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3 **Targeted gene expression profiling in European sea bass (*Dicentrarchus labrax*, L.)**
4 **follicles from primary growth to late vitellogenesis**

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19 **Abstract**

20 A real-time PCR-based gene expression survey was performed on isolated European sea
21 bass follicles from primary growth to late vitellogenesis. Expression levels of 18
22 transcripts with demonstrated relevance during oogenesis, encoding gonadotropin,
23 thyrotropin, estrogen, and androgen, and vitellogenin receptors, steroidogenesis-related as
24 well as growth and transcription factors were measured. Primary oocytes showed high
25 mRNA levels of *insulin-like growth factors 1* and *2*, *bone morphogenetic protein 4*,
26 *estrogen receptor 2b*, *androgen receptor b*, and *SRY-box containing gene 17* together
27 with low transcript amounts of gonadotropin receptors. Follicles at the lipid vesicles
28 stage (*i.e.*, the beginning of the secondary growth phase) showed elevated mRNA
29 amounts of *follicle stimulating hormone receptor (fshr)* and *anti-Mullerian hormone*.
30 Early-to-mid vitellogenic follicles showed high mRNA levels of *fshr* and *cytochrome*
31 *P450, family 19, subfamily A, polypeptide 1a* while mid-to-late vitellogenic follicles
32 expressed increasing transcript amounts of *luteinizing hormone/choriogonadotropin*
33 *receptor*, *steroidogenic acute regulatory protein*, and *estrogen receptors 1* and *2a*. The
34 molecular data presented here may serve as a solid base for future studies focused on

unravelling the specific mechanisms orchestrating follicular development in teleost fish.

Keywords: Teleost fish; ovary; follicle; folliculogenesis; oogenesis; gene expression.

1. Introduction

Oogenesis, *i.e.* the developmental process by which an oogonium becomes a fully-grown, mature, and fertilizable oocyte, takes place within the so-called “follicle complex” consisting of the oocyte, inner surrounding follicular or granulosa cells, outer theca layers (including blood vessels and extracellular matrix), and a basement membrane between granulosa and theca cells (Grier, 2000).

Oogenesis in teleost fish is divided in several successive steps including: (1) the formation of primordial germ-cells and oogonia, (2) the transformation of oogonia into primary oocytes with the onset of meiosis (also regarded as primary growth phase), (3) the secondary growth phase characterized by a significant oocyte enlargement due to the accumulation of lipids and yolk proteins (vitellogenesis) required for embryogenesis, (4) maturation characterized by the resumption of meiosis, germinal vesicle breakdown, lipid and yolk coalescence, and hydration (in pelagophil oocytes), and (5) ovulation (Patiño and Sullivan, 2002).

The process of oogenesis is controlled by an intricate regulatory network outside and within the follicle complex mainly comprising: (1) gonadotropin releasing hormone at brain level, (2) the pituitary gonadotropins, *i.e.* follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act directly at ovarian level regulating follicular functions, (3) steroid hormones acting both in the follicular environment as paracrine/autocrine factors and in extra-ovarian sites as endocrine hormones, and (4) peptide growth factors released from both the somatic components of the follicle complex and from the oocyte itself which exert their roles in a paracrine/autocrine fashion (Ge, 2005; Clelland and Peng, 2009; Lubzens et al., 2010; Zohar et al., 2010). Although intense research efforts at endocrine, cellular, and molecular levels have increased our knowledge about the specific roles of all these players (see references above), oogenesis is such a dynamic and complex physiological process that our current understanding of its regulation still has important gaps.

Different studies have investigated multiple aspects concerning the endocrine and molecular control of oocyte development and reproductive performance in European sea bass (*Dicentrarchus labrax*), one of the most important commercial teleost fish cultured

in the Mediterranean area (Zanuy et al., 2001; Taranger et al., 2010). To our knowledge, most of these studies were based on the assessment of gene expression levels in whole ovarian tissue samples collected at different moments along the reproductive cycle (*e.g.* Halm et al., 2008; Ibáñez et al., 2008; Rocha et al., 2009). However, the ovary of European sea bass has simultaneously several cohorts of follicles at different developmental stages, *i.e.* it shows a group-synchronous ovarian development (Mayer et al., 1990). Since expression levels of a particular gene are likely to depend on the developmental stage of the follicle, total ovarian gene expression will reflect the whole gonadal follicle composition. Hence, the objective of the current study is to analyze mRNA levels of multiple oogenesis-relevant transcripts, including gonadotropin receptors, steroid-related genes, and growth factors in homogeneous groups of follicles from primary growth until the end of vitellogenesis in order to determine their developmental expression profiles during European sea bass folliculogenesis.

2. Material and Methods

2.1. Fish and general rearing conditions

Adult female and male sea bass (3-4 years old) were maintained in 5 m³ tanks under natural photoperiod (Puerto Real, Cádiz, Spain; '36° 32' N, 06° 17'W) and approximately constant temperature (18-20 °C). Fish were fed *ad libitum* on commercial dry pellets (Vitalis Repro, Skretting; Burgos, Spain) three days a week. Fish euthanasia was accomplished by 2-phenoxyethanol overexposure (600 ppm) followed by decapitation. All experimental procedures involving care and use of live animals were carried out according to Spanish national and European bioethical regulations and were approved by the CSIC Bioethical Committee.

2.2. Isolation of ovarian follicles at different developmental stages

Ovarian follicles were collected from three mature females in December, *i.e.* at the peak of vitellogenesis (Prat et al., 1999). At this time, European sea bass ovaries contain a high number of follicles at all developmental stages from primary growth to late vitellogenesis (see below) and hence, ensuring sufficient sampling material. After dissection, ovaries from the different females were split in small pieces, mixed, and stored at 4°C in Allprotect™ Tissue Reagent (Qiagen; Hilden, Germany). Ovary pieces were then transferred to a culture dish and follicles at different developmental stages were manually isolated with watchmaker's forceps under a dissecting microscope

equipped with an ocular micrometer. Follicles were classified into 5 stages according to size and cytoplasm appearance (Mayer et al., 1988; Sorbera et al., 1999) (Fig. 1): (1) primary growth (Pg) stage consisting of chromatin nucleolar and perinucleolar follicles with a diameter lower than 120 μm ; (2) lipid vesicles stage (Lv) follicles with a diameter between 120 and 250 μm ; (3) early vitellogenic (Evit) follicles with a diameter between 250 and 400 μm ; (4) mid vitellogenic (Mvit) follicles with a diameter from 400 to 600 μm of diameter; and (5) late vitellogenic (Lvit) follicles with a diameter between 600 and 800 μm . Three different ovarian follicle pools each one containing around 100-150 isolated follicles were collected and processed for gene expression analysis.

2.3. Gene expression analyses

Total RNA was extracted from pools of isolated follicles using the NucleoSpin® RNA XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions (including an on-column DNase digestion). RNA integrity was checked using the Agilent RNA 6000 Nano kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), while RNA quantity and purity were analyzed spectrophotometrically. Average total RNA yield, ratio of absorbance at 260 and 280 nm in water, and RIN (RNA integrity number; Schroeder et al., 2006) for the different samples analyzed were 21.6 μg (range: 10.5-54.7 μg), 1.65 (range: 1.55-1.78), and 8.4 (range: 7.3-9.8), respectively. These parameters did not show any pattern of variation across different developmental stages (not shown).

For quantification of gene expression levels across follicular developmental stages, real-time PCR analysis using the relative standard curve method was carried out (for details see the Mastercycler ep realplex² Instruction Manual and Applied Biosystems, User Bulletin #2). After RNA extraction, a total RNA pool composed of equal sample amounts of different follicle stages was prepared to serve as standard. For each sample and for the standard RNA pool, 500 ng of total RNA was reverse-transcribed into cDNA in 20 μl reactions using the qScript™ cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Reverse transcription product from each sample was diluted 1/40 in water, and 8 μl was used for every real-time PCR. For the standard RNA pool, reverse transcription product was serially diluted in water to produce a standard cDNA curve with four points from 1/10 to 1/640 dilutions (4-fold dilution steps); 8 μl of each dilution was used for each standard curve real-time PCR. Reactions were performed in duplicate (final reaction volume: 20 μl) in a Mastercycler ep realplex² (Eppendorf,

Hamburg, Germany) using HPLC-purified primer pairs (Biomers, Ulm, Germany) specifically designed for each target gene (Supplemental Table 1) and the PerfeCTaTM SYBR® Green FastMixTM reaction mix (Quanta Biosciences). PCR conditions were: initial denaturation at 95°C for 5 min, 40 amplification cycles with one step at 95°C for 15 sec followed by a second step at 60°C for 30 sec. The absence of amplification in no template controls refuted the presence of contaminating genomic DNA (not shown). Melting curve analysis performed just after PCR indicated the specific amplification of individual products for each primer pair combination (not shown). PCR amplification efficiency (mean: 97.3±1.2%) was measured using the serially diluted cDNA standard curve, which was also used to calculate the amount of input RNA for every sample. Two endogenous reference genes previously used in European sea bass ovary, namely *18S ribosomal RNA (18s)* and *elongation factor 1-alpha (ef1a)* (e.g. Blázquez et al., 2008; Rocha et al., 2009) were measured but their expression levels showed significant variations among the different oocyte developmental stages studied (Fig. 2). This rendered both housekeeping genes unsuitable for the normalization procedures routinely used. In addition, other common reference genes, like *bactin* or *28S ribosomal RNA*, have already been proved not to be suitable for their use in European sea bass ovarian samples (Halm et al., 2008). Therefore, transcript levels calculated from the standard curve were analyzed in two ways: (1) non-normalized and expressed as raw arbitrary input amounts, and (2) input amount normalized against the adjusted expression of each endogenous reference gene measured. The second method involves the standardization of the reference gene expression by dividing individual values within a group by the quotient of dividing the mean value within a group by the mean value of a control group randomly chosen (Essex-Fraser et al., 2005). For representation, amounts for each transcript were expressed as fold changes relative to the follicle stage sample with lowest expression levels, which were set at 1.

2.4. Statistical Analyses

Significant differences among groups were identified by one-way ANOVA followed by the Student-Newman-Keuls test with a significance level (P) of 0.05. When necessary, data were log-transformed in order to comply with normality.

3. Results

The transcript levels of obtained in the present study using either non-normalized raw

input amounts or amounts normalized against the adjusted expression of *ef1a* and *18s* showed similar expression patterns during European sea bass folliculogenesis. According to this, the gene expression data presented hereinafter correspond solely to non-normalized raw input amounts.

Expression levels of gonadotropin and thyrotropin receptors are shown in Fig. 3. Transcript levels of *follicle stimulating hormone receptor (fshr)* were low at Pg and increased progressively until reaching a peak at Evit before declining again until Lvit. mRNA levels of *luteinizing hormone/choriogonadotropin receptor (lhcgrr)* remained low at Pg and Lv, but increased thereafter with highest levels in Lvit follicles. Transcript amounts of *thyroid stimulating hormone receptor (tshr)* were high at Pg, decreased significantly at Lv and reached low levels by Evit stage.

Fig. 4 shows the expression levels of several steroidogenesis-related genes and nuclear steroid receptors. mRNA amounts of *steroidogenic acute regulatory protein (star)* were low from Pg to Evit, and increased to maximum levels at Mvit, after which they decreased to intermediate levels in Lvit follicles. Transcript levels of *cytochrome P450, family 19, subfamily A, polypeptide 1a (cyp19a1a)* were low in Pg and Lv but increased significantly thereafter with highest levels in vitellogenic follicles. mRNA levels of *estrogen receptor 1 (esr1)* were low at Pg, Lv and Evit and increased thereafter until reaching a peak at Lvit follicles. Transcript amounts of *estrogen receptor 2a (esr2a)* were at intermediate levels in Pg and Lv oocytes, decreased significantly at Evit and increased thereafter with highest levels in Lvit follicles. mRNA levels of *estrogen receptor 2b (esr2b)* were highest at Pg, Lv and Lvit while low transcripts amounts were measured at Evit and Mvit follicles. Expression of *androgen receptor a (ara)* did not exhibit any significant changes during folliculogenesis. In contrast, transcripts amounts of *androgen receptor b (arb)* were high at Pg and Lv follicles but decreased significantly in vitellogenic follicles.

Expression levels of several growth factors are shown in Fig. 5. Transcripts amounts of *insulin-like growth factor 1 (igf1)* were high at Pg and Lv and decreased significantly during in vitellogenic follicles. Transcripts levels of *insulin-like growth factor 2 (igf2)* were high at Pg, decreased significantly at Lv and reached low levels by Evit stage. mRNA levels of *anti-Mullerian hormone (amh)* were low at Pg and peaked at Lv before reaching again low levels by Mvit. Transcript levels of *bone morphogenetic protein 4 (bmp4)* were highest in Pg and decreased significantly thereafter. Transcripts amounts of *transforming growth factor, beta 1a (tgfb1a)* were low from Pg to Mvit but increased

significantly at Lvit.

Fig. 6 shows the expression levels of *lipoprotein lipase (lpl)*, *vitellogenin receptor (vtgr)* and *SRY-box containing gene 17 (sox17)*. mRNA levels of *lpl* were low at Pg and increased progressively until reaching the highest levels in Mvit and Lvit follicles. Transcript amounts of *vtgr* and *sox17* were high at Pg, decreased significantly at Lv and reached low levels by Evit stage.

4. Discussion

Intense research efforts during the past years have contributed greatly to our understanding about the endocrine mechanisms orchestrating teleost fish oogenesis although we are still far from obtaining a comprehensive picture about this process. In this regard, the current study provides new molecular insights into European sea bass oogenesis by reporting expression level profiles of multiple genes in isolated and homogeneous follicular stages, a different strategy compared to the previously used approach of analyzing whole heterogeneous ovarian samples (*e.g.* Halm et al., 2008; Ibáñez et al., 2008; Rocha et al., 2009).

The use of housekeeping reference genes in real-time PCR quantification of transcript levels requires the stable expression of the selected gene(s) across the experimental samples analyzed. However, as regards gene expression analysis during fish folliculogenesis, several recent reports have showed that the genes traditionally used for this purpose, such as *bactin*, *18s*, *ef1a*, *glyceraldehyde-3-phosphate dehydrogenase*, *ribosomal protein L8*, or *acidic ribosomal phosphoprotein*, do change significantly during development (Ings and Van der Kraak, 2006; Halm et al., 2008; Rocha et al., 2009; Kroupova et al., 2011; this study) rendering this method of normalization inappropriate. Such variations are though to be originated by the changing total RNA composition of follicles during development, particularly by the influence of small RNA species abundantly present in primary follicles (Rocha et al., 2009; Kroupova et al., 2011). Messenger RNA has been used instead of total RNA for reverse-transcription to overcome this problem, although following this strategy, expression levels of several housekeeping reference genes still shows significant variations during fish folliculogenesis (Rocha et al., 2009; Kroupova et al., 2011). Provided the same amount of total RNA is used for reverse-transcription, normalization against total RNA input without any further relation to the expression of a reference gene has been also suggested as an appropriate method (Ings and Van der Kraak, 2006; Kroupova et al.,

2011). This method implies the analysis of data as non-normalized raw input amounts and has been previously used for reporting gene expression levels during fish follicular development (Ings and Van der Kraak, 2006; Kroupova et al., 2011). The use of non-normalized raw data is also supported by studies showing opposite trends between normalized and non-normalized data (Neuvians et al., 2005) in addition to contradictory results depending on the reference gene used for the same data set (Zhang et al., 2003). Another possibility for quantifying developmental changes in gene expression consists in standardizing the expression of the chosen reference gene in each sample at each stage to a randomly chosen control stage (Essex-Fraser et al., 2005). This strategy uses however “artificial” values for normalization and it has been furthermore shown that expression patterns were similar to those obtained using non-normalized data (Ings and Van der Kraak, 2006; this study). Accordingly, the above mentioned evidences support the appropriateness of using non-normalized raw data to describe the expression levels during fish folliculogenesis.

Pituitary gonadotropins, FSH and LH, acting through their receptors, *Fshr* and *Lhcgr* which are expressed by the granulosa and theca somatic cells (Yan et al., 1992; Rocha et al., 2007a; Andersson et al., 2009), are thought to be the main regulators of teleost fish oogenesis (reviewed by Ge, 2005; reviewed by Lubzens et al., 2010). However, the low transcript levels for both receptors reported for European sea bass (this study) and zebrafish follicles (Kwok et al., 2005; Tse and Ge, 2010) suggest that primary oocyte growth occurs in a gonadotropin-independent fashion. On the other hand and as previously proposed in teleost fish (Prat et al., 1996; Kwok et al., 2005; Luckenbach et al., 2008), *Fshr*-mediated signalling seems to be essential for the recruitment of oocytes into the secondary growth phase and vitellogenesis, and *Lhcgr*-mediated signalling may be relevant at mid-to-late vitellogenesis and further onwards in European sea bass.

Our study showed that *cyp19a1a* mRNA levels were high in vitellogenic follicles, which have been recently shown to possess a high capacity for synthesizing estrogens in European sea bass (Molés et al., 2008). A similar *cyp19a1a* expression pattern has been described for channel catfish ovary (Kumar et al. 2000b), although for zebrafish, *cyp19a1a* transcript levels were up-regulated at pre-vitellogenesis (Ings and Van der Kraak, 2006). Previous studies showed that ovarian *star* mRNA expression was greatly up-regulated during European sea bass oocyte maturation and ovulation, a mechanism probably involved in coping with the high and quick cholesterol demands required for the steroidogenic shifts driving both processes (Asturiano et al., 2002; Rocha et al.,

2009). Accordingly, the high *star* expression levels measured at mid-to-late vitellogenesis in the current study could be related to the steroid requirements for completion of vitellogenesis as well as may constitute a preparatory mechanism for further maturational steps.

The expression profile of the nuclear estrogen receptor *esr1* in sea bass follicles was similar to that described for rainbow trout (Nagler et al., 2000) suggesting a specific role for this receptor during mid-to-late vitellogenesis. On the other hand, the “U-shaped” patterns of *esr2a* and *esr2b* mRNA levels observed during sea bass follicular development may indicate their involvement not only in late vitellogenesis, but also in primary and early secondary growth. In fact, several studies have demonstrated the involvement of estrogens in early oocyte development and in favouring the transition from the primary into the secondary oocyte growth phase in fish (Khoo, 1979; Campbell et al., 2006; Miura et al., 2007). Also, non-aromatizable androgens have been experimentally implicated in the growth of primary oocytes and in lipid accumulation during early secondary growth in teleost fish (Lokman et al., 2007; Kortner et al., 2008). Thus, the high mRNA levels of *arb* measured in early European sea bass follicles suggest the involvement of this receptor in mediating such events.

In teleost fish, Igf1 and Igf2 are primarily synthesized by ovarian follicular cells (Reinecke, 2010). In European sea bass, primary and lipid vesicle stage follicles showed high levels of *igf1* and *igf2* mRNAs, suggesting their involvement in primary and early secondary oocyte growth. Also, there was an increased ovarian mRNA expression of *igf1* and *igf2* at the beginning of lipid uptake in coho salmon oocytes (Campbell et al., 2006). Interestingly, *in vitro* treatment of short finned eel oocytes with IGF1 resulted in an increased diameter of perinucleolar primary oocytes (Lokman et al., 2007). Similar to *igf1* and *igf2*, transcript levels of *tshr* and *bmp4* were elevated during the beginning of oocyte development in European sea bass. This contrasts with previous studies reporting highest *tshr* mRNA levels during vitellogenesis in several teleost fish species, including sea bass (Kumar et al. 2000a; Rocha et al., 2007b; Goto-Kazeto et al., 2009). On the other hand, the developmental expression pattern of *bmp4* reported here agrees with that previously described in rainbow trout (Lankford and Weber, 2010).

Within the zebrafish follicle, only granulosa cells express *amh* mRNA with maximum levels at the cortical alveoli stage (Rodríguez-Marí et al., 2005). Similarly, the transition from late perinucleolar to cortical alveoli oocyte stages in coho salmon was associated with an up-regulation of *amh* transcript levels (Luckenbach et al., 2008). Our results

fully agree with these reports suggesting a relevant role of *Amh* on early secondary oocyte growth in teleost fish.

Previous studies in zebrafish have reported decreasing *tgfb1a* mRNA levels from pre-vitellogenic to maturing follicles (Kohli et al., 2003). On the contrary, we have found higher *tgfb1a* transcript levels in European sea bass follicles at late vitellogenesis than in earlier stages. This discrepancy between the expression patterns in both species suggests that *Tgfb1a* may not be involved in preventing precocious oocyte maturation in European sea bass, as reported for zebrafish (Clelland and Peng, 2009), although further studies will have to demonstrate this assumption.

Expression patterns of *lpl* and *vtgr* described here were consistent with previous results reported in teleost fish, including European sea bass (Hiramatsu et al., 2004; Sánchez et al., 2004; Ibáñez et al., 2008; Luckenbach et al., 2008). The increasing *lpl* mRNA levels during sea bass oogenesis suggest an important role of *Lpl* in lipid uptake from the transition between primary and secondary growth onwards. On the other hand, the *vtgr* mRNA expression profile observed during sea bass oogenesis supports the concept that their transcripts are synthesized very early in development being stored for translation just prior to vitellogenesis (Hiramatsu et al., 2004).

A recent report has shown elevated *sox17* mRNA levels in female European sea bass during late ovarian differentiation (Navarro-Martín et al., 2009), when ovaries are occupied by oogonia and primary oocytes (Felip et al., 2002). Our study agrees with this information as regards the relatively high *sox17* mRNA amounts in primary oocytes.

Our expression survey was based on isolated follicles collected from late vitellogenic European sea bass ovaries, which contain oocytes at different developmental stages from the primary growth to late vitellogenesis. Thus, one question arising from this experimental setting is whether the expression levels of a given gene is similar in a given follicular stage collected from an ovary exclusively containing that stage or collected from an ovary containing a mixture of several developmental stages. In our case, one example could be to question whether the expression levels of a given gene in primary oocytes change if the oocytes are isolated from a primary growth ovary (containing exclusively primary oocytes) or from a vitellogenic ovary. Comparison of temporal/seasonal gene expression profiles in the ovary obtained in previous studies with other fish species with asynchronous or group-synchronous ovarian development (Kumar et al., 2000b; Hiramatsu et al., 2004; Sánchez et al., 2004; Kwok et al., 2005; Ibáñez et al., 2008; Rocha et al., 2009; Chauvigné et al., 2010), with the developmental

gene expression profiles reported here suggest that a given follicular stage seems to have a characteristic gene expression profile regardless the whole oocyte composition of the ovary. As regards European sea bass, primary oocytes contained in late vitellogenic ovaries still have elevated *vtgr* transcript levels and low mRNA amounts of *fshr*, *lhcg*, *star*, *cyp19a1a*, and *lpl* (this study) as do primary oocytes contained in primary pre-vitellogenic ovaries (Sánchez et al., 2004; Ibáñez et al., 2008; Rocha et al., 2009). In addition, expression levels of *lhcg*, *star*, *cyp19a1a*, and *lpl* are low in lipid vesicle stage follicles contained in late vitellogenic ovaries (this study) as in ovaries at late pre-vitellogenesis, where this stage represent the most advanced follicle class (Ibáñez et al., 2008; Rocha et al., 2009). These evidences may support the notion that fish follicles, as their mammalian counterparts (McGee and Hsueh, 2000), behave as “independent” functional units within the ovary in the way that the developmental progress of an individual follicle is determined by a cross-talk between the signals coming from its own intra-follicular environment and other stimuli with either paracrine (*e.g.* follicle-to-follicle) or endocrine origin. This would explain how a given follicle remains at primary growth stage while the adjacent ones progress through vitellogenesis in asynchronous or group-synchronous fish ovaries. The origin and identity of the signals determining the developmental progression of individual follicles, especially from early stages, remain however poorly understood in vertebrates (McGee and Hsueh, 2000).

In conclusion, the expression profiles of 18 oogenesis-relevant genes were analyzed in European sea bass follicles at different developmental stages. Although this study was exclusively based on the analysis of steady-state mRNA levels of selected target genes, and neither the respective protein amounts nor their functions were assessed experimentally, we believe that our data provide a solid base for future studies focused on unravelling the specific mechanisms orchestrating follicular development in teleost fish.

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References

- Andersson, E., Nijenhuis, W., Male, R., Swanson, P., Bogerd, J., Taranger, G.L., Schulz, R.W., 2009. Pharmacological characterization, localisation and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. Gen. Comp. Endocrinol. 163, 329-339.
- Asturiano, J.F., Sorbera, L.A., Ramos, J., Kime, D.E., Carrillo, M., Zanuy, S., 2002. Group-synchronous ovarian development. Ovulation and spermiation in the European sea bass (*Dicentrarchus labrax* L.) could be regulated by shifts in gonadal steroidogenesis. Sci. Mar. 66, 273-282.
- Blázquez, M., González, A., Papadaki, M., Mylonas, C.C., Piferrer, F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 95-101.
- Campbell, B., Dickey, J., Beckman, B., Young, G., Pierce, A., Fukada, H., Swanson, P., 2006. Previtellogenic Oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biol. Reprod. 75, 34-44.
- Chauvigné, F., Tingaud-Sequeira, A., Agulleiro, M.J., Calusinska, M., Gómez, A., Finn, R.N., Cerdà, J., 2010. Functional and evolutionary analysis of flatfish gonadotropin receptors reveals cladal- and lineage-level divergence of the teleost glycoprotein receptor family. Biol. Reprod. 82, 1088-1102.
- Clelland, E., Peng, C., 2009. Endocrine/paracrine control of zebrafish ovarian development. Mol. Cell. Endocrinol. 312, 42-52.
- Essex-Fraser, P.A., Steele, S.L., Bernier, N.J., Murray, B.W., Stevens, E.D., Wright, P.A., 2005. Expression of four glutamine synthetase genes in early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. J. Biol. Chem. 280, 20268-20273.
- Felip, A., Piferrer, F., Carrillo, M., Zanuy, S., 2002. Growth, gonadal development and sex ratios of meiogynogenetic diploid sea bass. J. Fish Biol. 61, 347-359.
- Ge, W., 2005. Intrafollicular paracrine communication in the zebrafish ovary: The state of the art of an emerging model for the study of vertebrate folliculogenesis. Mol. Cell. Endocrinol. 237, 1-10.

409 Goto-Kazeto, R., Kazeto, Y., Trant, J.M., 2009. Molecular cloning, characterization and
 410 expression of thyroid-stimulating hormone receptor in channel catfish. *Gen. Comp.*
 411 *Endocrinol.* 161, 313-319.

412 Grier, H., 2000. Ovarian germinal epithelium and folliculogenesis in the common
 413 snook, *Centropomus undecimalis* (Teleostei: Centropomidae). *J. Morphol.* 243, 265-
 414 281.

415 Halm, S., Ibañez, A.J., Tyler, C.R., Prat, F., 2008. Molecular characterisation of growth
 416 differentiation factor 9 (*gdf9*) and bone morphogenetic protein 15 (*bmp15*) and their
 417 patterns of gene expression during the ovarian reproductive cycle in the European sea
 418 bass. *Mol. Cell. Endocrinol.* 291, 95-103.

419 Hiramatsu, N., Chapman, R.W., Lindzey, J.K., Haynes, M.R., Sullivan, C.V., 2004.
 420 Molecular characterization and expression of vitellogenin receptor from white perch
 421 (*Morone americana*). *Biol. Reprod.* 70, 1720-1730.

422 Ibañez, A.J., Peinado-Onsurbe, J., Sánchez, E., Cerdá-Reverter, J.M., Prat, F., 2008.
 423 Lipoprotein lipase (LPL) is highly expressed and active in the ovary of European sea
 424 bass (*Dicentrarchus labrax* L.), during gonadal development. *Comp. Biochem.*
 425 *Physiol. A* 150, 347-354.

426 Ings, J.S., Van der Kraak, G.J., 2006. Characterization of the mRNA expression of
 427 StAR and steroidogenic enzymes in zebrafish ovarian follicles. *Mol. Reprod. Develop.*
 428 73, 943-954.

429 Khoo, K.H., 1979. The histochemistry and endocrine control of vitellogenesis in
 430 goldfish ovaries. *Can. J. Zool.* 57, 617-626.

431 Kohli, G., Hu, S., Clelland, E., Di Muccio, T., Rothenstein, J., Peng, C., 2003. Cloning
 432 of transforming growth factor-beta 1 (TGF-beta 1) and its type II receptor from
 433 zebrafish ovary and role of TGF-beta 1 in oocyte maturation. *Endocrinology* 144,
 434 1931-1941.

435 Kortner, T.M., Rocha, E., Silva, P., Castro, L.F.C., Arukwe, A., 2008. Genomic
 436 approach in evaluating the role of androgens on the growth of Atlantic cod (*Gadus*
 437 *morhua*) previtellogenic oocytes. *Comp. Biochem. Physiol. D* 3, 205-218.

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

441 Kumar, R.S., Ijiri, S., Kight, K., Swanson, P., Dittman, A., Alok, D., Zohar, Y., Trant,
 442 J.M., 2000a. Cloning and functional expression of a thyrotropin receptor from the

gonads of a vertebrate (bony fish): potential thyroid-independent role for thyrotropin in reproduction. *Mol. Cell. Endocrinol.* 167, 1-9.

Kumar, R.S., Ijiri, S., Trant, J.M., 2000b. Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. *Biol. Reprod.* 63, 1676-1682.

Kwok, H.F., So, W.K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors - evidence for their distinct functions in follicle development. *Biol. Reprod.* 72, 1370-1381.

Lankford, S.E., Weber, G.M., 2010. Temporal mRNA expression of transforming growth factor-beta superfamily members and inhibitors in the developing rainbow trout ovary. *Gen. Comp. Endocrinol.* 166, 250-258.

Lokman, P.M., George, K.N., Divers, S.L., Algie, M., Young, G., 2007. 11-ketotestosterone and IGF-I increase the size of previtellogenic oocytes from short-finned eel, *Anguilla australis*, *in vitro*. *Reproduction* 133, 955-967.

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: How fish eggs are formed. *Gen. Comp. Endocrinol.* 165, 367-389.

Luckenbach, J.A., Iliev, D.B., Goetz, F.W., Swanson, P., 2008. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. *Reprod. Biol. Endocrinol.* 6, 2.

Mayer, I., Shackley, S.E., Ryland, J.S., 1988. Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. I. An histological and histochemical study of oocyte development. *J. Fish Biol.* 33, 609-622.

Mayer, I., Shackley, S.E., Witthames, P.R., 1990. Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. II. Fecundity and pattern of oocyte development. *J. Fish Biol.* 36, 141-148.

McGee E.A., Hsueh A.J.W., 2000. Initial and cyclic recruitment of ovarian follicles. *Endocr. Rev* 21, 200-214.

Miura, C., Higashino, T., Miura, T., 2007. A progestin and an estrogen regulate early stages of oogenesis in fish. *Biol. Reprod.* 77, 822-828.

Molés, G., Gomez, A., Rocha, A., Carrillo, M., Zanuy, S., 2008. Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.* 158, 68-76.

Nagler, J.J., Krisfalusi, M., Cyr, D.G., 2000. Quantification of rainbow trout

477 (*Oncorhynchus mykiss*) estrogen receptor-alpha messenger RNA and its expression in
 478 the ovary during the reproductive cycle. J. Mol. Endocrinol. 25, 243-251.
 479 Navarro-Martín, L., Galay-Burgos, M., Sweeney, G., Piferrer, F., 2009. Different sox17
 480 transcripts during sex differentiation in sea bass, *Dicentrarchus labrax*. Mol. Cell.
 481 Endocrinol. 299, 240-251.
 482 Neuvians, T.P., Gashaw, I., Sauer, C.G., von Ostau, C., Kliesch, S., Bergmann, M.,
 483 Hacker, A., Grobholz, R., 2005. Standardization strategy for quantitative PCR in
 484 human seminoma and normal testis. J. Biotechnol. 117, 163-171.
 485 Patiño, R., Sullivan, C.V., 2002. Ovarian follicle growth, maturation, and ovulation in
 486 teleost fish. Fish Physiol. Biochem. 26, 57-70.
 487 Prat, F., Sumpter, J.P., Tyler, C.R., 1996. Validation of radioimmunoassay for two
 488 salmon gonadotropins (GTH I and GTH II) and their plasma concentrations
 489 throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus*
 490 *mykiss*). Biol. Reprod. 54, 1375-1382.
 491 Prat, F., Zanuy, S., Bromage, N., Carrillo, M., 1999. Effects of constant short and long
 492 photoperiod regimes on the spawning performance and sex steroid levels of female
 493 and male sea bass. J. Fish Biol. 54, 125-137.
 494 Reinecke, M., 2010. Insulin-like growth factors and fish reproduction. Biol. Reprod. 82,
 495 656-661.
 496 Rocha, A., Gómez, A., Zanuy, S., Cerdá-Reverter, J.M., Carrillo, M., 2007a. Molecular
 497 characterization of two sea bass gonadotropin receptors: cDNA cloning, expression
 498 analysis, and functional activity. Mol. Cell. Endocrinol. 272, 63-76.
 499 Rocha, A., Gómez, A., Galay-Burgos, M., Zanuy, S., Sweeney, G.E., Carrillo, M.,
 500 2007b. Molecular characterization and seasonal changes in gonadal expression of a
 501 thyrotropin receptor in the European sea bass. Gen. Comp. Endocrinol. 152, 89-101.
 502 Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal
 503 expression of gonadotropin receptors, steroidogenic acute regulatory protein and
 504 steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol. 162, 265-
 505 275.
 506 Rodríguez-Marí, A., Yan, Y.-L., BreMiller, R.A., Wilson, C., Cañestro, C.,
 507 Postlethwait, J.H., 2005. Characterization and expression pattern of zebrafish anti-
 508 Mullerian hormone (*amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad
 509 development. Gene Express. Patt. 5, 655-667.
 510 Sánchez, E., Ibáñez, A.J., Shaw, M., Martínez-Rodríguez, G., Zanuy, S., Carrillo, M.,

Prat, F., 2004. The vitellogenin receptor of European sea bass (*Dicentrarchus labrax* L.): Cloning, molecular characterization and developmental expression. Abstracts of the 5th International Symposium on Fish Endocrinology, Castellón, Spain, poster P88.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., Ragg, T., 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* 7.

Sorbera, L.A., Asturiano, J.F., Carrillo, M., Cerda, J., Kime, D.E., Zanuy, S., 1999. *In vitro* oocyte maturation in the sea bass: effects of hCG, pituitary extract and steroids. *J. Fish Biol.* 55, 9-25.

Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. *Gen. Comp. Endocrinol* 165, 483-515.

Tse, A.C.-K., Ge, W., 2010. Spatial localization of EGF family ligands and receptors in the zebrafish ovarian follicle and their expression profiles during folliculogenesis. *Gen. Comp. Endocrinol.* 167, 397-407.

Yan, L., Swanson, P., Dickhoff, W.W., 1992. A two-receptor model for salmon gonadotropins (GTH I and GTH II). *Biol. Reprod.* 47, 418-427.

Zanuy, S., Carrillo, M., Felip, A., Rodriguez, L., Blazquez, M., Ramos, J., Piferrer, F., 2001. Genetic, hormonal and environmental approaches for the control of reproduction in the European sea bass (*Dicentrarchus labrax* L.). *Aquaculture* 202, 187-203.

Zhang, Q.J., Chadderton, A., Clark, R.L., Augustine-Rauch, K.A., 2003. Selection of normalizer genes in conducting relative gene expression analysis of embryos. *Birth Defects Res. A Clin. Mol. Teratol.* 67, 533-544.

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

Figure legends

Figure 1. Morphology of European sea bass follicles at different developmental stages. The upper panels show the freshly isolated follicles, whereas the lower panels show the histological sections of the corresponding stages. Scale bars: 50 µm.

Figure 2. Stage-dependent relative expression levels (mean ± SEM) of the commonly used house-keeping reference genes *18S ribosomal RNA (18s)* and *elongation factor 1-*

alpha (ef1a) in isolated European sea bass follicles (n=3 for each stage): Pg, primary growth; Lv, lipid vesicles stage; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic. The target gene is indicated in every panel. For each target gene, expression levels are indicated as fold changes relative to the follicle stage sample with the lowest transcript amounts, which were set at 1. Different letters denote statistical significance.

Figure 3. Stage-dependent relative mRNA expression levels (mean $\pm$ SEM) of gonadotropin and thyrotropin receptors in isolated European sea bass follicles (n=3 for each stage). The target gene is indicated in every panel. See Fig. 2 for further details.

Figure 4. Stage-dependent relative mRNA expression levels of several steroidogenesis-related genes and nuclear steroid receptors in isolated European sea bass follicles. The target gene is indicated in every panel. See Fig. 2 for further details.

Figure 5. Stage-dependent relative mRNA expression levels of several growth factors in isolated European sea bass follicles. The target gene is indicated in every panel. See Fig. 2 for further details.

Figure 6. Stage-dependent relative mRNA expression levels of *lipoprotein lipase (lpl)*, *vitellogenin receptor (vtgr)*, and *SRY-box containing gene 17 (sox17)* in isolated European sea bass follicles. The target gene is indicated in every panel. See Fig. 2 for further details.

Figure 1

Figure 1 [grey scale]

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Supplemental Table 1. Primers used to quantify mRNA levels by real-time PCR in European sea bass isolated oocytes.

Target ¹	Forward (5' → 3')	Reverse (5' → 3')	Conc. ² (nM)	Amplicon size (bp)	GenBank Accession No.
<i>18s</i>	CGTGCATTTATCAGACCCAAA	AGTTGATAGGGCAGACATTCG	200	137	AY831388
<i>amh</i>	TGGTGGAGGAACAGTCTAATTG	TGACAGTACATAGCCTAAAGAGACC	200	114	AM232701
<i>ara</i>	AGTCCATTGAGCCTGAGGTG	CCCATTGACCACTTTGACC	200	123	DQ388346
<i>arb</i>	AGAAAGGGCTGGGAAGAGAG	ACTGAGCAGGGAGAGACAGG	200	127	DQ388347
<i>bmp4</i>	CTTCCACCATGAAGAGCACA	CGGTAGAGCCTCAGTTCAGC	100	135	AJ567451
<i>cyp19a1a</i>	GGATCACCAGGCATCTCAGT	CCCAATCCCAGACAGAAAGA	200	122	AJ311177
<i>efla</i>	GCTCATCGTTGGAGTCAACA	AGATGGGTACAAAGGCAACG	400	140	AJ866727
<i>esr1</i>	TGCACTACTCCGGTTCGTTT	TGCCACAATATGACCTAACACC	200	139	AJ505009
<i>esr2a</i>	TGCAGACAGACCAAACCTTGC	TTCTGTGGTGGTGCTACTGG	200	118	AJ489523
<i>esr2b</i>	GATGATGTCCCTCACCAACC	ACTTCAGCAGGTGGATCTGG	200	116	AJ489524
<i>fshr</i>	GCCAGATGGGTGGAGTTTAC	TCTTGGGACACCAGATGGAT	200	131	AY642113
<i>igf1</i>	ATTGTGGACGAGTGCTGCTT	CTCTTGGCATGTCTGTGTGG	200	124	AY800248
<i>igf2</i>	AATACGAGGTGTGGCAGAGG	TTGATCTTCTCCGCCTGTCT	200	100	AY839105
<i>lhcr</i>	AATTGTCCGCTTCATTCTGG	TCCTCCCACTACTCCACCA	100	106	AY642114
<i>lpl</i>	GATAGACAGCACCCAGCTCA	AGGCACAAGAGGAGGGAAAT	200	101	AM411614
<i>sox17</i>	GAACGCGTTTATGGTCTGG	TTTCCACGATTTCCCAACAT	200	106	EU761243
<i>star</i>	CCTGTGCTGAAGACCCAAAT	AAAGTCCACCTGCGTCTGAG	100	116	EF409994
<i>tgfb1a</i>	ACCTGGGATGGAAGTGGATA	GAGGCTCCTGGGTGTGAT	200	139	AM421619
<i>tshr</i>	CACAATTTCCGGCTGTGATGA	GCCAGTCCCTCCAAATACAA	200	115	DQ386646
<i>vtgr</i>	CTATGGTTGGGCATCGTACC	TCACAGCAACAGCACACAAA	200	140	FR717659

¹Complete gene names are given in the main text.

²Conc., final primer concentration for real-time PCR.