GROWTH DIFFERENTIATION FACTOR 9 AND BONE MORPHOGENETIC PROTEIN 15 mRNA AND PROTEIN: CELLULAR LOCALIZATION AND DEVELOPMENTAL EXPRESSION IN THE OVARY OF EUROPEAN SEA BASS

García-López, Á.[1], Sánchez-Amaya, M.I.[1], Halm, S.[1], Astola, A.[2], Prat, F[1].

[1]Instituto de Ciencias Marinas de Andalucía (CSIC), República Saharaui 2, 11510 Puerto Real, Cádiz, Spain, f.prat@csic.es, +34 956834701.

[2]Dept. Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain.

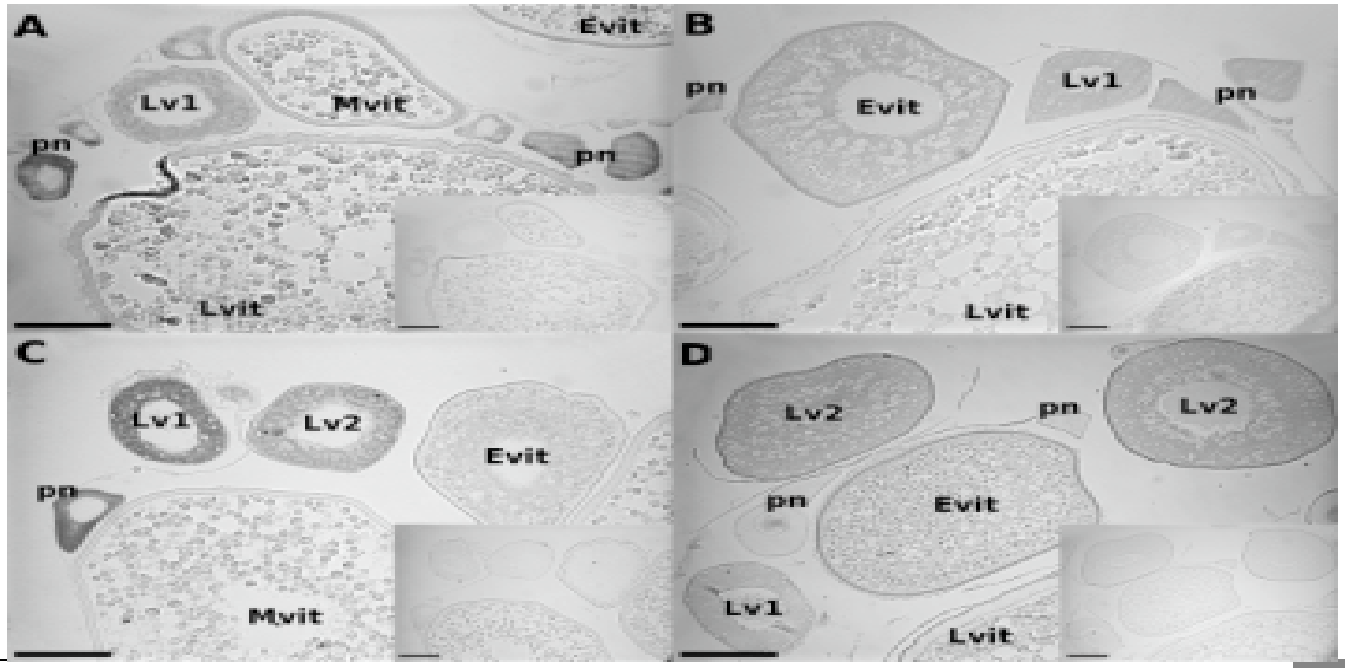
Introduction:

The two oocyte-secreted, transforming growth factor-beta superfamily members, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) have crucial roles in early follicular growth in mammals [1]: Gene knockouts or animals with inactivating mutations in the *gdf9* or *bmp15* genes are infertile with oocytes arrested at the early follicular stages. Recently, we cloned both genes in the European sea bass and expression studies showed high levels of *gdf9* and *bmp15* expression in ovaries containing exclusively pre-vitellogenic oocytes [2] suggesting a function in fish similar to what has been described for mammals. Here we present data about the cellular localization of the *gdf9* and *bmp15* mRNA and their respective proteins together with expression levels of *gdf9*/Gdf9 and *bmp15*/Bmp15 in follicles/oocytes at different developmental stages and protein expression in whole ovaries during the annual reproductive cycle of sea bass.

Methods:

In situ hybridization (ISH) was performed in ovaries of adult European sea bass using *gdf9 and bmp15* DIG-labeled riboprobes encoding the mature peptides. Immunohistochemistry (IHC) was carried out using species-specific Gdf9 antiserum (dilution: 1/2000) and Bmp15 antiserum (dilution: 1/500). Antisera were raised in rabbits using recombinant Gdf9 and Bmp15 mature proteins produced in *E. coli* BL21(DE3) cells as antigens. Expression levels of *gdf9* and *bmp15* mRNA in isolated follicles/oocytes was performed by qrtPCR, using specific primers, SYBR Green dye and cDNA retro-transcribed from total RNA as template. Total RNA was extracted from ovarian follicles collected from vitellogenic ovaries and classified into 5 stages according to size and cytoplasm appearance: perinucleolar/primary growth stage (pn/Pg), lipid vesicles stage (Lv), early vitellogenic (Evit), mid vitellogenic (Mvit), and late vitellogenic (Lvit) oocytes. Protein expression analysis was performed by Western blot analysis using protein extracts collected during total RNA extraction and Gdf9 and Bmp15 antisera at 1/6000 and 1/20000 dilutions, respectively. Protein expression in whole ovaries was also analyzed by Western blot using protein extracts of ovaries collected monthly

Fig. 1. Cellular localization of *gdf9*/Gdf9 (A,B) and *bmp15*/Bmp15 (C,D) mRNAs and proteins by *in situ* hybridization and immunohistochemistry. Insets correspond to negative controls. pn, perinucleolar stage; Lv1, lipid vesicle stage-1; Lv2, lipid vesicle stage-2; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic. Scale bars: 100 um.



during a reproductive cycle (n=5 per month).

Results:

ISH showed intense signals for *gdf9* and *bmp15* in the cytoplasm of the oocytes at the perinucleolar stage that decreased steadily until the early vitellogenic stage (Fig. 1A,C). IHC revealed an abundant Gdf9 presence in the cytoplasm of oocytes from perinucleolar to early vitellogenic stages (Fig. 1B). In contrast, signal for Bmp15 was absent in oocytes at the perinucleolar stage, and maximal at the lipid vesicle stage, decreasing in November (beginning of vitellogenesis) to April (spawning/postspawning period) (Fig. 3).

Conclusion:

In situ hybridization and the analyses of *gdf9* and *bmp15* transcripts in isolated oocytes confirm previous results obtained in the European sea bass [2], and indicate that the mRNAs are synthesized by the oocyte. The simultaneous expression of *gdf9* and Gdf9 furthermore suggests an important role for Gdf9 during the first stages of oogenesis. In contrast, although *bmp15*

Fig. 2. Expression levels of *gdf9*/Gdf9 (left) and *bmp15*/Bmp15 (right) mRNA and protein in isolated follicle/oocytes. Pn/Pg, perinucleolar/primary growth; LV, lipid vesicles; Evit, early vitellogenic; Mvit, mid vitellogenic; Lvit, late vitellogenic.

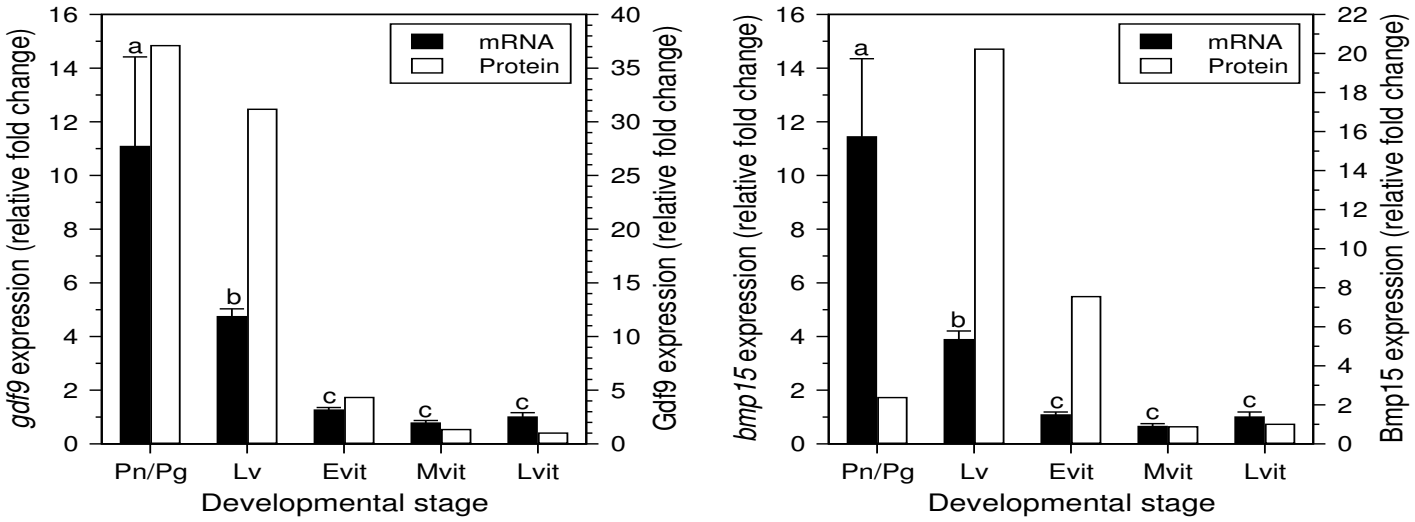
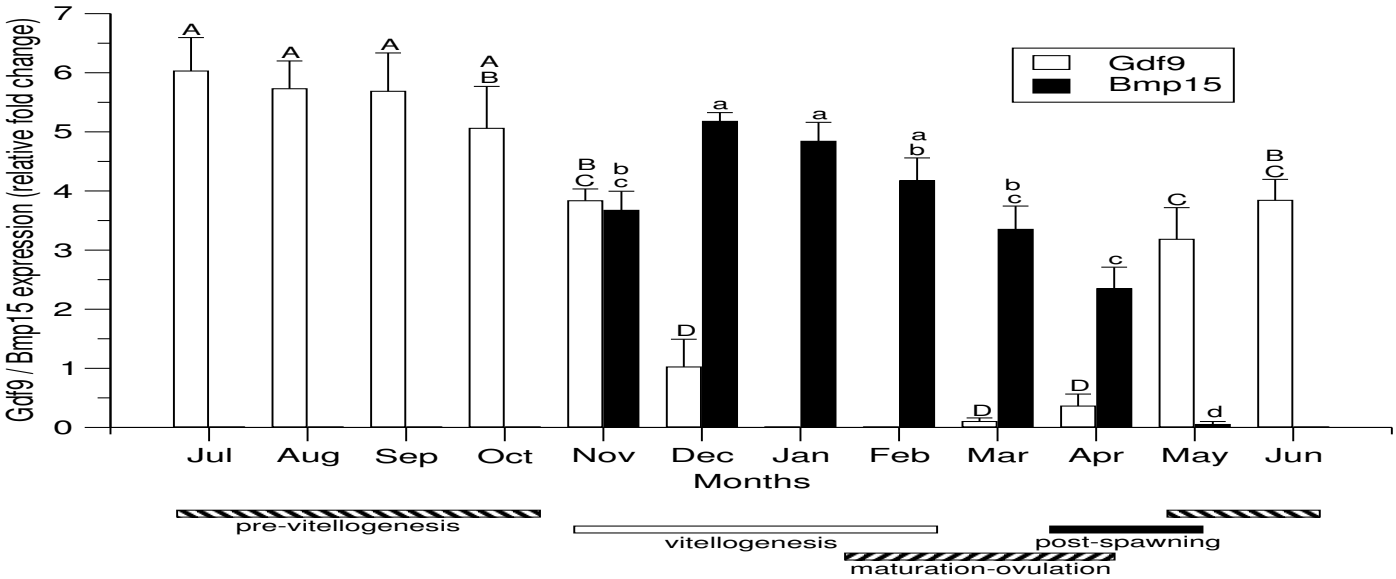


Fig. 3. Seasonal expression levels of Gdf9 and Bmp15 proteins in the ovary during the first reproductive cycle. Different letters indicate significant differences ($p < 0.05$).



early vitellogenic oocytes (Fig. 1D). Expression levels of *gdf9*/Gdf9 and *bmp15*/Bmp15 in isolated follicles/oocytes confirmed the results obtained by ISH and IHC (Fig. 2). During the reproductive cycle, expression levels of Gdf9 were high during pre-vitellogenesis while Bmp15 was only detected from mRNA expression is high in pre-vitellogenic oocytes and during pre-vitellogenesis of the reproductive cycle [2], high levels of Bmp15 protein are found from late pre-vitellogenesis and the beginning of vitellogenesis, suggesting a role for Bmp15 at later stages of development, maybe preventing premature oocyte maturation as it has been suggested in zebrafish [3].

Funded by the former Spanish Ministry of Education and Science. Grant AGL2007-61192/ACU to F.P. and CSIC-JAE program contract to Á.G.-L.

References:

[1]JUENGEL, J.L., McNATTY, K.P. 2005. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum. Reprod. Update 11: 144-161.

[2]HALM, S., IBAÑEZ, A.J., TYLER, C.R., PRAT, F. 2008. Molecular characterisation of growth differentiation factor 9 (*gdf9*) and bone morphogenetic protein 15 (*bmp15*) and their patterns of gene expression during the ovarian reproductive cycle in the European sea bass. Mol. Cell. Endocrinol., 291: 95-103.

[3]CLELLAND, E.S., TAN, Q., BALOFSKY, A., LACIVITA, R., PENG, C. 2007. Inhibition of premature oocyte maturation: A role for bone morphogenetic protein 15 in zebrafish ovarian follicles. Endocrinology, 148: 5451-5458.