-pituitary-gonad axis (Levavi-Sivan and Avitan, 2005). *aa-gnrhr1a* and *aa-gnrhr1b* had a wider distribution among non-reproductive tissues than *aa-gnrhr2*, the latter which was more restricted to the central nervous system and gonadal tissue. Comparable results were detected in sexually mature European sea bass, with a broad distribution for *gnrhr*s belonging to receptor type IA, while the *gnrhr*s type IIB was more restricted to the central nervous system (Moncaut et al., 2005). The observed presence of *aa-gnrhr*s in gill and kidney in the current study is in line with *gnrhr* expression in gill and kidney reported in other teleost species (Robison et al., 2001; Ikemoto and Park, 2005; Tello et al., 2008; Lin et al., 2010), and indicates a possible function of GnRH in osmoregulation.

4.3 Gene expression through gonad development

The increase in pituitary *aa-gnrhr2* expression during testis development suggests this as the primary Gnrhr mediating GnRH's reproductive function in male eels. This is in line with the increasing pituitary levels of GnRH1 peptide, considered as the hypophysiotropic form in the European eel, following induced maturation (Dufour et al., 1993), but not with the corresponding brain *gnrh1* transcript levels that remained stable throughout testis development (Peñaranda et al., 2010).

Analysis of *gnrhr* gene expression in females provides further insight into the function of these receptors during oogenesis and

ovulation, including their expression in pituitary and brain. In tilapia the receptor classified as receptor 1 (Type II *gnrhr2a*) was proposed to be the GnRH receptor involved in the control of gonadotropic function, while the receptor classified as receptor 2 (Type I *aa-gnrhr1b*) could play a role in modulating sensory inputs and body growth (Parhar et al., 2002; 2005; Chen and Fernald, 2006; Levavi-Sivan et al., 2006). Similarly, Type II *gnrhr2a* in pejerrey (*Odontesthes bonaerensis*) increased during gonad maturation in brain, pituitary and gonad, and variations were not observed for Type I *gnrhr1b* (Guilgur et al., 2009). Also, in the Atlantic cod, *gnrhr2a* is considered the main hypophysiotropic receptor (Hildahl et al 2011b; 2013). Similar results were obtained in the current work, with pituitary *aa-gnrhr2* transcript levels increasing with gonad development both in male and female eels.

aa-gnrhr1a and *1b* pituitary transcript levels remained stable during gonad development, except at the vitellogenic stage for *aa-gnrhr1b* in female pituitary. The increased levels of *aa-gnrhr1b* during vitellogenesis may be related to regulation of gonadotropins, but may also participate in regulating other pituitary hormones like growth hormone (Peter and Marchant, 1995; Melamed et al., 1998; Yaron et al., 2003; Onuma et al., 2005).

The expression of *aa-gnrhr1a* was not correlated with gonad development, but a significant decrease was observed from freshwater to seawater condition in female eels, again indicating that *aa-gnrhr1a* may be involved in osmoregulation. A positive effect of GnRH on prolactin release (hormone with osmoregulatory functions) was observed both *in vitro* (Weber et al., 1997; Tipsmark et al., 2005) and *in vivo* (Seale et al., 2002) in adult tilapia (Manzon, 2002). Freshwater control samples were not collected from male eels, as a consequence we cannot say whether the observed decrease in *aa-gnrhr1a* expression is common to both sexes, but a potential role for GnRH in eel freshwater

osmoregulation will be interesting to investigate further in the future.

Pituitary *aa-gnrhr2* gene expression increased during gonadal development in both sexes, suggesting a possible role during gonad maturation. In European silver eel *in vivo* experiments, E2 induced an increase in pituitary *lhb* expression (Schmitz et al., 2005), but this effect was not observed *in vitro* in pituitary cell culture from the silver eel stage (Aroua et al., 2007). These results suggest that E2 action *in vivo* is indirect, possibly via its stimulatory effect on Gnrh1 (Aroua et al., 2009). Higher E2 plasma levels were observed at the EV stage compared to PV stage in female eels reared at 20 ºC (Pérez et al., 2011), which could be responsible for the *aa-gnrhr2* increase observed at this stage in the di-mesencephalon, where Gnrh1-positive neurons are located in the eel (Montero et al., 1994). Supporting this hypothesis, Weltzien et al. (2006) reported that in prepubertal silver female eels, E2 treatment resulted in decreased gene expression of tyrosine hydroxylase (*th*) specifically in di- and mesencephalon. *th* expression reflects central dopaminergic activity (Goldstein et al., 1992), and dopamine is considered responsible for the inhibitory control of gonadotrope activity in many teleost species including the eel (Peter et al., 1986; Dufour et al., 2005; Aroua et al., 2009).

In conclusion, three *aa-gnrhr* mRNAs have been isolated and characterized from European eel. Phylogenetic and sequence analysis revealed two Type IA *gnrhr*s (putative *aa-gnrhr1a* and *aa-gnrhr1b*) and one Type IIB *gnrhr* (*aa-gnrhr2*). This is the first report of two Type I GnRH receptor subtypes in eels, supporting the notion that the teleost specific WGD occurred prior to the branching of the Elopomorpha. *aa-gnrhr2* is the most likely candidate to mediate gonadotropic function of GnRH in male European eel, while both the putative *aa-gnrhr1b* and *aa-gnrhr2* seem to play this role in female eel oogenesis.

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CHAPTER III

Variations in the gene expression of zona pellucida proteins, *zpb* and *zpc*, in female European eel (*Anguilla anguilla*) during induced sexual maturation

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Abstract

Vertebrate eggs are surrounded by an extracellular glycoprotein coat termed zona pellucida (ZP). Integrity of ZP is critical for a correct embryo development. Two zona pellucida protein genes (*zpb* and *zpc*) from European eel were characterized, specific qPCR assays developed and their expression in immature males and females carried out.

An experimental group of silver stage eel females was maintained at 18 ºC and hormonally induced to sexual maturation by weekly injections of carp pituitary extract during 12 weeks. Changes in *zpb* and *zpc* expression during sexual maturation were studied in liver and ovary by qPCR. In liver, no changes were recorded during hormonal treatment, while in ovary expression of both genes decreased during sexual development.

These results are a first step in the characterization of ZP in European eel and in the understanding of the mechanism underlying egg envelope formation.

Keywords: ZP proteins, screening, sexual development, qPCR, liver, ovary.

1. Introduction

European eel is a valuable species, both for economic and cultural reasons, but its wild population is suffering a rapid decline (Feunteun, 2002). Reproduction in captivity for this species has not been achieved yet and many studies have been carried out to better understand its reproductive physiology, both in males and females (e.g. Vidal et al., 2004; Sébert et al., 2008; Peñaranda et al., 2010; Pérez et al., 2011). Long-term hormonal treatments (fish pituitary extracts for females and human chorionic gonadotropin (hCG) for males) are necessary in *Anguilla spp*. to obtain gonadal maturation in captive eels (Fontaine, 1936; 1964), but eggs obtained by hormonal treatments are often of poor quality and it is one of the

main obstacles for reproduction (Ohta et al., 1996; Lokman and Young, 2000; Pedersen, 2003).

All vertebrate oocytes are surrounded by an extracellular glycoprotein coating. This coating is termed chorion in teleost fish, vitelline envelope in amphibians, perivitelline envelope in reptiles and birds, and zona pellucida in mammals (Spargo and Hope, 2003). In teleosts, the chorion proteins have been termed in different ways, such as zona pellucida proteins (ZPp) (Chang et al., 1996; 1997b; Wang and Gong, 1999), vitelline envelope proteins (VEP) (Hyllner et al., 2001) or zona radiata proteins (ZRp) (Arukwe et al., 2002). To avoid already existing confusions, Spargo and Hope (2003) suggested a standardization of the nomenclature by using ZP proteins and grouping them in 4 families: ZPA (not present in fish), ZPB, ZPC, and ZPX, the latter which is not present in amniotes. In the present work this last suggested nomenclature will be used and the proteins will be referred to as zona pellucida proteins (ZPp).

The inner layer of the fish chorion is closely related to the mammalian ZP, even if they serve different roles. In mammals, ZPp are involved in sperm binding during fertilization and a functional ZP is critical for fertilization, early embryogenesis and in the later stages of embryo development, as demonstrated in mice lacking ZPp or in women with ZP defects (Rankin et al., 1999; Conner et al., 2005).

In contrast to mammals, fertilization in fish takes place when a single sperm travels through the micropyle which leads directly to the oocyte membrane. So, ZPp in teleosts play mainly a structural role (Litscher et al., 2007) as well as presenting bactericidal properties and supplying a mechanical protection for the oocyte and the developing embryo. Following fertilization, ZPp are involved in chorion hardening and polyspermy prevention (Murata et al., 2003; Modig et al., 2007). For all these reasons, although no direct studies have been realized on ZPp lack or mutation in teleosts, a ZP

impairment or a no functional ZP can seriously compromise reproductive success.

Unlike mammals, where ZPp gene transcription takes place inside each oocyte, fish ZPp synthesis can occur in the maternal liver (Lyons et al., 1993; Hyllner et al., 2001), the ovary - oocyte itself (Chang et al., 1996; 1997b; Kudo et al., 2000; Modig et al., 2006) or in the surrounding granulosa cells (Modig et al., 2007) - or both (Kanamori et al., 2003; Modig et al., 2006). The reason of this double site of synthesis is unknown: probably a gene duplication event that led to expression in both ovary and liver was followed by the loss of one of the two genes in some species, perhaps in accordance to their reproductive strategy (Conner et al., 2003).

In some species, such as salmonids, turbot or gilthead sea-bream, ZPp synthesis is induced in the liver by 17β-estradiol (E2), including in males and juveniles following E2 treatment (Hyllner et al., 1991; Oppen-Berntsen et al., 1992; Del Giacco et al., 1998; Westerlund et al., 2001; Modig et al., 2006). Other hormones can also be involved in the regulation of ZPp synthesis, such as cortisol (Berg et al., 2004), although this is not widespread among teleosts (Modig et al., 2006). Ovarian ZPp gene expression in fish can either be under E2 control (Scholz et al., 2003; Modig et al., 2007) or not, as suggested by the presence of regulatory elements (called E-boxes) and promoter sequences lacking estrogen responsive elements (Mold et al., 2001; Kanamori et al., 2003; Liu et al., 2006).

Two ZPp homologous mRNAs have been detected in immature testis and ovary of Japanese eel (*Anguilla japonica*) and the corresponding proteins have been named eel spermatogenesis-related substances (eSRS) 3 and 4. In males, their expression is located in immature testis containing exclusively spermatogonia A and B and their transcription is suppressed by both 11-ketotestosterone (11-KT) and hCG, suggesting an inhibitory action on the initiation of spermatogenesis (Miura et al., 1998).

In artificially matured Japanese eel females, the expression of eSRS3 and eSRS4 mRNA decreased with the progression of oogenesis. Such mRNAs were located in the cytoplasm of previtellogenic oocytes (especially chromatine-nucleolus and perinucleolus stages) while protein was located in cytoplasm of previtellogenic oocytes and in the egg envelope from oocytes in the oil-droplet stage, as evidenced by *in situ* hybridization and immunohistochemistry, respectively (Kudo et al., 2000). No gene expression was found in the liver, neither in males nor in females (Miura et al., 1998; Kudo et al., 2000), indicating that ZPp gene expression in the Japanese eel occurs exclusively in the gonads (ovaries and immature testis). Subsequent studies showed that eSRS3 and eSRS4 proteins correspond to Zpb and Zpc, respectively, and that in Japanese eel females there are at least 5 ZPp (1 homologous to Zpb and 4 to Zpc), all of them expressed exclusively in the ovary (Sano et al., 2010).

Due to the lack of information on ZPp in European eel and to their suspected role during fertilization and embryonic development, the objective of the present work was to characterize 2 ZPp genes, determine their tissue distribution in immature male and female eels other than liver and gonad, and to quantify their mRNA expression by qPCR during induced sexual maturation in females. Of the 5 ZPp which were recently characterized in Japanese eel (Sano et al., 2010), the 2 quantitatively most important genes were chosen in an attempt to improve our knowledge about chorion formation in European eel, an endangered species and a suitable model to study fish reproduction.

2. Materials and methods

2.1. Fish handling

Thirty-nine silver-stage female eels (660 ± 162 g body weight) were caught by local fishermen between December and March

during their reproductive migration from the Albufera lagoon (Valencia, Spain) to the sea, and transported directly to the Universitat Politècnica de València (UPV, Spain) aquarium facilities.
Fish were placed in a tank of 1500 L recirculating freshwater and gradually acclimated to seawater salinity (37 g L^{-1}) and temperature (18 ± 1 ºC) over the course of two weeks. The tank was covered to maintain constant darkness, thereby reducing stress. Because eels stop eating from the start of their reproductive migration, they were fasted during the whole experiment. All the fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Hormonal treatment

After being anesthetized (benzocaine, 60 mg L^{-1}) and weighed to calculate the hormone dosage, female eels were treated weekly for 12 weeks with intra-peritoneal injections of carp pituitary extract (CPE; Catvis, Ltd The Netherlands) at a dose of 20 mg kg^{-1}. CPE was prepared as follows: 1 g of pituitary powder was diluted in 10 ml of NaCl solution (9 g L^{-1}) and centrifuged at 1260 *g* for 10 min. The supernatant was collected and stored at -20 ºC until use, between 1 to 3 weeks maximum.

2.3. Sampling

Eight females were euthanized to serve as freshwater control at the moment they arrived at the UPV facilities. After 14 days of acclimatization, 8 animals were sacrificed as seawater control group (W0). From this moment, the hormonal treatment started and 7-8 specimens were sacrificed every 4 weeks (W4, W8 and W12).
Animals were anesthetized (benzocaine, 60 mg L^{-1}) before being sacrificed by decapitation. Total body, liver and gonad weights were measured to calculate gonadosomatic index (GSI = 100 gonad weight x total body $weight^{-1}$) and hepatosomatic index (HSI = 100

liver weight x total body weight^{-1}). Total body length and vertical and horizontal eye diameter were measured to calculate eye index (EI = 100 π 0.25 (Dh+Dv)^2 x Lt^{-1}, where Dh = horizontal eye diameter, Dv = vertical eye diameter, and Lt = total length).

Gonad samples for histology were preserved in 10% buffered formalin (pH 7.4).

For RNA extraction and gene expression analyses, triplicate samples of gonad and liver were collected from each fish immediately after the sacrifice and stored in RNA later (Ambion Inc., Huntingdon, UK) at -20 ºC until further processing.

2.4. Gonad histology

After dehydration in ethanol, samples were embedded in paraffin and sections of 5-10 μm thickness were cut with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd, England). Slides were stained with hematoxylin and eosin and observed through a Nikon Eclipse E-400 microscope and pictures were taken with a Nikon DS-5M camera attached to the microscope, all from Nikon (Tokyo, Japan).

One hundred oocytes per specimen were measured, always selecting the biggest ones that showed a complete nucleus. The stage of oogenesis was determined according to Kayaba et al. (2001) and Selman and Wallace (1989). Perinucleolus, nucleolus, and oil droplet stages were grouped as previtellogenic stage (PV). Oocytes with small yolk globules located only at the periphery of the cytoplasm were classified as early vitellogenic stage (EV). Oocytes in the mid-vitellogenic stage (MV) showed bigger yolk globules distributed in the entire cytoplasm, but less numerous compared to the oil droplets. In the late vitellogenic stage (LV), yolk globules were more abundant than the oil droplets. The most advanced stage observed was the nuclear migration (NM), characterized by oocyte hydration and the migration of the nucleus towards the animal pole.

2.5. RNA extraction

Gonad and liver samples from 6 eels per sampling were homogenized in 1 ml of TRIzol reagent (Invitrogen, Belgium) in tubes containing ceramic lysing matrix (MP BIO, France) by shaking 20 s at 4 m s^{-1} or until complete homogenization (Fast-Prep24, MP BIO, France). After 5 min at room temperature, RNA was extracted using traditional phenol/chloroform method. DNase digestion (RNeasy Mini Kit, Qiagen, Germany) and RNA CleanUp (RNeasy Mini Kit, Qiagen, Germany) were performed according to the manufacturer's instructions. Following clean up, total RNA was diluted in 40 µl of nuclease-free water and stored at -80 ºC until further use.

RNA was quantified by Nanodrop spectrophotometry (Thermo Scientific, USA) and RNA integrity was checked by Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent RNA 6000 Nano, Germany). Samples used for gene expression analyses all had RIN values above 7.

2.6. cDNA synthesis

First-Strand cDNA was synthesized in 20 µl reactions with 1 µg (liver) or 2 µg (gonad) of total RNA as template, random hexamer primers and superscript III reverse transcriptase (Invitrogen, Belgium) according to the manufacturer's instructions. The mix was incubated at 25 ºC for 5 min and then at 50 ºC for 60 min. Reactions were heat inactivated at 70 ºC for 15 min. cDNA aliquots were stored at -20 ºC until further use.

2.7. RACE-PCR, cloning and sequencing

For isolation of the *Anguilla anguilla zpb* and *zpc* genes, RACE cDNA from liver samples was synthesized from total RNA by SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions.

Because of the high homology between European and Japanese eel, RACE-PCR primers were designed based on eSRS3 (*zpb*) and eSRS4 (*zpc*) Japanese eel sequences (GenBank accession number AB016041.1 and AB016042.1, respectively). Primers were purchased by Eurofins MWG (Germany). For *zpb* a 3'RACE-PCR with the forward primer 5'-GGGACAGTATTTATGAGCTGTCCTTCCAGTGCAGG-3' was run, while for *zpc* a 5' RACE-PCR with the reverse primer 5'-CATTGTGTAGGCTCAGGTAATGGCACTGGATGC-3' was run.

For each gene, 1 µl primer (10 pmol μl^{-1}), 5 µl 10X Universal Prime A Mix (UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' by Clontech, USA), 5 µl 10X AccuPrime PCR buffer, 1 µl dNTP mix, 1 µl AccuPrime TAQ Polymerase, 2.5 µl RACE cDNA from liver (3' RACE cDNA for *zpb* and 5'RACE cDNA for *zpc*) and 34.5 µl PCR grade water were used. Touchdown PCR was performed with the following conditions: after a denaturalization step at 94 ºC during 30 s, 7 cycles of 30 s at 94 ºC, 30 s at 72-65 ºC and 7 min at 72 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 65 ºC, 3 min at 72 ºC, and a final elongation step for 5 min at 72 ºC.

PCR products were checked by agarose gel (1%) electrophoresis. Because no bands were visible following the first PCR, a nested PCR was run using the following primers: *zpb* forward primer: 5'-GTAGCCGCTCCAGGGCCCCTTCGTGTTGAGCTCAGACTGGCTAGT-3': *zpc* reverse primer 5'-CATTGTGTAGGCTCAGGTAATGGCACTGGATGC-3' and Nested Universal Primer (NUP: 5'-AAGCAGTGGTATCAACGCAGAGT-3' by Clontech). For each gene, 1 µl primer (10 pmol μl^{-1}), 1 µl NUP, 2.5 µl 10X AccuPrime PCR buffer I, 0.5 µl AccuPrime TAQ Polymerase, 1 µl PCR product and 19 µl PCR grade water were used. After a denaturalization step at 94 ºC during 2 min, 35 PCR cycles of 15 s at 94 ºC, 15 s at 55 ºC and 1 min at 68 ºC, was followed by a final elongation step of 7 min at 68 ºC.

After the nested PCR, PCR products were checked by agarose gel (1%) electrophoresis and visible specific bands were cut out and purified (QIAquick Gel extraction kit, Qiagen, Germany). Purified PCR products were ligated into pGEM-T easy vector (Promega, USA) followed by transformation in competent *E. coli* JM109 cells (Promega). Positive white colonies were isolated and plasmids extracted by QIAgen Plasmid Purification Kit (Qiagen, Germany). The insert was sequenced in both directions using M13 and Sp6 primers. New RACE primers were designed based on the partial sequences obtained for *zpb* (3' end) and *zpc* (5' end). The following primers were used: *zpb* reverse primer 5'-GGGTCAGTCCTCTCCAAGATGCGCACTTCCACA-3' and *zpc* forward primer 5'-GGCTAAGCCTGATGCCGTGAAGGTCCACTGTGG-3'. For each gene, 1 µl primer (10 pmol μl^{-1}), 5 µl 10X Universal Prime A Mix (UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' by Clontech, USA), 5 µl 10X Advantage PCR buffer, 1 µl dNTP mix, 1 µl 50X Advantage 2 Polymerase mix, 2.5 µl RACE cDNA (from liver) and 34.5 µl PCR grade water were used. Touchdown PCR was performed as previously described. As no product was visible after agarose gel (1%) electrophoresis, nested PCR was performed using the following primers: *zpb* reverse primer 5'-CGCACTTCCACATACACAGGTTCCCGTAGGACC-3' and *zpc* forward primer 5'-TTCTGTTTGAGACTGAGCTCCATGACTGCGGC-3'. PCR was performed as previously described. As bands were visible, purification, ligation, transformation and preparation for sequencing were performed as previously described.

Sequence comparison was made using BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In the text, for gene and protein symbols following rules were used. Gene fish: all lowercase and italicized letters; protein fish: same symbol as the genes, but non-italic and the first letter is uppercase (https://wiki.zfin.org). Human and chicken: italicized, all upper case

letters (http://www.genenames.org/guidelines.html and http://www.agnc.msstate.edu/Guidelines.htm, respectively). Amphibians: lower case italic letters (http://www.xenbase.org/gene/static/geneNomenclature.jsp)

2.8. Sequence analysis

The partial sequences were combined and blast analysis was carried out at GeneBank (http://www.ncbi.nlm.nih.gov/) to confirm the identity of the gene. The full-length sequences and deduced amino acid sequences were then aligned with a sub-sample of zona pellucida (ZP) sequences from representative teleost orders; Perciformes (*Sparus aurata*), Salmoniformes (*Oncorhynchus mykiss*), Cypriniformes (*Carassius auratus*) and Anguilliformes (*Anguilla japonica*); and one representative mammalian (*Homo sapiens*), aves (*Gallus gallus*), and amphibian (*Xenopus laevis*) species ZP variants, using the CLC Main Workbench software (CLC bio, Denmark). The alignments were checked and manually adjusted for misaligned sequence. ZPb and ZPc variants were also aligned separately to assure appropriate alignment. The alignments were used to identify conserved domains. Pairwise analysis of the ZP domain amino acid sequence was used to determine the percent identity between the ZP variants in the selected representative species.

2.9. qPCR

In order to quantify the gene expression of *Anguilla anguilla zpb* and *zpc*, quantitative real time RT-PCR (qPCR) analyses were performed using a Light Cycler 480 system with SYBR Green I sequence-unspecific detection (Roche Diagnostics, France). As reference gene, *arp* (acidic ribosomal phosphoprotein P0) was used because its expression varies very little with experimental treatment (Weltzien et al., 2005b).

Primers were designed based on the obtained sequences using the Primer3 shareware (Whitehead Institute/MIT, USA). The obtained sequences were checked for homo/hetero dimer and hairpin formation by Vector NTI (Invitrogen, Belgium) and purchased from Eurofins MWG (Germany). To avoid detection of genomic DNA (gDNA), each primer was designed on one exon to span an exon-exon boundary.

The following pair of primers was selected for each gene:

- *zpb* Fw 5'-ACACTGCTGGGCTACATCCACC-3';
- *zpb* Rv 5'-AACGGTCGTCACGGTAGGGACA-3';
- *zpc* Fw 5'-GAGACTGAGCTCCATGACTGC-3';
- *zpc* Rv 5'-AGCACCAATGGCACTAGGTT-3'.

The expected product length for the *zpb* and *zpc* primer pairs was 89 and 99 bp, respectively. The primer pairs had a PCR efficiency of 1.990 (*zpb*) and 1.989 (*zpc*) based on cDNA dilution curves.

After a preincubation of 10 min at 95 ºC, qPCR analyses were performed under the following conditions: 10 s at 95 ºC, 10 s at 60 ºC and 5 s at 72 ºC. After 42 cycles, the machine performed a melting curve analysis by slowly increasing the temperature from 68 to 95 ºC, with a continuous registration of changes in fluorescent emission intensity.

The total volume for every qPCR was 10 µl, performed from diluted cDNA template (3 µl), forward and reverse primers (0.5 µM each) and SYBR Green Master Mix (5 µl) and water until reaching the final volume. Due to the high expression in gonad, cDNA was diluted 1/1000 in nuclease free water, while in liver cDNA dilution was 1/8. Target and reference genes in each samples was run in duplicate PCR reactions, and a cDNA pool from various samples was included in each run and acted as a calibrator. Quantification of the results was done using a relative standard curve method (Weltzien et al., 2005b). A no template control (NTC) with water replacing cDNA was used in every run to check for contamination. All primers were tested on gDNA and no-rt totalRNA to confirm that they would not

amplify potentially contaminating gDNA at the dilution used during analysis.

2.10. Tissue screening

Three prepubertal male and female silver eels from the fish farm Valenciana de Acuicultura, S.A. with average body weights of 118.0±14.67 and 632.0±46.46 g, respectively, were sacrificed in order to evaluate *zpb* and *zpc* expression in the following tissues: gonad, liver, pectoral fin, anterior kidney, posterior kidney, heart, olfactory bulbs, telencephalon, mesencephalon/diencephalon, cerebellum, medulla oblongata, pituitary and gills. Total RNA was extracted as described above and treated with DNase I (Turbo DNA-free, Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III reverse transcriptase (Invitrogen, Belgium) and random hexamer primers according to standard protocol. qPCR was performed as described above with a cDNA dilution of 1/1000.

2.11. Statistical analysis

After establishing data normality - variables that did not have a normal distribution were log-transformed and their normality was checked again -, a One-Way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test was carried out to compare results from morphological changes and mRNA expression. All values are expressed as mean ± standard error of mean (SEM). Differences were considered significant at $p < 0.05$. All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1. *zpb* and *zpc* sequences and tissue screening

Two full-length ZPp genes were identified in European eel by a combination of RT-PCR and RACE-PCR. Complete sequences of *zpb*

(1401 pb; GenBank accession number JN982278) and *zpc* (1619 pb; GenBank accession number JN982279) are shown in fig. 1A and B, respectively.

Percent identity of European eel ZP domain deduced amino acid sequence with each group of ZPp in fish and tetrapods are shown in table 1. The deduced amino acid sequence from European eel *zpb* shared 89% identity with Japanese eel eSRS3, and higher relative identity with vertebrate ZPb compared to the other groups of ZPp. The deduced amino acid sequence from European eel *zpc* shared 97% identity with Japanese eel eSRS4, and higher relative identity with other vertebrate ZPc compared to the other groups of ZPp.

Table 1. Percent identity shared between the ZP domain deduced amino acid sequence from European eel z*pb*, z*pc*, Japanese eel eSRS3 and eSRS4 and the four main groups of ZPp protein genes in representative teleost (Perciformes (*Gilthead seabream* – *zpb*, *zpc* and *zpx*), Salmoniformes (*Oncorhynchus mykiss* – *zpb* and *zpc*), Cypriniformes (*Carassius auratus* - *zpb* and *zpc*)) and tetrapods (*Homo sapiens* - *ZPA*, *ZPB* and *ZPC* -, *Gallus gallus* - *ZPA*, *ZPB* and *ZPC* - and *Xenopus laevis***-** *zpa*, *zpb* and *zpc*). Percent identity between homologous genes is given in bold.

Other sequences	**European eel sequences**	
	Zpb	***Zpc***
eSRS3	**89**	16
eSRS4	48	**97**
ZPa	31-33	12-13
ZPb	**41-68**	12-17
ZPc	13-17	**37-55**
ZPx	29-30	12-15

Deduced amino acid sequences from European eel ZPp genes possessed characteristic conserved domains (Fig 1). These included an N-terminal domain with the signal peptide and cleavage site, the ZP domain and a C-terminal domain with the transmembrane domain (TMD) and cleavage site. All ZPp possess a ZP domain, which consists of ~ 260 aa and is present in many extracellular

proteins with different roles (Jovine et al., 2005). ZP domains were identified in the European eel ZPp with 10 and 8 conserved Cys residues in the deduced amino acid sequence from *zpb* and *zpc*, respectively.

A trefoil domain, a module present in different extracellular proteins, often with binding function, and with 6 conserved Cys residues (Bork, 1993), was found in the deduced amino acid sequence from *zpb* upstream of the ZP domain. This structure is not present in the deduced amino acid sequence from *zpc*, which possesses five PQ rich repeat domains in the N-terminal region.

3.2. Gonadal development and morphological changes during induced maturation

Gonadal developmental stages during the hormonal treatment period are shown in fig. 2. Female eels responded positively to the treatment, as evidenced by the fact that vitellogenesis was stimulated. All freshwater and W0 specimens were in the previtellogenic stage (PV), while 86% of the specimens proceeded to the early vitellogenic stage (EV) following 4 weeks of treatment (4 injections). After 8 weeks of treatment, mid-vitellogenic (MV) stage appeared in 29% of the specimens. At W12, 75% of specimens were in the late vitellogenic stage (LV) and one specimen was in the nuclear migration stage (NM). The rapid development in the last month of treatment was supported by the GSI which increased slowly up until W8, while at W12 a sharp increase was recorded and statistically higher values were reached ($p < 0.0001$) (Fig. 2).

EI also increased during treatment with a statistical increase at W8 and a further increase observed at W12 ($p < 0.0001$) (fig 2). HSI was higher in freshwater control samples compared to at W0. From W0, HSI increased gradually along with the hormonal treatment,

reaching values similar to the freshwater control in the last sampling at W12 ($p < 0.005$) (Fig. 2).

Fig. 1. *Anguilla anguilla zpb* (A) and *zpc* (B, next page) complete mRNA sequences. The conserved sequences are identified as follows: trefoil domain (A, grey box) and PQ repeat sequences (B, grey box delineated by black borders); ZP domain (underlined); conserved cysteins (circled); putative c-terminal cleavage site (oval) and transmembrane domain (open box). An asterisk defines the stop codons.

A

```
acatggggagcaattgaccttctttcattatggcaagtattgtgcagcttggagtatggctttgtgttttgggcttt    48
                                  M  A  S  I  V  Q  L  G  V  W  L  C  V  L  G  F    16
gtcctggcacaggatcaagatcaatatttttgggtcaaaaggttcagatgcaacttttcatagatgccacgttggtggc  126
 V  L  A  Q  D  Q  D  Q  Y  F  G  S  K  G  S  D  A  T  F  H  R  C  H  V  G  G    42
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 F  A  R  V  P  C  G  D  P  A  I  S  S  T  D  C  E  A  L  N  C  C  F  E  Q  Q    68
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 C  Y  Y  A  N  E  V  T  V  H  C  L  R  D  G  L  F  M  V  V  A  S  R  A  A  T    94
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 S  R  V  E  P  K  V  *                                                        439
```
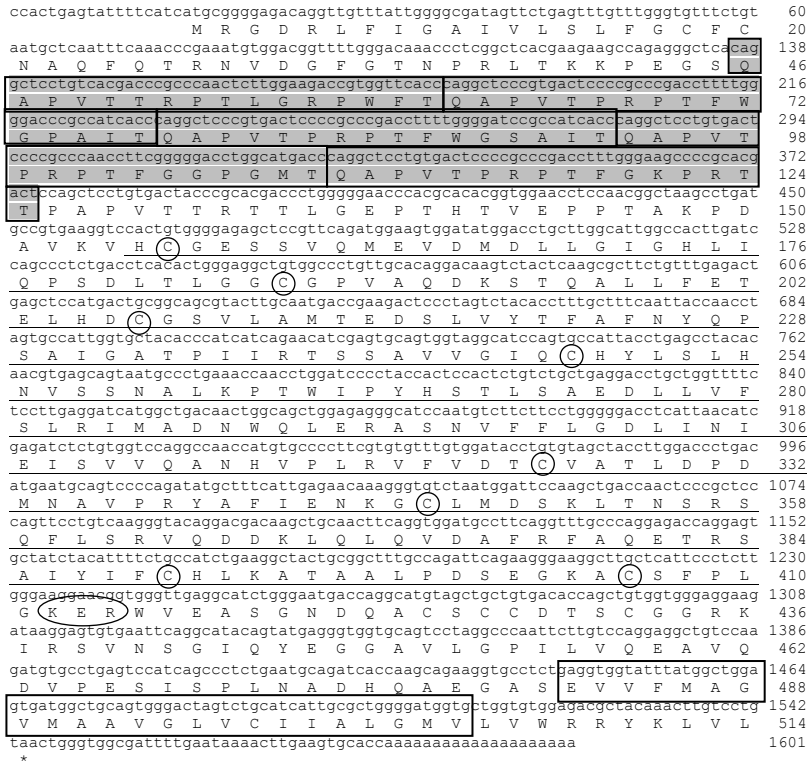

B



3.3. *zpb* and *zpc* gene expression

3.3.1. Tissue screening

qPCR analysis was utilized to characterize the male and female tissue-specific expression pattern of *zpb* and *zpc* in five parts of the dissected brain, in addition to the pituitary, gonad, liver, pectoral fin, anterior and posterior kidney, heart and gill. Differential expression was found between sexes (Fig. 3A and 3B). In immature females *zpb* and *zpc* expression was detected in all investigated tissues, with the exception of the olfactory bulbs, telencephalon and mesencephalon/diencephalon. However, gene expression was in general very low and the highest values were recorded in ovary and

kidney (Fig. 3A). Moreover, in females *zpc* expression was generally higher than *zpb*. In immature males, *zpb* and *zpc* expression was recorded only in the heart, at similar levels as in females (Fig. 3B).

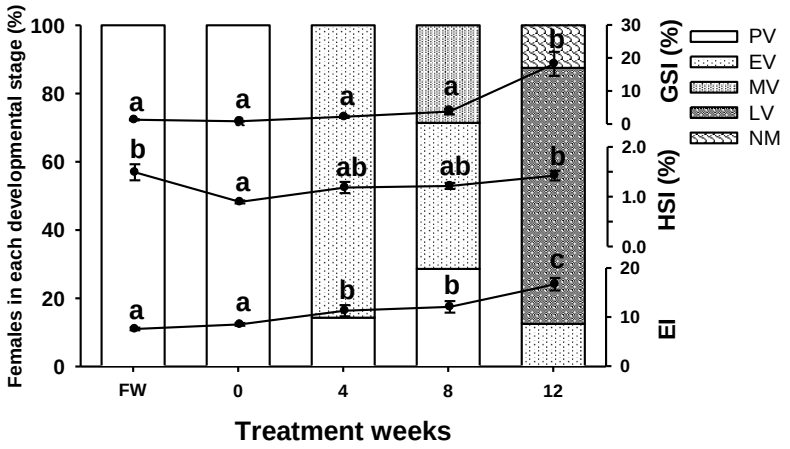


Fig. 2. Gonadosomatic Index (GSI = 100 gonad weight x total body weight^{-1}), Hepatosomatic Index (HSI = 100 liver weight x total body weight^{-1}), and Eye Index (EI = 100 π 0.25 (Dh+Dv)^2 x Lt^{-1}, Dh = horizontal eye diameter, Dv = vertical eye diameter and Lt = total length) evolution in freshwater control female eels (FW; n=8) and during hormonal treatment after 0 (n=8), 4 (n=8), 8 (n=7) or 12 (n=8) injections. PV: previtellogenic stage; EV: early vitellogenic stage; MV: mid-vitellogenic stage; LV: late vitellogenic stage; NM: nuclear migration. Different letters indicate statistical difference ($p < 0.05$).

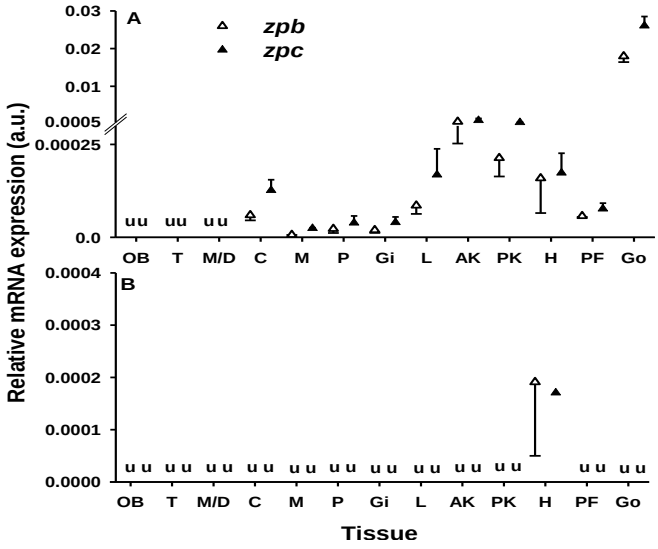


Fig. 3. *Anguilla anguilla zpb* and *zpc* gene expression in different tissues from immature female (A; n=3) and male (B; n=3) eels. OB: olfactory bulbs; T: telencephalon; M/D: mesencephalon/diencephalon; C: cerebellum, M: medulla oblongata; P: pituitary; Gi: gills; L: liver; AK: anterior kidney; PK: posterior kidney; H: heart; PF: pectoral fin; Go: gonad; u: undetectable.

3.3.2. *zpb* and *zpc* gene expression during induced sexual maturation

In the figures concerning *zpb* and *zpc* expression during hormonal treatment (figs. 4,5,6), data are shown normalized to the freshwater control group (FW), in order to make data interpretation easier. The only eel which had reached the nuclear migration stage was not considered in the gene expression analysis.

zpb and *zpc* expression levels in liver and ovary according to treatment week are shown in fig. 4 and 5.

In the liver (fig. 4A and 5A), neither *zpb* nor *zpc* expression varied statistically during treatment ($p > 0.20$ in both cases). However, *zpb* expression was in general lower during hormonal treatment than at FW control. On the other hand, *zpc* showed a low expression in all the samplings with exception of W8, when a sudden increase was recorded (not statistically significant).

In the ovary (fig. 4B and 5B), both *zpb* and *zpc* expression showed a general decreasing trend and the lowest gene expression value was recorded at W12 ($p < 0.05$ and $p < 0.01$ for *zpb* and *zpc*, respectively).

Gene expression results were analyzed also according to developmental stage (fig. 6), as not all the animals from the same week had reached the same developmental stage.

In liver, no differences were recorded among developmental stages neither in *zpb* nor *zpc* expression (data not shown), in agreement with the results obtained according to treatment week. In the ovary, the decrease in the expression of *zpb* ($p < 0.005$) and *zpc* ($p < 0.005$) matched with the passage from MV to LV, while no differences were recorded among other developmental stages (fig. 6A and B). However, the decrease in *zpb* and *zpc* expression followed two different trends; *zpb* expression showed a sharp decrease between MV and LV, while decrease in *zpc* expression was more gradual during development.

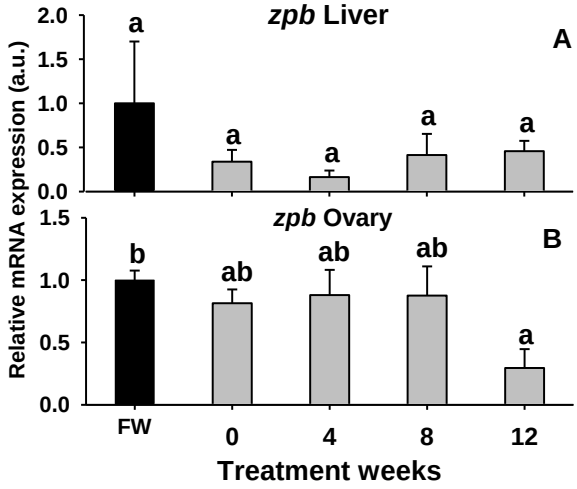


Fig. 4. *Anguilla anguilla zpb* gene expression in liver (A) and ovary (B) in freshwater control (FW; n=6) and after 0 (n=6), 4 (n=6), 8 (n_L=6; n_G=3) and 12 (n=6) weeks of hormonal treatment. $p < 0.05$.

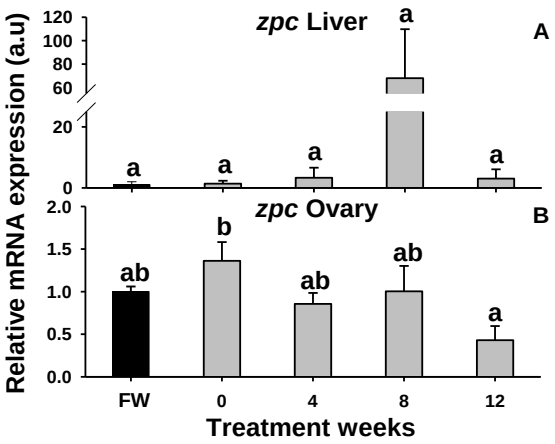


Fig. 5. *Anguilla anguilla zpc* gene expression in liver (A) and ovary (B) in freshwater control (FW; n=6) and after 0 (n=6), 4 (n=6), 8 (n_L=6; n_G=3) and 12 (n=6) weeks of hormonal treatment. $p < 0.05$.

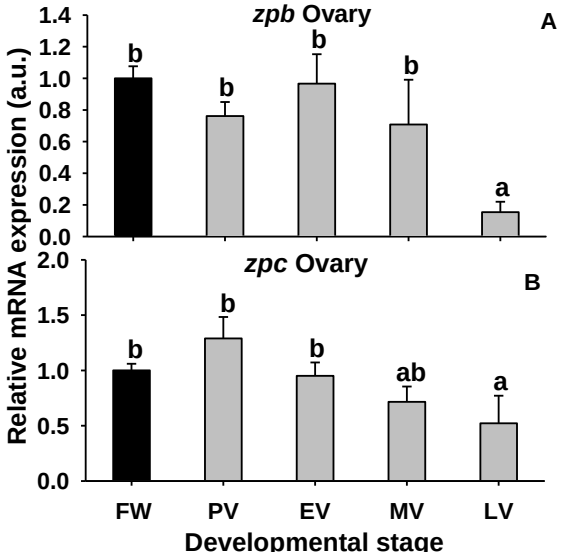


Fig. 6. Changes in *zpb* (A) and *zpc* (B) gene expression in ovary according to the developmental stage. FW: freshwater control (n=6); PV: previtellogenic stage (n=8); EV: early vitellogenic stage (n=6); MV: mid-vitellogenic stage (n=2); LV: late vitellogenic stage (n=5). $p < 0.05$.

4. Discussion

European eel *zpb* and *zpc* mRNAs have been cloned and characterized by RT-PCR and RACE-PCR. These genes have high identity with Japanese eel eSRS3 and eSRS4, respectively, and possess conserved structural motifs. The ZP domains with 10 and 8 conserved Cys residues in the deduced amino acid sequences from *zpb* and *zpc* genes, respectively, and the presence of a trefoil domain in *zpb* but not in *zpc* are similar to sequences identified in other species (Yonezawa and Nakano, 2003; Darie et al., 2004; Modig et al., 2006; Sano et al., 2010; Sun et al., 2010).

The functional role of the trefoil domain is unknown (Ohtsuki et al., 2008). In mammals, where it is present in ZPp isoforms not strictly involved in sperm binding, the trefoil domain has been related to a structural role as it give a higher resistance to proteolytic degradation and structural integrity (Bork, 1993; Rankin et al., 1999).

In *zpc*, a repeat sequence PQ-rich at the N-terminus was identified, which is not present in *zpb*. This difference between the two ZPp groups had already been described in Japanese eel (Sano et al., 2010). In other teleost species, a repeated domain at the N-terminus can be found in Zpb (Lyons et al., 1993; Chang et al., 1997b), in Zpc (Del Giacco et al., 2000; Sano et al., 2010) or in both of them (Litscher et al., 2007). Consensus sequence and length of the repeated sequence can vary among species and its function is not clear. However, PQ-rich sequences, such as the one found in European eel *zpc*, are probably related to the hardening of the eggshell (Litscher et al., 2007; Modig et al., 2007). Differences in the N-terminal region could be related to different roles played by distinct ZPp.

Among teleosts, a TMD was found only in Japanese eel ZPp, while this structure is common in other vertebrates (Modig et al., 2007; Sano et al., 2010). The presence of a TMD also in European eel ZPp at the C-terminus, as evidenced by the present study, confirms that

it is a characteristic of *Anguilla spp*. and suggests that it was present in ancient teleosts and lost during evolution.

In spite of the structural similarities, European and Japanese eel show differences in site of expression. In fact, unlike Japanese eel where ZPp genes are expressed only in the ovary (Miura et al., 1998; Kudo et al., 2000; Sano et al., 2010), European eel presented a ubiquitous expression pattern.

Indeed, ZPp genes were found to be expressed in other tissues outside the gonads. The different results can be due to the different methods used, as in the works on Japanese eel, gene expression analyses were performed by RT-PCR and Northern blot, while in the present study qPCR, a more sensitive technique, was employed.

Even if ZPp gene expression in teleosts occurs principally in liver or ovary, expression of ZPp mRNA in different tissues has been previously recorded in mature half smooth tongue sole (Sun et al., 2010). In this species, expression was more widely distributed in females compared to in males with the highest expression recorded in ovary and kidney, similar to what we found in the European eel.

The significance of expression in tissues other than ovary and liver in immature eels, especially males, needs further research.

In freshwater control eels, GSI and EI values match with values recorded in eels at the pre-migratory stage, while HSI values are more similar to the ones recorded for resident eels which have an HSI higher than pre-migratory eels (Durif et al., 2009b). This previous result matches with the lower HSI value recorded in the present work at W0 compared to in freshwater control. Hence, the decrease in HSI can be a first adaptive answer to seawater. The ensuing HSI increase is likely due to an active role of the liver during vitellogenesis and can also be a consequence of total body weight loss due to starvation and the high energetic cost of sexual maturation.

GSI and EI increased gradually during hormonal treatment. GSI increase is the result of gonad maturation, while increase in eye

dimensions is supposed to be an adaptive response to the increased darkness during the maturational oceanic migration (Rohr et al., 2001).

In maturing females, ZPp gene expression was recorded in both ovary and liver, even if expression in the ovary was higher than in the liver, leading to the different cDNA dilutions used for qPCR analyses, 1/1000 for ovary and 1/8 for liver samples.

The lower ZPp gene expression and the lack of variation in liver during sexual maturation, suggest that liver expression is independent of sexual maturation and that the principal site of synthesis of European eel ZPp is the ovary, in agreement with the situation in Japanese eel (Kudo et al., 2000). As already hypothesized due to the differences in the ZPp structure, also the differences in the hepatic gene expression between *zpb* (where gene expression results were constant during the whole treatment and lower than FW control) and *zpc* (where a peak was recorded at W8 even if not statistically significant) suggest that the 2 proteins could play different roles in eggshell formation.

In the present study, ovarian ZPp genes were expressed already prior to the beginning of sexual development and vitellogenesis, in agreement with results obtained in Japanese eel (Kudo et al., 2000), gilthead seabream (Modig et al., 2006) and carp (Chang et al., 1996; 1997b).

In Japanese eel, ZPp were located in the ooplasm already at oil-droplet stage oocytes (Kudo et al., 2000) and at this stage, a two-layer vitelline envelope is already present (Kayaba et al., 2001). In the following stages, its structure changes due to the appearance of a new layer and an increase in thickness (Kayaba et al., 2001).

The early formation of vitelline envelope and the changes in its structure during maturation in Japanese eel are in agreement with the results obtained in the present work with ZPp genes expressed already before the start of vitellogenesis and proceeding during oocyte maturation.

For the first time, two ZPp genes have been described in European eel and their expression in immature males and females in extra gonadal and extra hepatic tissues was investigated by qPCR. ZPp gene expression in ovary and liver from female eels during artificial sexual maturation has been quantified by qPCR. Similar to other species with ZPp gene expression localized to the ovary, the European eel ZPp expression commences prior to vitellogenesis. Further studies are necessary to elucidate the role of these genes in the eggshell, and to deepen our understanding of the mechanisms underlying ZPp gene expression and egg envelope formation, in particular to identify factors, endogenous and exogenous, responsible for improved egg quality.

Acknowledgements

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CHAPTER IV

Temperature modulates the progression of vitellogenesis in European eel

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Following one referee's request, the supplementary data were included in the text rather than shown in a specific section.

Abstract

Wild female European eels were matured with CPE (carp pituitary extract) under three thermal regimes, two of which were variable (T10-15 and T15-18, moving from 10 to 15 ºC and from 15 to 18 ºC, respectively) and one constant, at 18 ºC (T18). Before and during hormonal treatment, the eels were sampled and biometric measurements were taken. Immunoassays of sex steroids and vitellogenin were performed, as well as qPCR analyses of gene expression (ovarian *cyp19a1*) and ovarian histology. Prior to the hormonal treatment, the silver eels which had been maintained at 18 ºC showed lower 11-KT and E2 plasma levels compared to those maintained at 10 ºC. In addition, in the early vitellogenic stage, the androgen and *cyp19a1* levels were lower at 18 ºC than at 10 ºC. Both these results and the positive correlations found between GSI and 11-KT (at the PV stage) and between oocyte diameter and *cyp19a1* levels (in the EV stage), suggest that early ovarian development is facilitated at low temperatures. Vitellogenesis was induced by CPE in all the thermal groups, but progression to the mid-vitellogenic stage was only observed after an accumulation of 900-1200 ºD, at 15 or 18ºC, and progression to the late vitellogenic stage was only observed after an accumulation higher than 1300 ºD, at 18 ºC. Although temperature increased the rate of CPE-induced ovarian development, our results clearly indicate that this increase is not linear, but exponential, with acceleration in the increase of GSI at 18 ºC from the mid-vitellogenic stage, or after an accumulation of 1300 ºD. For the first time, a down-regulation of ovarian *cyp19a1* caused by high temperatures in CPE-treated eels was observed. These results demonstrate that temperature can modulate eel ovarian development both before and after exogenous hormonal stimulation, and this knowledge could be used to manipulate the timing of vitellogenesis progression under laboratory conditions.

Highlights

- Low temperatures induced steroidogenesis in previtellogenic eel ovaries.
- High temperatures down-regulate ovarian *cyp19a1* gene expression in CPE- treated eels during early ovarian development.
- Ovarian CPE-induced growth is accelerated by high temperatures or by an accumulation higher than 1300 ºD.

Keywords: *Anguilla anguilla*, thermal regime, sex maturation, sex steroids, *cyp19a1*

1. Introduction

European eels do not reproduce spontaneously in captivity, and their production in farms is still limited to the growing/rearing of glass eels caught in the wild, which is very expensive, as glass eel prices fluctuate between 400-700 €/kg (Nielsen and Prouzet, 2008). Eel aquaculture sustainability is also compromised by the dramatic decrease in the wild populations due to overfishing, habitat loss, and pollution (Feunteun, 2002). This has led the EU to recommend significant restrictions in European eel fishery. In order for the European eel aquaculture industry to have a future, it is therefore imperative to be able to close their life cycle under captive conditions.

It is known that the European eel perform a 4–6000 km reproductive migration from European coastal waters to their supposed spawning grounds in the Sargasso Sea (review van Ginneken and Maes, 2005). If prevented from carrying out this oceanic migration, the European eels remain at a pre-pubertal (silver) stage due to a dopaminergic blockage of pituitary gonadotropins in addition to a deficiency in gonadotropin stimulation by gonadotropin-releasing hormones (GnRH) (Dufour et al., 2003;

Vidal et al., 2004). Thus, long-term hormonal treatments (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are currently necessary to mature eels in captivity (Ohta et al., 1997; Lokman and Young, 2000; Asturiano et al., 2005; Palstra et al., 2005; Pérez et al., 2008; Peñaranda et al., 2010, Gallego et al., 2012). Such long-term hormonal treatments are expensive, and maturing one single female can cost between 50 and 100 € (taking into account only the hormones, own estimate). But, even with these treatments, the egg quality in European eel is still unpredictable (see review by Okamura et al., 2014).

Environmental factors, such as photoperiod and temperature, are the main natural triggers for reproduction in temperate fish species. The environmental conditions in which eels migrate from Europe to the spawning grounds have begun to be identified recently. The European eel migrates at depths of between 200-600 m, by performing daily vertical migrations, at temperatures between 10 and 12 ºC (Aarestrup et al., 2009). When eels leave the continental waters to enter the sea, they are still immature, with gonadosomatic indices (GSI= gonad weight x total body weight $^{-1}$) between 1-2.7% (Boëtius and Boëtius 1980; Durif et al., 2005). Thus, it is possible to suppose that early ovarian development in nature takes place at low temperatures. On the other hand, it is assumed that ovulation takes place at temperatures around 18-22 ºC, considering the water temperature in the supposed spawning areas of the Sargasso Sea (Friedland et al., 2007). Thus, by combining hormonal treatments with thermal profiles resembling those supposedly found in the wild, the quality of gonadal maturation in captivity could be improved. In a previous work (Pérez et al., 2011) we matured female European eels with carp pituitary extracts (CPE) under two thermal regimes; one variable regime increasing from 10 to 17 ºC, and one constant at 20 ºC. The results showed higher E2 plasma levels, as well as increased

expression of *fshb* and *lhb* in the pituitary, and of *estrogen receptor 1* in the ovary, in eels reared using the variable thermal regime, thus suggesting that a variable regime results in improved gonadal maturation.

The fish pituitary injections used to mature female eels provide exogenous gonadotropins directly to the ovaries, and subsequently stimulate ovarian steroid synthesis (Matsubara et al., 2003a; 2005), which not only directly stimulates the oocyte growth but also activates the entire reproductive endocrine axis through feedback mechanisms (review of Zohar et al., 2010). In most female fish the ovarian steroids involved in oocyte growth are testosterone (T) and 17β-estradiol (E2) although 11-ketotestosterone (11-KT) is also important, as it is related to oocyte growth and lipid uptake (Matsubara et al., 2003b; Lokman et al., 2007; Endo et al., 2008) in eel species. The steroid activity can be regulated at different levels, for example through changes in steroid receptor expression or ligand affinity, or changes in the expression or activity of steroidogenic enzymes, like P450aromatase, the enzyme responsible for transforming androgens into E2.

Water temperature can affect E2 levels through changes in the gene expression of the ovarian P450aromatase gene (*cyp19a1*), as has been shown in some fish species with temperature-dependant sex determination (reviews Ospina-Alvarez and Piferrer 2008, Miranda et al., 2013). We have previously observed lower E2 plasma levels in European eel females maintained at a high temperature (Pérez et al., 2011), suggesting that temperature has an effect on the activity or gene expression of the ovarian P450aromatase gene (*cyp19a1*) in this species.

In this study, we have used two thermal regimes in an attempt to simulate the thermal changes that eels probably experience during

their migration, with lower temperatures during the oceanic migration and higher temperatures at the spawning grounds in the Sargasso Sea. We have compared the results of these to those of a third thermal regime, with a constant temperature of 18 ºC. The aim was to discover whether temperature can modulate ovarian development induced by CPE through changes in steroid production, and gene expression of *cyp19a1.*

2. Materials and methods

2.1. Fish handling

One hundred and eleven silver female eels (mean body weight 750±22 g; mean length 72.2±0.6 cm) caught in the Albufera Lagoon (Valencia, Spain) during their migration to the sea were transported to the facilities of the Universitat Politècnica de València (UPV, Spain). Eight healthy eels were killed during the first 24 h upon arrival, to serve as freshwater controls (FW). The remaining eels were kept in two 1500 L tanks equipped with recirculating freshwater systems (18 ºC), and were gradually acclimated (over 10 days) to seawater, and moved to three 500 L tanks (1 experimental group/tank), with 34-35 fish/tank (18 ºC). Each 500 L tank had an independent seawater recirculation system and was equipped with two water chillers (Boyu L500). All the tanks were covered with a black waterproof sunshade to maintain semi-dark conditions. The experiment lasted from March to June 2009. The eels were not fed throughout the experiments. All the fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Mortality throughout the experiment was 13-14% (groups T10-15, T15-18, 4 out of 30 fish in each group; T18 group, 5 out of the 35 fish), without differences between the experimental groups.

2.2. Thermal treatments

Figure 1 shows the thermal regimes and the sampling points. Before the experiment started, the water temperature was gradually adjusted from 18 ºC (ambient temperature) to the experimental temperatures (10, 15, or 18 ºC) in order for the animals to gradually acclimate to the experimental conditions. Once the water had been maintained at those temperatures for six weeks, the temperature in regime T10-15 was increased from 10 to 15 ºC until the end of the experiment (Fig. 1). In addition, 10 weeks after the temperature had been maintained at 15 ºC, the temperature in group T15-18 was increased to 18 ºC until the end of the experiment. The design of these thermal profiles was based on previous research of ours (Pérez et al., 2011) and took into account the thermal profiles used for gonadal maturation in Japanese eel (Ijiri et al. 2011; Unuma et al., 2012).

2.3. Hormonal treatment

After maintaining the fish for 2 weeks at 10, 15 or 18 ºC, the hormonal treatment started (Fig. 1, injections 1-12). The hormonal treatment consisted of weekly intra-peritoneal injections of carp pituitary extract (CPE; Catvis, Ltd. The Netherlands) at a dose of 20 mg kg^{-1}. The CPE was prepared as follows: 1 g of CPE was diluted in 10 ml of NaCl solution (9 g L^{-1}) and centrifuged at 1260 *g* for 10 min. The supernatant was collected and stored at -20 ºC until use, between 1-4 weeks later. Every week, before injecting, the eels were anesthetized (benzocaine, 60 mg L^{-1}) and weighed to calculate the individual hormone dosage. Some females did not respond (or responded very slowly) to the hormonal treatment, as they were still in the previtellogenic stage even after 8 CPE injections. In total there were 6 females that did not respond to the treatment (2 from T10-T15, 1 from T15-18, 2 from T18). They were not included in the statistical analyses.

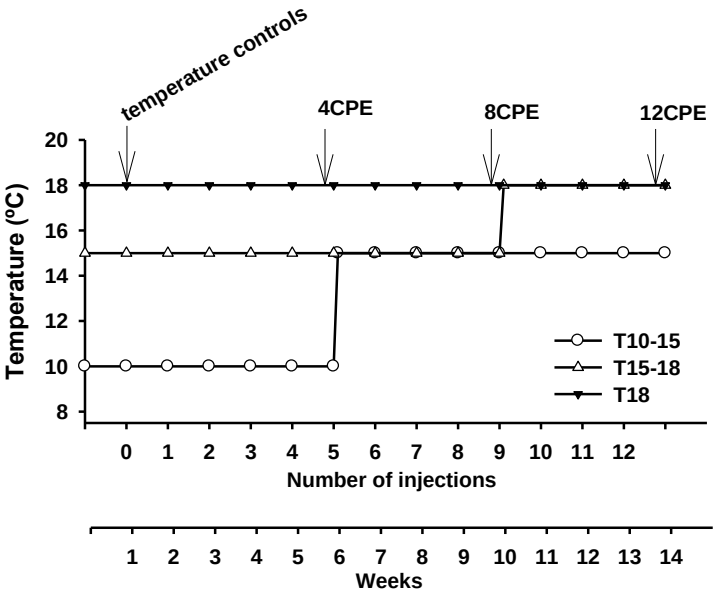

Fig. 1. Thermal regimes applied for each treatment (T10-15 open circle; T15-18 open triangle; T18 closed triangle). Arrows indicate sampling weeks: temperature controls (T0), and 7 days after the 4th, 8th and 12th CPE injections (4CPE, 8CPE, 12 CPE). Between 6-8 fish/treatment were sampled in each sampling point.

2.4. Fish sampling

Between 6 and 8 healthy females were sacrificed at each sampling point. Following the sampling for freshwater controls (FW) upon arrival at the UPV facilities, 8 eels were sacrificed following 7 days of temperature acclimation (10, 15 or 18 ºC), and used as temperature controls (T0, Fig. 1). Then, one week later, the hormonal treatment started in all the groups, with each fish receiving weekly CPE injections. Seven days after receiving the 4th, 8th and 12th injection at the different temperatures (sampling points

4CPE, 8CPE, 12CPE, Fig. 1) 6-8 fish were sacrificed from each group.

At each sampling, the eels were anesthetized (benzocaine, 60 mg L^{-1}) before being euthanized by decapitation. The gut was cut in the anal region and above the liver, and then weighed. Total body, gonad, and gut weights were recorded to calculate the gonadosomatic index (GSI=100 gonad weight x total body $weight^{-1}$) and Gut Index (GI=100 gut weight x total body $weight^{-1}$). In addition, total body length and eye diameter (vertical and horizontal) were measured to calculate the Eye Index (EI=100 ⊓ 0.25 $(Dh+Dv)^2$ x Lt^{-1}, where Dh = horizontal eye diameter, Dv=vertical eye diameter, and Lt=total body length (Pankhurst, 1982)). Blood was sampled from the caudal vasculature and centrifuged (3000 rpm, 15 min), and blood plasma was stored at -80 °C until analyses.

The gonad samples collected for histology were preserved in 10% buffered formalin. Triplicate samples from the gonad and liver were collected immediately after dissection from each fish, and then stored in RNA-later (Ambion Inc., Huntingdon, UK) at -20 ºC until RNA extraction and gene expression analyses by qPCR.

2.5. Gonad histology

After dehydration in ethanol, samples were embedded in paraffin and cut into 5-10 µm thick sections with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd. England). The slides were stained with haematoxilin and eosin and observed through a Nikon Eclipse E-400 microscope equipped with a Nikon DS-5M camera, all from Nikon (Tokyo, Japan).

One-hundred oocytes per specimen were measured (diameter), and the biggest ones were selected. The stages of oogenesis were determined according to Selman and Wallace (1989), Kayaba et al. (2001) and Pérez et al. (2011). In summary, the previtellogenic

stage included both the perinucleolar and lipid droplet stages; early vitellogenic oocytes contained small yolk globules restricted to the periphery of the oocyte, mid-vitellogenic oocytes showed abundant yolk vesicles and late vitellogenic oocytes showed more abundant yolk vesicles than lipid droplets.

2.6. RNA extraction and cDNA synthesis

2.6.1 Primer design

Eel acidic ribosomal phosphoprotein P0, *arp* (Table 1, Weltzien et al. 2005b; Aroua et al. 2007; Peñaranda et al. 2010) was used as the reference gene in the qPCR because its mRNA expression has been shown to be stable during experimental treatment (Weltzien et al. 2005b). The expression stability of the reference gene in the ovary was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1 (0.21; $p<0.05$) and Cq arithmetic mean of 10.1±0.72. The BestKeeper calculated variations in the reference gene based on the arithmetic mean of the Cq values. Genes with an 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 *cyp19a1* (Table 1). To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon-exon boundary. All primers were tested on gDNA and RNA to confirm that they would not amplify potentially contaminating gDNA. The specificity was confirmed by melting curve analysis, gel electrophoresis, and by the sequencing of the qPCR products.

Table 1. Primers used for qPCR analysis. Amplicon length and primer efficiency are given after Fw and Rv primer, respectively. GE=Gonad qPCR efficiency.

Gene	Sequence (5'- 3')	Orientation	Reference
cyp19a1	TTC AAG GGA ACG AAC ATC ATC	Fw (115 pb)	Tzchori et al. 2004
	AGA AAC GGT TGG GCA CAG T	Rv (GE=2.07)	
Arp	GTG CCA GCT CAG AAC ACG	Fw (107 pb)	Weltzien et al., 2005b
	ACA TCG CTC AAG ACT TCA ATG G	Rv (GE=2.18)	(AY763793.1)

2.6.2 SYBR Green assay (qPCR)

Total RNA was isolated from RNAlater preserved ovarian tissue following the method described by Hildahl et al. (2011a). The tRNA was then treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min. First-strand cDNA was synthesized from 2 µg total RNA, using random hexamer primers and superscript III reverse transcriptase (Invitrogen).

The qPCR assays were performed as described in Weltzien et al. (2005b), using a Light Cycler 480 system with SYBR Green I detection (Roche, Meylan, France). After an initial activation of *Taq* polymerase at 95 ºC for 10 min, 42 PCR cycles were performed using the following cycling conditions: 95 ºC for 10 s, 60 ºC for 10 s, and 72 ºC for 7 s. Each PCR reaction contained a total of 10 µl, comprising of 1:10 diluted cDNA template (3 µl), forward and reverse primers (250 nM each), and SYBR Green Master Mix (5 µl). Transcript levels were determined as Weltzien et al. (2005b), using an efficiency-adjusted relative quantification method (Pfaffl, 2001). Briefly, it was calculated from the formula:

Relative expression= $ET^{CpT(C) - CpT(S)} \times ER^{CpR(S)-CpR(C)}$

Where ET/ER is the efficiency of target/reference amplification and CpT/CpR is the cycle number at target/reference detection threshold. C is representing the calibrator and S the sample. Target and reference genes in unknown samples were run in duplicate PCR reactions, and a cDNA pool from ovarian samples was included in each run and acted as a calibrator (*cyp19a1*: 25.16±0.15; *arp*: 14.93±0.04). Non-template control (cDNA was replaced by water) for each primer pair were run on all plates.

2.7. Immunoassays for T, 11-KT, E2 and VTG

Testosterone (T) plasma levels were measured in 25µl duplicates of each sample using a competitive testosterone ELISA KIT (Eurobio AbCys, Les Ulis, France), with a sensitivity of 0.07 ng ml^{-1}, and an intra-assay variation of 6-10 %. The standard curve was between 0.008 and 16 ng ml^{-1}. All plasma samples were assayed in the same test.

11-KT plasma levels were measured in 25 µl duplicates of each plasma sample using an 11-KT ELISA Kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), with a sensitivity of 1-2 pg/ml and an intra-assay variation of 10-15%. The standard curve was between 0.78 and 100 pg ml^{-1}. All plasma samples were assayed in the same test.

T and 11 KT immunoassays were validated for eel plasma by performing the following tests: an assay of serial dilutions of various eel plasma samples and a validation of the parallelism with the standard curve; the addition of known amounts of steroid to eel plasma samples and the validation of the recovery; the addition of eel plasma to each standard dose and the validation of the recovery. These steroid immunoassays were previously carried out on the eels to measure the increases in androgen plasma levels during the transition from the juvenile yellow stage to the prepubertal silver stage (silvering; Aroua et al., 2005), and during

experimental maturation (Peñaranda et al., 2010; Jeng et al., 2012).

Vitellogenin (VTG) plasma levels were assayed using a homologous ELISA previously developed for the European eel. Details and validation of the assay have already been described (Burzawa-Gérard et al., 1991). Each plasma sample was assayed at serial dilutions in duplicates. The sensitivity of the ELISA was 1.7 ng ml^{-1}. The intra and inter-assay variation coefficients were 6.2% and 9.1%, respectively. This VTG assay had previously been carried out to measure the increase in VTG plasma levels during silvering (Sbaihi et al., 2001; Aroua et al., 2005), and during experimental maturation (Vidal et al., 2004; Durif et al., 2006; Pierron et al., 2008).

17β-estradiol (E2) plasma concentrations were measured by means of radioimmunoassay (RIA), according to the method described by Schulz (1984). In summary, free (i.e. not conjugated) steroids were extracted from 200 µl plasma with 4 ml diethylether after vigorously shaking for 4 min. The aqueous phase was frozen in liquid nitrogen, while the organic phase was transferred to a glass tube, evaporated in a water bath at 45 ^{o}C and then reconstituted through the addition of 600 µl assay buffer, and then assayed for E2. Cross-reactivities of the E2 antiserum have previously been examined by Frantzen et al. (2004)**.** The limit for the assay was 0.2 ng ml^{-1}. To validate E2 recovery from plasma in the eel assay, plasma pools were spiked with 5 and 15 ng E2 ml^{-1} of plasma and then subjected to ether extraction as described above. The resulting products from the different treatments were then assayed by the E2 RIA at two different dilutions. A plasma E2 dilution curve parallel to that of the assay standard curve was established. In addition, to test E2 extraction from plasma, radiolabelled steroid (c. 100000 c.p.m.) was added to 200 µl aliquots (n=8) of plasma and then ether extracted. Steroid recovery after ether extraction was 85.6±1.0%. E2 values were corrected for recovery losses. The inter- and intra-

assay coefficients of variation (CV) for the E2 assay were 9.4% (n=4) and 5.2% (n=10), respectively.

2.8. Statistical analysis

Each variable was analysed first for normality by the asymmetry standard coefficient and Curtosis coefficient. The variables that did not have a normal distribution were log-transformed and their normality was checked again. Then, a two-way ANOVA (treatment, sampling point) was performed to discover whether each variable was affected by the experimental treatments and/or by the sampling point. One-way ANOVA analyses were then performed to compare thermal treatments in the same sampling time. Variance homogeneity was checked using the Bartlett test. The one-way ANOVA analyses were followed by a Newman-Keuls post-hoc test. If normality failed following the log transformation, a non-parametric test was carried out (Kruskal-Wallis test), followed by a Dunn's test.

Pearson linear correlations between the different variables were calculated using the statistical software provided by Statgrafics Plus 5. Simple and multivariate regression analyses were performed to study the relationship between the GSI and the accumulated degrees day (ºD) and the accumulated CPE-doses, using the software provided by Statgrafics Plus 5. All the values are expressed as mean ± standard error of mean (SEM). Differences were considered significant when $p<0.05$. All the statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1. Morphological changes and gonadal development

Figure 2 shows the percentage of females in each developmental stage after ovarian histology observation.

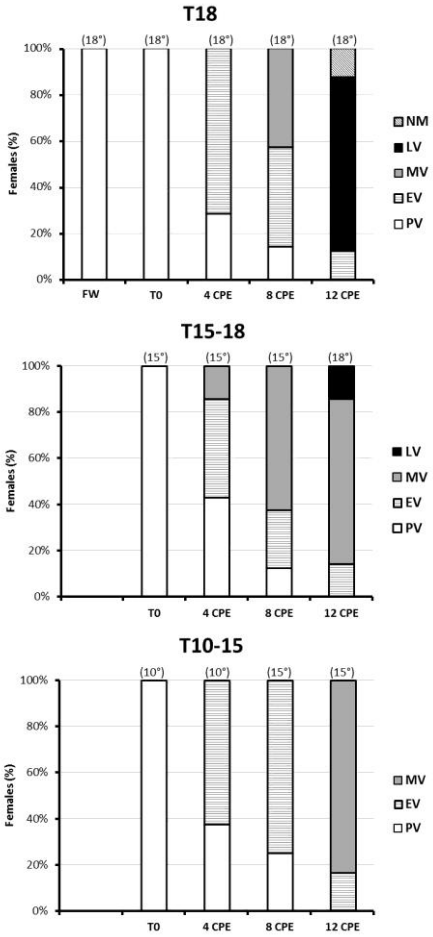

Fig. 2. Effect of hormonal treatment and thermal regime on ovarian development in freshwater (FW) control, temperature control (T0), and after 4, 8 or 12 CPE injections in each thermal treatment (n=6-8/group). PV: previtellogenic stage (white color); EV: early vitellogenic stage (horizontal lines); MV: mid vitellogenic stage (grey color); LV: late vitellogenic stage (black color); NM: nuclear migration stage (diagonal lines).

Before starting the hormonal treatment, all the eel oocytes were in the PV stage (FW and T0 controls). After 4 CPE injections (4CPE), ovaries in the early vitellogenic stage (EV) were present in all the groups, and the mid-vitellogenic (MV) stage was even observed in 14% of T15-18 females. Four weeks later (8CPE), females from groups T15-18 and T18 were in the MV stage (62% and 43% respectively), while females from T10-15 only developed to the EV stage. In the last sampling (12CPE) most females from T18 were in the late vitellogenic stage (LV), while in the other groups they were mostly in the MV stage (Fig. 2).

Figure 3 shows the changes of the biometric parameters throughout the experiment. The GSI and oocyte diameter gradually increased throughout the experiment ($p<0.01$). At the 12CPE, the highest GSI ($p<0.01$) and oocyte diameter ($p<0.05$) were observed in group T18 (Fig. 3A, B). The Eye Index (EI, Fig. 2C) increased from FW to SW, and at this point it was lower in the high temperature group, T18 ($p<0.001$), than in the other two groups. Gut Index was higher ($p<0.01$) in FW eels than in the T0 controls.

3.2. Steroid and vitellogenin plasma levels

11-ketotestosterone (11-KT) and testosterone (T) plasma levels (Fig. 4A, B) were in general lower in group T18 than in the other groups. 11-KT plasma levels (Fig. 4A) were lower in the T18 group in SW control ($p<0.01$), and at 4CPE both 11-KT and T were lower in T18 compared to group T10-15 ($p<0.01$).

The thermal treatment also affected the E2 plasma levels (Fig. 4C), which were lower in group T18 compared to group T10-15 in two time-points: T0 controls, and 8CPE ($p<0.05$). In general, E2 levels decreased after SW and temperature adaptation, followed by an increase after 4 and 8 CPE injections, and a new decrease after 12 injections ($p< 0.01$). Similar to E2, VTG plasma levels decreased after SW and temperature adaptation, but showed a high increase

after 4 CPE injections, reaching peak values after 12 CPE injections. The thermal treatments affected the VTG plasma levels, which were higher in group T18 both in SW and after 12 CPE injections ($p<0.01$).

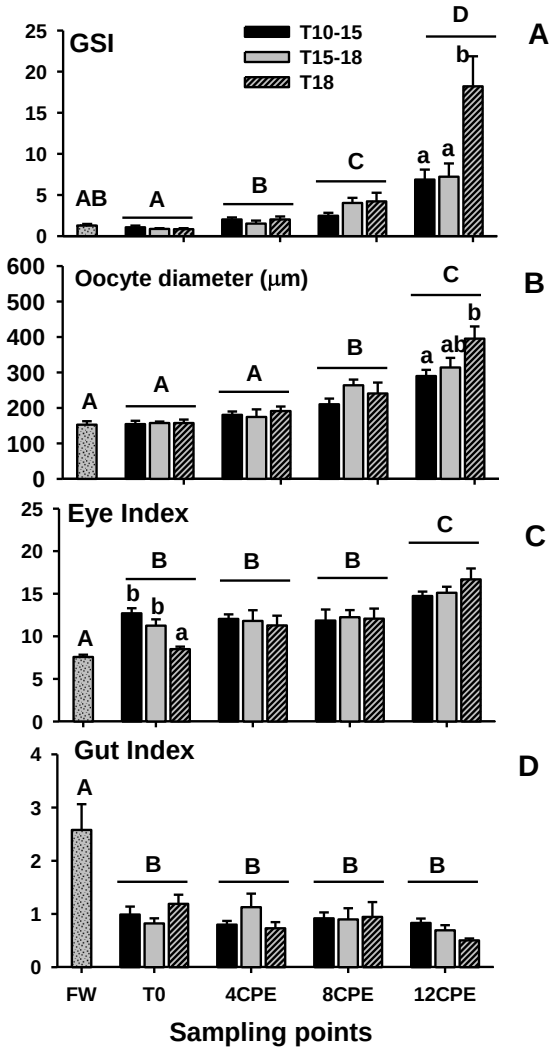

Fig. 3. Effect of hormonal treatment and thermal regime on biometric parameters: Gonadosomatic Index (GSI; A), oocyte diameter (B) Eye Index (EI; C) and Gut Index (D) in freshwater (FW) control, temperature control (T0), and after 4, 8 or 12 CPE injections in each thermal treatment (n=6-8/group). Small letters indicate significant differences between the thermal treatments in a same sampling point ($p<0.05$, df=2). Capital letters indicate significant differences through time considering all thermal treatments ($p<0.05$, df=4).

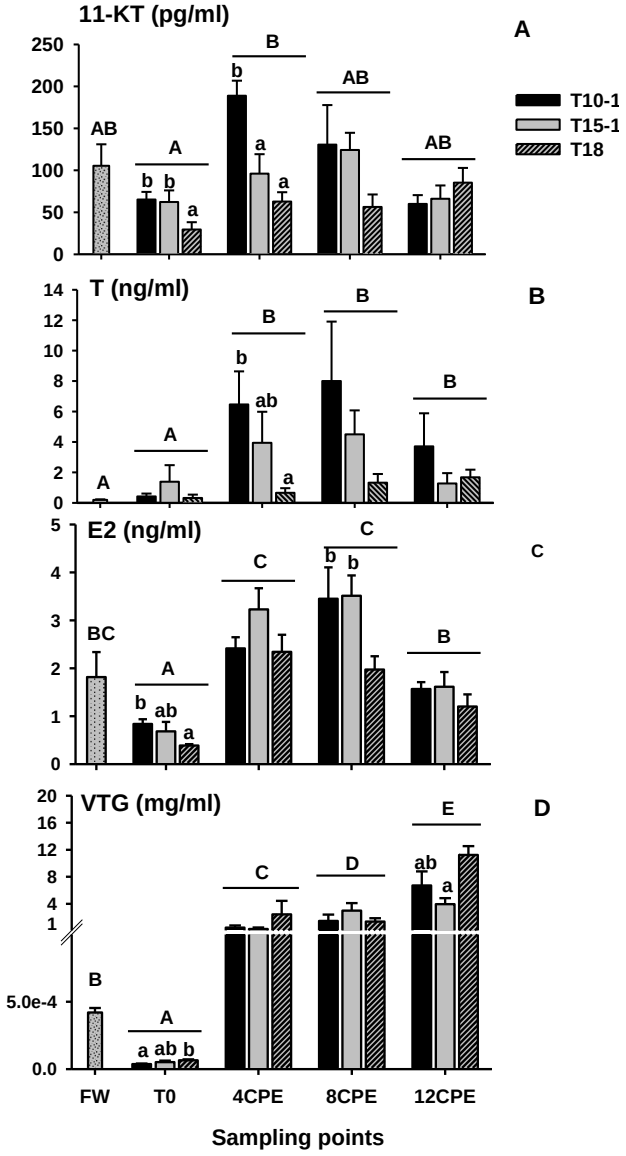

Fig. 4. Effect of hormonal treatment and thermal regime on 11-ketotestosterone (11-KT, A), testosterone (T, B), 17-β-estradiol (E2, C) and vitellogenin (VTG, D) plasma levels in freshwater (FW) control, temperature control (T0), and after 4, 8 or 12 CPE injections in each thermal treatment (n=6/group). Small letters indicate significant differences between the thermal treatments in a same sampling point ($p<0.05$, $df=2$). Capital letters indicate significant differences through time considering all thermal treatments ($p<0.05$, $df=4$).

3.2. Ovarian *cyp19a1* expression

Ovarian *cyp19a1* expression (Fig. 5) increased progressively throughout the experiment.

Group T18 showed reduced a *cyp19a1* expression at 4CPE ($p<0.01$) compared to the other groups. If we take into account only the previtellogenic females (Fig. 6), treatments T10-15 and T15-18 induced a significantly higher expression of *cyp19a1* than T18 ($p< 0.01$). In addition, T levels were lower in group T18 compared to the lower temperature group, T10-15. When examining only the early vitellogenic females, it was observed that a high constant temperature (T18) caused a low expression of *cyp19a1* ($p<0.01$) compared to group T10-15, and lower T levels (and a similar, not significant trend in E2) compared to the other two groups.

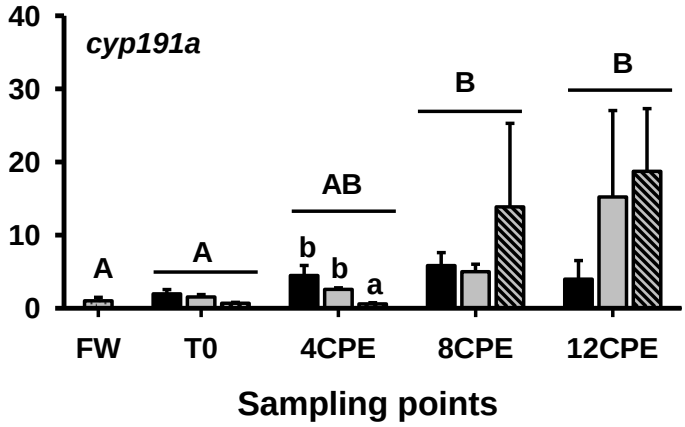

Fig. 5. Effect of thermal and hormonal treatment on ovarian *cyp19a1* gene expression in freshwater control (FW), temperature control (T0), and after 4, 8 and 12 CPE injections. The relative expression was normalized to the abundance of *arp*. Results are expressed as mean of fold change ± SEM (n=6) with respect to the FW control, which has been set at 1. Small letters indicate significant differences between the thermal treatments at the same sampling point ($p<0.05$, df=2). Capital letters indicate significant differences through time considering all thermal treatments ($p<0.05$, df=4).

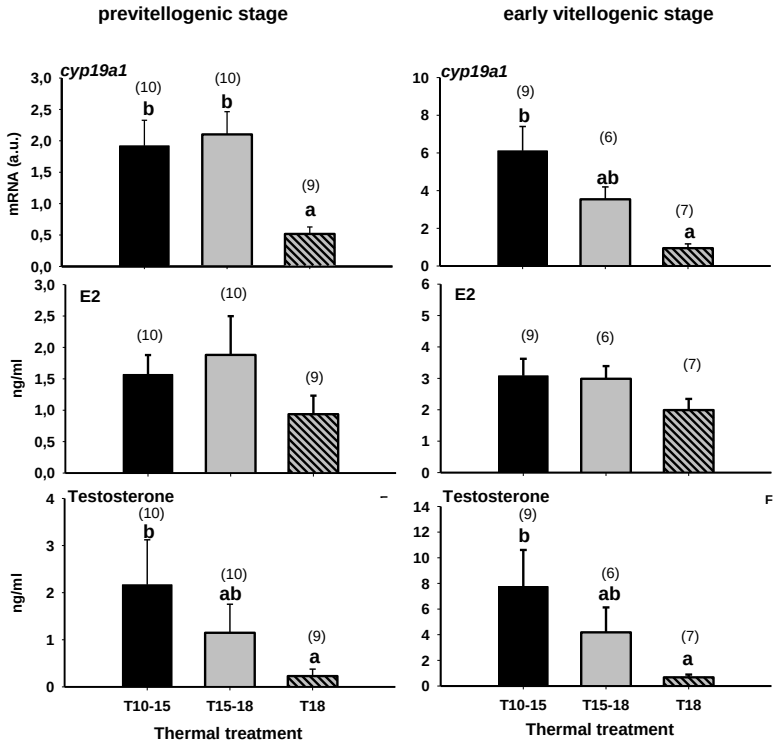

Fig. 6. Previtellogenic and early vitellogenic levels of ovarian *cyp19a1* gene expression, plasma 17β-estradiol (E2) and testosterone, in each thermal treatment. Small letters indicate significant differences between the thermal treatments ($p<0.05$, $df=2$). Number of fish/group is show in brackets.

4. Discussion

4.1. Low temperatures induced steroidogenesis prior to hormonal treatment

Ovaries in previtellogenic stage were observed in the FW control group, as well as in the eels sampled at T0, after having spent one month in SW. This corroborates the idea that captive eels experience a gonadotropin insufficiency (Dufour et al., 1989) or dopamine blockage of the reproductive neuroendocrine axis (Dufour et al., 2005; Vidal et al., 2004). The FW control eels showed higher

E2 and VTG levels than the eels from T0, indicating that seawater alone does not facilitate vitellogenesis but, on the contrary, may reinforce the previtellogenic blockage.

Interestingly, before starting the hormonal treatments, water temperature alone affected several parameters including Eye Index and 11-KT and E2 plasma levels. These were all lower in the group kept at 18 ºC compared to 10 ºC. It has been shown that 11-KT promotes previtellogenic oocyte growth in shortfinned eel (*A. australis*; Lokman et al., 2007) and coho salmon (Campbell et al., 2006; Forsgren and Young, 2012), and also potentiates the effect of E2 in stimulating hepatic synthesis of VTG in Japanese eel (Asanuma et al., 2003). Regarding E2, apart from the known role it plays during vitellogenesis, it has been linked to oogonial proliferation in Japanese eel (Miura et al., 2007) and to previtellogenic oocyte growth in coho salmon, (Campbell et al., 2006; Forsgren and Young, 2012). In Japanese eel it has been suggested that a decrease in water temperature induced an early stage of ovarian development, with the thermal reduction from 25 to 15 ºC increasing 11-KT and E2 levels and the oil droplet number in PV oocytes (Sudo et al., 2011). While the oil drop number was not measured in this experiment, other parameters suggest that low temperatures can facilitate the oocyte growth at the PV stage. For instance, the GSI values were higher (but without statistical differences) in the eels maintained at 10 ºC compared to the eels maintained at 18 ºC (1.08 vs 0.86, respectively), and the GSI showed a positive correlation with 11-KT levels ($r=0.58$; $p=0.006$; Table 2), which were higher at low temperatures.

On other hand, the Eye Index, which is an indicator of the onset of eel puberty (Aroua et al., 2005), was lower in female eels maintained at 18 ºC compared to the other temperatures. This thus supports the idea that maintaining female eels at this temperature in the PV stage does not facilitate the previtellogenic growth. Similar to the results found at low temperatures, an increase in Eye Index,

11-KT plasma levels, and GSI were also observed after maintaining European eels swimming during long periods (reviewed by Palstra et al., 2009).

Table 2. Correlation cofficients at PV stage (n= 29)

	oocyte diameter	T	11-KT	E2	*cyp191a*	VTG
GSI	0,397 0,027	0,308 n.s.	**0,579** 0,000	0,238 n.s.	0,162 n.s.	0,309 n.s.
Oocyte diameter		0,2837 n.s.	0,308 0,092	-0,077 n.s.	0,184 n.s.	-0,112 n.s.
T			0,6486 0,000	0,388 0,0310	0,215 n.s.	0,111 n.s.
11-KT				0,212 n.s.	0,071 n.s.	0,002 n.s.
E2					0,395 0,028	**0,700** 0,000
cyp191a						0,388 0,031

Thus, both low temperatures and swimming could be promoting previtellogenic growth, which seems logical as eels in nature should experience both parameters (swimming at low temperatures) at the same time.

Our results show, for the first time, a down-regulation of ovarian *cyp19a1* expression at 18 ºC compared to lower temperatures in

adult European eel at the previtellogenic stage. In Japanese eel, Ijiri et al. (2003) demonstrated a strong correlation between ovarian aromatase gene expression and aromatase enzyme activity from ovarian follicles, strongly suggesting that aromatase enzyme activity would be lower at 18 ºC in the ovaries of the European eel females analysed in the present work. This corresponds very well with the lower E2 levels observed at high temperatures, also at the previtellogenic stage.

4.2. High temperatures reduce CPE-induced steroidogenesis and *cyp191a* expression at the early vitellogenic stage

Carp pituitary injections (CPE) provide exogenous gonadotropins directly to the ovaries, and result in the subsequent stimulation of ovarian steroid synthesis (Matsubara et al., 2003a; 2005) and the activation of the entire reproductive neuroendocrine axis through feedback mechanisms (review of Zohar et al., 2010). Thus, ovaries from eels maintained at different temperatures showed different responses to CPE treatment, as evidenced by ovarian development and steroid plasma levels.

In this experiment, early vitellogenic CPE-treated eels kept at constant high temperatures (T18) showed reduced *cyp19a1* gene expression levels and lower androgen plasma levels (T, 11-KT) than fish maintained at 10-15 ºC (Group T10-15). Vitellogenesis is an E2-dependent process, and aromatase is the enzyme which converts androgens (mainly T) into E2. Aside from this, 11-KT also enhances E2-induced VTG synthesis (Asanuma et al., 2003). Thus, the highest steroid and *cyp191a* expression levels observed at low temperatures in the EV stage suggest that CPE-induced early vitellogenic growth could be facilitated by low temperatures. This agrees with the positive correlation found between oocyte diameter and *cyp19a1* expression at this stage ($r=0.67$, $p< 0.001$, Table 3).

Table 3. Correlation cofficients at EV stage (n= 22)

	oocyte diameter	T	11KT	E2	*cyp191a*	VTG
GSI	**0,687**	-0,151	0,398	-0,195	0,254	0,248
	0,002	n.s.	n.s.	n.s.	n.s.	n.s.
Oocyte diameter		-0,337	0,290	-0,155	**0,665**	0,562
		n.s.	n.s.	n.s.	0,003	0,015
T			0,243	0,342	0,122	-0,420
			n.s.	n.s.	n.s.	n.s.
11KT				-0,063	0,405	-0,084
				n.s.	n.s.	n.s.
E2					-0,015	-0,403
					n.s.	n.s.
cyp191a						**0,476**
						0,046

Previous research on other fish species have shown reductions in E2 plasma levels at high temperatures during vitellogenesis (striped bass *Morone saxatilis*, Clark et al., 2005; Atlantic salmon, review by Pankhurst and King, 2010; pikeperch *Sander lucioperca*, Hermelink et al., 2013), but only a few studies on adult fish have previously demonstrated an inhibition of the expression of aromatase by thermal regimes. For the first time, we have demonstrated a down-regulation of *cyp19a1* in CPE-treated female European eels at high temperatures. Similarly, a reduced aromatase expression at high

temperatures has been observed in adult red seabream (*Pagrus major*) and Atlantic salmon, in previtellogenic and vitellogenic stages, respectively (Lim et al., 2003; Anderson et al., 2012).

The E2 profile during sex maturation was similar in all the thermal groups, increasing during early-mid vitellogenesis (weeks 4-8) and decreasing thereafter, in fish which were either in the MV or LV stage. A similar increase in E2 levels during vitellogenesis has previously been observed in European eel (Pérez et al., 2011), and in New Zealand long-finned eels (*A. dieffenbachii*, Lokman et al., 2001). Nevertheless, the E2 levels of Japanese eel matured at 20 ºC were low during vitellogenesis, and increased only in the LV stage (Matsubara et al., 2003a) or later (Ijiri et al., 1995; Suetake et al., 2002). Such differences in the E2 response to pituitary treatments could be species-specific.

VTG and E2 showed a high degree of correlation in the PV stage (0.70, $p<0.01$, table 2), but the VTG profiles did not follow the same pattern as the E2 profiles. While the E2 plasma levels decreased at the end of the hormonal treatment, the VTG plasma levels increased, corroborating the ovarian histological observations. A lack of consistency between the E2 and VTG plasma levels has already been observed in a number of fish species (reviewed by Pankhurst, 2008), and may be due to the short half-life of steroids in the plasma (Pankhurst, 2008), or to the time lapse between the increase in plasma E2 and the release of vitellogenin to blood plasma. Classical steroid actions occur through several steps, and it takes from hours to days between steroid synthesis and the appearance of its biological effect (reviewed in Norris, 1996).

The 11-KT levels found in this research study were lower than those previously reported by van Ginneken et al. (2007) or Palstra et al. (2009) in their studies on European eel females, but similar to those previously reported by Sébert et al. (2007; 2008), or Aroua et al.

(2005). The differences may be due to the different methods used to measure 11-KT, i.e. radioimmunoassay vs ELISA.

4.3. Temperature modulates the progression of vitellogenesis

The results of this research confirm that thermal regimes affect ovarian development in the European eel, agreeing with our previous results (Pérez et al., 2011). The present results allow us to take a closer look at the combined effect of temperature and hormonal treatment on the progression of vitellogenesis in the European eel. In this research we have shown that, during hormonal treatment, early vitellogenesis can be reached at 10, 15, or 18 ºC, as evidenced by the histological features and the GSI increase after 4CPE. However, further development to the mid-vitellogenic stage is delayed in eels maintained at 10 ºC during the first weeks of hormonal treatment even if they are then transferred to 15 ºC, as results from 8CPE show.

On the other hand, ovarian development up to the mid-vitellogenic stage was as fast at 15 ºC as at 18 ºC, as the histological results from 8CPE show (comparison between T15-18 and T18). However, further development to the late vitellogenic stage was delayed in the eels maintained for 8 weeks at 15 ºC compared to eels maintained at a constant 18 ºC, even when both groups were maintained at the same temperature (18 ºC) during the last part of the experimental period, from 8CPE to 12CPE. Thus, the results obtained can only be explained on the basis of the thermal period experienced by the eels prior to each sampling. Table 4 indicates the degrees day (ºD) experienced by the female eels before they reached the different development stages, and the accumulated CPE dose received until that time.

Table 4 shows that the MV stage was reached after 1120 ºD (group T10-15), 840-1340 ºD (group T15-18), and 1008 ºD (group T18).

Also, the LV stage was reached after 1512 ºD (group T18 at 12CPE), but not after 1340 ºD (group T15-18 at 12CPE) or 1120 ºD (group T10-15 at 12CPE).

Table 4. Degrees day experienced by female eels before reaching the different development stages, and accumulated CPE dose received until that time (mg kg $^{-1}$). EV: dominance of early vitellogenic stage; MV: dominance of mid-vitellogenic stage; LV: dominance of late vitellogenic stage. Present experiment. Pérez et al., (2011) and Vílchez et al. (2013) were calculated from our original data. Mordenti et al. (2013) was estimated from the data provided in Mordenti et al. (2013).

	EV	**MV**	**LV**
Present experiment			
T10-15	280 ºD (80 mg) 700 ºD (160 mg)	1120 ºD (240 mg)	
T15-18	420 ºD (80 mg)	840 ºD (160 mg) 1260 ºD (240 mg)	
T18	504ºD (80 mg)	1008 ºD (160 mg)	1512 ºD (240 mg)
Pérez et al. (2011)	640 ºD (106 mg)	987 ºD (200 mg)	1680 ºD (240 mg)
Vílchez et al. (2013)		1173 ºD (240 mg)	1672 ºD (320 mg)
Mordenti et al. (2013)			1628 º D (420 mg)

These results suggest that, in CPE-induced eel gonadal maturation, an accumulation of 900-1200 ºD (and 160-240 mg CPE kg^{-1}) should facilitate development up to the mid-vitellogenic stage, while an accumulation of more than 1300 ºD would facilitate development up to the late vitellogenic stage. This agrees with previous results of ours (Pérez et al., 2011), where the MV stage was reached after a mean accumulation of 987 ºD or 1172 ºD (Pérez et al., 2011, Vílchez et al. 2013), while the LV stage was the dominant stage after an accumulation of 1680 ºD, although not observed after 1220

ºD. Also, Mordenti et al. (2013) observed LV stages in CPE-treated European eels after an accumulation of 1628 ºD (our own calculations, Table 4), but not after 1085 ºD.

Although it is clear that temperature increased the rate of ovarian development, our results clearly indicate that this increase is not linear. A significant exponential regression ($p<0.001$) between GSI and ºD was found (Fig. 7).

Thus, the GSI of CPE-treated eels increased exponentially with the ºD experienced, thus indicating that the GSI growth accelerated from an accumulation of about 1300 ºD. When data from other experiments (Pérez et al., 2011; Vílchez et al. 2013) was added to this model, the significance of the exponential regression model increased ($R^2=0.95$; $r=0.97$ $p<0.001$, Fig. 7B).

Nevertheless, at the same time as the ºD accumulated, the fish received additional doses of CPE. A significant exponential regression ($p<0.001$) was also seen between the GSI and the accumulated CPE dose. However, this exponential correlation between the GSI and the accumulated CPE-dose was weaker ($R^2= 0.81$; $r=0.90$) than the correlation between the GSI and the accumulated ºD ($R^2= 0.93$; $r=0.96$). Also, when a multivariate regression model was applied to explain the GSI variation from both ºD and accumulated CPE-dose variables, only the first variable was significant (data not shown). While it is impossible to differentiate between the effects of the accumulated ºD day and the accumulated CPE-doses, it would appear that the increase in GSI accelerates from a certain level of ºD (about 1300 ºD) or accumulated dose of CPE (about 240 mg). This knowledge could be applied in the design of thermoperiods for the induction of eel maturation, as well as to manipulate the timing of the progression of vitellogenesis in laboratory conditions.

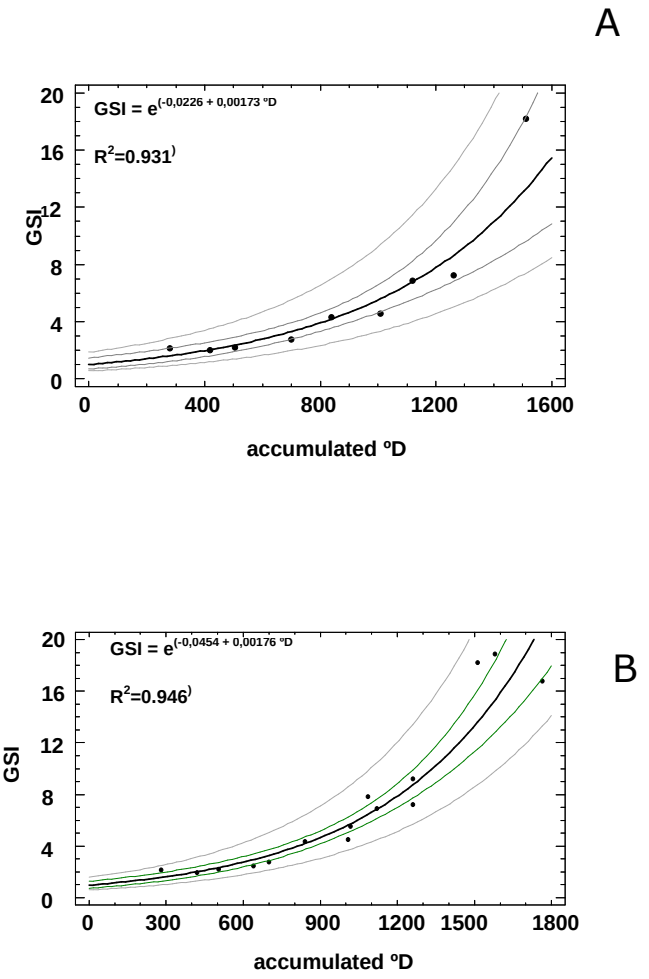

Fig. 7. A. Relationship between GSI and degrees day (ºD) accumulated from the start of hormonal treatment (time-points 4CPE, 8CPE, 12CPE). Significant positive correlation was found between GSI and ºD ($r=0.96$; $p< 0.001$). B. Relationship between GSI and degrees day (ºD) using the data from present experiment and from Pérez et al. (2011), and Vílchez et al., (2013).

5. Conclusions

For the first time in the European eel, a down-regulation of ovarian aromatase gene expression (*cyp191a*) by high temperatures has

been demonstrated. This study has also proved that low temperatures alone induced steroidogenesis in previtellogenic eel ovaries, but high temperatures during CPE treatment caused an acceleration of ovarian growth to late vitellogenic stage.

The dual role of low and high temperatures on eel maturation suggested in this study may reflect the natural ecophysiological situation. The progression of vitellogenesis, likely impairing swimming capacities, would be prevented by the low temperatures encountered during the transoceanic migration, while the high temperature of the spawning ground would facilitate the late vitellogenic stages and the final ovarian maturation of the European eel.

Acknowledgements

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GENERAL DISCUSSION

The results obtained and shown in the present thesis provide a further step in the knowledge of European eel vitellogenesis and the physiological changes occurring during the different phases of gonadal development.

Previtellogenesis and early vitellogenesis

Going from previtellogenesis (PV) to early vitellogenesis (EV) stage, changes in gene expression at brain level are recorded in olfactory bulbs (OB) and mi-diencephalon (MD), with an increase of *aa-cyp19a1* in the former and *aa-arb* gene expression in both of them. One possible explanation of this aromatase and *aa-arb* increase is that it could be related with an increase of 17β-estradiol (E2) resulting in a positive effect on maturation. Androgen receptors (ARs) would guarantee a higher detection of testosterone (T) which could increase E2 synthesis, indispensable for the following gonadal development. However, the higher T detection due to the increase in *aa-arb* gene expression in OB and MD could also increase the dopamine (DA) inhibition in the forebrain and block the maturation (Dufour et al., 1988; 2005; Linard et al., 1996; Montero et al., 1996; Vidal et al., 2004), also considering that the forebrain is the region with the highest level of tyrosinase hydroxilase, the limiting enzyme for DA synthesis (Weltzien et al., 2006). Also, the changes recorded in OB can be explained because forebrain is the region which mainly responds to environmental stimuli and to pheromones, as evidenced in goldfish (Fujita et al., 1991).

An explication for this apparent contradiction can be that at the beginning of vitellogenesis, European eels start to respond to the hormonal treatment getting prepared for the following maturation as suggested by the possible E2 synthesis, but the DA blockage is still strong and prevents the maturation since in the nature this step

would match with the migration. Compared to other anguillid species, European eel has to face one of the longest migration (Aoyama et al., 2003) and the degree of gonad development at the beginning of the migration is inversely related with the migration distance (Sudo and Tsukamoto, 2013). It can be suggested that the maturation parameters found in other *Anguilla* spp at the beginning of the migration could give an idea of those of European eel at a more advanced gonadal development, when specimens are not easily fished

In the gonads, an increase in *aa-arb* expression has been recorded and it can be related with the previtellogenic oocyte growth, due to the role that androgens play in this event (Rohr et al., 2001; Matsubara et al., 2003a,b; 2004; Lokman et al., 2007; Kazeto et al., 2011; Setiawan et al., 2012).

So, it can be resumed that at the beginning of the vitellogenesis eels are receptive to environmental stimuli which trigger the first physiological changes bringing to vitellogenesis, but still suffer DA inhibition at brain level which prevents maturation.

Vitellogenesis

Changes during the hormonal treatment are mainly recorded during the passage from EV to vitellogenesis (V) and involve brain, pituitary and gonads.

The decrease of *aa-ara* gene expression in the forebrain suggests a removal of the DA blockage. The DA mechanism is one of the strongest obstacles to maturation (Weltzien et al., 2009) and obtained results confirm it is removed only approaching vitellogenesis.

At the same time, a general increase in aromatase gene expression was recorded, suggesting that there is an intense E2 synthesis

which could have a positive effect on eel maturation and on *aa-gnrh1* gene expression (Montero et al., 1995).

In agreement with the possible increase of *aa-gnrh1*, an increase in *aa-gnrhr1b* and *aa-gnrhr2* gene expression was recorded in pituitary, being the receptors indispensable for GnRH (Gonadotropin Releasing Hormone) action and gonadotropin release. In pituitary, also *aa-arb* and *aa-cyp19a1* gene expression increase was recorded, and the parallel increase of both gene expressions suggests that E2 is synthesized from T.
To validate this idea, studies involving other tecnhinque rather than qPCR are necessary. So, it would be useful obtaining information not only abut the temporal gene expression, but also the effective protein synthesis and localization.

The same mechanism can be deduced also for the gonads where ARs and aromatase gene expression increase in this step. The higher levels of E2 could have a positive feedback effect on GnRH-R (GnRH-Receptor) gene expression in the pituitary, as evidenced in other teleost species (Levavi-Silvan et al., 2006; Lin et al., 2010). Also, E2 could have a positive feedback effect on aromatase gene expression in the brain (but not in the ovaries), as demonstrated in the Japanese eel (Jeng et al., 2012).
In the future, special attention should be paid to ZP gene expression, both in males and females. In immature males, their expression was not found in testis (Mazzeo et al., 2012, chapter III), which is the common situation in fish, unlikely what was found in Japanese eel (Miura et al., 1998). Regarding the females, present results confirm that ovary is the main site of ZP production in European eel, while, because of the really lower expression level, the synthesis of ZPs in the liver is probably not related with the sexual maturation. However, due to the important role that ZPs play in oocyte formation, it would be very important to understand ZP

role and their synthesis regulation. In the same way, since many other ZP genes were identified in European eel after our experiment (Van den Thillart and Dirks, personal communication), and in spite of having studied in the present work the most important ZPs (at least accordingly with previous studies in Japanese eel), it can be concluded that further studies, focused on egg formation and quality, are needed.

Temperature effect

Studying temperature effect on vitellogenesis, it was observed that high constant temperature causes a faster maturation, but results from gene expression and steroid levels suggest that lower and increasing starting temperature really influences eel vitellogenesis and could have a positive effect on maturation and egg quality.

This idea is confirmed also by results obtained at brain level (Mazzeo et al., unpublished results). In fact, a general higher expression of genes involved in vitellogenesis was observed at the beginning of the vitellogenic process. In particular, a higher *aa-arb* gene expression in the brain and pituitary was found at lower temperatures at the beginning of the vitellogenesis. It can be deduced that lower temperatures increase DA inhibition since the animal perceives low temperatures as the signal that migration has not finished yet and so it is not worth of starting vitellogenesis. Moreover, DA has been positively related to eel orientation, crucial for a successful reproductive migration (Westin, 1990; Sébert et al., 2008; Pasqualini et al., 2009), so its higher synthesis can have a positive effect on eel orientation and it is enhanced at lower temperatures, one of the environmental factors triggering eel migration.
Also brain *aa-gnrhr1b* and *aa-gnrhr2* gene expression was higher at lower temperatures at the beginning of vitellogenesis, suggesting

again that low temperatures can have a positive effect on eel maturation.
All these results together suggest that lower temperatures reinforce DA blockage while, at the same time, the obtained steroid plasma levels suggest that low temperatures promote early vitellogenic phases and the previtellogenic oocyte growth. However, if the higher androgen levels generated by low temperatures promote the early development, higher temperatures are necessary for the vitellogenesis going on.

Thermal regimes with initial low and increasing temperatures are more similar to the environmental stimuli and, as consequence, it could result in a better oocyte and likely embryo quality.
It should also be considered that maturation under artificial conditions is quite different to the natural one. In fact, by hormonal treatment at 18 ºC, maturation can be reached in 3 months, depending on the individuals, while in the wild eels migrate during several months under no constant conditions and experiment a temperature increase approaching the spawning areas at the Sargasso Sea (van Ginneken and Maes, 2005). Hence, it cannot be discarded the traditional maturation protocol used in this species is too fast and brings to an impaired vitellogenesis.
As negative point, starting with lower temperatures makes the hormonal treatment and the eel maturation still longer, which can be counter-productive, especially if the new findings are going to be applied at aquaculture level.

So, a longer maturation at lower temperature could be physiologically recommended. However, further researches are necessary to evaluate its true applicability and usefulness, especially considering the costs which a longer hormonal treatment would imply. For this purpose, it would be very important obtaining eggs and embryos from eels matured under different thermal

regimes. It would allow evaluating if these differences at physiological level result in a different embryo quality and survival, which is one of the final goals of the studies in eel reproduction.

CONCLUSIONS

1. Six new genes have been characterized and described: *aa-cyp19a1* (Accession Number: KF990052.1), *aa-gnrhr1a* (AN: JX567769.1), *aa-gnrhr1b* (AN: JX567770.1), *aa-gnrhr2* (AN: JX567771.1), *aa-zpb* (AN: 982278.1) and *aa-zpc* (AN: 982279.1).
2. Among GnRH-Rs, *aa-gnrhr1b* and *aa-gnrhr2* are probably mainly involved in vitellogenesis, while for *aa-gnrhr1a* a role in osmoregulation rather than in gonadal development was suggested. Also ARs play different roles, being *aa-arb* probably more involved in sexual maturation due to its higher gene expression in pituitary and gonads. In the brain, a decrease in *aa-ara* and an increase in *aa-cyp19a1* gene expression were found, suggesting that decreased androgen and increased estrogen levels are necessary for advancement of the maturation process. During vitellogenesis, in pituitary and ovary, *aa-ar*, *aa-arb*, and *aa-cyp19a1* expression levels increased in line with gonad maturation.
3. On one hand high and constant temperature results in a faster vitellogenic process, on the other hand eels submitted at lower temperatures at the beginning of the maturation present stronger signals of previtellogenic oocyte growth that could improve oocyte quality.

FUTURE PERSPECTIVES

Obtained results indicate that temperature affects vitellogenesis in European eel. However, with the available data it is not possible to establish if these differences result in differences in gamete quality as only one group reached late vitellogenic stage and no gametes were obtained at all.
However, temperature proves to be an important environmental factor regulating vitellogenesis and going on experimenting on its modulator effect could supply useful information on eel reproductive physiology.

Since eels apparently suffer a daily temperature change and oscillation rather than a progressive increase of temperature from the starting point to the Sargasso Sea, it could be useful studying how these changes affect eel vitellogenesis.

More attention should be paid also to see if these differences in vitellogenesis progression result, at the end of the maturation, in the production of higher quality gametes and an increase in spawning rate and embryo survival. In fact, obtaining good quality and progeny is the main goal of studying vitellogenesis in European eel and of improving the maturation protocol in order to close the reproductive cycle of this species.

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