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Temperature modulates testis steroidogenesis in European eel

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ABSTRACT

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

1. Introduction

The European eel (*Anguilla anguilla*) is a teleost fish with a peculiar life cycle in which pubertal individuals undertake, apparently in 6–7 months, a transatlantic migration to the spawning areas in the Sargasso Sea (Tesch, 1978). The precise route that they take and the depth they swim at are not well known. However, satellite tags, used to document the oceanic migratory route up to 1300 km off the European coasts, have shown that they make daily vertical migrations between depths of 200 and 1000 m. It appears that they swim in shallower and warmer waters through the night (means of 282 m and 11.7 °C), while at dawn they descend to deeper and colder waters (means of 564 m and 7–10 °C) (Aarestrup et al., 2009). However, several authors have expressed doubts regarding the validity of the data gathered by satellite tags as they may have a negative effect on the swimming performance and energetics of the fish (Methling et al., 2011).

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

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

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

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

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1967), and that is the reason why the maturation of males and females of this species has traditionally been performed in water of that temperature (Peñaranda et al., 2010; Pérez et al., 2009). However, the influence of temperature on the maturation process of the European eel has recently been noted both in females (Mazzeo et al., 2014; Pérez et al., 2011) and in males (Baeza et al., 2015; Tanaka et al., 2001).

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

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

Regarding androgens, 11-ketotestosterone (11-KT) is considered to be the most important in teleosts (Miura and Miura, 2003) and is biosynthesized from testosterone (T) by two enzymes, 11β-hydroxylase (cytochrome P450-11β) and 11β-hydroxysteroid dehydrogenase (11β-HSD; Jiang et al., 2003). In teleosts, 11β-HSD sequence is similar to mammalian 11β-HSD type 2 (Albiston et al., 1994). Some examples can be found in the rainbow trout (*Oncorhynchus mykiss*, Kusakabe et al., 2003), tilapia (*Oreochromis niloticus*) and Japanese eel (Jiang et al., 2003). In eel, two homologous genes of mammalian 11β-HSD type 2 are present in the testis: 11β-HSD (Albiston et al., 1994; Jiang et al., 2003; Kusakabe et al., 2003) and 11β-HSD short form (11β-HSDsf) (Ozaki et al., 2006), both enzymes with 11β-dehydrogenase activity.

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

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

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

2. Materials and methods

2.1. Fish maintenance, hormonal treatment and sampling

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

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

Each week groups of 5–8 eels per thermal regime were anesthetized with benzocaine dissolved in seawater (>60 ppm) and sacrificed by decapitation (total amount: ~273 fish). Total weights and gonad weights were recorded to calculate the gonadosomatic index (GSI = 100 gonad weight × total body weight^{-1}). In addition, samples from the testis were collected and stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at −20 °C until the extraction of total RNA (Peñaranda et al., 2010).

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

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

2.2. Human and animal rights

This study was carried out in strict accordance with the recommendations laid out in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (BOE, 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (Permit Number: 2014/VSC/PEA/00147). The fish were sacrificed under anesthesia with benzocaine (>60 ppm), and