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Additional Information

Temperature modulates the progression of vitellogenesis in European eel

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35 **Abstract**

36 Wild female European eels were matured with CPE (carp pituitary extract) under three
37 thermal regimes, two of which were variable (T10-15 and T15-18, moving from 10 to
38 15 ºC and from 15 to 18 ºC, respectively) and one constant, at 18 ºC (T18). Before and
39 during hormonal treatment, the eels were sampled and biometric measurements were
40 taken. Immunoassays of sex steroids and vitellogenin were performed, as well as qPCR
41 analyses of gene expression (ovarian *cyp19a1*) and ovarian histology. Prior to the
42 hormonal treatment, the silver eels which had been maintained at 18 ºC showed lower
43 11-KT and E2 plasma levels compared to those maintained at 10 ºC. In addition, in the
44 early vitellogenic stage, the androgen and *cyp19a1* levels were lower at 18 ºC than at 10
45 ºC. Both these results and the positive correlations found between GSI and 11-KT (at
46 the PV stage) and between oocyte diameter and *cyp19a1* levels (in the EV stage),
47 suggest that early ovarian development is facilitated at low temperatures. Vitellogenesis
48 was induced by CPE in all the thermal groups, but progression to the mid-vitellogenic
49 stage was only observed after an accumulation of 900-1200 ºD, at 15 or 18ºC, and
50 progression to the late vitellogenic stage was only observed after an accumulation
51 higher than 1300 ºD, at 18 ºC. Although temperature increased the rate of CPE-induced
52 ovarian development, our results clearly indicate that this increase is not linear, but
53 exponential, with acceleration in the increase of GSI at 18 ºC from the mid-vitellogenic
54 stage, or after an accumulation of 1300 ºD. For the first time, a down-regulation of
55 ovarian *cyp19a1* caused by high temperatures in CPE-treated eels was observed. These
56 results demonstrate that temperature can modulate eel ovarian development both before
57 and after exogenous hormonal stimulation, and this knowledge could be used to
58 manipulate the timing of vitellogenesis progression under laboratory conditions.

59

60 Highlights

61 • Low temperatures induced steroidogenesis in previtellogenic eel ovaries.

62 • High temperatures down-regulate ovarian *cyp19a1* gene expression in CPE-
63 treated eels during early ovarian development.

64 • Ovarian CPE-induced growth is accelerated by high temperatures or by an
65 accumulation higher than 1300 ºD.

66

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

1. Introduction

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

It is known that the European eel perform a 4–6000 km reproductive migration from European coastal waters to their supposed spawning grounds in the Sargasso Sea (review van Ginneken & 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 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 200-600 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 1-2.7 %

102 (Boëtius & Boëtius 1980; Durif et al., 2005). Thus, it is possible to suppose that early
103 ovarian development in nature takes place at low temperatures. On the other hand, it is
104 assumed that ovulation takes place at temperatures around 18-22ºC, considering the
105 water temperature in the supposed spawning areas of the Sargasso Sea (Friedland et al.,
106 2007). Thus, by combining hormonal treatments with thermal profiles resembling those
107 supposedly found in the wild, the quality of gonadal maturation in captivity could be
108 improved. In a previous work (Pérez et al., 2011) we matured female European eels
109 with carp pituitary extracts (CPE) under two thermal regimes; one variable regime
110 increasing from 10 to 17 ºC, and one constant at 20 ºC. The results showed higher E2
111 plasma levels, as well as increased expression of *fshb* and *lhb* in the pituitary, and of
112 *estrogen receptor 1* in the ovary, in eels reared using the variable thermal regime, thus
113 suggesting that a variable regime results in improved gonadal maturation.
114
115 The fish pituitary injections used to mature female eels provide exogenous
116 gonadotropins directly to the ovaries, and subsequently stimulate ovarian steroid
117 synthesis (Matsubara et al., 2003a, 2005), which not only directly stimulates the oocyte
118 growth but also activates the entire reproductive endocrine axis through feedback
119 mechanisms (review of Zohar et al., 2010). In most female fish the ovarian steroids
120 involved in oocyte growth are testosterone (T) and 17β-estradiol (E2) although 11-
121 ketotestosterone (11-KT) is also important, as it is related to oocyte growth and lipid
122 uptake (Endo et al., 2008, Lokman et al., 2007; Matsubara et al., 2003b) in eel species.
123 The steroid activity can be regulated at different levels, for example through changes in
124 steroid receptor expression or ligand affinity, or changes in the expression or activity of
125 steroidogenic enzymes, like P450aromatase, the enzyme responsible for transforming
126 androgens into E2.
127
128 Water temperature can affect E2 levels through changes in the gene expression of the
129 ovarian P450aromatase gene (*cyp19a1*), as has been shown in some fish species with
130 temperature-dependant sex determination (reviews Ospina-Alvarez & Piferrer 2008,
131 Miranda et al., 2013). We have previously observed lower E2 plasma levels in European
132 eel females maintained at a high temperature (Pérez et al., 2011), suggesting that
133 temperature has an effect on the activity or gene expression of the ovarian
134 P450aromatase gene (*cyp19a1*) in this species.
135

136 In this study, we have used two thermal regimes in an attempt to simulate the thermal
137 changes that eels probably experience during their migration, with lower temperatures
138 during the oceanic migration and higher temperatures at the spawning grounds in the
139 Sargasso Sea. We have compared the results of these to those of a third thermal regime,
140 with a constant temperature of 18 ºC. The aim was to discover whether temperature can
141 modulate ovarian development induced by CPE through changes in steroid production,
142 and gene expression of *cyp19a1*.
143

144 **2. Materials and methods**

145 **2.1. Fish handling**

146 One hundred and eleven silver female eels (mean body weight 750±22 g; mean length
147 72.2±0.6 cm) caught in the Albufera Lagoon (Valencia, Spain) during their migration to
148 the sea were transported to the facilities of the Universitat Politècnica de València
149 (UPV, Spain). Eight healthy eels were killed during the first 24 h upon arrival, to serve
150 as freshwater controls (FW). The remaining eels were kept in two 1500 L tanks
151 equipped with recirculating freshwater systems (18 ºC), and were gradually acclimated
152 (over 10 days) to seawater, and moved to three 500 L tanks (1 experimental group/tank),
153 with 34-35 fish/tank (18 ºC). Each 500 L tank had an independent seawater recirculation
154 system and was equipped with two water chillers (Boyu L500). All the tanks were
155 covered with a black waterproof sunshade to maintain semi-dark conditions. The
156 experiment lasted from March to June 2009. The eels were not fed throughout the
157 experiments. All the fish were handled in accordance with the European Union
158 regulations concerning the protection of experimental animals (Dir 86/609/EEC).
159 Mortality throughout the experiment was 13-14 % (groups T10-15, T15-18, 4 out of 30
160 fish in each group; T18 group, 5 out of the 35 fish), without differences between the
161 experimental groups.
162

163 **2.2. Thermal treatments**

164 Figure 1 shows the thermal regimes and the sampling points. Before the experiment
165 started, the water temperature was gradually adjusted from 18 ºC (ambient temperature)
166 to the experimental temperatures (10, 15, or 18 ºC) in order for the animals to gradually
167 acclimate to the experimental conditions. Once the water had been maintained at those
168 temperatures for six weeks, the temperature in regime T10-15 was increased from 10 to
169 15 ºC until the end of the experiment (Fig.1). In addition, 10 weeks after the

170 temperature had been maintained at 15 ºC, the temperature in group T15-18 was
171 increased to 18 ºC until the end of the experiment. The design of these thermal profiles
172 was based on previous research of ours (Pérez et al., 2011) and took into account the
173 thermal profiles used for gonadal maturation in Japanese eel (Unuma et al., 2012; Ijiri et
174 al. 2011).

175

176 **2.3. Hormonal treatment**

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

189

190 **2.4. Fish sampling**

191 Between 6 and 8 healthy females were sacrificed at each sampling point. Following the
192 sampling for freshwater controls (FW) upon arrival at the UPV facilities, 8 eels were
193 sacrificed following 7 days of temperature acclimation (10, 15 or 18 ºC), and used as
194 temperature controls (T0, Fig. 1). Then, one week later, the hormonal treatment started
195 in all the groups, with each fish receiving weekly CPE injections. Seven days after
196 receiving the 4th, 8th and 12th injection at the different temperatures (sampling points
197 4CPE, 8CPE, 12CPE, Fig. 1) 6-8 fish were sacrificed from each group.

198

199 At each sampling, the eels were anesthetized (benzocaine, 60 mg L^{-1}) before being
200 sacrificed by decapitation. The gut was cut in the anal region and above the liver, and
201 then weighed. Total body, gonad, and gut weights were recorded to calculate the
202 gonadosomatic index (GSI = 100 gonad weight x total body $weight^{-1}$) and Gut Index
203 (GI = 100 gut weight x total body $weight^{-1}$). In addition, total body length and eye

204 diameter (vertical and horizontal) were measured to calculate the Eye Index (EI = 100 π
205 0.25 $(Dh+Dv)^2$ x Lt^{-1}, where Dh = horizontal eye diameter, Dv = vertical eye diameter,
206 and Lt = total body length (Pankhurst, 1982)). Blood was sampled from the caudal
207 vasculature and centrifuged (3000 rpm, 15 min), and blood plasma was stored at -80 °C
208 until analyses.
209
210 The gonad samples collected for histology were preserved in 10% buffered formalin.
211 Triplicate samples from the gonad and liver were collected immediately after dissection
212 from each fish, and then stored in RNA-later (Ambion Inc., Huntingdon, UK) at -20 ºC
213 until RNA extraction and gene expression analyses by qPCR.
214

215 **2.5. Gonad histology**

216 After dehydration in ethanol, samples were embedded in paraffin and cut into 5-10 µm
217 thick sections with a Shandon Hypercut manual microtome (Shandon, Southern
218 Products Ltd. England). The slides were stained with haematoxilin and eosin and
219 observed through a Nikon Eclipse E-400 microscope equipped with a Nikon DS-5M
220 camera, all from Nikon (Tokyo, Japan).
221
222 One-hundred oocytes per specimen were measured (diameter), and the biggest ones
223 were selected. The stages of oogenesis were determined according to Selman and
224 Wallace (1989), Kayaba et al. (2001) and Pérez et al. (2011). In summary, the
225 previtellogenic stage included both the perinucleolar and lipid droplet stages; early
226 vitellogenic oocytes contained small yolk globules restricted to the periphery of the
227 oocyte, mid-vitellogenic oocytes showed abundant yolk vesicles and late vitellogenic
228 oocytes showed more abundant yolk vesicles than lipid droplets.
229

230 **2.6. RNA extraction and cDNA synthesis**

231 **2.6.1 Primer design**

232 Eel acidic ribosomal phosphoprotein P0, *arp* (Table 1, Aroua et al. 2007; Peñaranda et
233 al. 2010; Weltzien et al. 2005) was used as the reference gene in the qPCR because its
234 mRNA expression has been shown to be stable during experimental treatment (Weltzien
235 et al. 2005). The expression stability of the reference gene in the ovary was determined
236 using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation
237 (SD[±Cq]) lower than 1 (0.21; $p<0.05$) and Cq arithmetic mean of 10.1±0.72. The

BestKeeper calculated variations in the reference gene based on the arithmetic mean of the Cq values. Genes with an SD value higher than 1 are defined as unstable. The Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers for *cyp19a1* (Table 1). To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon-exon boundary. All primers were tested on gDNA and RNA to confirm that they would not amplify potentially contaminating gDNA. The specificity was confirmed by melting curve analysis, gel electrophoresis, and by the sequencing of the qPCR products.

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 ºC for 10 min, 42 PCR cycles were performed using the following cycling conditions: 95 ºC for 10 s, 60 ºC for 10 s, and 72 ºC for 7 s. Each PCR reaction contained a total of 10 µl, comprising of 1:10 diluted cDNA template (3 µl), forward and reverse primers (250 nM each), and SYBR Green Master Mix (5 µl). Transcript levels were determined as Weltzien et al. (2005), using an efficiency-adjusted relative quantification method (Pfaffl, 2001). Briefly, it was calculated from the formula:

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

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

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

Testosterone (T) plasma levels were measured in 25µl duplicates of each sample using a

272 competitive testosterone ELISA KIT (Eurobio AbCys, Les Ulis, France), with a
273 sensitivity of 0.07ng/ml, and an intra-assay variation of 6-10 %. The standard curve was
274 between 0,008 and 16 ng/ml. All plasma samples were assayed in the same test.

275 11-KT plasma levels were measured in 25 µl duplicates of each plasma sample using an
276 11-KT ELISA Kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), with a
277 sensitivity of 1-2 pg/ml and an intra-assay variation of 10-15%.. The standard curve was
278 between 0.78 and 100 pg/ml. All plasma samples were assayed in the same test.

279 T and 11 KT immunoassays were validated for eel plasma by performing the following
280 tests: an assay of serial dilutions of various eel plasma samples and a validation of the
281 parallelism with the standard curve; the addition of known amounts of steroid to eel
282 plasma samples and the validation of the recovery; the addition of eel plasma to each
283 standard dose and the validation of the recovery. These steroid immunoassays were
284 previously carried out on the eels to measure the increases in androgen plasma levels
285 during the transition from the juvenile yellow stage to the prepubertal silver stage
286 (silvering; Aroua et al., 2005), and during experimental maturation (Peñaranda et al.,
287 2010; Jeng et al., 2012).

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

296

297 17β-estradiol (E2) plasma concentrations were measured by means of
298 radioimmunoassay (RIA), according to the method described by Schulz (1984). In
299 summary, free (i.e. not conjugated) steroids were extracted from 200µl plasma with 4
300 ml diethylether after vigorously shaking for 4 min. The aqueous phase was frozen in
301 liquid nitrogen, while the organic phase was transferred to a glass tube, evaporated in a
302 water bath at 45°C and then reconstituted through the addition of 600 µl assay buffer,

303 and then assayed for E2. Cross-reactivities of the E2 antiserum have previously been
304 examined by Frantzen et al. (2004). The limit for the assay was 0.2 ng ml^{-1}. To validate
305 E2 recovery from plasma in the eel assay, plasma pools were spiked with 5 and 15 ng
306 E2 ml^{-1} of plasma and then subjected to ether extraction as described above. The
307 resulting products from the different treatments were then assayed by the E2 RIA at two
308 different dilutions. A plasma E2 dilution curve parallel to that of the assay standard
309 curve was established. In addition, to test E2 extraction from plasma, radiolabelled
310 steroid (c. 100000 c.p.m.) was added to 200µl aliquots (n=8) of plasma and then ether
311 extracted. Steroid recovery after ether extraction was 85.6±1.0%. E2 values were
312 corrected for recovery losses. The inter- and intra-assay coefficients of variation (CV)
313 for the E2 assay were 9.4% (n=4) and 5.2% (n=10), respectively.
314
315 **2.8. Statistical analysis**
316 Each variable was analysed first for normality by the asymmetry standard coefficient
317 and Curtosis coefficient. The variables that did not have a normal distribution were log-
318 transformed and their normality was checked again. Then, a two-way ANOVA
319 (treatment, sampling point) was performed to discover whether each variable was
320 affected by the experimental treatments and/or by the sampling point. One-way
321 ANOVA analyses were then performed to compare thermal treatments in the same
322 sampling time. Variance homogeneity was checked using the Bartlett test. The one-way
323 ANOVA analyses were followed by a Newman-Keuls post-hoc test. If normality failed
324 following the log transformation, a non-parametric test was carried out (Kruskal-Wallis
325 test), followed by a Dunn's test.
326
327 Pearson linear correlations between the different variables were calculated using the
328 statistical software provided by Statgrafics Plus 5. Simple and multivariate regression
329 analyses were performed to study the relationship between the GSI and the accumulated
330 degrees day (ºD) and the accumulated CPE-doses, using the software provided by
331 Statgrafics Plus 5. All the values are expressed as mean ± standard error of mean
332 (SEM). Differences were considered significant when $p<0.05$. All the statistical
333 procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville,
334 MO, USA).
335
336

3. Results

3.1. Morphological changes and gonadal development

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

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

3.2. Steroid and vitellogenin plasma levels

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

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

Similar to E2, VTG plasma levels decreased after SW and temperature adaptation, but showed a huge increase after 4 CPE injections, reaching peak values after 12 CPE injections. The thermal treatments affected the VTG plasma levels, which were higher in group T18 both in SW and after 12 CPE injections ($p<0.01$).

371 ### 3.2. Ovarian *cyp19a1* expression

372 Ovarian *cyp19a1* expression (Fig. 5) increased progressively throughout the experiment.
373 Group T18 showed reduced a *cyp19a1* expression at 4CPE ($p<0.01$) compared to the
374 other groups. If we take into account only the previtellogenic females (Fig. 6),
375 treatments T10-15 and T15-18 induced a significantly higher expression of *cyp19a1*
376 than T18 ($p< 0.01$). In addition, T levels were lower in group T18 compared to the
377 lower temperature group, T10-15. When examining only the early vitellogenic females,
378 it was observed that a high constant temperature (T18) caused a low expression of
379 *cyp19a1* ($p<0.01$) compared to group T10-15, and lower T levels (and a similar, not
380 significant trend in E2) compared to the other two groups.

381

382 ## 4. Discussion

383 ### 4.1. Low temperatures induced steroidogenesis prior to hormonal treatment

384 Ovaries in previtellogenic stage were observed in the FW control group, as well as in
385 the eels sampled at T0, after having spent one month in SW. This corroborates the idea
386 that captive eels experience a gonadotropin insufficiency (Dufour et al., 1989) or
387 dopamine blockage of the reproductive neuroendocrine axis (Dufour et al., 2005; Vidal
388 et al., 2004). The FW control eels showed higher E2 and VTG levels than the eels from
389 T0, indicating that seawater alone does not facilitate vitellogenesis but, on the contrary,
390 may reinforce the previtellogenic blockage.

391 Interestingly, before starting the hormonal treatments, water temperature alone affected
392 several parameters including Eye Index and 11-KT and E2 plasma levels. These were
393 all lower in the group kept at 18 ºC compared to 10 ºC. It has been shown that 11-KT
394 promotes previtellogenic oocyte growth in shortfinned eel (*A. australis*; Lokman et al.,
395 2007) and coho salmon (Campbell et al., 2006; Forsgren and Young, 2012), and also
396 potentiates the effect of E2 in stimulating hepatic synthesis of VTG in Japanese eel
397 (Asanuma et al., 2003). Regarding E2, apart from the known role it plays during
398 vitellogenesis, it has been linked to oogonial proliferation in Japanese eel (Miura et al.,
399 2007) and to previtellogenic oocyte growth in coho salmon, (Campbell et al., 2006;
400 Forsgren & Young, 2012). In Japanese eel it has been suggested that a decrease in water
401 temperature induced an early stage of ovarian development, with the thermal reduction
402 from 25 to 15 ºC increasing 11-KT and E2 levels and the oil droplet number in PV
403 oocytes (Sudo et al., 2011). While the oil drop number was not measured in this
404 experiment, other parameters suggest that low temperatures can facilitate the oocyte

405 growth at the PV stage. For instance, the GSI values were higher (but without statistical
406 differences) in the eels maintained at 10 ºC compared to the eels maintained at 18 ºC
407 (1.08 vs 0.86, respectively), and the GSI showed a positive correlation with 11-KT
408 levels (r= 0.59; p= 0.006; Supplementary table 1), which were higher at low
409 temperatures. On other hand, the Eye Index, which is an indicator of the onset of eel
410 puberty (Aroua et al., 2005), was lower in female eels maintained at 18 ºC compared to
411 the other temperatures. This thus supports the idea that maintaining female eels at this
412 temperature in the PV stage does not facilitate the previtellogenic growth. Similar to the
413 results found at low temperatures, an increase in Eye Index, 11-KT plasma levels, and
414 GSI were also observed after maintaining European eels swimming during long periods
415 (reviewed by Palstra et al., 2009). Thus, both low temperatures and swimming could be
416 promoting previtellogenic growth, which seems logical as eels in nature should
417 experience both parameters (swimming at low temperatures) at the same time.
418
419 Our results show, for the first time, a down-regulation of ovarian *cyp19a1* expression at
420 18 ºC compared to lower temperatures in adult European eel at the previtellogenic stage.
421 In Japanese eel, Ijiri et al. (2003) demonstrated a strong correlation between ovarian
422 aromatase gene expression and aromatase enzyme activity from ovarian follicles,
423 strongly suggesting that aromatase enzyme activity would be lower at 18 ºC in the
424 ovaries of the European eel females analysed in the present work. This corresponds very
425 well with the lower E2 levels observed at high temperatures, also at the previtellogenic
426 stage.
427
428 **4.2. High temperatures reduce CPE-induced steroidogenesis and *cyp191a***
429 **expression at the early vitellogenic stage.**
430 Carp pituitary injections (CPE) provide exogenous gonadotropins directly to the
431 ovaries, and result in the subsequent stimulation of ovarian steroid synthesis (Matsubara
432 et al., 2003a, 2005) and the activation of the entire reproductive neuroendocrine axis
433 through feedback mechanisms (review of Zohar et al., 2010). Thus, ovaries from eels
434 maintained at different temperatures showed different responses to CPE treatment, as
435 evidenced by ovarian development and steroid plasma levels.
436 In this experiment, early vitellogenic CPE-treated eels kept at constant high
437 temperatures (T18) showed reduced *cyp19a1* gene expression levels and lower
438 androgen plasma levels (T, 11-KT) than fish maintained at 10-15 ºC (Group T10-15).

439 Vitellogenesis is an E2-dependent process, and aromatase is the enzyme which converts
440 androgens (mainly T) into E2. Aside from this, 11-KT also enhances E2-induced VTG
441 synthesis (Asanuma et al., 2003). Thus, the highest steroid and *cyp191a* expression
442 levels observed at low temperatures in the EV stage suggest that CPE-induced early
443 vitellogenic growth could be facilitated by low temperatures. This agrees with the
444 positive correlation found between oocyte diameter and *cyp19a1* expression at this stage
445 (r=0.67, $p < 0.001$, Supplementary table 2).
446
447 Previous research on other fish species have shown reductions in E2 plasma levels at
448 high temperatures during vitellogenesis (striped bass *Morone saxatilis*, Clark et al.,
449 2005; Atlantic salmon, review by Pankhurst and King, 2010; pikeperch *Sander*
450 *lucioperca*, Hermelink et al., 2013), but only a few studies on adult fish have previously
451 demonstrated an inhibition of the expression of aromatase by thermal regimes. For the
452 first time, we have demonstrated a down-regulation of *cyp19a1* in CPE-treated female
453 European eels at high temperatures. Similarly, a reduced aromatase expression at high
454 temperatures has been observed in adult red seabream (*Pagrus major*) and Atlantic
455 salmon, in previtellogenic and vitellogenic stages, respectively (Lim et al., 2003;
456 Anderson et al., 2012).
457
458 The E2 profile during sex maturation was similar in all the thermal groups, increasing
459 during early-mid vitellogenesis (weeks 4-8) and decreasing thereafter, in fish which
460 were either in the MV or LV stage. A similar increase in E2 levels during vitellogenesis
461 has previously been observed in European eel (Pérez et al., 2011), and in New Zealand
462 long-finned eels (*A. dieffenbachii*, Lokman et al., 2001). Nevertheless, the E2 levels of
463 Japanese eel matured at 20 ºC were low during vitellogenesis, and increased only in the
464 LV stage (Matsubara et al., 2003a) or later (Ijiri et al., 1995; Suetake et al., 2002). Such
465 differences in the E2 response to pituitary treatments could be species-specific.
466
467 VTG and E2 showed a high degree of correlation in the PV stage (0.70, $p < 0.01$,
468 Supplementary table 1), but the VTG profiles did not follow the same pattern as the E2
469 profiles. While the E2 plasma levels decreased at the end of the hormonal treatment, the
470 VTG plasma levels increased, corroborating the ovarian histological observations. A
471 lack of consistency between the E2 and VTG plasma levels has already been observed
472 in a number of fish species (reviewed by Pankhurst, 2008), and may be due to the short

473 half-life of steroids in the plasma (Pankhurst, 2008), or to the time lapse between the
474 increase in plasma E2 and the release of vitellogenin to blood plasma. Classical steroid
475 actions occur through several steps, and it takes from hours to days between steroid
476 synthesis and the appearance of its biological effect (reviewed in Norris et al., 1996).
477
478 The 11-KT levels found in this research study were lower than those previously
479 reported by van Ginneken et al. (2007) or Palstra et al. (2009) in their studies on
480 European eel females, but similar to those previously reported by Sebert et al. (2007,
481 2008), or Aroua et al. (2005). The differences may be due to the different methods used
482 to measure 11-KT, i.e. radioimmunoassay vs ELISA.
483

484 **4.3. Temperature modulates the progression of vitellogenesis**

485 The results of this research confirm that thermal regimes affect ovarian development in
486 the European eel, agreeing with our previous results (Pérez et al., 2011). The present
487 results allow us to take a closer look at the combined effect of temperature and
488 hormonal treatment on the progression of vitellogenesis in the European eel. In this
489 research we have shown that, during hormonal treatment, early vitellogenesis can be
490 reached at 10, 15, or 18 ºC, as evidenced by the histological features and the GSI
491 increase after 4CPE. However, further development to the mid-vitellogenic stage is
492 delayed in eels maintained at 10 ºC during the first weeks of hormonal treatment even if
493 they are then transferred to 15 ºC, as results from 8CPE show.
494 On the other hand, ovarian development up to the mid-vitellogenic stage was as fast at
495 15 ºC as at 18 ºC, as the histological results from 8CPE show (comparison between
496 T15-18 and T18). However, further development to the late vitellogenic stage was
497 delayed in the eels maintained for 8 weeks at 15 ºC compared to eels maintained at a
498 constant 18 ºC, even when both groups were maintained at the same temperature (18 ºC)
499 during the last part of the experimental period, from 8CPE to 12CPE. Thus, the results
500 obtained can only be explained on the basis of the thermal period experienced by the
501 eels prior to each sampling. Table 3 indicates the degrees day (ºD) experienced by the
502 female eels before they reached the different development stages, and the accumulated
503 CPE dose received until that time.
504 Table 2 shows that the MV stage was reached after 1120 ºD (group T10-15), 840-1340
505 ºD (group T15-18), and 1008 ºD (group T18). Also, the LV stage was reached after
506 1512 ºD (group T18 at 12CPE), but not after 1340 ºD (group T15-18 at 12CPE) or 1120

507 ºD (group T10-15 at 12CPE). These results suggest that, in CPE-induced eel gonadal
508 maturation, an accumulation of 900-1200 ºD (and 160-240 mg CPE kg^{-1}) should
509 facilitate development up to the mid-vitellogenic stage, while an accumulation of more
510 than 1300 ºD would facilitate development up to the late vitellogenic stage. This agrees
511 with previous results of ours (Pérez et al., 2011), where the MV stage was reached after
512 a mean accumulation of 987 ºD or 1172 ºD (Pérez et al., 2011, Vílchez et al. 2013),
513 while the LV stage was the dominant stage after an accumulation of 1680 ºD, although
514 not observed after 1220 ºD. Also, Mordenti et al. (2013) observed LV stages in CPE-
515 treated European eels after an accumulation of 1628 ºD (our own calculations, Table 3),
516 but not after 1085 ºD.
517
518 Although it is clear that temperature increased the rate of ovarian development, our
519 results clearly indicate that this increase is not linear. A significant exponential
520 regression ($p<0.001$) between GSI and ºD was found (Figure 7). Thus, the GSI of CPE-
521 treated eels increased exponentially with the ºD experienced, thus indicating that the
522 GSI growth accelerated from an accumulation of about 1300 ºD. When data from other
523 experiments (Pérez et al., 2011, Vílchez et al. 2013) was added to this model, the
524 significance of the exponential regression model increased (R^2=0.95; r=0.97 $p<0.001$,
525 Fig. 7B).
526 Nevertheless, at the same time as the ºD accumulated, the fish received additional doses
527 of CPE. A significant exponential regression ($p<0.001$) was also seen between the GSI
528 and the accumulated CPE dose. However, this exponential correlation between the GSI
529 and the accumulated CPE-dose was weaker (R^2= 0.81; r=0.90) than the correlation
530 between the GSI and the accumulated ºD (R^2= 0.93; r=0.96). Also, when a multivariate
531 regression model was applied to explain the GSI variation from both ºD and
532 accumulated CPE-dose variables, only the first variable was significant (data not
533 shown). While it is impossible to differentiate between the effects of the accumulated
534 ºD day and the accumulated CPE-doses, it would appear that the increase in GSI
535 accelerates from a certain level of ºD (about 1300 ºD) or accumulated dose of CPE
536 (about 240 mg). This knowledge could be applied in the design of thermoperiods for the
537 induction of eel maturation, as well as to manipulate the timing of the progression of
538 vitellogenesis in laboratory conditions.
539
540

541 **5. Conclusions**

542 For the first time in the European eel, a down-regulation of ovarian aromatase gene
543 expression (*cyp191a*) by high temperatures has been demonstrated. This study has also
544 proved that low temperatures alone induced steroidogenesis in previtellogenic eel
545 ovaries, but high temperatures during CPE treatment caused an acceleration of ovarian
546 growth to late vitellogenic stage.

547

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

554

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565

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814 **Tables**

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

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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
	ACA TCG CTC AAG ACT TCA ATG G	Rv (GE=2.18)	(AY763793.1)

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842 Table 2. Degrees day experienced by female eels before reaching the different
843 development stages, and accumulated CPE dose received until that time (mg kg $^{-1}$). EV:
844 dominance of early vitellogenic stage; MV: dominance of mid-vitellogenic stage; LV:
845 dominance of late vitellogenic stage. Present experiment. Pérez et al., (2011) and
846 Vílchez et al. (2013) were calculated from our original data. Mordenti et al. (2013) was
847 estimated from the data provided in Mordenti et al. (2013).
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	EV	**MV**	**LV**
Present experiment			
T10-15	280 ºD (80 mg) 700 ºD (160 mg)	1120 ºD (240 mg)	
T15-18	420 ºD (80 mg)	840 ºD (160 mg) 1260 ºD (240 mg)	
T18	504ºD (80 mg)	1008 ºD (160 mg)	1512 ºD (240 mg)
Pérez et al. (2011)	640 ºD (106 mg)	987 ºD (200 mg)	1680 ºD (240 mg)
Vílchez et al. (2013)		1173 ºD (240 mg)	1672 ºD (320 mg)
Mordenti et al. (2013)			1628 º D (420 mg)

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863 **Figure captions**

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

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

875

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

883

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

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892 Fig. 5. Effect of thermal and hormonal treatment on ovarian *cyp19a1* gene expression in
893 freshwater control (FW), temperature control (T0), and after 4, 8 and 12 CPE injections.
894 The relative expression was normalized to the abundance of *arp*. Results are expressed
895 as mean of fold change ± SEM (n=6) with respect to the FW control, which has been set

896 at 1. Small letters indicate significant differences between the thermal treatments at the
897 same sampling point ($p<0.05$, df=2). Capital letters indicate significant differences
898 through time considering all thermal treatments ($p<0.05$, df=4).
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900 Figure 6. Previtellogenic and early vitellogenic levels of ovarian *cyp19a1* gene
901 expression, plasma 17β-estradiol (E2) and testosterone, in each thermal treatment. Small
902 letters indicate significant differences between the thermal treatments ($p<0.05$, df=2).
903 Number of fish/group is show in brackets.
904
905 Figure 7. A. Relationship between GSI and degrees day (ºD) accumulated from the start
906 of hormonal treatment (time-points 4CPE, 8CPE, 12CPE). Significant positive
907 correlation was found between GSI and ºD ($r=0.96$; $p< 0.001$). B. Relationship between
908 GSI and degrees day (ºD) using the data from present experiment and from Pérez et al.
909 (2011), and Vílchez et al., (2013).
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932 **Figures**

933

934 Figure 1
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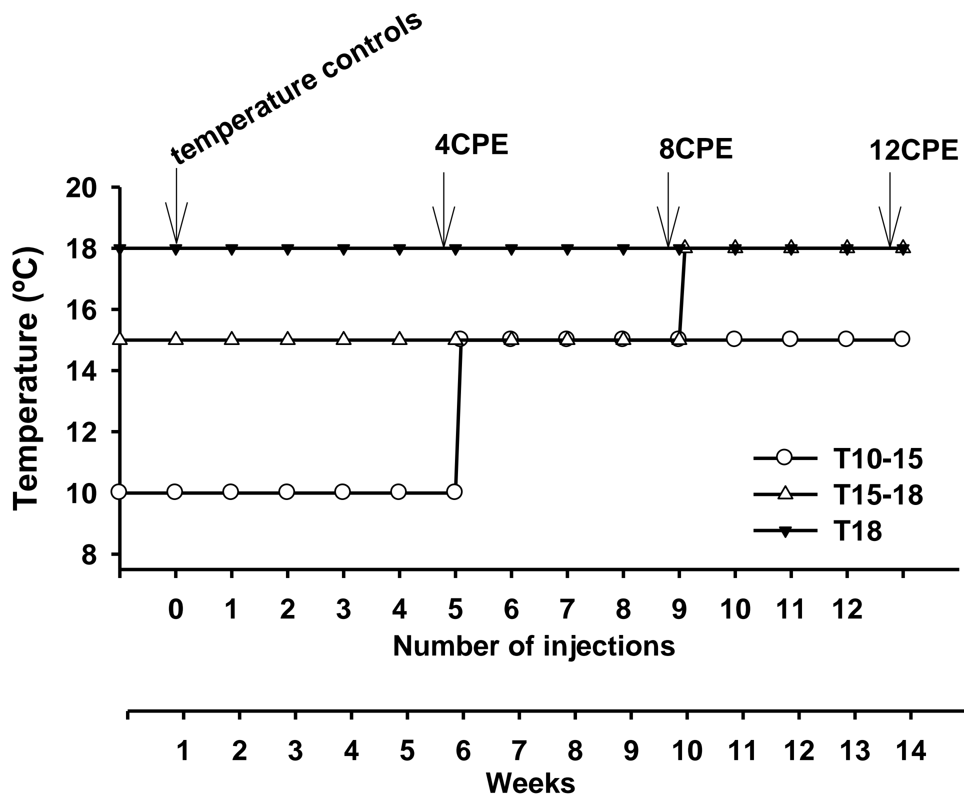

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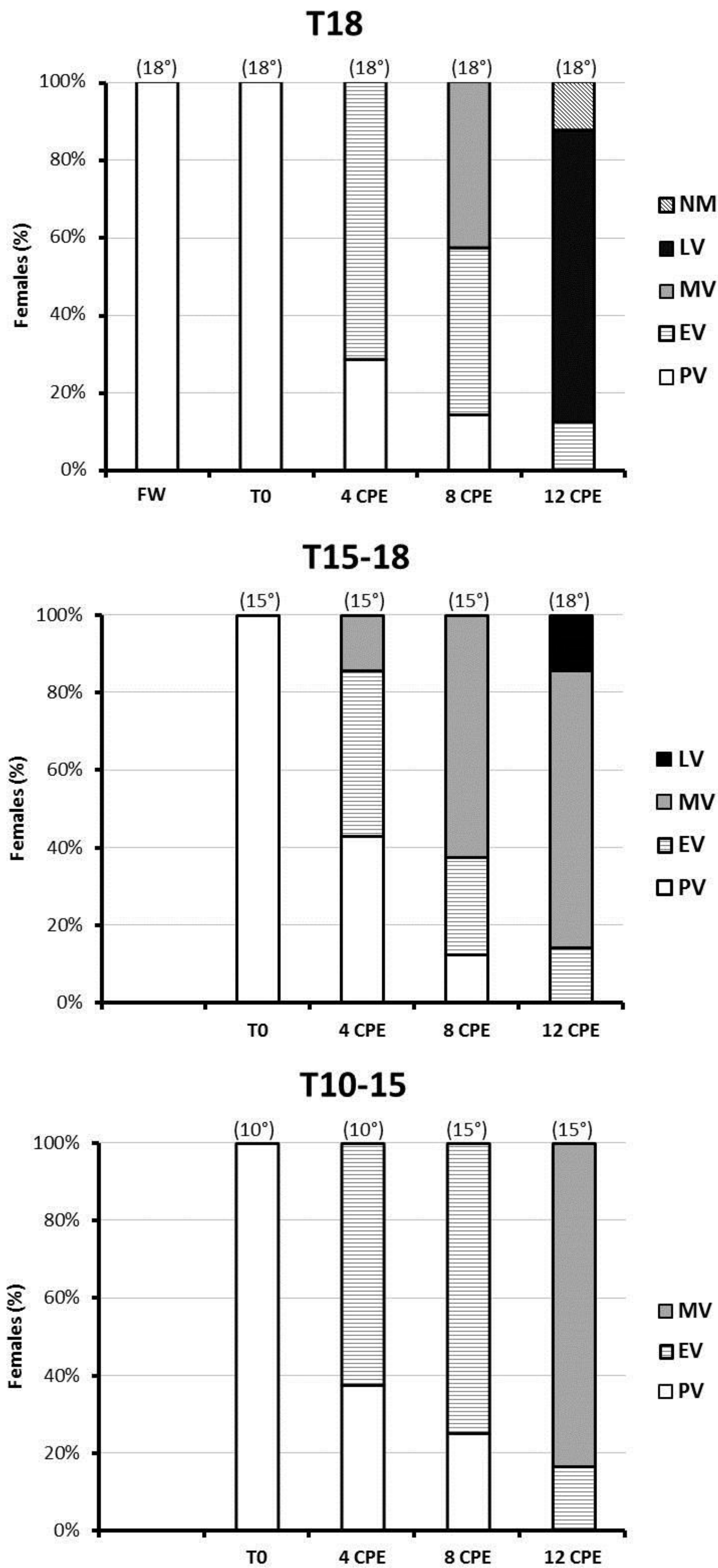

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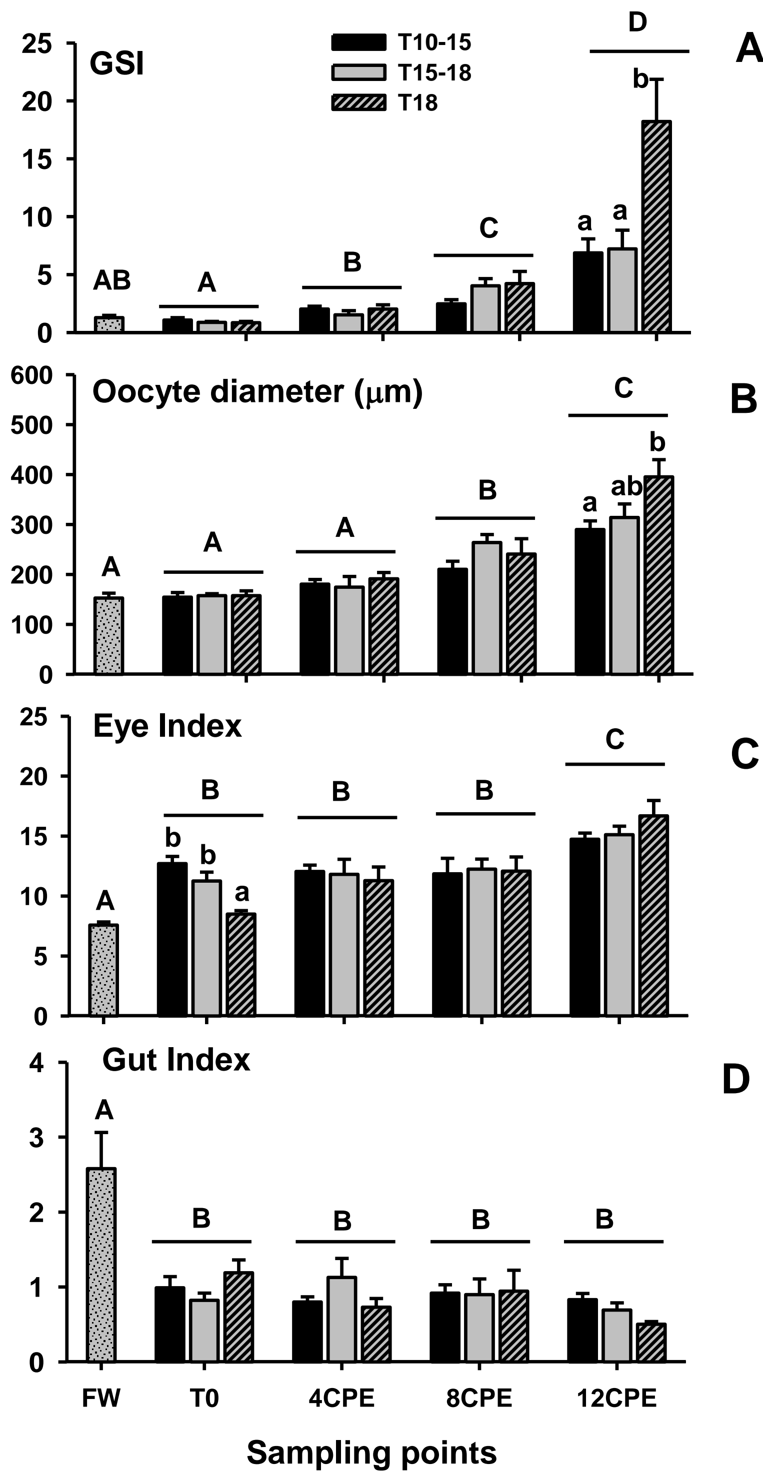

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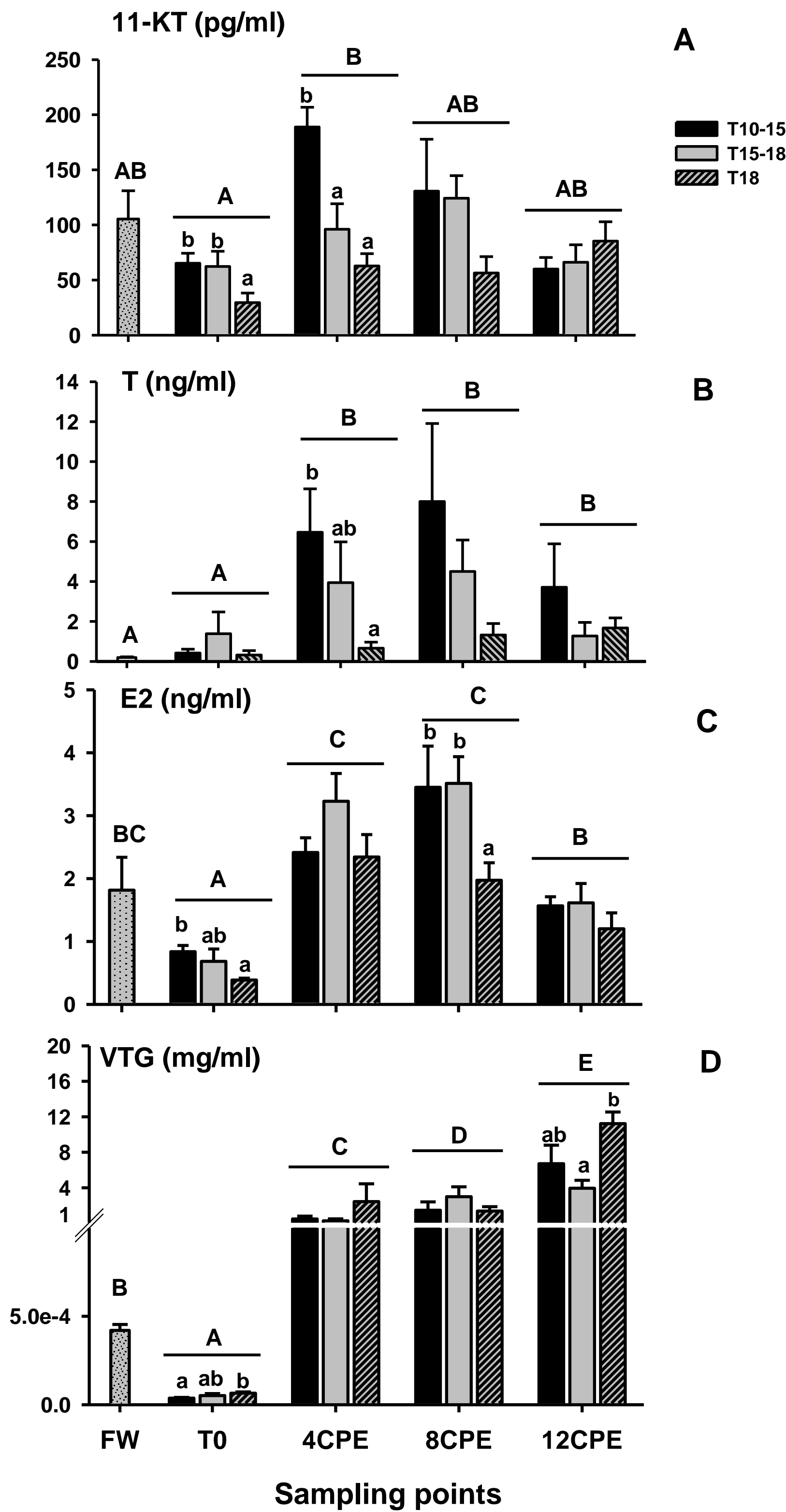

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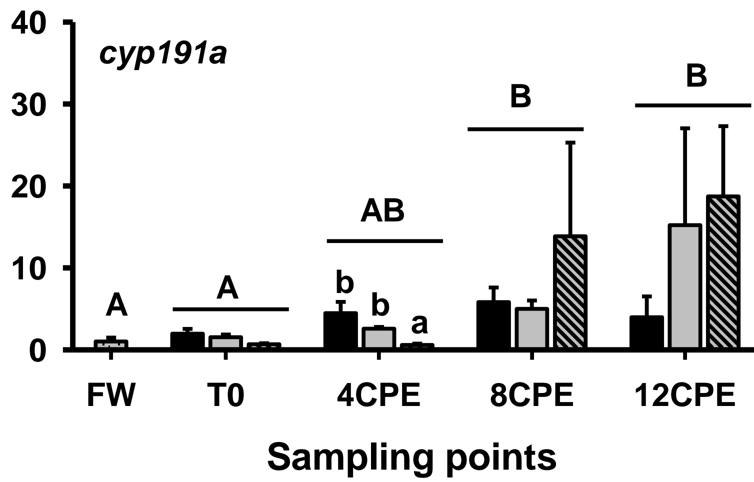

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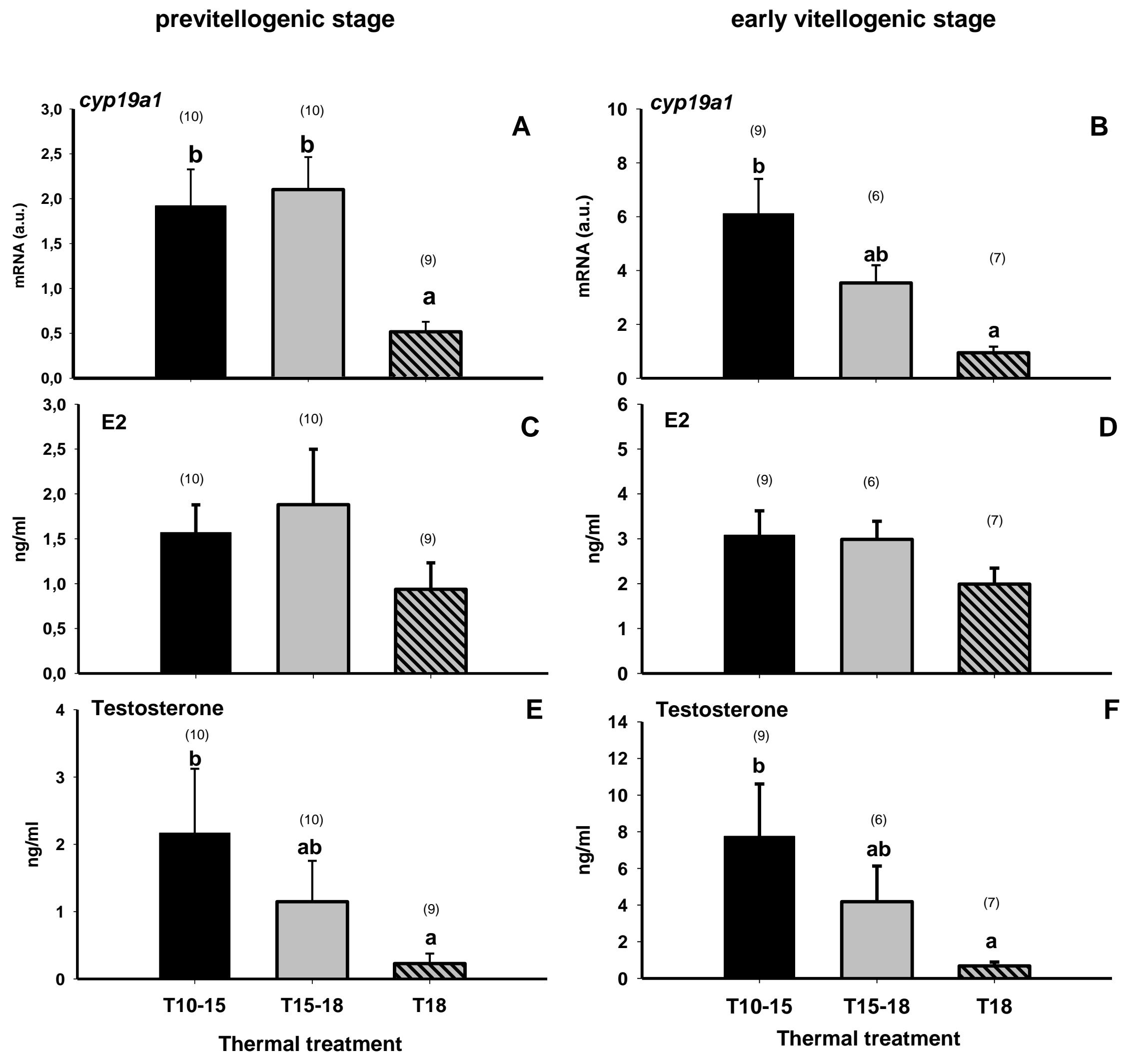

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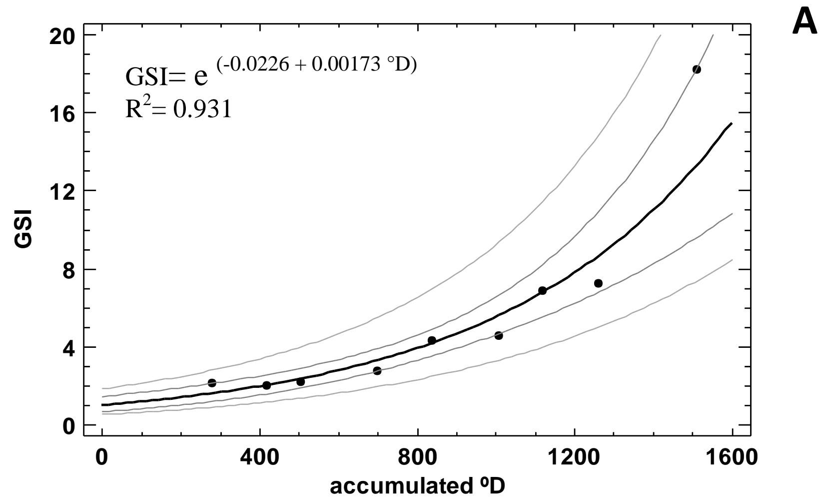

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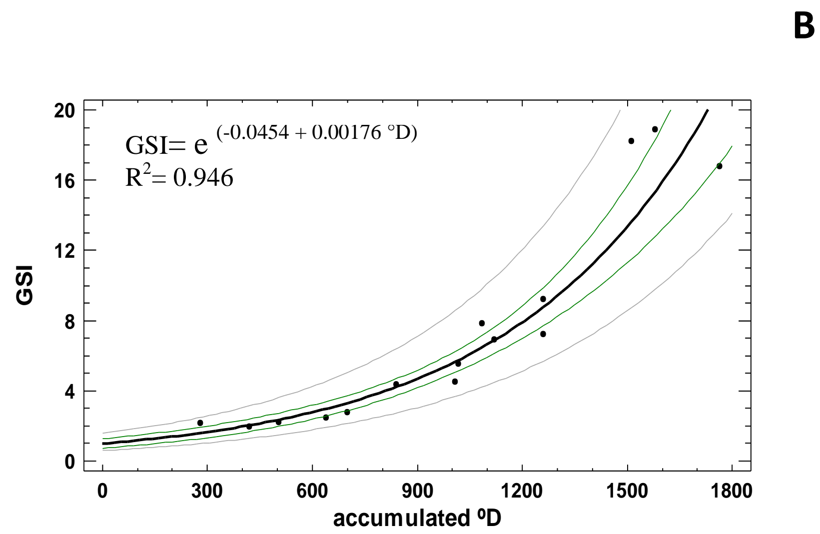

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1073 **Supplementary Table 1. Correlation cofficients at PV stage (n= 29)**

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

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1078 **Supplementary Table 2. Correlation cofficients at EV stage (n= 22)**
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	oocyte diameter	T	11KT	E2	*cyp191a*	VTG
GSI	**0,687** 0,002	-0,151 n.s.	0,398 n.s.	-0,195 n.s.	0,254 n.s.	0,248 n.s.
diameter		-0,337 n.s.	0,290 n.s.	-0,155 n.s.	**0,665** 0,003	0,562 0,015
T			0,243 n.s.	0,342 n.s.	0,122 n.s.	-0,420 n.s.
11KT				-0,063 n.s.	0,405 n.s.	-0,084 n.s.
E2					-0,015 n.s.	-0,403 n.s.
cyp191a						**0,476** 0,046

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