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1 **Bone morphogenetic protein 15 and growth differentiation factor 9 expression in**
2 **the ovary of European sea bass (*Dicentrarchus labrax*): Cellular localization,**
3 **developmental profiles, and response to unilateral ovariectomy**
4
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21 **Abstract**

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

43

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

46

47 **1. Introduction**

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

54 Among mammalian oocyte-derived growth factors, two members of the transforming
55 growth factor, beta (TGFB) superfamily, namely bone morphogenetic protein 15
56 (BMP15) and growth differentiation factor 9 (GDF9), have received particular attention
57 and numerous studies have demonstrated their essential roles in regulating ovarian
58 follicular functions and development. *In vitro* treatment with BMP15 or GDF9 has been
59 shown to enhance oocyte developmental competence [24] and to promote granulosa and
60 theca cells proliferation together with a reduction of follicular steroidogenesis
61 [14,43,50]. Moreover, complete neutralization of BMP15 or GDF9 results in a
62 disruption of follicular growth from a very early stage of development, while partial
63 neutralization leads to enhanced ovulation rates probably as a consequence of an
64 increased sensitivity to follicle stimulating hormone (FSH; [29,30,40]). The use of
65 'knock-out' mice models has also shown that BMP15 regulates the fertilization
66 potential of oocytes, while GDF9 is essential for normal follicular development
67 [6,12,53].

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

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

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

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

97 stages. mRNA and protein expression sites for both factors were furthermore localized
98 in the ovary by *in situ* hybridization and immunohistochemistry, respectively. Finally,
99 the changes in ovarian mRNA and protein levels of both factors in response to unilateral
100 ovariectomy (ULO), a surgical procedure implying the removal of one the gonads [17],
101 were analyzed in order to evaluate a potential involvement of Bmp15 and Gdf9 in the
102 growth compensation observed in the remaining ovary.
103
104 **2. Material and Methods**
105 2.1. Fish handling
106 All experimental procedures involving care and use of live animals were carried out
107 according to Spanish national and European bioethical regulations and were approved
108 by the CSIC Bioethical Committees. Prior to every sampling, fish were sedated with 2-
109 phenoxyethanol at a 300 ppm dose in order to reduce handling stress. For euthanasia,
110 the double anaesthetic dose was applied followed by decapitation of the fish.
111
112 2.2. Anti-serum production
113 For polyclonal anti-serum production, recombinant European sea bass Bmp15 and Gdf9
114 peptides were produced in *Escherichia coli* BL21(DE3) cells (Novagen; Madison, WI,
115 USA) transfected with the pBiEx-1 expression vector (Novagen) containing the cDNA
116 sequences encoding the respective mature peptides (amino acids 303-459 for *bmp15*;
117 amino acids 305-438 for *gdf9*; [22]). cDNA sequences were inserted in the *BamHI-*
118 *HindIII* (for Bmp15 construct) and *KpnI-XhoI* (for Gdf9 construct) restriction sites of
119 pBiEx-1, allowing the resulting recombinant peptides to include a N-terminal 6× His
120 tag. Correct arrangements of expression constructs were confirmed by DNA
121 sequencing. Bacterial inclusion bodies (containing the peptides) were isolated using

122 BugBuster® reagent (Novagen) and solubilized in PBS containing 4% v/v 2-
123 mercaptoethanol. Purification of recombinant peptides was achieved by SDS-PAGE
124 (12.5% gels; see section 2.3.) under reducing and denaturing conditions and subsequent
125 excision of the respective protein bands from the gel. Bands were thoroughly minced,
126 resuspended in 1mM EDTA plus complete protease inhibitor cocktail (Roche,
127 Mannheim, Germany), and then incubated with agitation at 37ºC overnight for protein
128 elution. Batches of eluted proteins were pooled, concentrated using Amicon ultra-15
129 centrifugal filter devices (10 KDa MWCO membrane; Millipore; Billerica, MA, USA),
130 and stored at -80ºC. Anti-serum production was performed by Biomedal S.L. (Seville,
131 Spain). Specificity of peptide and anti-serum production was ascertained by Coomassie
132 blue staining and Western blot analysis using an anti-6×His epitope tag antibody (600-
133 401-382; Rockland; Gilbertsville, PA, USA) and the specific anti-sera as described
134 under section 2.3.
135
136 <u>2.3. SDS-PAGE and Western blot analysis</u>
137 Protein samples were quantified using the Pierce® BCA protein assay kit (Thermo
138 Fisher Scientific; Waltham, MA, USA) and then submitted to electrophoresis in 4%
139 stacking and 10-12.5% resolving polyacrylamide gels (0.1% w/v SDS) under denaturing
140 and reducing or non-reducing conditions (10 min at 95°C in the presence or absence of
141 4% v/v 2-mercaptoethanol, respectively). Gels were stained with 0.1% w/v Coomassie
142 brilliant blue R-250 (Fluka; Sigma-Aldrich, Madrid, Spain) in a 40% v/v methanol and
143 10% v/v acetic acid solution. A broad range prestained SDS-PAGE standard (Bio-Rad
144 Laboratories; Hercules, CA, USA) was used as molecular weight marker.
145 For Western blot analysis, electrophoresed protein samples were blotted onto 0.45µm
146 Amersham Hybond^{TM}-C Extra nitrocellulose membranes (GE Healthcare; Chalfont St.

147 Giles, UK). Membranes were blocked in 5% w/v non-fat milk for 60 min and then
148 probed with anti-6×His epitope tag antibody (Rockland; 1/3,000), European sea bass
149 Bmp15 anti-serum (1/20,000), or European sea bass Gdf9 anti-serum (1/6,000) for 60
150 min. For signal detection, membranes were incubated with donkey anti-rabbit IgG
151 horseradish peroxidase-linked secondary antibody (GE Healthcare, NA934; 1/5,000) for
152 60 min using the Amersham ECL system (GE Healthcare). Finally, chemiluminescent
153 signals were visualized and quantified using a ChemiDoc™ XRS+ Molecular Imager
154 equipped with the Image Lab™ software (Bio-Rad Laboratories).
155
156 <u>2.4. Seasonal expression levels of Bmp15 and Gdf9 proteins in the ovary during the first</u>
157 <u>reproductive cycle</u>
158 Female European sea bass, obtained from a stock raised in the facilities of the Instituto
159 de Acuicultura Torre de la Sal (Castellón, Spain), were monthly sampled during the first
160 sexual maturation period. At each sampling point, five fish were euthanized and their
161 ovaries dissected. Part of the ovarian tissue was frozen in liquid nitrogen for protein
162 extraction (see below) and part was processed for histological analyses [46].
163 Crude protein extracts were obtained by disrupting around 100 mg ovarian tissue in 1
164 ml of buffer containing 72 mM Tris pH 6.8, 18.2% v/v glycerol, 3.64% w/v SDS. After
165 centrifugation (15 min, 4ºC, 12,000 ×g), supernatants were collected, the total protein
166 content determined, and then each sample (50 μg of total protein per lane) was
167 subjected to SDS-PAGE and Western blot analysis as described under section 2.3.
168
169 <u>2.5. Cellular localization of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the</u>
170 <u>ovary</u>
171 *In situ* hybridization and immunohistochemistry were performed on 6 μm paraffin

172 sections obtained from 4% paraformaldehyde fixed ovarian samples collected at
173 different developmental stages [25].
174
175 *2.5.1.* In situ *hybridization*
176 *In situ* hybridization was performed as described in [41], but increasing the
177 hybridization temperature to 50ºC. Sense and anti-sense cRNA probes were synthesized
178 by *in vitro* transcription of European sea bass *bmp15* and *gdf9* cDNA sequences
179 encoding the mature peptides (nucleotides 1041-1514 for *bmp15*; nucleotides 953-1357
180 for *gdf9*; [22]) as reported in [41].
181
182 *2.5.2. Immunohistochemistry*
183 For immunohistochemistry, sections were first submitted to an antigen retrieval
184 treatment with 10 mM sodium citrate pH 6.0, 0.05% v/v Tween 20 at 95ºC for 20 min
185 (only performed for Gdf9 detection). Then, slides were incubated with 0.35% v/v
186 hydrogen peroxide for 15 min for endogenous peroxidase blocking, treated with 3% w/v
187 bovine serum albumin for 60 min, and probed overnight with European sea bass Bmp15
188 anti-serum (1/500), European sea bass Gdf9 anti-serum (1/2,000) or pre-immune sera
189 (at the same respective dilutions). For signal detection, sections were incubated with
190 goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma-
191 Aldrich; A9169; 1/1,000) for 120 min and thereafter with 1.4 mM 3,3'-
192 diaminobenzidine tetrahydrochloride hydrate, 0.01% v/v hydrogen peroxide.
193

194 2.6. Developmental expression levels of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and
195 proteins in isolated follicular stages and ovulated oocytes

196

197 *2.6.1. Isolation of ovarian follicles and ovulated oocytes*

198 Ovarian follicles were collected from vitellogenic females in December. At this time,
199 European sea bass ovaries contain great numbers of follicles at all developmental stages
200 from primary growth to late vitellogenesis (see below) and hence, ensure sufficient
201 sampling material. After dissection, ovaries were quickly split in small pieces,
202 submerged in Allprotect™ Tissue Reagent (Qiagen; Hilden, Germany), and stored at
203 4ºC. Ovary pieces were then carefully transferred to a culture dish and follicles were
204 manually isolated with watchmaker´s forceps under a dissecting microscope equipped
205 with an ocular micrometer. Follicles were classified into 5 stages according to size and
206 cytoplasm appearance [36,49]): (1) primary growth (Pg) stage consisting of chromatin
207 nucleolar and perinucleolar follicles with a diameter lower than 120 µm; (2) lipid
208 vesicles stage (Lv) follicles with a diameter between 120 and 250 µm; (3) early
209 vitellogenic (Evit) follicles with a diameter between 250 and 400 µm; (4) mid
210 vitellogenic (Mvit) follicles with a diameter from 400 to 600 µm of diameter; and (5)
211 late vitellogenic (Lvit) follicles with a diameter between 600 and 800 µm. Ovulated
212 oocytes (Ov) were furthermore collected from spawning European sea bass females in
213 March and stored in Allprotect™ Tissue Reagent at 4ºC until further subsampling.
214 Three different pools each one containing around 100-150 follicles/oocytes were
215 collected and processed for mRNA and protein expression analyses.

216

217 *2.6.2. mRNA expression analysis*

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

219 RNA XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's
220 instructions (including an on-column DNase digestion) and further processed for
221 quantifying *gdf9* and *bmp15* mRNA levels by real-time PCR as reported [18]. For
222 *bmp15* analysis the primers GGCAGATTTGATGGGTCATT (forward) and
223 CTTTAACAGGAACGGCGAAG (reverse) were used at 100 nM (amplicon size 117
224 bp), while for *gdf9* analysis the primers TCACAGGTGGACTCTTTCCA (forward) and
225 GCTGCTCCAGATCAAACTTCTT (reverse) were used at 200 nM (amplicon size 104
226 bp). In addition, expression levels of the endogenous reference genes *18S ribosomal*
227 *RNA* (*18s*) and *elongation factor 1-alpha* (*ef1a*) were measured as described [18]. The
228 relative amount of each transcript in every sample was determined using the relative
229 standard curve method as reported [18]; as standard, serially diluted cDNA was used
230 that had been prepared from a pool of total RNA of different follicle/oocyte stages [18].
231
232 *2.6.3. Protein expression analysis*
233 To ensure sufficient protein amounts from each stage for Western blot analysis, the
234 ethanolic lysates obtained from total RNA extraction of the different follicle/oocyte
235 pools were combined resulting in only one pooled sample per stage of development.
236 Samples were first submitted to diafiltration against a buffer containing 100 mM Tris
237 pH 6.8, 0.3% w/v SDS using Amicon ultra-15 centrifugal filter devices. Subsequently,
238 the buffer was exchanged for 100 mM Tris pH 6.8, 0.3% w/v SDS, 10% v/v glycerol,
239 the supernatants obtained by centrifugation (15 min, 4ºC, 12,000 ×g) collected and the
240 total protein content determined. SDS-PAGE and Western blot were performed as
241 described under section 2.3. except different total protein loads per lane (2-50 μg) were
242 used due to differences in total protein yield from follicles/oocytes at each
243 developmental stage. Relative protein expression levels were obtained by correcting

244 band intensities by the respective protein load.

245

246 <u>2.7. Expression of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the ovary of</u>

247 <u>European sea bass in response to ULO</u>

248 The changes in ovarian mRNA and protein levels of both factors in response to ULO

249 was analyzed in order to evaluate a potential involvement of Bmp15 and Gdf9 in the

250 growth compensation observed in the remaining ovary [17]. On 6th October 2009 (day

251 0), *i.e.* one month before the expected onset of vitellogenesis [45], randomly selected

252 pit-tagged adult female European sea bass (mean total body weight: 1.77±0.09 kg) were

253 either submitted to ULO operation (n=8; ULO group), sham operated (n=4; SHAM

254 group), or euthanized (n=5; INITIAL control group) as described [17]. Both ovarian

255 lobes were excised from euthanized fish in the INITIAL group. Sixty nine days after

256 operations, on 14th December 2009, females in the ULO and SHAM groups were

257 euthanized and the ovarian lobes dissected. Part of the ovarian tissue was frozen in

258 liquid nitrogen for protein and RNA extraction and part was processed for histological

259 analysis as described [17].

260 The starting point was chosen in accordance to results in rainbow trout showing that the

261 mid vitellogenesis was the critical developmental stage for inducing a new recruitment

262 of primary oocytes in the remaining ovarian lobe after ULO [52]. The end point of the

263 experiment was chosen in accordance to the vitellogenic growth period in European sea

264 bass [45], *i.e.* and at the time when most of the oocytes of the first main egg clutch are

265 expected to have reached the mid-late vitellogenesis stage [2,37].

266 Ovarian expression levels of *bmp15* and *gdf9* mRNAs in response to ULO were

267 determined by real-time quantitative PCR as reported in [18] and using the primer pairs

268 specified under section 2.6.2. In addition, expression levels of the endogenous reference

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

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

272

273 <u>2.8. Protein/gene expression data normalization, representation and statistical analyses</u>

274 For normalization of gene expression data, two endogenous reference genes previously
275 used in European sea bass tissues were considered: *18s* and *ef1a* (*e.g.* [3,47]). However,
276 and even though identical RNA amounts and cDNA synthesis procedures were
277 meticulously used for every sample analyzed none of them showed a constitutive
278 expression in the sample sets analyzed (Supplementary Fig. 1). In addition, other
279 common reference genes, like bactin or 28S ribosomal RNA, have already been proved
280 not to be suitable for their use in European sea bass ovarian samples [22]. Thus, we
281 decided to use non-normalized relative transcript amounts for the target genes studied as
282 recently reported [17,18]. This strategy is supported by studies showing opposite trends
283 between normalized and non-normalized data [42], contradictory results depending on
284 the reference gene used for the same data set [56], as well as the validity of non-
285 normalized expression data to explain the molecular changes associated with
286 oocyte/ovarian development in teleost fish [22,26]. Following the same rationale,
287 protein expression levels were not normalized to any housekeeping reference protein,
288 but only corrected in respect to total protein load.

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

291 All the numeric data are expressed as mean ± standard error of mean (SEM). Significant
292 differences between groups were identified by one-way ANOVA followed by the
293 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

344 3.4. Developmental expression levels of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and
345 proteins in isolated follicular stages and ovulated oocytes

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

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

356

357 3.5. Expression of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in the ovary of
358 European sea bass in response to ULO

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

365

366 **4. Discussion**

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

369 (particularly sex steroids and peptide growth factors); nevertheless it is now well known
370 that mammalian oocytes actively secrete a variety of growth factors with essential
371 functions in this process [20]. Although experimental information is lacking for most if
372 not all teleost species, the recent identification and description of the expression patterns
373 within the piscine ovary of several oocyte-derived growth factors with essential roles in
374 mammalian folliculogenesis, such as Bmp15, Gdf9 or epidermal growth factors, have
375 suggested that fish oocytes participate actively in the regulation of its own development
376 as well [9,12,19,22,51]. In order to shed more light on the potential roles of these
377 growth factors in piscine oogenesis, we provide here information on Bmp15 and Gdf9
378 transcript and protein levels in the ovary of European sea bass.
379 To study the expression patterns of Bmp15 and Gdf9 proteins, specific polyclonal anti-
380 sera were developed and validated for their use in Western blots and
381 immunohistochemistry using European sea bass ovarian samples. Single protein bands
382 were obtained for each factor by Western blot using both reducing and non-reducing
383 conditions and according to their sizes were attributed to Bmp15 and Gdf9 full pre-
384 propeptide precursor forms, which have predicted weights of 51.7 KDa and 50.1 KDa,
385 respectively [22]. Similarly, Lokman et al. [34] reported the detection of the pre-
386 propeptide of Gdf9 as unique protein form in ovarian extracts of short-finned eel
387 (*Anguilla australis australis*). On the other hand, three different Bmp15 protein bands,
388 corresponding in size to the monomeric and dimeric mature domain and the precursor
389 forms of the protein, were detected in zebrafish oocytes artificially over-expressing the
390 *bmp15* gene, while only the precursor form of Bmp15 was detected in untreated oocytes
391 [8]. Altogether, this information suggests that the pre-propeptide precursors of Bmp15
392 and Gdf9 are the predominant forms of both proteins in fish ovaries. The mature forms
393 of fish Bmp15 and Gdf9, on the other hand, do not seem to be produced in a regular

394 manner as they are not readily detectable in ovarian/oocyte extracts under a variety of
395 experimental conditions [this study, 8, 34]. This could be supported by observations
396 reported in the mammalian ovary for several members of the TGFB superfamily,
397 including BMP15, GDF8 and anti-Müllerian hormone, in which the production of
398 mature peptides by dimerization and proteolytic cleavage of the precursors is tightly
399 controlled in a timely and developmentally regulated manner ensuring that the active
400 mature ligand is only present at a location and at a precise moment where it is required
401 [11,55]. Further experimental work is required to determine if such situation applies to
402 teleost Bmp15 and Gdf9 proteins as well (which may complicate to a great extent the
403 detection of the mature peptides) or the lack of detection of the mature peptides is due to
404 technical problems associated with the different polyclonal anti-sera used.
405 In teleost fish, several studies have analyzed ovarian mRNA levels of *bmp15* and *gdf9*
406 during the course of follicular development. In most species, transcripts amounts of
407 both factors are high during primary pre-vitellogenic growth, but decrease significantly
408 with the progression of follicular development [22,32-34]. In contrast, there is little
409 information on Bmp15 and Gdf9 protein expression levels during piscine oogenesis.
410 Using specific anti-sera and European sea bass ovarian samples collected throughout an
411 annual reproductive cycle and isolated follicles at different developmental stages, we
412 found that Gdf9 protein followed similar temporal and developmental expression
413 patterns as its transcript, with decreasing levels from primary growth and pre-
414 vitellogenesis to late vitellogenesis [22, this study]. For Bmp15 protein, on the other
415 hand, expression in the ovary/oocyte was undetectable/low during the primary growth
416 phase but increased sharply during the beginning of secondary growth (lipid vesicles
417 stage), *i.e.* when *bmp15* transcript levels began to decrease significantly [22, this study].
418 To our knowledge, this is the first time that *bmp15* mRNA and Bmp15 protein have

419 been reported to follow incongruent expression profiles, as in all species studied until
420 now, *bmp15*/Bmp15 mRNA and protein (as well as *gdf9* and Gdf9) have been shown to
421 possess overlapping spatiotemporal expression patterns in the ovary/oocyte (*e.g.*, goat
422 [48], mouse [13,16,38,39], rat [43], hen [28], zebrafish [7], or short-finned eel [34]).
423 Our results suggest that Gdf9 may play a role during the primary oocyte growth phase
424 as proposed previously in European sea bass [22], while Bmp15 could participate in the
425 control of more advances stages of follicular development as suggested in zebrafish [8].
426 *In situ* hybridization and immunohistochemistry demonstrated that both *bmp15*/Bmp15
427 and *gdf9*/Gdf9 mRNAs and proteins are exclusively expressed in European sea bass
428 oocytes as reported for zebrafish [33], rainbow trout (*Oncorhynchus mykiss*) [4], short-
429 finned eel [34], human [1], mouse [13,16,38,39], rat [27,43], sheep [5], and hen [15].
430 The mRNAs and/or the proteins of both factors have also been detected in follicular
431 somatic cells in some species, such as zebrafish [7,33], rainbow trout [32], goat [48],
432 and hen [15,29], however in most cases at much lower amounts in comparison to
433 oocytes. Taken together, this information indicates that the oocyte is the primary
434 production site of BMP15/Bmp15 and GDF9/Gdf9 proteins in the ovary of vertebrates,
435 including European sea bass.
436 According to the expression patterns of Bmp15 and Gdf9 proteins during European sea
437 bass follicular development, it seems evident that the regulatory mechanisms controlling
438 the protein amounts of each factor in sea bass oocytes are different. The surge of Bmp15
439 protein expression in the ovary/follicles coincides with the decrease of *gdf9*/Gdf9
440 mRNA and protein amounts and with a sharp increase of *follicle stimulating hormone*
441 *receptor* (*fshr*) mRNA levels during the reproductive cycle/follicular development in
442 this species [this study,17,46]. Interestingly, the expression levels of BMP15 protein (as
443 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

469 functions in the process.

470 In conclusion, the results of the present study show that the oocyte is the primary 471 production site of Bmp15 and Gdf9 in the ovary of European sea bass. *bmp15* and *gdf9* 472 transcripts exhibit almost identical spatial, temporal, and developmental expression 473 profiles in the ovary; however, the disparate expression patterns of their respective 474 proteins suggest that the mechanisms regulating the Bmp15 and Gdf9 amounts in the 475 oocyte as well as their functions in oogenesis are different. The reduced mRNA 476 amounts of both factors found in the ovary of hemi-castrated females were not 477 accompanied by the decrease in their respective protein amounts and, thus, it remains 478 unclear at present if Bmp15 and Gdf9 are involved in the regulatory network 479 orchestrating the compensatory ovarian growth observed after ULO in European sea 480 bass.

481

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486

487 **References**

488 [1] J. Aaltonen, M.P. Laitinen, K. Vuojolainen, R. Jaatinen, N. Horelli-Kuitunen, L. 489 Seppa, H. Louhio, T. Tuuri, J. Sjoberg, R. Butzow, O. Hovatta, L. Dale, O. Ritvos, 490 Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B 491 are expressed in oocytes during early folliculogenesis, J. Clin. Endocrinol. Metab. 492 84 (1999) 2744-2750.

493 [2] J.F. Asturiano, L.A. Sorbera, J. Ramos, D.E. Kime, M. Carrillo, S. Zanuy,

494 Hormonal regulation of the European sea bass reproductive cycle: an
495 individualized female approach, J. Fish Biol. 56 (2000) 1155-1172.
496 [3] M. Blázquez, A. González, M. Papadaki, C.C. Mylonas, F. Piferrer, Sex-related
497 changes in estrogen receptors and aromatase gene expression and enzymatic
498 activity during early development and sex differentiation in the European sea bass
499 (*Dicentrarchus labrax*), Gen. Comp. Endocrinol. 158 (2008) 95-101.
500 [4] J. Bobe, T. Nguyen, S. Mahé, P. Monget, In silico identification and molecular
501 characterization of genes predominantly expressed in the fish oocyte, BMC
502 Genomics 9 (2008) 499.
503 [5] K.J. Bodensteiner, C.M. Clay, C.L. Moeller, H.R. Sawyer, Molecular cloning of
504 the ovine growth/differentiation factor-9 gene and expression of
505 growth/differentiation factor-9 in ovine and bovine ovaries, Biol. Reprod. 60
506 (1999) 381-386.
507 [6] H. Chang, C.W. Brown, M.M. Matzuk, Genetic analysis of the mammalian
508 transforming growth factor-β superfamily, Endocr. Rev. 23 (2002) 787-823.
509 [7] E. Clelland, G. Kohli, R.K. Campbell, S. Sharma, S. Shimasaki, C. Peng, Bone
510 morphogenetic protein-15 in the zebrafish ovary: Complementary
511 deoxyribonucleic acid cloning, genomic organization, tissue distribution, and role
512 in oocyte maturation, Endocrinology 147 (2006) 201-209.
513 [8] E.S. Clelland, Q. Tan, A. Balofsky, R. Lacivita, C. Peng, Inhibition of premature
514 oocyte maturation: A role for bone morphogenetic protein 15 in zebrafish ovarian
515 follicles, Endocrinology 148 (2007) 5451-5458.
516 [9] E. Clelland, C. Peng, Endocrine/paracrine control of zebrafish ovarian
517 development, Mol. Cell. Endocrinol 312 (2009) 42-52.
518 [10] E.S. Clelland, S.P. Kelly, Exogenous GDF9 but not Activin A, BMP15 or TGFβ

519 alters tight junction protein transcript abundance in zebrafish ovarian follicles,
520 Gen. Comp. Endocrinol. 171 (2011) 211-217.
521 [11] N. di Clemente, S.P. Jamin, A. Lugovskoy, P. Carmillo, C. Ehrenfels, J.-Y. Picard,
522 A. Whitty, N. Josso, R.B. Pepinsky, R.L. Cate, Processing of anti-Mllerian
523 hormone regulates receptor activation by a mechanism distinct from TGF-β, Mol.
524 Endocrinol. 24 (2010) 2193-2206.
525 [12] J. Dong, D.F. Albertini, K. Nishimori, T.R. Kumar, N. Lu, M.M. Matzuk, Growth
526 differentiation factor-9 is required during early ovarian folliculogenesis, Nature
527 383 (1996) 531-535.
528 [13] J.L. Dube, P. Wang, J. Elvin, K.M. Lyons, A.J. Celeste, M.M. Matzuk, The bone
529 morphogenetic protein 15 gene is X-linked and expressed in oocytes, Mol.
530 Endocrinol. 12 (1998) 1809-1817.
531 [14] S.J. Edwards, K.L. Reader, S. Lun, A. Western, S. Lawrence, K.P. McNatty, J.L.
532 Juengel, The cooperative effect of growth and differentiation factor-9 and bone
533 morphogenetic protein (BMP)-15 on granulosa cell function is modulated
534 primarily through BMP receptor II, Endocrinology 149 (2008) 1026-1030.
535 [15] S. Elis, J. Dupont, I. Couty, L. Persani, M. Govoroun, E. Blesbois, F. Batellier, P.
536 Monget, Expression and biological effects of bone morphogenetic protein-15 in
537 the hen ovary, J. Endocrinol. 194 (2007) 485-497.
538 [16] J.A. Elvin, A.T. Clark, P. Wang, N.M. Wolfman, M.M. Matzuk, Paracrine actions
539 of growth differentiation factor-9 in the mammalian ovary, Mol. Endocrinol. 13
540 (1999) 1035-1048.
541 [17] Á. García-López, M.I. Sánchez-Amaya, C.R. Tyler, F. Prat, Mechanisms of oocyte
542 development in European sea bass (*Dicentrarchus labrax*, L.): Investigations via
543 application of unilateral ovariectomy, Reproduction 142 (2011) 243-253.

544 [18] Á. García-López, M.I. Sánchez-Amaya, F. Prat, Targeted gene expression
545 profiling in European sea bass (*Dicentrarchus labrax*, L.) follicles from primary
546 growth to late vitellogenesis, Comp. Biochem. Physiol. A 160 (2011) 374-380.
547 [19] W. Ge, Intrafollicular paracrine communication in the zebrafish ovary: The state of
548 the art of an emerging model for the study of vertebrate folliculogenesis, Mol.
549 Cell. Endocrinol. 237 (2005) 1-10.
550 [20] R.B. Gilchrist, L.J. Ritter, D.T. Armstrong, Oocyte-somatic cell interactions
551 during follicle development in mammals, Anim. Reprod. Sci. 82/83 (2004) 431-
552 446.
553 [21] X. Guéripel, V. Brun, A. Gougeon, Oocyte bone morphogenetic protein 15, but not
554 growth differentiation factor 9, is increased during gonadotropin-induced follicular
555 development in the immature mouse and is associated with cumulus oophorus
556 expansion, Biol. Reprod. 75 (2006) 836-843.
557 [22] S. Halm, A.J. Ibañez, C.R. Tyler, F. Prat, Molecular characterisation of growth
558 differentiation factor 9 (*gdf9*) and bone morphogenetic protein 15 (*bmp15*) and
559 their patterns of gene expression during the ovarian reproductive cycle in the
560 European sea bass, Mol. Cell. Endocrinol. 291 (2008) 95-103.
561 [23] S.G. Hillier, Gonadotropic control of ovarian follicular growth and development,
562 Mol. Cell. Endocrinol. 179 (2001) 39-46.
563 [24] T.S. Hussein, J.G. Thompson, R.B. Gilchrist, Oocyte-secreted factors enhance
564 oocyte developmental competence, Dev. Biol. 296 (2006) 514-521.
565 [25] A.J. Ibáñez, J. Peinado-Onsurbe, E. Sánchez, J.M. Cerdá-Reverter, F. Prat,
566 Lipoprotein lipase (LPL) is highly expressed and active in the ovary of European
567 sea bass (*Dicentrarchus labrax* L.), during gonadal development, Comp. Biochem.
568 Physiol. A 150 (2008) 347-354.

569 [26] J.S. Ings, G.J. Van der Kraak, Characterization of the mRNA expression of StAR
570 and steroidogenic enzymes in zebrafish ovarian follicles, Mol. Reprod. Develop.
571 73 (2006) 943-954.
572 [27] R. Jaatinen, M.P. Laitinen, K. Vuojolainen, J. Aaltonen, H. Louhio, K.
573 Heikinheimo, E Lehtonen, O. Ritvos, Localization of growth differentiation factor-
574 9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and
575 its novel homolog GDF-9B, Mol. Cell. Endocrinol. 156 (1999) 189-193.
576 [28] P.A. Johnson, M.J. Dickens, T.R. Kent, J.R. Giles, Expression and function of
577 growth differentiation factor-9 in an Oviparous species, *Gallus domesticus*, Biol.
578 Reprod. 72 (2005) 1095-1100.
579 [29] J.L. Juengel, N.L. Hudson, D.A. Heath, P. Smith, K.L. Reader, S.B. Lawrence,
580 A.R. O'Connell, M.P.E. Laitinen, M. Cranfield, N.P. Groome, O. Ritvos, K.P.
581 McNatty, Growth differentiation factor 9 and bone morphogenetic protein 15 are
582 essential for ovarian follicular development in sheep, Biol. Reprod. 67 (2002)
583 1777-1789.
584 [30] J.L. Juengel, N.L. Hudson, M. Berg, K. Hamel, P. Smith, S.B. Lawrence, L.
585 Whiting, K.P. McNatty, Effects of active immunization against growth
586 differentiation factor 9 and/or bone morphogenetic protein 15 on ovarian function
587 in cattle, Reproduction 138 (2009) 107-114.
588 [31] P.G. Knight, C. Glister, TGF-β superfamily members and ovarian follicle
589 development, Reproduction 132 (2006) 191-206.
590 [32] S.E. Lankford, G.M. Weber, Temporal mRNA expression of transforming growth
591 factor-beta superfamily members and inhibitors in the developing rainbow trout
592 ovary, Gen. Comp. Endocrinol. 166 (2010) 250-258.
593 [33] L. Liu, W. Ge, Growth differentiation factor 9 and its spatiotemporal expression

594 and regulation in the zebrafish ovary, Biol. Reprod. 76 (2007) 294-302.
595 [34] P.M. Lokman, Y. Kazeto, Y. Ozaki, S. Ijiri, R. Tosaka, M. Kohara, S.L. Divers, H.
596 Matsubara, L.G. Moore, S. Adachi, Effects of reproductive stage, GH, and 11-
597 ketotestosterone on expression of growth differentiation factor-9 in the ovary of
598 the eel, *Anguilla australis*, Reproduction 139 (2010) 71-83.
599 [35] J.A. Luckenbach, J.T. Dickey, P. Swanson, Follicle-stimulating hormone
600 regulation of ovarian transcripts for steroidogenesis-related proteins and cell
601 survival, growth and differentiation factors in vitro during early secondary oocyte
602 growth in coho salmon, Gen. Comp. Endocrinol. 171 (2011) 52-63.
603 [36] I. Mayer, S.E. Shackley, J.S. Ryland, Aspects of the reproductive biology of the
604 bass, *Dicentrarchus labrax* L. I. An histological and histochemical study of oocyte
605 development, J. Fish Biol. 33 (1988) 609-622.
606 [37] I. Mayer, S.E. Shackley, P.R. Witthames, Aspects of the reproductive biology of
607 the bass, *Dicentrarchus labrax* L. II. Fecundity and pattern of oocyte development,
608 J. Fish Biol. 36 (1990) 141-148.
609 [38] S.A. McGrath, A.F. Esquela, S.J. Lee, Oocyte-specific expression of growth
610 differentiation factor-9, Mol. Endocrinol. 9 (1995) 131-136.
611 [39] M. Monti, C.A. Redi, Oogenesis specific genes (Nobox, Oct4, Bmp15, Gdf9,
612 Oogenesin1 and Oogenesin2) are differentially expressed during natural and
613 gonadotropin-induced mouse follicular development, Mol. Reprod. Dev. 76 (2009)
614 994-1003.
615 [40] R.K. Moore, S. Shimasaki, Molecular biology and physiological role of the oocyte
616 factor, BMP-15, Mol. Cell. Endocrinol. 234 (2005) 67-73.
617 [41] H.M. Murray, S.E. Douglas, J.W. Gallant, J.C. Pérez-Casanova, S.C. Johnson,
618 Ontogeny of lipase expression in winter flounder, *Pseuodopleuronectes*

619 *americanus*, J. Fish Biol. 62 (2003) 816-833.
620 [42] T.P. Neuvians, I. Gashaw, C.G. Sauer, C. von Ostau, S. Kliesch, M. Bergmann, A.
621 Hacker, R. Grobholz, Standardization strategy for quantitative PCR in human
622 seminoma and normal testis, J. Biotechnol. 117 (2005) 163-171.
623 [43] F. Otsuka, Z. Yao, T. Lee, S. Yamamoto, G.F. Erickson, S. Shimasaki, Bone
624 morphogenetic protein-15. Identification of target cells and biological functions, J.
625 Biol. Chem. 275 (2000) 39523-39528.
626 [44] C. Peng, E. Clelland, Q. Tan, Potential role of bone morphogenetic protein-15 in
627 zebrafish follicle development and oocyte maturation, Comp. Biochem. Physiol. A
628 153 (2009) 83-87.
629 [45] F. Prat, S. Zanuy, S., N. Bromage, M. Carrillo, Effects of constant short and long
630 photoperiod regimes on the spawning performance and sex steroid levels of female
631 and male sea bass, J. Fish Biol. 54 (1999) 125-137.
632 [46] A. Rocha, A. Gómez, M. Galay-Burgos, S. Zanuy, G.E. Sweeney, M. Carrillo,
633 Molecular characterization and seasonal changes in gonadal expression of a
634 thyrotropin receptor in the European sea bass, Gen. Comp. Endocrinol. 152 (2007)
635 89-101.
636 [47] A. Rocha, S. Zanuy, M. Carrillo, A. Gómez, Seasonal changes in gonadal
637 expression of gonadotropin receptors, steroidogenic acute regulatory protein and
638 steroidogenic enzymes in the European sea bass, Gen. Comp. Endocrinol. 162
639 (2009) 265-275.
640 [48] J.R.V. Silva, R.V.D. Hurk, H.T.A. van Tol, B.A.J. Roelen, J.R. Figueiredo,
641 Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein
642 15 (BMP15), and BMP receptors in the ovaries of goats, Mol. Reprod. Dev. 70
643 (2005) 11-19.

644 [49] L.A. Sorbera, J.F. Asturiano, M. Carrillo, J. Cerda, D.E. Kime, S. Zanuy, *In vitro*
645 oocyte maturation in the sea bass: effects of hCG, pituitary extract and steroids, J.
646 Fish Biol. 55 (1999) 9-25.
647 [50] L.J. Spicer, P.Y. Aad, D.T. Allen, S. Mazerbourg, A.H. Payne, A.J.. Hsueh,
648 Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits
649 steroidogenesis by bovine theca cells: Influence of follicle size on responses to
650 GDF9, Biol. Reprod. 78 (2008) 243-253.
651 [51] A.C.-K. Tse, W. Ge, Spatial localization of EGF family ligands and receptors in
652 the zebrafish ovarian follicle and their expression profiles during folliculogenesis,
653 Gen. Comp. Endocrinol. 167 (2010) 397-407.
654 [52] C.R. Tyler, J.J. Nagler, T.G. Pottinger, M.A. Turner, Effects of
655 unilateralovariectomy on recruitment and growth of follicles in the rainbow trout,
656 *Oncorhynchus mykiss*, Fish Physiol. Biochem. 13 (1994) 309-316.
657 [53] C. Yan, P. Wang, J. DeMayo, F.J. DeMayo, J.A. Elvin, C. Carino, S.V. Prasad,
658 S.S. Skinner, B.S. Dunbar, J.L. Dube, A.J. Celeste, M.M. Matzuk, Synergistic
659 roles of bone morphogenetic protein 15 and growth differentiation factor 9 in
660 ovarian function, Mol. Endocrinol. 15 (2001) 854-866.
661 [54] Y. Yang, A. Balla, N. Danilovich, M.R. Sairam, Developmental and molecular
662 aberrations associated with deterioration of oogenesis during complete or partial
663 follicle-stimulating hormone receptor deficiency in mice, Biol. Reprod. 69 (2003)
664 1294-1302.
665 [55] O. Yoshino, H.E. McMahon, S. Sharma, S. Shimasaki, A unique preovulatory
666 expression pattern plays a key role in the physiological functions of BMP-15 in the
667 mouse, Proc. Natl. Acad. Sci. USA 103 (2006) 10678-10683.
668 [56] Q.J. Zhang, A. Chadderton, R.L. Clark, K.A. Augustine-Rauch, Selection of

669 normalizer genes in conducting relative gene expression analysis of embryos, Birth
670 Defects Res. A Clin. Mol. Teratol. 67 (2003) 533-544.
671

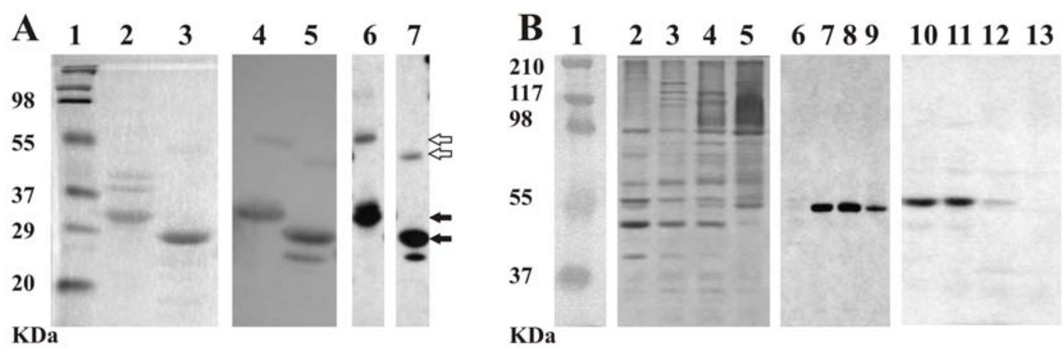
672 **Figure legends**

673 **Figure 1.** Production of recombinant European sea bass Bmp15 and Gdf9 peptides in *E.*
674 *coli* for polyclonal anti-serum generation (A) and validation of a Western blot procedure
675 for detecting both proteins in European sea bass ovarian samples (B). Lanes in panel A
676 correspond to: 1, standard (12.5% reducing and denaturing gel); 2 and 3, SDS-PAGE
677 (12.5% gel) of purified Bmp15 and Gdf9 peptides (respectively) stained with
678 Coomassie blue; 4 and 5, Western blot of purified Bmp15 and Gdf9 peptides
679 (respectively) probed with an anti-6× His epitope tag antibody; and, 6 and 7, Western
680 blot of purified Bmp15 and Gdf9 peptides (respectively) probed with their specific anti-
681 sera. Black arrows depict monomeric forms of the purified peptides, while white arrows
682 depict aggregated forms of them. Lanes in panel B correspond to: 1, standard (10%
683 reducing and denaturing gel); 2-5, SDS-PAGE (10% gel) of ovarian extracts collected
684 monthly on October, November, December, and January (respectively) stained with
685 Coomassie blue; 6-9 and 10-13, Western blots of the same extracts probed with specific
686 Bmp15 and Gdf9 anti-serum, respectively.
687

688 **Figure 2.** Seasonal expression levels of Bmp15 (A) and Gdf9 (B) proteins in the ovary
689 of European sea bass during the first reproductive cycle (n=5 per month). Data are
690 expressed as fold change in respect to the mean values for April (Bmp15) or December
691 (Gdf9), which were set at 1. For each parameter, different superscripts denote
692 significant differences between groups ($p<0.05$).
693

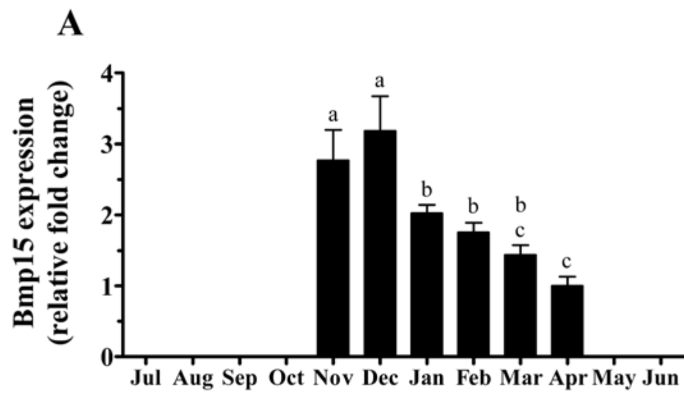
694 **Figure 3.** Cellular localization of *bmp15*/Bmp15 and *gdf9*/Gdf9 mRNAs and proteins in
695 the ovary of European sea bass by *in situ* hybridization (A,C) and
696 immunohistochemistry (B,D). Insets correspond to sections incubated with sense probes
697 (A,C) or pre-immune sera (B,D). pn, perinucleolar; 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

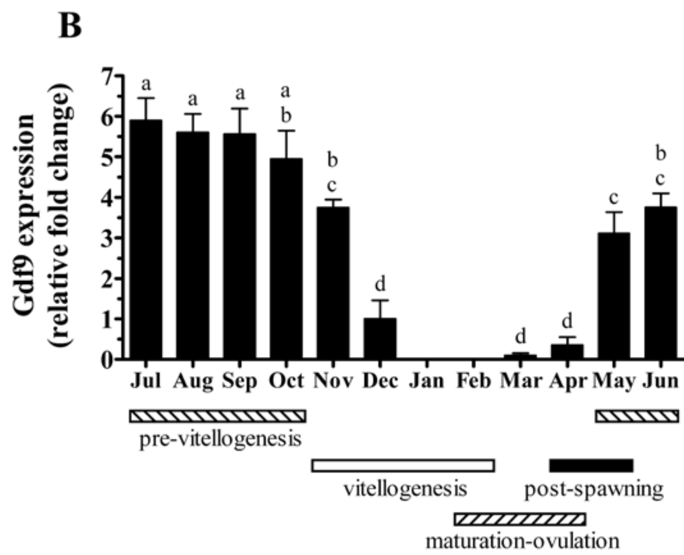
714



715 Figure 1

716

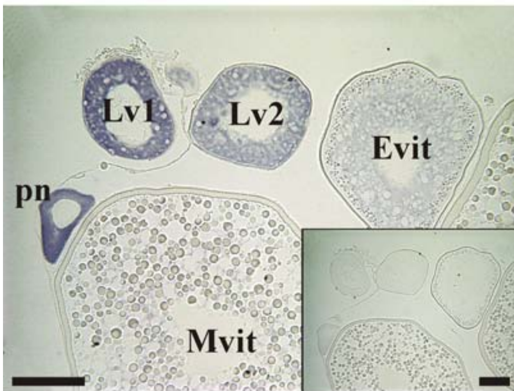




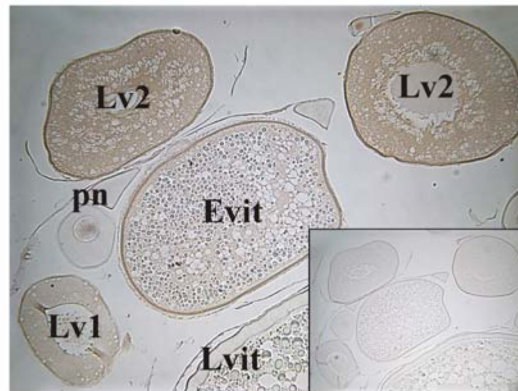
717

718 Figure 2

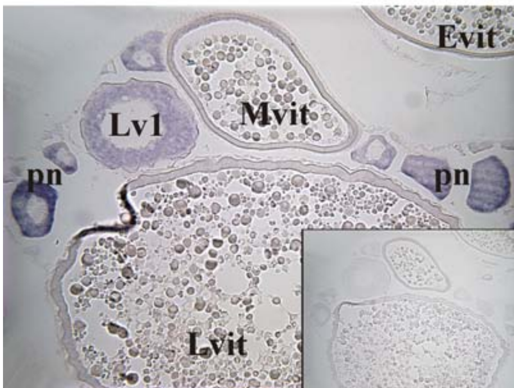
A *bmp15* mRNA



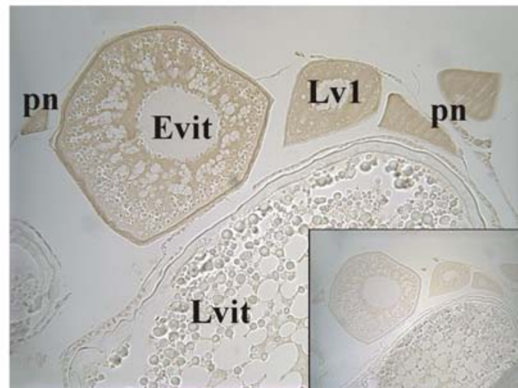
B Bmp15 protein



C *gdf9* mRNA



D Gdf9 protein

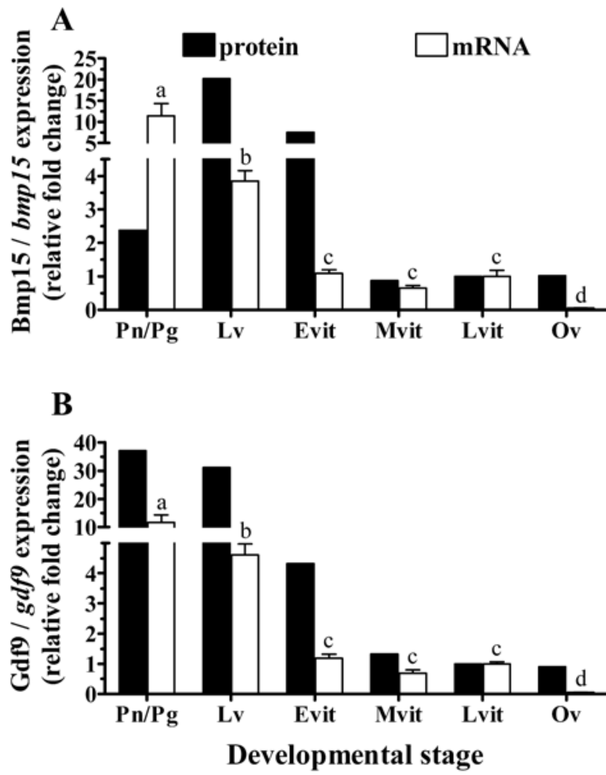


719

720 Figure 3

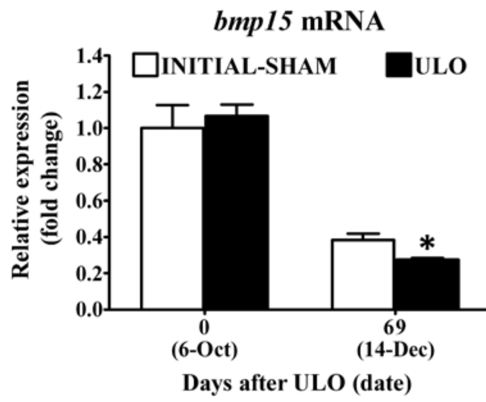
721

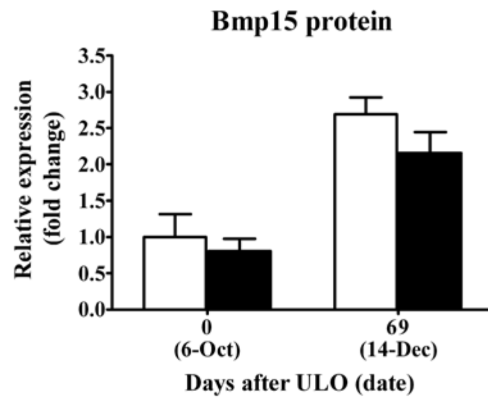
722

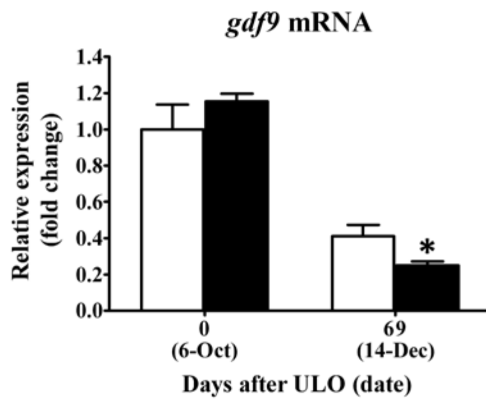


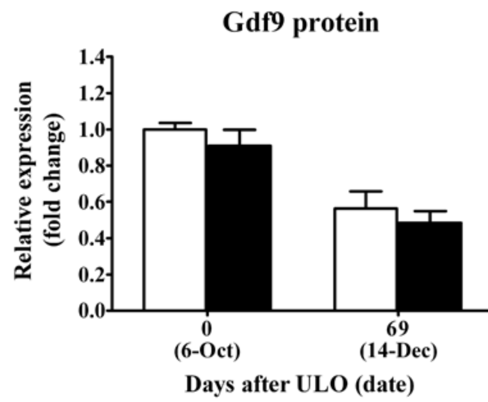
723 Figure 4

724









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726 Figure 5