Bone morphogenetic protein 15 and growth differentiation factor 9 expression in the ovary of European sea bass (*Dicentrarchus labrax*): Cellular localization, developmental profiles, and response to unilateral ovariectomy

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Abstract

Vertebrate oocytes actively contribute to follicle development by secreting a variety of growth factors, among which bone morphogenetic protein 15 (BMP15/Bmp15) and growth differentiation factor 9 (GDF9/Gdf9) have been paid particular attention. In the present study, we describe the cellular localization, the developmental profiles, and the response to unilateral ovariectomy (a procedure implying the surgical removal of one of the ovaries) of protein and mRNA steady-state levels of Bmp15 and Gdf9 in the ovary of European sea bass, an important fish species for marine aquaculture industry. *In situ* hybridization and immunohistochemistry demonstrated that the oocyte is the main production site of Bmp15 and Gdf9 in European sea bass ovary. During oocyte development, Bmp15 protein expression started to be detected only from the lipid vesicle stage onwards but not in primary pre-vitellogenic (*i.e.* perinucleolar) oocytes as the *bmp15* mRNA already did. Gdf9 protein and *gdf9* mRNA expression were both detected in primary perinucleolar oocytes and followed similar decreasing patterns thereafter. Unilateral ovariectomy induced a full compensatory growth of the remaining ovary in the 2-month period following surgery (Á. García-López, M.I. Sánchez-Amaya, C.R. Tyler, F. Prat 2011). The compensatory growth elicited different changes in the expression levels of mRNA and protein of both factors, although the involvement of Bmp15 and Gdf9 in the regulatory network orchestrating such process remains unclear at present. Altogether, our results establish a solid base for further studies focused on elucidating the specific functions of Bmp15 and Gdf9 during primary and secondary oocyte growth in European sea bass.

Keywords: Teleost fish; oocyte; folliculogenesis; oogenesis; paracrine actions; Tgfb superfamily

1. Introduction

It is widely accepted that growth factors locally produced within the ovary play essential roles in controlling the development of vertebrate oocytes [19,23,24,31]. Some of these factors are produced early during oocyte development, regulating follicular growth in a gonadotropin-independent manner, and/or at later stages contributing to oocyte growth by modulating or mediating several gonadotropin activities within the follicle [19,23,24,31].

Among mammalian oocyte-derived growth factors, two members of the transforming growth factor, beta (TGFB) superfamily, namely bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), have received particular attention and numerous studies have demonstrated their essential roles in regulating ovarian follicular functions and development. *In vitro* treatment with BMP15 or GDF9 has been shown to enhance oocyte developmental competence [24] and to promote granulosa and theca cells proliferation together with a reduction of follicular steroidogenesis [14,43,50]. Moreover, complete neutralization of BMP15 or GDF9 results in a disruption of follicular growth from a very early stage of development, while partial neutralization leads to enhanced ovulation rates probably as a consequence of an increased sensitivity to follicle stimulating hormone (FSH; [29,30,40]). The use of 'knock-out' mice models has also shown that BMP15 regulates the fertilization potential of oocytes, while GDF9 is essential for normal follicular development [6,12,53].

As observed for mammals, the oocyte seems to be the primary expression site of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in teleost fish [4,7,32-34]. Even though mRNA expression profiles during follicular development suggest the involvement of both factors in early oogenesis stages in fish [22,32-34], experimental

evidence supporting this idea is lacking at present. Nevertheless, results of *in vitro* assays using zebrafish follicles suggest that Bmp15 plays a role in promoting secondary follicle growth while suppressing precocious oocyte maturation [7,8], whereas Gdf9 has been reported to modulate transcript abundance of tight junction complex components and steroidogenic acute regulatory protein [10].

Ligands of the TGFB superfamily in mammals are translated as pre-propeptide precursors consisting of an N-terminal signal peptide followed by a prodomain and a mature domain [6]. The biologically active form is obtained after dimerization of the protein precursor and cleavage of the mature peptide domain in homo-dimeric form [6]. After proteolytic cleavage, however, the prodomains of many TGFB ligands remain non-covalently associated in a complex with their mature domains; such association modulates the access of the peptides to their cognate receptors, even totally preventing the ligand-receptor interactions [11]. Dissociation of these complexes is tightly regulated by extrinsic biological processes, ensuring that the active mature ligand is only released at a location and at a moment where it is required [11,55]. Based on structural-sequence homologies, it is assumed that piscine Bmp15 and Gdf9 are similarly processed as their mammalian counterparts. Nevertheless, the expression of a homo-dimer has been only reported for zebrafish Bmp15 oocytes artificially over-expressing the *bmp15* gene [8].

Recently, we cloned the full cDNA sequences encoding *bmp15* and *gdf9* in European sea bass (*Dicentrarchus labrax*), one of the most important commercial marine fish species in the Mediterranean area, and found both transcripts highly expressed during early ovarian development [22]. In this study, European sea bass specific Bmp15 and Gdf9 anti-sera were produced in order to quantify protein levels throughout the annual ovarian development cycle as well as in isolated follicles at different developmental

stages. mRNA and protein expression sites for both factors were furthermore localized in the ovary by *in situ* hybridization and immunohistochemistry, respectively. Finally, the changes in ovarian mRNA and protein levels of both factors in response to unilateral ovariectomy (ULO), a surgical procedure implying the removal of one the gonads [17], were analyzed in order to evaluate a potential involvement of Bmp15 and Gdf9 in the growth compensation observed in the remaining ovary.

2. Material and Methods

2.1. Fish handling

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 Committees. Prior to every sampling, fish were sedated with 2-phenoxyethanol at a 300 ppm dose in order to reduce handling stress. For euthanasia, the double anaesthetic dose was applied followed by decapitation of the fish.

2.2. Anti-serum production

For polyclonal anti-serum production, recombinant European sea bass Bmp15 and Gdf9 peptides were produced in *Escherichia coli* BL21(DE3) cells (Novagen; Madison, WI, USA) transfected with the pBiEx-1 expression vector (Novagen) containing the cDNA sequences encoding the respective mature peptides (amino acids 303-459 for *bmp15*; amino acids 305-438 for *gdf9*; [22]). cDNA sequences were inserted in the *BamHI-HindIII* (for Bmp15 construct) and *KpnI-XhoI* (for Gdf9 construct) restriction sites of pBiEx-1, allowing the resulting recombinant peptides to include a N-terminal 6× His tag. Correct arrangements of expression constructs were confirmed by DNA sequencing. Bacterial inclusion bodies (containing the peptides) were isolated using

BugBuster® reagent (Novagen) and solubilized in PBS containing 4% v/v 2-mercaptoethanol. Purification of recombinant peptides was achieved by SDS-PAGE (12.5% gels; see section 2.3.) under reducing and denaturing conditions and subsequent excision of the respective protein bands from the gel. Bands were thoroughly minced, resuspended in 1mM EDTA plus complete protease inhibitor cocktail (Roche, Mannheim, Germany), and then incubated with agitation at 37ºC overnight for protein elution. Batches of eluted proteins were pooled, concentrated using Amicon ultra-15 centrifugal filter devices (10 KDa MWCO membrane; Millipore; Billerica, MA, USA), and stored at -80ºC. Anti-serum production was performed by Biomedal S.L. (Seville, Spain). Specificity of peptide and anti-serum production was ascertained by Coomassie blue staining and Western blot analysis using an anti-6×His epitope tag antibody (600-401-382; Rockland; Gilbertsville, PA, USA) and the specific anti-sera as described under section 2.3.

2.3. SDS-PAGE and Western blot analysis

Protein samples were quantified using the Pierce® BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, USA) and then submitted to electrophoresis in 4% stacking and 10-12.5% resolving polyacrylamide gels (0.1% w/v SDS) under denaturing and reducing or non-reducing conditions (10 min at 95°C in the presence or absence of 4% v/v 2-mercaptoethanol, respectively). Gels were stained with 0.1% w/v Coomassie brilliant blue R-250 (Fluka; Sigma-Aldrich, Madrid, Spain) in a 40% v/v methanol and 10% v/v acetic acid solution. A broad range prestained SDS-PAGE standard (Bio-Rad Laboratories; Hercules, CA, USA) was used as molecular weight marker.

For Western blot analysis, electrophoresed protein samples were blotted onto 0.45µm Amersham Hybond^{TM}-C Extra nitrocellulose membranes (GE Healthcare; Chalfont St.

Giles, UK). Membranes were blocked in 5% w/v non-fat milk for 60 min and then probed with anti-6×His epitope tag antibody (Rockland; 1/3,000), European sea bass Bmp15 anti-serum (1/20,000), or European sea bass Gdf9 anti-serum (1/6,000) for 60 min. For signal detection, membranes were incubated with donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibody (GE Healthcare, NA934; 1/5,000) for 60 min using the Amersham ECL system (GE Healthcare). Finally, chemiluminescent signals were visualized and quantified using a ChemiDoc™ XRS+ Molecular Imager equipped with the Image Lab™ software (Bio-Rad Laboratories).

2.4. Seasonal expression levels of Bmp15 and Gdf9 proteins in the ovary during the first reproductive cycle

Female European sea bass, obtained from a stock raised in the facilities of the Instituto de Acuicultura Torre de la Sal (Castellón, Spain), were monthly sampled during the first sexual maturation period. At each sampling point, five fish were euthanized and their ovaries dissected. Part of the ovarian tissue was frozen in liquid nitrogen for protein extraction (see below) and part was processed for histological analyses [46].

Crude protein extracts were obtained by disrupting around 100 mg ovarian tissue in 1 ml of buffer containing 72 mM Tris pH 6.8, 18.2% v/v glycerol, 3.64% w/v SDS. After centrifugation (15 min, 4ºC, 12,000 ×g), supernatants were collected, the total protein content determined, and then each sample (50 µg of total protein per lane) was subjected to SDS-PAGE and Western blot analysis as described under section 2.3.

2.5. Cellular localization of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the ovary

In situ hybridization and immunohistochemistry were performed on 6 µm paraffin

sections obtained from 4% paraformaldehyde fixed ovarian samples collected at different developmental stages [25].

2.5.1. In situ *hybridization*

In situ hybridization was performed as described in [41], but increasing the hybridization temperature to 50ºC. Sense and anti-sense cRNA probes were synthesized by *in vitro* transcription of European sea bass *bmp15* and *gdf9* cDNA sequences encoding the mature peptides (nucleotides 1041-1514 for *bmp15*; nucleotides 953-1357 for *gdf9*; [22]) as reported in [41].

2.5.2. Immunohistochemistry

For immunohistochemistry, sections were first submitted to an antigen retrieval treatment with 10 mM sodium citrate pH 6.0, 0.05% v/v Tween 20 at 95ºC for 20 min (only performed for Gdf9 detection). Then, slides were incubated with 0.35% v/v hydrogen peroxide for 15 min for endogenous peroxidase blocking, treated with 3% w/v bovine serum albumin for 60 min, and probed overnight with European sea bass Bmp15 anti-serum (1/500), European sea bass Gdf9 anti-serum (1/2,000) or pre-immune sera (at the same respective dilutions). For signal detection, sections were incubated with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich; A9169; 1/1,000) for 120 min and thereafter with 1.4 mM 3,3'-diaminobenzidine tetrahydrochloride hydrate, 0.01% v/v hydrogen peroxide.

2.6. Developmental expression levels of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in isolated follicular stages and ovulated oocytes

2.6.1. Isolation of ovarian follicles and ovulated oocytes

Ovarian follicles were collected from vitellogenic females in December. At this time, European sea bass ovaries contain great numbers of follicles at all developmental stages from primary growth to late vitellogenesis (see below) and hence, ensure sufficient sampling material. After dissection, ovaries were quickly split in small pieces, submerged in Allprotect™ Tissue Reagent (Qiagen; Hilden, Germany), and stored at 4ºC. Ovary pieces were then carefully transferred to a culture dish and follicles 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 [36,49]): (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. Ovulated oocytes (Ov) were furthermore collected from spawning European sea bass females in March and stored in Allprotect™ Tissue Reagent at 4ºC until further subsampling. Three different pools each one containing around 100-150 follicles/oocytes were collected and processed for mRNA and protein expression analyses.

2.6.2. mRNA expression analysis

Total RNA was extracted from isolated follicles/oocytes pools using the NucleoSpin®

RNA XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions (including an on-column DNase digestion) and further processed for quantifying *gdf9* and *bmp15* mRNA levels by real-time PCR as reported [18]. For *bmp15* analysis the primers GGCAGATTTGATGGGTCATT (forward) and CTTTAACAGGAACGGCGAAG (reverse) were used at 100 nM (amplicon size 117 bp), while for *gdf9* analysis the primers TCACAGGTGGACTCTTTCCA (forward) and GCTGCTCCAGATCAAACTTCTT (reverse) were used at 200 nM (amplicon size 104 bp). In addition, expression levels of the endogenous reference genes *18S ribosomal RNA* (*18s*) and *elongation factor 1-alpha* (*ef1a*) were measured as described [18]. The relative amount of each transcript in every sample was determined using the relative standard curve method as reported [18]; as standard, serially diluted cDNA was used that had been prepared from a pool of total RNA of different follicle/oocyte stages [18].

2.6.3. Protein expression analysis

To ensure sufficient protein amounts from each stage for Western blot analysis, the ethanolic lysates obtained from total RNA extraction of the different follicle/oocyte pools were combined resulting in only one pooled sample per stage of development. Samples were first submitted to diafiltration against a buffer containing 100 mM Tris pH 6.8, 0.3% w/v SDS using Amicon ultra-15 centrifugal filter devices. Subsequently, the buffer was exchanged for 100 mM Tris pH 6.8, 0.3% w/v SDS, 10% v/v glycerol, the supernatants obtained by centrifugation (15 min, 4ºC, 12,000 ×g) collected and the total protein content determined. SDS-PAGE and Western blot were performed as described under section 2.3. except different total protein loads per lane (2-50 µg) were used due to differences in total protein yield from follicles/oocytes at each developmental stage. Relative protein expression levels were obtained by correcting

band intensities by the respective protein load.

2.7. Expression of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the ovary of European sea bass in response to ULO

The changes in ovarian mRNA and protein levels of both factors in response to ULO was analyzed in order to evaluate a potential involvement of Bmp15 and Gdf9 in the growth compensation observed in the remaining ovary [17]. On 6th October 2009 (day 0), *i.e.* one month before the expected onset of vitellogenesis [45], randomly selected pit-tagged adult female European sea bass (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 euthanized (n=5; INITIAL control group) as described [17]. Both ovarian lobes were excised from euthanized fish in the INITIAL group. Sixty nine days after operations, on 14th December 2009, females in the ULO and SHAM groups were euthanized and the ovarian lobes dissected. Part of the ovarian tissue was frozen in liquid nitrogen for protein and RNA extraction and part was processed for histological analysis as described [17].

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 [52]. The end point of the experiment was chosen in accordance to the vitellogenic growth period in European sea bass [45], *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 [2,37].

Ovarian expression levels of *bmp15* and *gdf9* mRNAs in response to ULO were determined by real-time quantitative PCR as reported in [18] and using the primer pairs specified under section 2.6.2. In addition, expression levels of the endogenous reference

genes *18s* and *ef1a* were measured as described [18].

Quantification of Bmp15 and Gdf9 protein levels in the ovary was performed as described under sections 2.3. and 2.4.

2.8. Protein/gene expression data normalization, representation and statistical analyses

For normalization of gene expression data, two endogenous reference genes previously used in European sea bass tissues were considered: *18s* and *ef1a* (*e.g.* [3,47]). However, and even though identical RNA amounts and cDNA synthesis procedures were meticulously used for every sample analyzed none of them showed a constitutive expression in the sample sets analyzed (Supplementary Fig. 1). 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 [22]. Thus, we decided to use non-normalized relative transcript amounts for the target genes studied as recently reported [17,18]. This strategy is supported by studies showing opposite trends between normalized and non-normalized data [42], contradictory results depending on the reference gene used for the same data set [56], as well as the validity of non-normalized expression data to explain the molecular changes associated with oocyte/ovarian development in teleost fish [22,26]. Following the same rationale, protein expression levels were not normalized to any housekeeping reference protein, but only corrected in respect to total protein load.

Protein and gene expression levels are reported as fold change in respect to the values of a selected data group, whose average value was set to 1.

All the numeric data are expressed as mean ± standard error of mean (SEM). Significant differences between groups were identified by one-way ANOVA followed by the Student-Newman-Keuls test or by two-way ANOVA followed by the Bonferroni post

294 test. All statistical analyses were carried out adopting a significance level (p) of 0.05.

295

296 **3. Results**

297 3.1. Recombinant peptide production, anti-serum development, and validation of a
298 Western blot procedure

299 Bmp15 and Gdf9 recombinant peptides showed relative molecular weights of 32 KDa
300 and 28 KDa, respectively (Fig. 1A). In Western blots, both peptides were labelled with
301 an anti-6×His epitope tag antibody as well as with the respective specific anti-sera (Fig.
302 1A). In addition, Bmp15 and Gdf9 anti-sera bound to secondary products with
303 approximate double molecular weights (Fig. 1A), which are thought to correspond to
304 aggregates of the main peptide forms.

305 Western blots using European sea bass ovarian extracts, both under reducing and non-
306 reducing conditions, retrieved single protein bands of approximately 52 KDa for Bmp15
307 anti-serum and 54 KDa for Gdf9 anti-serum (Fig. 1B), while no specific signals were
308 obtained when extracts were probed with the pre-immune sera (not shown).

309

310 3.2. Ovarian expression levels of Bmp15 and Gdf9 proteins during the first reproductive
311 cycle

312 Bmp15 protein was not detected at pre-vitellogenesis, but was evident at the transition
313 to vitellogenesis (*i.e.* in November) (Fig. 2A). Thereafter, Bmp15 protein levels
314 remained high at the peak of vitellogenic period (*i.e.* in December), decreased as late
315 vitellogenesis, maturation-ovulation and post-spawning proceeded (*i.e.* from January to
316 April), and finally reached undetectable levels at late post-spawning/pre-vitellogenesis
317 (*i.e.* in May).

318 Gdf9 protein levels were high during pre-vitellogenesis (*i.e.* from July to October), but

319 decreased significantly with the progression of vitellogenesis until January, when Gdf9
320 protein remained undetectable (Fig. 2B). At maturation-ovulation (*i.e.* from February to
321 April), Gdf9 protein levels remained low/undetectable but increased again during late
322 post-spawning and pre-vitellogenesis (*i.e.* from May to June).
323 Representative histological sections of ovarian development during European sea bass
324 reproductive cycle are shown in Supplementary Fig. 2.
325
326 3.3. Cellular localization of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the
327 ovary
328 When European sea bass ovarian sections were hybridized with a *bmp15* anti-sense
329 probe, positive signals were exclusively found in the cytoplasm of oocytes (Fig. 3A).
330 The staining intensity was maximal at the perinucleolar stage and decreased steadily as
331 oocyte development progressed with hardly detectable signals at mid-late vitellogenesis.
332 Bmp15 protein was not detected at the perinucleolar stage, slightly appeared in the
333 cytoplasm of lipid vesicles stage-1 oocytes and reached maximum staining intensity in
334 oocytes at lipid vesicles stage-2 (Fig. 3B). Thereafter, signal amounts decreased in early
335 vitellogenic oocytes being hardly detectable at mid to late vitellogenesis. No signals
336 were detected in the follicular cells.
337 The cellular expression patterns of *gdf9* mRNA (Fig. 3C) and Gdf9 protein (Fig. 3D)
338 were similar to that described above for *bmp15* mRNA, although in case of Gdf9
339 protein, the decrease in the signal intensity from perinucleolar to early vitellogenic
340 oocytes was not so evident. No *gdf9*/Gdf9 signals were detected in the follicular cells.
341 In no case, unspecific labelling was observed when the respective sense probe or pre-
342 immune sera were used (inset panels in Fig. 3).
343

3.4. Developmental expression levels of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in isolated follicular stages and ovulated oocytes

Bmp15 protein levels were low in follicles at the perinucleolar stage (primary growth), sharply increased at the lipid vesicles stage, and decreased progressively during vitellogenesis (Fig. 4A). mRNA levels of *bmp15* (Fig. 4A) and *gdf9*/Gdf9 protein and mRNA amounts (Fig. 4B) followed approximately similar profiles during folliculogenesis with high expression levels in perinucleolar primary oocytes and progressively decreasing amounts in follicles from the lipid vesicles stage to late vitellogenesis.

Ovulated oocytes expressed significantly lower amounts of mRNAs for both factors than late vitellogenic follicles, while the levels of the respective proteins were approximately similar in both stages (Fig. 4).

3.5. Expression of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the ovary of European sea bass in response to ULO

There were no significant differences in the mRNA and protein expression levels of both factors between INITIAL control and ULO females at day 0 (Fig. 5). At 69 days after surgery, transcript levels for *bmp15* and *gdf9* were significantly reduced (approximately 1.5- and 1.6-fold, respectively) in ULO females compared to the SHAM control group. There were no significant differences for Bmp15 and Gdf9 ovarian protein levels between treatments.

4. Discussion

Follicular development has been historically regarded as being passively regulated by endocrine (mainly pituitary gonadotropins) and follicular somatic cell derived hormones

(particularly sex steroids and peptide growth factors); nevertheless it is now well known that mammalian oocytes actively secrete a variety of growth factors with essential functions in this process [20]. Although experimental information is lacking for most if not all teleost species, the recent identification and description of the expression patterns within the piscine ovary of several oocyte-derived growth factors with essential roles in mammalian folliculogenesis, such as Bmp15, Gdf9 or epidermal growth factors, have suggested that fish oocytes participate actively in the regulation of its own development as well [9,12,19,22,51]. In order to shed more light on the potential roles of these growth factors in piscine oogenesis, we provide here information on Bmp15 and Gdf9 transcript and protein levels in the ovary of European sea bass.

To study the expression patterns of Bmp15 and Gdf9 proteins, specific polyclonal anti-sera were developed and validated for their use in Western blots and immunohistochemistry using European sea bass ovarian samples. Single protein bands were obtained for each factor by Western blot using both reducing and non-reducing conditions and according to their sizes were attributed to Bmp15 and Gdf9 full pre-propeptide precursor forms, which have predicted weights of 51.7 KDa and 50.1 KDa, respectively [22]. Similarly, Lokman et al. [34] reported the detection of the pre-propeptide of Gdf9 as unique protein form in ovarian extracts of short-finned eel (*Anguilla australis australis*). On the other hand, three different Bmp15 protein bands, corresponding in size to the monomeric and dimeric mature domain and the precursor forms of the protein, were detected in zebrafish oocytes artificially over-expressing the *bmp15* gene, while only the precursor form of Bmp15 was detected in untreated oocytes [8]. Altogether, this information suggests that the pre-propeptide precursors of Bmp15 and Gdf9 are the predominant forms of both proteins in fish ovaries. The mature forms of fish Bmp15 and Gdf9, on the other hand, do not seem to be produced in a regular

manner as they are not readily detectable in ovarian/oocyte extracts under a variety of experimental conditions [this study, 8, 34]. This could be supported by observations reported in the mammalian ovary for several members of the TGFB superfamily, including BMP15, GDF8 and anti-Müllerian hormone, in which the production of mature peptides by dimerization and proteolytic cleavage of the precursors is tightly controlled in a timely and developmentally regulated manner ensuring that the active mature ligand is only present at a location and at a precise moment where it is required [11,55]. Further experimental work is required to determine if such situation applies to teleost Bmp15 and Gdf9 proteins as well (which may complicate to a great extent the detection of the mature peptides) or the lack of detection of the mature peptides is due to technical problems associated with the different polyclonal anti-sera used.

In teleost fish, several studies have analyzed ovarian mRNA levels of *bmp15* and *gdf9* during the course of follicular development. In most species, transcripts amounts of both factors are high during primary pre-vitellogenic growth, but decrease significantly with the progression of follicular development [22,32-34]. In contrast, there is little information on Bmp15 and Gdf9 protein expression levels during piscine oogenesis. Using specific anti-sera and European sea bass ovarian samples collected throughout an annual reproductive cycle and isolated follicles at different developmental stages, we found that Gdf9 protein followed similar temporal and developmental expression patterns as its transcript, with decreasing levels from primary growth and pre-vitellogenesis to late vitellogenesis [22, this study]. For Bmp15 protein, on the other hand, expression in the ovary/oocyte was undetectable/low during the primary growth phase but increased sharply during the beginning of secondary growth (lipid vesicles stage), *i.e.* when *bmp15* transcript levels began to decrease significantly [22, this study]. To our knowledge, this is the first time that *bmp15* mRNA and Bmp15 protein have

been reported to follow incongruent expression profiles, as in all species studied until now, *bmp15*/Bmp15 mRNA and protein (as well as *gdf9* and Gdf9) have been shown to possess overlapping spatiotemporal expression patterns in the ovary/oocyte (*e.g.*, goat [48], mouse [13,16,38,39], rat [43], hen [28], zebrafish [7], or short-finned eel [34]). Our results suggest that Gdf9 may play a role during the primary oocyte growth phase as proposed previously in European sea bass [22], while Bmp15 could participate in the control of more advances stages of follicular development as suggested in zebrafish [8].

In situ hybridization and immunohistochemistry demonstrated that both *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins are exclusively expressed in European sea bass oocytes as reported for zebrafish [33], rainbow trout (*Oncorhynchus mykiss*) [4], short-finned eel [34], human [1], mouse [13,16,38,39], rat [27,43], sheep [5], and hen [15]. The mRNAs and/or the proteins of both factors have also been detected in follicular somatic cells in some species, such as zebrafish [7,33], rainbow trout [32], goat [48], and hen [15,29], however in most cases at much lower amounts in comparison to oocytes. Taken together, this information indicates that the oocyte is the primary production site of BMP15/Bmp15 and GDF9/Gdf9 proteins in the ovary of vertebrates, including European sea bass.

According to the expression patterns of Bmp15 and Gdf9 proteins during European sea bass follicular development, it seems evident that the regulatory mechanisms controlling the protein amounts of each factor in sea bass oocytes are different. The surge of Bmp15 protein expression in the ovary/follicles coincides with the decrease of *gdf9*/Gdf9 mRNA and protein amounts and with a sharp increase of *follicle stimulating hormone receptor* (*fshr*) mRNA levels during the reproductive cycle/follicular development in this species [this study,17,46]. Interestingly, the expression levels of BMP15 protein (as well as of its mRNA) increase in response to FSH treatment in mouse ovary, but are

444 down-regulated in both FSH receptor null and +/- gonads [54]. In contrast, neither in
445 mouse nor in pre-vitellogenic coho salmon (*Oncorhynchus kisutch*) ovarian *Gdf9*/*gdf9*
446 expression has been shown to be modulated by FSH treatment *in vitro* [21,35]. Finally,
447 it has been demonstrated that mice lacking GDF9 show increased expression of *bmp15*
448 mRNA compared to wild type siblings, while BMP15 null and +/- mouse ovaries
449 exhibit normal *Gdf9* transcript levels [53]. Although further specific studies must be
450 performed for obtaining a solid conclusion, these evidences together with the expression
451 patterns of Bmp15 and Gdf9 proteins during follicular development lead us to
452 hypothesize that in European sea bass, Bmp15 oocyte levels could be regulated by both
453 FSH- and Gdf9-dependent signaling, while Gdf9 oocyte levels do not seem to be
454 modulated by FSH.
455 Finally, we analyzed the ovarian expression levels of *bmp15*/Bmp15 and *gdf9*/Gdf9
456 mRNAs and proteins in females subjected to ULO, a surgical procedure recently
457 applied for the first time in European sea bass by our laboratory [17]. Removal of one of
458 the ovarian lobes induced a full compensatory growth in the remaining lobe due to an
459 increased number of early perinucleolar oocytes and mid to late vitellogenic follicles
460 [17]. Results of gene expression analyses pointed towards members of the FSH-
461 dependent signaling pathway as the main players in orchestrating such an ovarian
462 compensatory growth [17]. We report here that ovarian expression levels of *bmp15* and
463 *gdf9* mRNAs were significantly down-regulated in hemi-castrated females, although
464 such changes were not associated with the decrease in the amounts of the respective
465 proteins. Thus, the potential involvement of both Bmp15 and Gdf9 in the compensatory
466 ovarian growth following ULO in European sea bass remains unclear at present. Further
467 experimental studies, for instance combining ULO with protocols directed to block
468 Bmp15- and/or Gdf9-dependent signalling, are required to identify their specific

functions in the process.

In conclusion, the results of the present study show that the oocyte is the primary production site of Bmp15 and Gdf9 in the ovary of European sea bass. *bmp15* and *gdf9* transcripts exhibit almost identical spatial, temporal, and developmental expression profiles in the ovary; however, the disparate expression patterns of their respective proteins suggest that the mechanisms regulating the Bmp15 and Gdf9 amounts in the oocyte as well as their functions in oogenesis are different. The reduced mRNA amounts of both factors found in the ovary of hemi-castrated females were not accompanied by the decrease in their respective protein amounts and, thus, it remains unclear at present if Bmp15 and Gdf9 are involved in the regulatory network orchestrating the compensatory ovarian growth observed after ULO in European sea bass.

Acknowledgements

Funding for the present work was provided by the Spanish Ministry of Education and Science to F.P through the grant AGL2007-61192/ACU. Á.G.-L. was supported by a contract from the CSIC-JAE program.

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Figure legends

Figure 1. Production of recombinant European sea bass Bmp15 and Gdf9 peptides in *E. coli* for polyclonal anti-serum generation (A) and validation of a Western blot procedure for detecting both proteins in European sea bass ovarian samples (B). Lanes in panel A correspond to: 1, standard (12.5% reducing and denaturing gel); 2 and 3, SDS-PAGE (12.5% gel) of purified Bmp15 and Gdf9 peptides (respectively) stained with Coomassie blue; 4 and 5, Western blot of purified Bmp15 and Gdf9 peptides (respectively) probed with an anti-6× His epitope tag antibody; and, 6 and 7, Western blot of purified Bmp15 and Gdf9 peptides (respectively) probed with their specific anti-sera. Black arrows depict monomeric forms of the purified peptides, while white arrows depict aggregated forms of them. Lanes in panel B correspond to: 1, standard (10% reducing and denaturing gel); 2-5, SDS-PAGE (10% gel) of ovarian extracts collected monthly on October, November, December, and January (respectively) stained with Coomassie blue; 6-9 and 10-13, Western blots of the same extracts probed with specific Bmp15 and Gdf9 anti-serum, respectively.

Figure 2. Seasonal expression levels of Bmp15 (A) and Gdf9 (B) proteins in the ovary of European sea bass during the first reproductive cycle (n=5 per month). Data are expressed as fold change in respect to the mean values for April (Bmp15) or December (Gdf9), which were set at 1. For each parameter, different superscripts denote significant differences between groups ($p<0.05$).

694 **Figure 3.** Cellular localization of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in
695 the ovary of European sea bass by *in situ* hybridization (A,C) and
696 immunohistochemistry (B,D). Insets correspond to sections incubated with sense probes
697 (A,C) or pre-immune sera (B,D). pn, perinucleolar; Lv1, lipid vesicles stage-1; Lv2,
698 lipid vesicles stage-2; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late
699 vitellogenic. Scale bars: 100 µm.
700
701 **Figure 4.** Developmental expression levels of *bmp15*/Bmp15 (A) and *gdf9*/Gdf9 (B)
702 mRNAs (n=3) and proteins (n=1) in isolated European sea bass follicles and ovulated
703 oocytes. Pn/Pg, perinucleolar stage (primary growth); Lv, lipid vesicles stage; Evit,
704 early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic; Ov, ovulated oocytes.
705 Data are expressed as fold change in respect to the mean values for Lvit, which were set
706 at 1. Different superscripts denote significant differences between groups ($p<0.05$).
707
708 **Figure 5.** Expression of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the
709 ovary of European sea bass in response to unilateral ovariectomy (ULO). Data are
710 expressed as fold change in respect to values for INITIAL females (day 0), which were
711 set at 1. An asterisk denotes a significant difference compared to INITIAL-SHAM
712 operated females at the same sampling date ($p<0.05$).
713

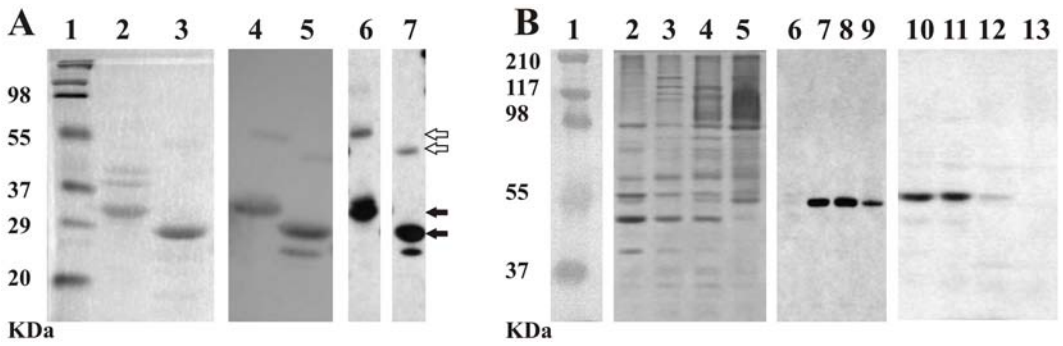
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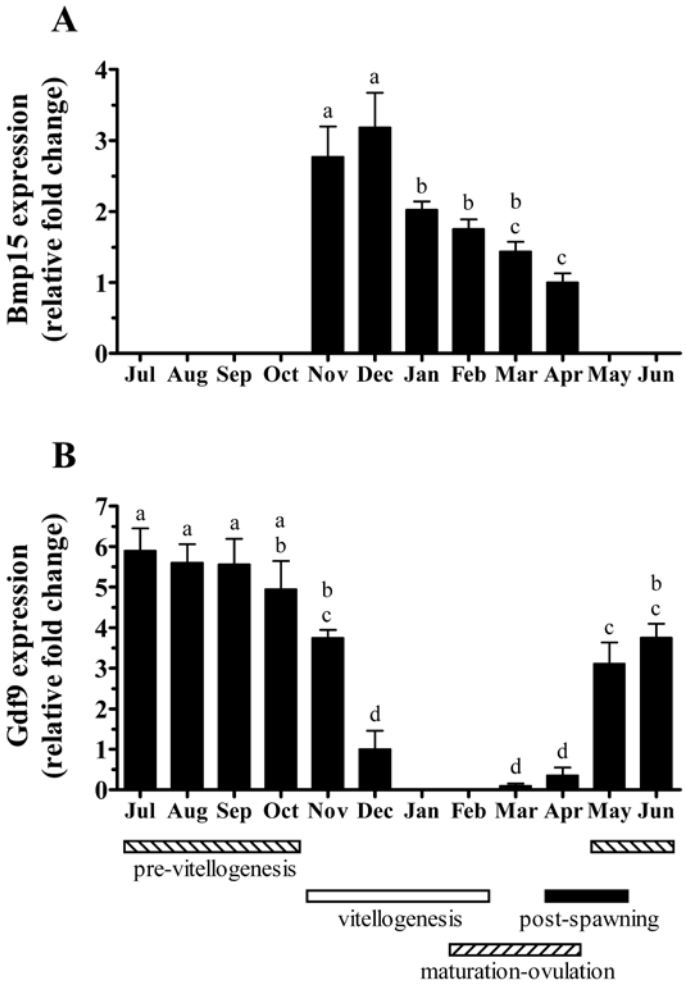
Figure 1



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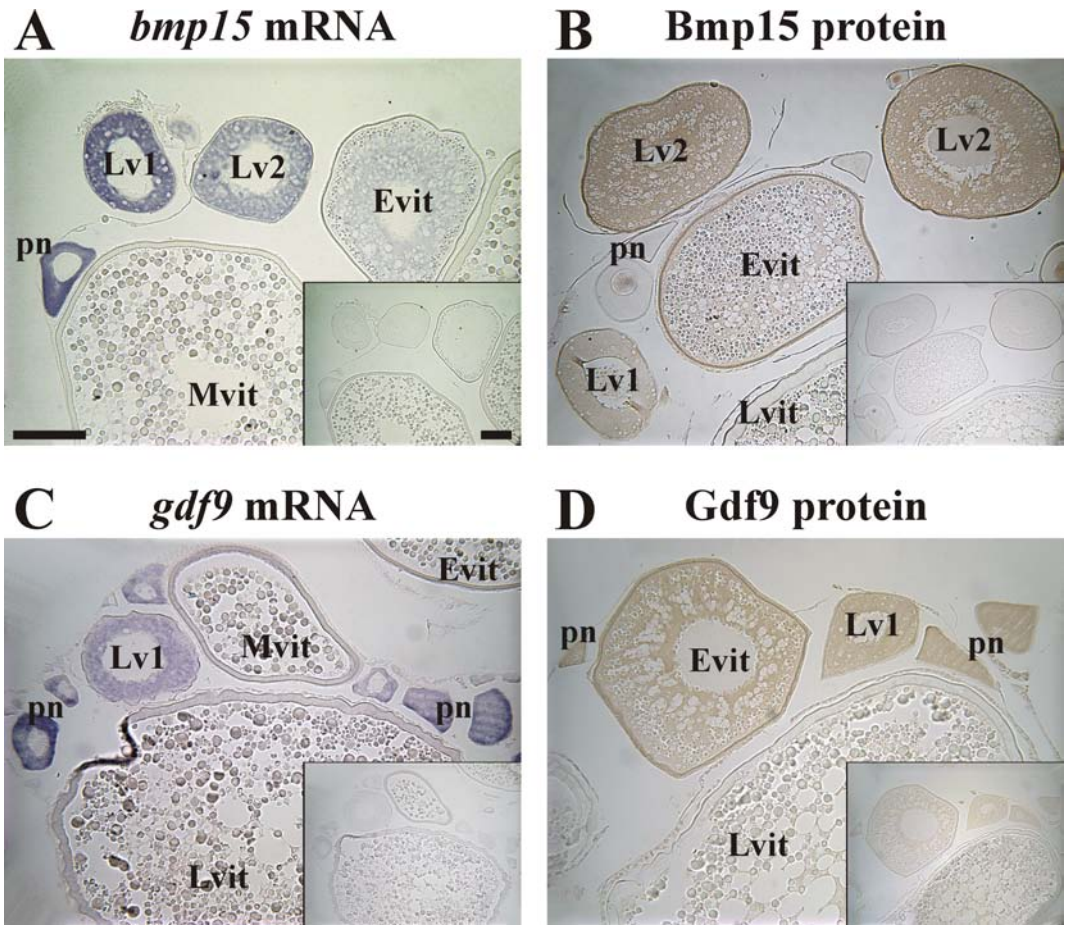

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720 Figure 3

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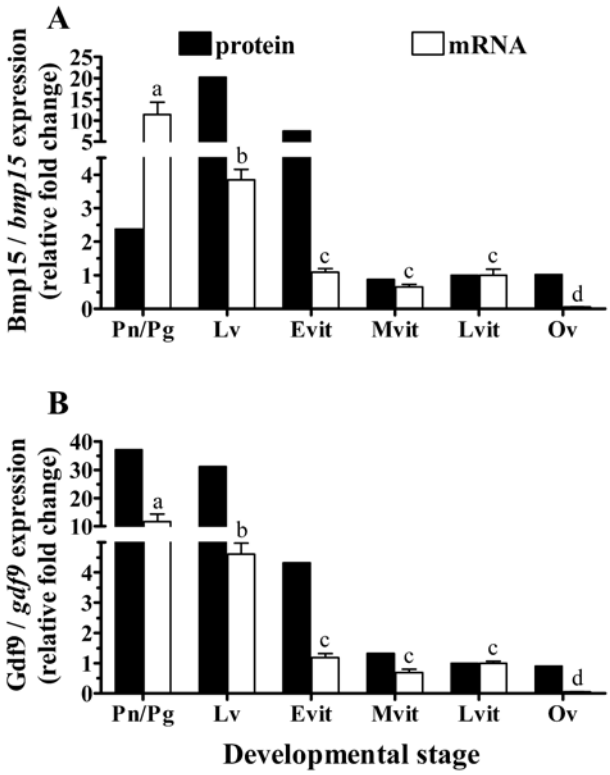

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723 Figure 4

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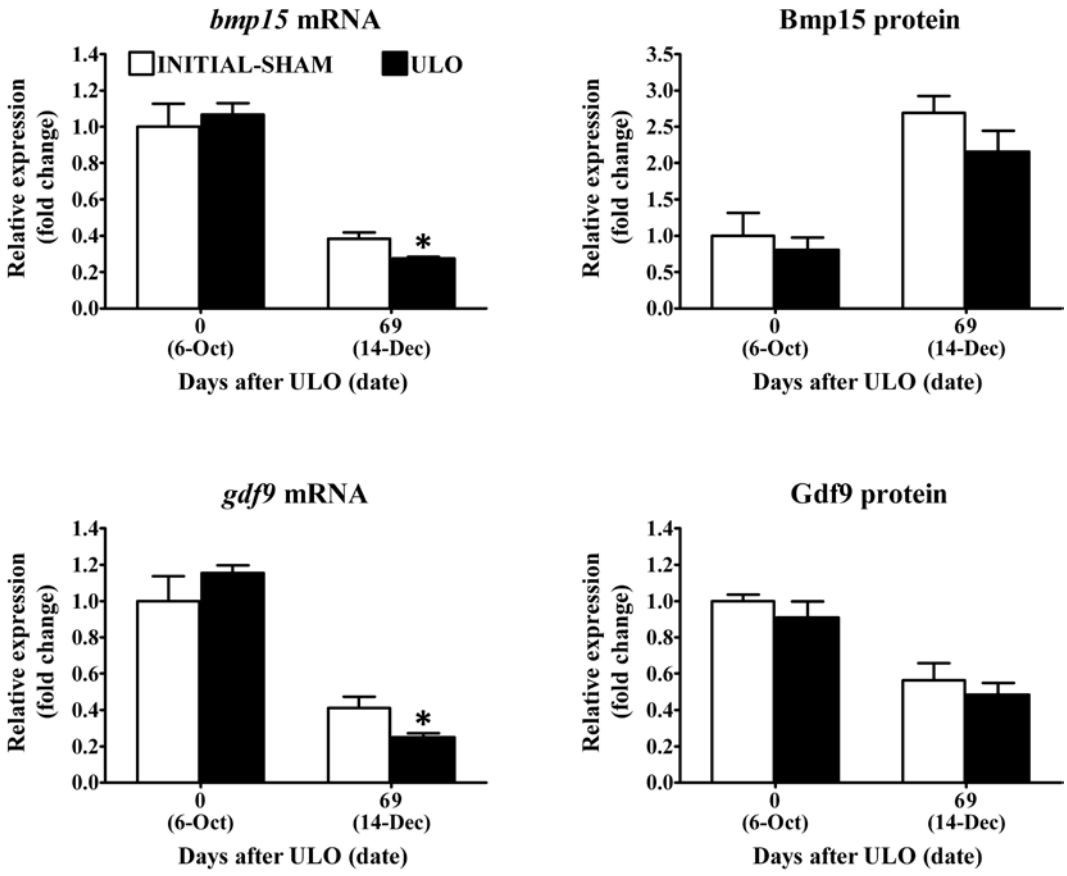

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726 Figure 5