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Exploring correlations between sex steroids and fatty acids and their potential roles in the induced maturation of the male European eel

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ABSTRACT

The present study was undertaken to evaluate the correlations between the fatty acids in the liver and testis and the plasma levels of the hormonal steroids used during eel spermatogenesis, in order to clarify the physiological roles fatty acids play in the spermatogenetic process. The stages of testis development (S1–S5) were assessed by histological observations in order to classify the different phases of hormonally-induced spermatogenesis and evaluate the possible relationships between the hormones and fatty acids in each stage.

The highest plasma levels of 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT) were found in S1, when spermatogonial proliferation occurs. A correlation was found between 17α-20β-dihydroxy-4-pregnen-3-one (DHP) levels and some fatty acids during the proliferation and growing phases (S1–2), suggesting that DHP might modulate lipid metabolism in the liver during early spermatogenesis. The DHP levels increased significantly during the growing phase (S2) and remained at high levels throughout the subsequent development stages (S3–S5).

Similar to results found in mammals, our results show that in the eel there are regulatory mechanisms, including eicosapentaenoic acid (20:5-n3, EPA) and docosahexaenoic acid (22:6-n3, DHA), which act as modulators in the synthesis of androgens, particularly during the final phase of sperm maturation. Our results suggest that the fact that EPA, ARA and DHA concentrations in the eel testis remain constant/stable during spermiation could be related to the subsequent union of the spermatozoa and the egg. The findings from this research provide new insights for further studies about the possible effect of steroids on desaturase activity and highlight the importance of the effect of lipid metabolism during male eel spermatogenesis.

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1. Introduction

European eel (*Anguilla anguilla*) is seriously under threat and has declined notably in recent years (Nielsen and Prouzet, 2008). The decline in the health of the spawners occurs as a consequence of pollution, diseases, migration barriers, restriction of habitat, etc. Together these stressors decrease the chances of successful migration and reproduction, thus affecting, egg and larval development in wild (ICES, 2013). Eel is a valued product with a high commercial demand in both Europe and Asia, and recently standardized artificial fertilization techniques have been developed for this species (Butts et al., 2014).

To date, eels do not mature spontaneously in captivity, and many studies have focused on achieving this goal. However, maturity can be induced by long-term hormonal treatments in female (Dufour et al., 2003; Mazzeo et al., 2012; Peñaranda et al., 2013a, 2013b; Pérez et al., 2011) and male broodstock (Asturiano et al., 2006; Gallego et al., 2012; Müller et al., 2005; Peñaranda, et al., 2010).

Eels do not feed during their transoceanic migration, as such the food available during the growing phase of their life cycle provides them with the fat stores which they later use for their reproductive migration and gonadal development (Van Ginneken and Van den Thillart, 2000). Fatty acids affect gametogenesis, and we know that in males they have two specific functions: to regulate steroid production and to ensure the appropriate composition of the sperm cell membranes (Dupont et al., 2014). Unsaturated fatty acids provide the sperm plasma membrane with the fluidity required for membrane fusion, an event associated with fertilization (Whates et al., 2007). Numerous studies have focused on the modulatory effects dietary fatty acids have on steroid production in terrestrial animals (Castellano et al., 2011; Kelton et al., 2013; Zhang et al., 1992) and aquatic animals (Asturiano et al., 2000; Cerdá et al., 1995, 1997; Martín et al., 2009; Navas et al., 1998). In terms of steroidogenesis, most research has focused on arachidonic acid (20:4-n6, ARA) because there is clear evidence that it can influence steroid output at a cellular level and is considered to be one of the most important factors in successful fish reproduction (Alorend, 2004; Furuita et al., 2003; Norambuena et al., 2013). ARA is the precursor for

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some prostaglandins which are active biological substances involved in reproduction (Sargent et al., 2002). For instance, in vitro, ARA promotes testicular synthesis of testosterone in goldfish (*Carassius auratus*) stimulating prostaglandin synthesis (Wade et al., 1994). Asturiano et al. (2000) demonstrated that in European sea bass (*Dicentrarchus labrax*) ARA stimulated a significant increase in prostaglandin E_2 production, in a dose- and time-dependent manner, and suggested that it may have important effects on steroidogenesis and spermiation. On the other hand, series 3 fatty acids can influence both the prostaglandin and steroid pathways involved in the regulation of the reproductive function, as well as the fatty acid composition and fertilizing capacity of sperm (Whates et al., 2007).

Another important factor is the influence of steroids on the metabolism of fatty acids. In the case of tilapia (*Oreochromis mossambicus*), an increase in desaturase activity was seen in fish treated with E2 injections. This was reflected in the decrease in the saturated fatty acids and the increase in the monounsaturated fatty acids (Hsieh et al., 2004). In rats, testosterone can cause an increase or decrease in various desaturase activities, thus modifying the fatty acid profile in the testis (Hurtado and Gómez, 2005). In humans, Burdge (2006) suggested that estrogen has a regulatory effect on the conversion of linolenic acid (18:3n3, ALA) to eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA).

The objective of this research was to investigate the correlations between liver and testis fatty acids and sex steroids at different stages of gonadal development (S1–S5) during hormonally-induced sexual maturation in male European eels.

2. Material and methods

2.1. Fish acclimatization and hormonal treatment

Three hundred and seventeen male eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A., (Puçol, Valencia; East coast Spain) were transported to the Aquaculture Laboratory at the Polytechnic University of Valencia. They were housed in six 200 L aquaria, each was equipped with separate recirculation systems and covered to maintain constant darkness. The fish were gradually acclimatized over the course of two weeks from freshwater to seawater (37 ± 0.3 g L^{-1}). Once a week they were anesthetized with benzocaine (60 ppm) and weighed before being administered human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Argent Chemical Laboratories, USA) by intraperitoneal injection (Pérez et al., 2000).

2.2. Thermal regimes

The fish underwent three thermal regimes: T10 (10 °C for the first 6 weeks, 15 °C for the next 3 weeks and 20 °C for the last 6 weeks); T15 (15 °C for the first 6 weeks and 20 °C for the last 9 weeks) and T20 (20 °C throughout the whole experimental period; Gallego et al., 2012). Two aquaria were used for each treatment and 50 eels per aquaria, thus, 100 eels per thermal regime.

When the means of the fatty acids and of the hormones in each of the thermal regimes were compared by stage of development, no differences between the thermal treatments were found (P-value <0.05, see Supplementary Tables 1–3). Thus, in order to increase the number of samples and having checked beforehand that there were no differences, all the analyses were carried out independently of the thermal regime.

2.3. Sampling

When the animals arrived at our facilities, and before starting any treatment, eight animals were sacrificed as freshwater controls. Fish sacrifice was carried out by decapitation, after having previously been anesthetized with benzocaine (60 ppm). Blood samples were collected in heparinized vials and centrifuged at 3000 r.p.m. for 5 min, and the blood plasma was stored at −80 °C until analysis.

A small sample of testis (0.5 g) from each male was preserved in 10% buffered formalin for histology processing. For each thermal regime, liver and testis samples from eight animals were obtained during the first eight weeks of treatment, and from five animals in the last five weeks. The first testis samples were collected as soon as they had reached an appropriate size for analysis. All the testis and liver samples were stored at −80 °C until lipid extraction and fatty acid quantification.

2.4. Fat extraction and fatty acid quantification

The fat extraction of the liver and testis was carried out using a modified version of the method described by Baeza et al. (2014). The fatty acid quantification was carried out by gas chromatography as described by Baeza et al. (2014).

2.5. Steroids

Plasma concentrations of 17α-20β-dihydroxy-4-pregen-3-one (DHP), 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) were measured by means of radioimmunoassay, as described previously (Frantzen et al., 2004; Schulz, 1985). Assay characteristics and cross-reactivities of the E2 and T antisera have previously been examined by Frantzen et al. (2004) and further validated for eel plasma by Mazzeo et al. (2014). The characteristics and cross-reactivities of the DHP assay have previously been described by Tveiten et al. (2010) and validated for eel plasma by Peñaranda et al. (2010). The cross-reactivities of a new 11-KT antiserum used in this work have previously been described by Johnsen et al. (2013). To validate the recovery of 11-KT from plasma in the eel assay, a plasma pool was spiked with 45 ng 11-KT pr. ml of plasma and then underwent ether extraction as described below. The resulting product was then assayed by the 11-KT RIA at three different dilutions. The dilutions were found to be parallel to the standard assay curve. Steroid recovery after ether extraction was 71.9 ± 2.8.0%. The 11-KT values were corrected for recovery losses. The inter and intra assay coefficients of variation (CV) for the 11-KT assay were 15.4% ($n = 7$) and 5.3% ($n = 10$), respectively.

2.6. Gonad histology

After fixation in 10% buffer formalin (pH 7.4), a small section of testis was dehydrated in ethanol and embedded in paraffin. The samples were sectioned to thicknesses of 5 and 10 μm. The sections were stained using the current hematoxylin and eosin method. The slides were observed using a Nikon Eclipse E-400 microscope and the images were taken with a Nikon DS-5M camera.

The maturation stages were determined using the following criteria: Stage 1 (S1) was characterized by the dominance of spermatogonia; some spermatocytes can be present but not dominant; Stage 2 (S2), with spermatocytes as the dominant cell. Some spermatids can be present in low numbers. The dominant process in this stage is meiosis; Stage 3 (S3) was characterized by the dominance of spermatids. The dominant process in this stage is spermiogenesis (spermatid maturation). Males in non-spermiating stage; if some milt was produced, it is of a low volume (<0.5 ml) and low motility (<10 %); Stage 4 (S4), abundant sperm cells present inside the tubule lumen. Tubule lumen delimited by a multiple germ cell layer. Males in early spermiation stage; Stage 5 (S5), was characterized by a dominance of spermatozoa and a low proportion of other germ cells and luminal fusion. Males showing high sperm motility and high sperm volume. Stage of maximal spermiation. (Fig. 1).

Once the fatty acid and steroid analyses had been carried out, the results were classified into the different development stages of the testis. These were assigned once the animals had been sacrificed.

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