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

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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.

1. Introduction

The European eel stock has been in gradual decline for at least half a century (van Ginneken and Maes, 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 Ginneken and Maes, 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 regard, Tanaka and coworkers were able to obtain leptocephali larvae of Japanese eel (*Anguilla japonica*) in captivity in 2003, taking them through metamorphosis to obtain glass eels (Tanaka et al., 2003; 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.

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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 and Durif, 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 and Wessel, 2005; Lyman-Gingerich and Pelegri, 2007). Ribosomes are formed by the assembly of ribosomal rRNAs (28S, 18S, 5.8S and 5S rRNA) and ribosomal proteins (Rpl) (Lyman-Gingerich and Pelegri, 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 and Romaniuk, 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; Szymański 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 °C, 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. 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