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Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females

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Abstract

We examined the effect of temperature in European silver eels during their maturation induced by injections of carp pituitary extract on endocrine parameters: pituitary *fshβ* and *lhβ* expression, plasma 17β-estradiol (E2) and vitellogenin, estrogen receptor 1 (*esr1*), and vitellogenin2 (*vtg2*) expression in liver. A variable thermal regime (T10) that increased from 10º to 14º and 17ºC was compared with a constant 20ºC regime (T20) during 12 weeks. T10 caused a faster development until week 8, higher *fshβ*, *lhβ*, *esr1* expression, and higher E2 levels. The results strongly suggest that T10 is inducing a higher endogenous FSH level which increases the E2 circulating level during vitellogenesis. A variable thermal regime induced an *fshβ* expression and E2 profile in vitellogenic hormonally matured eel females that were more similar to the profile observed in other naturally maturing fish.

Keywords: Eel, temperature, vitellogenesis, *fshβ*, *lhβ*, E2, *esr1*, *vtg2*, mRNA.

1. Introduction

The European eel migrates across the Atlantic Ocean for supposedly 6-7 months to reach the spawning area, in the Sargasso Sea [78]. When they leave the rivers and estuaries to enter the sea, they are still immature, with a gonadosomatic index (GSI = 100 x gonad weight x total body weight^{-1}) lower than 2% [4, 23], although one migrating female with a GSI =10% was caught at a depth of 500 m near the Azores Islands [10], 1500 km away from the Iberian coast. This means that the vitellogenic growth of the oocytes may be initiated during their journey. Recently the European eel migratory route has been followed by tagging eels with pop-up satellite archival transmitters to the first 1300 km of their 6000 km migration [1]. It has been demonstrated that eels undertook diel vertical migrations predominantly between depths of 200 and 1000 m. At night eels swim in shallow water (mean 282 m, 11.7 ±0.5ºC) while at dawn they dive to the cooler disphotic zone (mean 564 m, 10.12 ± 0.9ºC, minimum 7.1ºC). Such data agree corroborates the diel migrations in Western Spain [76] and the Mediterranean [77], where eels swim in the hypolimnion, at 13ºC, but migrate at night to warmer surface waters. In the Sargasso Sea, the presumed spawning area, once hormonally matured silver eels were released, they dived up to 700 m [77], or to 250-270 m [27], where water temperature was around 19ºC.

Eel species (*Anguilla* spp.) do not mature spontaneously in captivity due to a dopaminergic inhibition in addition to a deficient stimulation of gonadotropin-releasing hormone (GnRH) [21, 22, 79, 82]. Maturity can be induced with long-term gonadotropic treatments, such as injections of carp or salmon pituitary extracts in females of European eel [26] and Japanese eel [49], or of human chorionic gonadotropin (hCG) in males of both species [25, 51]. Such methods have been widely used both for European eel males [5, 6, 7, 8, 37, 41, 48, 49, 57, 58, 74], and for European eel females [4, 20, 53, 55, 56, 59, 70]. In the case of Japanese eel, with some modifications to these methods, it has been possible to produce glass eels [33, 75]. However, fish pituitary injections used to mature female eels caused abnormal gonadotropin profiles [50, 68, 72].

Classically, the water temperature in the hypothetical spawning area of the European eel has been considered to be at 20 ºC [11, 12]. Probably for this reason European eels have been

matured at a constant water temperature around 20ºC [4, 53, 55, 57, 80]. However, it seems probable that at sea the gonadal development occurs at lower temperatures, and the spawning at warmer temperatures.

The vitellogenesis is a complex process which hormonal control has been studied in fish as well as in other vertebrates [62]. Moreover, it is well known that fish vitellogenesis is affected by the water temperature. In striped bass, *Morone saxatilis* [16], and Senegalese sole, *Solea senegalensis* [28], constant warm temperatures reduced steroid production and impaired vitellogenesis in females compared with groups kept under naturally changing thermoperiods. In female Japanese eel, treatment with salmon gonadotropins at low temperatures (10ºC) induced lower vitellogenin levels and ovarian development than treatment at high temperatures (20ºC) [67]. Changes in water temperature could also be important. In one specimen of Japanese eel, ovarian development (GSI= 8) was obtained without hormonal treatment, only by changing the water temperature daily between 5 and 15ºC, while eels maintained at constant temperatures did not show any sign of maturation [46].

Our hypothesis is that maintaining female eels at constant high temperatures during induced hormonal maturation could be partly responsible for the abnormal hormonal profile (pituitary FSHβ underexpression and LHβ overexpression) observed in Japanese eels [50, 68, 72]. To test this hypothesis, endocrine parameters related to vitellogenesis, such as gonadotropin subunits expression, 17β-estradiol and vitellogenin plasma levels, and liver expression of *estrogen receptor 1* (*esr1*) and *vitellogenin 2* (*vtg2*) were analysed in two groups of eels: one maintained at constant 20 ºC, and another in a variable thermal regime intended to simulate the lower temperatures experienced during natural migration and the higher temperatures encountered at the spawning grounds.

2. Material and methods

2.1. Experimental fish and temperature treatments

Fifty-four wild female eels (mean body weight 847±28 g; mean length 71.4±0.8 cm) caught in the Albufera Lagoon during their migration to sea were transported to the Aquaculture Laboratory at the Polytechnic University of Valencia. They were kept in two 500 l fibreglass tanks equipped with separate recirculation systems and covered to maintain constant darkness. The females were tagged with passive integrated transponders (PIT tags) injected into epaxial muscle for individual identification. The fish were acclimatised for two weeks from freshwater to seawater (salinity 37.0±0.3 ‰), after which the water temperature was progressively changed from ambient temperature (18 ºC) to 10 or 20 ºC. The change was done at a rate of 1ºC/ day. One week having been maintained at these temperatures, 5 females from each temperature regime were sacrificed (week 0), and then the hormonal treatment started. In the case of remaining fish the group kept at 20 ºC (T20) was maintained at this temperature for the rest of the experiment. The T10 group was kept in a variable thermal regime (Figure 1). The fish were not fed throughout the experiment. 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 treatments was lower in T10 group (2 of 29 fish; 7%) than in the T20 group (7 of 25 fish; 28%).

2.2. Hormonal treatments

Females were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, Catvis, Ltd) at a dose of 20 mg/kg body weight [35, 55, 56, 68, 70], until the end of the experiment. The pituitary powder was diluted in NaCl 0.9 g/l, centrifuged (1260 g, 10 min) and the supernatant stored at -20 ºC until use, between 1 and 4 weeks later. All the fish were anaesthetised with benzocaine (60 ppm) and weighed before the injections to calculate the individual CPE dose.

2.3. Sampling and biometry

Groups of 5 eels from both the T10 and T20 group were anaesthetised and sacrificed by decapitation at 0, 4, 8 and 12 weeks of treatment. The external morphological parameters

140 measured in sacrificed fish were: body weight, total length, eye diameter (vertical and
141 horizontal) and pectoral fin length. Blood samples were obtained from the caudal vasculature,
142 and the plasma obtained by centrifugation (3000 rpm, 15 min) was stored at -80 ºC. The
143 pituitary gland was removed quickly, preserved in liquid nitrogen and stored at -80 ºC. The
144 gonad and liver were weighed, and samples of gonad were preserved in 10% buffered formalin
145 for histological procedures. The gonadosomatic index (GSI = 100 gonad weight x total body
146 weight^{-1}), and hepatosomatic index (HSI= 100 liver weight x total body weight^{-1}) were
147 calculated.
148
149 *2.4 Histological procedures*
150 The gonad tissue was embedded in paraffin wax, and sectioned to thickness of 5 and 10 µm.
151 Sections were stained by the current haematoxylin and eosin method. Slides were observed
152 using a Nikon Eclipse E-400 microscope prior to being photographed using a DS-5M camera
153 connected with a DS Camera Control Unit DS-L1, all from Nikon (Tokyo, Japan). Around fifty
154 measurements of larger oocyte diameters were performed for each eel with the Camera Control
155 Unit software. The evaluation of the maturation stages was performed according to previous
156 works [35, 69].
157
158 *2.5. Immunoenzymatic assays of oestradiol and vitellogenin*
159 Plasma levels of 17β-estradiol (E2) were assayed using specific EIA kit according to the
160 manufacturer's instructions (AbCyss, Paris, France).
161 Vitellogenin plasma levels were assayed using a homologous ELISA previously developed for
162 the European eel [15].
163
164 *2.6. Gene expression analyses by quantitative real-time PCR (qrtRT-PCR)*
165 *RNA extraction and first strand cDNA synthesis.* Total RNA was extracted from the pituitaries
166 and liver portions using traditional phenol/chloroform extraction [60].
167

Primers and reference gene. The primers are shown in Table 1. Acidic ribosomal phosphoprotein P0 for *fshβ* and *lhβ* (ARP) [2, 92], and *β-actin* for *esr1* and *vtg2* [54] were used as reference genes in the respective quantitative real time Reverse Trancriptase-Polymerase chain reactions (qrtRT-PCR).

SYBR Green assay. The quantitative assays of eel gonadotropin subunit expression (*fshβ* and *lhβ*) were performed using a Light Cycler system with SYBR Green I sequence-unspecific detection (Roche, Meylan, France), using the method developed by [2]. The *fshβ* and *lhβ* expressions data were normalised by dividing by the ARP expression. The quantitative assays of eel *esr1* and *vtg2* were performed as described in [54], and expression levels were normalized to the expression level of *β-actin* (between *x*% and 100%).

The genetic sequence deposits used to design the gene specific primers were as follows: *Anguilla anguilla fshβ* total cDNA: AY169722 [70]; *lhβ* cDNA: X61039 [65]; *ARP* cDNA: AY763793 [82]; *esr1* cDNA EU073125, *vtg2* cDNA: EU073128 [54], *A. japonica β-actin* partial cDNA: AB074846.

2.7. Ovulation and fertilization assays

In order to ensure that final maturation and ovulation can be achieved with a variable thermal regime, CPE treatment was continued with a group of six females from T10. Ripe females were observed between the 16^{th} to 20^{th} weeks of treatment. Ovulation was induced by injecting 17α-20β-dihydroxy-4-pregnen-3-one (DHP, Sigma Chemicals) at a dose of 2 µg/g body weight [51]. DHP was diluted in 96% ethanol (4 mg/ml) and each dose diluted 1:1 with NaCl 0.9% (v/v) before abdominal injection. After stripping, females were sampled as described in section 2.3.

Samples of eggs were fertilized with fresh sperm obtained from hCG treated males [57] and incubated in Petri dishes containing artificial seawater at 20ºC, in the dark.

2.8. Statistics

Each variable was first analysed to check normality by the asymmetry standard coefficient and Curtosis coefficient. Variables that did not have a normal distribution were log-transformed and

their normality was checked again. Data from T20 and T10 (excluding post-ovulation results) were first analysed by a two-way ANOVA (temperature and development stage) followed by one-way ANOVA to evaluate differences within each treatment (T10 or T20) over time. Ovulation results were compared only with results from T10 group. Comparison of means between treatments or times was done using a Newman-Kewls multiple comparison test. Variance homogeneity was checked with the Bartlett test. P values <0.05 were considered to indicate significant differences. All statistical procedures were performed using Statgraphics Plus® 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

3. Results

3.1. *Gonadosomatic index*

Figure 2 shows the evolution of the gonadosomatic index throughout the experimental period. In both groups a progressive increase was observed ($p < 0.001$). At week 8 the GSI in T10 group was significantly higher than in T20 ($p < 0.001$).

3.2. Histology

The ovarian development (Figure 3) observed throughout the 12 weeks of treatments was classified in 5 stages:

- Previtellogenic (PV): in perinucleolar stage with none or few lipid droplets, or in lipid droplet stage but without yolk vesicles (Fig 3A, B).
- Early vitellogenic (EV): small yolk vesicles restricted to the periphery of the oocyte (Fig 3C).
- Mid-vitellogenic: characterised by abundant yolk vesicles, which were clearly visible and distributed in the cytoplasm from the membrane to the nucleus. Lipid droplets more abundant than yolk vesicles (Fig. 3D, E).
- Late Vitellogenic: yolk vesicles enlarged and more abundant than in the previous stage (Fig 3F).

3.3. Development stage and ovulation

The percentage of each development stage at each week of treatment can be seen in Figure 4. Before the hormonal treatment all the females were in previtellogenic stage (PV). After 4 CPE injections 100% of T10 females were in early vitellogenesis (EV), whereas in T20 20% were still in PV stage (Fig. 3B). Also, at week 8, 100% of T10 females were in mid-vitellogenesis (MV), but only 20% of T20 females were in that stage, while the rest were less developed, in EV stage. At week 12, 100% of T10 females and 66.6% of T20 females were still in MV, while in T20 33.3% of them were in late vitellogenesis.

Six females from T10 were further treated with CPE and 5 of them ovulated after 16-20 CPE injections. One female spawned spontaneously in the tank, and the eggs were not collected for fertilization. Four eels responded to the DHP injection after between 9 and 12 h and the egg batches were fertilized with fresh sperm obtained from hCG treated males [57]. Female 1 oocytes did not show cell cleavage; Female 2 showed some oocytes which developed until morula stage, while in Females 3 and 4 some oocytes developed until blastula stage (Fig. 5).

3.4. Comparison between thermal treatments

In order to further analyse the effect of temperature regimes we compared biometric and hormonal parameters at the same developmental stage. The divisions that were carried out were validated by the fact that oocyte diameter increased steeply in both groups along the development (Fig 6A). Figure 6B shows the evolution of the hepatosomatic index (HSI) throughout the treatments. HSI was higher in early vitellogenic T10 females than in EV T20 females ($p<0.05$) and did not change with the ovarian development.

3.5. Pituitary fshβ and lhβ mRNA

Both at previtellogenic, early and mid-vitellogenic stages, T10 females showed higher *fshβ* mRNA levels than T20 females ($p<0.05$; Fig 7A). At PV stage, *fshβ* expression in T10 was 4.5 times higher than in T20, while at EV it was 2.8 times higher, and finally at MV it was 2.4 times

higher than in T20. In both groups, a steep increase in *fshβ* expression was noted between stages PV, EV and mid-vitellogenic.

Pituitary *lhβ* mRNA in T10 group at EV stage was 12 times higher than in T20 females ($p < 0.01$; Fig 7B). Pituitary *lhβ* profile was different between the temperature groups; in group T10 it started to increase at EV, while in T20 it increased later, at MV stage.

3.6. 17β-Estradiol and vitellogenin plasma levels

17β-Estradiol (E2) increased from PV to vitellogenic stages in both groups ($p < 0.01$, Fig. 8A). However, in T10 group E2 plasma levels at EV and MV were higher (3.3 and 3.5 times, respectively) than in T20 females ($p < 0.05$).

No differences in vitellogenin plasma levels were observed between experimental groups at any development stage (Fig. 8B). In both groups Vtg increased steeply from previtellogenic to vitellogenic stages. Vitellogenin was in average around 40900 times higher in EV females than in PV females (32767 and 49372 times in T10 and T20, respectively; $p<0.01$). A new increase in Vtg was observed between EV and MV stages, the latter showing levels 5 times higher than those of EV stage ($p < 0.01$).

3.7. Liver expression of estrogen receptor 1 (esr1) and vitellogenin 2 (vtg2)

At EV stage liver *esr1* was 4 times more expressed in T10 than in T20 females (Fig. 9A). Profiles from PV to MV were different in both groups; in T20 there were no significant changes during the oocyte growth, while in T10 group *esr1* mRNA peaked at EV stage ($p<0.05$) and decreased at MV stage.

Liver expression of *vitellogenin2* gene [47, 54] in T10 females was higher (x 13.6) than in T20 females ($P<0.001$), at the EV stage. Liver expression profiles were similar between groups; an increase in *vtg2* expression was observed from PV to vitellogenic stages.

The temperature regime affected HSI, *fshβ* mRNA, *lhβ* mRNA, E2 plasma levels, and liver *esr1* mRNA, all of which were higher in T10 group (Table 2). Table 2 shows the significance of the effects of temperature and the development stage on all the parameters studied by two-way

ANOVA. Pituitary *lhβ* mRNA, E2, and *vtg2* mRNA levels were also affected by the ovarian development. Vitellogenin plasma levels were not affected by temperature, but changed with the development stage.

3.8. Comparison between post-ovulation and vitellogenesis

Table 3 shows the comparison of the endocrine parameters between vitellogenic and postovulatory T10 females. It can be seen that *fshβ* expression, E2 and *esr1* mRNA were lower at postovulation than at vitellogenesis, while expression of *lhβ* and *vtg2*, as well as the Vtg plasma levels were higher at postovulation than at vitellogenesis.

4. Discussion

The results of this work indicate that the thermal regime affected the ovarian development velocity, as well as important reproductive parameters. *fshβ* and *lhβ* pituitary mRNA, plasma E2, HSI, and *estrogen receptor 1* liver expression reached higher levels when a variable thermal regime or low temperatures were applied to previtellogenic or early vitellogenic European eels. Our results are consistent, as differences between thermal treatments have been observed even in the same stage of ovarian development.

A faster sex development was observed with the T10 regime from 4 to 8th week of hormonal treatments. The temperature in T10 during the period of weeks 4 to 8 was between 14-15ºC, and females developed from early to mid vitellogenesis. However, from weeks 8 to 12, oocyte growth in T20 accelerated, while in T10 it increased at a slower rate. Our hypothesis is that lower temperatures promoted the first vitellogenic steps, while higher temperatures, when applied to EV or more developed ovaries (GSI>4; oocytes > 270-280 µm) caused an acceleration in the last vitellogenic stages of development. This seems to coincide with our results from a new experiment (not published yet) where we have observed the same sequence: an initial fast ovarian development both at 15 or 18 °C until EV-MV stage (GSI 4.2-4.7), and a sudden increase later (GSI, vitellogenin) at the highest temperature.

306 Pituitary *fshβ* expression was higher in T10 than in T20 females, both in previtellogenic, early
307 and mid-vitellogenic females. Thermal regime affected also pituitary *lhβ* expression in EV
308 females, being higher in T10 than in T20 females.
309 The profiles of variation of *fshβ* mRNA throughout the treatments were different to previous
310 studies. In Japanese and European eel, *fshβ* gene expression decreased after treatments with fish
311 pituitary [32, 68, 70], while high *lhβ* expression was observed from vitellogenesis to maturation.
312 However, in this study an increase was noted in the expression of *fshβ* gene during the
313 development from previtellogenesis to mid-vitellogenesis, like in salmonids (reviewed by [38,
314 66]), or in naturally maturing anguillids such as New Zealand long-finned eels *Anguilla*
315 *dieffenbachii* [68], and Japanese conger *Conger myriaster* [34]. Also, in marble eel (*Anguilla*
316 *marmorata*) treated with CPE plus LHRHa and hCG, an increase in *fshβ* from PV to MV has
317 been observed [30].
318 It seems that a low thermal regime induces gonadotropic expression profiles which resemble
319 more to the natural profiles than those observed in eels at constant high temperature regime.
320 The results observed suggest that a higher endogenous FSH level is inducing a higher E2
321 production in T10 group, as E2 levels, as well as the *fshβ* expression were higher in T10 than in
322 T20. It still remains to be seen whether the *fshβ* expression actually reflects the FSH circulating
323 level. The E2 profile observed in T10 was similar to that observed in Japanese conger *Conger*
324 *myriaster* [34], and other naturally maturing fish, where plasma E2 levels increase during
325 vitellogenesis and decline during maturation [9]. In contrast, E2 levels were low during
326 vitellogenesis in Japanese eels treated with salmon pituitary extracts at 20ºC [31, 44, 72] and
327 they increased only at final maturation stages.
328 Although E2 negative feedbacks on *fshβ* in other fish species [19, 36, 43, 71] including
329 Japanese eel [32] have been observed, in this study we could not see any evidence supporting a
330 negative feedback of E2 on *fshβ* expression, as the highest E2 levels (in T10 at mid-vitellogenic
331 stage) coincided with the highest *fshβ* mRNA levels. In previous works E2 treatment *in vivo* did
332 not decrease *fshβ* expression in the European eel [2, 70], and even E2 had a stimulatory effect
333 on *fshβ* expression in pituitary cultures of immature female eels [2].

The lower E2 levels observed in T20 could be related to a lower activity of ovarian P450 aromatase enzyme, which converts testosterone in E2, as it was observed that high temperatures reduced its activity and expression [18, 29, 62]. Constant high temperatures during vitellogenesis reduced E2 plasma levels in striped bass [16], and Atlantic salmon [52], and suppressed aromatase mRNA in adult red sea bream *Pagrus major* [39]. The lower temperatures experienced by T10 group could allow for a higher expression and/or activity of ovarian P450 aromatase as such a higher E2 production in response to FSH signalling.

Vitellogenesis includes the binding of E2 to their nuclear receptors in the hepatocytes. The estrogen receptors in fish liver are Esr1 (or ERα) and Esr2 (or ERβ). Estradiol-17β stimulates the *Esr1* expression in the liver of tilapia, zebrafish, medaka, or rainbow trout [13, 17, 45, 83], and is considered the main inducer of vitellogenin synthesis. In this study hepatic *esr1* expression was higher in T10 group (at EV stages). This could be related to the higher *vtg2* expression and HSI in T10 females at EV stage. To summarise, it seems that the higher E2 in T10 induced a higher *esr1* liver expression that in turn induced a higher *vtg2* mRNA production in the liver at EV stage.

In this study, no differences in plasma vitellogenin were observed between the temperature groups. The Vtg plasma levels reflect the balance between liver production/release and the Vtg uptake by the oocyte. One explanation for the discrepancy between E2, *esr1* mRNA, *vtg2* mRNA and Vtg plasma levels could be that the liver Vtg production was higher in T10 but also was its uptake by oocytes, leading to similar plasma Vtg levels as in T20 group. However, if this was the case oocyte diameter should have been higher in T10. In fact, this was not observed. Although *vtg2* mRNA was higher in T10, the vitellogenin plasma level was not higher in this group. We have not analyzed *vtg1* mRNA, but if its profile was similar to the *vtg2* mRNA, we could hypothesize the following regarding T10 group: 1) *vtg* mRNA has not been completely translated to vitellogenin or 2) vitellogenin has been synthesized, but not completely released to blood plasma. These hypotheses agree with a previous report indicating that Vtg gene translation increased with the water temperature in rainbow trout [40]. The hypothesis of an accumulation of E2 related products is supported by the observed Vtg plasma protein/mRNA

362 *Vtg2* ratio, which was 88 vs 19 in T20 and T10, respectively. The high Vtg level observed at
363 post-ovulation could be due to an accumulation in blood plasma (with the high *vtg2* mRNA at
364 post-ovulation suggesting high synthesis) because of the end of their uptake by the oocytes, as
365 demonstrated in other species such as European sea bass [3].
366 The gonadotropin profiles observed in this work agree in general with the model present in
367 salmonids and other fish species (reviewed by [38, 66]), where blood FSH (or high levels of
368 *fshβ* mRNA) predominated in vitellogenic stages, while circulating levels of LH (or *lhβ* mRNA)
369 predominates later. However, we do not have evidences of a second FSH increase at ovulation,
370 which was observed in rainbow trout [14, 63], but not in coho salmon [67]. Taken together, our
371 results suggest that FSH should have an important role on the vitellogenesis of European eel,
372 while LH should be more important in the advanced stages of vitellogenesis or ovulation.
373 A higher mortality in T20 group was observed throughout the treatments. Although all the eels
374 were healthy when the experiment started, it is possible that they carried some bacteria from the
375 lake were they were caught, where *Vibrio vulnificus*, *Edwardsiella tarda* and *Aeromonas* spp
376 [24] are relatively common. The higher mortality in T20 could be related to the higher
377 temperatures, as the development and pathogenity of those bacteria increase with higher
378 temperatures [42, 84].
379 We have shown that it is possible to obtain eggs and some embryonic development in female
380 eels treated with CPE under a variable thermal regime. The effect of thermal regimes on egg
381 quality should be tested, as no comparison between experimental treatments has been carried
382 out.
383 It has been demonstrated that thermoperiod affects vitellogenesis in European eel through
384 changes in gonadotropins expression, estradiol receptor expression and E2 levels. It seems that a
385 step-wise temperature increase during maturation with pituitary hormones induced an endocrine
386 profile (*fshβ* expression and E2) in eel females which resembles that observed in naturally
387 maturing fish. If a higher gamete quality could be obtained from these apparently more natural
388 endocrine profiles, is a question that should be addressed in the future.
389

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Table 1. Primer sequences used in the RT-PCR assays.

Gene	Primer 5’	Primer 3’	Amplicon size
lhβ	TCACCTCCTTGTTTCTGCTG	TAG CTT GGG TCC TTG GTG ATG	149 bp
fshβ	TCTCGCCAACATCTCCAT C	AGAATCCTGGAAGCACA	100 bp
ARP	GTG CCA GCT CAG AAC ACT G	ACATCGCTCAAGACTTCAATGG	107 bp
esr1	GTCGAGGACAAAGCCATCAT	CCGATCATCAGCACCTCCAG	318 bp
vtg2	CGAGGATGCTCCCCTAAAGT	CCCCCTCAGCTGTGGTAATA	220 bp
βactin	CTCCCTGGAGAAGAGCTACG	GGAGTTGAAGGTGGTCTCGT	153 bp

Table 2. Two-way ANOVA analysing the effect of temperature treatment and weeks of treatment on biometric parameters, pituitary *fshβ* and *lhβ* expression, 17-βestradiol, vitellogenin plasma levels, liver estradiol receptor 1 mRNA (*esr1*), vitellogenin 2 mRNA (*vtg2*). n.s. indicates non significant differences.

	Temperature	Stage	Interaction
GSI (%)	n.s.	P=0.0000	n.s.
HSI (%)	P=0.0015	n.s.	n.s.
Oocyte diameter (µm)	n.s.	P=0.0000	n.s.
fshβ mRNA (a.u)	P=0.027	n.s.	n.s.
lhβ mRNA (a.u)	P= 0.0031	P= 0.0051	n.s.
Vitellogenin (µg/ml)	n.s.	P=0.0000	n.s.
E2 (pg/ml)	P=0.037	P=0.0094	n.s.
esr1 mRNA (ng mRNA)	P=0.0048	n.s.	n.s.
vtg2 mRNA (ng mRNA)	n.s.	P=0.0000	0.0039

Table 3. Comparison between postovulation and vitellogenic endocrine parameters in T10 group (mean ± SEM; vitellogenesis n=15; post-ovulation n=5)

	Vitellogenesis	Postovulation	P-value
fshβ mRNA (a.u)	2.758 ± 0.696 b	0.077 ± 1.064 a	0.0001
lhβ mRNA (a.u)	0.865 ± 0.328 a	2.588 ± 0.500 b	0.0057
E2 (pg/ml)	2961.8 ± 512.4 b	849.9 ±782.6 a	0.0001
Vitellogenin (µg/ml)	23473 ± 5114 a	118527 ± 7811 b	0.0000
Esr1 mRNA (ng mRNA)	$2.23x10^{-11}$ ± $1.79x10^{-11}$ b	$1.85x10^{-10}$ ±$2.83x10^{-11}$ a	0.0001
vtg2 mRNA (ng mRNA)	0.045 ± 0.17 a	1.54 ± 0.27 b	0.0002

719 **Figure captions**

720

721 Figure 1. Water temperature treatments during treatment with carp pituitary extract (CPE) in
722 female silver eels. Arrows indicate the sampling times at weeks 0, 4^{th}, 8^{th} and 12^{th}. Line
723 indicates the period when ovulations were obtained in T10 group.

724

725 Figure 2. Evolution of gonadosomatic index (GSI) along the treatments. Means are given ±
726 SEM (n= 5 eels/group). Asterisks show significant differences between T10 and T20. Capital
727 letters show significant differences within T10 group. Small letters show significant differences
728 within T20 group.

729

730 Figure 3. Histological sections of oocytes at different times during hormonal and temperature
731 treatments. A) Previtellogenic oocytes at lipid droplet stage, week 0. B) Lipid droplet stage after
732 4 weeks of CPE treatment. C) Mid-vitellogenic oocyte, week 8, T10 group. D) Early
733 vitellogenic oocyte; week 8, T20 group. E) Mid-vitellogenic oocyte, week 12, T10 treatment. F)
734 Late-vitellogenic oocyte, week 12, T20 treatment. Scale bar: A, B, E, F: 100 µm; C, D: 100 µm.

735

736 Figure 4. Percentage of the different stages of ovarian development at 0, 4^{th}, 8^{th} and 12^{th} weeks
737 of CPE treatment (n=10/week) in each temperature treatment. PV= previtellogenic stage; EV=
738 early vitellogenic stage; MV= mid-vitellogenic stage; LV= late vitellogenic stage.

739

740 Figure 5. Blastula stages observed after fertilization. (A) from Female 3, 24 h after fertilization;
741 (B) from Female 4, 33 h after fertilization. Scale bar: 200 µm.

742

743 Figure 6. Effect of thermal treatment on the hepatosomatic index (HSI) and oocyte diameter
744 along the ovarian development. Capital letters show significant differences within T10 group.
745 Small letters show significant differences within T20 group. Asterisks show significant
746 differences between T10 and T20 in each stage (*$p<0.05$, **$p< 0.01$ANOVA). Means are given

± SEM. PV= previtellogenic stage (n=11); EV= early vitellogenic stage (n=12); MV= mid-vitellogenic stage (n=16).

Figure 7. Effect of thermal treatment on pituitary gonadotropin expression (*fshβ* and *lhβ* subunits) along the ovarian development. Data are normalised to eel ARP. Asterisks show significant differences between T10 and T20 in each stage (*p<0.05, **p< 0.01ANOVA). Means are given ± SEM. PV= previtellogenic stage (n=11); EV= early vitellogenic stage (n=12); MV= mid-vitellogenic stage (n=16).

Figure 8. Effect of thermal treatment on blood plasma 17-β-estradiol (E2), and vitellogenin (Vtg) along the ovarian development. Asterisks show significant differences between T10 and T20 in each stage (*p<0.05, **p< 0.01ANOVA). Means are given ± SEM. PV= previtellogenic stage (n=11); EV= early vitellogenic stage (n=12); MV= mid-vitellogenic stage (n=16).

Figure 9. Effect of thermal treatment on liver *estrogen receptor 1* expression (*esr1*) mRNA and *vitellogenin 2* mRNA (*vtg2*) along the ovarian development. Asterisks show significant differences between T10 and T20 in each stage (*p<0.05, **p< 0.01ANOVA). Means are given ± SEM. PV= previtellogenic stage (n=11); EV= early vitellogenic stage (n=12); MV= mid-vitellogenic stage (n=16).

Figure 1

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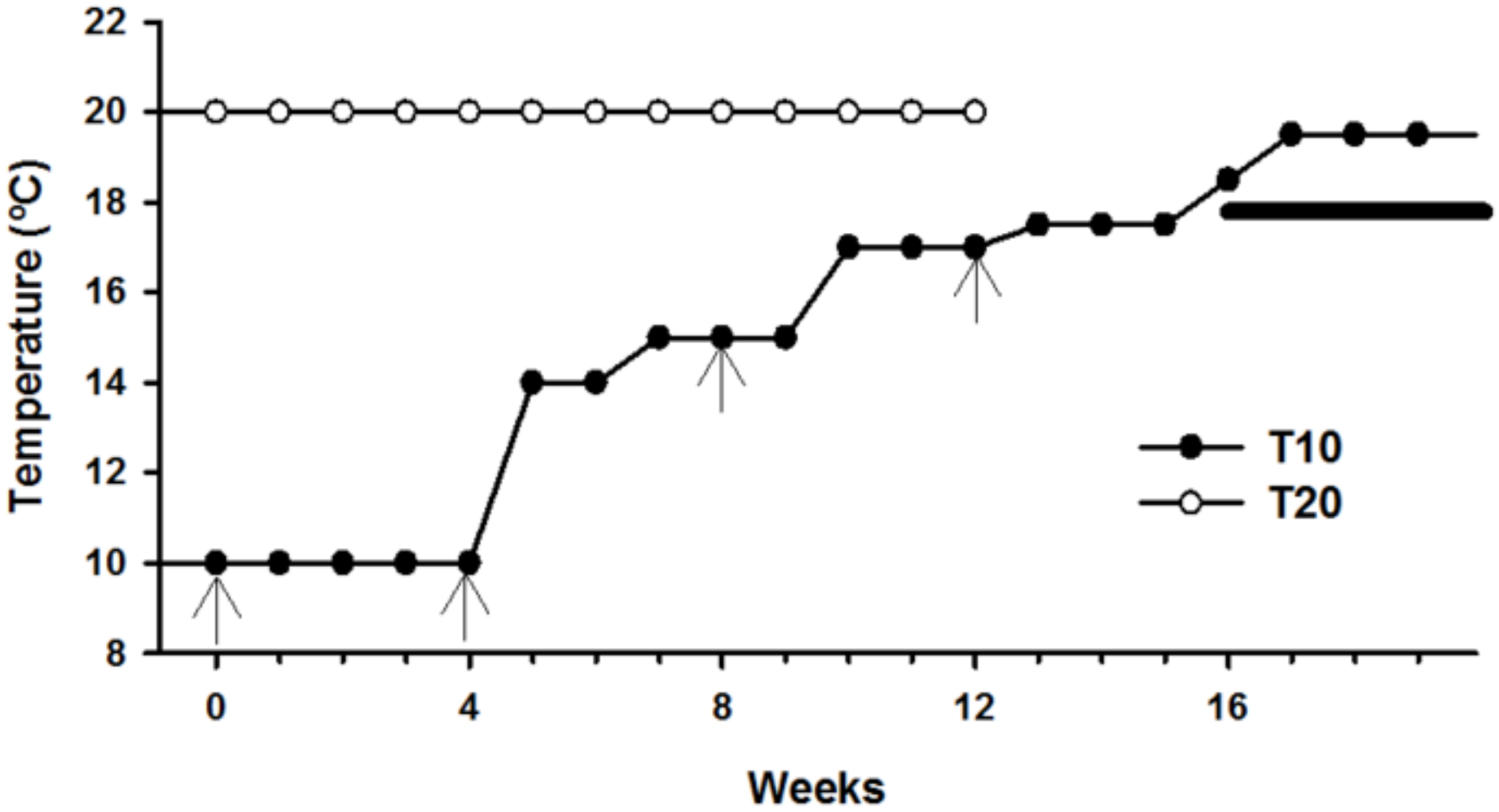

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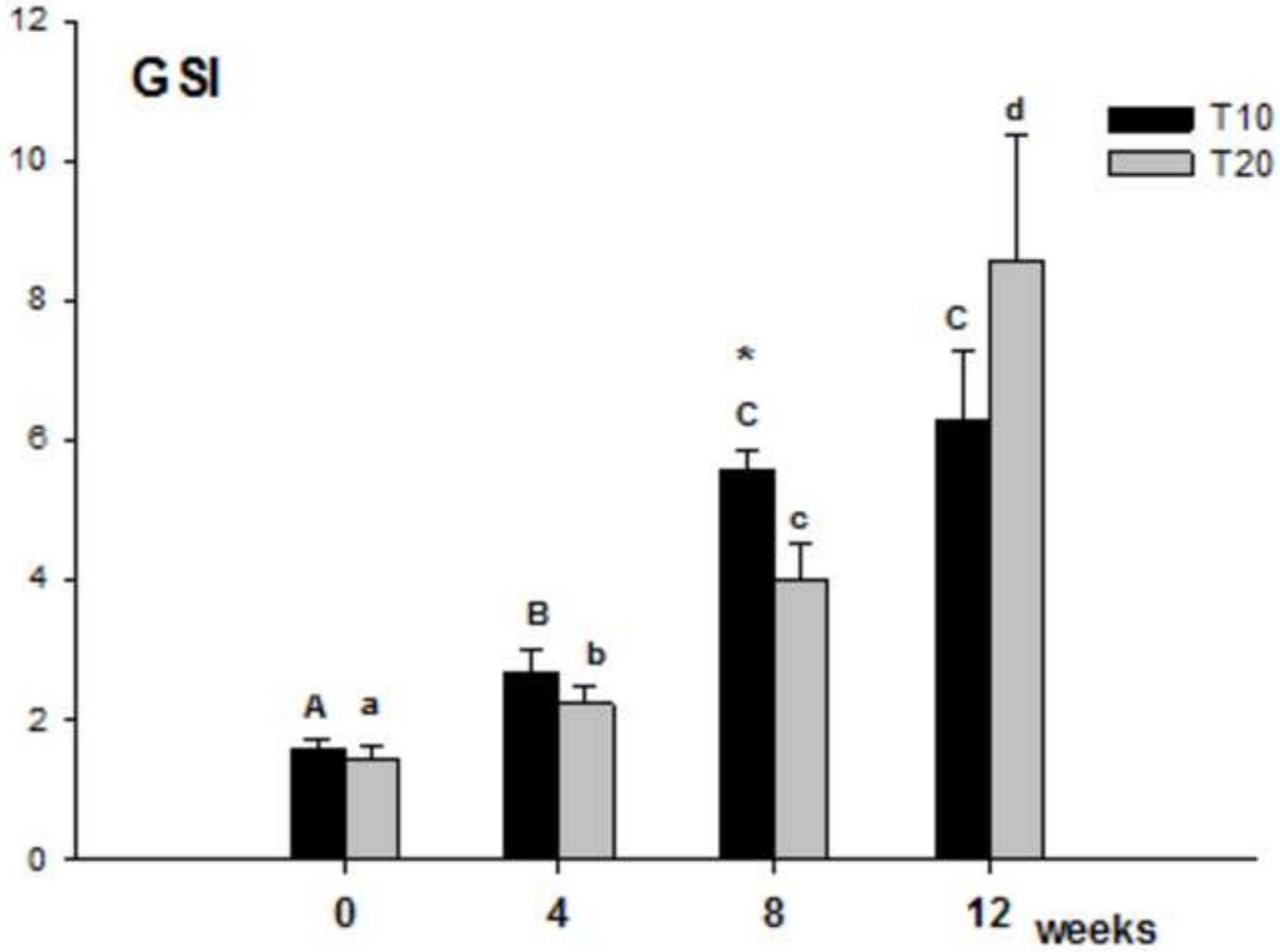

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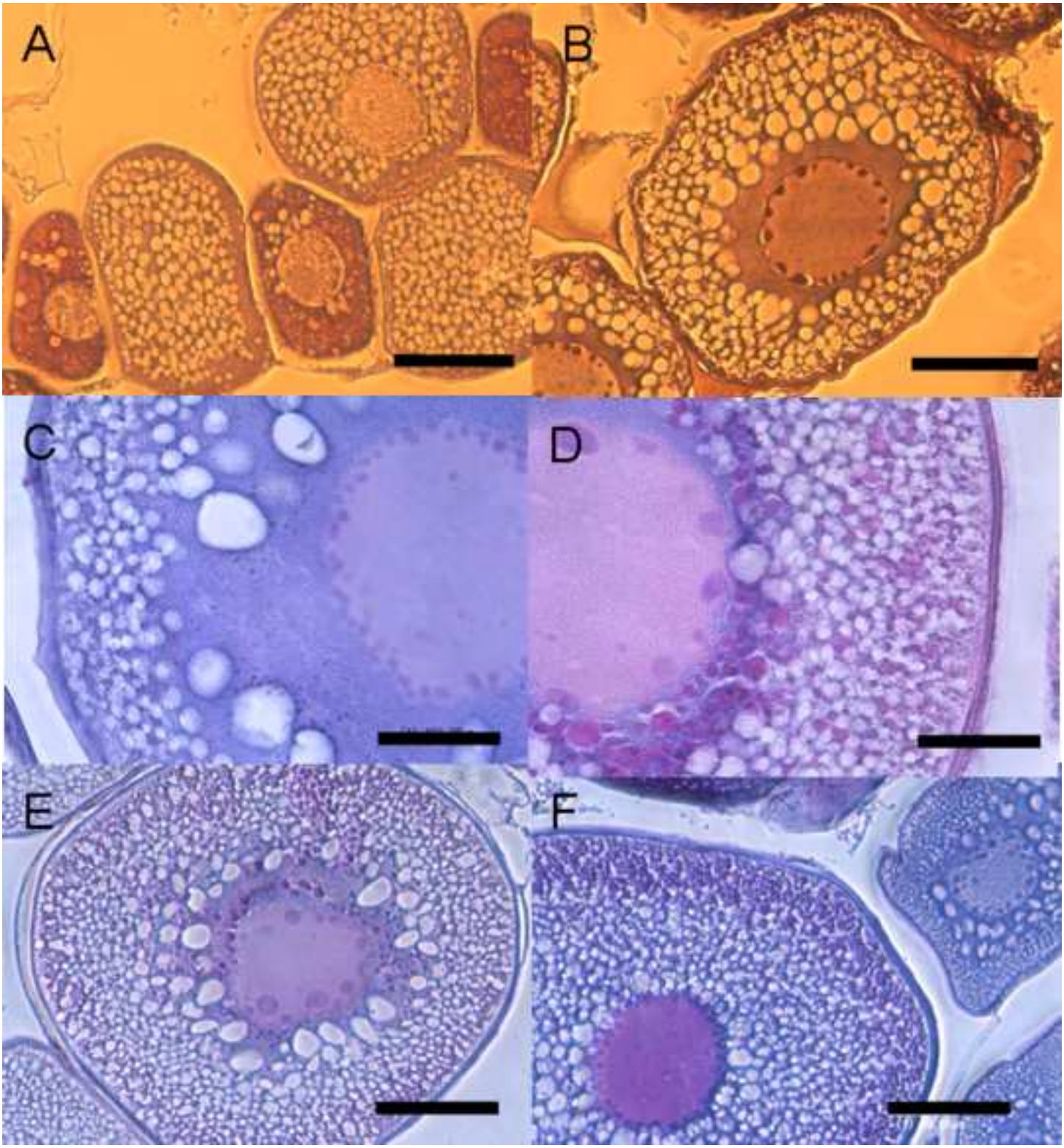

Figure 4

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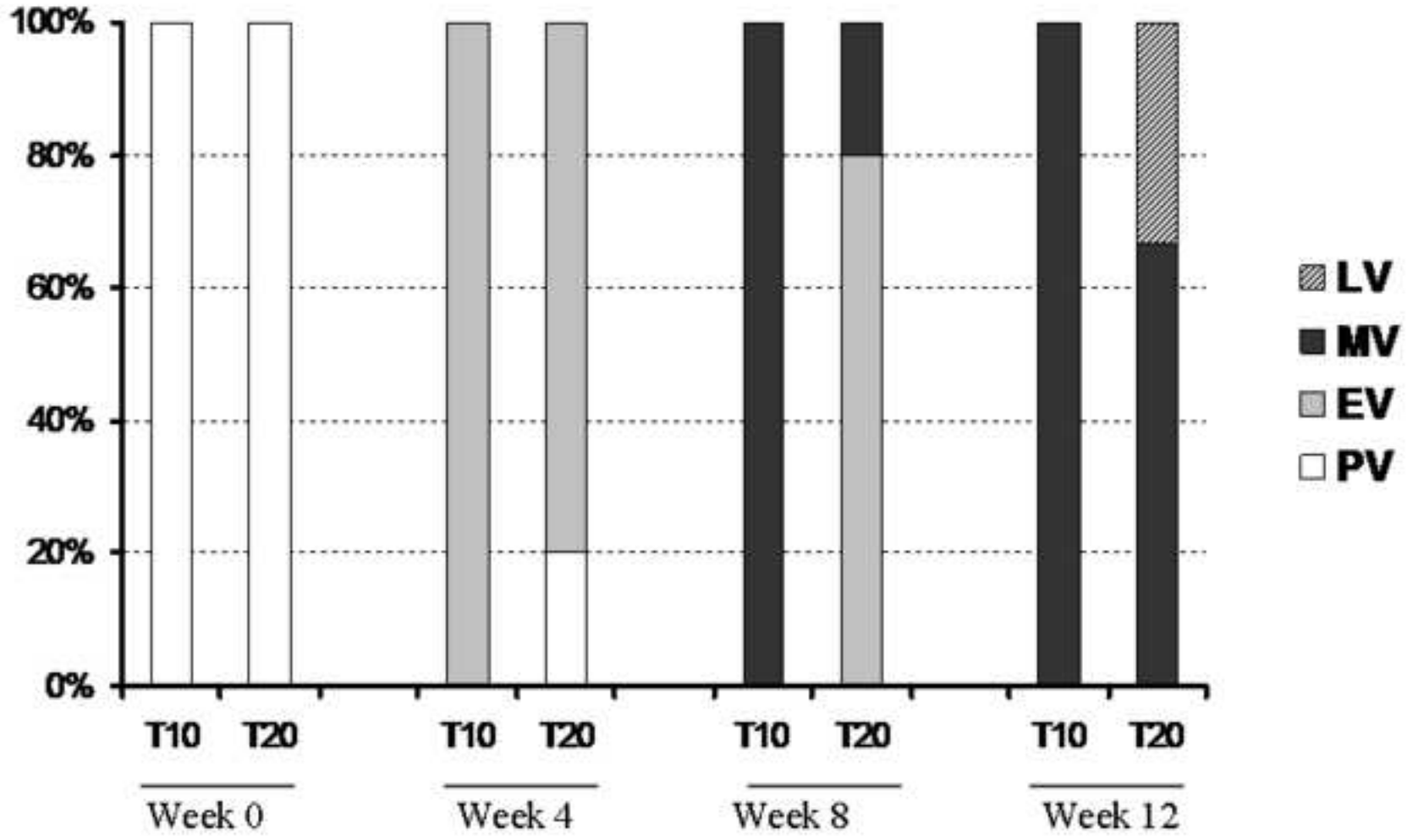

Figure 5

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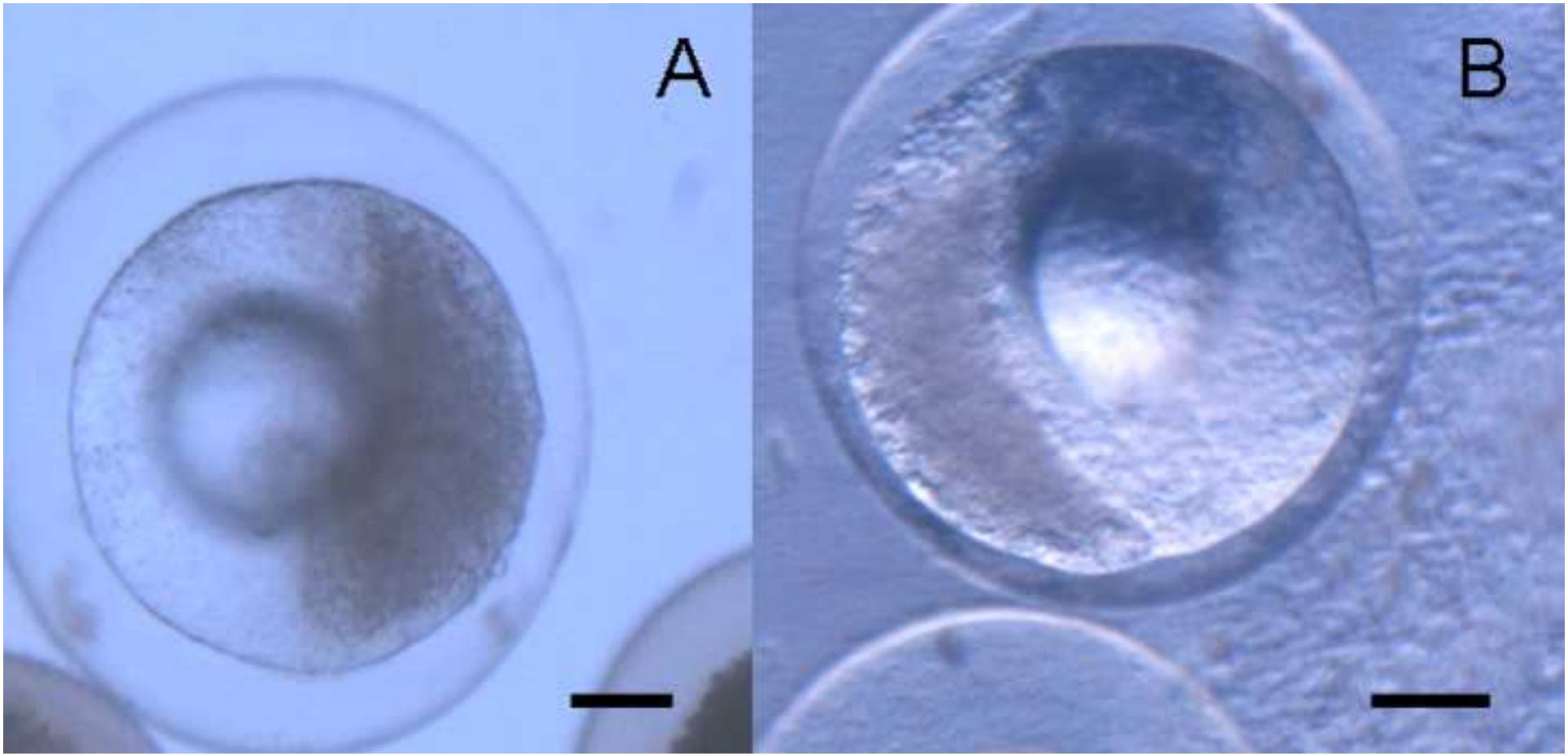

Figure 6

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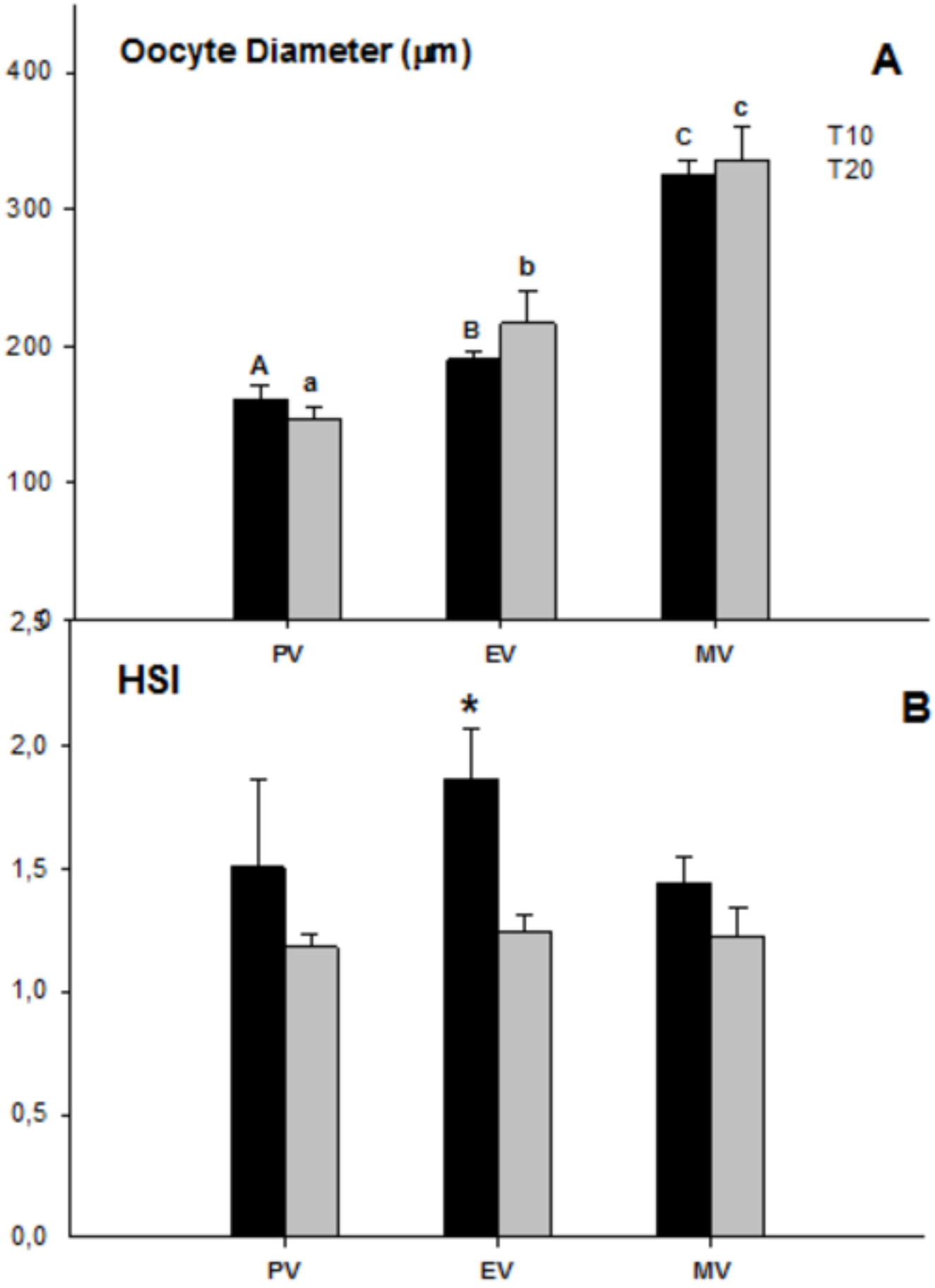

Figure 7

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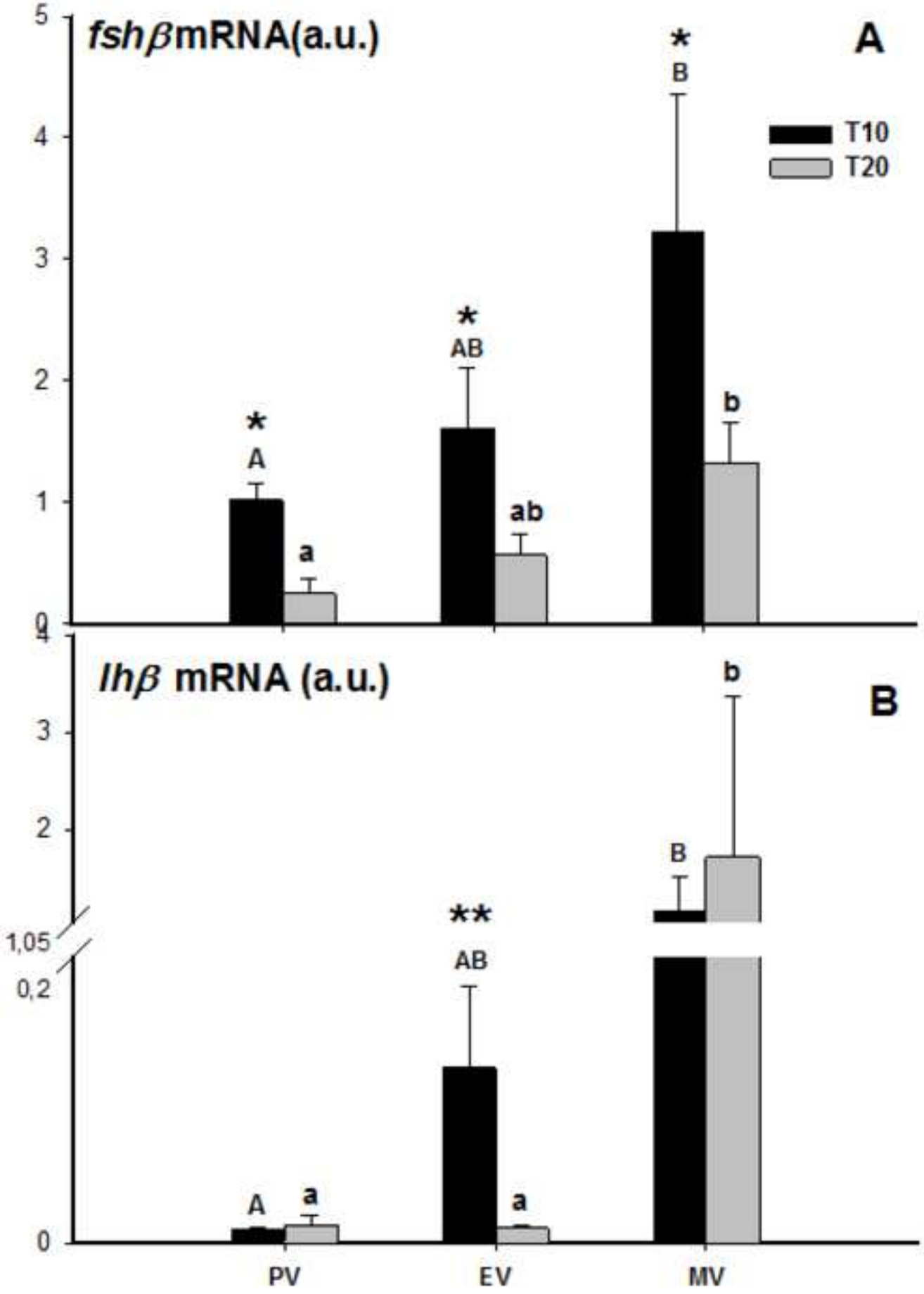

Figure 8

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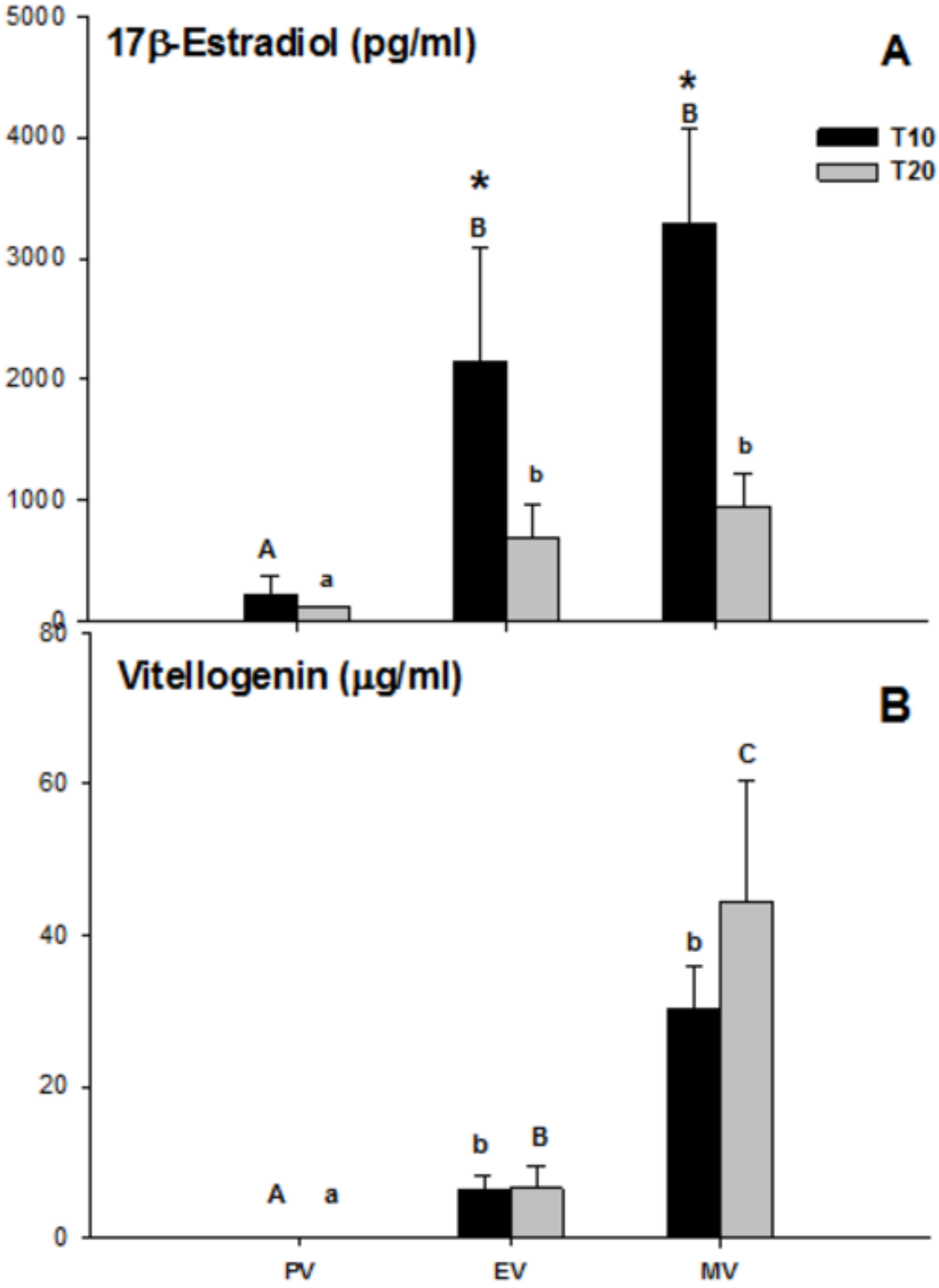

Figure 9

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