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Differential expression of gonadotropin and estrogen receptors and oocyte cytology during follicular maturation associated with egg viability in European eel (*Anguilla anguilla*)

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Abstract

In captivity, oogenesis and ovarian follicle maturation in European eel can be induced experimentally using hormonal therapy. The follicle's ability to respond effectively to the induction of maturation and ovulation, resulting in viable eggs, depends on the oocyte stage at the time of induction. We hypothesized that variation in the expression of key hormone receptors in the ovary and size of oocyte lipid droplets are associated with changes in oocyte stage. Thus, we induced ovarian follicle maturation using a priming dose of fish pituitary extract followed by an administration of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP). Females were then strip-spawned, the eggs were fertilized *in vitro*, incubated and larval survival was recorded at 3 days post hatch (dph). The expression of gonadotropin receptors (*fshr*, *lhcgr1* and *lhcgr2*) and estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gpera* and *gperb*) was quantified and the size of oocyte lipid droplets measured. Larval survival at 3 dph was used to differentiate high- and low-quality egg batches. Results showed significantly higher abundance of *lhcgr1* and *esr2a* at priming for high-quality egg batches whereas *fshr* and *gperb* transcripts were significantly higher at DHP injection for low-quality egg batches. Therefore, high levels of *lhcgr1* and *esr2a* may be important for attaining follicular maturational competence, while high *fshr* and *gperb* mRNA levels may indicate inadequate maturational competence. Furthermore, lipid droplet size at DHP and in ovulated eggs was significantly smaller in high-quality egg batches than in low-quality, which indicates that droplet size may be a useful marker of follicular maturational stage.

Key words: estrogen receptors, gonadotropin receptors, lipid droplet, follicular maturation, egg quality

1. Introduction

The oceanic, reproductive stages of European eel (*Anguilla anguilla*) remain undiscovered and sexual maturation neither occurs naturally in their continental habitats nor in captivity. This arrested development results from a strong dopaminergic inhibition (Dufour et al., 1988; Vidal et al., 2004) and a deficient pituitary gonadotropic function, i.e. both gonadotropin synthesis and release are low (Dufour et al., 1983). Experimentally, gonadal development can be induced using hormonal treatments based on fish pituitary extracts in females and human chorionic gonadotropin in males (Fontaine et al. 1964; Yamamoto and Yamauchi, 1974; Dufour et al., 1989; Ohta et al., 1996; Pedersen, 2003). In females, such treatment leads to oocyte growth until maturation. Induction of follicular maturation and ovulation generally requires an additional dose of pituitary extract and an injection of a maturation-inducing steroid (MIS) (Yamauchi, 1990; Ohta et al., 1996; Pedersen, 2003) such as 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) in the case of the eel. Recent advances in eel assisted reproductive technology have enabled the production of viable eggs and yolk-sac larvae (Butts et al., 2016; Sørensen et al., 2016). However, resulting egg quality is variable and low fertilization rate and poor larval survival are often observed. Variation in egg quality is partly related to the timing induction of ovarian follicle maturation and ovulation, as previous studies have shown that hormonal treatment given too early or too late in the reproductive cycle can be ineffective or inefficient (Palstra et al. 2005; Mylonas et al., 2010; Unuma et al., 2011). Because hormones need to bind to receptors to exert their biological function, differences in responsiveness to hormonal treatment could be due to differences in hormone receptor expression.

In teleost fishes, as in other vertebrates, the two pituitary gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play major roles in the regulation of oogenesis and

production of sex steroids (Pierce and Parsons, 1981). Gonadotropic function is mediated by specific membrane receptors, the FSH receptor (FSHR) and the LH receptor (LHCGR), which corresponds to the human LHCGR. These are mainly expressed in the somatic cells of the gonads (Rosenfeld et al., 2007), though they can also be expressed in germ cells (Chauvigné et al., 2014). Until recently, it was accepted that teleosts had a single FSHR and a single LHCGR encoded by *fshr* and *lhcgr* genes, respectively. However, in addition to *fshr*, Maugars and Dufour (2015) identified and characterized two *lhcgr* genes (*lhcgr1* and *lhcgr2*) in some fish species, including the European eel. European eel *lhcgr1* corresponds to the LHCGR previously characterized in the Japanese eel (*Anguilla japonica*) (Kazeto et al, 2012). In comparison to the many studies on FSH and LH, knowledge about their receptors in teleost species is still limited.

Sex steroids, such as estrogens, are also well-known for their role in reproductive function. In female fish, estrogens (primarily estradiol-17β, E2) are involved in the regulation of oogenesis and vitellogenesis (Wallace, 1985) as well as in the feedback control of gonadotropin expression and release (Zohar et al., 2010). Estrogens can diffuse through the cell membrane and bind to nuclear estrogen receptors (ESRs). Two distinct subtypes of nuclear ESRs, *esr1* and *esr2*, have been cloned from several mammalian and non-mammalian vertebrates (Mosselman et al. 1996; Hawkins et al., 2000). In teleosts, including the European eel, two paralogs of *esr2* have been reported, *esr2a* (also named *erβ2*) and *esr2b* (also named *erβ1*) (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002; Nagler et al., 2007; Lafont et al., 2016). In addition, estrogens can also activate receptors on the cell surface, initiating rapid and often non-genomic biological responses (Watson and Gametchu, 1999; Falkenstein et al., 2000; Norman et al., 2004). The human G-protein coupled receptor 30 (GPER30 or GPER) was shown to have the binding characteristics of an E2 membrane receptor (Revankar et al.,

2005; Thomas et al., 2005). Subsequent studies in zebrafish, Atlantic croaker (*Micropogonias undulatus*) and common carp (*Cyprinus caprio*) have also shown that estrogens produced by follicle cells inhibit or delay spontaneous follicular maturation via the activation of membrane receptors (Pang et al., 2008; Pang and Thomas, 2009; Peyton and Thomas, 2011; Majumder et al., 2015). In the European eel and some other teleosts, two paralogous *gper* genes (*gpera* and *gperb*) have been recently identified (Lafont et al. 2016) but their roles have not yet been investigated.

During follicular maturation, cytological changes in the oocytes take place. This includes migration of the germinal vesicle towards the oocyte periphery followed by breakdown of the nuclear envelope, and meiotic resumption (Lubzens et al., 2017). In many teleosts, including the eel, another feature of oocyte cytoplasmic maturation is the coalescence of lipid droplets to form one or a few large oil globules (Kagawa, 2013). These cytological changes have been used as biomarkers for assessment of oocyte maturational status in relation to assisted reproduction of eel (Palstra et al., 2005; Unuma et al., 2011).

The main objective of this study was to assess the expression of gonadotropin receptors (*fshr*, *lhcgr1* and *lhcgr2*) and estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gpera and gperb*) during induced maturation and ovulation and investigate their relation with subsequent egg quality, ultimately estimated as fertilization rate, hatching success and early larval survival. Finally, concomitant changes in oocyte lipid droplet size were evaluated as potential cytological biomarker of follicular maturational status.

2 Materials and Methods

2.1 Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental breeding protocols were approved by the

Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2010/561-1783). All efforts were made to minimize animal handling and stress.

2.2 Experimental animals and rearing conditions

Female silver eels (n=10; mean length and weight ± SD were 72 ± 11 cm and 781 ± 393 g, respectively) were caught from a freshwater lake (Vandet Sø) in northern Jutland (Denmark) and transported to a research facility of the Technical University of Denmark located at Lyksvad Fish Farm (Vamdrup, Denmark). Eels were randomly distributed into duplicate 300 L tanks equipped with a recirculation system and gradually acclimated to artificial saltwater over a period of two weeks, i.e. freshwater adjusted to 36 ppt salinity using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany). Thirty male eels (body weight 106 ± 13 g; body length 38 ± 2 cm) reared on DAN-EX 2848 (BioMar A/S, Brande, Denmark) were obtained at a commercial eel farm (Stensgård Eel Farm A/S, Randbøl, Denmark), transported to Lyksvad Fish Farm and kept in separate tanks under the same conditions as the female eels. At the onset of hormonal treatment, the eels were anaesthetized individually in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg/L, Sigma-Aldrich, Germany), tagged with a passive integrated transponder (pit-tag) in the abdominal muscle, and body weight and length were measured**.** Throughout the experiment, all fish were maintained at ~36 ppt salinity, ~20 °C, and a natural local daily photoperiod. No feed was provided during experiments since eels in the migratory, silvering stage cease feeding (Lokman et al., 2003).

2.3 Induction of gametogenesis and sampling

Females received weekly intramuscular injection of salmon pituitary extract (SPE) at a constant dosage of 18.75 mg/kg initial body weight to induce and sustain follicular development and vitellogenesis (Kagawa et al., 2005; Tomkiewicz et al., 2012). The first injection was given concurrent with pit-

tagging and regular treatment lasted 16-20 weeks depending on the responsiveness of the females. Pituitary extract was prepared using freeze-dried salmon pituitaries (Argent Chemical Laboratories, Washington, USA) that were grinded, diluted in NaCl 0.9 g/L and centrifuged according to Ohta et al. (1996, 1997). Supernatants were stored at -20 °C until use. Females were weighed at the weekly injections to follow changes in body weight.

Individual treatment for follicular maturation and ovulation was initiated at first signs of the onset of oocyte hydration, i.e. a body weight increase of 10 – 15 % compared to the initial weight and a soft abdomen (Pedersen, 2003, 2004). To assess oocyte developmental stage, each female was anaesthetized in an aqueous solution of benzocaine, and an ovarian biopsy (~0.2 ml) was obtained, using a sterile disposable injection needle (16G x 1 ½"). The biopsy was taken at a standard location on the left side of the body ~5 - 10 cm anterior to the genital pore, relative to female size, and the female thereafter transferred to a separate tank under the same conditions for individual care. The biopsy was inspected under the microscope and oocyte development graded on a scale from 1-7 according to Palstra et al. (2005). Progression of oocyte maturation varied in time and homogeneity, so each female was followed until the most developed oocytes exhibited characteristics close to stage 4, i.e., fully transparent oocyte with nucleus at periphery (Fig. 1). At this stage, an additional SPE injection as primer was given to females to sustain and boost follicular development (Pedersen, 2004; Kagawa et al., 2005). To complete follicular maturation and induce ovulation, the eel maturation inducing steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim, Germany) was given ~24 h later at a dose of 2 mg/kg present body weight (Ohta et al., 1996). Prior to DHP injection, a new biopsy (~0.2 ml) was obtained to evaluate the progression of oocyte development. DHP was injected into the ovarian tissue under anaesthesia (Palstra et al. 2005). Female eels ovulated

12 to 15 hours after DHP injection and at that time the eggs were stripped by applying gentle pressure along the abdomen of the fish. The volume of ovulated eggs collected was recorded.

Induction of spermatogenesis in the male eels started 4 weeks after the induction of oogenesis in females, as males only need 7-9 weeks of treatment to reach spermiation (Tomkiewicz et al. 2011). Each week, males received an intramuscular injection of human chorionic gonadotropin (hCG, Sigma Aldrich Denmark, A/S) at dose of 1.5 IU hCG per kg initial body weight (Butts et al., 2014). An additional hCG injection was given 12 h prior to milt collection in order to enhance sperm production.

2.4 Sampling for gene expression and cytological analysis

Sampling for each female comprised: 1) the ~0.2 ml ovarian biopsy obtained ~1h before the SPE priming injection (SPE); 2) the ~0.2 ml biopsy obtained prior to DHP injection (DHP) and 3) a sample of unfertilized eggs collected immediately after stripping (EGG). Each sample included digital images of oocytes/eggs to measure lipid droplet diameter and a preserved sample (~0.1 ml) for gene expression analysis. Prior to photography, the sampled oocytes/eggs were cleared in Serra's liquid (ethanol:formalin:acetic at 6:4:1, diluted 20X in phosphate buffered saline, PBS, see Stoeckel (2000)) for staining of the germinal vesicle. The pictures were taken at 20X magnification using a digital camera (Digital Sight DS-Fi1, Nikon Corporation, Japan) connected to an optical microscope (Eclipse 55i, Nikon Corporation, Japan) for measurement of lipid droplet diameter inside the oocytes. Samples taken for analysis of gene expression were preserved in RNA-later (Ambion Inc., Huntingdon, UK), refrigerated at 5 °C for 24 h and then frozen at -20 °C until RNA extraction.

2.5 Fertilization rate, hatching success and larval survival

In order to evaluate the egg viability, eggs were fertilized *in vitro*, and fertilization rate, hatching success, and larval survival was determined. For each female, milt from 4-5 males was collected, leaving three week intervals between individual male stripping. The spermatocrit was estimated (pooled milt mixture according to Sørensen et al. (2013) and used to dilute milt in an immobilizing diluent (Asturiano et al., 2004; Peñaranda et al., 2010). The diluted milt at a standardized concentration was used for fertilization within 4 hours after collection (Butts et al., 2014).

Immediately after stripping, eggs were mixed with the milt solution and added natural seawater for sperm activation (Butts et al. 2014). After 5 min gamete contact time, a sub-sample of eggs was transferred to a 100 ml graduated cylinder glass to estimate percent floating eggs. The remaining eggs were transferred to 10 L containers for separation of floating and sinking eggs. The eggs were kept at 20 °C and the fertilization rate and floating percent was accessed 3-5 hours post fertilization (HPF). To estimate fertilization success, a sample of 100-150 floating eggs was photographed using an optical microscope (Eclipse 55i, Nikon Corporation, Japan) at 20X magnification and a digital camera (Digital Sight DS-Fi1, Nikon Corporation, Japan). Fertilized eggs were identified by the presence of blastomere cleavage (minimum 4 cell stage), while those that had not reached the 4-cell stage were considered unfertilized.

For estimation of hatching success, eggs (~200 eggs in triplicate, for each female) were collected from the floating layer after 3-5 HPF and incubated at 20 °C in flasks (Nunc® 75 cm2 flasks, non-treated with ventilated caps, Thermo Scientific) (Sørensen et al. 2014). Each flask contained 250 ml of seawater (36 ppt) ampicillin (50 mg/L) and rifampicin (50 mg/L). Flasks stayed undisturbed inside a dark and closed incubator at 20 °C until the number of hatched larvae was counted at 55 HPF. To

estimate larval survival, ~300 g of eggs from the floating layer were incubated in a 60 L incubator with natural seawater at ~20 °C. After hatch, when available ~3000 larvae in triplicate were stocked in 40 L tanks of an aquaculture recirculation system containing natural seawater adjusted to 36 ppt salinity using Tropic Marin Sea Salt and reared at 20 °C. Survival was measured as the percentage of still living larvae at 3 days post hatch (dph).

For statistical analysis, egg batches were categorized into two groups: i) high-quality and ii) low-quality based on larval survival. Hereby, survival beyond the first 3 dph (high mortality period) was used to differentiate the groups (Table 2).

2.6 Gene expression analysis

RNA extraction and cDNA synthesis:

After Proteinase K treatment, total RNA was purified from ovarian biopsies and ovulated eggs by homogenization of 30 mg of tissue in 700 µl TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). TRIzol/chloroform separation was performed to remove DNA and proteins. The aqueous-phase was transferred to a new tube and 500 µl of isopropanol were added for RNA precipitation. Genomic DNA contamination was removed by treating the total RNA with the TURBO DNA-free kit (Ambion). Total RNA quality and concentration was measured spectrophotometrically using NanoDrop (Saveen Werner AB, Limhamn, Sweden).

RNA was then reverse transcribed into cDNA using Hight Capacity RNA-to-cDNA kit (AB) according to manufacturer's protocol. In summary, reaction volumes of 20 µl contained 450 ng total RNA, 1X RT buffer, 1X enzyme mix. The following cycling parameters were used: 37 °C for 60 min, 95 °C for 5 min and hold at 4 °C, using a 2720 Thermal cycler, AB.

211 Quantitative real-time PCR (qPCR):

212 The following gene specific primers were previously designed based on the nucleotide sequence of the
213 European eel: gonadotropin receptors, *fshr*, *lhcgr1* and *lhcgr2* (Maugars and Dufour, 2015); estrogen
214 receptors, *esr1*, *esr2a*, *esr2b*, *gpera and gperb* (Lafont et al., 2016). Specific primers for European eel
215 18S ribosomal RNA gene (*18S*) were designed in this study (Table 1), and their specificity and
216 efficiency was tested in qPCR.

217 The qPCRs were performed with a lightcycler (Roche, Ltd. Basel, Switzerland), using SYBR Green I.
218 Each reaction was prepared with 4 µl of diluted cDNA template, 2 µl PCR grade water, 2 µl of SYBR
219 Green master mix and 1 µl of each forward and reverse primers (0.5 pmol each at final concentration).

220 The following qPCR conditions were applied: polymerase activation step of 10 min at 95 °C, followed
221 by 51 cycles of 10 sec of denaturizing at 95 °C, 5 sec of annealing at 60 °C, 6 sec of elongation at 72
222 °C for *esr1*, *esr2a*, *esr2b*, *gpera and gperb* or 10 sec at 72 °C for *18S*, *lhcgr1*, *lhcgr2* and *fshr*. The
223 programs ended with a melting curve analysis by slowly increasing the temperature (0.1 °C/sec) from
224 65 °C to 95 °C, with a continuous registration of changes in fluorescent emission intensity. This last
225 step aimed at ensuring the presence of only one amplified product. Each qPCR run contained a non-
226 template control (cDNA was substituted by water) for each primer pairs to confirm that the qPCR mix
227 was not contaminated. Serial dilutions of a pool of ovary biopsies and ovulated egg cDNAs were used
228 as a standard curve for each gene. One dilution was included in each run as a calibrator. Normalization
229 of the qPCR data was performed using *18S* as a reference gene since it was stable through all sampling
230 points with a coefficient of variance at SPE, DHP and OV of 40%, 40% and 48%, respectively.

2.7 Measurement of lipid droplet diameter

Using the digital images of each sample (before SPE priming, before DHP injection and ovulated eggs), 10 oocytes were randomly selected among those at the most advanced stage of development. Here, ten of the largest lipid droplets were measured using the free software ImageJ (1.48d) and the maximum five values averaged (Unuma et al., 2011). For each lipid droplet, the diameter was calculated by the average of two diameter measurements. At the final stages of coalescence, only a few droplets became larger while the others became smaller (Fig. 1), in these stages, the diameter was based on the diameter of the largest droplets only (Unuma et al., 2011).

2.8 Statistical analysis

Statistical analysis was performed using R version 3.1.3 (R core team, 2015). Statistical differences in gene expression and lipid droplet diameter between quality groups (high- and low-quality) in relation to sampling time (before SPE, before DHP and after stripping) were evaluated using linear mixed-effects (LME) models. The female ID was included in the models as within-subjects variable to account for the repeated measurements taken on each female through the sampling points. Model assumptions of normality and equal variance were checked using Q-Q plots and by observation of the residuals versus fitted values plot. Square root and logarithmic transformations were applied to gene expression and droplet diameter data whenever data deviated from a normal distribution. Linear regression analysis was used to determine whether there were significant correlations between gene expression at each sampling time and fertilization rate and hatching success. The expression of each gene was screened for outliers using the Tukey's method. This method uses the interquartile range (IQR) to identify the outliers range above and below the 1.5X IQR. A significance level (p) of 0.05 was applied in all tests.

3 Results

3.1 Egg production, fertilization and hatching success

The females studied produced on 327 ± 152 g eggs corresponding to 29-56 % of the initial body weight (Table 2). The percent floating eggs varied from 25 to 100 %, while the fertilization rate ranged between 15 to 99 % and the hatching rate between 0 to 80 %. Batch size was positively related to initial female body weight ($R = 0.94$; $p < 0.001$) and length ($R = 0.95$; $p < 0.001$). Fertilization rate was not correlated with female length ($R = -0.03$; $p = 0.930$) nor initial weight ($R = 0.09$; $p = 0.798$). Hatching success ranged from 19 to 80 % and 0 to 5 % in the high- and low-quality groups, respectively (Table 2). There was also no significant correlation between hatching success and female length ($R = -0.03$; $p = 0.927$) nor initial weight ($R = 0.05$; $p = 0.901$).

3.2 Expression of gonadotropin receptors

All three gonadotropin receptors (*fshr*, *lhcgr* and *lhcgr2*) were detected in the ovarian biopsies with genes differentially expressed across sampling points from the induction of oocyte maturation, SPE and DHP, and EGG (Fig. 2). Overall, transcript levels of *fshr* did not vary during oocyte maturation, i.e. between the ovarian biopsy taken before SPE and the biopsy taken before DHP ($p > 0.05$). However, *fshr* levels dropped down below the level of detection of qRT-PCR detection in the eggs (at least 534 times less expressed than in the ovarian biopsies). Within sampling points, the expression levels of *fshr* were similar between the high- and low-quality groups in the biopsies obtained prior to SPE priming ($p > 0.05$; Fig. 2). However, before DHP, *fshr* transcript levels were significantly lower in the high-quality group than in the low-quality group ($p < 0.001$). Present results also indicated a significant negative correlation between *fshr* before DHP injection and hatching success ($R = -0.79$; $p = 0.012$; Table 3).

Overall, *lhcgr1* mRNA levels did not vary significantly ($p > 0.05$) during maturation (from SPE to DHP injection). In contrast, transcript levels were around 150 times lower in ovulated eggs than in the ovarian biopsies ($p < 0.001$). Moreover, in the ovarian biopsies collected before SPE priming, *lhcgr1* expression was significantly higher in the high-quality group compared to the low-quality ($p = 0.002$; Fig. 2). Present results also showed a significant positive correlation between *lhcgr1* at SPE and hatching success ($R = 0.68$, $p = 0.032$; Table 3). There was no difference between the two groups in the ovary samples taken before DHP injection and in ovulated eggs ($p > 0.05$).

Regardless of egg quality, *lhcgr2* transcript levels were similar between the ovarian biopsies taken before SPE and DHP ($p > 0.05$) but significantly less expressed in the ovulated eggs (at least 5 times less expressed than in the ovarian biopsies, $p < 0.001$). Within sampling points, transcript levels of ovarian *lhcgr2* at SPE priming and DHP injection did not differ significantly between the high and low quality groups ($p > 0.05$; Fig. 2). However, *lhcgr2* mRNA levels in unfertilized egg samples were significantly higher in the high-quality than in the low-quality group ($p = 0.013$). There were no significant correlations between *lhcgr2* transcript levels and hatching success or fertilization rate ($p > 0.05$; Table 3).

3.3 Expression of estrogen receptors

The nuclear receptor *esr1* was expressed in the ovarian samples obtained during oocyte maturation without significant variation between the ovarian samples before SPE priming and DHP ($p > 0.05$; Fig. 3). After stripping, transcript levels were below the level of detection of qRT-PCR in unfertilized eggs (at least 40 times less expressed than in the ovarian biopsies). Moreover, *esr1* expression was similar between the high- and low-quality groups within the SPE and DHP sampling points ($p > 0.05$). There

294 were no significant correlations between *esr1* and hatching success or fertilization rate ($p > 0.05$; Table
295 3).

296 From the two nuclear *esr2* receptors, only *esr2a* was detected in the ovary during the induction of
297 oocyte maturation. Irrespective of hatching success, transcript levels of *esr2a* did not vary during
298 maturation treatment ($p > 0.05$), but the gene was much less expressed in unfertilized eggs (at least 67
299 times less expressed than in the ovarian biopsies, $p < 0.001$). In the biopsies taken before SPE priming,
300 transcript levels of *esr2a* were significantly higher in the high-quality group compared to the low-
301 quality group ($p = 0.020$; Fig. 3). In the ovarian biopsies taken before DHP and ovulated eggs, *esr2a*
302 expression levels were similar between groups ($p > 0.05$). We did not find any significant correlations
303 between *esr2a* transcript levels and hatching success or fertilization rate ($p > 0.05$; Table 3). Transcript
304 levels of *esr2b* were below the qRT-PCR detection threshold in all samples and could not be measured.

305 The two estradiol membrane receptors, *gpera* and *gperb*, were detectable and presented a different
306 expression pattern during induced oocyte maturation and after stripping (Fig. 3). Irrespective of egg
307 quality group, *gpera* transcript levels did not vary during maturation treatment ($p > 0.05$) but were
308 significantly lower in the eggs ($p < 0.001$). Expression of *gpera* did not differ significantly between
309 high- and low-quality in neither the ovarian biopsies before SPE and DHP injection, nor in ovulated
310 eggs ($p > 0.05$; Fig. 3). There were no significant correlations between *gpera* transcript levels and
311 hatching success or fertilization rate ($p > 0.05$; Table 3). Overall, there was no significant difference in
312 *gperb* mRNA expression levels during maturation ($p > 0.05$) while transcript levels of *gperb* dropped
313 to a level below the qRT-PCR detection threshold in ovulated eggs (at least 375 times less expressed
314 than in the ovarian biopsies). The expression levels of *gperb* before SPE administration did not differ
315 between the high-quality and low-quality group ($p > 0.05$) while levels were significantly lower in the

high-quality before the DHP administration ($p = 0.010$; Fig. 3). Present results did not show any significant correlations between *gperb* levels and hatching success or fertilization rate ($P > 0.05$; Table 3).

3.4 Lipid-droplet related oocyte maturation status

Image analysis of the ovarian biopsies collected during the course of oocyte maturation induction revealed 2-3 cohorts of oocytes of different sizes and stages of development (Fig. 4). Oocytes at the most advanced stage of development were at the germinal vesicle migration stage, characterized by a transparent cytoplasm and peripheral germinal vesicle visible before SPE and DHP injection (Fig. 1a and b), similar to stage 4 and 5 according to the classification developed by Palstra et al. (2005), respectively. No germinal vesicle could be observed in ovulated eggs after clearing in Serra's liquid (Fig. 1c). As lipid droplets coalesced, their diameter increased significantly throughout oocyte maturation and stripped eggs ($p < 0.001$). In the high-quality group, mean ± SD lipid droplet diameter was 89 ± 23 µm at SPE priming, 136 ± 14 µm at DHP injection and 156 ± 15 µm at EGG. In the low-quality group, lipid droplet diameter was 110 ± 34 µm at SPE priming, 194 ± 72 µm at DHP injection and 248 ± 74 µm at EGG. Overall, mean lipid droplet diameter was significantly higher and with wide size variations in the low-quality than in the high-quality group ($p = 0.028$; Fig. 5), and mean size increased significantly over time in both groups (from SPE to EGG, $p < 0.001$). Lipid droplet size in the low-quality group also demonstrated an increasingly larger inter-female size variation over time, compared to the high-quality group, where there were less lipid droplet size variation at all times. We did not find a significant difference in droplet diameter between high- and low-quality at SPE priming ($p > 0.05$) but at DHP and EGG, lipid droplet diameter was significantly higher in the low-quality group ($p = 0.042$ and $p = 0.005$, respectively).

4 Discussion

4.1 Differential expression of gonadotropin receptors

Gonadotropin receptors mediate the biological effects exerted by gonadotropin hormones. In this study, we analyzed for the first time the expression pattern of the *fshr* and the duplicate *lhcgr* during the induction of follicular maturation and ovulation of European eel using SPE treatment. The expression of the two genes encoding distinct LHCGR (*lhcgr1* and *lhcgr2*) was also detected in the ovary of immature eels (Maugars and Dufour, 2015). In the present case, both *lhcgr1* and *lhcg2* were well expressed in the ovary during induced maturation. Since the report of the coexistence of duplicated *lhcgr* in teleosts (Maugars and Dufour, 2015), their expressions have not yet been compared in any other teleost species. In fishes, there are few studies about the expression of gonadotropin receptors during follicular maturation and ovulation and none concerning gonadotropins and estradiol receptors multiple paralogs. A previous study showed a high expression of ovarian *lhcgr* orthologous to eel *lhcgr2*, in mature female Atlantic salmon (*Salmo salar*) caught during the upstream migration (Maugars and Schmitz, 2006), which appears characteristic of the maturation stage prior to ovulation. Moreover, we found that both genes were significantly less expressed in ovulated eggs. This agrees with the location of LH binding sites found in the granulosa and thecal cells in European eel (Salmon et al., 1988). Similarly, *lhcgr* orthologous to eel *lhcgr1* was found to be expressed in granulosa and weakly in theca cells in chub mackerel (*Scomber japonicus*) (Nyuji et al., 2013) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008). Therefore, a drop in mRNA levels would be expected in successfully ovulated eggs due to the loss of the follicle layers that remain inside the ovary after the oocyte is extruded. Nevertheless, the drop in expression in ovulated eggs was more substantial for *lhcgr1* (150 less expressed) than for *lhcgr2* (5 times less expressed), suggesting that *lhcgr1* is mostly expressed in follicular cells.

We hypothesized that differences in responsiveness to hormonal treatments during oocyte maturation may be related to differences in hormone receptor expression, which consequently could affect embryonic development. To test this, we calculated hatching success and larval survival as measures of embryonic developmental competence (egg quality). Transcript levels of *lhcgr2* during induction of oocyte maturation were similar regardless of hatching success. However, *lhcgr2* mRNA in ovulated eggs was significantly higher in the high-quality group when compared to the low-quality group, suggesting that *lhcgr2* could have a role in embryonic development. Gonadotropin receptor transcripts are also present in mouse oocyte and preimplantation embryo, with a potential beneficial role in oocyte maturation and early embryonic development (Patsoula et al., 2001).

Previous studies have shown that gonadotropic treatment induces an increase in mRNA levels of *lhβ* as well as an increase in LH hormone in the pituitary in both European (Dufour et al., 1989; Schmitz et al., 2005) and Japanese eel (Nagae et al., 1996; Saito et al., 2003; Jeng et al., 2007). This increase in pituitary content of LH is likely necessary for the LH surge (in this case mimicked by the LH present in the SPE) triggering ovulation. However, it is still uncertain if this observation is due to the effect of gonadotropins alone, as both SPE and CPE treatments contain other components (neurotransmitters, steroids) that can affect receptor expression. Our results showed that ovarian levels of *lhcgr1* at the time of SPE primer administration were both positively correlated with hatching success, and were significantly higher in the high-quality group than in the low-quality group at SPE. This suggests that females with higher hatching success had higher sensitivity to LH at the time SPE was administrated. In contrast, the significantly lower levels of *lhcgr1* expression in females with low-quality eggs could have limited their response to the LH in the SPE primer. This is particularly important, in a first stage of oocyte maturation, LH regulates the follicle's ability to produce maturation-inducing steroid (MIS)

and the oocyte's response to MIS (i.e. oocyte maturational competence); on a second stage the follicle cells produce MIS (Patiño et al., 2001). Thus, failure to respond to LH at the first stage could subsequently compromise the oocyte's ability to respond to the MIS (i.e. the DHP injection). Additionally, LH signalling can stimulate other pathways than those leading to DHP synthesis. This has been observed in zebrafish, where the expression of *ptgs2a* (a cox-2 gene) induced by LH was necessary for ovulation to occur (Tang et al., 2017). Since oocyte maturation and ovulation may be independently/differently regulated by the same ligand, the two processes may come out of synchrony with each other, and this may influence the developmental capacity of the egg/embryo. In this case, the mismatch between *lhcgr1* expression and the administration of the SPE primer may have affected the follicle's ability to respond to DHP in females in the low-quality group. Hence, a high expression of *lhcgr1* before SPE priming seems to be a good biomarker of high maturational competence.

Overall, *fshr* was well expressed in the mature ovary at all sampling times during the induction of oocyte maturation. In Japanese eel, 17 weeks of treatment with pituitary homogenates also increased ovarian levels of *fshr* (Jeng et al., 2007). However, the physiological relevance of high *fshr* expression during ovarian maturation in teleosts is still unclear. In the case of the eel, gonadotropins contained in exogenous pituitary homogenates (SPE in this case) could up-regulate the expression of both gonadotropin receptors. Alternatively, in the case of multiple spawning, an ovulatory surge of FSH could play an important role in the follicle recruitment for the next reproductive cycle (Prat et al., 1996; Tyler et al., 1997; Sambroni et al., 2007). In this study, oocyte development presented a group-synchronous pattern (Wallace and Selman, 1981), with 2-3 cohorts of oocytes of different size and stage of development present in the ovarian biopsies taken during the induction of oocyte maturation and ovulation. Thus, a high expression of *fshr* in the ovary during maturation could be related to the

405 regulatory role of FSH on the less developed oocyte batches also present in the biopsies. Alternatively,
406 it is also possible that FSHR has an important function on maturing oocytes (as occurs in mammals,
407 where FSH up-regulates LHCGR (Zeleznik et al., 1974). After ovulation, levels of *fshr* mRNA level
408 dropped below the level of detection, indicating that *fshr* is also mostly expressed in the follicle cells
409 surrounding the oocyte. This is in agreement with the observations by *in situ* hybridization of *fshr*
410 expression in granulosa and theca cells in vitellogenic follicles in salmon (Andersson et al., 2009).

411 In relation to hatching success, present results showed that transcript levels of *fshr* before the DHP
412 injection were negatively correlated with hatching success, and females with low quality eggs had
413 significantly higher transcript levels of *fshr*. This indicates that females in the low-quality group were
414 more sensitive to FSH at the time DHP was administrated. However, while *lhβ* mRNA levels tend to
415 increase, *fshβ* levels significantly decrease with gonadotropic treatment in both Japanese (Yoshiura et
416 al., 1999) and European eel females (Schmitz et al., 2005). Therefore, increased sensitivity to FSH at
417 the time of DHP injection does not appear to be determinant for successful oocyte maturation and
418 ovulation treatment in European eel. In contrast, increasing mRNA levels of *fshr* were associated to a
419 better competence of the oocyte to mature following pituitary hormone induction in rainbow trout
420 (*Oncorhynchus mykiss*) (Bobe et al., 2003). However, differences in hormone receptor expression
421 among species are likely influenced by differences in the reproductive strategies. For example, in
422 rainbow trout which is considered as total spawner (Mylonas and Zohar, 2007), *fshr* mRNA levels peak
423 at maturation and ovulation while *lhcgr* increased significantly later after ovulation (Sambroni et al.,
424 2007). In contrast, in zebrafish, which is a multiple batch spawner, *fshr* transcripts peaked at mid-
425 vitellogenesis and dropped at the end of vitellogenesis (Kwok et al., 2005). Overall, a combination of
426 low expression of ovarian *lhcgr1* before SPE priming and high expression of *fshr*, when DHP is

administrated, could be indicators of an ineffective response to oocyte maturation treatment resulting in low egg quality.

4.2 Differential expression of estrogen receptors

In this study, we quantified gene expression of three nuclear (*esr1*, *esr2a*, *esr2b*) and two membrane (*gpera* and *gperb*) estrogen receptors, during hormonal induction of oocyte maturation in European eel. We found that transcripts of *esr1* were quite similar in the eel ovary throughout induced maturation, with no significant differences between the two hatching groups. This suggests that *esr1* mRNA is not a limiting factor for the development of maturational competence. Nevertheless, up-regulation of *esr1* transcripts in the ovary of matured eels after ovulation (Lafont et al, 2015) suggests that this receptor might play an important role during oocyte maturation. Regulation of *esr1* transcript levels may be associated with an increase in circulatory levels of estradiol-17β (E_2) during oocyte maturation stages (da Silva et al., 2016). In particular, E_2 plasma concentration increases two to five times after SPE priming in European eel (H. Tveiten, unpubl. results). Up-regulation of *esr1* was associated with an increase of circulatory estrogens before ovulation also *in vitro* in eel hepatocytes (Lafont et al, 2015). In contrast, *esr1* expression in the testis of male eels was markedly expressed in early stages of spermatogenesis but significantly down-regulated in late stages of spermatogenesis (Morini et al., 2017). This indicates a differential expression pattern of *esr1* between oogenesis and spermatogenesis in eels. Differences in hepatic *esr1* expression between sexes have been observed in goldfish and are likely related to differences in basal levels of E_2 between males and females (Nelson and Habibi, 2010). In unfertilized eggs, *esr1* transcript levels were below the level of detection, suggesting that this estrogen receptor is mostly expressed in the follicular cells.

Before SPE priming, *esr2a* transcripts were significantly more expressed in females with higher hatching success which may indicate that this receptor has an important role during oocyte maturation. In previous studies, the expression of *esr2a* in mature female eels after spawning was similar to controls (Lafont et al., 2015) and in male eels, it was down-regulated during final stages of spermatogenesis (Morini et al., 2017). Transcripts of *esr2a* were below the level of detection of the qRT-PCR in ovulated eggs, suggesting that they are mainly expressed in follicular cells, as *esr1*. Transcripts of *esr2b* were below the level of detection of the qRT-PCR in all samples, i.e. undetectable during follicular maturation and ovulation. In male eels, *esr2b* transcripts in the testis were significantly down-regulated throughout spermatogenesis (Morini et al., 2017). Thus, it is likely that *esr2b* does not play a key role during the final stages of gametogenesis in European eel.

It is well known that in female fish GPER mediates the E_2-induced meiotic arrest of oocytes, e.g. zebrafish, Atlantic croaker and common carp (Majumder et al., 2015; Pang and Thomas, 2009; Pang et al., 2008; Peyton and Thomas, 2011). In these previous studies, only one GPER gene was characterized, known now to be the orthologous to teleost *gpera*. We found that *gpera* transcripts were expressed at similar levels in the ovary throughout the oocyte maturation (all stages), without significant differences between the high-quality and low-quality group. Levels of *gpera* transcript showed only a moderate decrease in ovulated eggs (not significant), indicating that *gpera* transcripts are largely present in the eel oocyte itself, in contrast to all the other estradiol receptors. In contrast, expression of *gperb* before DHP injection was significantly lower in females exhibiting higher egg viability. Furthermore, *gperb* was the only estrogen receptor that was less expressed in females exhibiting high quality eggs. This may suggest that *gperb* plays an important role in mediating the inhibitory effect of estrogen during oocyte maturation also in European eel, where estrogen-dependent

oocyte growth is arrested at the first meiotic prophase by high levels of intracellular cyclic AMP (cAMP) (Conti et al., 2002). In the pre-ovulatory phase, a LH surge causes a shift in the steroidogenic pathway to the production of the MIS. When the MIS production (DHP injection in this case) is sufficient to overcome the estrogen inhibitory effect, the binding of MIS to a G protein-coupled progestin membrane receptor triggers meiosis resumption and GVBD by causing a decrease in cAMP concentrations (Jalabert and Finet 1986; Finet et al., 1988). Moreover, in Japanese eel, increased E_2 in the late stages of oocyte development inhibits the production of DHP (Ijiri et al., 1995). Thus, a lower expression of *gperb* in the HEQ group suggests a lower sensitivity to estrogen and subsequently a lower estrogen inhibition, which may have enhanced the effect of DHP. In male eels, both *gpera* and *gperb* transcripts significantly increased during spermatogenesis suggesting that GPERs may be involved in final sperm maturation (Morini et al., 2017). Overall, our results raise the possibility that an E_2 pathway, possibly regulated by *esr2a* and *gperb,* is involved in the control of oocyte maturation also in European eel. However, we did not find any significant correlation between the expression of these two genes and other egg quality indicators such as fertilization and hatching success. Thus, evidence is weak and further investigation is necessary to clarify their role during oocyte maturation and subsequent egg quality.

4.3 Cytological indicators of oocyte maturation status

An early index of maturational acquisition is the onset of germinal vesicle migration, which is accompanied by the rise in LH at the end of follicle growth (Lubzens et al., 2010). In this study, a peripheral germinal vesicle in the most advanced oocytes was used as an indicator that females had progressed into initial stages of oocyte maturation before SPE priming. The appearance of oil droplets was used as a marker of the maturation progress to initiate SPE priming or DHP injection (Palstra et al.

2005; Unuma et al., 2011). Here, the coalescence of the oil droplets reflects the hormonal processes during final maturation resulting in decreasing numbers and increasing size of the lipid droplets. Interestingly in this study, the oocytes of females in the high-quality group had on average smaller lipid droplets with less size variation compared to those with low-quality group. Especially at DHP and ovulation the difference between the groups was significant. The observed variation in lipid droplet size, in conjunction with the hormone receptor expression pattern in the same females, suggests that treatment administrated at an earlier stage enhanced maturational competence and the subsequent developmental capacity of the egg/embryo. In general, limited information is available on the mechanisms of oocyte lipid coalescence as well as on the role of this physiologically important process in the subsequent survival and development of the eggs and embryos. Our findings, substantiating insight in the underlying hormonal processes, suggest lipid droplet diameter to be an accurate, quantitative indicator of maturation status in European eel.

In conclusion, ovarian *fshr*, *lhcgr1*, *esr2a* and *gperb* were differentially expressed across sampling points between females presenting high and low egg quality. A mismatch between the timing of hormone injections and the expression of some of these genes may therefore influence the follicle's ability to respond to treatment. Furthermore, changes in hormone receptor expression were associated with changes in oocyte maturation status. Here, the average size of lipid droplets in advanced oocytes was smaller in the high- compared to low-quality group throughout the maturation process, indicating that the timing of hormonal treatments can be optimized, thereby optimizing procedures in assisted reproduction of the endangered European eel.

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737

Legends

Figure 1: Oocytes at each sampling point.

Photomicrographs showing examples of oocytes at the most advanced stage of development from the biopsy samples taken a) before SPE priming, b) before DHP injection and c) ovulated eggs cleared in Serra's liquid. GV: germinal vesicle and LD: lipid droplet.

Figure 2: Expression of gonadotropin receptors.

Boxplots representing expression of gonadotropin receptors (*fshr*, *lhcgr1* and *lhcgr2*) in ovarian biopsies taken at the time of administration of the SPE primer (SPE) and DHP injection (DHP) as well as ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5) groups. The bold line inside each box represents the median, lower and upper sides of each box represent the lower and upper quartile (25% and 75%) and whiskers extend to the most extreme data point, no more than 1.5 times the interquartile distance. Data points outside the boxplot are classed as "outliers". Significant differences between groups are identified with "*" when $p < 0.05$; "**" when $p < 0.01$ and "***" when $p < 0.001$.

Figure 3: Expression of estrogen receptors.

Boxplots representing expression of nuclear (*esr1, esr2a*) and membrane estrogen receptors (*gpera* and *gperb*) in ovarian biopsies taken at the time of administration of the SPE primer (SPE) and DHP injection (DHP) as well as in ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5) groups. The bold line inside each box represents the median, lower and upper sides of each box represent the lower and upper quartile (25% and 75%) and whiskers extend to the most extreme data point, no more than 1.5 times the interquartile distance. Significant differences between groups are identified with "*" when $p < 0.05$ and "**" when $p < 0.01$.

Figure 4:

Oocytes in three different maturational stages from an ovarian biopsy taken before the SPE priming injection. 1: small and opaque oocytes; 2: large oocytes with darker cytoplasm; 3: large oocytes with transparent cytoplasm and migratory nucleus.

Figure 5: Lipid droplet diameter.

Boxplot representing the lipid droplet diameter at the time of administration of the SPE priming (SPE) and DHP injection (DHP), as well as in ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5) groups. The bold line inside each box represents the median, the lower and upper sides of each box represent the lower and upper quartile (25% and 75%) and whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile distance. Data points outside the boxplot are classed as “outliers”. Significant differences between egg groups are identified with an “*” when $p < 0.05$.

Figure 1

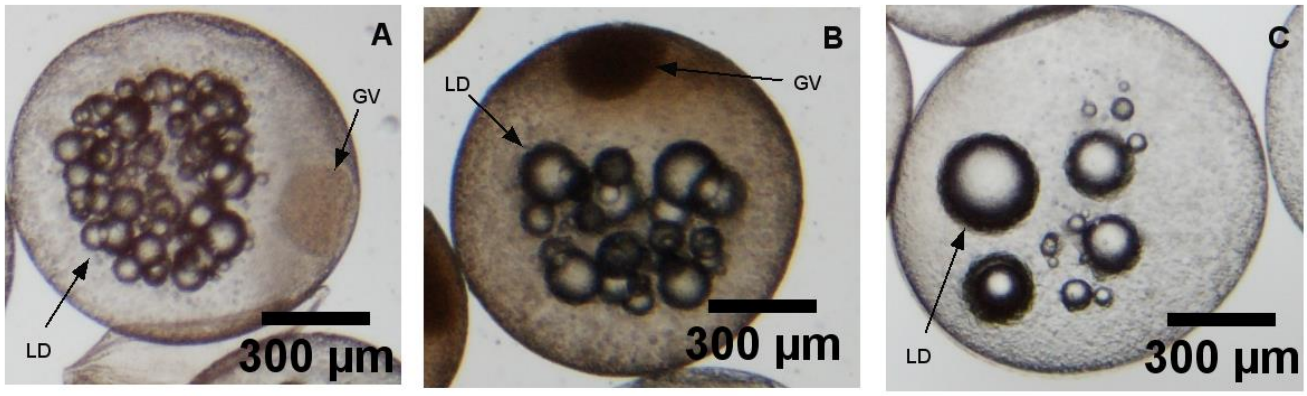

Figure 2

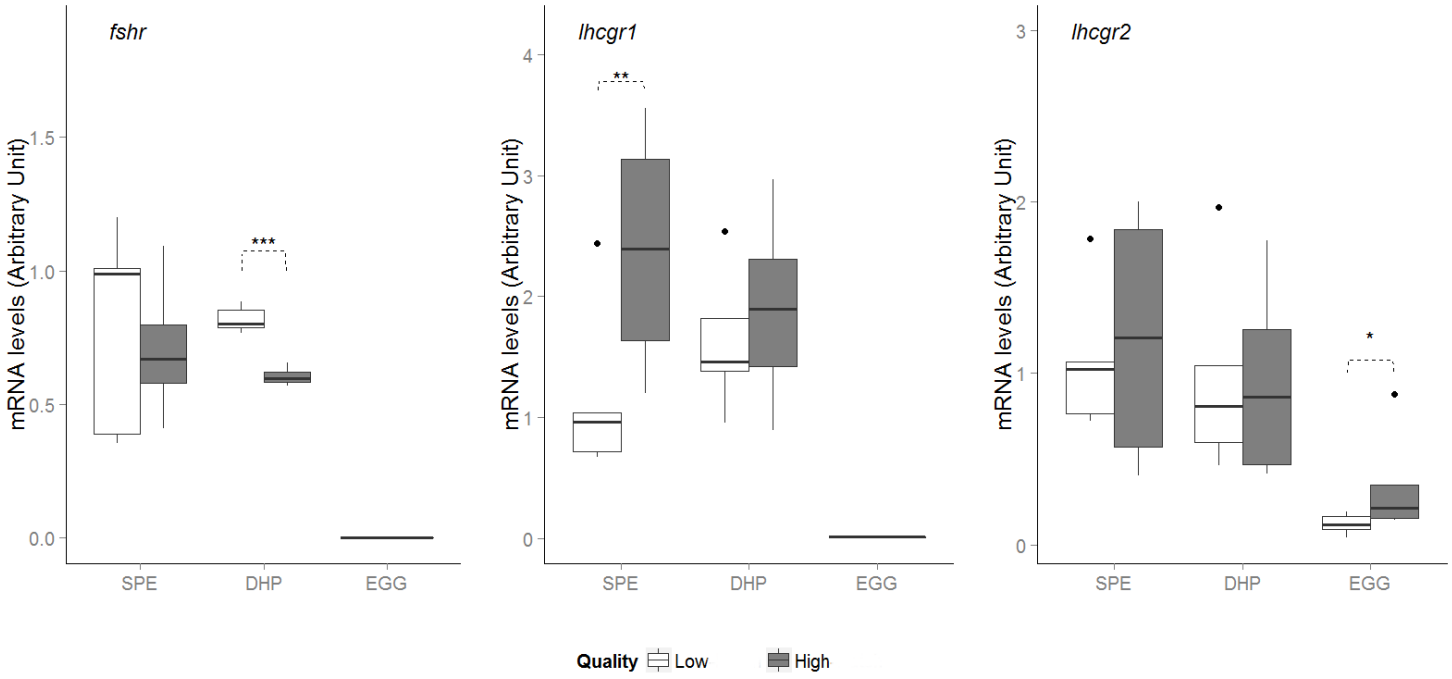
fshr
lhcgr1
lhcgr2
mRNA levels (Arbitrary Unit)
SPE
DHP
EGG
***
**
*
Quality
Low
High

Figure 3

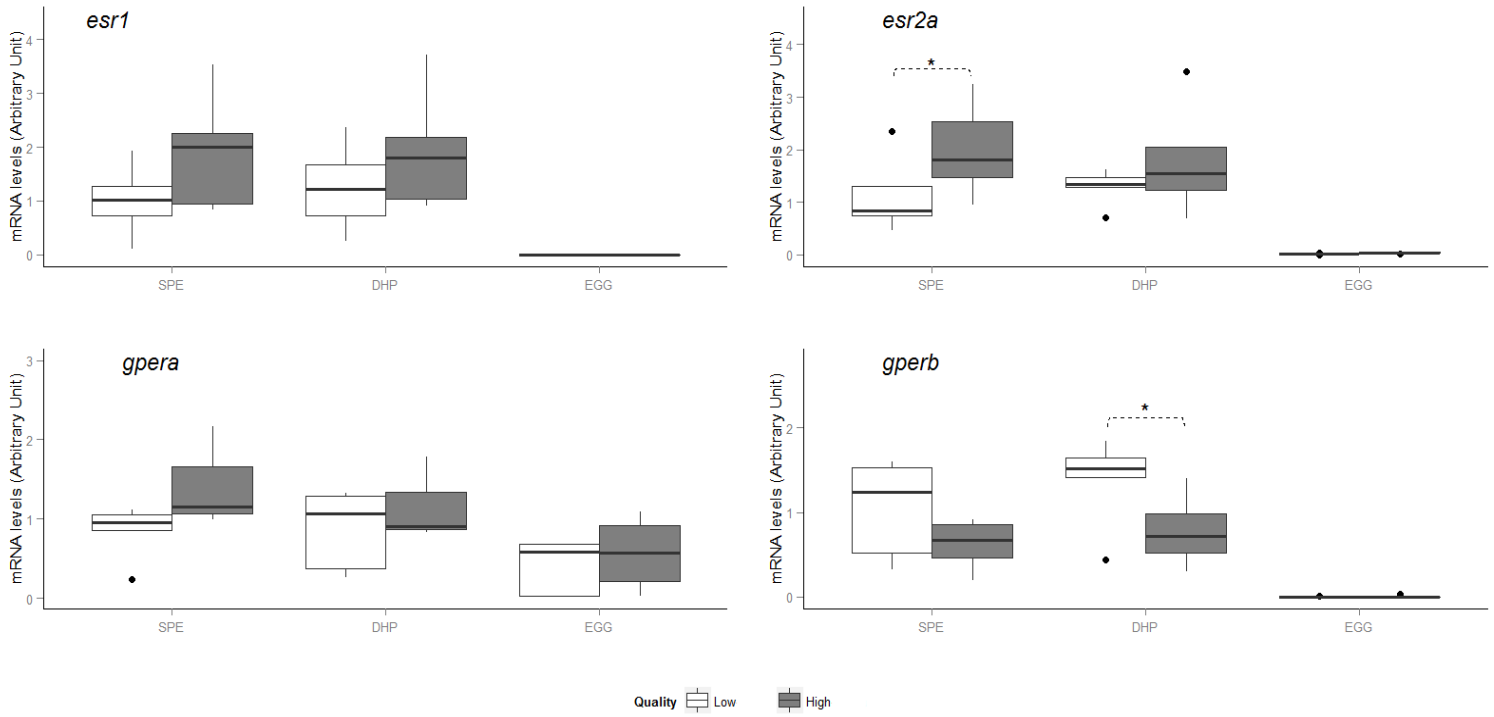
esr1
esr2a
gpera
gperb
mRNA levels (Arbitrary Unit)
SPE
DHP
EGG
*
Quality
Low
High

Figure 4

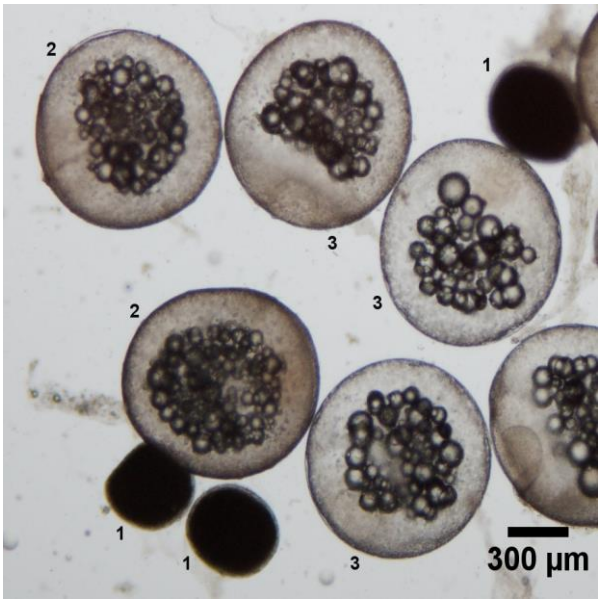

Figure 5

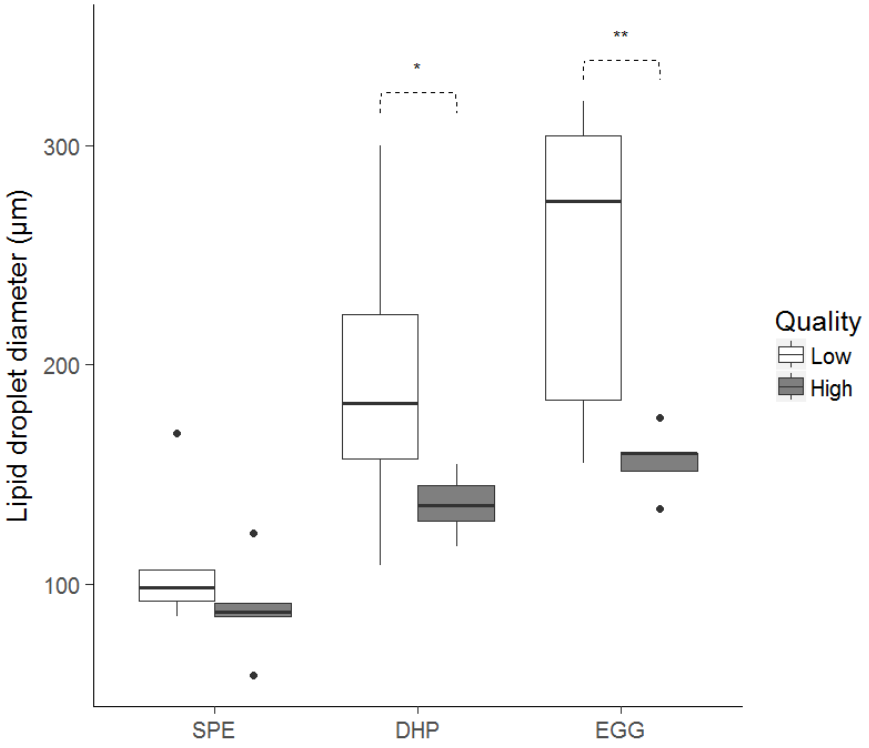
Lipid droplet diameter (µm)
300
200
100
*
**
SPE
DHP
EGG
Quality
Low
High

Table 1 Quantitative PCR primer sequences for gonadotropin receptors (*fshr*, *lhcgr1* and *lhcgr2*), nuclear estrogen receptors (*esr1*, *esr2a*, *esr2b*), membrane estrogen receptors (*gpera* and *gperb*) and reference gene (*18s*).

Name	Sequence (5'- 3')	Orientation	Accession number	Amplicon size (bp)	Reference
fshr	CCTGGTCGAGATAACAATCACC	Forward	LN831181	148	Maugars and Dufour (2015)
	AATCTTGGAGAAATCAGGCAGT	Reverse			
lhcgr1	GCGGAAACACAGGGAGAAC	Forward	LN831182	155	Maugars and Dufour (2015)
	GGTTGAGGTACTGGAAATCGAAG	Reverse			
lhcgr2	GTTTCCTGACCTATCGGCTATT	Forward	LN831183	132	Maugars and Dufour (2015)
	GGTTGAGGTACTGGAAATCGAAG	Reverse			
esr1	GCCATCATACTGCTCAACTCC	Forward	CUH82767	76	Lafont et al., (2015)
	CCGTAAAGCTGTCGTTCAGG	Reverse			
esr2a	TGTGTGCCTCAAAGCCATTA	Forward	CUH82768	169	Lafont et al., (2015)
	AGACTGCTGCTGAAAGGTCA	Reverse			
esr2b	TGCTGGAATGCTGCTGGT	Forward	CUH82769	123	Lafont et al., (2015)
	CCACACAGTTGCCCTCATC	Reverse			
gpera	CAACTTCAACCACCGGGAGA	Forward	CUH82770	170	Lafont et al., (2015)
	TGACCTGGAGGAAGAGGGACA	Reverse			
gperb	AACCTGAACCACACGGAAA	Forward	CUH82771	170	Lafont et al., (2015)
	TGACCTGGAAGAAGAGGGACA	Reverse			
18s	CTCAACACGGGAAACCTCAC	Forward	FM946070	118	
	AGACAAATCGCTCCACCAAC	Reverse			

Table 2 Information and group assignment about female eels and resulting egg batches used in the present study, including initial length, (L_i) and body weight (BW_i), volume of eggs stripped, floating fraction (%), fertilization success (%), hatching success (%) and larval survival (%) at 3 days post hatch (dph).

Female No.	L_i (cm)	BW_i (g)	Eggs (g)	Floating	Fertilization	Hatching	Survival 3 dph	Group
1.	70	693	322	99	68	65	67	high-quality
2.	67	574	324	99	99	80	93	high-quality
3.	60	433	229	99	69	55	83	high-quality
4.	78	1014	335	95	97	19	13	high-quality
5.	86	1444	571	50	80	67	78	high-quality
6.	79	1019	372	25	84	4	0	low-quality
7.	63	414	200	90	15	5	0	low-quality
8.	92	1334	586	99	20	0	0	low-quality
9.	62	426	195	50	25	0	0	low-quality
10.	60	459	133	100	96	4	0	low-quality

Table 3 Pearson's correlation coefficient R and *p*-value for the linear regressions between gene expression at the time of SPE priming (SPE), DHP injection (DHP) and ovulated eggs (EGG), and hatching and fertilization success (n=10).

Gene	Time point	Hatching		Fertilization	
		R	*p*	R	*p*
fshr					
	SPE	0.23	0.529	-0.20	0.577
	DHP	-0.79	0.012	-0.46	0.213
	EGG	NA	NA	NA	NA
lhcgr1					
	SPE	0.68	0.032	0.10	0.780
	DHP	0.36	0.314	-0.38	0.281
	EGG	-0.17	0.637	-0.17	0.636
lhcgr2					
	SPE	0.00	0.999	0.42	0.223
	DHP	-0.24	0.512	0.40	0.250
	EGG	0.05	0.892	0.32	0.397
esr1					
	SPE	0.54	0.111	-0.11	0.762
	DHP	0.41	0.236	-0.33	0.351
	EGG	NA	NA	NA	NA
esr2α					
	SPE	0.60	0.067	0.05	0.894
	DHP	0.49	0.154	-0.11	0.762
	EGG	0.60	0.064	0.35	0.316
gpera					
	SPE	0.34	0.331	0.56	0.094
	DHP	0.29	0.419	0.46	0.183
	EGG	0.06	0.865	0.38	0.275
gperb					
	SPE	-0.44	0.204	-0.62	0.058
	DHP	-0.31	0.413	-0.21	0.584
	EGG	NA	NA	NA	NA