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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

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Abstract

Members of the transforming growth factor- β superfamily, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), have crucial roles in primary follicle growth in mammals. To initiate investigations into their significance in teleost oogenesis, we set out to clone and characterise the cDNAs of *gdf9* and *bmp15* and analysed their patterns of gene expression during the ovarian reproductive cycle in the European sea bass (*Dicentrarchus labrax*). Sea bass *gdf9* and *bmp15* cDNAs were 2200 and 2049 bp long, coding for 438 and 459 amino acids, respectively, and were most similar to zebrafish *gdf9* and *bmp15* (64.4 and 56.1%, respectively). By Northern analysis, sea bass *gdf9* and *bmp15* mRNA transcripts were detected in the ovary only of the tissues analysed and their sizes were 2.2 and 2.1 kb, respectively. Dot-blot analysis revealed high levels of *gdf9* and *bmp15* expression in the ovary during primary oocyte growth and previtellogenesis (July to October), with a significant decline at the onset of vitellogenesis (November) and remaining low until the beginning of new oocyte growth (April/May). There was a highly significant positive correlation ($r = 0.939$) between *gdf9* and *bmp15* gene expression in individual samples. The high levels of *gdf9* and *bmp15* mRNA transcripts in the ovary, especially during the previtellogenic growth period suggests an important role for these factors in early primary oocyte growth in the European sea bass.

Key words: cloning, folliculogenesis, *gdf9b*, teleost fish, TGF-beta superfamily

1 Introduction

The recent isolation and functional characterisation of oocyte-secreted transforming growth factor β (TGF- β) superfamily members has brought about a re-examination of the role the germ cell plays in folliculogenesis. Historically regarded as being passively regulated by endocrine and ovarian somatic cell derived hormones and growth factors, it is now well-known that the oocyte secretes a variety of growth factors that possess vital functions for normal follicle development (Gilchrist et al., 2004). Particular attention has been paid to the two TGF- β superfamily members growth differentiation factor 9 (GDF9/*gdf9*) and bone-morphogenetic factor 15 (BMP15/*bmp15*; also called growth differentiation factor 9b; GDF9b/*gdf9b*), both of which have key roles in early follicle growth in mammals (Vitt and Hsueh, 2002; Juengel et al., 2004; Moore and Shimasaki, 2005).

Isolated and characterised for the first time in 1993 by McPherron and Lee, Dong and coworkers (1996) subsequently proved GDF9 essential for early follicle growth by generating *gdf9* deficient female mice that contained ovaries arrested at the primary follicle stage, rendering these animals infertile. Moreover, injection of ovaries of immature rats with recombinant *gdf9* resulted in an increased number of primary follicles while the pool of primordial follicles was decreased (Vitt et al., 2000a).

In mammals, GDF9 is predominantly expressed in the ovary and the same is true for its closest homologue, BMP15, which exhibits gene and protein expression patterns closely resembling those for GDF9 (McGrath et al., 1995; Dube et al., 1998; Jaatinen et al., 1999). The role of BMP15 in folliculogenesis and fertility has been studied in naturally occurring strains of sheep with point mutations in *bmp15*. Homozygous carriers are infertile and follicular growth is arrested at the primary stage of follicular development (Galloway et al., 2000). Heterozygous mutant carriers, in contrast, exhibit

higher ovulation rates and litter sizes than their wild-type counterparts. Unlike sheep, effects of BMP15 in mice are more subtle. Homozygous *bmp15* knock-out females show decreased fertility due to lower ovulation and fertilisation rates; however a block in folliculogenesis is not apparent. Moreover, heterozygous females do not exhibit any aberrant phenotype (Yan et al., 2001).

gdf9/GDF9 and *bmp15*/BMP15 are expressed in oocytes from the primordial follicle stage in sheep (Bodensteiner et al., 1999), goat (Silva et al., 2005), bovine (Bodensteiner et al., 1999), and brushtail possum (Eckery et al., 2002), while in human (Aaltonen et al., 1999), mouse (McGrath et al., 1995; Dube et al., 1998; Elvin et al., 1999), and rat (Hayashi et al., 1999; Jaatinen et al., 1999) expression does not commence until the primary follicle stage. Both growth factors are known to stimulate the proliferation of granulosa cells, but they also suppress follicle stimulating hormone (FSH) -induced granulosa cell differentiation (Vitt et al., 2000b; Otsuka 2002). The effect of GDF9 on FSH action is reflected by its ability to inhibit FSH-dependent lutenising hormone (LH) receptor expression, cAMP production, and progesterone and oestradiol synthesis (Elvin et al., 1999; Vitt et al., 2000a; Spicer et al., 2006). Similar to GDF9, BMP15 is an inhibitor of FSH-induced progesterone synthesis and *in vitro* studies have demonstrated that this effect is mediated through the suppression of FSH receptor gene expression (Otsuka et al., 2001).

Information on GDF9 and BMP15 in other classes of vertebrates, other than in mammals, is very limited. In 2005, the isolation and characterisation of *gdf9* from the ovary of chicken was reported (Johnson et al., 2005), and *in vitro* experiments showed that oocyte-conditioned medium was capable of enhancing granulosa cell proliferation, while preincubation with a chicken-specific GDF9 antibody abolished this effect. Recently, *gdf9* and *bmp15* were isolated from zebrafish ovaries (Clelland et al., 2006;

Liu and Ge, 2007), and first results from *in vitro* and *in vivo* experiments point towards an important role for both growth factors in ovarian development and oocyte maturation in teleost fish (Liu and Ge, 2007; Clelland et al., 2007).

The European sea bass *Dicentrarchus labrax*, is the most important commercial fish species cultured in the Mediterranean area. Consequently, various aspects concerning the endocrine, genetic, and environmental control of sexual differentiation, development and reproductive performance have been studied in this species (Zanuy et al., 2001; Pifferer et al., 2005). Studies investigating factors potentially involved in the early phase of oocyte growth in sea bass, however, are scarce. As a first step to identify growth factors potentially involved in early oocyte growth, we set out to isolate and characterise *gdf9* and *bmp15* for the first time in a marine teleost, the European sea bass, and to analyse their patterns of gene expression in relation with the ovarian reproductive cycle.

2 Materials and Methods

2.1 Isolation of sea bass *gdf9* and *bmp15* cDNAs

An ovary sea bass cDNA library constructed into the Uni-ZAP XR vector was screened with zebrafish *gdf9* and *bmp15* cDNA probes which had been synthesised by RT-PCR from zebrafish ovarian total RNA. The probes contained nucleotide (nt) positions 313-1330 and 655-1263 of zebrafish *gdf9* and *bmp15* (GenBank accession numbers [AY833104](#) and [AY954923](#), respectively). Half a million pfu were screened with ³²P- α -dCTP labelled probes, and hybridisation was performed under low stringent conditions (30% formamide, 600 mM NaCl, 40 mM sodium phosphate buffer, pH 7.4; 2.5 mM EDTA, 1% SDS) at 42°C overnight. Membranes were washed with 2x SSPE, 0.1% SDS at room temperature for 30 min and exposed to a film at – 80°C for 4 days. After three

rounds of screening, positive clones were excised into pBluescript SK(-), rescued into *E. coli* SOLR cells and sequenced at the Sequencing service at the University of Valencia.

2.2 Sequence analysis

Sequence analysis and comparisons of homology between *gdf9* and *bmp15* sequences were performed using European Molecular Biology Open Software Suite (EMBOSS). Amino acid alignments were generated using Clustal W. Phylogenetic analysis was done by multiple alignments of deduced amino acid sequences using the Neighbour-Joining method (Saitou and Nei, 1987) within the Clustal W programme. Evolutionary trees were constructed using TreeView 1.6.6.

2.3 Gene expression of sea bass *gdf9* and *bmp15* in gonadal and somatic tissues

2.3.1 Total RNA extraction

Total RNA was extracted from adult sea bass ovary, testis, liver, heart, kidney, brain, pituitary, muscle, fat and spleen using Tri-Reagent (Molecular Research Center, INC., Cincinnati, Ohio, USA), and following the manufacturer's instructions. Apart from testis, tissues were derived from a female during previtellogenesis. RNA integrity was checked by running a few microlitres of each sample in an agarose gel and its quantity and purity was analysed spectrophotometrically.

2.3.1.1 Northern blot

10 µg of total RNA of each tissue were denatured in 50% DMSO and 1 M deionised glyoxal at 50°C for 50 min, loaded into an 1% agarose gel and run in 1x MOPS [3-(N-morpholino)-propanesulphonic acid] buffer at 50 V for four hours. After electrophoresis,

the gel was soaked in 50 mM NaOH for 15 min and the RNA transferred to a Hybond-N nylon membrane (GE Healthcare Europe GmbH; Barcelona, Spain) in 10x SSC overnight. The RNA was cross-linked by UV and its amount was confirmed by staining the membrane with methylene-blue. The membrane was used for hybridisation with both probes, i.e. sea bass *gdf9* and (after stripping) with sea bass *bmp15* (see 2.3.2).

2.3.1.2 RT-PCR

2 µg of total RNA of each tissue was transcribed into cDNA using 40 ng of random hexamer primers and 200 units of Superscript III (Invitrogen, Inchinnan, Paisley, UK) following the manufacturer's instructions. 1 µl of cDNA was added to PCR reactions containing 10 µM of sea bass *gdf9* and *bmp15* specific primers (*gdf9* sense primer: nt 552-572 and *gdf9* antisense primer: nt 1017-1037 of GenBank accession number [AM933667](#); *bmp15* sense primer: nt 682-701 and *bmp15* antisense primer: 1457-1477 of GenBank accession number [AM933668](#)), 1.25 mM dNTPs, 2.5 mM MgCl₂ and the buffer provided with the DFS-Taq DNA Polymerase (Bioron GmbH, Ludwigshafen, Germany). Reactions were run for 30 cycles with an annealing temperature of 58°C and an extension time of 1 min. 5 µl of each PCR reaction were run on a 1% agarose gel and afterwards, the gel was denatured in 0.5 N NaOH for 30 min and the amplified cDNA fragments blotted on N-Hybond nylon membranes in 10x SSC overnight. cDNA fragments were UV cross-linked.

2.3.2 Hybridisation

Membranes were hybridised with sea bass *gdf9* and *bmp15* specific probes (that had been synthesised by RT-PCR using the primers as described under 2.3.1.2) labelled with ³²P-α-dCTP. Hybridisation was carried out at 42°C overnight (in 50% formamide, 6x

SSPE, 0.5% SDS, 5x Denhardt's, 0.1 mg/ml RNA type III from baker's yeast) and washes were performed at high stringencies, with final washes of 0.1x SSPE/0.1% SDS at 65°C for 1-3 hours, depending on the strength of the signal. Membranes were exposed to a phosphor imager screen for several hours and analysed on a Personal Imager System (Bio-Rad, Hercules, California, USA).

2.4 Sea bass *gdf9* and *bmp15* gene expression in the ovary during the reproductive cycle

2.4.1 Sampling

Five adult female sea bass were anaesthetised, killed and sampled each month between July 2003 and June 2004 in accordance with the regulations of the Bioethical Committee of the CSIC. Their weight was recorded and their gonadosomatic index (GSI) determined according to the formula: $GSI (\%) = [(gonad\ weight/body\ weight) \times 100]$. Part of the gonadal tissue was frozen in liquid nitrogen for RNA extraction and part was fixed in 4% paraformaldehyde for histological analysis.

2.4.2 Dot-blot analysis

Total RNA was extracted and its quantity and integrity analysed from each sample as described under 2.3.1. For dot-blot analysis, 3.5 µg of total RNA of each sample was denatured in 2.2 M formaldehyde and 50% formamide at 55°C for 15 min. After addition of ice-cold 10x SSC (final concentration), the samples were applied to a Bio-Dot microfiltration apparatus (Bio-Rad) and bound to a Hybond-N membrane under vacuum. The wells were washed twice with 10x SSC and the membrane was subsequently UV-cross-linked. The membrane was hybridised consecutively with the sea bass *gdf9* and *bmp15* specific probe as described under 2.3.2 and exposed to a

phosphor imager screen. Radioactive signals of samples were quantified using the Personal Imager System (Bio-Rad) and, after background correction, expressed as adjusted volume intensities (optical intensity \times mm²).

Data were not normalised to any housekeeping gene, since the three housekeeping genes tested, i.e. β -actin, 18S rRNA and 28S rRNA showed differential expression in sea bass ovary. However, to assure that similar amounts of RNA had been loaded onto the membrane, it was stained with 0.08% methylene blue after blotting (Fig. 5).

2.4.3 Data representation and statistics

Data are presented as means \pm standard error of the mean (SEM). Since the data were not normally distributed, a nonparametric test (Kruskal Wallis) was employed to compare differences between groups. If differences were found ($p < 0.05$), a multiple comparison test according to Conover (1980) was applied. Correlation analysis was performed by ranks according to Spearman. All statistical analyses were carried out using SPSS software programme (SPSS Inc, Chicago, IL, USA).

3 Results

3.1 Isolation and sequence characterisation of sea bass *gdf9* and *bmp15* cDNAs

After three rounds of cDNA library screening, for each probe ten positive clones were obtained of which seven contained inserts showing highest sequence similarity to *gdf9* or *bmp15* in other species (BlastX). The largest-size inserts were subsequently fully sequenced. Sea bass *gdf9* cDNA (GenBank accession number [AM933667](#)) was 2200 bp long with an open reading frame (ORF) of 1317 nt. The 5-untranslated region (UTR) was 40 nt long while the 3-UTR contained 843 nt and ended with a polyA-tail. A

polyadenylation signal (AATAAA) was present 44 nt upstream of the beginning of the polyA-tail. In comparison to *gdf9* cDNA, sea bass *bmp15* cDNA (GenBank accession number [AM933668](#)) was slightly smaller, consisting of 2049 bp. Five- and 3-UTR were 134 and 535 nt long, respectively, flanking an ORF of 1380 bp. Sea bass *bmp15* cDNA ended with a polyA-tail, and several polyadenylation signals (all AATAAA) were present at nt positions 1547, 1892, 1905 and 2026.

The ORFs of sea bass *gdf9* and *bmp15* encoded proteins of 438 and 459 amino acids (aa), respectively (Fig. 1 and 2). Using ProP 1.0 (Duckert et al., 2004) for predicting potential signal peptide and propeptide cleavage sites, we observed potential signal peptide cleavage sites between aa 33 and 34 for both amino acid sequences, while putative propeptide cleavage sites were present at aa 301 and 304 (RKKR and RWKR) for *gdf9* and at aa 300 (RHRR) for *bmp15*. A conserved N-glycosylation site was present in sea bass *gdf9* at aa position 228.

In sea bass *bmp15*, there was a stretch of serine residues in both the propeptide and mature peptide region, (Fig. 2), which were absent in all other *bmp15* sequences. Sea bass *gdf9* and *bmp15* both contained the conserved, TGF- β superfamily member domain in the mature peptide region, including six conserved cysteine residues (Fig. 1 and 2). Highest amino acid similarities for sea bass *gdf9* and *bmp15* were found with their respective counterparts in zebrafish, and were 64.4 and 56.1%, respectively (Table 1). In comparison with mammals and birds, amino acid similarities were lower, i.e. between 42.0 (sea bass *bmp15* compared to human *bmp15*) and 52.4% (sea bass *gdf9* compared to chicken *gdf9*). Amino acid similarities were always higher when comparing the (conserved) mature peptide regions only.

In a phylogenetic tree (Fig. 3), sea bass *gdf9* and *bmp15* were grouped together with their respective orthologues in *gdf9*- and *bmp15*-specific clades, both originating

from a common root and separated from the cluster of *gdf5* sequences. Sea bass *gdf9* and *bmp15* were grouped most closely with zebrafish *gdf9* and *bmp15*, respectively; however, the bootstrap value for the sea bass/zebrafish *bmp15* subclade was only 535 (out of 1000 replicates).

3.2 Gene expression of sea bass *gdf9* and *bmp15* in gonadal and somatic tissues

Sea bass *gdf9* and *bmp15* specific probes strongly hybridised to RNA transcripts of 2.2 and 2.1 kb in size, respectively, from ovary (Fig. 4A and 4B; upper panels). Using RT-PCR as a more sensitive detection method, in addition to ovarian expression, *gdf9* and *bmp15* were also detected in testis and in somatic tissues, including (female) liver, heart, kidney, brain, pituitary, muscle, fat and spleen (Fig. 4A and 4B; lower panels).

3.3 Expression of sea bass *gdf9* and *bmp15* during ovarian development

Sea bass *gdf9* and *sbsbmp15* mRNA transcript levels were high during previtellogenesis (July to October), but declined significantly at the onset of vitellogenesis (November) to reach their lowest levels at the time of oocyte maturation, during January and February (Fig. 5). Levels of expression increased significantly again in March to reach the highest levels in May, at the beginning of the period of new oocyte growth. There was a highly significant positive correlation ($r = 0.939$, $p < 0.001$; Spearman rank correlation) between *gdf9* and *bmp15* transcript levels in individual ovary samples (Fig. 6).

4 Discussion

In this study we isolated two sea bass cDNAs encoding *gdf9* and *bmp15*. The lengths of the cloned cDNAs of 2200 and 2049 bp corresponded to the sizes of *gdf9* and *bmp15* transcripts detected by Northern hybridisation, suggesting that we had cloned the full-

length cDNAs of both genes. Sea bass *gdf9* and *sbsbmp15* cDNAs are larger than most cDNAs cloned for either of the two genes in other animals; i.e. human *gdf9* (2020 bp) (McPherron and Lee, 1993), bovine *gdf9* (1816 bp) (Pennetier et al., 2004), chicken *gdf9* (1646 bp) (Johnson et al., 2005), zebrafish *gdf9* (2072 bp) (Liu and Ge, 2007), chicken *bmp15* (1875 bp; Genbank accession number [AY725199](#)) and zebrafish *bmp15* (1611 bp) (Clelland et al., 2006). An exception to this is mouse *bmp15*, which due to a 3-UTR of about 1.5 kb, is 3086 bp long (Dube et al., 1998).

For sea bass *gdf9*, the length of the ORF is similar to that of *gdf9* in other species. In contrast, the ORF of sea bass *bmp15* is exceptionally large and contains up to 327 additional nucleotides (when compared to chicken *bmp15*). Most interestingly, there is a nucleotide insertion just downstream of the putative cleavage site in sea bass *bmp15*, which is not found in other *bmp15* sequences. Part of this nucleotide sequence encodes a stretch of serine residues and a similar polyserine track is present further upstream in the propeptide region of sea bass *bmp15* (Fig. 2). Vertebrate and insect vitellogenins, i.e. egg yolk proteins, also contain stretches of serines which in some insects are located up- and downstream of putative cleavage sites; that is, at positions similar to sea bass *bmp15*. It has been speculated that these polyserine tracks have arisen because of amplification of trinucleotide repeats encoding serine (Chen et al., 1997). It is tempting to suggest that the serine runs present in sea bass *bmp15* might have evolved in a similar way, as all serines in these stretches are encoded by the trinucleotides TCX, being indicative of a serial amplification of this particular codon. In vertebrate vitellogenins, it has been proposed that these serines could have a role as a nutrient carrier, binding calcium phosphate for the provision of Ca^{2+} for bone formation, and other physiological functions, in the embryo (Wahli et al., 1988). Whether these polyserine tracks have a particular function in sea bass *bmp15* is not known.

The deduced amino acid sequences of both cDNAs possess the characteristics typical for members of the TGF- β superfamily (Shimasaki et al., 2004), including a potential signal peptide sequence for secretion, a putative tetrabasic proteolytic processing site, and a COOH-terminal region containing the conserved, TGF- β superfamily domain. A distinguishing feature normally found in members of the TGF- β superfamily is the presence of seven conserved cysteines in the TGF- β superfamily domain. While six of these cysteines participate in giving the molecule its unique three-dimensional structure, the remaining, seventh cysteine is responsible for the formation of intermolecular, disulfide bonds between monomers of TGF- β superfamily members (Xiao et al., 2007). However, neither sea bass *gdf9* nor *bmp15* contains this seventh cysteine (being replaced by a serine residue in both cases) and the same has been observed for *gdf9* and *bmp15* in other species (McPherron and Lee, 1993; Jaatinen et al., 1999). Consequently, it is believed that if homo- or heterodimer formations between GDF9 and BMP15 monomers do occur, they have to be of non-covalent nature (Liao et al. 2003).

Members of the TGF- β superfamily belong to a large group of secretory proteins with diverse physiological functions. The prodomain of TGF- β family members is only poorly conserved, sometimes even for members of the same subfamily, and structural homology is mainly observed for the carboxyterminal domain coding for the mature protein (Kingsley, 1994). Consequently, low evolutionary conservancy among members of a particular TGF- β subfamily can sometimes complicate their identification (Miura et al., 2002; Sawatari et al., 2007). *gdf9* is fairly conserved among mammalian species, but sequences become more divergent across the vertebrate kingdom. This is even more evident for *bmp15*, which only shares 61% amino acid similarity between human and chicken compared to 73% for *gdf9* between the same species. Similarly, sea bass *bmp15*

only shares 56.1% amino acid similarity with zebrafish *bmp15*, while for *gdf9* amino acid identities are 64.4% between both species. However, although amino acid similarities are evidently low, phylogenetic analysis clustered sea bass *gdf9* and *bmp15* together with their respective counterparts in mammals and birds, thereby confirming their identity.

In mammals, GDF9 and BMP15 are indispensable for early ovarian development and their absence leads to an ablation of early folliculogenesis and hence infertility in some species (Dong et al., 1996; Galloway et al., 2000). The predominant presence and high expression of *gdf9* and *bmp15* in the ovary of sea bass, in particular during primary oocyte growth (1.25 μ g of total RNA were still sufficient to detect both transcripts in a dot-blot analysis; results not shown) suggests an important role for both growth factors during early ovarian development in this species. Similar to sea bass, *gdf9* transcript levels in zebrafish have been shown to be highest during primary growth and to decrease significantly with the progression of vitellogenesis (Liu and Ge, 2007). Transcript levels of a rainbow trout *gdf9*-annotated expressed sequence tag are also high during early oogenesis, and furthermore androgen treatment of genetic all-female populations has shown to downregulate *gdf9* expression in these animals (Baron et al., 2008). Similarly, *gdf* mRNA levels in chicken are highest in small follicles (< 1 mm) (Johnson et al., 2005). In mammals, *gdf9* and *bmp15* transcripts were found to be abundantly present in primary oocytes in humans and in rats (Aaltonen et al., 1999; Jaatinen et al., 1999), while in mouse *gdf9* mRNA was detected in oocytes at all stages of follicular development with no obvious correlation between *gdf9* expression levels and developmental stage (McGrath et al., 1995).

While the later stages of folliculogenesis are under control of the pituitary gonadotropins FSH and LH, the very early stages of oogenesis are gonadotropin-

independent and regulated by growth factors produced locally in the ovary, including GDF9 and BMP15 (Findlay et al., 2002). With the onset of gonadotropin receptor expression, however, cells become responsive to gonadotropins. Recently, the gene expression of FSH and LH receptors (*fshr* and *lhr*) were analysed during the reproductive season in the ovary of sea bass and expression of both receptors was high during early vitellogenesis (Rocha et al., 2007), i.e. when sea bass *gdf9* and *bmp15* transcript levels start to decline sharply. A similar reciprocal pattern of gene expression has been observed for *gdf9* compared with *fshr* and *lhr* in zebrafish (Ge, 2005), suggesting transcriptional control of gonadotropin receptor gene expression by GDF9 and BMP15 and/or vice versa. In support of this view human chorionic gonadotropin (hCG), which is known to act via the LH receptor in zebrafish, reduced *gdf9* expression in fully grown oocytes of zebrafish *in vitro* (Liu and Ge, 2007).

Intriguingly, sea bass *gdf9* and *bmp15* transcript levels in individual ovaries were highly and positively correlated. A similar, congruent pattern of *gdf9* and *bmp15* gene expression in the ovary has, to our knowledge, not reported in any fish species before. In fact, contrasting with this, in zebrafish while *gdf9* was predominantly expressed in primary oocytes (Liu and Ge, 2007), *bmp15* transcript levels did not vary significantly in oocytes of different developmental stages when analysed by real-time PCR (Clelland et al., 2006). The parallel patterns of expression observed for *gdf9* and *bmp15* in the ovary of sea bass suggests that both genes might have cooperative roles in this tissue. In support of this view, *in vitro* recombinant murine or ovine *gdf9* and ovine *bmp15* have been shown to have strong synergistic effects on granulosa cell proliferation and progesterone and inhibin production (McNatty et al., 2005). Furthermore, *in vivo*, sheep with mutations in the *gdf9* and *bmp15* genes have a greater ovulation rate than animals with a mutation for only one of the two genes (Hanrahan et

al., 2004). It will therefore be of great interest to investigate the combined effects of GDF9 and BMP15 on ovarian development in sea bass *in vitro* and *in vivo* in future studies.

Using RT-PCR as a more sensitive detection method, expression of sea bass *gdf9* and *bmp15* was also detected in testis as well as in female liver, heart, kidney, brain, pituitary, muscle, adipose tissue, and spleen. For mammalian *gdf9* and *bmp15*, gene expression has been described exclusively for ovary in some cases (McPherron and Lee, 1993; Dube et al., 1998; Galloway et al., 2000), but other studies have demonstrated their presence in extra-ovarian tissues including testis, pituitary, liver, uterus, placenta, and bone marrow (Fitzpatrick et al., 1998; Aaltonen et al., 1999; Eckery et al., 2002). In zebrafish, expression of *gdf9* was detected in ovary and testis only (Liu and Ge, 2007), while *bmp15* was expressed more widely and detected in brain, liver, gut, heart, and muscle (Clelland et al., 2006). GDF9 and BMP15 are both thought to control FSH signalling (Vitt et al., 2000a; Otsuka et al., 2001), and hence their presence in the brain and pituitary in sea bass as well as in other species might point towards an autocrine feedback control of the hypothalamus-pituitary-gonadal reproductive axis. A functional autocrine/paracrine system of pituitary FSH control has been described for activin and inhibin, other members of the TGF- β superfamily and which originally were isolated from the ovary (Ying, 1988), but which now are known to be locally produced in the pituitary (Bilezikjian et al., 2006).

In summary, cDNAs encoding *gdf9* and *bmp15* were isolated for the first time from a marine teleost species, the European sea bass. The high mRNA transcript levels for *gdf9* and *bmp15* in the ovary compared with other tissues and their predominant expression during previtellogenesis suggests an important role for these growth factors in early folliculogenesis in this species. Furthermore, the highly similar expression

profiles of *gdf9* and *bmp15* during the ovarian cycle let suggest that these factors might act cooperatively in their ovarian function(s) in the European sea bass.

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Figure legend

Figure 1: Alignment of deduced amino acids of sea bass (sbs; [CAP71884](#)), zebrafish (zsh; [AAV91155](#)), chicken (chk; [AAT74587](#)) and human (hum; [AAH96229](#)) *gdf9*. The signal peptide, propeptide and mature peptide regions are depicted according to human *gdf9*. A potential signal peptide region for sea bass *gdf9* is shown with an open box. The cleavage site of human *gdf9* (RRTR) and putative cleavage sites of sbs, zfs, and chicken *gdf9* are boxed and grey-shaded. The six conserved cysteines found in the TGF- β superfamily domain (underlaid in light-grey) are also boxed and grey-shaded. The arrow denotes the position where a seventh conserved cysteine can be found in other members of the TGF- β superfamily. A conserved potential N-glycosylation site is marked with a triangle. Identical and similar amino acids are indicated by asterix and dots (: = strong score, . = weak score according to comparison matrix Gonnet Pam250), respectively.

Figure 2: Alignment of deduced amino acids of sea bass (sbs; [CAP71885](#)), zebrafish (zsh; [AAI15086](#)), chicken (chk; [AAU94351](#)) and human (hum; [O95972](#)) *bmp15*. The signal peptide, propeptide and mature peptide regions are shown according to human *bmp15*. A potential signal peptide region for sea bass *bmp15* is shown with an open box. The cleavage site of human *bmp15* (RRTR) and putative cleavage sites of sbs, zfs, and chicken *bmp15* are boxed and grey-shaded. The six conserved cysteines found in the mature protein are also boxed and grey-shaded. The arrow denotes the position where a seventh conserved cysteine can be found in other members of the TGF- β superfamily. The two long stretches of serine residues found in sea bass *bmp15* are marked by a dotted line. Identical and similar amino acids are indicated by asterix and dots (: =

strong score, . = weak score according to comparison matrix Gonnet Pam250), respectively.

Figure 3: Phylogenetic tree based on an amino acid alignment for GDF9 and BMP15 amino acid sequences in mammals, birds, and teleost fish; i.e. GDF9 of human (AAH96229), mouse (AAH52667), pig (AAT67460), bovine (Q9GK68), sheep (O77681), chicken (AAT74587), zebrafish (AAV91155) and sea bass (CAP71884) as well as BMP15 of human (O95972) mouse (AAH55363) pig (AAL58885), bovine (Q6PX77), sheep (AAF81688), chicken (AAU94351), zebrafish (AAI15086), and sea bass (CAP71885). For comparison, amino acid sequences for TGF- β superfamily member GDF5 in human (CAA56874), mouse (AAH34546) and chicken (AAD30451) were added to the alignment. Sea bass anti-Müllerian hormone (AMH; CAJ78431) was used as an outgroup. Bootstrap values are indicated (1000 replicates).

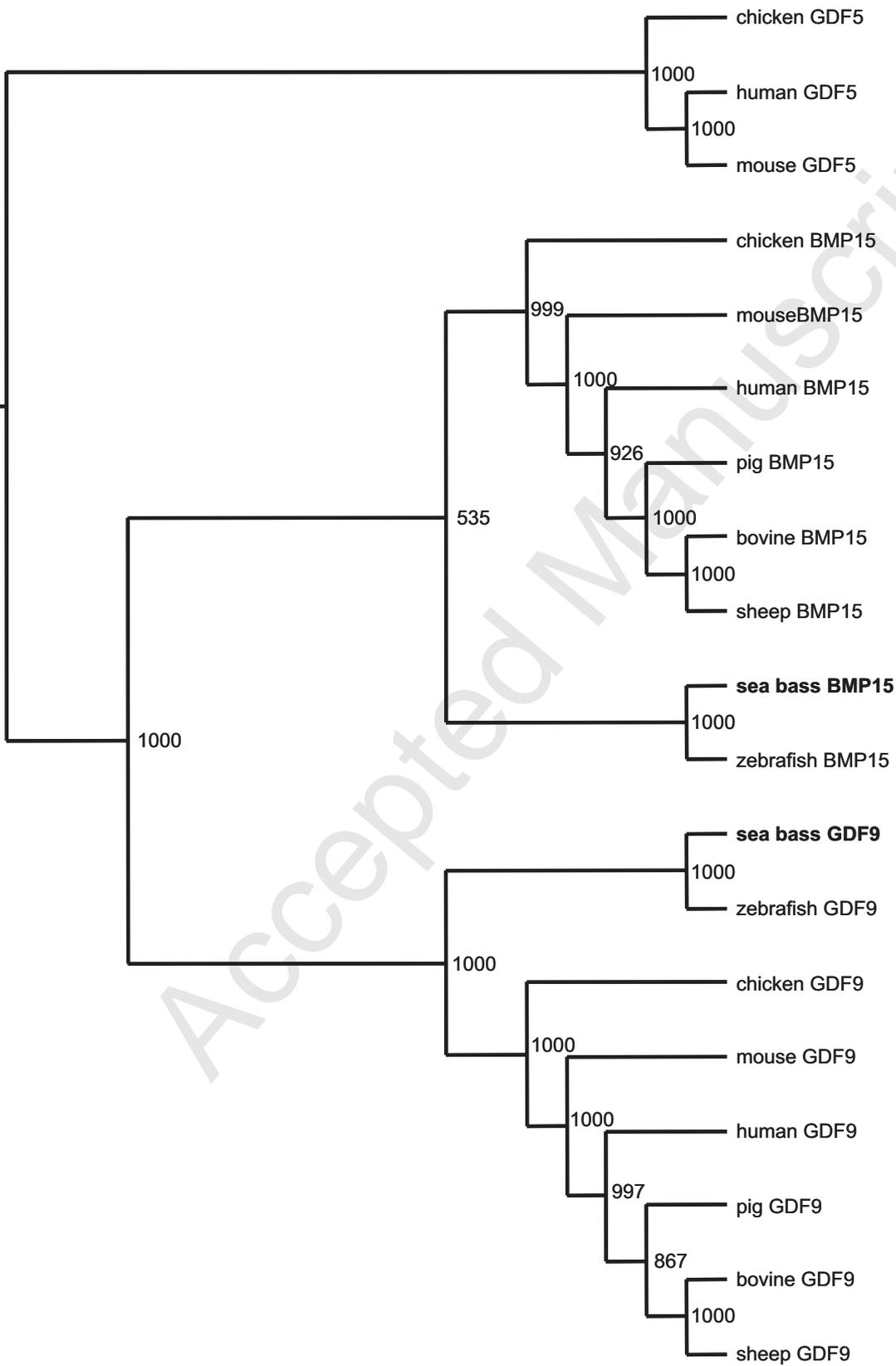
Figure 4: Tissue-specific sea bass *gdf9* (A) and *bmp15* (B) gene expression analysed by Northern blot (10 μ g of total RNA) and blotting of RT-PCR (2 μ g of total RNA; 30 cycles) products followed by hybridisation with sea bass *gdf9* or *bmp15* specific probes, respectively. Northern blot (including the stained total RNA of each preparation): upper panels; RT-PCR blot: lower panels. ov: ovary; te: testis; li: liver; he: heart; ki: kidney; br: brain; pi: pituitary; mu: muscle; ad: adipose; sp: spleen.

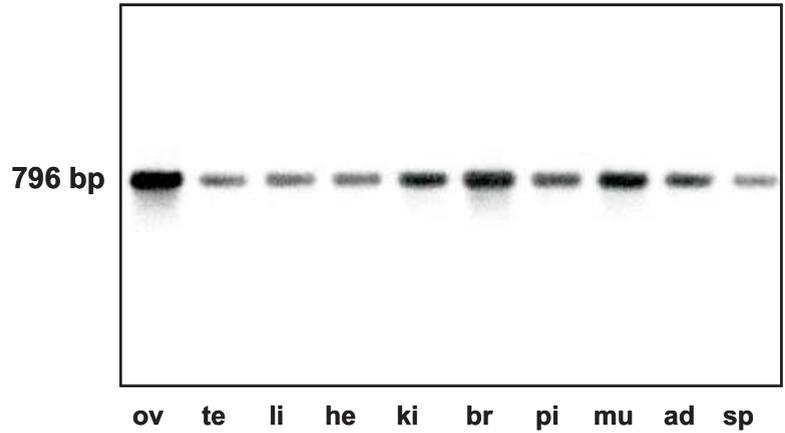
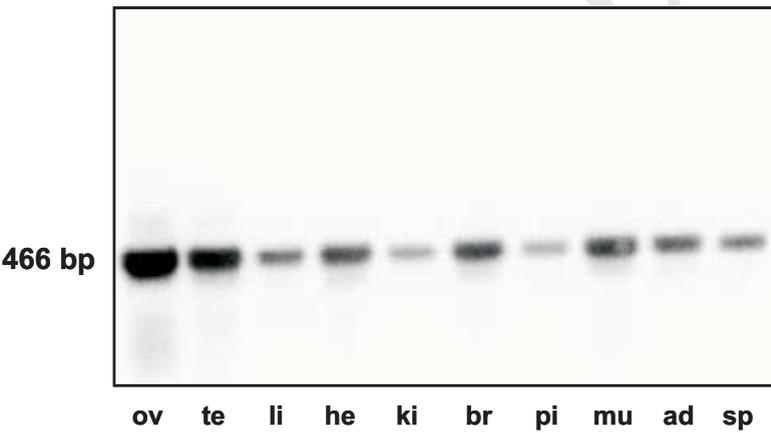
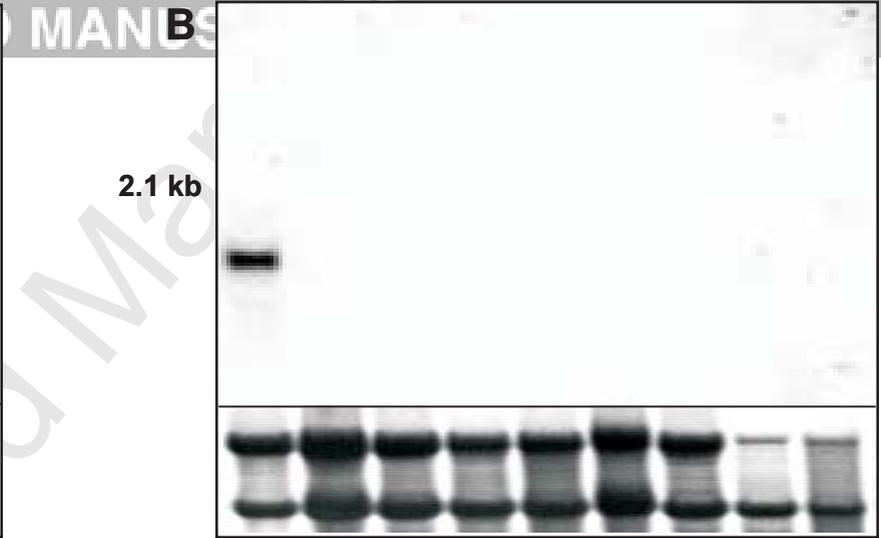
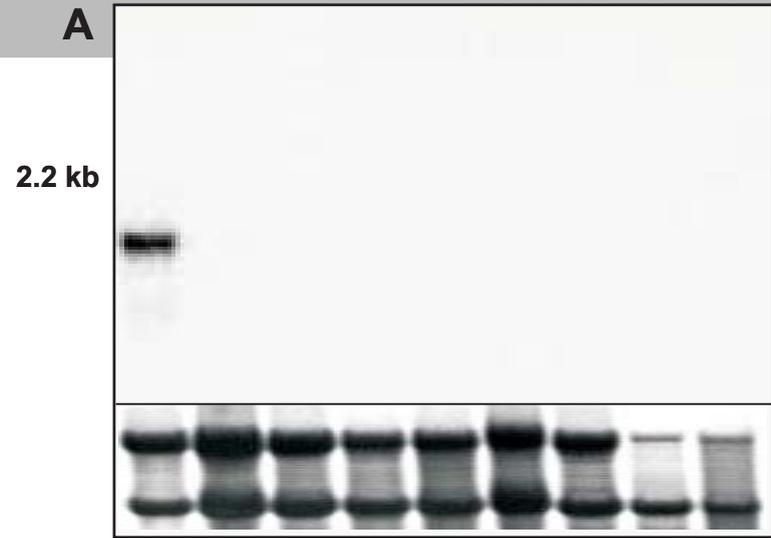
Figure 5: (A) Sea bass *gdf9* and *bmp15* gene expression in the ovary during the reproductive cycle analysed by dot-blot hybridisation. Depicted are the fold-changes of adjusted volume intensities (means and SEM) for each group (n = 5) compared to the group with the lowest values (group January for *gdf9* and group February for *bmp15*), which was set at 1. (B) Membrane used for dot-blot analysis stained with methylene blue to verify similar loadings of total RNA per sample.

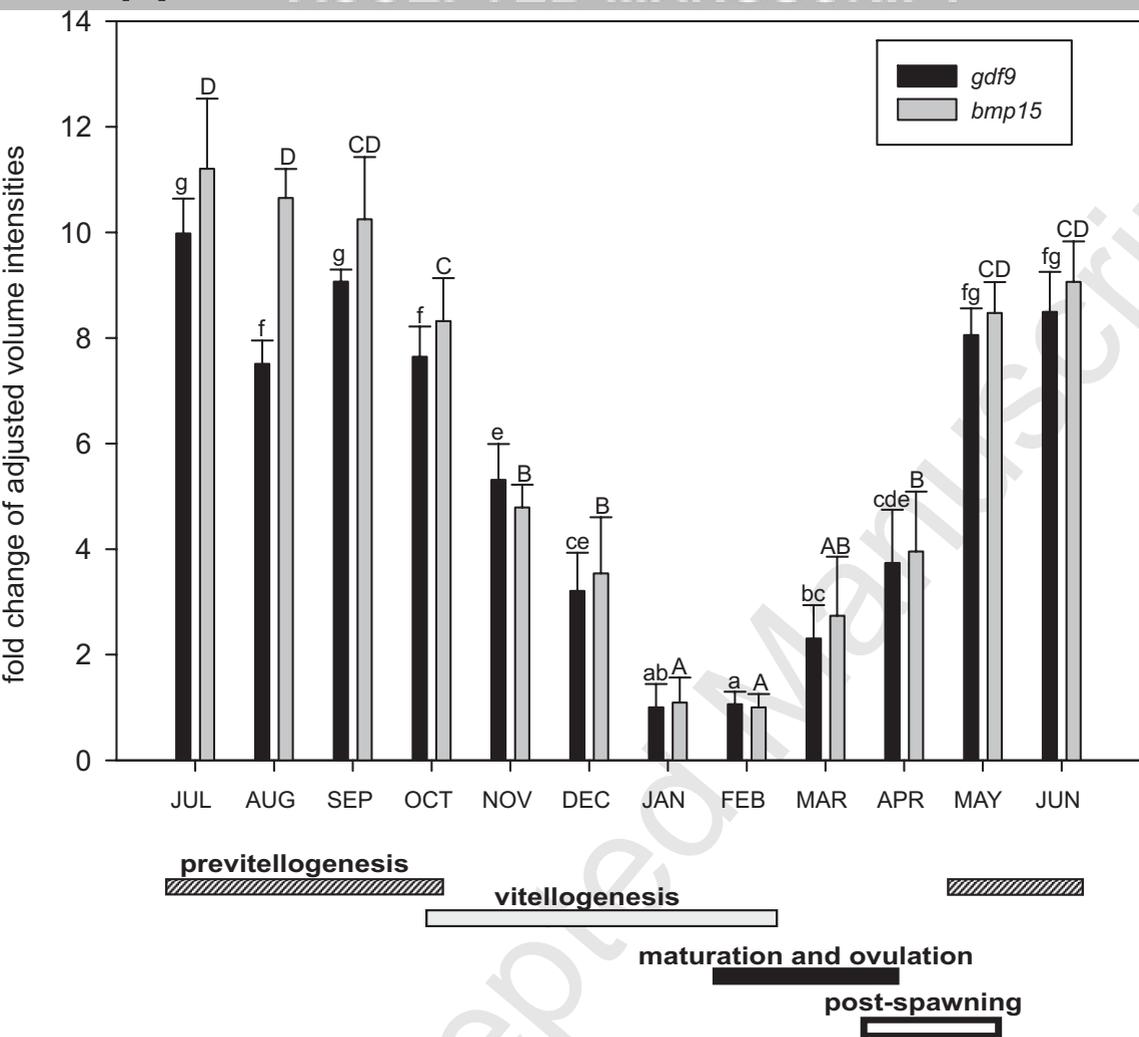
Figure 6: Spearman-rank correlation of sea bass *gdf9* and *bmp15* gene expression during the reproductive cycle. Depicted are the ranks of adjusted volume intensities of individual samples (n = 60) for each gene.

Table 1: Deduced amino acid similarities (%) of the prepropeptide/mature peptide coding region of sea bass *gdf9* and *bmp15* compared with those of human, mouse, pig, chicken, and zebrafish *gdf9* and *bmp15*.

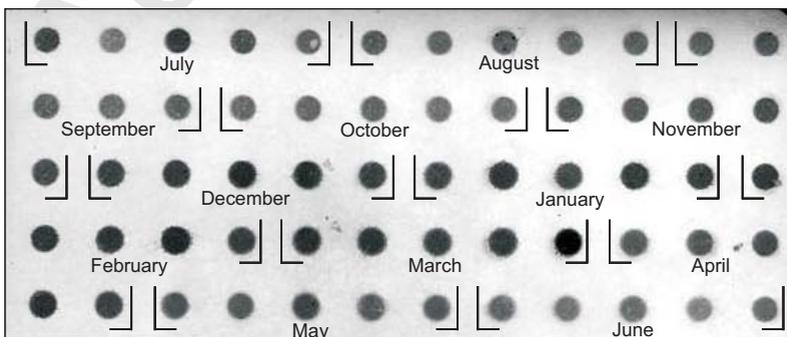
species	sea bass <i>gdf9</i>		sea bass <i>bmp15</i>	
	<i>gdf9</i>	<i>bmp15</i>	<i>bmp15</i>	<i>gdf9</i>
human	48.0/70.7	45.7/59.0	42.0/54.1	39.6/52.2
mouse	46.4/66.2	42.7/59.7	42.5/56.1	42.0/52.9
pig	50.9/71.9	42.9/62.7	42.5/56.3	40.3/53.5
chicken	52.4/69.9	42.6/67.2	43.1/57.3	41.8/54.8
zebrafish	64.4/77.3	40.9/49.3	56.1/61.4	38.0/49.0







B



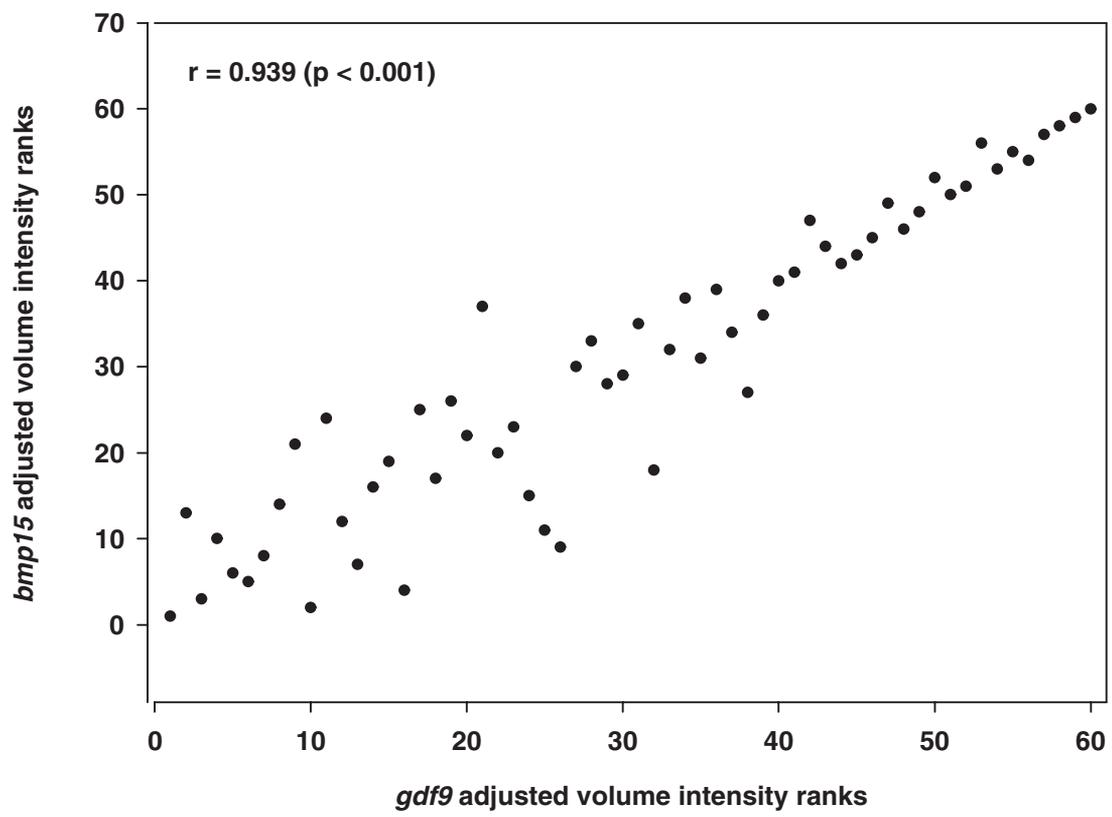


Figure 6