I. Rojo-Bartolomé; Martínez-Miguel, L.; Lafont, A.; Vilchez Olivencia, MC.; Asturiano Nemesio, JF.; Pérez Igualada, LM.; Cancio, I. (2017). Molecular markers of oocyte differentiation in European eel during hormonally induced oogenesis. Comparative Biochemistry and Physiology Part A Molecular & Integrative Physiology. 211:17-25. doi:10.1016/j.cbpa.2017.05.018

Molecular markers of oocyte differentiation in European eel during hormonally induced oogenesis

Iratxe Rojo-Bartolomé[a], Leticia Martínez-Miguel[a], Anne-Gaëlle Lafont[b], M. Carmen Vílchez[c], Juan F. Asturiano[c], Luz Pérez[c], Ibon Cancio[a*]

[a]*CBET Research Group, Dept. of Zoology and Animal Cell Biology, Research Centre for Experimental Marine Biology and Biotechnology of Plentzia (PIE-UPV/EHU), University of the Basque Country (UPV/EHU), Areatza, z/g E-48620 Plentzia, Basque Country*

[b]*Muséum National d'Histoire Naturelle, Sorbonne Universités, Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, CNRS 7208, IRD 207, UPMC, UCN, UA, Paris, France*

[c]*Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Edificio 7G. Camino de Vera, s/n, 46022, Valencia, Spain*

Running title: Ribosomal biogenesis genes during oogenesis in eels

*Corresponding author

Dr. Ibon Cancio;

CBET Research Group, Dept. of Zoology and Animal Cell Biology, Research Centre for Experimental Marine Biology and Biotechnology of Plentzia (PIE-UPV/EHU), University of the Basque Country (UPV/EHU),

Areatza, z/g E-48620 Plentzia, Basque Country

Email: ibon.cancio@ehu.es

Abstract

Reproduction in captivity is a key study issue in *Anguilla anguilla* as a possible solution for its dwindling population. Understanding the mechanisms controlling the production of ribosomal building blocks during artificially induced oocyte maturation could be particularly interesting. Transcription levels of ribosomal biogenesis associated genes could be used as markers to monitor oogenesis. Eels from the Albufera Lagoon were injected with carp pituitary extract for 15 weeks and ovaries in previtellogenic (PV) stage (non-injected), in early-, mid-, late-vitellogenesis (EV, MV, LV), as well as in migratory nucleus stage (MN) were analysed. 5S rRNA and related genes were highly transcribed in ovaries with PV oocytes. As oocytes developed, transcriptional levels of genes related to 5S rRNA production (*gtf3a*), accumulation (*gtf3a*, *42sp43*) and nucleocytoplasmic transport (*rpl5, rpl11*) and the 5S/18S rRNA index decreased (PV>EV>MV>LV>MN). On the contrary, 18S rRNA was at its highest at MN stage while *ubtf1* in charge of activating RNA-polymerase I and synthesising 18S rRNA behaved as 5S related genes. Individuals that did not respond (NR) to the treatment showed 5S/18S index values similar to PV females, while studied genes showed EV/LV-like transcription levels. Therefore, NR females fail to express the largest rRNAs, which could thus be taken as markers of successful vitellogenesis progression. In conclusion, we have proved that the transcriptional dynamics of ribosomal genes provides useful tools to characterize induced ovarian development in European eels. In the future, such markers should be studied as putative indicators of response to hormonal treatments and of the quality of obtained eel oocytes.

Key words: *Anguilla anguilla*, Carp pituitary extract, oocyte growth, 5S rRNA, 5S/18S rRNA index, RNA Polymerases I and III, ribosomal biogenesis genes.

1. Introduction

The European eel stock has been in gradual decline for at least half a century (van Ginneken et al. 2005; Pujolar et al. 2012; ICES 2013) with numbers dropping as much as 99% since the 1980s (ICES 2013) so understanding the mechanisms triggering sexual maturation in European eels has become a focus of economic and scientific interest (van van Ginneken et al. 2005). Developing new methodologies to control reproductive maturation of eels in captivity could allow establishing a self-sustained aquaculture rather than the nowadays applied culture system dependent on fishing and growing of wild glass eels (Dirks et al. 2014).

In order to establish an efficient aquaculture activity, high-quality eggs and sperm are needed to produce viable juveniles. Attempts to reproduce eels in captivity have largely been unsuccessful (Boëtius and Boëtius, 1980; Pedersen, 2004; Palstra et al. 2005; Palstra and van den Thillart, 2010; Pérez et al. 2011). In this respect[JF1], Tanaka and co-workers were able to obtain leptocephali larvae of Japanese eel (*Anguilla japonica*) in captivity in 2003, taking them through metamorphosis to obtain glass eels (Ijiri et al. 2011; Okamura et al. 2014). In the case of the European eel successful fertilization and hatching, taking the larvae through the yolk-sac stage was recently reported (Butts et al. 2014; Sørensen et al. 2014). However, with the existing breeding protocols, most fertilized eggs do not develop and all larvae die prematurely. Considering this, it is imperative to enhance the knowledge base on eel reproduction and develop the technology needed to produce good quality gametes and viable offspring that would allow to rear larvae beyond the first feeding stage.

Gonadotropins (luteinising hormone, LH; and follicle-stimulating hormone, FSH) positively control the development and activity of gonads in all vertebrates (Dufour et al. 2003; Levavi-Sivan et al., 2010; Zohar et al. 2010). In some teleosts, gonadotropin

secretion at the pituitary is under the control of gonadotropin-releasing hormone (GnRH) that exerts a stimulatory control, and dopamine, kisspeptins and gonadotropin inhibitory hormone, with an inhibitory effect (Dufour et al. 2003; Zohar et al., 2010; Pasquier et al. 2011). Eels do not mature in their continental water habitats due to a strong dopaminergic inhibition and a deficient stimulation of gonadotropin-releasing hormone (GnRH) release (Dufour et al. 2003; Vidal et al. 2004). Therefore, eels will not become sexually mature until they are in the open ocean, under the influence of still unknown environmental factors (Bruijs et al. 2009; Mazzeo et al. 2014). Research on the control of this blockade of sexual maturation has allowed inducing maturation in captivity, applying hormonal treatments mainly consisting of injections of fish pituitary extracts to silver eels captured in transition from freshwaters to the ocean (reviewed in Okamura et al. 2014). In this way, the maturation in female European eels is based on weekly injections of such extracts administered for periods of 10-20 weeks (Butts et al. 2014). The same is applied to males injecting human chorionic gonadotropins (Asturiano et al. 2005; Okamura et al. 2014). There are obvious disadvantages to this method. In this sense, individuals need to be manipulated weekly, while cost is high and gamete quality is unpredictable (Okamura et al. 2014). Besides, in many circumstances some individuals do not respond even after 6 months of injections (Dirks et al. 2014).

Oocyte differentiation and maturation in fish relies on an intense incorporation of a large quantity of molecules into the cell. This involves a specific expression regulation of the oocyte genome, although many of the molecules are incorporated from surrounding ovarian somatic cells or from other organs such as the liver. Our previous studies have revealed that 5S ribosomal RNA (5S rRNA) and accompanying proteins are good markers of female fish oocyte differentiation due to their transcription level dynamics changing as oocyte grows (Diaz de Cerio et al. 2012; Rojo-Bartolomé et al. 2016). For instance,

high levels of 5S rRNA in oocytes allows identification of intersex testis in fish that due to exposure to xenoestrogenic chemicals develop oocytes in their spermatic cysts (Diaz de Cerio et al. 2012; Ortiz-Zarragoitia et al. 2014).

All the molecular machinery necessary for 5S rRNA transcription and accumulation is also required in great quantities in the oocyte (Song et al. 2005; Lyman-Gingerich et al. 2007). Ribosomes are formed by the assembly of ribosomal rRNAs (28S, 18S, 5.8S and 5S rRNA) and ribosomal proteins (Rpl) (Lyman-Gingerich et al. 2007). With the exception of 5S rRNA, all other rRNAs are produced in the nucleolus as a single 45S rRNA precursor by RNA polymerase I (Pol I). This Pol I is controlled by the upstream binding transcription factor 1 (Ubtf1). In contrast, 5S rRNA is transcribed in the nucleus by RNA polymerase III regulated by the general transcription factor IIIA (Gtf3a) (Szymanski et al. 2003; Ortiz-Zarragoitia et al. 2014). Gtf3a binds 5S rRNA within the nucleus and the complex is transported to the cytosol where it is accumulated in the form of small 7S ribonucleoprotein particles (RNP) (Szymanski et al. 2003). Although a big proportion of the cytosolic 5S rRNA appears as 7S RNP, it can also be accumulated as 42S RNP associated to P43 (P43 5S RNA-binding protein or 42Sp43) (Picard et al. 1980; Zhang et al. 1995; Ortiz-Zarragoitia et al. 2014). Ribosomal protein l5 (Rpl5) can then bind 5S rRNA accumulated as 7S or 42S RNPs to stabilize 5S rRNA and forming a pre-ribosomal RNP that will migrate to the nucleus for ribosome assembly when bound to yet another ribosomal protein, Rpl11 (Ciganda and Williams, 2011).

In *Xenopus*, *gtf3a* mRNA levels are approximately 1 million times higher in oocytes than in somatic cells, *42sp43* transcript levels being also very high in oocytes (Allison et al. 1995; Penberthy et al. 2003; Szymanski et al. 2003). The levels of *gtf3a* and *42sp43* mRNA mirror those of total 5S rRNA also in fish ovaries (Diaz de Cerio et al. 2012). *gtf3a* is overexpressed early in oogenesis, constituting a high proportion of total

cytoplasmic mRNA and protein in oocytes of anurans and fish, and then decreases manifold during vitellogenesis (Penberthy et al. 2003; Rojo-Bartolomé et al. 2016). In this way, gtf3a is also a potent molecular marker of oocytes in many teleost fish species (Rojo-Bartolomé et al. 2016). Additionally, it has been observed that the expression of 5S rRNA predominates in ovaries with oocytes in previtellogenic stages, while vitellogenesis marks the onset of the transcription and accumulation of 18S rRNA in fish. Therefore, a simple calculation of the ratio 5S to 18S rRNA allows ranking fish ovaries according to their developmental stage (Rojo-Bartolomé et al. 2016). In this molecular context, it could be hypothesised that oocytes in fish need to accumulate ribosomal intermediates in order to quickly assemble ribosomes in case of being fertilized (Diaz de Cerio et al. 2012). This would allow sustaining protein synthesis during embryogenesis.

Our aim in the present study was to characterize the profile of ribosomal RNA incorporation into experimentally matured European eel oocytes. For that purpose, we calculated the ovarian 5S/18S index using total RNA as a molecular biomarker of oocyte development and differentiation, and quantified the transcription levels of different genes related with ribosomal biogenesis, chosen according to their association with the activity of RNA polymerases I and III (*18S rRNA*, *ubtf1* and *gtf3a*) and 5S rRNA accumulation in the cell (*gtf3a*, *42sp43*, *rpl5* and *rpl11*). Such transcripts could provide quantitative information of the effect of hormonal treatments on the differentiation process of oocytes in European eels.

2. Material and methods

2.1. Samples and hormonal treatment

Wild female European eels (*Anguilla anguilla* L.) were captured by local fishermen in the Valencia Albufera lagoon during their migrating phase as silver eels and transferred

to the Aquaculture Laboratory of the Universitat Politècnica de València, where they were maintained in 500 l tanks equipped with recirculation system, heating/cooling systems, and black covers to reduce light intensity. After acclimation from freshwater to seawater conditions at 15-20 ºC, weekly hormonal treatments started. Female eels were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, Catvis, Ltd.) at a dose of 20 mg/kg body weight (Pérez et al. 2011; Mazzeo et al. 2014), until the end of the experiment, after 15 weeks. As the eels stop feeding at the silver stage and throughout sexual maturation, they were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana; Permit Number: 2014/VSC/PEA/00147). All efforts were made to minimize animal suffering and stress.

2.2. Histological analysis and staging

Animals (n=33) were dissected, as described in Mazzeo et al. (2014), previous to the first injection (0) and after 4, 8 and 12 weeks of hormonal treatment with CPE. For histological analysis the ovaries were fixed with 10% (v/v) buffered formalin and embedded in paraffin wax. Then, sections of 5 to 10 µm in thickness were produced. Sections were stained with haematoxylin and eosin using standard procedures. Slides were observed using a Nikon Eclipse E-400 microscope (Nikon, Tokyo; Japan) and the evaluation of the maturation stages was performed according to Pérez et al. (2011). After histological

observation the samples were classified into five stages of ovarian development (Fig. 1). Non-injected eels showed previtellogenic oocytes (PV), displaying lipid droplets but without observable yolk vesicles (Fig. 1a). Females in early-vitellogenic stage (EV) were obtained after 4 weeks of injections, and their gonads showed EV oocytes with small yolk vesicles restricted to the cell cortex (Fig. 1b). Ovaries with mid-vitellogenic (MV) oocytes were obtained after 8 CPE injections (Fig. 1c), and showed abundant yolk vesicles. Characteristic and abundant yolk vesicles were distributed throughout the cytoplasm advancing inwards towards the nucleus. Late-vitellogenic (LV) ovaries were obtained after 12 CPE injections, showing oocytes with enlarged and more abundant yolk vesicles (Fig. 1d). Post-vitellogenic ovarian samples were obtained after 12-15 weeks; they showed the nucleus migrating from the centre of the oocyte to the periphery, during the process leading to final oocyte maturation (Fig. 1e). Some ovaries showed no evidence of having initiated oocyte differentiation after 8 and 12 weeks of injection, non-responders (NR), with the oocytes resting in early oogenesis stages, most of them at PV stage (Fig. 1f).

2.3. RNA extraction, quantification and quality assessment: 5S/18S rRNA index

A portion (less than 40 mg) of each of the 33 ovaries analysed histologically was embedded in RNA Later (Sigma-Aldrich, St. Louis, Missouri, USA) and frozen in liquid nitrogen until needed for RNA extraction. Total RNA was then isolated using TRI® reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions. Dry RNA pellet was resuspended in 80 µL RNase-free water and stored at -80ºC.

RNA amount and quality were assessed with a cuvette photometer (Biophotometer plus, Eppendorf AG, Hamburg, Germany) and also using 2100 Bioanalyzer Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California, USA). A_{260}/A_{280} ratio values

around 2 and A_{260}/A_{230} ratio values around 1.8 were considered acceptable. The RIN value of all samples was calculated when possible with the Bioanalyzer (RIN values in the range of 7.2-9.4 are considered acceptable). This ratio was impossible to measure in PV, EV and NR ovaries due to the high signal detected in the 5S rRNA region. In these cases the 28S/18S ratio was used as quality indicator, with values around 2 indicating good quality.

Electropherograms provided by the Bioanalyzer were also used to quantify the relative concentration of the bands corresponding to 5S rRNA and 18S rRNA in each sample. The Time Corrected Area of each rRNA peak was used to calculate the 5S/18S rRNA ratio and Log_2 of this value provided the 5S/18S rRNA index (Rojo-Bartolomé et al. 2016).

2.4. cDNA synthesis and quantitative PCR (qPCR) analysis

First-strand cDNA was synthesized using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) in the 2720 Applied Biosystems Thermal Cycler (Life Technologies). Retrotranscription was performed according to manufacturer's instructions using random primers using a maximum of 2 µg total RNA in a reaction volume of 20 µl (100 ng/µL final theoretical cDNA concentration).

The concentration of single stranded cDNA (ssDNA) was quantified by fluorescence in the Synergy HT Multi-Made Microplate Reader (BioTek, Winoosky, USA) using Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, Life Technologies). The quantification was run in triplicates, in a reaction volume of 100 µl, with a theoretical cDNA concentration of 0.2 ng/µL. The fluorescence was measured at 485/20 nm excitation and 528/20 nm emission wavelengths. Real cDNA concentration was calculated using the high-range standard curve according to the manufacturer's instructions. Once cDNA concentration was calculated, the exact amount of cDNA loaded in the qPCR reactions

was calculated adjusting the dilution used for each gene.

2.5. Gene transcription analyses by qPCR

Sequences for *A. anguilla ubtf1* (GeneBank Accession number: KX132907), *rpl5* (KU140416) and *rpl11* (KU140415) were obtained from the European eel transcriptome database available in our laboratory after pyrosequencing the multi-tissue eel transcriptome (data not published). 18S rRNA sequence was obtained from the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/) (FM946070). Information allowing to clone and sequence partial fragments of *A. anguilla gtf3a* (KX132905) and *42sp43* (KX132906) sequences were obtained from the European eel genome sequence repository (http://www.zfgenomics.com/sub/eel). Annotation of sequences was performed through homology search using the BlastN and BlastX analysis from NCBI (Table 1).

Primers 15-25 bp long were designed to obtain amplicons of around 200 bp. They were also designed to span exon-exon boundaries to avoid amplification of genomic DNA. The obtained primer sequences (Table 2) were evaluated for homo/hetero-dimers and hairpin formations using the IDT online primer design tool https://eu.idtdna.com/calc/analyzer). Primers were purchased from Eurofins MWG (https://www.eurofinsgenomics.eu/). To verify specificity of the primers, conventional PCRs were performed as follows: 94ºC for 2 min, denaturation at 94ºC for 30 s, annealing step (temperature for each primer set in table 2) for 30 s, elongation at 72ºC for 30 s. PCR was finalised at 72ºC for 8 min. PCR products were visualized in 1.5% (w/v) agarose gels stained with ethidium bromide.

Transcription levels of *gtf3a*, *ubtf1*, *42sp43*, *rpl5*, *rpl11* and 18S rRNA were determined using SYBR Green PCR Master Mix (Roche, Indianapolis, USA) in all 33 individuals analysed histologically. Optimal concentrations of primers (25 mM for *ubtf1* and 12.5

mM for other genes) and samples (20 ng/µL) were used for each gene. Samples were run in triplicates in a 7300 PCR thermal cycler (Applied Biosystems, Forster City, California, USA) using a final reaction volume of 20 µL, containing 2 µL of appropriately diluted sample. Reaction conditions were as follows: 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and annealing step of 60 s at appropriate temperature (Table 2). Amplification reaction was followed by a dissociation stage to obtain a dissociation curve, which would allow checking the specificity of each primer set and ensuring that only the specific transcript was amplified.

Transcription levels were normalized in reference to the amount of cDNA loaded for each sample as measured by fluorescence. All gene transcription results were normalized with the amount of cDNA charged in the qPCR according to Rojo-Bartolomé et al. (2016) using as adapted ΔCT formula (*RQ*) with eficiency correction (*E*):

$$E = \left[10^{-1/m}\right] - 1$$

m being the slope of the standard curve of the qPCR reaction.

$$RQ = Log_2\left[\frac{(1 + Eficiency)^{|\Delta CT|}}{ng\ cDNA}\right]$$

Where $\Delta CT = CT\ sample - CT\ plate\ internal\ control$

2.6. Statistical analysis

The statistical analyses were undertaken using SPSS (SPSS Inc., Chicago, Illinois, USA). For the statistical analysis data were tested for normality by the Shapiro-Wilk ($n<30$) test. Equality of variance was also tested applying the Levene's test, both at a 0.05 significance level ($p<0.05$). Then, data were subjected to analysis of variance by One-way ANOVA to identify significant differences between the ovarian developmental stages. The one-way ANOVA analyses were followed by a Tukey post-hoc test to compare all of the

groups in pairs. In the case of the 5S/18S rRNA index ANOVA could not be applied and non-parametric Kruskal-Wallis test was used ($p<0.05$).

3. Results

3.1. RNA analysis and 5S rRNA

RNA quality was checked by spectrophotometry and capillary electrophoresis. The mean value of A_{260}/A_{280} ratio was 1.71, with quite similar values for all the samples (Table 3). The total RNA quality assessment provided by the RIN algorithm could not be calculated for any of the PV and NR samples, and for some of the EV, MV and LV ones. In the cases when the RIN was measurable, only ovaries with LV and MN oocytes displayed values acceptable under the commonly used standards (Table 3).

In the less developed ovaries total RNA electrophoresis always showed a prominent band in the region around 120 nucleotides that corresponds to 5S rRNA. Meanwhile the typical bands for 18S rRNA and 28S rRNA were very faint, increasing its presence along vitellogenesis. Electropherograms provided by the Agilent 2100 Bioanalyzer allowed to distinguish these differences in transcript profiles for ovaries depending on the developmental stage of the oocytes they contained (Fig. 2). 5S rRNA peak was very high in ovaries with previtellogenic oocytes (PV and NR, Fig. 2A and 2F). Subsequently, 18S and 28S rRNA peaks progressively gained importance with respect to the 5S rRNA peak as vitellogenesis (EV<MV<LV; Fig 2B to 2D) advanced towards oocyte maturation (MN, Fig 2E).

3.2. 5S/18S rRNA index during oocyte development

The 5S/18S rRNA index, calculated measuring the area contained within the 5S rRNA and 18S rRNA specific peaks in the electropherograms, allowed distinguishing ovaries

according to their developmental stage (Fig. 3). The highest index values were shown by the ovaries with oocytes in PV stage, differing significantly from ovaries with oocytes at other developmental stages as a result of the high prevalence of 5S rRNA. The index value decreased as the oocytes entered vitellogenesis and started to produce 18S rRNA and 28S rRNA, till reaching final maturation. Some of the animals did not respond to the hormonal stimulus and did not progress from early oogenesis, this being reflected in a lack of activation of 18S rRNA production. In these NR samples, the 5S/18S rRNA index was high, resembling the index values of non-treated ovaries (Figs. 1A and 1F, 2A and 2F and 3). Therefore, after only 4 weeks of injection the 5S/18S rRNA index allows to identify that ovarian recrudescence cannot be triggered by the hormonal treatment in NR female eels.

3.3. Transcription levels of ribosome biogenesis related genes through ovary development

The transcription levels of genes related with the 5S rRNA production by RNA Polymerase III (*gtf3a*), cytosol stockpiling (*gtf3a* itself and *43sp42*) and nuclear transport for ribosome assembly (*rpl5* and *rpl11*) were measured by qPCR (Fig. 4A). All genes showed identical transcription pattern. The highest transcription levels in all the cases were recorded during early oocyte developmental stages (PV and EV). On the contrary, when the oocytes were mature, at MN stage, the lowest transcription levels were measured. NR individuals showed transcription levels among EV, MV or LV groups (Fig. 4).

Also, genes related with RNA Polymerase I activity and 18S rRNA production were studied transcriptionally (*ubtf1* and 18S rRNA) (Fig. 4B). In the case of *ubtf1*, the transcription levels followed the same trend observed for the genes related to 5S rRNA,

with decreasing levels during vitellogenesis. The NR group showed the same transcription levels of LV and MN groups. On the contrary, 18S rRNA transcription levels were at their lowest at PV stage and increased while oogenesis progressed to maturity, in accordance with observations in the total RNA electrophoretic analysis. Transcription levels in the NR individuals resembled those recorded in PV individuals (Fig. 4B).

4. Discussion

4.1. Ovarian development and 5S rRNA accumulation in European eel

Vitellogenesis in European eels inhabiting continental waters is blocked mainly due to dopaminergic inhibition (Dufour et al. 2003; Vidal et al. 2004). Thus, treatments with fish pituitary extract can artificially stimulate oocyte growth (Pasquier et al. 2011; Pérez et al. 2011). The electrophoretic analysis of total RNA extracted from eel ovaries showed that previous to initiation of vitellogenesis, oocytes accumulated high relative amounts of 5S rRNA in PV oocytes; whereas 18S and 28S rRNA progressively gained in importance as vitellogenesis advanced towards final oocyte maturation. This oocyte specific accumulation of 5S rRNA was first described in anuran frogs, where 5S rRNA can constitute 75% of the ovarian total RNA content (van den Eynde et al. 1989), and it has been also demonstrated in many teleost fish species with different ovarian maturation mechanisms; asynchronous vs synchronous (Diaz de Cerio et al. 2012; Rojo-Bartolomé et al. 2016).

These observations have important methodological implications when applied to the analysis of gene transcription profiles in fish ovaries. The gold standard for the analysis of the quality of total RNA is given by the RNA Integrity Number (RIN) obtained using the 2100 Bioanalyzer of Agilent Technologies. This value is obtained using the 28S to 18S rRNA ratio. Acceptable values for downstream qPCR analysis are above 7-7.8. In

our eel samples, with oocytes in stages MV to MN, RIN values were normally above 7. In contrast, in many samples with oocytes in PV or EV, RIN values could not be calculated or were below 7, due to the lack of prominent 18S/28S rRNA bands. We have previously seen in ovaries of different fish species that RIN values cannot be calculated when ovaries are in early oogenesis stages (Rojo-Bartolomé et al. 2016). Kroupova et al. (2011) and Manousaki et al. (2014) have also described this problem. Manousaki et al. (2014) concluded that it was not possible to calculate the RIN value in the ovaries of the proterandric fish *Diplodus puntazzo*. These authors mentioned specific efforts to improve sub-optimal RIN number values, due to the presence of a prevalent peak of around 100 nucleotides (5S rRNA). In the future, fish reproductive physiologists and endocrinologists should consider that RIN calculation is not representative of RNA quality in fish ovaries, especially in immature individuals.

4.2. 5S/18S rRNA index during oogenesis in European eels

As described in other teleost species, *Engraulis encrasicolus* and *Lepidorhombus whiffiagonis* (Rojo-Bartolomé et al. 2016), PV stages in eels displayed the highest 5S/18S rRNA index values, while ovaries containing MN oocytes ranked the lowest, due to the increased transcription of 18S rRNA during vitellogenesis. Therefore, 5S/18S rRNA index values calculated on electropherograms are diagnostic to identify quantitatively the developmental stage of the ovaries.

Oogenesis involves oocyte growth, meiosis, and synthesis and storage of organelles and new molecules (Song et al. 2006; Kleppe et al. 2014). This is evident in the case of 5S rRNA in PV oocytes. Ribosome biogenesis is dependent of rRNA synthesis, which is itself subjected to the availability of nutrients (Murray et al. 2003). Oocytes would need to accumulate rRNAs in order to quickly assemble ribosomes in case of being fertilized

and allow protein synthesis during early embryogenesis (Ortiz-Zarragoitia et al. 2014). It could be hypothesised that early production and stockpiling of 5S rRNA in PV oocytes occurs first due to the reduced energetic demand in comparison to the investment required for the production of the largest rRNA molecules. In this way, the oocytes would save energy during previtellogenesis until reproduction is envisaged to occur under favourable conditions (Rojo-Bartolomé et al. 2016). With the onset of the energetically expensive vitellogenesis, the production of the larger rRNA molecules would be also put in place. This is more evident in yellow eels that live in continental waters at prepubertal stage for years. Mechanistically, the present study suggests that blockade of vitellogenesis in European eels, also affects oocyte 18S and 28S rRNA production. Thus, gonadotropins (or the sex steroids induced by them) would release this inhibition during gonadal recrudescence.

On the other hand, these results fully disregard 18S rRNA as a useful housekeeping gene for qPCR results along fish oogenesis. In this sense, we consider that there are no adequate reference genes for the study of the dynamic transcriptional processes happening during oogenesis, so we recommend normalizing qPCR results to the exact amount of cDNA loaded per sample and amplification (Rojo-Bartolomé et al., 2016).

4.3. Transcription levels of genes controlling rRNA synthesis in European eel ovaries

5S rRNA is produced by RNA Polymerase III (Pol III) in eukaryotic cells. In turn, Pol III activation is controlled by Gtf3a (Szymanski et al. 2003). Additionally, Gtf3a also binds 5S rRNA for its stockpiling in the cytosol and it has been shown to be highly transcribed in ovaries in contrast to testis in fish (Rojo-Bartolomé et al. 2016). *gtf3a* transcripts also accumulate in ovaries of eels and, as it occurs in ovaries from megrim (Rojo-Bartolomé et al. 2016), transcription levels decreased at vitellogenesis allowing the ranking of the

ovarian developmental stages. PV individuals displayed the highest *gtf3a* transcription levels and MN ones the lowest. NR females, despite long hormonal treatment, showed *gtf3a* transcription levels similar to those observed in EV and LV. Thus, transcription of *gtf3a* seems somehow regulated by the hormonal treatment itself, although full regulation is a consequence of oocyte differentiation occurring after CPE treatment.

18S rRNA and other ribosomal RNAs, in contrast to 5S rRNA, are synthesised by RNA polymerase I (Pol I) which produces the 45S pre-rRNA in the nucleolus (Drygin et al. 2010). Ubtf1 is the transcription factor that enables the access of the Pol I machinery to the 45S rDNA genes (Bazett-Jones et al. 1994; Reeder et al. 1995). In cancer cells, where a high demand of ribosomes for protein production occurs, upregulation of *ubtf1* has been observed (Drygin et al. 2010). On the other hand, post-translational regulation of Ubtf1 activity by (de)phosphorylation and (de)acetylation processes has been defended to predominate (Russell and Zomerdijk, 2005). In the present study, qPCR analysis showed no match between the profiles of *ubtf1* and 18S rRNA transcription. In eel ovaries *ubtf1* transcript levels were at their highest during PV stage, when, as reported in *Xenopus*, Pol I activity would be very low (Roger et al. 2002). On the contrary, it was significantly downregulated during MN phase, when Pol I activity should be maximal, according to 18S rRNA levels in the present study and as reported for *Xenopus* oocytes (Ginsberg et al. 1984). As the production of 45S rRNA precursor must be fast before meiosis resumption, Pol I could be controlled mainly by post-translational modifications of Ubtf1. Thus, we propose that gonadotropins within CPE, or the ovarian estradiol production triggered by them, would regulate Pol I activation during secondary oocyte growth. In NR females the impossibility to respond to CPE would be reflected in an impossibility to activate Pol I and to produce 18S rRNA.

426 *4.4. Transcription levels of other genes involved in 5S rRNA subcellular localization*

427 It could be expected that genes related to ribosome biogenesis and 5S rRNA handling
428 would be also transcribed very early during oocyte development (Diaz de Cerio et al.
429 2012). For instance, 42sp43 forms 42S RNP storage particles with 5S rRNA in the cytosol
430 (Ciganda and Williams, 2011), and as previously observed in thicklip grey mullet ovaries
431 (Diaz de Cerio et al. 2012), *42sp43* showed the transcription pattern of 5S rRNA and
432 *gtf3a* during artificially induced eel maturation. *rpl5* and *rpl11* also showed the same
433 transcription pattern during maturation. The lowest levels were recorded in the most
434 mature ovaries, and the highest in ovaries with PV oocytes. NR females always showed
435 higher transcription levels than responding ones. It must be remembered that both
436 ribosomal proteins are responsible for the nuclear transportation and incorporation of 5S
437 rRNA into the nucleolus (Szymanski et al. 2003; Donati et al. 2013; Tang et al. 2015),
438 which is the main morphological and functional feature of fish perinucleolar PV oocytes.
439 The overall gene transcription profile hereby, with the exception of 18S rRNA, shows a
440 downregulation during oocyte development. It could be deduced that this downregulation
441 is a consequence of a general turning off of the transcription machinery, occurring with
442 whole chromatin assembly prior to final meiotic division in oocytes. However, fish
443 oocytes are known to be transcriptionally active until the MN stage, and many genes are
444 actively upregulated during vitellogenesis (Kleppe et al. 2014). In a microarray based
445 transcriptome comparison of Atlantic cod (*Gadus morhua*) follicles in different oogenesis
446 stages, including ovulated eggs, Kleppe et al. (2014) demonstrated a continuous pattern
447 of mRNA accumulation and degradation along oogenesis. In general, more transcripts
448 were downregulated than upregulated from PV to EV follicles (555 transcripts down *vs*
449 349 up), or from EV to LV stages (532 down *vs* 376 up), but transcription was still active
450 at LV. The general shut down of transcription occurred at ovulation (MN) but with still

149 upregulated genes (647 down). Studies performed with the same eel samples studied here, have demonstrated constant transcript levels of zona pellucida genes until the MN stage (Mazzeo et al. 2012, 2014).

Thus, we can conclude that 5S rRNA is highly expressed in European eel ovaries with the highest relative expression levels in ovaries with PV oocytes and the lowest in ovaries with MN oocytes. CPE, probably gonadotropins therein or the estrogens they induce to produce in the ovary, positively regulates vitellogenesis in European eel, controlling directly or indirectly also the inactivation of Pol III and the activation of the Pol I pathway. In female eels therefore, identification of a decrease in 5S/18S rRNA index and in *gtf3a* transcript levels could be taken as an indication of ovarian recrudescence and initiation of vitellogenesis. This could be a very useful tool in the research of eel reproduction, endocrinology and physiology. However, the observed regulation of ribosomal genes cannot be considered a direct consequence of CPE injections, but a consequence of the oocyte differentiation/maturation triggered by the treatment. NR females, treated during 8-12 weeks, never reach the gene transcription levels in the MN group. In any case, non-differentiated NR females show transcription levels most similar to MV suggesting that hormonal treatment in itself, has some transcription regulation role on such genes. Maybe full regulation of the genes requires induction of steroidogenesis in the ovary. This would explain that while *gtf3a* is downregulated to EV-LV values, 5S/18S rRNA index is still as high as in PV. Therefore, this index identifies NR females after only 4 weeks of injection. Long-term hormonal treatments are expensive, and maturing one single female eel can cost between 50-100 € (Mazzeo et al. 2014). To be able to predict that a batch of treated female eels will not be able to mature properly after 4-5 injections could save a considerable amount of money. In conclusion, this study provides important basic knowledge on the transcriptional regulation of ribosomal genes during oocyte

development in fish and could have important implications for the study of European eel endocrinology in basic research and, although based on an invasive technical approach, also in a future aquaculture production context.

Declaration of interest: The authors declare that there is no conflict of interest that could be prejudicing the impartiality of the research reported.

Acknowledgements. This work was supported by EU 7th Framework Program (PRO-EEL, grant agreement n°245257 & AQUAGAMETE COST Action FA1205), Spanish MINECO (AGL2012-33477 and AGL2015-63936-R), Basque Government (S-PE12UN086 & IT810-13) & UPV/EHU (UFI 11/37).Some fish were supplied by the Hunting and Continental Fishing Service of Generalitat Valenciana. I.R.B holds a PhD fellowship of the Basque Government.

References

Allison, L.A., North, M.T., Neville, L.A., 1995. Differential binding of oocyte-type and somatic-type 5S rRNA to TFIIIA and ribosomal protein L5 in *Xenopus* oocytes specialization for storage versus mobilization. Dev. Biol. 168, 284-295.

Asturiano, J.F., Pérez, L., Garzón, D., Peñaranda, D.S., Jiménez, F.M., Martínez-Llorens, S., Tomás A., Jover, M. 2005. Effect of different methods for the induction of spermiation on semen quality in European eel. Aquat. Res. 36, 1480-1487.

Bazett-Jones, D.P., Leblanc, B., Herfort, M., Moss, T., 1994. Short-range DNA looping by the *Xenopus* HMG-box transcription factor, xUBF. Science 264, 1134-1137.

499 Boëtius, I., Boëtius, J., 1980. Experimental maturation of female silver eels, *Anguilla*
500 *anguilla* Estimates of fecundity and energy reserves for migration and spawning.
501 Dana 1, 1–28.

502 Bruijs, M.C.C., Durif, C.M.F. 2009. Silver eel migration and behaviour. In: van den
503 Thillart, G.E.E.J.M., Dufour, S., Rankin, J.C., (eds). Spawning Migration of the
504 European eel, Vol 30 pp 65-95. Netherlands: *Springer*.

505 Butts, I.A.E., Sørensen, S.R., Politis, S., Pitcher, T.E., Tomkiewicz, J. 2014.
506 Standardization of fertilization protocols for the European eel, *Anguilla anguilla.*
507 Aquaculture 426-427, 9-13.

508 Ciganda, M., Williams, N., 2011. Eukaryotic 5S rRNA biogenesis. *Wiley*
509 *Interdisciplinary Reviews RNA* 2, 523–533

510 Diaz de Cerio, O., Rojo-Bartolomé, I., Bizarro, C., Ortiz-Zarragoitia, M., Cancio, I. 2012.
511 5S rRNA and accompanying proteins in gonads: powerful markers to identify sex
512 and reproductive endocrine disruption in fish. Environ. Sci. Technol. 46, 7763-
513 7771.

514 Dirks R.P., Burgerhout E., Brittijn S.A., de Wijze D.L., Ozupek H., Tuinhof-Koelma N.,
515 Minegishi Y., Jong-Raadsen S.A., Spaink H.P., van den Thillart G.E.E.J.M., 2014.
516 Identification of molecular markers in pectoral fin to predict artificial maturation
517 of female European eels (*Anguilla anguilla*). Gen. Comp. Endocrinol. 204, 267-
518 276.

519 Donat,i G., Peddigari, S., Mercer, C.A., Thomas, G., 2013. 5S ribosomal RNA is an
520 essential component of a nascent ribosomal precursor complex that regulates the
521 Hdm2-p53 checkpoint. Cell Rep. 4, 87-98.

522 Drygin, D., Rice, W.G., Grummt, I., 2010. The RNA polymerase I transcription
523 machinery: An emerging target for the treatment of cancer. Ann. Rev. Pharmacol.
524 Toxicol. 50, 131-156.

525 Dufour, S., Burzawa-Gerard, E., Le Belle, N., Sbaihi, M., Vidal, B., 2003. Reproductive
526 endocrinology of the European eel, *Anguilla anguilla.* In *Eel Biology,* pp 373-386.
527 Eds K Aida, K Tsukamoto and K Yamauchi. Tokyo: *Springer.*

528 Ginsberg, A.M., King, B.O., Roeder, R.G., 1984. *Xenopus* 5S gene transcription factor,
529 TFIIIA Characterization of a cDNA clone and measurement of RNA levels
530 throughout development. Cell 39, 479-489.

531 ICES 2013. Report of the 2013 Session of the Joint EIFAC/ICES Working Group on
532 Eels (WGEEL) ICES CM 2013/ACOM: 18.

533 Ijiri, S., Tsukamoto, K., Chow, S., Kurogi, H., Adachi, S., Tanaka, H., 2011. Controlled
534 reproduction in the Japanese eel (*Anguilla japonica*), past and present. Aquaculture
535 *Europe.* 36, 13–17.

536 Kleppe, L., Edvardsen, R.B., Furmanek, T., Taranger, G.L., Wargelius, A., 2014. Global
537 transcriptome analysis identifies regulated transcripts and pathways activated
538 during oogenesis and early embryogenesis in Atlantic cod. Mol. Reprod. Dev. 81,
539 619-635.

540 Kroupova, H., Trubiroha, A., Wuertz, S., Kloas, W., 2011. Stage-dependent differences
541 in RNA composition and content affect the outcome of expression profiling in
542 roach (*Rutilus rutilus*) ovary. Comp. Biochem. Physiol. 159A, 141–149.

543 Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010.
544 Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol.,
545 165 412–437

546 Lyman-Gingerich, J., Pelegri, F., 2007. Materal factors in fish oogenesis and embryonic
547 development. In *The Fish Oocyte,* pp 142-149. Eds P.F. Babin, J. Cerdà, E-
548 Lubzens.
549 Mairy, M., Denis, H., 1971. Biochemical studies on oogenesis. I. RNA synthesis and
550 accumulation during oogenesis of the South African toad *Xenopus laevis.* Dev.
551 Biol. 24, 143-165.
552 Manousaki, T., Tsakogiannis, A., Lagnel, J., Sarropoulou, E., Xiang, J.Z.,
553 Papandroulakis, N., Mylonas, C.C., Tsigenopoulos, C.S., 2014. The sex-specific
554 transcriptome of the hermaphrodite sparid sharpsnout seabream (*Diplodus*
555 *puntazzo*). BMC Genomics 15, 655.
556 Mazzeo, I., Peñaranda, D,S,, Gallego, V,, Hildahl, J,, Nourizadeh-Lillabadi, R,,
557 Asturiano, J.F., 2012. Variations in the gene expression of zona pellucida proteins,
558 zpb and zpc, in female European eel (*Anguilla anguilla*) during induced sexual
559 maturation. Gen. Comp. Endocrinol. 178, 338-346.
560 Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-lillabadi, R., Tveiten,
561 H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature
562 modulates the progression of vitellogenesis in the European eel. Aquaculture 434,
563 38-47.
564 Murray, H.D., Schneider, D.A., Gourse, R.L., 2003. Control of rRNA expression by
565 small molecules is dynamic and nonredundant. Mol. Cell. 12:125-134.
566 Okamura, A., Horie, N., Mikawa, N., Yamada, Y., Tsukamoto, K., 2014. Recent
567 advances in artificial production of glass eels for conservation of anguillid eel
568 populations. Ecol. Fresh. Fish. 23, 95-110.

569 Ortiz-Zarragoitia, M., Bizarro, C., Rojo-Bartolomé, I., Diaz de Cerio, O., Cajaraville,
570 M.P., Cancio, I., 2014. Mugilid fish are sentinels of exposure to endocrine
571 disrupting compounds in coastal and estuarine waters. Mar. Drugs 12, 4756-4782.
572 Palstra, A.P., Cohen, E.G.H., Niemantsverdriet, P.R.W., van Ginneken, V.J.T., van den
573 Thillart, G.E.E.J.M., 2005. Artificial maturation and reproduction of European
574 silver eel: development of oocytes during final maturation. Aquaculture 249, 533-
575 547.
576 Palstra, A.P., van den Thillart, G.E.E.J.M., 2010 Swimming physiology of European
577 silver eels (*Anguilla anguilla* L.): energetic costs and effects on sexual maturation
578 and reproduction. Fish Physiol. Biochem. 36, 297-322.
579 Pasquier, J., Lafont, A.G., Leprince, J., Vaudry, H., Rousseau, K., Dufour, S., 2011. First
580 evidence for a direct inhibitory effect of kisspeptins on LH expression in the eel,
581 *Anguilla anguilla.* Gen. Comp. Endocrinol. 173, 216-225.
582 Pedersen, B.H., 2004. Fertilisation of eggs, rate of embryonic development and hatching
583 following induced maturation of the European eel *Anguilla anguilla.* Aquaculture
584 237, 461-473.
585 Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S.,
586 Asturiano, J.F., 2010. Molecular and physiological study of the artificial
587 maturation process in European eel males: from brain to testis. Gen. Comp.
588 Endocrinol. 166, 160-171.
589 Penberthy, W.T., Griffin, D., Hall, R.K., Taylor, W.L., 2003. The *Xenopus* B2 factor
590 involved in TFIIIA gene regulation is closely related to Sp1 and interacts in a
591 complex with USF. Gene 305, 205-215.
592 Pérez. L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., van den Thillart,
593 G.E.E.J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine

parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol. 174, 51-59.

Picard, B., le Maire, M., Wegnez, M., Denis, H., 1980. Biochemical research on oogenesis. Composition of the 42-S storage particles of *Xenopus laevis* oocytes. Eur. J. Biochem. 109, 359-368.

Pujolar, J.M., Marino, I.A.M., Milan, M., Coppe, A., Maes, G.E., Capoccioni, F., Ciccotti, E., Bervoets, L., Covaci, A., Belpaire, C., Cramb, G., Patarnello, T., Bargelloni, L., Bortoluzzi, S., Zane, L., 2012. Surviving in a toxic world: transcriptomics and gene expression profiling in response to environmental pollution in the critically endangered European eel. BMC Genomics 13, 507.

Reeder, R.H., Pikkard, C.S., McStay, B., 1995. UBF, an architectural element for RNA polymerase I promoters In: Eckstein F, Lilley DMJ (eds) Nucleic Acids and Molecular Biology, Vol 9, pp 251-263. Verlag: Springer.

Roger, B., Moisand, A., Amalric, F., Bouvet, P., 2002. rDNA Transcription during *Xenopus laevis* oogenesis. Biochem. Biophys. Res. Comm. 290, 1151-1160.

Rojo-Bartolomé, I., Diaz de Cerio, O., Diez, G., Cancio, .I, 2016. Identification of sex and female's reproductive stage in commercial fish species through the quantification of ribosomal transcripts in gonads. Plos ONE 11, e0149711.

Russell, J., Zomerdijk, J., 2005. RNA-polymerase-I-directed rDNA transcription, life and works. Trends Biochem. Sci. 30, 2.

Song, J.L., Wessel, G.M., 2005. How to make an egg: transcriptional regulation in oocytes. Differentiation 73, 1-17.

Song, J.L., Wong, J.L., Wessel, G.M., 2006. Oogenesis: Single cell development and differentiation. Dev. Biol. 300, 385-405.

618 Sørensen, S.R., Skov, P.V., Lauesen, P., Tomkiewicz, J., Bossier, P., Schryver, P.D.,
619 2014. Microbial interference and potential control in culture of European eel
620 (*Anguilla anguilla*) embryos and larvae. Aquaculture 42, 426-427.

621 Stefanovsky, V.Y., Moss, T., 2008. The splice variants of UBF differentially regulate
622 RNA polymerase I transcription elongation in response to ERK phosphorylation.
623 Nucleic Ac. Res. 36, 5093-5101.

624 Szymański, M., Barciszewska, M.Z., Erdmann, V.A., Barciszewski, J., 2003. 5S rRNA:
625 structure and interactions. Biochem. J. 371, 641-651.

626 Tanaka, H., Kagawa, H., Ohta, H., Unuma, T., Nomura, K., 2003. The first production
627 of glass eel in captivity: fish reproductive physiology facilitates great progress in
628 aquaculture. Fish Physiol. Biochem. 28, 493-497.

629 Tang, J., Di, J., Cao, H., Bai, J., Zheng, J., 2015. p53-mediated autophagic regulation: A
630 prospective strategy for cancer therapy. Cancer Lett. 363, 101-107.

631 van den Eynde, H., Mazabraud, A., Denis, H., 1989. Biochemical research on oogenesis.
632 RNA accumulation in the oocytes of the newt *Pleurodeles waltl.* Development 106,
633 11-16.

634 van Ginneken, V.J.T., Maes, G.E., 2005. The European eel (*Anguilla anguilla*,
635 Linnaeus), its lifecycle, evolution and reproduction: a literature review. Rev. Fish
636 Biol. Fish. 15, 367-398.

637 Vidal, B., Pasqualini, C., Le Belle, N., Holland, M.C.H., Sbaihi, M., Vernier, P., Zohar,
638 Y., Dufour, S., 2004. Dopamine inhibits luteinizing hormone synthesis and release
639 in the juvenile European eel: a neuroendocrine lock for the onset of puberty. Biol.
640 Reprod. 71, 1491-1500.

641 Zhang, W.Q., Romaniuk, P.J., 1995. Characterization of the 5S RNA binding activity of
642 *Xenopus* zinc finger protein p43. J. Mol. Biol. 245, 549-558.

643 Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of
644 reproduction in teleost fish. Gen. Comp. Endocrinol 165, 438-455.

645

646 **Legends**

647 **Figure 1. European eel ovarian developmental stages as analysed through ovary**
648 **histology.** Micrographs illustrate the developmental stage of oocytes in the eel ovaries
649 along the carp pituitary extract (CPE) treatment. (a) Ovary with previtellogenic (PV)
650 oocytes in perinucleolar stage in a control female that received no CPE injections. (b)
651 Ovary with oocytes in early-vitellogenesis (EV) stage after 4 CPE injections. (c) Oocytes
652 in MV stage after 8 CPE injections. (d) Late-vitellogenesis (LV) stage oocytes after 12
653 CPE injections. (e) Ovary with oocytes in nuclear migratory (NM) stage (12 CPE
654 injections). (F) Ovary after 8 CPE injections in a NR female showing previtellogenic
655 stage oocytes. Scale bars: a, c, d, e and f=100 µm; b=50 µm.

656

657 **Figure 2. Total RNA extracted from eel ovaries at different developmental stages**
658 **after hormonal treatment and analyzed through capillary electrophoresis using the**
659 **2100 Agilent Bionalyzer.** (a) Electropherogram corresponding to an ovary with oocytes
660 in previtellogenic (PV) stage with a prominent peak (high RNA concentration) belonging
661 to 5S rRNA. 18S and 28S rRNA levels are so low that they can be hardly recognised. (b,
662 c and d) Electropherograms of ovaries with oocytes at early- (b), mid- (c) and late-
663 vitellogenic (d) stages. (e) Electropherogram of an ovary with oocytes in nuclear
664 migratory (NM) stage. (f) Ovary from a non-responding (NR) female with PV oocytes.

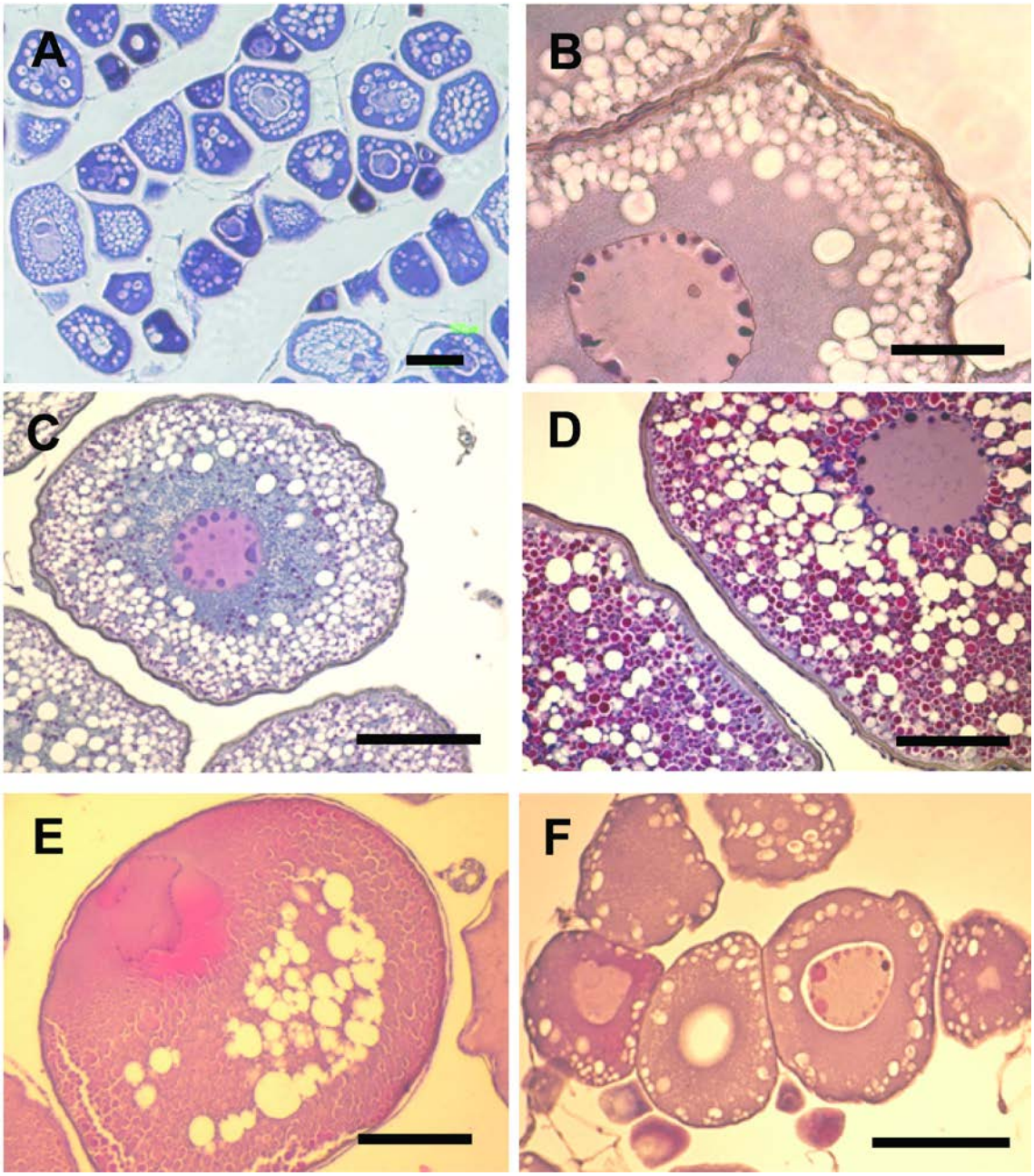
665

666 **Figure 3. 5S/18S rRNA index during the European eel ovarian development as**
667 **calculated from the total RNA electropherograms.** Each dot identifies the index value

of one individual. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p<0.05$). The number of carp pituitary extract injections received by each individual is indicated "x number". In the non-responding (NR) group, 3 of the eels received 8 injections while the forth one received 12 injections.

Figure 4. Box plots representing relative quantification of the transcription levels of different genes related to the biogenesis of ribosomes in ovaries of European eels injected with CPE. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. (a) Transcript levels of 5S rRNA related genes (*gtf3a, 42sp43, rpl5 and rpl11*) (b) Transcript levels of RNA Polymerase I related genes (*ubtf1* and 18S rRNA). Different letters indicate significant differences between means (ANOVA, $p<0.05$).

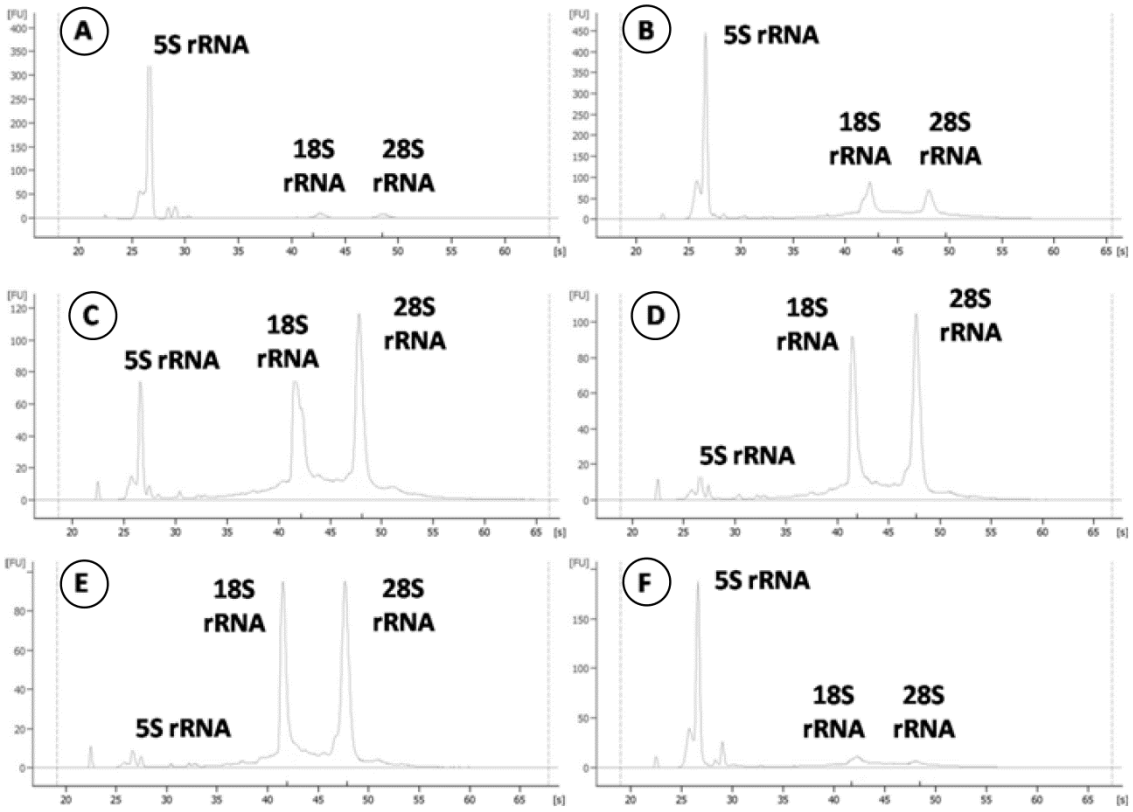
684 Fig. 1.



685

686

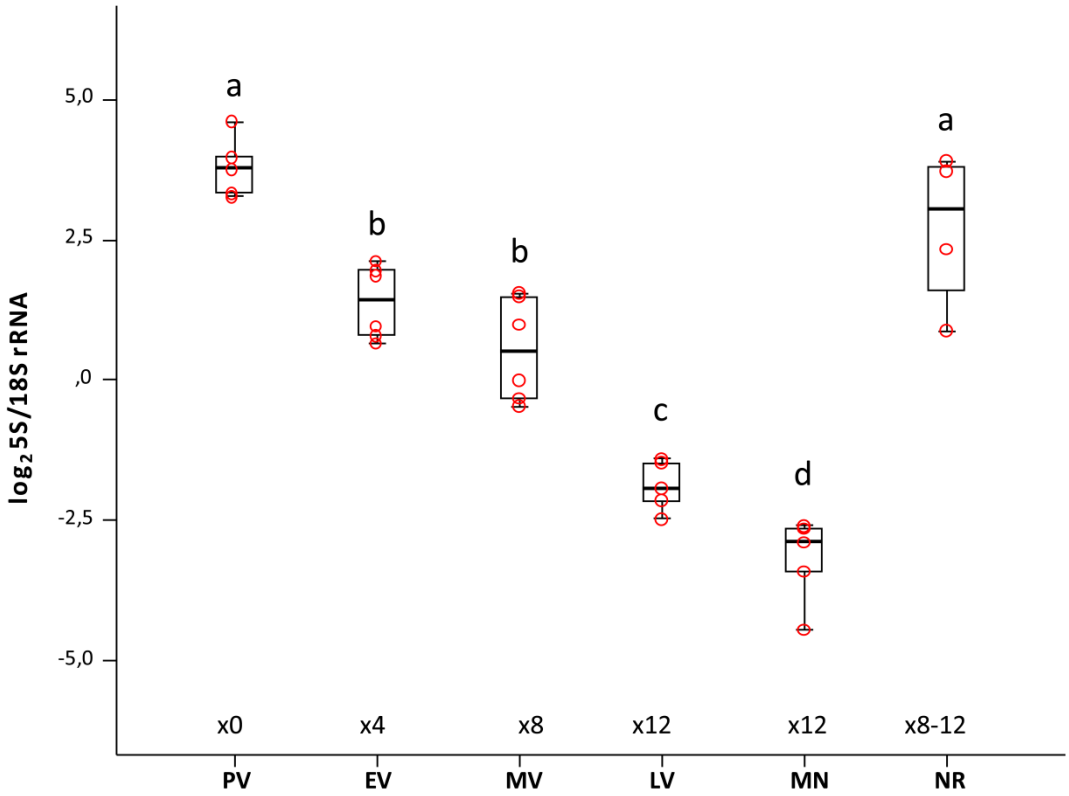
687 Fig.2



688

689

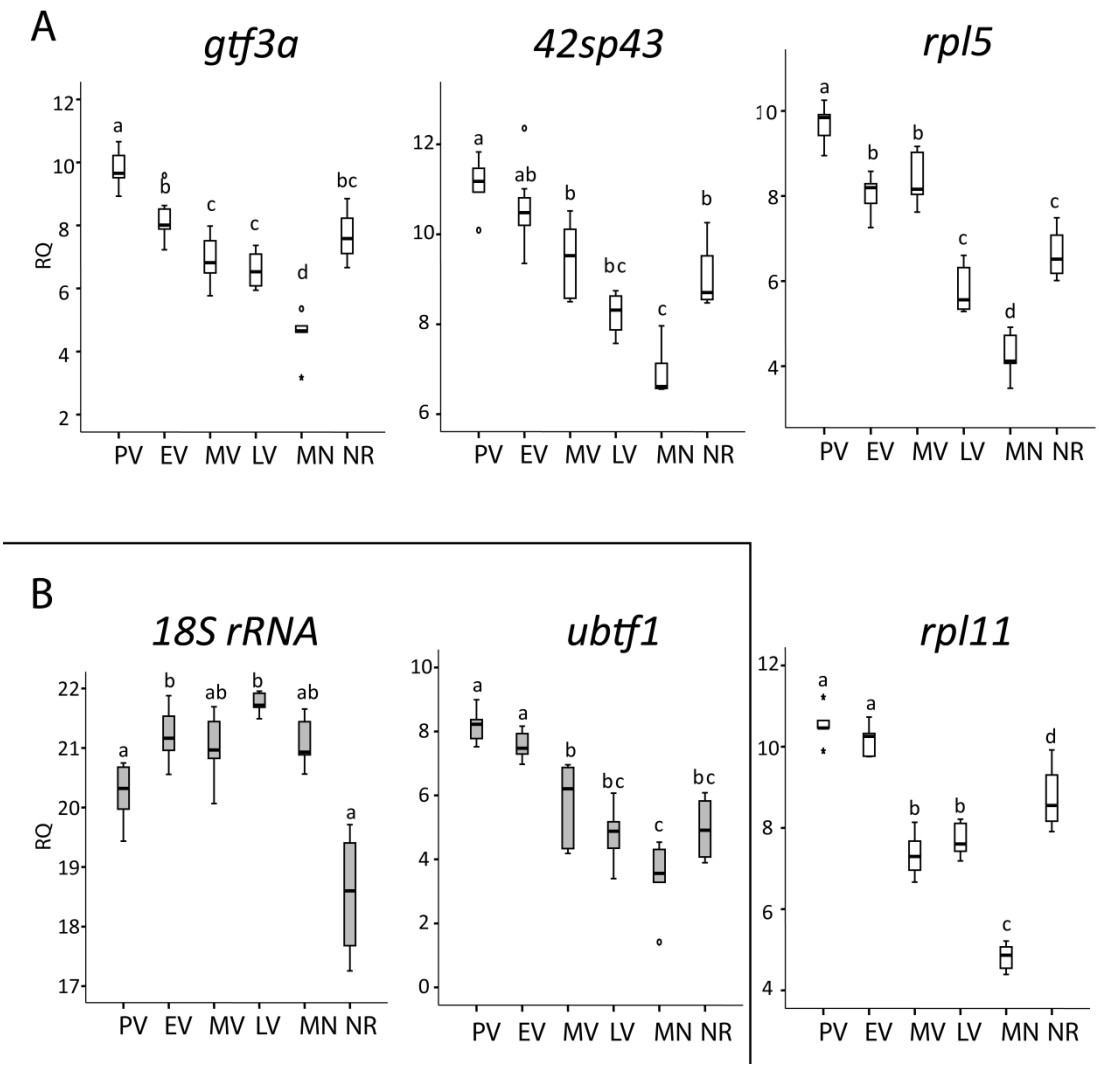
690 Fig. 3



691

692

693 Fig. 4


A
gtf3a
42sp43
rpl5
RQ
PV EV MV LV MN NR
B
18S rRNA
ubtf1
rpl11
RQ
PV EV MV LV MN NR

694

Tables

Table 1 European eel sequences cloned and sequenced for this study and identity values (through Blastx) with the most similar ortholog sequences available in GenBank. The percentage of the sequenced total gene cds is provided for each gene.

Gene name & NCBI accession number	Aminoacid identity (%)	E value (Blastx)	Most similar ortholog in GenBank	CDS%
A. anguilla upstream binding factor 1 (*ubtf1*) KX132907	97	2E-145	Upstream binding transcription factor, RNA polymerase I [*Danio rerio*] NP_001005395	73
A. anguilla transcription factor III A (*gt3a*) KX132905	62	E-116	General transcription factor IIIAa [*Danio rerio*] AAH95553	97
A. anguilla 42sp43 KX132906	64	3E-143	PREDICTED: P43 5S RNA-binding protein-like [*Esox lucius*] XP_012991469	96
A. anguilla ribosomal protein l5 (*rpl5)* KU140416	95	0	60S ribosomal protein L5 [*Salmo salar*] ACI66198	96
A. anguilla ribosomal protein l11 (*rpl11*) KU140415	99	2E-54	60S ribosomal protein L11 [*Platichthys plesus*] CAH57695	92

Table 2 Primer sequences used for the qPCR analysis in European eel ovaries.

Gene	Forward sequence (5'-3')	Reverse sequence(5'-3')	Amplicon size (bp)	Annealing temp (°C)
18SrRNA	GAGGCCCTGTAATTGGAATGAG	TAATATACGCTATTGGAGCTGGAATT	110	60
42sp43	CCTGCTTCTCCACCACCTT	CAGACTCTCCTGCATGGCA	124	58
gtf3a	AGGGTTGCGACAAGAGTTTCTGC	GAACACTTTTCCACAGCCCTCATA	214	61
rpl5	AGCAGTTCTCCCGCTTCAT	GACTGGGTTCTCACGGATA	96	56
rpl11	ATCGGCATCTACGGCTTGGA	GCCTCCTCCTTGCGGATG	119	59
ubtf1	ACCACTGCTAAAGATCAAGCCTG	CTGCAGAGTAGTGATTGAATGCC	154	61

Table 3 RNA quality parameters as they were estimated spectrophotometrically measuring the absorbance at A_{260}/A_{280} and at A_{260}/A_{230} and as measured through the calculation of the RNA Integrity Number (RIN) after capillary electrophoresis in the 2100 Bioanalyzer.

Sample	A_{260}/A_{280}	A_{260}/A_{230}	RIN
PV1	1.85	2.22	-
PV2	1.89	2.03	-
PV3	1.89	1.97	-
PV4	1.76	2.35	-
PV5	1.83	1.89	-
EV1	1.80	2.14	2.5
EV2	1.61	0.56	4.9
EV3	1.80	0.97	5.5
EV4	1.89	0.37	2.8
EV5	1.73	1.56	-
EV6	1.65	1.98	-
EV7	1.79	2.08	-
EV8	1.63	1.36	5.5
MV1	1.71	2.06	-
MV2	1.65	2.03	7.4
MV3	1.68	1.89	-
MV4	1.63	2.23	-
MV5	1.74	1.54	8.7
MV6	1.75	1.84	8.2
LV1	1.70	1.04	2.4
LV2	1.63	1.31	8.7
LV3	1.63	2.05	8.5
LV4	1.55	2.05	8.8
LV5	1.61	1.19	9.1
LV6	1.63	1.42	8.9
LV7	1.67	1.65	-
MN1	1.60	1.18	9.4
MN2	1.89	1.98	7.2
MN3	1.75	1.87	9.0
MN4	1.69	1.67	9.3
MN5	1.70	1.65	9.0
NR1	1.63	1.78	-
NR2	1.59	2.30	-
NR3	1.59	2.30	-
NR4	1.68	1.20	-