Temperature modulates the progression of vitellogenesis in the European eel

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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 °C and from 15 to 18 °C, respectively) and one constant, at 18 °C (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 °C showed lower 11-KT and E2 plasma levels compared to those maintained at 10 °C. In addition, in the early vitellogenic stage, the androgen and cyp19a1 levels were lower at 18 °C than at 10 °C. 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 °D, at 15 or 18 °C, and progression to the late vitellogenic stage was only observed after an accumulation higher than 1300 °D, at 18 °C. 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 °C from the mid-vitellogenic stage, or after an accumulation of 1300 °D. 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 stimulations, and this knowledge could be used to manipulate the timing of vitellogenesis progression under laboratory conditions.

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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 and 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 performs 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 (Asturiano et al., 2005; Gallego et al., 2012; Lokman and Young, 2000; Ohta et al., 1997; Palstra et al., 2005; Peñaranda et al., 2010; Pérez et al., 2008). 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 the European eel is still unpredictable (see review by Okamura et al., 2013).

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 and 600 m, by performing daily vertical migrations, at temperatures between 10 and 12 °C (Aarestrup et al., 2009). When eels leave the continental waters to enter the sea, they are still immature, with gonadosomatic indices (GSI = gonad weight ∗ total body weight^{-1}) between 1 and 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 °C, 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 extract (CPE) under two thermal regimes; one variable regime increasing from 10 to 17 °C, and one constant at 20 °C. 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 (Endo et al., 2008; Lokman et al., 2007; Matsubara et al., 2003b) 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-Álvarez 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 °C. 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 °C), 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 °C). 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

Fig. 1 shows the thermal regimes and the sampling points. Before the experiment started, the water temperature was gradually adjusted from 18 °C (ambient temperature) to the experimental temperatures (10, 15, or 18 °C) 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 °C until the end of the experiment (Fig. 1). In addition, 10 weeks after the temperature had been maintained at 15 °C, the temperature in group T15–18 was increased to 18 °C 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 the 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 °C, 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 °C until use, between 1 and 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
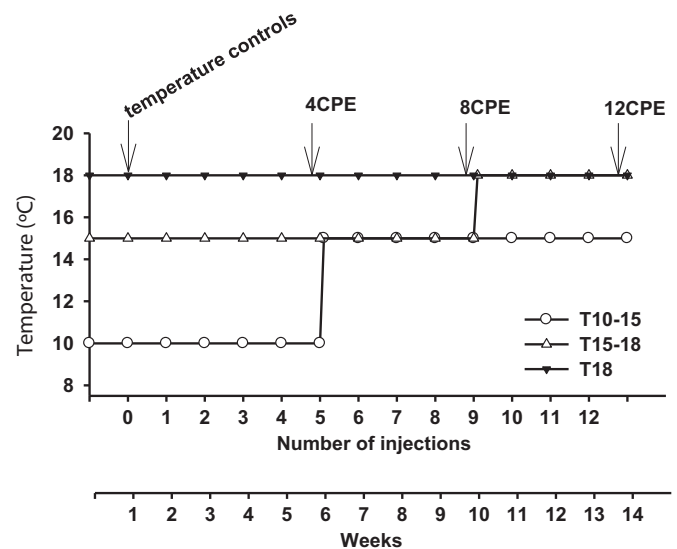

Fig. 1. Thermal regimes applied for each treatment (T10–15, open circle; T15–18, open triangle; T18, closed triangle). Arrows indicate sampling weeks: temperature controls (T0), and 7 days after the 4th, 8th and 12th CPE injections (4CPE, 8CPE, 12 CPE). Between 6 and 8 fish/treatment were sampled in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

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 T10–15, 1 from T15–18, 2 from T18). They were not included in the statistical analyses.

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 °C), 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 injections 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 sacrificed 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 × total body weight^{-1}) and Gut Index (GI = 100 gut weight × total body weight^{-1}). In addition, total body length and eye diameter (vertical and horizontal) were measured to calculate the Eye Index (EI = $100\pi[0.25(Dh + Dv)]^2 \times Lt^{-1}$, 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 RNAlater (Ambion Inc., Huntingdon, UK) at −20 °C 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 hematoxylin 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, Aroua et al., 2007; Peñaranda et al., 2010; Weltzien et al., 2005) was used as the reference gene in qPCR because its mRNA expression has been shown to be stable during experimental treatment (Weltzien et al., 2005). 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[$\pm$Cq]) lower than 1 (0.21; p < 0.05) and Cq arithmetic mean of 10.1 $\pm$ 0.72. 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 the sequencing of the qPCR products.

2.6.2. SYBR Green assay (qPCR)

Total RNA was isolated from RNAlater preserved ovarian tissue following the method described by Hildahl et al. (2011). 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. (2005), using a Light Cycler 480 system with SYBR Green I detection (Roche, Meylan, France). After an initial activation of Taq polymerase at 95 °C for 10 min, 42 PCR cycles were performed using the following cycling conditions: 95 °C for 10 s, 60 °C for 10 s, and 72 °C 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. (2005), using an efficiency-adjusted relative quantification method (Pfaffl, 2001). Briefly, it was calculated from the formula:

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

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 $\pm$ 0.15; arp: 14.93 $\pm$ 0.04). Non-template controls (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 25 μL duplicates of each sample using a competitive testosterone ELISA kit (Eurobio AbCys, Les Ulis, France), with a sensitivity of 0.07 ng/mL, and an intra-assay variation of 6–10%. The standard curve was between 0.008 and 16 ng/mL. 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. 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

Table 1
Primers used for qPCR analysis. Amplicon length and primer efficiency are given after Fw and Rv primers, 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. (2005) (AY763793.1)
	ACA TCG CTC AAG ACT TCA ATG G	Rv (GE = 2.18)	

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

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. 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 °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. 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, radiolabeled steroid (c. 100,000 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 analyzed first for normality by the asymmetry standard coefficient and Kurtosis 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 Statgraphics Plus 5. Simple and multivariate regression analyses were performed to study the relationship between the GSI and the accumulated degree days (°D) and the accumulated CPE-doses, using the software provided by Statgraphics 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

Fig. 2 shows the percentage of females in each developmental stage after ovarian histology observation. Before starting the hormonal treatment, all the eel oocytes were in the PV stage (FW and T0). After 4 CPE
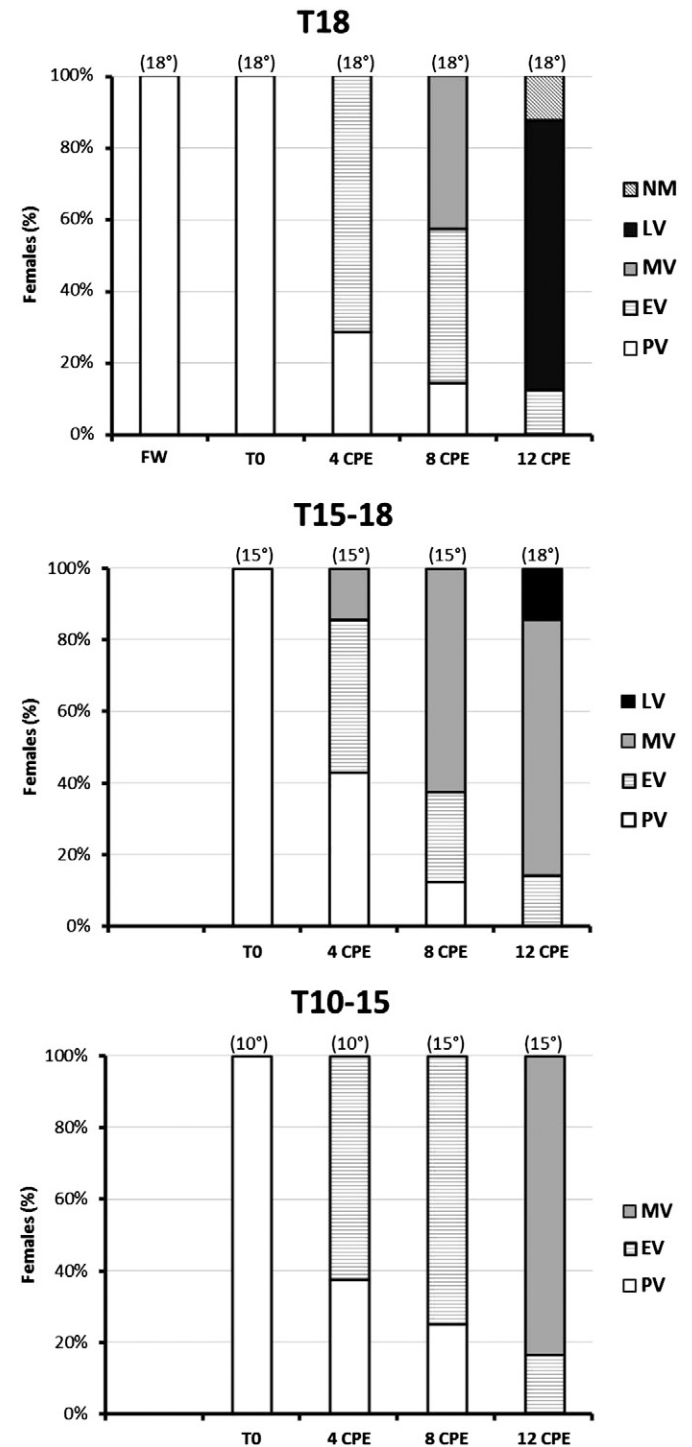

Fig. 2. Effect of hormonal treatment and thermal regime on ovarian development in freshwater controls (FW), temperature controls (T0), and after 4, 8 or 12 CPE injections in each thermal treatment (n = 6–8/group). PV: previtellogenic stage (white color); EV: early vitellogenic stage (horizontal lines);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

injections (4CPE), ovaries in the early vitellogenic (EV) stage 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 point (12CPE) most females from T18 were in the late vitellogenic (LV) stage, while in the other groups they were mostly in the MV stage (Fig. 2).

Fig. 3 shows the evolution of the biometric parameters throughout the experiment. The GSI and oocyte diameter gradually increased throughout the experiment ($p < 0.01$). At 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 than in T0.
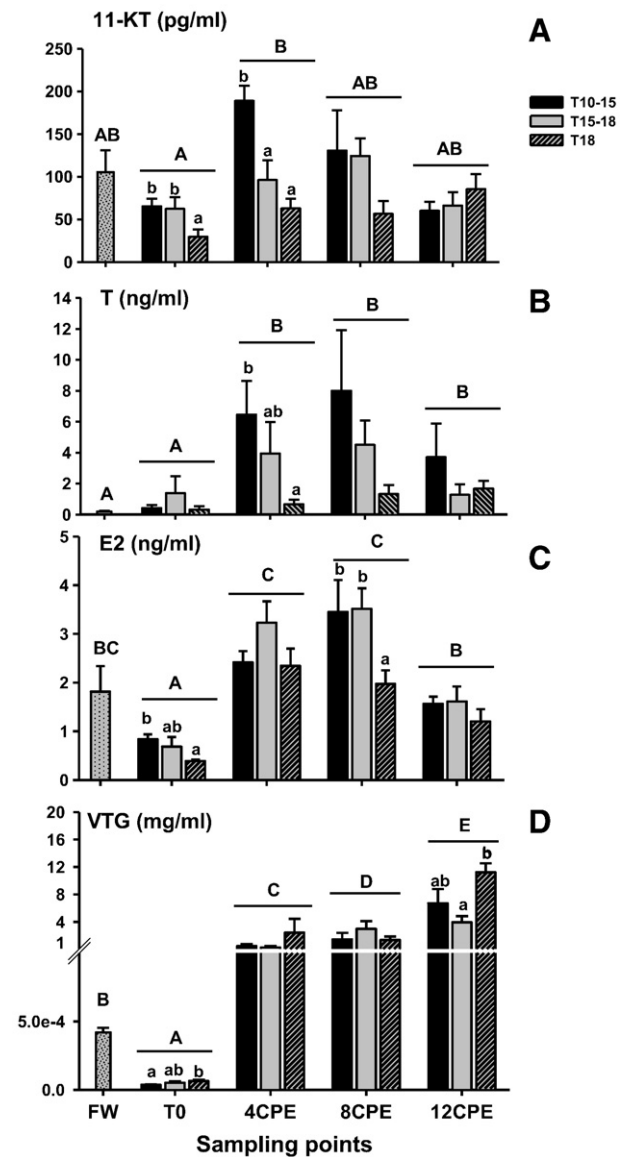
3.2. Steroid and vitellogenin plasma levels

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, 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 huge increase after 4 CPE injections,
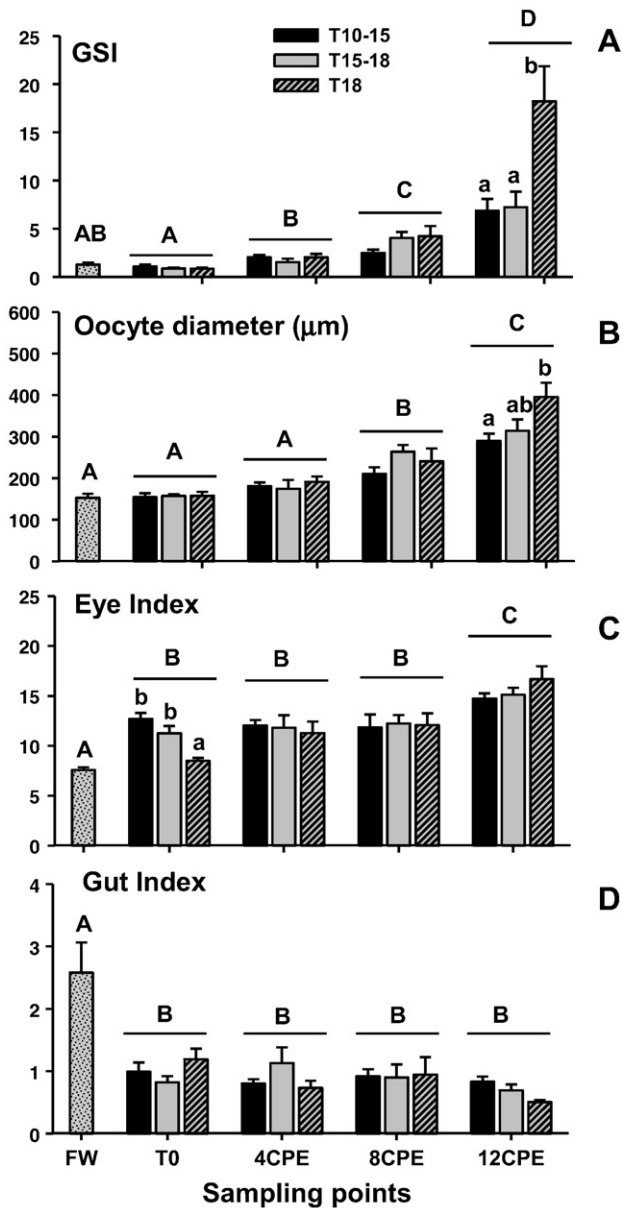
Fig. 3. Effect of hormonal treatment and thermal regime on biometric parameters: Gonadosomatic Index (GSI; A), oocyte diameter (B), Eye Index (EI; C) and Gut Index (D) in freshwater controls (FW), temperature controls (T0), and after 4, 8 or 12 CPE injections in each thermal treatment (n = 6–8/group). 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 controls (FW) control, temperature controls (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).

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).

3.2. Ovarian cyp19a1 expression

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

Group T18 showed a reduced *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.

4. Discussion

4.1. Low temperatures induced steroidogenesis prior to hormonal treatment

Ovaries in the previtellogenic stage were observed in FW, as well as in 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 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 °C compared to 10 °C. It has been shown that 11-KT promotes previtellogenic oocyte growth in shortfinned eel (*Anguilla 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 the Japanese eel (Asanuma et al., 2003). Regarding E2, apart from the known role that it plays during vitellogenesis, it has been linked to oogonial proliferation in the Japanese eel (Miura et al., 2007) and to previtellogenic oocyte growth in coho salmon, (Campbell et al., 2006; Forsgren & Young, 2012). In the 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 °C 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 °C compared to the eels maintained at 18 °C (1.08 vs 0.86, respectively), and the GSI showed a positive correlation with 11-KT levels (r = 0.59; p = 0.006; Supplementary Table 1), which were higher at low temperatures. On the 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 °C 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, increases 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). 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 °C compared to lower temperatures in adult European eels at the previtellogenic stage. In the 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 °C in the ovaries of the European eel females analyzed 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 cyp19a1 expression at the early vitellogenic stage

Carp pituitary extract (CPE) injections 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 °C (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 *cyp19a1* 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, Supplementary Table 1).

Previous research on other fish species has 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 (Anderson et al., 2012; Lim et al., 2003).
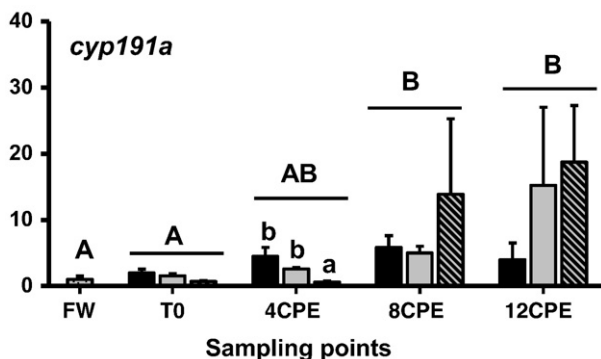
Fig. 5. Effect of thermal and hormonal treatments on ovarian *cyp19a1* gene expression in freshwater controls (FW), temperature controls (T0), and after 4, 8 and 12 CPE injections. The relative expression was normalized to the abundance of *arp*. Results are expressed as mean of fold change ± SEM (n = 6) with respect to the FW, 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).

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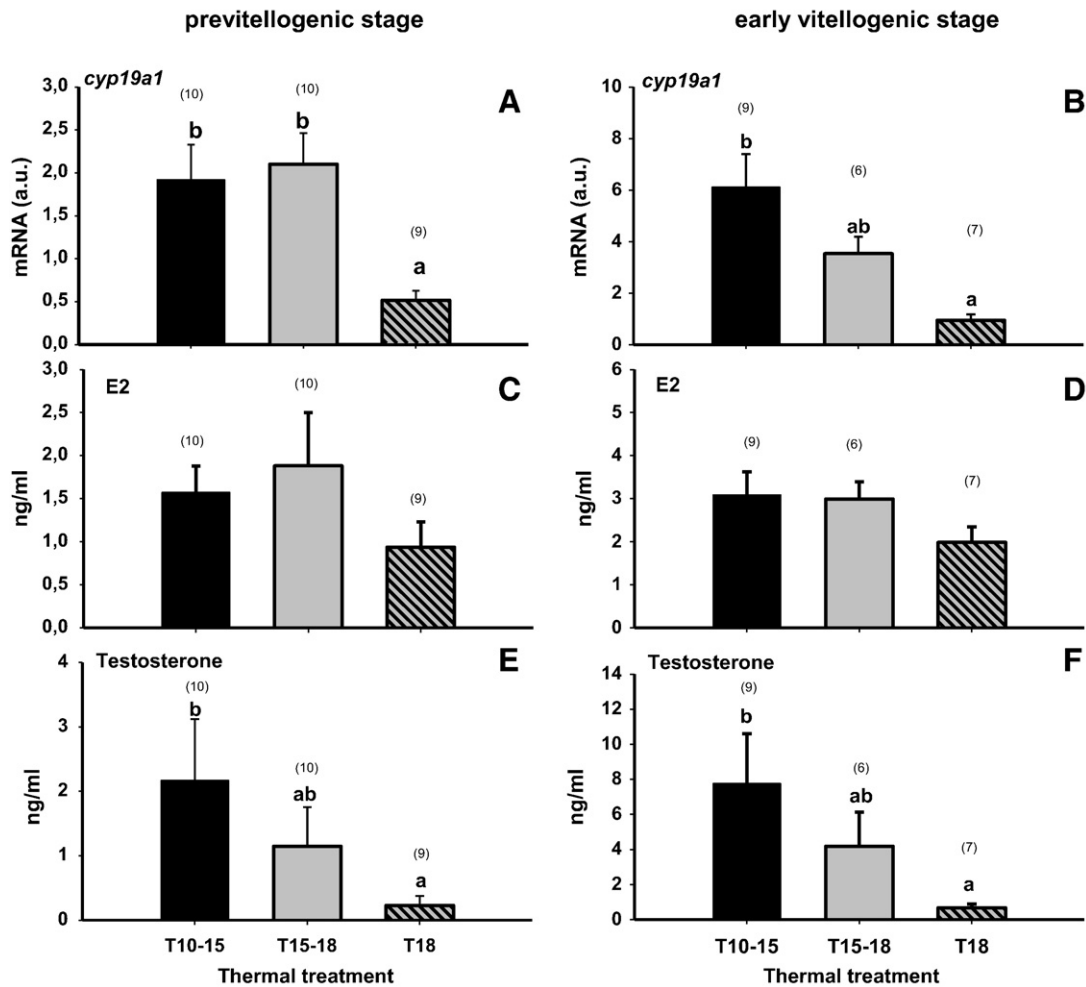
Fig. 6. Previtellogenic and early vitellogenic levels of ovarian *cyp19a1* gene expression, plasma 17β-estradiol (E2) and testosterone, in each thermal treatment. Small letters indicate significant differences between the thermal treatments (p < 0.05, df = 2). Number of fish/group is show in brackets.

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 the European eel (Pérez et al., 2011), and in New Zealand long-finned eels (*Anguilla dieffenbachii*, Lokman et al., 2001). Nevertheless, the E2 levels of the Japanese eel matured at 20 °C 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, Supplementary Table 1), 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 and Maes (2005) 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 °C, 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 °C during the first weeks of hormonal treatment even if they are then transferred to 15 °C, as results from 8CPE show.

On the other hand, ovarian development up to the mid-vitellogenic stage was as fast at 15 °C as at 18 °C, 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 °C compared to the eels maintained at a constant temperature of 18 °C, even when both groups were maintained at the same temperature (18 °C) 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 2 indicates the degree days (°D)

Table 2

Degree days experienced by female eels before reaching the different development stages, and accumulated CPE dose received until that time (mg kg^{-1}).

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

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).

experienced by the female eels before they reached the different development stages, and the accumulated CPE dose received until that time.

Table 2 shows that the MV stage was reached after 1120 °D (group T10–15), 840–1340 °D (group T15–18), and 1008 °D (group T18). Also, the LV stage was reached after 1512 °D (group T18 at 12CPE), but not after 1340 °D (group T15–18 at 12CPE) or 1120 °D (group T10–15 at 12CPE). These results suggest that, in CPE-induced eel gonadal maturation, an accumulation of 900–1200 °D (and 160–240 mg CPE kg^{-1}) should facilitate development up to the mid-vitellogenic stage, while an accumulation of more than 1300 °D 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 °D or 1172 °D (Pérez et al., 2011; Vílchez et al., 2013), while the LV stage was the dominant stage after an accumulation of

1680 °D, although not observed after 1220 °D. Also, Mordenti et al. (2013) observed LV stages in CPE-treated European eels after an accumulation of 1628 °D (our own calculations, Table), but not after 1085 °D.

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 °D was found (Fig. 7). Thus, the GSI of CPE-treated eels increased exponentially with the °D experienced, thus indicating that the GSI growth accelerated from an accumulation of about 1300 °D. 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 °D accumulated, the fish received additional doses of CPEs. 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 °D (R^2 = 0.93; r = 0.96). Also, when a multivariate regression model was applied to explain the GSI variation from both °D 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 °D and the accumulated CPE-doses, it would appear that the increase in GSI accelerates from a certain level of °D (about 1300 °D) 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.

5. Conclusions

For the first time in the European eel, a down-regulation of ovarian aromatase gene expression (cyp19a1) 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.
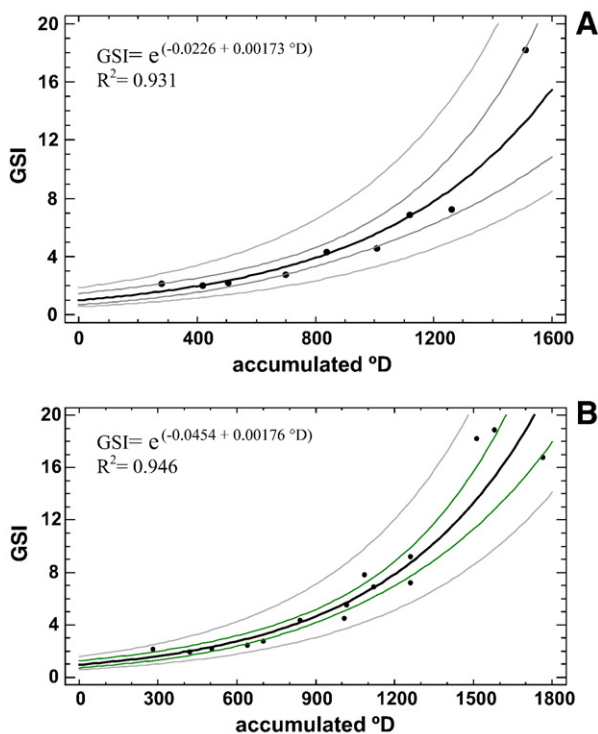
The dual role of low and high temperatures on eel maturation suggested in this study may reflect the natural ecophysiological situation. The progression of vitellogenesis, likely impairing swimming capacities, would be prevented by the low temperatures encountered during the transoceanic migration, while the high temperature of the spawning ground would facilitate the late vitellogenic stages and the final ovarian maturation of the European eel.

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2014.07.020.

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