Mechanisms of oocyte development in European sea bass (*Dicentrarchus labrax* L.): investigations via application of unilateral ovariectomy

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Abstract

Unilateral ovariectomy (ULO) was performed in European sea bass (*Dicentrarchus labrax* L.) during late pre-vitellogenesis/early vitellogenesis. Plasma steroid levels and the expression of a suite of potential oogenesis-relevant genes in the ovary, brain, and pituitary were evaluated with the aim of understanding their involvement in the compensatory oocyte development occurring within the remaining ovarian lobe. After 69 days of surgery the remaining ovarian lobe in ULO fish was gravimetrically equivalent to an intact-paired ovary of sham operated, control fish. This compensatory ovarian growth was based on an increased number of early perinucleolar oocytes and mid-late stage vitellogenic follicles without an apparent recruitment of primary oocytes into the secondary growth phase. Plasma steroid levels were similar in ULO and control females at all time points analyzed, suggesting an increased steroid production of the remaining ovarian lobe in hemi-castrated females. Results of the gene expression survey conducted indicate that the signaling pathways mediated by Fsh and GnRH1 constitute the central axes orchestrating the observed ovarian compensatory growth. In addition, steroid receptors, Star protein, Igfs, and members of the transforming growth factor beta superfamily including anti-Müllerian hormone and bone morphogenetic protein 4 were identified as potentially relevant players within this process, although their specific actions and interactions remain to be established. Our results demonstrate that ULO provides an excellent *in vivo* model for elucidating the interconnected endocrine and molecular mechanisms controlling oocyte development in European sea bass.

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Introduction

Oogenesis in teleost fish is a highly complex process including several successive steps, i.e. 1) mitotic proliferation of oogonia; 2) transformation of oogonia into primary oocytes with the onset of meiosis (primary growth phase); 3) secondary growth phase characterized by a significant oocyte enlargement due to the accumulation of lipids and yolk proteins (vitellogenesis); 4) maturation characterized by the resumption of meiosis, germinal vesicle breakdown, lipid and yolk coalescence, and hydration; and 5) ovulation (Patiño & Sullivan 2002). The process of oogenesis is controlled by an intricate regulatory network both outside and within the follicle complex, the latter consisting of the oocyte, an inner surrounding granulosa cells, and two outer theca layers (Grier 2000). The main players within this regulatory network include GnRH in the brain, the pituitary gonadotropins, i.e. Fsh and Lh, as well as sex steroids and peptide growth factors acting locally in the ovary in an autocrine or paracrine fashion or, in case of

steroids, on extra-ovarian sites as endocrine hormones (e.g. regulating the hepatic synthesis of vitellogenin or exerting feedback actions on GnRH and gonadotropin production and release at the brain and pituitary; Ge 2005, Lubzens *et al.* 2010). Despite our knowledge of the specific roles of the above-mentioned factors, oogenesis is a dynamic and complex process and, thus, many aspects of its regulation remain to be elucidated.

In teleost fish, as for other vertebrate taxa, the surgical removal of one of the ovarian lobes, referred to as unilateral ovariectomy (ULO) or hemi-castration, induces a compensatory growth in the remaining ovarian lobe ultimately generating a single lobe equivalent in mass to the intact paired ovary of control individuals (Goswami & Sundaraj 1968a, Dadzie & Hyder 1976, Tyler *et al.* 1994, 1996, 1997, Luckenbach *et al.* 2008). The mechanisms involved in such compensatory growth, which may vary across species and with the reproductive status of the animal, include increased mitotic activity of oogonia, new recruitment of primary follicles into the secondary growth phase, increased size of pre-existing

follicles, and/or reduced follicular atresia (see references above). In teleost fish, the endocrine processes driving these compensatory mechanisms are not fully understood, although the results of ULO studies in rainbow trout (*Oncorhynchus mykiss*) point toward Fsh as one of the main regulators (Tyler *et al.* 1997). Based on the dramatic physiological changes that ULO provokes, this procedure constitutes a powerful *in vivo* technique for studying multiple aspects of the integral regulatory mechanisms driving oogenesis, especially the early stages of oocyte development, which are largely unexplored in teleost fish.

The European sea bass (*Dicentrarchus labrax*) is one of the most important commercial teleost fish cultured in the Mediterranean area. Ovarian development of this species is defined as group-synchronous, i.e. follicles at different developmental stages are simultaneously present in the ovary and are spawned progressively in three to four batches during a *ca.* 2 months reproductive period in mid winter (Mayer *et al.* 1990, Asturiano *et al.* 2000, 2002). Diverse descriptive and experimental studies have provided some information concerning the physiological control of European sea bass reproductive processes, including oocyte development (Zanuy *et al.* 2001, Taranger *et al.* 2010), but there are still significant knowledge gaps on the controlling

mechanisms. To start to address some of these knowledge gaps, ULO was applied as an experimental tool to study the integrated regulatory mechanisms spanning the ovary, pituitary, and brain, responsible for mediating oocyte development in European sea bass.

Results

Morphological and morphometrical evaluation

At the time of ULO operations, fish in both experimental groups showed no significant differences in total body weight (mean: 1.77 ± 0.09 kg), ovarian lobe weight (Fig. 1A), gonadosomatic index (GSI; Fig. 1B), total number of oocytes, or number of oocytes at different stages of follicular development (Fig. 1D). Ovaries at this time were mainly comprised of oocytes at early and late perinucleolar and lipid vesicles stages together with very low numbers of early vitellogenic follicles (Fig. 1D and F).

After 69 days of surgical procedures, the ovarian lobe weights of ULO females were 2.3-fold higher ($P < 0.05$) than those in the sham-operated control (SHAM) individuals (Fig. 1A and C), while total body weights (mean: 1.88 ± 0.08 kg) and GSI values (calculated taking into account both ovarian lobes in SHAM females but only the

Figure 1 Morphological and morphometrical changes in European sea bass ovary in response to unilateral ovariectomy (ULO). Bars in panels A, B, D, and E represent the mean $\pm$ S.E.M. Panel C shows the macroscopic view of ovaries collected 69 days after surgery. Panels D and E represent the total number of oocytes at different stages of follicular development corrected for total body weight of the fish. For INITIAL (0 days) and SHAM females (69 days after surgery), the total oocyte number was calculated by summing the oocyte number contained in both ovarian lobes. For ULO females, the total oocyte number at 0 days was calculated by doubling the values obtained for the excised ovary, while at 69 days after surgery, the oocyte number in the remaining left ovary was considered as the total number of oocytes. White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. For the sake of clarity, numbers of each follicular stage are shown using different potentials (indicated in the top of the panels). GSI, gonadosomatic index; Epn, early perinucleolar; Lpn, late perinucleolar; Lv1, lipid vesicles stage 1; Lv2, lipid vesicles stage 2; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic. An asterisk denotes a significant difference compared with the respective parameter in INITIAL-SHAM females at each sampling date ($P < 0.05$). Scale bars: 2 cm (C); 200 μ m (F–H).

remaining ovarian lobe in ULO fish) were similar in both groups (Fig. 1B). Evaluation of oocyte stages present in the ovary showed that ULO females (in comparison to SHAM fish) had on average 1.6-fold fewer total number of oocytes ($P<0.05$) in concordance with 1.9-fold fewer late perinucleolar oocytes ($P<0.05$), 1.5-fold fewer lipid vesicles stage 1 oocytes ($P<0.05$), 1.6-fold fewer lipid vesicles stage 2 oocytes ($P<0.05$), and 2.2-fold lower number of early vitellogenic follicles ($P<0.05$; Fig. 1E, G and H). In contrast, ovaries of ULO females had similar numbers of early perinucleolar oocytes and mid- and late-stage vitellogenic follicles compared with those of SHAM individuals (Fig. 1E, G and H). Incidence of oocyte atresia was low and similar in both experimental groups (not shown).

Total number of oocytes contained in the paired ovaries of control fish showed an average 33% decrease from day 0 (INITIAL group) to day 69 (SHAM group) (Fig. 1D and E). This decrease was associated with a weight increase of the fish (oocyte numbers are corrected for individual body weight) together with a decline of the estimated number of early perinucleolar oocytes. Due to the small size of this stage of development and their particular arrangement within sections from vitellogenic ovaries (they are scarce and scattered among much bigger follicles), we found that our counting strategy may have underestimated their numbers in vitellogenic ovaries.

Linear regression analyses (Table 1) showed positive correlations between the GSI and the number of lipid vesicles stage 2 oocytes at the time of ULO, between the GSI 69 days after ULO and both the GSI and the number of lipid vesicles stage 2 oocytes at the time of ULO, between the total number of oocytes 69 days after ULO and both the total number of oocytes and the number of late perinucleolar oocytes at the time of ULO, and

between the GSI and the number of mid and late vitellogenic oocytes 69 days after ULO. Correlations between the GSI, the total number of oocytes, and the number of oocytes at any developmental stage other than that mentioned above were not significant.

Plasma steroid levels

The temporal profiles for circulating levels of 17β -estradiol (E_2) and testosterone were similar in both INITIAL-SHAM and ULO females (Fig. 2); low steroid levels were measured at days -25 , 0 , and 21 after operations, with increasing concentrations of steroids at days 47 and 69 after ULO. There were no significant differences in steroid concentrations between the two groups at any sampling point, although there appeared to be a possible tendency toward relatively lower levels in operated females 47 days after ULO.

Gene expression analysis in the ovary, pituitary, and brain

The expression levels of all the genes analyzed in the ovary were similar in both groups at the time of ULO operations (Fig. 3). At 69 days after surgery, ovarian expression levels of Fsh receptor (*fshr*), *star*, androgen receptor α (*ara*), bone morphogenetic protein 4 (*bmp4*), and lipoprotein lipase (*lpl*) were significantly higher in ULO females compared with SHAM individuals. In contrast, ovarian transcripts amounts for insulin-like growth factor 1 (*igf1*), anti-Müllerian hormone (*amh*), and vitellogenin receptor (*vtgr*) were all significantly downregulated. There were no significant changes for all other transcripts analyzed in the ovary, including

Table 1 Correlation analyses between different morphometrical parameters of European sea bass ovary at the time of unilateral ovariectomy (ULO) and 69 days thereafter.

		At ULO									
		GSI	Total oocytes	Epn	Lpn	Lv1	Lv2	Evit			
At ULO	GSI	–	NS	NS	NS	NS	$r^2=0.51$ $P<0.01$	NS			
69 days after ULO	GSI	$r^2=0.81$ $P<0.01$	NS	NS	NS	NS	$r^2=0.58$ $P<0.05$	NS			
	Total oocytes	NS	$r^2=0.65$ $P<0.05$	NS	$r^2=0.70$ $P<0.05$	NS	NS	NS			
		69 days after ULO									
		GSI	Total oocytes	Epn	Lpn	Lv1	Lv2	Evit	Mvit	Lvit	M/Lvit
69 days after ULO	GSI	–	NS	NS	NS	NS	NS	NS	$r^2=0.55$ $P<0.01$	$r^2=0.57$ $P<0.01$	$r^2=0.83$ $P<0.0001$

For analysis, oocyte numbers were corrected for total body weight of the fish. Epn, early perinucleolar; Lpn, late perinucleolar; Lv1, lipid vesicles stage 1; Lv2, lipid vesicles stage 2; Evit, early vitellogenic; Mvit, mid vitellogenic; Lv2, late vitellogenic; M/Lvit, mid plus late vitellogenic; GSI, gonadosomatic index (%); NS, not significant.

Figure 2 Time course of plasma levels of 17 β -estradiol (E_2) and testosterone in response to unilateral ovariectomy (ULO) in European sea bass. Values are expressed as mean $\pm$ S.E.M. White bars represent combined data from INITIAL and SHAM groups at -25 and 0 days and only from SHAM group at 21, 47, and 69 days. Different letters denote significant differences during the experimental period within each group ($P < 0.05$). No significant differences between the two groups were found at any sampling point ($P < 0.05$).

LH/choriogonadotropin receptor (*lhcr*), TSH receptor (*tshr*), cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), estrogen receptors 1 (*esr1*), 2a (*esr2a*), and 2b (*esr2b*), *arb*, *igf2*, transforming growth factor, beta 1a (*tgfb1a*), and SRY-box containing gene 17 (*sox17*).

In the pituitary of ULO females (Fig. 4), transcripts levels of glycoprotein hormones, alpha polypeptide (*cga*), Fsh, beta polypeptide (*fshb*), and GnRH receptor 2a (*gnrh2a*) were significantly higher compared with SHAM control fish, while there were no significant changes in the expression levels of Lh, beta polypeptide (*lhb*), *esr1*, *esr2a*, *esr2b*, *ara*, and *arb*.

In the brain of ULO females, mRNA expression of *gnrh1* was significantly upregulated, while *esr1* and *esr2a* transcripts amounts were significantly down-regulated compared to SHAM control fish. Transcript levels for *gnrh3*, *cyp19a1b*, and *esr2b* were similar in both groups (Fig. 5).

Discussion

Although research efforts have contributed significantly in widening-up our knowledge about the mechanisms regulating teleost fish oogenesis, we are still far from understanding the complete process. Here, we report the regulatory changes observed in European sea bass reproductive axis in response to ULO, a surgical procedure used for the first time in this species and with a strong potential as an *in vivo* model for investigating factors involved in the regulation of oocyte development in teleost fish.

Morphological responses to ULO

Macroscopically, the response of European sea bass to ULO was similar to those previously reported for other fish species (Goswami & Sundaraj 1968a, Dadzie & Hyder 1976, Tyler *et al.* 1994, 1996, 1997, Luckenbach *et al.* 2008), i.e. the ovarian lobe remaining after surgery underwent a compensatory growth ultimately reaching

a total mass equivalent to that of intact paired gonads. The mechanism by which such compensatory growth is accomplished seems to vary across fish species and have been shown to depend on the ovarian stage at the time of surgery. In European sea bass, which were submitted to ULO at late pre-vitellogenesis/early vitellogenesis, we found that the number of early perinucleolar and mid-late vitellogenic oocytes was fully compensated 2 months after surgery, but oocyte stages from late perinucleolar to early vitellogenic (as well as total oocyte number) were not. This may indicate that even though new early perinucleolar oocytes were originated in response to ULO, as reported in stinging catfish

Figure 3 Expression of target genes (shown in the individual panels) in the ovary of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days after operations are indicated in arbitrary units (mean $\pm$ S.E.M.). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in INITIAL-SHAM-operated females at each sampling date ($P < 0.05$).

Figure 4 Expression of target genes (shown in the individual panels) in the pituitary of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days after the operations are indicated in arbitrary units (mean $\pm$ S.E.M.). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in SHAM-operated females at 69 days after the operations ($P < 0.05$).

(*Heteropneustes fossilis*; Goswami & Sundaraj 1968a) and blue tilapia (*Oreochromis aureus*; Dadzie & Hyder 1976), these new oocytes did not progress through the primary nor the secondary growth phases during the study period. Therefore, and unlike that reported for rainbow trout hemi-castrated at pre- or early vitellogenesis (Tyler *et al.* 1994, 1996, 1997), the compensatory ovarian growth observed in European sea bass after ULO was not associated with recruitment of a complete new batch of primary oocytes into the secondary growth phase. Rather, such compensation was based on an extra recruitment of follicles at mid and late vitellogenesis, probably derived from the pool of early vitellogenic oocytes already existing in the ovary.

European sea bass undertake several spawning events deriving the eggs from three to four different batches of post-vitellogenic oocytes. The first batch contains more oocytes than the successive ones (up to 50% of total oocytes to be spawned), which mature and are ovulated in unison (Mayer *et al.* 1990, Asturiano *et al.* 2002). Given these findings, we speculate that the extra recruitment of mid-to-late vitellogenic follicles observed in hemi-castrated European sea bass occurs to ensure a full complement of oocytes for spawning in the first batch of eggs and thus ensuring maintenance of fertility for the first main ovulatory episode. Further studies would be required, however, covering the full spawning period to assess for any overall effects on fertility collectively over the three to four spawning events.

In rainbow trout subjected to ULO before mid vitellogenesis, the compensatory ovarian growth was

achieved by the recruitment of new primary oocytes (Tyler *et al.* 1994, 1996). In contrast, when surgery was performed at later stages, the compensation was based on an over-enlargement of pre-existing follicles without any further recruitment of primary oocytes into the maturing pool. Consequently, the term 'critical window for recruitment' was introduced and assigned to mid-vitellogenesis in this species (Tyler *et al.* 1994, 1996). Whether such a critical window exists for European sea bass is less clear, but if such a window does exist, it must occur before the appearance of lipid vesicles stage 2/early vitellogenesis, i.e. before the developmental time point when ULO was performed in this study. Our results support the idea that the presence of lipid vesicle stage 2 oocytes at ULO favored the further development of the pre-existing follicles rather than the formation of new ones. This is based on the findings that 1) the number of lipid vesicle stage 2 oocytes at ULO was correlated with the GSI 69 days after and 2) the weight of the ovary after ULO was determined by the number of mid and late vitellogenic oocytes and not by the total number of oocytes present. In contrast, the positive correlation between the number of late perinucleolar oocytes at the time of ULO and the total number of oocytes 69 days after may indicate that the presence of such staged oocytes favored the formation of additional oocytes. In fact, when blue tilapia females were hemi-castrated at the protoplasmic stage (i.e. ovaries containing oocytes with vacuole-free cytoplasm (perinucleolar stage) as most advanced stage), new recruitment of both primary and secondary oocytes and accelerated vitellogenic development were evident, which ultimately led to complete compensatory ovarian growth (Dadzie & Hyder 1976). Further studies in European sea bass are needed to determine whether the ovary has sufficient

Figure 5 Expression of target genes (shown in the individual panels) in the brain of European sea bass in response to unilateral ovariectomy (ULO). For each gene, the expression levels at 0 and 69 days after the operations are indicated in arbitrary units (mean $\pm$ S.E.M.). White bars represent data from INITIAL group at 0 days and from SHAM group at 69 days. An asterisk denotes a significant difference compared to the respective parameter in SHAM-operated females at 69 days after the operations ($P < 0.05$).

plasticity to recruit a new full complement of oocytes after ULO and to identify unequivocally the critical stage of ovarian development when this may occur.

Endocrine responses to ULO

Pituitary gonadotropins, Fsh, and Lh, acting through their respective receptors Fshr and Lhcgr, are the main direct regulators of teleost fish gonadal functions (Ge 2005, Lubzens *et al.* 2010). In this regard, the patterns of gene expression of gonadotropin subunits in the pituitary and gonadotropin receptors in the ovary of ULO females suggest that the Fsh/Fshr-mediated signaling may constitute the central gonadotropic axis orchestrating the ovarian compensatory growth observed in response to ULO in European sea bass. Nevertheless, the involvement of Lh-dependent signaling in the process cannot be ruled out taking into account the higher *cga* levels measured in ULO females, which together with the relatively higher levels of expression of *lhb* seen in the pituitary could lead to an over-production of Lh. In support of this hypothesis, previous reports studying the effects of ULO in teleost fish have demonstrated the involvement of gonadotropins, particularly Fsh, in the gonadal compensation observed in response to hemi-castration (Goswami & Sundaraj 1968b, Dadzie & Hyder 1976, Tyler *et al.* 1997).

Assessment of temporal profiles of plasma concentrations of E₂ and testosterone showed no significant differences between operated and control fish, in spite of the removal of half the mass of the gonadal tissue resulting from hemi-castration. This suggests that, as pointed out previously for rainbow trout (Tyler *et al.* 1997), the dynamics of steroid synthesis and release by ovarian tissue of ULO females differed from that of the intact control fish; the production of sex steroids by the remaining ovary in the ULO fish must have far exceeded than that in the intact fish. Star serves to translocate cholesterol into the mitochondria, a process referred to as the rate-limiting step in steroidogenesis (Stocco *et al.* 2005). Thus, the almost twofold higher amount of *star* transcripts in the ovary of ULO fish suggest the involvement of Star in effecting the compensatory responses of circulating steroid concentrations in hemi-castrated European sea bass. Previous studies have shown that Fsh-dependent signaling is involved in the upregulation of *star* transcription during teleost fish follicular development (Lubzens *et al.* 2010, Luckenbach *et al.* 2011), findings supporting a putative influence of the Fsh/Fshr axis on Star actions in our study. In European sea bass females, expression of *star* is approximately eight times higher in mid and late vitellogenic follicles compared with earlier stages (Á García-López, MI Sánchez-Amaya & F Prat 2010, unpublished observations) and thus the elevated *star* mRNA levels observed in ULO females could also

constitute a reflection of the proportionally higher number of these type of follicles.

The upregulation in the transcription of *gnrh1* in the brain and of *gnrhr2a* in the pituitary suggests the involvement of the GnRh system in the ovarian compensatory response in hemi-castrated European sea bass. Interestingly, GnRh1 has been identified as the most relevant GnRh form controlling oocyte development and reproductive activity in several fish species (Holland *et al.* 1998, Andersson *et al.* 2001, Zmora *et al.* 2002, Okuzawa *et al.* 2003, Molés *et al.* 2007). Previous studies have reported overlapping expression of *gnrhr2a* with both *fshb* and *lhb* in pituitary cells of European sea bass (González-Martínez *et al.* 2004), findings supporting a putative influence of GnRh-derived signaling on gonadotropin subunits transcription and gonadotropin release. However, such an effect has only been verified experimentally in European sea bass for *cga* and *lhb* mRNAs and for Lh secretion, while *fshb* transcription was shown to be unaffected by GnRh treatment (Forniés *et al.* 2003, Mateos *et al.* 2002) and no information for Fsh release is presently available. These findings agree with data obtained for zebrafish (Lin & Ge 2009), although other studies have reported significant effects of GnRh treatments on pituitary *fshb* mRNA levels and Fsh release in female teleost fish (Levavi-Sivan *et al.* 2010). Collectively, such results suggest, however, the involvement of the GnRh1/GnRh2a signaling cascade in the stimulation of *cga* transcription observed in the pituitary of hemi-castrated European sea bass, while the involvement of such pathway in the upregulation of *fshb* transcription also observed in our study remains to be proven experimentally.

Changes in the transcription levels of estrogen and androgen receptors in the brain and ovary of ULO females observed in our study suggest that modulation of cellular responsiveness to steroid stimulation was one of the mechanisms involved in the regulatory response leading to the ovarian compensatory growth in European sea bass. At the level of the ovary, upregulation of *ara* transcription observed in ULO females suggests some, as yet uncharacterized, role(s) of androgen-dependent signaling in gonadal compensation. Our own unpublished experiments showed that the number of androgen and estrogen receptor transcripts varies differentially according to the follicle stage in European sea bass, although the significance of such changes for teleost fish follicular development remains to be established. In addition, estrogens and androgens are able to exert negative and positive feedback effects on the activity of multiple reproduction-relevant neural systems, including the GnRh producing neurons, depending on the physiological-reproductive status of the animal (Klenke 2006, Zohar *et al.* 2010). In the case of our study, it is tempting to hypothesize that the downregulation of *esr1* and *esr2a* in the brain of hemi-castrated European sea bass was part of a steroidal feedback loop ultimately

leading to the stimulation of *gnrh1* transcription. Previous studies in stinging catfish submitted to ULO showed that administration of estradiol benzoate (an estrogenic compound) effectively blocked the compensatory growth of the remaining ovary (Goswami & Sundaraj 1968b). Collectively, these and our data support the concept that correctly balanced steroid signaling is essential for the proper compensatory development of oocytes after hemi-castration in teleost fish.

Expression of both *igf1* and *igf2* normally show progressive decreasing levels during European sea bass oocyte development (Á García-López, MI Sánchez-Amaya & F Prat 2010, unpublished observations) but, interestingly, here they showed opposing modulatory responses after ULO. This indicates that their respective encoded proteins may serve disparate regulatory functions within the compensatory ovarian growth in European sea bass. Although the specific biological actions of Igf-related peptides have not been clearly established in any fish species, such functions could include the stimulation of primary oocyte growth, the modulation of pituitary *fshb* expression, and/or the regulation of follicular steroidogenesis (Lin & Ge 2009, Lankford & Weber 2010, Reinecke 2010).

AMH has been shown to decrease follicular sensitivity to FSH as well as the expression of *Lhcgr* and *Cyp19a1a* in mouse ovary (Di Clemente *et al.* 1994, Durlinger *et al.* 2001). Accordingly, the threefold downregulation of *amh* transcripts observed in hemi-castrated European sea bass suggests that *Amh* plays a role within the local interplay ultimately leading to increased *fshr* mRNA levels observed after ULO and consequently to an increased sensitivity of follicles to Fsh. Recombinant BMP4 has been found to modulate FSH signaling in a way that promotes E_2 production in cultured rat granulosa cells (Shimasaki *et al.* 2004). Hence, elevated *bmp4* transcript levels in hemi-castrated European sea bass suggest *Bmp4* to be important in compensating steroid levels after ULO.

Finally, in the regulatory network of responses to ULO, ovarian transcription of *vtgr*, which mediates the endocytic uptake of vitellogenin through the oocyte membrane (Perazzolo *et al.* 1999, Hiramatsu *et al.* 2004), was downregulated, while *lpl*, which is involved in supplying the oocyte with fatty acids (Ibáñez *et al.* 2008), was upregulated. As *vtgr* transcripts decreases as translation of the receptor and its translocation to the oocyte membrane takes place, i.e. as oocyte development progresses (Perazzolo *et al.* 1999, Hiramatsu *et al.* 2004; Á García-López, MI Sánchez-Amaya & F Prat 2010, unpublished observations), the responses observed for both *vtgr* and *lpl* could be associated with the fact that there were proportionally more follicles actively incorporating both vitellogenin and fatty acids in the ovary of hemi-castrated European sea bass (i.e. mid and late vitellogenic follicles). Although the

respective circulating levels of vitellogenin and fatty acids were not measured in our study, previous reports suggest that they may not be modified as circulating vitellogenin amounts do not change in response to ULO in rainbow trout (Tyler *et al.* 1996) and plasma total lipid levels remain stable during vitellogenesis in European sea bass (Cerdà *et al.* 1995).

Conclusions

ULO in European sea bass at the late pre-vitellogenesis/early vitellogenesis stage resulted in a compensatory ovarian growth and this was the result of increased numbers of early perinucleolar oocytes and mid-late vitellogenic follicles, without an apparent recruitment of primary oocytes into the secondary growth phase. Targeted gene expression analyses across the brain–pituitary–ovary axis indicated that *Fsh/Fshr* and the *Gnrh1/Gnrhr2a* signaling pathways acted as the main axes orchestrating the ovarian compensatory growth. Steroid receptors, steroidogenesis-related proteins, including *Star*, Igf-related peptides, and members of the transforming growth factor, beta superfamily, such as *Amh* and *Bmp4*, were also identified as potentially relevant players in the compensatory response process, although any direct connections between them remain to be established. We have shown that the ULO technique provides a highly tractable approach for elucidating the interconnected endocrine and molecular mechanisms controlling oocyte development in teleost fish.

Materials and Methods

Fish and general rearing and handling conditions

Pit-tagged adult female and male European sea bass (3–4 years old) were maintained in 5 m³ tanks under natural photoperiod (Puerto Real, Cádiz, Spain; 36° 36' N, 6° 13' W) and constant temperature (18–20 °C). Fish were fed *ad libitum* on commercial dry pellets (Vitalis Repro, Skretting; Burgos, Spain) 3 days a week. Prior to sampling, fish were sedated with 2-phenoxyethanol at a 300 ppm dose in order to reduce handling stress. For killing, a lethal anesthetic dose (600 ppm) was applied followed by decapitation of the fish. 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.

Surgical procedures

On 6th October 2009 (day 0), i.e. 1 month before the expected onset of vitellogenesis (Prat *et al.* 1999), randomly selected females (mean total body weight: 1.77 ± 0.09 kg) were either submitted to ULO operation ($n=8$; ULO group), sham operated ($n=4$; SHAM group), or killed ($n=5$; INITIAL control group). The starting point was chosen in accordance to results

in rainbow trout showing that the mid vitellogenesis was the critical developmental stage for inducing a new recruitment of primary oocytes in the remaining ovarian lobe after ULO (Tyler *et al.* 1994, 1996). One ULO female died within 24 h following the operation, but no further mortalities occurred during the time-course of the experiment.

Ovarian lobes, whole brain, and pituitary were excised from fish in the INITIAL group. Extraction of the right ovary in females of the ULO group was accomplished by two 3–4 cm longitudinal incisions made into the abdominal cavity. A cephalic incision was made in order to ligate and cut the vessel connecting the liver and the ovary and to detach the anterior part of the gonad from the body wall. Then, the whole right ovarian lobe was eased outward through the caudal incision, the joint with the left lobe was clamped with hemostatic forceps, and finally the ovary was excised fully. Following this procedure, blood loss was minimal and thus no cauterization was required. Fish in the SHAM group received only one incision into the abdominal cavity. All incisions were closed with non-absorbable silk sutures, which were removed 21 days later as healing of the incisions proceeded correctly and no herniation was evident.

Sixty-nine days after operations, on 14th December 2009, the experiment was finished; females in the ULO and SHAM groups were killed and ovarian lobes, whole brain, and pituitary dissected out. The end point of the experiment was chosen in accordance to the vitellogenic growth period in European sea bass (Prat *et al.* 1999), i.e. and at the time when

most of the oocytes of the first main egg clutch are expected to have reached the mid-late vitellogenesis stage (500–800 µm diameter; Mayer *et al.* 1990, Asturiano *et al.* 2000).

Ovarian lobes collected at days 0 and 69 days after surgery were weighed individually, their volume determined by water displacement, and divided into several subsamples for histological and molecular analyses. As no differences in weight or volume between both ovarian lobes were found in females of the INITIAL and SHAM groups (results not shown), total ovarian weight and volume of ULO fish at the time of operations was calculated by doubling the value obtained from the single ovarian lobe dissected. At 69 days, the weight of the single ovarian lobe was considered as total gonad weight in ULO females. Before 25 days of surgery and thereafter at 21–26 days intervals until the termination of the experiment (see dates in Fig. 2), fish were weighed and blood sampled. The GSI was calculated as $100 \times \text{total gonad weight} \times \text{total body weight}^{-1}$.

Histological and stereological procedures

Ovarian fragments were processed for plastic embedding and histological examination as described in García-López *et al.* (2007), except that the tissue was fixed in 4% glutaraldehyde in PBS pH 7.4 at 4 °C for 24 h.

To estimate the total number of oocytes at each developmental stage, the stereological method of Weibel (1979) as described in Medina *et al.* (2002) and García-López *et al.*

Table 2 Target transcripts, target tissue, and primer sequences for the quantification of mRNA levels in European sea bass brain (B), pituitary (P), and ovary (O).

Transcript ^a	Tissue	Forward (5' → 3')	Reverse (5' → 3')	Amplicon size (bp)	GenBank accession numbers
<i>18s</i>	B, P, O	CGTGCAATTTATCAGACCCAAA	AGTTGATAGGGCAGACATTCG	137	AY831388
<i>amh</i>	O	TGGTGGAGGAACAGTCTAATTG	TGACAGTACATAGCCTAAAGAGACC	114	AM232701
<i>ara</i>	O, P	AGTCCATTGAGCCTGAGGTG	CCCATTGACCACCTTTGACC	123	DQ388346
<i>arb</i>	O, P	AGAAAGGGCTGGGAAGAGAG	ACTGAGCAGGAGAGACAGG	127	DQ388347
<i>bactin</i>	B, P, O	CACCGCAATGCTTCTAACA	GCGCCTGAATGTGTATGAGA	114	AJ537421
<i>bmp4</i>	O	CTTCCACCATGAAGAGCACA	CGGTAGAGCCTCAGTTCAGC	135	AJ567451
<i>cga</i>	P	AAGGCCATGAAGACGATGAC	TGTGTGGTTTCGCACCTTTA	114	AF269157
<i>cyp19a1a</i>	O	GGATCACCAGGCATCTCAGT	CCCAATCCCAGACAGAAAGA	122	AJ311177
<i>cyp19a1b</i>	B	CTCGAACAGCACAACTTCA	TTAGCTCAGCAGGCTCATCC	128	AY138522
<i>ef1a</i>	B, P, O	GCTCATCGTTGGAGTCAACA	AGATGGGTACAAAGGCAACG	140	AJ866727
<i>esr1</i>	B, P, O	TGCACTACTCCGGTTCGTTT	TGCCACAATATGACCTAACACC	139	AJ505009
<i>esr2a</i>	B, P, O	TGCAGACAGACCAACTTGC	TTCTGTGGTGGTGCTACTGG	118	AJ489523
<i>esr2b</i>	B, P, O	GATGATGTCCCTCACCAACC	ACTTCAGCAGGTGGATCTGG	116	AJ489524
<i>fshb</i>	P	AAGGTTGTCCAGTGGGTGTC	GCTGGGTATGTCTCCAGGAA	107	AF543314
<i>fshr</i>	O	GCCAGATGGGTGGAGTTTAC	TCTTGGGACACCAGATGGAT	131	AY642113
<i>gnrhr2a</i>	P	GCTGGGTGGGAAATACAGAA	GAGGACTGAAACGAGAAGACAGA	116	AJ419594
<i>gnrh1</i>	B	GAGGGAAGAGGGAAGTGGAC	GGTGATTCCTCTGCACAACC	112	AF224279
<i>gnrh3</i>	B	GTTGTTGGCGTTGGTGGT	TCCTGATGGTTGCCTCTAGC	104	AF224280
<i>igf1</i>	O	ATTGTGGAGAGTGTGCTT	CTCTTGGCATGTCTGTGTGG	124	AY800248
<i>igf2</i>	O	AATACGAGGTGTGGCAGAGG	TTGATCTTCTCCGCTGTCT	100	AY839105
<i>lhb</i>	P	TTTCTGGGAGCCACATCTTC	ACTTGGGACAACCCTCCTTT	115	AF543315
<i>lhcg</i>	O	AATTGTCCGTTCTATTCTGG	TCCTCCCAACTACTCCACCA	106	AY642114
<i>lpl</i>	O	GATAGACAGCACCCAGCTCA	AGGCACAAGAGGAGGGAAT	101	AM411614
<i>sox17</i>	O	GAACGCGTTTATGGTCTGG	TTTCCACGATTTCCCAACAT	106	EU761243
<i>star</i>	O	CCTGTGCTGAAGACCCAAAT	AAAGTCCACCTGCGTCTGAG	116	EF409994
<i>tgfb1a</i>	O	ACCTGGGATGGAAGTGGATA	GAGGCTCCTGGGTTGTGAT	139	AM421619
<i>tshr</i>	O	CACAATTCGGCTGTGATGA	GCCAGTCCCTCCAAATACAA	115	DQ386646
<i>vtgr</i>	O	CTATGGTTGGGCATCGTACC	TCACAGCAACAGCACACAAA	140	FR717659

^aComplete transcript names are given in the text.

(2007) was applied. Oocyte classification was based on Mayer *et al.* (1988) and included the following stages: early perinucleolar (20–50 µm diameter), late perinucleolar (50–110 µm diameter), lipid vesicles stage 1 (100–160 µm diameter), lipid vesicles stage 2 (140–250 µm diameter), early vitellogenic (250–400 µm diameter), mid vitellogenic (400–600 µm diameter), and late vitellogenic (600–800 µm diameter). Ovarian fragments analyzed were taken from the central part of each lobe and regarded as being representative for the whole ovarian lobe (no longitudinal differences in oocyte composition are found in European sea bass ovary; Navas 2003). No significant differences in oocyte composition between the left and the right ovarian lobes were found in the fish of the INITIAL and SHAM groups (not shown) and thus the total oocyte number of the ULO-operated fish at 0 days was calculated by doubling the values obtained for the excised ovary. For ULO females at 69 days after surgery, the oocyte number in the left ovary was considered as the total number of oocytes. Data are reported as total numbers of oocytes at different stages of follicular development corrected for the total body weight of the fish ('relative fecundity'). Thus, total numbers of oocytes at 69 days after surgery were calculated taking into account both ovarian lobes in SHAM females but only the remaining ovarian lobe in ULO fish.

Steroid immunoassays

Plasma was obtained by centrifugation (3000 g; 4 °C; 10 min) of blood samples and stored at –80 °C. Levels of E₂ and testosterone in plasma were then measured by commercially available enzyme immunoassays (Cayman Chemical Company, Ann Arbor, MI, USA) validated for their use in European sea bass in our laboratory (results not shown). The sensitivity of the assays, defined as 90% binding, were 98 pg/ml for the E₂ assay and 55 pg/ml for the testosterone assay. Respective inter-assay coefficients of variation (calculated at 50% binding) were 10.4 and 4.2%.

Gene expression analyses

Brain, pituitary, and ovary samples were snap frozen in liquid nitrogen and stored at –80 °C. Total RNA was extracted using the NucleoSpin RNA II and RNA XS kits (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. RNA integrity was checked using the Agilent RNA 6000 Nano kit in a Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), while RNA quantity and purity were analyzed spectrophotometrically.

For each sample, 500 ng total RNA was transcribed into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). RT products were diluted to 1/40, and 8 µl were used for each real-time PCR, which were performed in duplicate measurements on an ep realplex² Mastercycler (Eppendorf, Hamburg, Germany) using primer pairs specifically designed for each target gene (100–400 nM; Table 2) and PerfeCTa SYBR Green FastMix 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 s followed by a second step at 60 °C

for 30 s. To correct for variability in amplification efficiency among samples, standard curves were prepared for each tissue analyzed by serially diluting cDNA samples obtained from retro-transcribed total RNA pools of all experimental samples. The relative amount of each transcript for each sample was determined from the corresponding standard curve.

For data normalization, expression levels of three previously used endogenous reference genes in European sea bass tissues were measured: 18S ribosomal RNA (*18s*), elongation factor 1-alpha (*ef1a*), and *bactin* (e.g. Blázquez *et al.* 2008, Mitter *et al.* 2009, Rocha *et al.* 2009). However, as none of them showed a constitutive expression among the experimental samples analyzed, we opted for reporting non-normalized relative amounts for every target gene studied. This strategy is 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).

Statistical analyses

Significant differences between groups were identified by the Student's unpaired *t*-test (comparison between two groups) or by two-way ANOVA followed by the Bonferroni post-test (comparison among multiple groups along time). When necessary, data were log transformed in order to comply with normality. Correlations between different parameters were evaluated by linear regression analysis. All statistical analyses were carried out adopting a significance level (*P*) of 0.05.

Declaration of interest

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

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References

- Andersson E, Fjelldal PG, Klenke U, Vikingstad E, Taranger GL, Zohar Y & Stefansson SO 2001 Three forms of GnRH in the brain and pituitary of the turbot, *Scophthalmus maximus*: immunological characterization and seasonal variation. *Comparative Biochemistry and Physiology Part B* **129** 551–558. (doi:10.1016/S1096-4959(01)00363-3)

- Asturiano JF, Sorbera LA, Ramos J, Kime DE, Carrillo M & Zanuy S 2000 Hormonal regulation of the European sea bass reproductive cycle: an individualized female approach. *Journal of Fish Biology* **56** 1155–1172. (doi:10.1111/j.1095-8649.2000.tb02131.x)
- Asturiano JF, Sorbera LA, Ramos J, Kime DE, 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. *Scientia Marina* **66** 273–282.
- Blázquez M, González A, Papadaki M, Mylonas CC & 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*). *General and Comparative Endocrinology* **158** 95–101. (doi:10.1016/j.ygcen.2008.06.001)
- Cerdà J, Zanuy S, Carrillo M, Ramos J & Serrano R 1995 Short- and long-term dietary effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profiles of lipids and sex steroids in relation to reproduction. *Comparative Biochemistry and Physiology Part C* **111** 83–91. (doi:10.1016/0305-0491(94)00224-I)
- Dadzie S & Hyder M 1976 Compensatory hypertrophy of the remaining ovary and the effects of methallibure in the unilaterally ovariectomized *Tilapia aurea*. *General and Comparative Endocrinology* **29** 433–440. (doi:10.1016/0016-6480(76)90026-5)
- Di Clemente N, Goxe B, Remy JJ, Cate R, Josso N, Vigier B & Salesse R 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. *Endocrine* **2** 553–558.
- Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA *et al.* 2001 Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* **142** 4891–4899. (doi:10.1210/en.142.11.4891)
- Forníes MA, Carrillo M, Mañanós E, Sorbera L, Zohar Y & Zanuy S 2003 Relative potency of the forms of GnRH and their analogs on LH-secretion in sea bass (*Dicentrarchus labrax*). *Journal of Fish Biology* **63** 73–89. (doi:10.1046/j.1095-8649.2003.00130.x)
- García-López Á, Couto E, Canario AVM, Sarasquete C & Martínez-Rodríguez G 2007 Ovarian development and plasma sex steroid levels in cultured female Senegalese sole *Solea senegalensis* Kaup. *Comparative Biochemistry and Physiology Part A* **146** 342–354. (doi:10.1016/j.cbpa.2006.11.014)
- 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. *Molecular and Cellular Endocrinology* **237** 1–10. (doi:10.1016/j.mce.2005.03.012)
- González-Martínez D, Madigou T, Mañanós E, Cerdá-Reverter JM, Zanuy S, Kah O & Muñoz-Cueto JA 2004 Cloning and expression of gonadotropin-releasing hormone receptor in the brain and pituitary of the European sea bass: an *in situ* hybridization study. *Biology of Reproduction* **70** 1380–1391. (doi:10.1095/biolreprod.103.022624)
- Goswami SV & Sundararaj BI 1968a Compensatory hypertrophy of the remaining ovary after unilateral ovariectomy at various phases of the reproductive cycle of catfish, *Heteropneustes fossilis* (Bloch). *General and Comparative Endocrinology* **11** 401–413. (doi:10.1016/0016-6480(68)90097-X)
- Goswami SV & Sundararaj BI 1968b Effect of estradiol benzoate, human chorionic gonadotropin, and follicle-stimulating hormone on unilateral ovariectomy-induced compensatory hypertrophy in catfish, *Heteropneustes fossilis* (Bloch). *General and Comparative Endocrinology* **11** 393–400. (doi:10.1016/0016-6480(68)90096-8)
- Grier H 2000 Ovarian germinal epithelium and folliculogenesis in the common snook, *Centropomus undecimalis* (Teleostei: Centropomidae). *Journal of Morphology* **243** 265–281. (doi:10.1002/(SICI)1097-4687(200003)243:3<265::AID-JMORA>3.0.CO;2-I)
- Hiramatsu N, Chapman RW, Lindzey JK, Haynes MR & Sullivan CV 2004 Molecular characterization and expression of vitellogenin receptor from white perch (*Morone americana*). *Biology of Reproduction* **70** 1720–1730. (doi:10.1095/biolreprod.103.023655)
- Holland MCH, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A & Zohar Y 1998 Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. *General and Comparative Endocrinology* **112** 394–405. (doi:10.1006/gcen.1998.7138)
- Ibáñez AJ, Peinado-Onsurbe J, Sánchez E, Cerdá-Reverter JM & Prat F 2008 Lipoprotein lipase (LPL) is highly expressed and active in the ovary of European sea bass (*Dicentrarchus labrax* L.), during gonadal development. *Comparative Biochemistry and Physiology Part A* **150** 347–354. (doi:10.1016/j.cbpa.2008.04.598)
- Klenke U 2006 Gonadal and steroid feedback regulation of the hypothalamus–pituitary axis in striped bass (*Morone saxatilis*). *PhD Dissertation*. University of Maryland.
- Lankford SE & Weber GM 2010 Temporal mRNA expression of transforming growth factor-beta superfamily members and inhibitors in the developing rainbow trout ovary. *General and Comparative Endocrinology* **166** 250–258. (doi:10.1016/j.ygcen.2009.09.007)
- Levavi-Sivan B, Bogerd J, Mañanós EL, Gómez A & Lareyre JJ 2010 Perspectives on fish gonadotropins and their receptors. *General and Comparative Endocrinology* **165** 412–437. (doi:10.1016/j.ygcen.2009.07.019)
- Lin S-W & Ge W 2009 Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish – an *in vitro* approach. *General and Comparative Endocrinology* **160** 183–193. (doi:10.1016/j.ygcen.2008.11.020)
- Lubzens E, Young G, Bobe J & Cerdà J 2010 Oogenesis in teleosts: how fish eggs are formed. *General and Comparative Endocrinology* **165** 367–389. (doi:10.1016/j.ygcen.2009.05.022)
- Luckenbach JA, Kusakabe M, Swanson P & Young G 2008 Unilateral ovariectomy increases egg size and reduces follicular atresia in the semelparous coho salmon, *Oncorhynchus kisutch*. *Journal of Experimental Zoology* **309A** 468–476. (doi:10.1002/jez.476)
- Luckenbach JA, Dickey JT & Swanson P 2011 Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors *in vitro* during early secondary oocyte growth in coho salmon. *General and Comparative Endocrinology* **171** 52–63. (doi:10.1016/j.ygcen.2010.12.016)
- Mateos J, Mananós E, Carrillo M & Zanuy S 2002 Regulation of follicle stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean sea bass. *Comparative Biochemistry and Physiology Part B* **132** 75–86. (doi:10.1016/S1096-4959(01)00535-8)
- Mayer I, Shackley SE & Ryland JS 1988 Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. I. An histological and histochemical study of oocyte development. *Journal of Fish Biology* **33** 609–622. (doi:10.1111/j.1095-8649.1988.tb05504.x)
- Mayer I, Shackley SE & Witthames PR 1990 Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. II. Fecundity and pattern of oocyte development. *Journal of Fish Biology* **36** 141–148. (doi:10.1111/j.1095-8649.1990.tb05590.x)
- Medina A, Abascal FJ, Megina C & García A 2002 Stereological assessment of the reproductive status of female Atlantic northern bluefin tuna during migration to Mediterranean spawning grounds through the Strait of Gibraltar. *Journal of Fish Biology* **60** 203–217. (doi:10.1111/j.1095-8649.2002.tb02398.x)
- Mitter K, Kotoulas G, Magoulas A, Mulero V, Sepulcre P, Figueras A, Novoa B & Sarropoulou E 2009 Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European sea bass (*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology Part B* **153** 340–347. (doi:10.1016/j.cbpb.2009.04.009)
- Molés G, Carrillo M, Mananós E, Mylonas CC & Zanuy S 2007 Temporal profile of brain and pituitary GnRHs, GnRH-R and gonadotropin mRNA expression and content during early development in European sea bass (*Dicentrarchus labrax* L.). *General and Comparative Endocrinology* **150** 75–86. (doi:10.1016/j.ygcen.2006.07.012)
- Navas JM 2003 Efecto del contenido lipídico de las dietas administradas a reproductores de lubina (*Dicentrarchus labrax* L.) sobre el proceso reproductor y sobre la calidad y composición de los huevos. *PhD Dissertation*. University of Valencia, Spain.
- Neuhaus TP, Gashaw I, Sauer CG, von Ostau C, Kliesch S, Bergmann M, Hacker A & Grobholz R 2005 Standardization strategy for quantitative PCR in human seminoma and normal testis. *Journal of Biotechnology* **117** 163–171. (doi:10.1016/j.jbiotec.2005.01.011)

- Okuzawa K, Gen K, Bruysters M, Bogerd J, Gothilf Y, Zohar Y & Kagawa H 2003 Seasonal variation of the three native gonadotropin-releasing hormone messenger ribonucleic acids levels in the brain of female red seabream. *General and Comparative Endocrinology* **130** 324–332. (doi:10.1016/S0016-6480(02)00629-9)
- Patino R & Sullivan CV 2002 Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry* **26** 57–70. (doi:10.1023/A:1023311613987)
- Perazzolo LM, Coward K, Davail B, Normand E, Tyler CR, Pakdel F, Schneider WJ & Le Menn F 1999 Expression and localization of messenger ribonucleic acid for the vitellogenin receptor in ovarian follicles throughout oogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biology of Reproduction* **60** 1057–1068. (doi:10.1095/biolreprod60.5.1057)
- Prat F, Zanuy S, Bromage N & Carrillo M 1999 Effects of constant short and long photoperiod regimes on the spawning performance and sex steroid levels of female and male sea bass. *Journal of Fish Biology* **54** 125–137. (doi:10.1111/j.1095-8649.1999.tb00617.x)
- Reinecke M 2010 Insulin-like growth factors and fish reproduction. *Biology of Reproduction* **82** 656–661. (doi:10.1095/biolreprod.109.080093)
- Rocha A, Zanuy S, Carrillo M & Gómez A 2009 Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. *General and Comparative Endocrinology* **162** 265–275. (doi:10.1016/j.ygcen.2009.03.023)
- Shimasaki S, Moore RK, Otsuka F & Erickson GF 2004 The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews* **25** 72–101. (doi:10.1210/er.2003-0007)
- Stocco DM, Wang X, Jo Y & Manna PR 2005 Multiple signalling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Molecular Endocrinology* **19** 2647–2659. (doi:10.1210/me.2004-0532)
- Taranger GL, Carrillo M, Schulz RW, Fontaine P, Zanuy S, Felipe A, Weltzien F-A, Dufour S, Karlsen Ø, Norberg B *et al.* 2010 Control of puberty in farmed fish. *General and Comparative Endocrinology* **165** 483–515. (doi:10.1016/j.ygcen.2009.05.004)
- Tyler CR, Nagler JJ, Pottinger TG & Turner MA 1994 Effects of unilateral ovariectomy on recruitment and growth of follicles in the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry* **13** 309–316. (doi:10.1007/BF00003435)
- Tyler CR, Pottinger TG, Santos E, Sumpter JP, Price S-A, Brooks S & Nagler JJ 1996 Mechanisms controlling egg size and number in the rainbow trout, *Oncorhynchus mykiss*. *Biology of Reproduction* **54** 8–15. (doi:10.1095/biolreprod54.1.8)
- Tyler CR, Pottinger TG, Coward K, Prat F, Beresford N & Maddix S 1997 Salmonid follicle-stimulating hormone (GtH I) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. *Biology of Reproduction* **57** 1238–1244. (doi:10.1095/biolreprod57.5.1238)
- Weibel ET 1979 *Stereological Methods. Vol. 1: Practical Methods for Biological Morphometry*, London: Academic Press.
- Zanuy S, Carrillo M, Felipe A, Rodríguez L, Blázquez 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. (doi:10.1016/S0044-8486(01)00771-2)
- Zhang QJ, Chadderton A, Clark RL & Augustine-Rauch KA 2003 Selection of normalizer genes in conducting relative gene expression analysis of embryos. *Birth Defects Research. Part A, Clinical and Molecular Teratology* **67** 533–544. (doi:10.1002/bdra.10075)
- Zmora N, González-Martínez D, Muñoz-Cueto JA, Madigou T, Mañanós-Sánchez E, Doste SZ, Zohar Y, Kah O & Elizur A 2002 The GnRH system in the European sea bass (*Dicentrarchus labrax*). *Journal of Endocrinology* **172** 105–116. (doi:10.1677/joe.0.1720105)
- Zohar Y, Muñoz-Cueto JA, Elizur A & Kah O 2010 Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology* **165** 438–455. (doi:10.1016/j.ygcen.2009.04.017)

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