

Participation of the insulin-like growth factor system in the early maturity in juvenile male European sea bass

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ABSTRACT

It is known that the insulin-like growth factor (Igf) system is involved in gonadal development and, in turn, reproduction in vertebrates. In this study, the circulating levels of Igf-1 and follicle-stimulating hormone (Fsh) were assessed as physiological signals related to gonadal maturation stage in juvenile male European sea bass at 1 year of age. The seasonal profiles of Igf-1 and Fsh were analyzed in non-precocious (NP) and precocious (PR) fish during the early onset of puberty. Subsequently, gonadal expression patterns of the three *igf* genes and their two receptors, *igf-1r* and *igf-2r*, were studied during their first year of life. The analysis was continued to evaluate the mRNA expression profiles of three genes (*sox17*, *scf*, *nf2*) mediating spermatogenesis progression. Collectively, these results demonstrated that PR males have higher circulating levels of Igf-1 as compared to NP fish before and during the putative gonadal development period (PGD). Plasma Fsh levels were significantly higher in PR males as compared to NP fish before the start of the PGD period, although this was followed by a decrease in Fsh levels and a subsequent recovery, coinciding with the reproductive season (February). Results showed that the total variance associated with the appearance of precocity may be explained by the contribution of factors such as body size and plasma levels of Igf-1, Fsh and sex steroids in male sea bass. Expression of *igfs* was high during stage I, and this was significantly correlated with the expression of genes related to cell cycle progression, including *sox17*, *scf* and *nf2*. Finally, the decreased expression of all these gonadal factors in precocious animals suggests their potential involvement in regulating the proliferative growth of spermatogonia and supporting cells during the early stages of spermatogenesis, thus evoking the onset of puberty in male sea bass.

1. Introduction

The insulin-like growth factor (Igf) system, which includes the participation of three ligands (Igf-1, Igf-2, Igf-3), six binding proteins (Igfbps), and two receptors (Igf-1r, Igf-2r), is well conserved in vertebrates (Griffeth et al., 2014; Neirijnck et al., 2018). The Igf system is recognized to have an important role in growth regulation in mammals and fish, although it is also involved in other biological processes such as cellular differentiation, proliferation, reproduction, metabolism among others (Ndandala et al., 2022). In the case of reproduction, it is known that Igf-1 affects the hypothalamic-pituitary-gonadal axis at different levels (Daftary and Gore, 2005; Gu et al., 2021). Although its effects on the reproductive function have been studied mainly in mammals,

exploration into the specific role of Igfs in fish gonadal growth and development is less extensive and still far from clear (Chandrashekar et al., 2004; Reinecke, 2010; Ndandala et al., 2022; Zhao et al., 2023). Therefore, the elucidation of the role that the Igf system may play in fish reproduction, as well as the potential involvement of these peptides in regulating the timing of puberty onset merit to be investigated (Schulz et al., 2019; Dees et al., 2021).

It is reported that plasma levels of Igf-1 increase at the time of puberty in several vertebrate species such as mouse (DiVall et al., 2010), sheep (Roberts et al., 1990) and primates (Copeland et al., 1982; Juul et al., 1994). Igf-1 signaling is essential to stimulate the follicle-stimulatory hormone (Fsh) and, in turn, evoking steroidogenic gene expression in granulosa cells in mouse, rat and humans (Zhou et al.,

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2013). In addition, Igf-1 promotes development of spermatogonia to primary spermatocytes in mouse (Shen et al., 2014). In teleosts, it has been reported that *igf-1* and *-2* genes are widely expressed in various tissues, however, the expression of the *igf-3* gene has been specifically observed in the brain and gonads where it plays a remarkable role in spermatogenesis and oogenesis (Yang et al., 2015; Song et al., 2016; Gu et al., 2021). An increase in plasma Igf-1 has been observed in Chinook salmon during the early mitotic stages of spermatogenesis (Campbell et al., 2003) and it has been considered as a growth factor influencing ovary development in salmonids (Campbell et al., 2006). Studies in the zebrafish (*Danio rerio*) have found that gonadotropin and steroids are involved in the differential regulation of ovarian Igf system (Nelson and Van Der Kraak, 2010). In fact, Fsh stimulates Sertoli cell production of Igf-3, which promotes the progression of type A spermatogonia into meiosis through Igf receptors (Nóbrega et al., 2015; Morais et al., 2017). On the other hand, mRNA expression of three ligands and two receptors in brain, liver and gonads in fish of both sexes in the silver pomfret (*Pampus argenteus*) suggests that this system is involved in gonadal growth and development in this species (Gu et al., 2021). In the orange-spotted grouper females (*Epinephelus coioides*), all three ovarian *igfs* show differential gene expression depending on the gonadal stage, where the *igf-1* and *-2* genes respond to growth hormone (Gh), but not *igf-3*. In humans, chorionic gonadotropin (hCG) effects might be mediated by ovarian *igf-1* and *igf-3* (Yang et al., 2015). Collectively, these studies emphasize the participation of the Igf system in gonadal development, although the specific role of the three Igfs in oogenesis and spermatogenesis and their relationship with somatic growth, reproduction and other physiological functions in fish need to be further explored (Ndandala et al., 2022).

The European sea bass, *Dicentrarchus labrax* (hereinafter, sea bass) is a teleost fish mostly farmed in the Mediterranean area. The sex ratio in captivity is highly biased towards males, which are usually smaller than females at harvest (Felip and Piferrer, 2018). Furthermore, early sexual maturation affects both sexes, a situation that leads to differences in growth, thus reducing biomass, which results in a financial problem in terms of production (Felip et al., 2006; Sempere et al., 2023). It has been reported that feed restriction in males entering puberty affects circulating Igf-1 plasma levels and reduces the gonadal *igf* expression and the proliferation of spermatogonia (Escobar-Aguirre et al., 2020). This suggests that the Igf system may play a crucial role in reproductive function in this species. Recently, the study by Sempere et al. (2023) has shown that circulating levels of Igf-1 increase in females at the time of early onset puberty. Accordingly, this study reports the seasonal profiles of circulating levels of Igf-1 and Fsh as indicators of the gonadal maturation stage of non-precocious (NP) and precocious (PR) 1-year-old males. Subsequently, the gonadal expression patterns of *igf/igfr* system were investigated in juveniles during their first reproductive cycle to evaluate their involvement in sea bass gonadal function. The changes in the expression of cell cycle progression-related genes and their correlation to the expression profiles of *igfs* were also evaluated to assess its involvement in the progression of spermatogenesis. These genes included the Sry-related HMG-box transcription factor *sox17* (*sox17*) as a mitotic proliferation factor, the stem cell factor (*scf*) as a cell survival and proliferation marker and the moesin-ezrin-radixin-like (MERLIN) tumor suppressor gene (*nf2*) as a proliferation factor. The findings of this work support the role that Igfs may play at the onset of puberty of male sea bass.

2. Material and methods

2.1. Animals

Sea bass specimens hatched at the Institute of Aquaculture Torre de la Sal (IATS) (40° N and 0° E) were used in this study. Larval and fish rearing was carried out following standard procedures for this species as previously described in Sempere et al. (2023). Briefly, juvenile sea bass

($n = 71$ males in total, >47 g) were individually tagged at 183 days post-hatching (dph) and histologically identified when the fish were terminally sampled over the course of their first year of life (Sempere et al., 2023). The animals were handled according to the guidelines for animal experiments set out in Spanish Royal Decree 53/2013 (RD, 2013) and European legislation (Directive 2010/63/EU) (EC, 2010) for the use of laboratory animals for scientific purposes.

2.2. Gonad histology

Small pieces of testes were collected, fixed and processed as previously described in Sempere et al. (2023) and the stage of testicular development was determined according to Begtashi et al. (2004). Accordingly, fish of 1 year old in stages IV and V were considered as maturing males that achieved early onset puberty and they were designated as precocious fish (PR) ($n = 9$) (Rodríguez et al., 2012). On the other hand, males in the I, II or III stages were designed as non-precocious fish (NP) ($n = 8$) in February, coinciding with the maximum period of sea bass testicular growth. Furthermore, the gonads of 1-year-old males ($n = 54$) were collected during the putative gonadal development (PGD) period (December–February, 18 fish/month), including fish in stage I ($n = 13$, immature), stage II ($n = 12$, early recrudescence), stage III ($n = 15$, mid recrudescence), stage IV ($n = 9$, late recrudescence) and stage V ($n = 5$, fully spermiating testes).

2.3. Evaluation of key performance indicators

Samplings were periodically performed from 183 to 351 dph. Fish were anesthetized with MS-222 (0.1 g l^{-1} of sea water) (Sigma-Aldrich; Merck group, Darmstadt, DE) before sampling and body weight (W), fork length (L) and Fulton's condition factor (K) of NP and PR fish were individually measured. Blood samples were collected and subsequently plasma was separated and stored at -20°C until analyzed (Sempere et al., 2023). Circulating plasma levels of insulin-like growth factor-1 (Igf-1) (Vega-Rubín de Celis et al. (2004) and follicle-stimulating hormone (Fsh) (Molés et al., 2012) were determined by radioimmunoassay (RIA) and a specific enzyme-linked immunosorbent assay (ELISA), respectively, for use in the sea bass. Changes in plasma levels were individually analyzed between both male groups throughout their first reproductive cycle. In addition, plasma testosterone (T), 11-ketotestosterone (11-KT) and 17β -estradiol (E_2) were measured at 245 and 351 dph using a conventional enzyme immunoassay (EIA) (Rodríguez et al., 2000, 2001; Molés et al., 2011). Details on the sensitivity of these assays, as well as the percentage of binding and the inter- and intra-assay coefficients are described in Sempere et al. (2023). At the end of the first year of life, fish were sacrificed using an overdose of anesthetic and the gonadosomatic index (GSI), viscerosomatic (VSI) and hepatosomatic (HSI) indexes were calculated (Escobar-Aguirre et al., 2020).

2.4. Search for the most stable reference gene

A total of five reference genes were selected for qPCR gene expression analysis in sea bass testes, including the ribosomal protein 18 s (*18s rRNA*), the elongation factor 1α (*ef1\alpha*), ubiquitin-like fubi-ribosomal protein ES30 fusion protein (*fau*), ribosomal protein L13 (*l13*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). Their stable expression across different conditions was analyzed with the Bestkeeper, GeNorm, NormFinder and RefFinder software, as described by De Spiegelaere et al. (2015) (Supplementary Data Table S1). In each case, the genes were ranked from most stable to least stable based on the values yielded by the different methods for each candidate reference gene. Comparisons between methods were performed on samples including stage I (NP, $n = 8$) and stage IV-V (PR, $n = 9$), representing the first and the late stages of gonadal development, respectively. The analyses were carried out using $1 \mu\text{l}$ of the final 1:500 dilution (*18s*) or 1:10 dilution (*ef1\alpha* and *gapdh*) of cDNA samples or pure cDNA (*l13* and *fau* genes). The

qPCR was performed using 5× PyroTaqEvaGreen qPCR Mix Plus (NO ROX) (Cultek Molecular Bioline) for *fau*, *l13* and *gapdh*, while the 5× PyroTaq PROBE qPCR Mix Plus (NO ROX) (Cultek Molecular Bioline) was used for *ef1a* and *18srRNA* genes. The thermocycling conditions for the PyroTaqEvaGreen analyses were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min. A melting-curve analysis was run after each real-time experiment for each gene, thus evidencing the specificity of the real-time qPCR. For the Taqman probe-based assays, the thermocycling conditions were: initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min. Tenfold serial dilutions of known concentrations of the plasmids containing each of the genes were used. The specific primers used are shown in Supplementary Data Table S2. Amplification reactions were carried out in a CFX Connect™ Realtime System (Bio-Rad Laboratories, S.A.), using 96-well white plates (Abgene, UK), and data analyzed using CFX Maestro™ Software version 1.1 provided by the manufacturer.

2.5. Quantitative real-time PCR (qPCR) of gonadal factors

Sea bass total RNA was isolated from the gonads of 1-year-old males ($n = 54$) showing different testicular stages during the PGD period and a total of 17 prepubertal males designated as NP ($n = 8$) and PR ($n = 9$) in this study. mRNA expression of three *igfs* and their two receptors, as well as *sox17*, *scf* and *nf2*, was analyzed by qPCR. The specific primers used are shown in Supplementary Data Table S2. Total RNA was extracted as previously described in Alvarado et al. (2013) and samples were treated with TURBO DNA-free™ Kit (Invitrogen) to remove RNA contamination when detected. A total volume of 20 µl of reaction, including 1 µg of total RNA and random hexamer primers, was prepared for reverse-transcription reactions (SuperScript IV Reverse Transcriptase, Invitrogen, LifeTechnologies). The qPCR mixture consisted of 1 µl of cDNA sample in a final volume of 20 µl. Quantification was performed using 1 µl of the final 1:25 dilution (for *igfs* and their receptors at stages I to V of testicular development) and 1:50 dilution of cDNA sample (for progression-related genes and *18s rRNA*). In the case of NP and PR males, 1 µl of the final 1:5 dilution or 1:25 dilution of cDNA samples was used to quantify *igfs* and their receptors or cell cycle progression-related genes and *18s rRNA* respectively. The qRT-PCR of *igfs*, *igfrs* and cell cycle progression-related genes were performed using the thermocycling conditions for the PyroTaqEvaGreen analyses, as previously described in this study. All gonadal factor gene transcripts were quantified using a pool of the cDNA samples ($n = 54$ for testicular development and $n = 17$ for both male groups, NP and PR) to prepare the standard curves producing a 5-fold dilution series over 6 points according to the $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). The standard curves covered all potential template concentrations that may be encountered during the study. All samples and negative controls were run in duplicate. Data were expressed as relative fold change values of mRNA for each target gene/*18s* mRNA (starting quantity mean $\pm$ standard error of the mean, SEM) (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Data are represented as the mean $\pm$ standard error of the mean (SEM). One-way ANOVAs were performed with the AOV function from the package stats to test differences between NP and PR males for body weight, fork length, K, plasma levels of sex steroids and body indexes. Mixed two-way ANOVAs were performed with the anova.test function from the rstatix package, in order to test differences over time between NP and PR males in terms of Igf-1 and Fsh plasma levels, which were repeated measures. A principal component analysis (PCA) of body weight, fork length and plasma levels of Igf-1, Fsh, T, 11-KT and E_2 was used to split the total variance into components that were used to plot NP and PR male sea bass at 245 dph, using the PCA and fviz_pca_biplot

function from the FactoMineR and FactoExtra packages, respectively. The PCA was validated by performing a MANOVA, using the MANOVA function from the MANOVA.RM package. Gene expression differences between samples of *igfs* and their receptors, as well as *sox17*, *scf* and *nf2*, were tested with a one-way ANOVA. Relative differences were expressed as a proportion of the mean value in the PR group for comparison between NP and PR fish and as a proportion of the mean value in the IV stage for male sea bass sampled during the PGD period. In addition, another one-way ANOVA was used to select the most stable reference gene to be used for gene expression normalization in this study. The association between pairs of parameters (gene expression levels, body size and plasma levels) was evaluated by the correlation coefficient r , using the Hmisc and corrplot packages. All the analyses in this study were performed with R-project (R Core Team, 2022), including the detection of outliers using the package outliers and the calculation of missing data using the imputeTS package. Before the analysis, normality was tested with Shapiro-Wilk (shapiro.test function) or Kolmogorov-Smirnov (ks.test function) tests for mixed ANOVA and one-way ANOVA, respectively. Homoscedasticity was checked with the Bartlett test (bartlett.test function). Logarithmic transformation of data was performed to meet normality and homoscedasticity requirements, and alternatively using Kruskal-Wallis Rank Sum Test (kruskal.test from stats package) when necessary. Statistical differences were considered significant when $P < 0.05$.

3. Results

3.1. Growth and circulating plasma levels of Fsh and Igf-1

The reproductive performance of juvenile male sea bass was assessed in NP and PR fish, finding that the mean body weight and fork length of PR males were significantly higher than those of NP fish. Similarly, statistically significant differences were observed in K and body indexes between both male groups (Supplementary Data Table S3). No significant differences in the circulating plasma levels of sex-steroids were observed, except for higher E_2 levels in NP males at 245 dph (November). On the other hand, circulating plasma levels of Igf-1 were higher in PR fish, which reached values up to 10.27–12.53 ng/ml from 183 to 245 dph (September–November), coinciding with the growth and sex differentiation period in the sea bass. These levels then decreased during the PGD period (December–February) onwards (7.01 ± 0.70 – 8.86 ± 1.29 ng/ml) (Fig. 1A). In the case of NP fish, Igf-1 circulating levels ranged from 4.99 ± 0.48 to 9.99 ± 1.81 ng/ml. No statistical differences were detected in the interaction between the fish testicular stage and their age. Regarding the circulating Fsh levels, significantly higher levels were observed in PR males in comparison to those of NP males at 245 dph in November and at 351 dph in March ($P < 0.05$) (Fig. 1B). Nevertheless, the plasma levels of Fsh showed statistical differences depending on the age of fish, as well as in the interaction between testicular stage and age of males ($P < 0.05$). The total of variance associated to the appearance of precocity was 73 %, with a contribution of the gonadal stage that accounted for 53.1 % and 19.9 % of total variance along the X- and Y-axis, respectively (Fig. 2). The percentage of variation explained by each factor is indicated in the Fig. 2 that showed NP and PR fish clustering into two groups. Based on these findings, these factors might be considered as biomarkers of early onset puberty in male sea bass.

3.2. Analysis of the reference genes

A ranking of stability values was generated for each method and gene considered in this study (Supplementary Data Table S1). The *18s rRNA* appears ranked first by BestKeeper (coefficient of variation), NorFinder and RefFinder, and ranked second by GeNorm and BestKeeper (standard deviation). The gonadal expression levels of *18s rRNA* were not significantly different ($P = 0.607$) in NP versus PR fish. On the other hand,

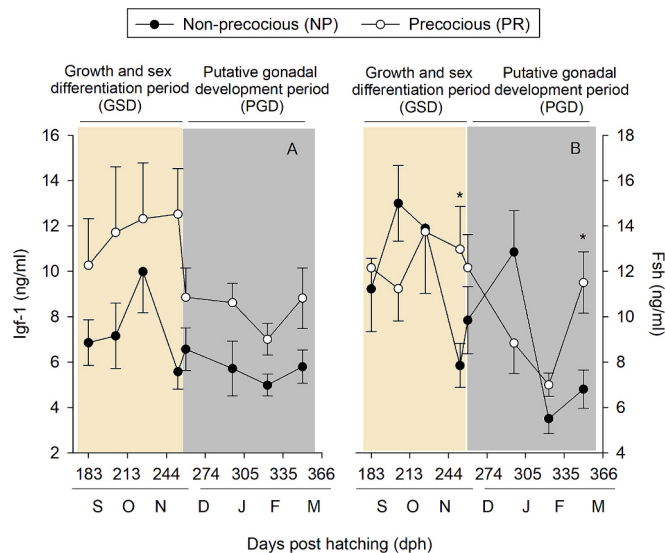


Fig. 1. Changes in the plasma circulating levels of insulin-like growth factor-1, Igf-1 (**A**) and follicle-stimulating hormone, Fsh (**B**) in non-precocious (NP) ($n = 8$) (closed circles) and precocious (PR) ($n = 9$) (open circles) juvenile male sea bass during their first year of life. Data are expressed as the mean $\pm$ SEM. Igf-1 showed significant differences according to the testicular stage ($P = 0.004$) and age ($P = 0.014$), but not for their interaction ($P = 0.668$). Fsh showed significant differences according to age ($P = 0.003$), but not for the testicular stage ($P = 0.532$), although statistical differences were observed between NP and PR at two sampling points (*). Significant differences were also observed for the interaction between the gonadal stage and the age of fish ($P = 0.043$). Shaded areas indicate the growth and sex differentiation (GSD) period and the putative gonadal development (PGD) period one year before full spermiation.

significant changes in the expression of *18S rRNA* were seen during gonadal development ($P = 0.00207$), although the difference between the stage I and V, which corresponded to the highest and the lowest values observed, respectively, was lower than twofold (Supplementary Data Fig. S1). Based on that, normalization of testis samples in this study was performed using *18S rRNA* as the reference gene.

3.3. Gonadal expression of *igf/igfr* and cell cycle progression-related genes

Changes in gonadal expression of the genes of interest during the PGD period were first examined in juvenile male sea bass. The expression of *igf-1* was high during the early stages of gonadal development, whereas a non-significant decline was observed during mid-recrudescence that was followed by significantly lower levels at the late recrudescence stage (Fig. 3A). Then, expression of *igf-1* increased during the full spermiating stage, although not significantly so from the previous stage. *Igf-2* expression was also high during the early stages of gonadal development (Fig. 3B). The expression of *igf-2* significantly declined during the mid- and late recrudescence stages, as compared to that of the immature stage. During the full spermiating stage, *igf-2* expression was not significantly different from the previous stage. Regarding the expression of *igf-3*, values were the highest in the mid-recrudescence stage, with a significant decrease at full spermiating stage (Fig. 3C). *Igf-1r* levels remained high during the immature stage and, as spermatogenesis progressed its expression decreased (Fig. 3D).

The expression of *igf-2r* showed non-significant differences (Fig. 3E). In addition, mRNA levels of *sox17* (Fig. 4A), *scf* (Fig. 4B) and *nf2* (Fig. 4C) were high in the immature stage, as compared to the other stages. In the case of *sox17*, expression levels decreased during early recrudescence, although they were not significantly different from the previous stage, followed by a decline during mid-recrudescence and with significantly lower levels at the late recrudescence stage. Levels

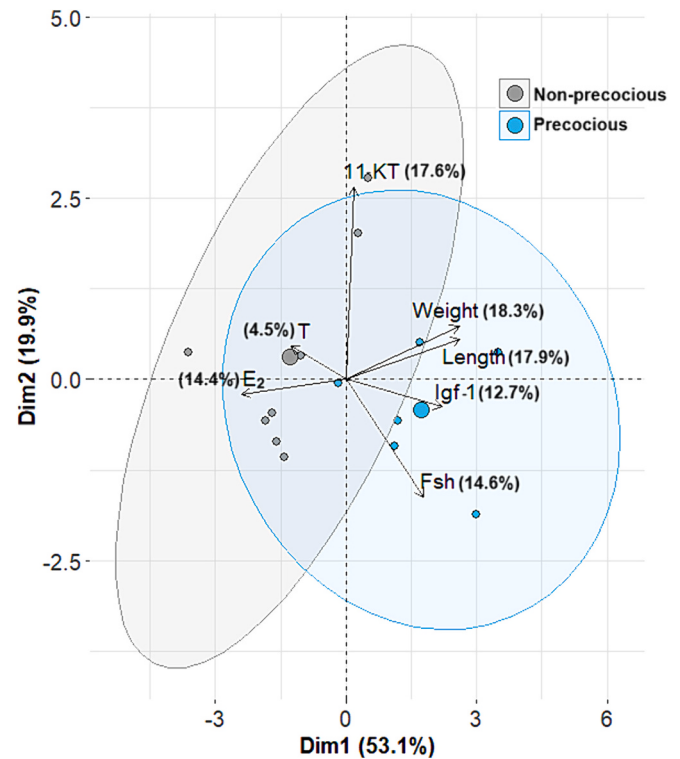


Fig. 2. Biplot of principal component analysis based on key parameters measured in male sea bass during their first year of life. Samples clustered into two groups: the non-precocious (immature fish) (grey circles) and precocious (maturing fish) (blue circles) males. Percentage values represent the variation explained by each parameter considered ($P = 3.26E-04$). The ovals represent the concentration ellipse (light color) and the confidence ellipse (dark color) that is plotted around of each group with a security of 95 %. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during the full spermiating stage were not statistically different from early recrudescence onwards. Overall, the expression pattern of the *igfs/igfr* system, except for *igf-3* and *igf-2r*, and the expression of *sox17*, *scf* and *nf2* genes reached the maximum at the immature stage during spermatogenesis, and their expression decreased as spermatogenesis progressed (Figs. 3 and 4).

3.4. Quantification of gene expression in the testes of NP and PR males

High levels of all five genes were expressed in NP fish, including the three *igf* ligands, *igf-1* (Fig. 5A), *igf-2* (Fig. 5B), *igf-3* (Fig. 5C), and *sox17* (Fig. 5D), *scf* (Fig. 5E) and *nf2* (Fig. 5F) ($P < 0.05$), suggesting that these gonadal factors might play an important role in the spermatogenesis at early onset puberty in sea bass, during its initiation and maintenance.

3.5. Correlation analysis

Correlation analysis of body size, plasma reproductive hormones and Igf-1 (Fig. 6A) and expression of *igfs* and cell cycle progression-related genes (Fig. 6B) between NP and PR males identified significant relationships among all the factors analyzed in this study. Weight and length were positively correlated with plasma Igf-1 and E_2 levels ($P < 0.05$). Changes in Igf-1 were found to be positively correlated with changes in Fsh, but negatively correlated with changes in E_2 . Other correlations, including androgen plasma levels, were not significant in this study. Gonadal *igf-1* expression was positively correlated with *igf-2*, *igf-3*, *sox17* and *scf* ($P < 0.05$). A significant positive correlation was seen between *igf-2* and -3 , which were found to correlate with *sox17* and *scf*

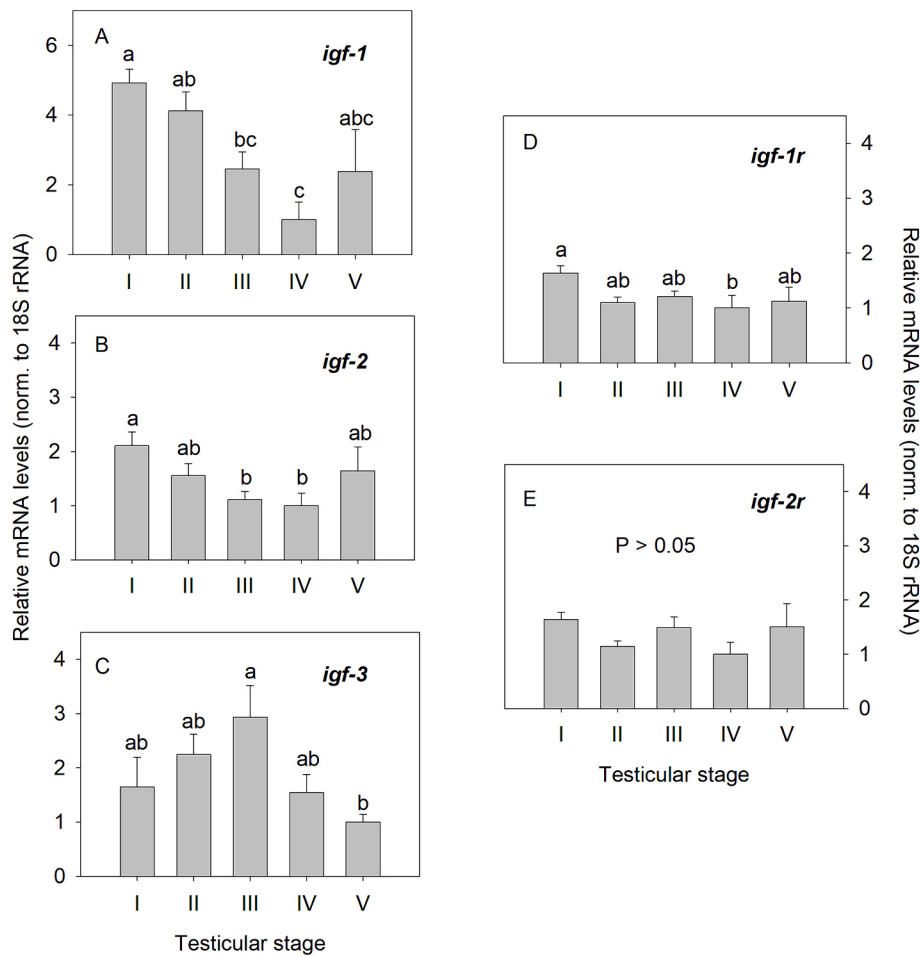


Fig. 3. Relative changes in testicular expression of *igf-1* (A), *igf-2* (B), *igf-3* (C), *igf-1r* (D) and *igf-2r* (E) in juvenile male sea bass sampled during their first year of life. Values are shown as the mean $\pm$ SEM and represented by stages of gonadal development as determined by histology. Males: stage I ($n = 13$), immature; stage II ($n = 12$), early recrudescence; stage III ($n = 15$), mid-recrudescence; stage IV ($n = 9$), late recrudescence; stage V ($n = 5$) and full spermiating testes. Testicular expression values were normalized to *18S rRNA*, which was adjusted to compensate for changes in expression across stages, and expressed as fold change with respect to that stage, with the lowest mean value for each gene; this was considered as the reference and had a value of 1. Statistically significant differences are indicated with different letters above the bars. The P -values for *igf-1*, -2 and -3 showed significant differences according to the testicular stage, $P = 5.64 \times 10^{-5}$, 0.00791 and 0.03619 , respectively. Significant differences were also reported for *igf-1r* ($P = 0.0267$) and *igf-2r* ($P = 0.142$).

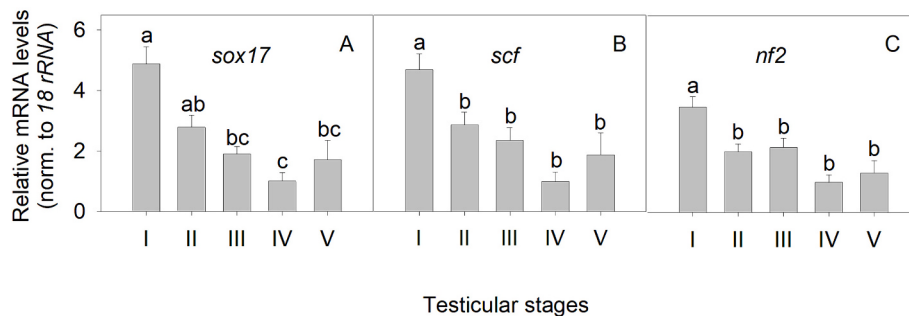


Fig. 4. Relative changes in testicular expression of cell cycle progression-related genes, *sox17* (A), *scf* (B) and *nf2* (C) in juvenile male sea bass sampled during their first year of life. Values are shown as the mean $\pm$ SEM and represented by stages of gonadal development as determined by histology. The legend is the same as in Fig. 3. The P -values for *sox17* ($P = 6.36 \times 10^{-7}$), *scf* ($P = 2.63 \times 10^{-5}$) and *nf2* ($P = 3.84 \times 10^{-5}$) showed significant differences according to the testicular stage.

transcript levels. Changes in *nf2* expression were positively correlated with changes in transcript levels of *igf-2*, although negatively correlated with *sox17* and *scf* ($P < 0.05$).

4. Discussion

The size differences between NP and PR male sea bass observed in this study are consistent with those reported in previous studies on this teleost fish (Begtashi et al., 2004; Rodríguez et al., 2012), thus showing that PR juvenile males of 1-yr old are usually larger than NP fish. It has

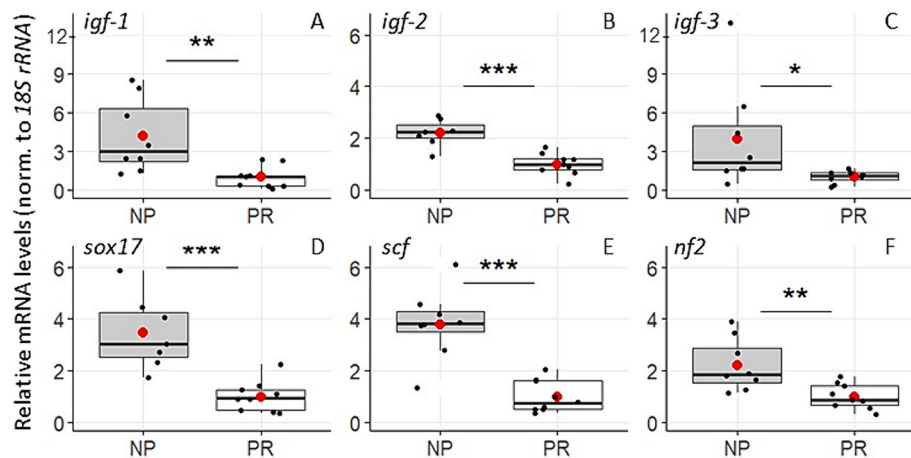


Fig. 5. Comparison and expression analysis of Igf system and cell cycle progression-related genes between non-precocious (NP) ($n = 8$) and precocious (PR) ($n = 9$) juvenile male sea bass: *igf-1* (A), *igf-2* (B), *igf-3* (C), *sox17* (D), *scf* (E) and *nf2* (F). Testicular expression values were normalized to *18S rRNA*, which was adjusted to compensate for changes in expression across stages (NP, stage I and PR, stage IV-V). This was expressed as fold change with respect to the stage with the lowest mean value for each gene, which was considered as the reference and had the value of 1. Data are represented in box-and-whisker plots: the boxes delimit the 25 and 75 percentiles, and the lines crossing them represent the median value; vertical bars indicate the 5–95 percentile range and the dots represent outliers. Asterisks indicate statistical differences between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

been suggested that growth can potentially affect the timing of maturity, although the relationship existing between fish body size and age at maturation is unresolved (Martyniuk et al., 2003; Bastien et al., 2011). A number of studies have shown the value of Igf signaling (Igfs, Igfbps) to evaluate growth, reproduction and gonadal development and, in turn, for its utility as an endocrine biomarker for somatic growth performance in fish and shellfish (Picha et al., 2008; Pérez-Sánchez et al., 2018; Chandhini et al., 2021). The temporal profile of Igf-1 and Fsh in PR and NP prepubertal males showed higher circulating Igf-1 levels in PR fish before and during the PGD period, whereas increased Fsh levels occurred before the start of the PGD period and during the reproductive season. We have found a positive correlation between weight, length and plasma Igf-1. Igf-1 plasma levels also correlate with circulating levels of Fsh in juvenile male sea bass. Furthermore, the total variance associated with gonadal development between NP and PR evidenced for a possible interaction between these two productive traits, growth and maturity. The endocrine changes in Igf-1 and Fsh we have seen are in accordance with previous results obtained in female sea bass (Sempere et al., 2023), supporting the role of plasma Igf-1 and Fsh levels as putative biomarkers of the onset of puberty in fish (Campbell et al., 2003, 2006).

Our findings show the relevance of the Igf system in order to understand the relationship between the somatotrophic and reproductive axes at the time of puberty (Chandrashekar et al., 2004; Cannarella et al., 2018; Chandhini et al., 2021). A large number of studies have revealed that the *igfs* display an important role in gonadal development in mammal and non-mammal species (Nelson and Van Der Kraak, 2010; Pitetti et al., 2013; Griffeth et al., 2014; Shen et al., 2014; Nóbrega et al., 2015; Neirijnck et al., 2018; Gu et al., 2021; Safian et al., 2017; Nandala et al., 2022; Zhao et al., 2023), including invertebrates such as oysters (Moon and Choi, 2020). Our study demonstrates that sea bass gonadal expression patterns of the *igf/igfr* system during spermatogenesis depend on the developmental stage. The expression levels observed in early-mid stages of spermatogenesis (stages I, II and III) as compared to those observed in late recrudescence and full spermiation (stages IV and V) suggest a potential role for *igfs* in the development of germ cells, as well as in the proper progression of spermatogenesis. This is supported by a previous study in sea bass, using a laser-capture microdissection method combined with quantitative-PCR, which showed that spermatogonia exhibited a high expression of *igf-1* (Viñas and Piferrer, 2008). Accordingly, in males of silver pomfret (*Pampus argenteus*) a high expression of the elements of *igf/igfr* system occurred during

spermatogonia proliferation. In turbot males (*Scophthalmus maximus*), however, increased expression of *igf-3* was seen in the testes during the proliferation of spermatogonia, whereas the highest levels of *igf-1* and *igf-2* were detected at the beginning of spermatogenesis and spermiation (Zhao et al., 2023). In the present study, the expression variation dynamics of *igfr* receptors in the gonad of male sea bass showed a more stable expression level than those of the ligands throughout testicular development in this teleost fish. These findings need further investigation being also of interest to determine the autocrine/paracrine signaling of the Igf system in the gonads as well as the molecular mechanisms of interaction with gonadotroping hormonal action as previously observed in mammalian spermatogenesis (Zhou et al., 2013). The highest expression of the mitotic proliferation marker *sox17* in male sea bass was found at stage I, coinciding with increased *igf-1* and *igf-2* transcripts, although its levels were not statistically different from those at stage II. This agrees with previous sea bass studies in which *sox17* was observed to be restricted to spermatogonia (Viñas and Piferrer, 2008), the type of germ cell that is distinctive at stages I and II during testicular development. Of note, the transition between these two stages is characterized by an increase in the mitotic proliferation of spermatogonial cells, when spermatogonia are converted from type A into B (Begtashi et al., 2004). The significant positive correlation observed between the expression of *igfs* and *sox17*, *scf* and *nf2* thus suggests that all these gonadal factors might be regulating the same part of sea bass spermatogenesis, including proliferation and maintenance of cell survival. In other words, these gonadal factors might stimulate the early stages of spermatogenesis, when mitosis of spermatogonia and supporting cells occurs in the testis (Campbell et al., 2003; Wang et al., 2015). The stage-specific gene expression of both mitogen factors *scf* and *nf2* revealed a profile similar to that of *sox17* during the progression of spermatogenesis in this species. Interestingly, the highest levels of *igf-3* at stage III –although not statistically different from previous stages– and its significant correlation with the expression of *sox17* and *igfs*, suggests that *igf-3* might be responsible for the entrance of germ cells into the first meiotic division (Begtashi et al., 2004).

Collectively, these results show the potential involvement that Igf-1 and its related genes might have as paracrine/autocrine regulators of gonadal growth and differentiation of the testes, thus triggering the meiosis events in teleost fish (Dubois and Callard, 1993; Berishvili et al., 2006). We found a correlation among *scf*, *sox17* and *igfs*, suggesting the important role of the Scf system in the regulation of cell proliferation during testicular development, which agrees with the function of this

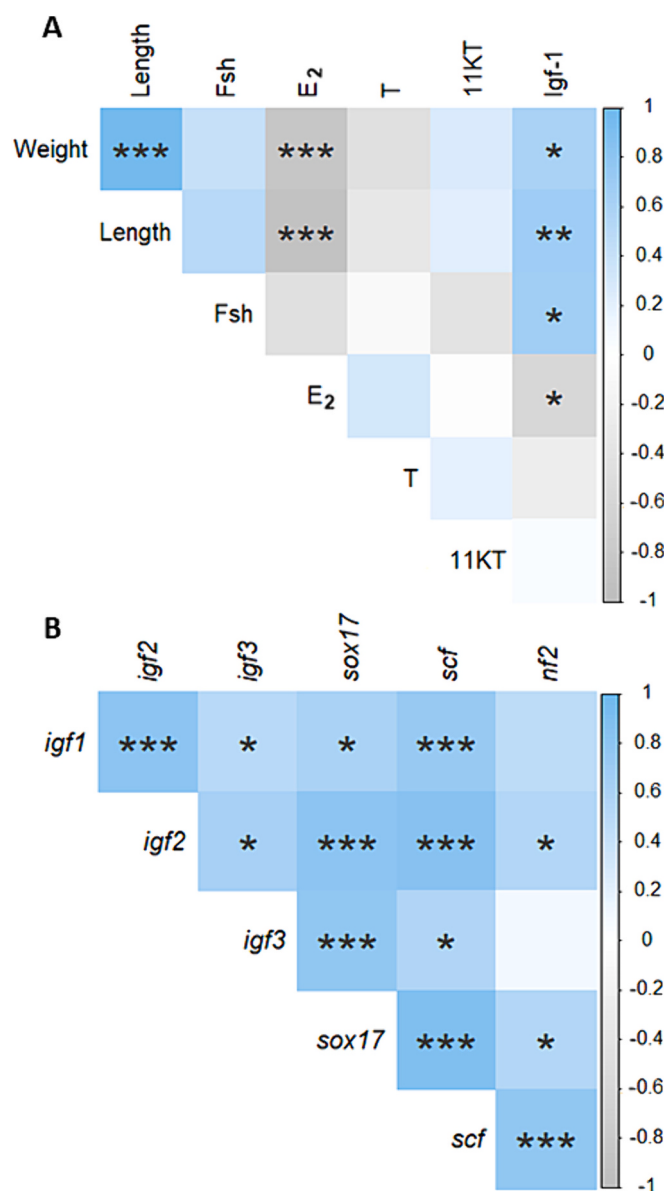


Fig. 6. Plot showing correlation analysis among weight, length and circulating plasma levels (A) and changes in relative expression levels of cell cycle progression-related genes (B) in individual non-precocious ($n = 8$) and precocious ($n = 9$) juvenile male sea bass. Asterisks indicate the level of significance of the correlation (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The color legend indicates the Pearson correlation coefficient.

paracrine regulator in mammal spermatogenesis (Hakovirta et al., 1999; Mauduit et al., 1999; Cannarella et al., 2022). Interestingly, *nf2* was significantly down-regulated in pubertal as compared to prepubertal testes in Atlantic salmon (*Salmo salar*) (Kjærner-Semb et al., 2018). Here, we also show a positive correlation of *nf2* with *igf-2*, although the expression profiles of both *sox17* and *scf* genes were negatively correlated, suggesting that this factor might regulate cell proliferation, albeit the mechanism is not well understood. Therefore, a more comprehensive approach is needed to know how gonadal factors interact to control the early male germ cell development in fish. In this sense, *igf-1*, *-2* and *-3* might be considered as putative candidates to regulate proliferative growth of germ cells during gonad development in juvenile male sea bass, an action that might require the sequential modulation of mitogen gene expression (*sox17*, *scf* and *nf2*). Of note, the quantification of these six gene transcripts in sea bass testes is useful to monitor the cells state and, in turn, candidate gonadal markers of early puberty. These and

other previously reported findings, which evidenced the involvement of *ff1b*, *gsdf1* and *star* genes in this biological process (Crespo et al., 2013), provide potential targets for early sexual maturation control in this teleost fish.

In summary, this work reports that *Igf-1* and *Fsh* plasma levels increase before and during the time of puberty in juvenile male sea bass, and might be potentially used as biomarkers for the identification of puberty in this aquaculture marine fish. Although further characterization of their co-regulation is required to understand the link existing between the somatotrophic and reproductive axes in fish (Reinecke, 2010), this study highlights the *igfs* shows differential gene expression throughout sea bass spermatogenesis. In addition, the gonadal expression of *igfs* is positively correlated with proliferation-related genes thus evidencing for a key participation of these factors in spermatogenesis progression in sea bass. Accordingly, we have achieved to identify six putative gonadal markers that play a role in the onset of early maturity in juvenile males. The molecular mechanisms by which these mitogen factors regulate the survival of spermatogonia and their proliferation remains as an interesting subject for future studies in European sea bass.

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CRediT authorship contribution statement

Laura Sempere: Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Carlos Fernández:** Writing – review & editing, Methodology. **Soledad Ibáñez:** Methodology, Formal analysis. **Conrado Marín:** Methodology, Formal analysis. **Gregorio Molés:** Writing – review & editing, Methodology. **Jaume Pérez-Sánchez:** Writing – review & editing, Methodology. **Paulino Martínez:** Writing – review & editing, Methodology. **Ana Viñas:** Writing – review & editing, Methodology. **Alicia Felip:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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