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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 ºC, maturation stopped in the A spermatogonial stage (SPG1), and no further maturation was observed until the temperature was ≥15 ºC. 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 ºC.

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 oestrogen 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.

Keywords: *Anguilla anguilla*, temperature, steroid, spermatogenesis, gene expression

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

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

To date, most of the steroidogenic enzymes have been cloned and their expression has been analysed by both PCR approaches and *in situ* hybridisation (Tokarz et al., 2013). The P450scc enzyme (cyp11a1) acts as the catalyst for the first and rate-limiting step in steroidogenesis, and is responsible for the conversion of cholesterol into pregnenolone. In teleosts (Tokarz et al., 2013), including the Japanese eel (*Anguilla*

japonica; Ijiri et al., 2006), its gene expression and immunolocalization are located in the Leydig cells. One of the enzymes responsible for metabolising pregnenolone is the cytochrome P450c17 (cyp17) enzyme. Two forms of P450c17 (I and II) were discovered in medaka (*Oryzias latipes*; Zhou et al., 2007). P450c17-I was identified as being responsible for 17β-estradiol (E_2) production while P450c17-II played a key role in the production of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Zhou et al., 2007). P450c17 (*cyp17-I*) was cloned and characterised in Japanese eel by Kazeto et al. (2000a), who reported a significant increase in its gene expression after salmon pituitary extract injections in female eels.

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

Cytochrome P450 aromatase (cyp19) acts as a catalyst for the synthesis of oestrogens, 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 synthesising 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), which was identified as the MIS in the perciform family Sciaenidae (Trant and Thomas, 1989). In some species, both steroids appear to participate in regulating oocyte maturation (Asturiano et al., 2000; Ohta et al., 2002), but until now with eels, the cyp21 gene has been linked to cortisol production through the conversion of progesterone into 11-deoxycorticosterone (Li et al., 2003) in head kidney.

If we consider the limited knowledge available to us on the reproductive migration of this species, it seems probable that gonadal development, which takes several months, happens at low temperatures, while the spawning and the spermiation happen at higher temperatures. Therefore, our hypothesis is that temperature could play a crucial role in regulating the progress of maturation during reproductive migration, inhibiting or inducing the gene expression of steroidogenic enzymes through androgen synthesis at low temperatures and oestrogen 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 ºC, thus once the fish arrived at our facilities, they were acclimated at 20 ºC 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 ºC) over the course of another week and randomly distributed in six 200-L aquaria (approximately 100 males per treatment). Finally, the animals were underwent three thermal regimes: T10: 10 °C (first 4 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15: 15 °C (first 4 weeks) and 20 °C (last 9 weeks); and T20: 20 °C during the whole experimental period.

As previously described by Gallego et al. (2012), after being anaesthetized with benzocaine dissolved in

seawater (60 ppm) the males were administered weekly intraperitoneal injections of recombinant human chorionic gonadotropin (rechCG; 1.5 IU g^{-1} fish; Ovitrelle®, *Merck Serono Europe Limited*, UK) in order to induce maturation and spermiation.

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

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

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

2.2. Human and Animal Rights

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

2.3 Histology processing

The testis samples were processed and analysed as described by Baeza et al. (2015). The maturation stages (Figure 1) were determined using the following criteria: dominance of germ cell types, degree of development of the seminal tubules, GSI and sperm production by the male sampled in the same week of the sacrifice. In summary, Stage SPG0: non-injected fish. Dominance of A spermatogonia. Absence of

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

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

2.4.1 Primer design

Eel acidic ribosomal phosphoprotein P0, *aa-arp* (Table 1) was used as a reference gene in the qPCR analysis because its mRNA expression is stable throughout hormonal treatment (Weltzien et al., 2005). The expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1 (0.94; $p<0.05$) and a Cq arithmetic mean of 24.7±1.93 in the testis. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. The Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers for *aacyp11a1, aacyp17-I, aa11βHSD, aaCR20β-HSD and aacyp21* (Table 1). To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon-exon boundary. All the

primers were tested on gDNA and RNA to confirm that potentially contaminant gDNA was not amplified. The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the qPCR products.

2.4.2 SYBR Green assay (qPCR)

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

In order to quantify the gene expression, qPCR assays were developed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas GMBH). The PCR protocol included an initial step of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 1 s and 60 °C for 30 s. To evaluate assay specificity, the machine performed a melting curve analysis directly following PCR by slowly (0.1 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 20 µl, performed from diluted (1:10) DNA template (5 µl), forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Weltzien et al. (2005). Target and reference genes in unknown samples were run in duplicate PCR reactions, and a cDNA pool from various samples was included in each run as a calibrator. Non-template control (cDNA was replaced by water) for each primer pair was run in duplicate on all plates.

2.5 Steroids

Plasma concentrations of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) were measured by mean of radioimmunoassays, as described previously (Frantzen et al., 2004; Schulz, 1985). Assay characteristics and cross-reactivities of the E2 and T antisera have previously been examined by Frantzen et al. (2004) and further validated for eel plasma

by Mazzeo et al. (2014). The characteristics and cross-reactivities of the DHP assay have previously been described by Tveiten et al. (2010) and validated for eel plasma by Peñaranda et al. (2010). The cross-reactivities of a new 11-KT antiserum used in this work have previously been described by Johnsen et al. (2013). To validate the recovery of 11-KT from plasma in the eel assay, a plasma pool was spiked with 45 ng 11-KT/ml of plasma and then underwent ether extraction as described below. The resulting product was then assayed by the 11-KT RIA at three different dilutions. The dilutions were found to be parallel to the standard assay curve. Steroid recovery after ether extraction was 71.9±2.8%. The 11-KT values were corrected for recovery losses. The inter- and intra-assay coefficients of variation (CV) for the 11-KT assay were 15.4% (n=7) and 5.3% (n=10), respectively.

2.6 Statistical analysis

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

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

3. Results

3.1 Testis development

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

Seawater acclimation did not induce any gonad development (SPG 0). At 20 ºC and 15 °C, after the third week of treatment no fish were found in SPG2 (Figures. 2B and 2C). Moreover, it was necessary to

increase the temperature to 15 ºC in the T10 group for the fish to reach SPG2 (6th week, Figure 2A).

The onset of SPC1 and SPC2 was delayed when using thermal regimes of lower temperatures. In T20 fish reached stages SPC1 and SPC2 between the 2nd and 4th week, in T15 between the 4th and 7th week , and in T10 between the 7th and 10th week. At 20 ºC, numerous spermatid cells, characteristic of the SD stage, were observed from the 4th week of the treatment (until the 7th week), and again the appearance of these cells was delayed at lower temperatures (6th-8th week in T15 and 9th-10th week in T10).

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

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

3.2.1 Androgen synthesis

Temperature modulated the expression of testis steroidogenic enzyme genes. The cyp11a1 enzyme is responsible for the synthesis of pregnenolone from cholesterol and cyp17-I mediates the synthesis of 17α-OH-pregnenolone from pregnenolone, opening the androgen synthetic pathway. The increased expression of both genes was not linked to temperature, with the highest values being recorded in the first few weeks of treatment and after seawater acclimation (up to 37‰; Figure 3). But these high values were maintained for longer at lower temperatures (Figures. 3C and 3F); until the 6th week in T10, in contrast to the 3rd and 2nd weeks in T15 and T20, respectively (Figures. 3A-B, 3D-E). Likewise, the increase in T plasma levels (Figures. 3G-I) wasn´t linked to temperature. One hormone injection was enough to induce the highest T values in all three thermal regimes, decreasing progressively throughout spermatogenesis (Figures. 3G-I).

11β-OHT is converted into 11-KT by the 11β-HSD enzyme. A parallel increase was observed between the expression of *aa11βHSD* and 11-KT plasma levels (Figure 4). In T20 and T15, one hCG injection was enough to increase *aa11βHSD* expression and 11-KT plasma levels (Figures. 4A-B and 4D-E). Although the increase of both the gene expression and 11-KT plasma levels happened regardless of the temperature, the peak in the expression of *aa11βHSD* was delayed by one week at lower temperatures, with it being recorded in the 2nd week of hormonal treatment in T15 and T10, and in the 1st week in T20. The

289 subsequent decrease in *aa11βHSD* expression and 11-KT plasma levels was less pronounced in T10
290 (Figures. 4C and 4F) than in the other two groups.
291
292 ### *3.2.2 Oestrogen and progestin synthesis*
293 cyp19a1 is the limiting enzyme for E_2 synthesis. However, an increase in E_2 plasma levels took place prior
294 to the peak in *aacyp19a1*expression in the testis in all the thermal regimes (Figure 5). The E_2 peak was
295 observed after seawater acclimation, regardless of the temperature, and decreased throughout the course
296 of spermatogenesis.
297 In contrast, the expression of *aacyp19a1* in the testis was modulated by temperature. Lower temperatures
298 delayed the peak in *aacyp19a1*, recorded in the 2nd, 3rd and 6th weeks in T20, T15 and T10, respectively
299 (Figures. 5A-C). It is remarkable that the increase in *aacyp19a1* expression took place when the
300 temperature was over 10 ºC (Figure 5C).
301 Cyp21 is the enzyme which mediates the pathway for the production of 20βS, and 20βHSD is responsible
302 for DHP synthesis. No significant differences in the expression of *aaCR20β-HSD* in any of the thermal
303 regimes were recorded at any point in the hormonal treatment, compared to freshwater conditions (data
304 not shown). Again, no significant differences in *aacyp21* gene expression were observed in T20 (Figure
305 6A) throughout the treatment, but in T15 a progressive increase was registered up until the 4th week of
306 treatment. The levels then decreased when the water temperature was increased from 15 to 20 ºC (Figure
307 6B). In T10, low values of *aacyp21* mRNA transcript were observed until the temperature was increased
308 to 15 ºC (from week 4), the levels then decreased after the 7th week (Figure 6C), when the water
309 temperature was increased again to 20 ºC.
310 Fish with significantly higher DHP plasma values than untreated fish were observed in the 3rd week of
311 treatment in T20 (Figure 6D). Lower temperatures delayed this increase to the 5th and 6th weeks in T15
312 and T10, respectively (Figures. 6E and 6F). Also, the highest DHP levels were delayed at lower
313 temperatures, with them being reached in week 4 in T20 in contrast to week 10 in the T15 and T10.
314
315 ### *3.2.3 Steroid and gene expression throughout spermatogenesis*
316 Firstly, the seawater acclimation was enough to increase the E_2 plasma levels (Figure 7A), which then
317 decreased in the following stages. This decrease was fastest in T10. Thereafter, the T and 11KT plasma

318 levels increased at SPG1 (Figures. 7B and 7C). This increase was higher in T20, but the levels of both
319 androgens decreased in the subsequent stages in all three thermal regimes. There were two registered
320 increases in progestin production, at SPG2 (compared to freshwater conditions) and at SD stage
321 (compared to the previous stages), with no significant difference recorded between the groups (Figure
322 7D).
323 Regarding gene expression, the highest levels of *cyp11a1* and *cyp17-I* were registered at SPG1, with a
324 progressive decrease in the subsequent stages (Figures 8A and 8B). This increase, in both genes, was
325 greater at lower temperatures (T20 and T15). Similar results were observed for *aa11βHSD* gene
326 expression (Figure 8C), but with higher values at high temperatures (T20 and T15).
327 In contrast, the peaks in *aacyp19a1* and *aacyp21* gene expression were observed at SPG2 (Figures 8D and
328 8E), with lower values also being registered in the subsequent stages. In this case, fish from lower
329 temperature regimes (T10 group) showed a higher *aacyp21* gene expression increase at SPG 2 stage in
330 comparison to those from higher temperature groups (Figure 8E).
331

332 ## 4. Discussion

333

334 Temperature exercised a clear effect on eel maturation, delaying spermatogenesis in thermal regimes with
335 lower temperatures. The gonad development of the fish reared at 20 ºC throughout the whole treatment
336 showed similar results to those observed in previous studies (Peñaranda et al., 2010). Nevertheless, the
337 fish reared at 10 ºC arrested their maturation at SPG1, and no further maturation was observed until the
338 temperature was increased (15 ºC). In hormonally-treated female eels, a delay in gonad maturation caused
339 by low rearing temperatures was also observed (Mazzeo et al. 2014; Pérez et al., 2011; Sudo et al., 2011).
340 In the European female eel, both high (20 ºC) and low (10 °C) temperatures induced steroidogenesis in
341 previtellogenic eel ovaries, but high temperatures during hormonal treatment caused an acceleration of
342 ovarian growth from the mid to late vitellogenic stage (Mazzeo et al. 2014; Pérez et al. 2011). In Japanese
343 female eel, fish maintained at 10 ºC did not reach ovulation; however when the water temperature was
344 increased from 10 to 20 ºC, the fish ovulated in the subsequent weeks (Sato et al. 2006). Sudo et al.
345 (2011) observed lower gonadotropins expression in fish reared with a gradual temperature decrease from
346 25 to 15 ºC compared to fish reared at 25 ºC. These results from female eels appear to corroborate our

study, since the first stages of maturation are possible independently of the temperature, even when this temperature is low (10 °C), but higher temperatures being necessary for further maturation.

4.1 Androgen synthesis

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

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

As was observed for *aacyp11a1* and *aacyp17-I* gene expression, the increase of *aa11βHSD* gene expression was not temperature dependent. The *aa11βHSD* gene expression reported in the current study is the sum of both 11β-HSD type 2 subtypes (11β-HSD and 11β-HSDsf) present in eels. In the Japanese eel, *11β-HSD* mRNA transcripts were not found in immature eel testis, but appeared at day 1, and peaked at day 3 after the administering of hCG injections. Thereafter, the levels decreased rapidly from day 6, and fell very low after day 12 (Jiang et al., 2003). As aforementioned, the difference could be that maturation of the Japanese eel was induced using a single injection, while we applied weekly hCG injections. Since the 11-KT plasma levels increased in tandem with the *aa11βHSD* gene expression, it is likely that both 11β-HSD type 2 subtypes play an important role in 11-KT synthesis. The increase in *aa11βHSD* transcripts and 11-KT plasma levels were observed at SGP1 when A spermatogonia cells were predominant in the testes, and decreased with the spermatogenesis progression. These results corroborate the role of 11-KT during spermatogenesis as proposed by Schulz et al. (2010).

4.2 Oestrogens and progestin synthesis

As reported in previous studies (Quérat et al., 1987), the increase of salinity to seawater conditions (37%)

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

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

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

The expression of the *aacyp21* gene seems to be regulated by temperature, since the values were low at

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

The aa*cyp21* gene expression increased at SPG2, when A spermatogonia were predominant and some germ cells started to differentiate in B spermatogonia. Also, at this stage (SPG2), higher DHP plasma levels were found, suggesting that progestins can be involved in spermatogonial differenciation or in the testis morphogenesis, since tubule lumen formation was observed at this stage (SPG2). These new possible roles of progestins need to be further studied in the future.

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

5. Conclusion

In conclusion, temperature modulates steroidogenesis, the gonad maturation and the spermiation process in eels. Irrespective of the temperature, the onset of spermatogenesis was characterised by an increase in oestrogen and androgen plasma levels, which stimulated the proliferation of spermatogonia. Nevertheless, at 10 °C maturation was arrested at SPG1, and for spermatogenesis to advance further, it was necessary to increase the temperature to 15 °C. In fact, this temperature (15 °C) was required to induce a shift in steroidogenesis from androgen synthesis to oestrogen and progestin synthesis, with *aacyp19a1* and aa*cyp21* gene expression increasing at 15 ºC. It could be hypothesized that the testis of male eels migrating at low temperatures develop up to spermatogonia proliferation, but no further maturation occurs until the fish reach higher temperatures, in the Sargasso Sea, and then spermatogenesis can be completed.

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600

601 ## Legends

602

603 **Figure 1 Histological sections of eel testis at different developmental stages during human chorionic**
604 **gonadotropin (hCG) hormonal treatment.** A) Testis at Freshwater and Seawater conditions (SPG0). B)
605 Testis at SGP1. C) Testis at SGP2. D) Testis at SPC1. E) Testis at SPC2. F) Testis at SD. G) Testis at
606 SZ1. H) Testis at SZ2. See main text for definition of gonad developmental stages. SPG= Spermatogonia;
607 SPC: Spermatocyte; SD: Spermatid; SZ: Spermatozoa. Scale bars, 25 μm (A, B, C, D, E, F) and 100 μm
608 (G, H).

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

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

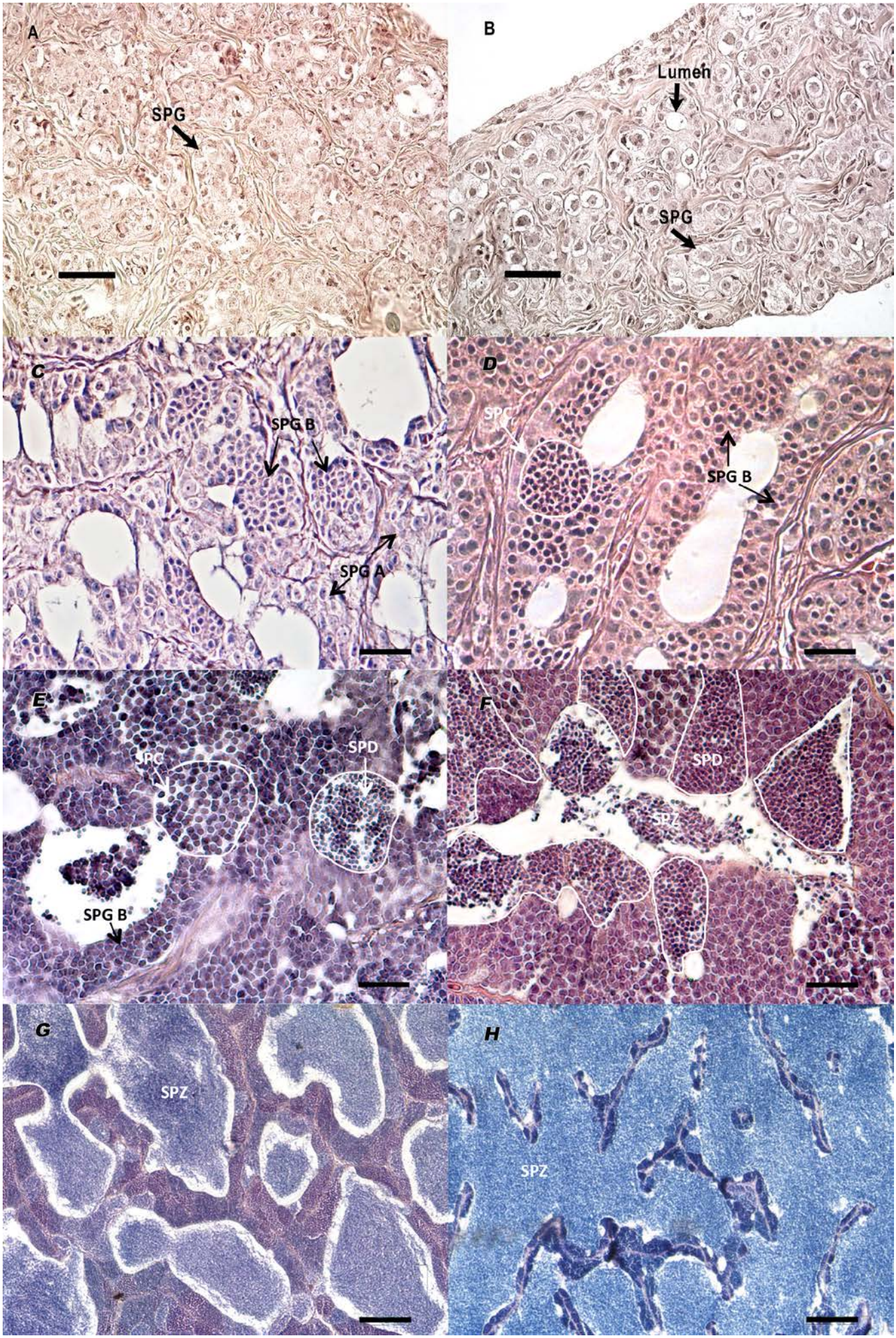
Figure 4 Gene expression of *Anguilla anguilla 11βHSD* (*aa11HSD*; A, B and C) and 11-ketotestosterone (11KT; D, E and F) plasma levels during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented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

Figure 5 Gene expression of *Anguilla anguilla aromatase P450 a1* (*aacyp19a1*; A, B and C) and estradiol (E_2; D, E and F) plasma levels during the weeks of treatment in the three thermal regimes. Different superscripts mean significant differences ($p<0.05$; n=6-15). The temperature regime has been represented as following, red colour: 20 ºC, orange colour: 15 ºC and blue colour: 10 ºC.

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

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

Figure 8 A) Gene expression of *Anguilla anguilla P450c17-I* (*aacypc17-I*), B) *Anguilla anguilla P450scc* (*aacyp11a1*), C) *Anguilla anguilla 11βHSD* (*aa11HSD*), D) *Anguilla anguilla aromatase P450 a1* (*aacyp19a1*) and E) *Anguilla anguilla cyp21* (*aacyp21*) at the different stages of gonad development. Different superscripts mean significant differences ($p<0.05$; n=6-15) through spermatogenesis considering the three thermal regimes as a unique group in each stage and asterisk reports significant differences ($p<0.05$; n=5-8) between thermal regimes (red line: T20 group; orange line: T15 group; blue line: T10 group). See main text for definition of gonad developmental stages. FW= Freshwater, SW= Seawater, SPG1= Spermatogonia 1 stage, SPG2= Spermatogonia 2 stage, SPC1= Spermatocyte 1 stage, SPC2= Spermatocyte 2 stage, SD= Spermatid stage, SZ1= Spermatozoa 1 stage and SZ2= Spermatozoa 2 stage


A
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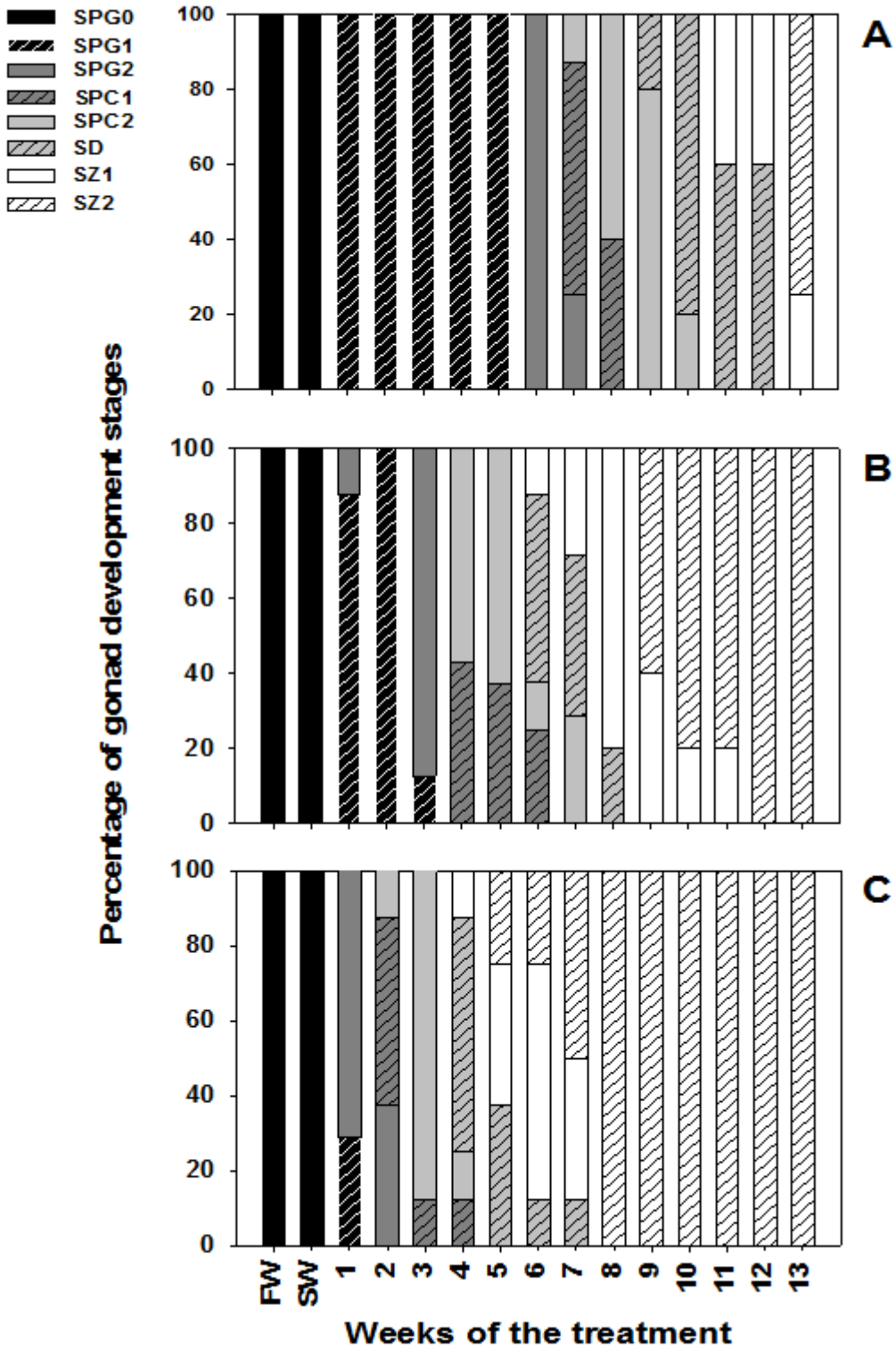
SPG0
SPG1
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SPC2
SD
SZ1
SZ2
Percentage of gonad development stages
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Weeks of the treatment

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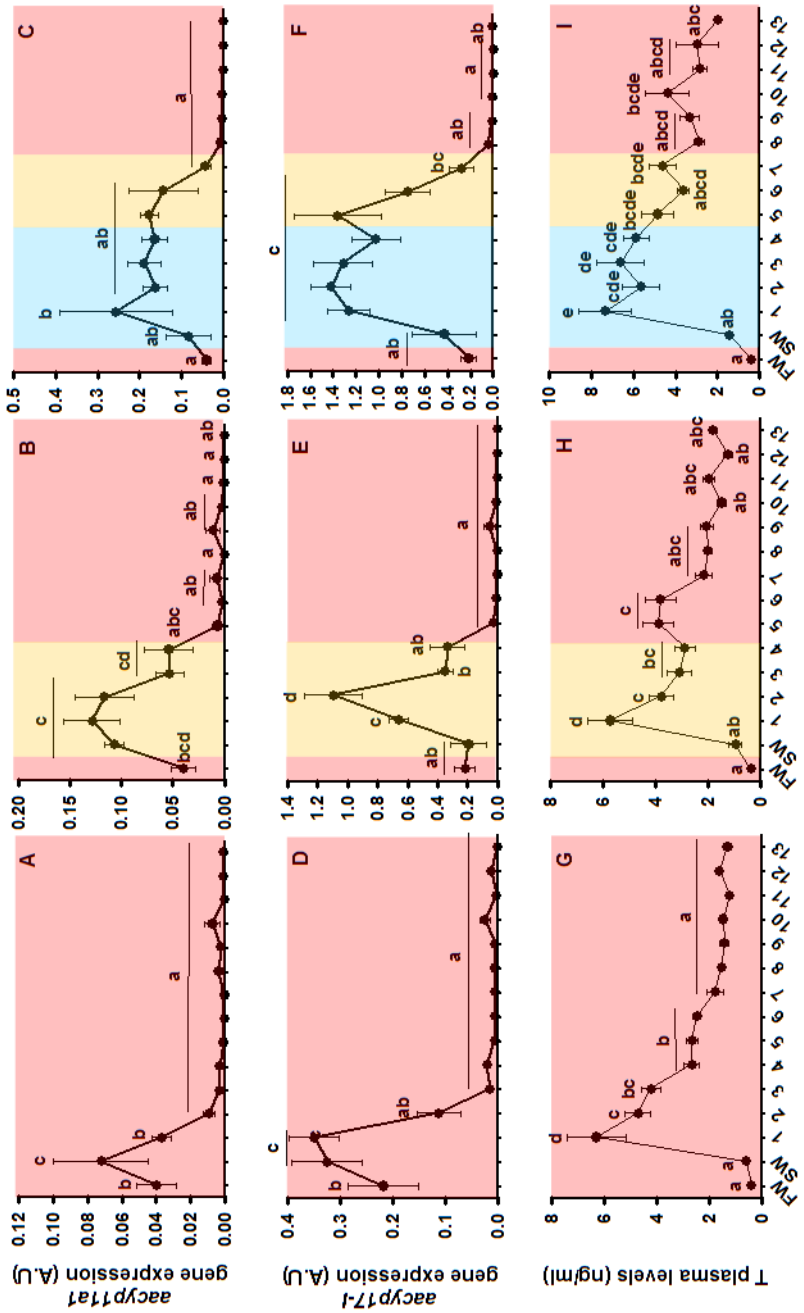
Fig 3


A
B
C
D
E
F
G
H
I
aacyp17a1 gene expression (A.U)
aacyp17-I gene expression (A.U)
T plasma levels (ng/ml)
FW
SW

671 Fig 4

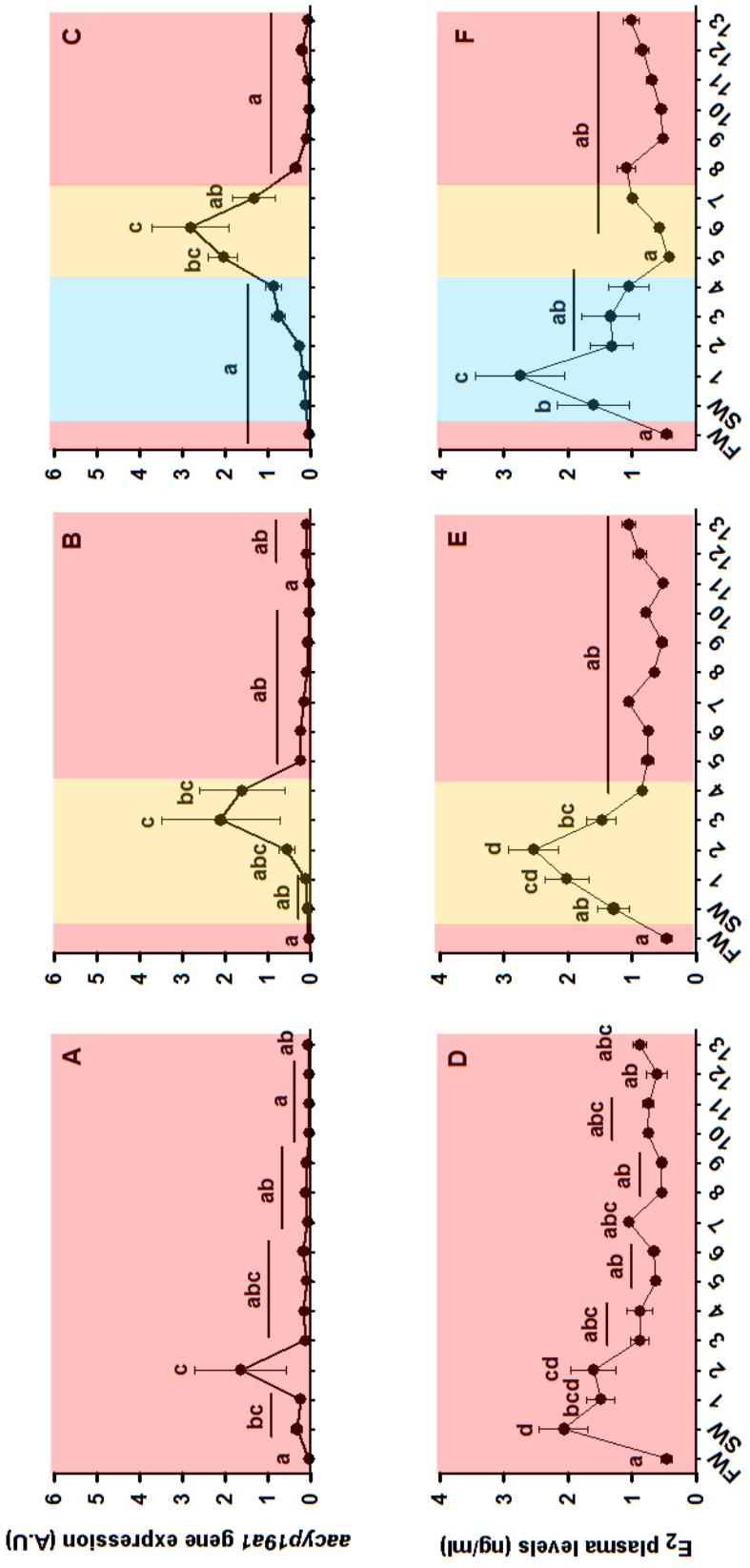
672

aa11βHSD gene expression (A.U)
11-KT plasma levels (ng/ml)
A
B
C
D
E
F
FW
SW
1
2
3
4
5
6
7
8
9
10
11
12
13

673

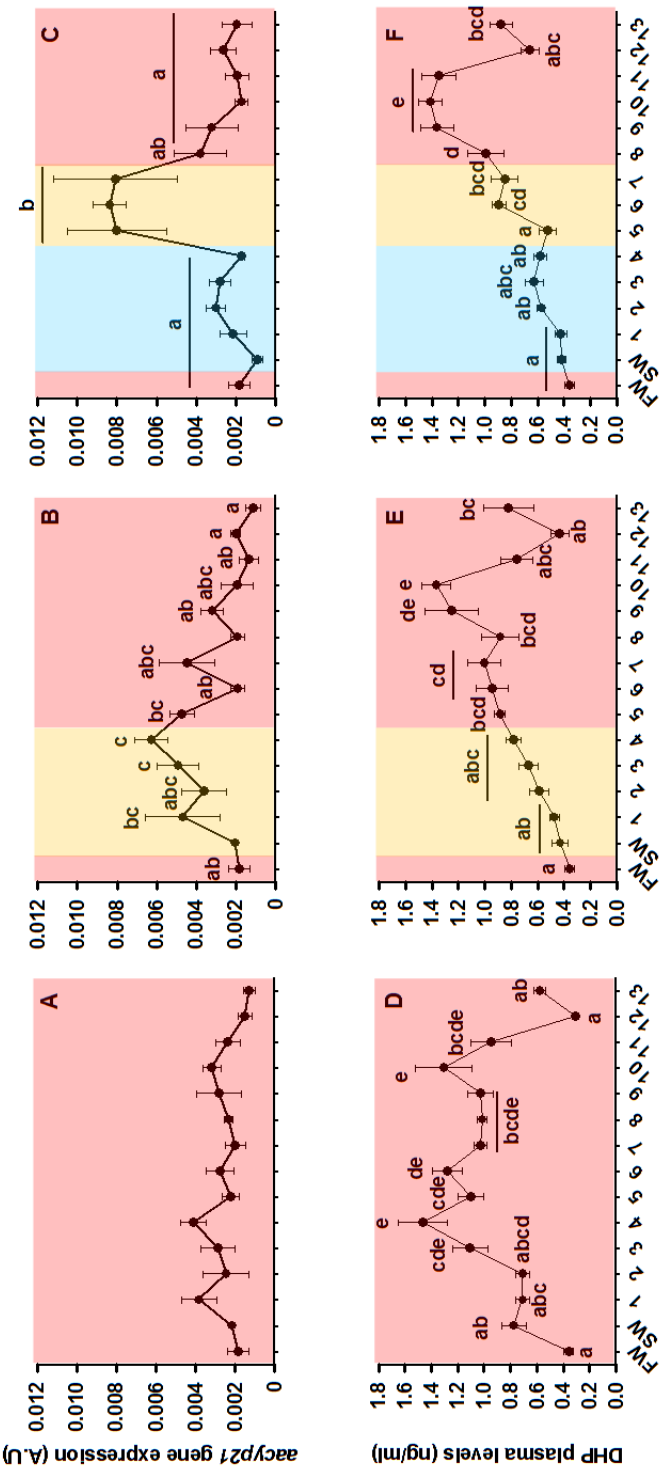
674 Fig 5

675


A
B
C
D
E
F
aacyp19a1 gene expression (A.U)
E2 plasma levels (ng/ml)
FW
SW

676

677 Fig 6



678

679

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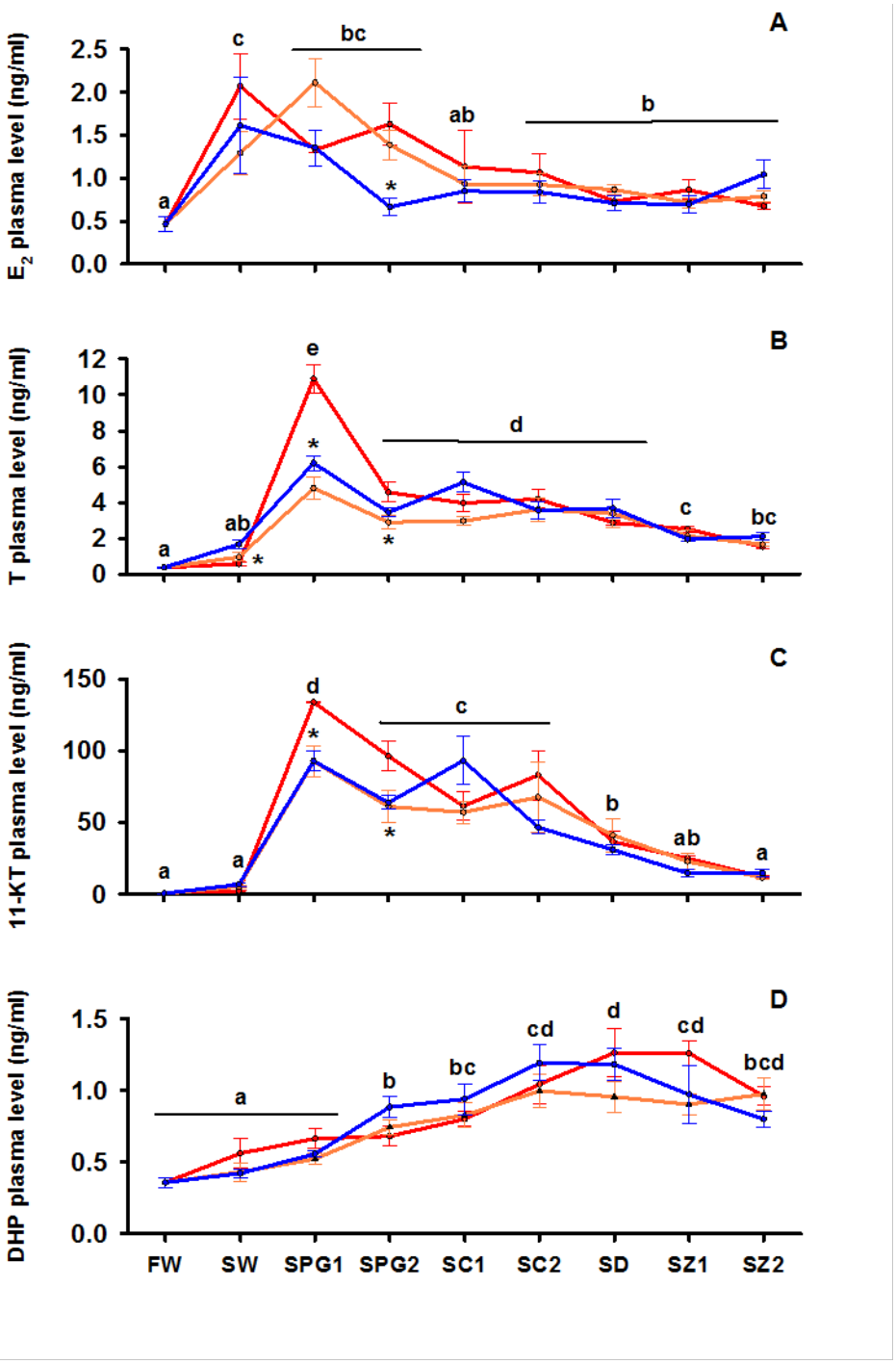
681 Fig 7

682

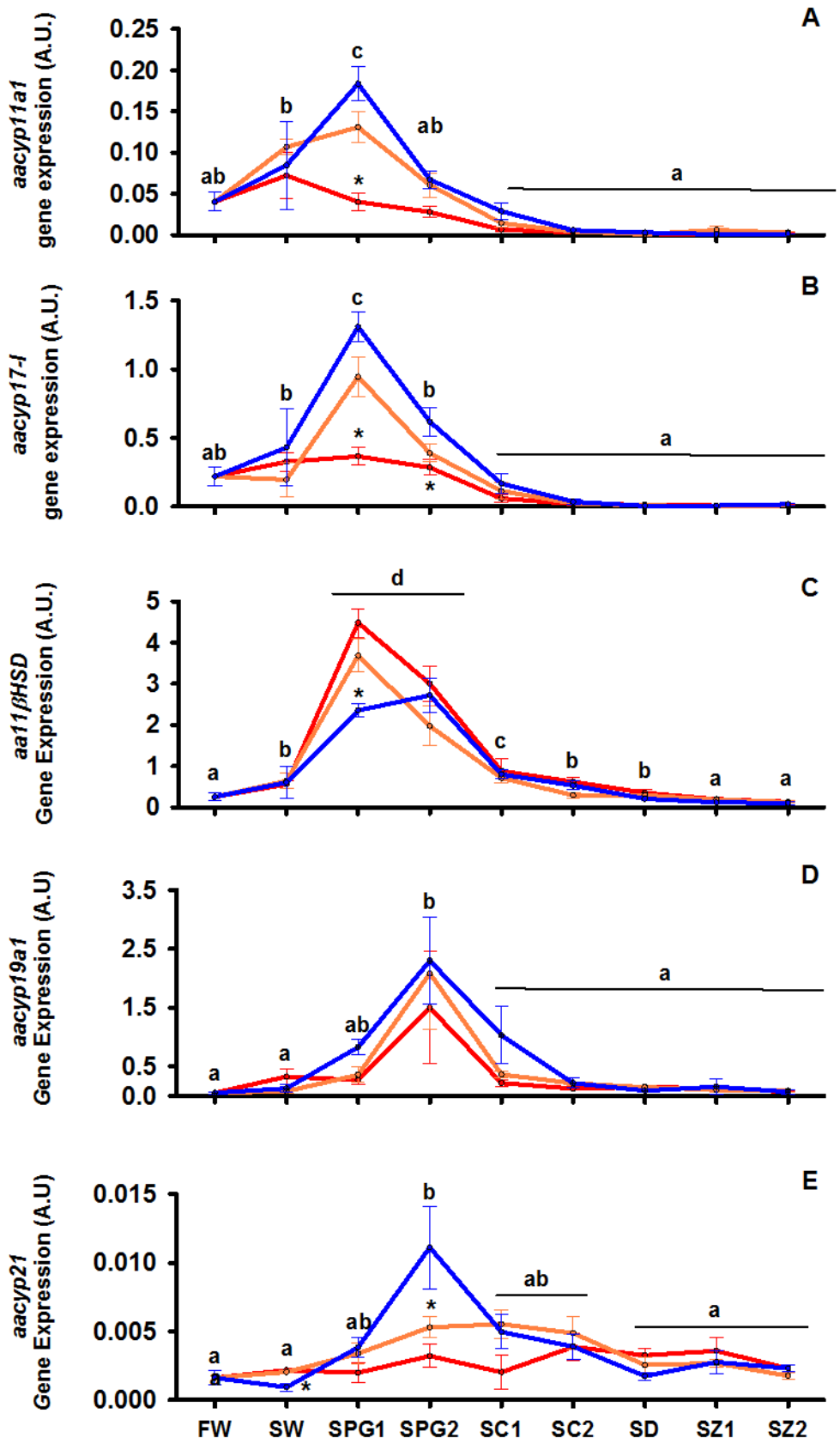


683

684

685

686 Fig 8


A
aacyp11a1
gene expression (A.U.)
0.25
0.20
0.15
0.10
0.05
0.00
ab
b
c
ab
a
*
B
aacyp17-I
gene expression (A.U.)
1.5
1.0
0.5
0.0
ab
b
c
b
a
*
*
C
aa11βHSD
Gene Expression (A.U.)
5
4
3
2
1
0
a
b
d
c
b
b
a
a
*
D
aacyp19a1
Gene Expression (A.U)
3.5
2.5
1.5
0.5
0.0
a
a
ab
b
a
E
aacyp21
Gene Expression (A.U)
0.015
0.010
0.005
0.000
a
a
ab
b
ab
a
*
*
FW
SW
SPG1
SPG2
SC1
SC2
SD
SZ1
SZ2

687

Table 1. Primer sequences used in quantitative PCR.

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

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