Comparative Biochemistry and Physiology, Part A 221 (2018) 44–54

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

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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ARTICLE INFO

Keywords:
Estrogen receptors
Gonadotropin receptors
Lipid droplet
Follicular maturation
Egg quality

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 the administration of a 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) injection. 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.

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

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https://doi.org/10.1016/j.cbpa.2018.03.010
Received 12 October 2017; Received in revised form 15 March 2018; Accepted 15 March 2018
Available online 26 March 2018

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 et al., 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, 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 × 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 to 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