Molecular performance of Prl and Gh/Igf1 axis in the Mediterranean meagre, *Argyrosomus regius*, acclimated to different rearing salinities

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Note: This paper follow the ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines)

Abstract

Aquaculture industry in the Mediterranean region exhibits a growing interest for the Mediterranean meagre *Argyrosomus regius*. Some preliminary works showed a good growth performance of the species in nearly isosmotic salinities. However, the patterns of alteration of prolactin (Prl) as well as growth hormone (Gh)/insulin growth factor-1 (Igf1) axis at the molecular level are not yet described in this species. Therefore, we cloned and sequenced partial cDNAs for pituitary prolactin (*prl*) and growth hormone (*gh*), hepatic insulin-like growth factor (*igf1*), and β-actin (*actb*). Expression patterns of these transcripts were tested in juveniles of *A. regius* acclimated to four different environmental salinities: i) 5 ‰ (hyposmotic); ii) 12 ‰ (isosmotic); iii) 38 ‰ (hyperosmotic; seawater control); and iv) 55‰ (extremely hyperosmotic). All investigated transcripts shared high sequence identities with their counterparts in other perciformes. *prl* mRNA levels showed inverse pattern with increasing salinities. *gh* mRNA enhanced significantly in both 12 ‰ and 55 ‰ salinity groups in comparison to the control group, while *igf1* showed its maximum expression levels under the nearly isosmotic environment. The results indicated clear sensitivity of *prl, gh* and *igf1* to changes in environmental salinity, which can possibly control the euryhalinity capacity of this species.

Key words: actb, Argyrosomus regius, euryhaline, gh, igf1, prl, qPCR, salinity.

1- Introduction

The Mediterranean meagre, *Argyrosmus regius,* belongs to the family Sciaenidae that consists of 282 species, contributing 20 % to the world aquaculture industry. It is the second globally identified group of economically important aquaculture species after the groupers (family Serranidae) (Cárdenas 2010). In its normal habitats, *A. regius* is well known as a species with good euryhaline capability, performing spring-summer spawning migration of adults for reproduction in estuaries (brackish waters), and then getting back in autumn-winter to deep waters. Juveniles remain in estuaries as nursery beds for the whole summer season. They migrate later to coastal waters at the end of the summer (FAO 2005-2015). The environment of this species is then dynamically-changing, moving between seawater and brackish waters. These migrations should be aided by the presence of an osmoregulatory system with a reasonable degree of preparedness for coping with such varying ecological niches, as in other euryhaline perciformes that exhibit similar migratory life pattern from the estuaries to the open seawater and vice versa (e.g., see Dean and Woo 2004; Mancera and McCormick 2007).

A. regius hatchery production started in 1996 in the south of France. Since then, the hatchery production of this species has been slowly extending in the area (FAO 2015). Due to its rapid growth rate, especially in the isosmotic salinities at 7-18 psu (Muñoz et al. 2008), easiness in processing, low fat content and somewhat rigid texture, it gained good popularity for both aquaculture producers and consumers (Monfort 2010). This species has been cultured in cages and earthen ponds in both brackish water and

70 seawater conditions (Jiménez et al. 2005; El-Shebly and El-Kady 2007; Vargas-Chacoff
71 et al. 2014).

72 To our knowledge, there is no information on the endocrine control of the
73 euryhalinity on *A. regius*. Several key hormones are identified for the major roles they
74 perform in triggering the fish species acclimation to different environmental salinities,
75 mainly the hypophyseal prolactin (Prl) and growth hormone (Gh), as well as the hepatic
76 insulin-like growth factor type 1 (Igf1) (Takei and McCormick 2013). Prl is well-known as
77 a freshwater-adapting hormone. Its hyperosmoregulatory role aids the tissues to reduce
78 their general permeability, ion loss under highly ion-deficient environments, and
79 regulation of essential enzymes, channels and transporters that regulate ions passage
80 (Sakamoto and McCormick 2006; Mancera and McCormick 2007; Whittamore 2012;
81 Breves et al. 2014). These processes seem to be highly conserved on very
82 phylogenetically different euryhaline species. Gh and Igf1 roles in teleost fish
83 osmoregulation seem to be more established and understood in salmonid rather than
84 non-salmonid fishes. In salmonids, Gh/Igf1 axis exhibits plasma-hypoosmoregulatory
85 actions (Sakamoto et al. 1990; Sakamoto and McCormick 2006), increasing opercular
86 chloride cell numbers, gill Na^+,K^+-ATPase activity and mRNA expression of
87 Na^+,K^+-ATPase subunits, as well as salinity tolerance when administered (McCormick
88 1995, 2001). However, in non-salmonid fishes, this role is still controversial due to the
89 contradictory results obtained among different teleosts (Mancera and McCormick 1998).
90 For this reason, more research on both euryhaline and stenohaline species to determine
91 the widespread osmoregulatory actions of the Gh/Igf1 axis is required (Sakamoto and
92 McCormick 2006; Mancera and McCormick 2007; Mohammed-Geba et al. 2015).

This study aimed to investigate the osmoregulatory sensitivity of Prl and Gh/Igf1 axis in *A. regius* juveniles under different environmental salinities (5, 12, 38 and 55 ‰). In order to do so, partial cDNAs from *prl*, *gh* and *igf1*, together with a reference gene (β-actin, *actb*), were obtained by polymerase chain reaction (PCR), cloned and sequenced. The obtained gene sequences were phylogenetically analyzed to infer some common pattern of expression of such transcripts in different environmental salinities among similar fish species to *A. regius*, especially the ones with estuarine life stages. The expression patterns of *prl*, *gh* and *igf1* mRNAs were assessed in individuals acclimated to different environmental salinities using semi-quantitative real time PCR (qPCR). The results are discussed in relation to the good euryhaline capacity of this species.

2. Materials and Methods

2.1. Animals and experimental protocol

Juveniles of *A. regius* (n= 32, 150-180 g body mass) were provided by IFAPA Centro “El Toruño” (El Puerto de Santa María, Cádiz, Spain) and transferred to the wet laboratories in the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz), where they were acclimated during 7 days to 38 ‰ salinity and 21-22 ºC temperature. After this time, 8 animals (4 per tank) were transferred to two tanks equilibrated at 5 ‰ (140 mOsm kg^{-1} H_2O), 12 ‰ (364 mOsm kg^{-1} H_2O), 38‰ (control, 1049 mOsm kg^{-1} H_2O) and 55 ‰ (1546 mOsm kg^{-1} H_2O). The experimental salinities were achieved by mixing seawater (SW) with dechlorinated tap water (until reaching 5 ‰ and 12 ‰ salinities) or with natural marine salt (Salina de la Tapa, El Puerto de Santa María, Cádiz, Spain) until reaching 55 ‰ salinity. Each tank was maintained in a closed recirculating water system, and a 20 % of the water was replaced every two days. To ensure optimal water conditions water quality criteria (hardness, and levels of O_2, CO_2, H_2S, NO^{2-}, NO^{3-}, NH^{4+},Ca^{2+}, Cl^- and suspended solids) were monitored and no major changes were observed during the experiment or between salinity treatments. Water salinity was checked daily and, when necessary, adjusted to the nominal salinity by regulation of the flux of dechlorinated tap water or SW. Fish were fed a daily ration of 1 % of their body mass with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain). Every morning before feeding, rearing tanks were checked and no food was left. No mortality was observed during the acclimation period. Fish were fasted for 1 day before sampling. After 14 days of

127 acclimation, fish were netted, anesthetized with 2 mL L^{-1} of 2-phenoxyethanol (Sigma-
128 Aldrich, Madrid, Spain), weighed, heads separated from trunks, and organs sampled. The
129 entire pituitary gland and biopsies from the liver of each animal were immediately
130 preserved in 5-10 volumes (w/v) of RNA*later*® (Ambion-LifeTechnologies, Madrid, Spain),
131 kept overnight at 4 ºC and then transferred to -20 ºC until further analysis. All experimental
132 procedures complied with the Guidelines of the European Union (2010/63/UE) and the
133 Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.
134
135 *2.2.* Cloning of *A. regius prl, gh, igf1* and *actb* partial cDNAs
136
137 Gene-specific or degenerate primers for *prl, gh, igf1* and *actb* were designed from
138 published cDNA sequences, especially from perciformes, after performing an initial
139 alignment of mRNA sequences (GenBank accession numbers or NCBI Reference
140 Sequences are shown between parenthesis) using ClustalX2.1. *prl* sequences used were
141 those of *Oreochromis mossambicus* (KC702508), *Acanthopagrus schlegelii* (EU165342),
142 and *Perca flavescens* (AY332491). For *gh*, sequences used were those of *Nibea coibor*
143 (FJ375311), *Pseudosciaena crocea* (AF231941), *Rhabdosargus sarba* (AY553207),
144 *Sparus aurata* (U01301), *Pagrus major* (X06962), *Sciaenops ocellatus* (AF063834), and
145 *Lepomis cyanellus* (AY530822). For *igf1*, the sequences aligned for primer design were
146 those from *S. aurata igf1* (total, EF563837; isoform a, Y996779; isoform b, EF688015;
147 isoform c, EF688016), *S. ocellatus* (total, GU175982; isoform ea2, GQ443298; isoform
148 ea3, GQ443299; isoform ea4, GQ443297), *Umbrina cirrosa* (AY941254), *Larimichthys*
149 *crocea* (JN565945), *Perca fluviatilis* (AJ586908), and *P. flavescens igf1b* (AY332492).

For *actb*, the aligned sequences were from *Danio rerio* (NM 131031), *S. aurata* (X89920), *P. fluviatilis* (EU664997*), P. flavescens* (AY332493), and *L. crocea* (GU584189). Sequences of degenerate and conserved cloning primers are shown in Table 1. All primers used were purified by desalting and purchased from biomers.net (Germany). All kits were used according to manufacturer's instructions; otherwise any modification will be mentioned.

Total RNA was extracted with the NucleoSpin® RNA XS kit and the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany), using single pituitary glands and liver biopsies, respectively. Each organ was homogenized with an IKA® Ultra-Turrax®T8 (IKA-Werke, Staufen im Breisgau, Germany), and including the on-column DNA digestion using the RNase-free DNase provided with the kit. RNA quality was checked in the Bioanalyzer 2100 system with the RNA 6000 Pico kit for pituitary and RNA 6000 Nano kit for liver (Agilent Technologies, Life Sciences, Santa Clara, California). RNA quantity was measured spectrophotometrically at 260 nm with a BioPhotometer Plus (Eppendorf, Hamburg, Germany). cDNA synthesis proceeded with samples with a RNA integrity number (RIN) higher than8, and total RNA concentration higher than 100 ng μL^{-1} for liver or 10 ng μL^{-1} for pituitary.

cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, ThermoFisher Scientific, Madrid, Spain), with ~3 µg of total RNA from liver and ~100 ng from pituitary. 1 U of BIOTAQ™ DNA polymerase (Bioline, Berlin, Germany) was used in each PCR reaction applied for the amplification of the target genes in a total volume of 25 µL. The PCRs were accomplished in a Mastercycler®pro (Eppendorf, Hamburg, Germany). The PCR program is shown in Table 2. Fresh PCR products were directly

cloned into the pCR®4-TOPO cloning vector (Invitrogen, ThermoFisher Scientific, Madrid, Spain) and sequenced in the Unidad de Genómica of the University of Córdoba, Spain. For all putative clones, forward and reverse sequencing were carried out using the dideoxynucleotide chain-termination method with T3 and T7 universal primers.

2.3. Sequence identification and phylogenetic analyses

The obtained sequences were compared to GenBank database using Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast) to confirm their identity with other *prl, gh, igf1, and actb* cDNA sequences available there. Sequences of these genes belonging to various fish species were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/) and aligned using ClustalW integrated into the software Mega 5.0 (Tamura et al. 2011). The results were used for constructing neighbor-joining trees, after determining the best nucleotide substitution model. One thousand (1,000) bootstraps were applied for enhancing the reliability of the test.

2.4. Total RNA extraction and quantitative real time polymerase chain reaction (qPCR)

Total RNA from liver and pituitary was extracted using the same kits mentioned in section 2.2, following manufacturer protocol. RNA concentration and quality were assessed as previously described. All samples had RNA integrity number (RIN) values >8. 250 ng from pituitaries total RNA and 500 ng from liver were separately used for cDNA synthesis using qScript™ cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg,

196 Maryland, USA). Generated cDNAs were stored at –20°C for a period never exceeding
197 one month.

198 All qPCR steps were performed using PerfeCTa™ SYBR®Green FastMix™
199 (Quanta BioSciences, Gaithersburg, Maryland, USA), with cycling conditions detailed in
200 Table 2. The qPCR primers (Table 3) were designed using the software primer3
201 (http://frodo.wi.mit.edu/primer3/) based on the cDNA sequences described above and
202 published in GenBank (http://www.ncbi.nlm.nih.gov/nuccore): *A. regius prl* (KP984534),
203 *gh* (KM402037), shared regions between *igf1_ea2* (KM402035) and *igf1_ea4*
204 (KM402036), unique zone of *igf1_ea4*, and *actb* (KM402038). All qPCR primers were
205 purified by HPLC and purchased from biomers.net (Germany).

206 qPCR reactions (10 µL), composed of 400 pg cDNA template, 5 µL PerfeCTa™
207 SYBR®Green FastMix, and 0.5 µL from each primer, were performed with the Master
208 cycler®ep Realplex^2 operated with Realplex 2.2 software (Eppendorf, Hamburg,
209 Germany). Reactions, ran in triplicate, were incubated at 95 °C for 5 min, followed by 40
210 cycles of 95 °C for 15 s and 60 °C for 1 min. Non-template controls (NTCs) and non-
211 reverse transcribed RNA were used as negative controls in every experiment. A single-
212 peak melting curve was used to check for the absence of primer-dimer artifacts and non-
213 specific amplifications. *actb* was used as the internal reference gene for normalizing
214 mRNA expression data, owing its low C_T variability as we found during the qPCR runs
215 (not exceeding 0.5 C_T differences among different salinities). Relative gene quantification
216 was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

217

218 *2.5. Statistics*

Statistical analyses were performed using one way analysis of variance (ANOVA) and Tukey-HSD Post-Hoc test, after checking the normal distribution of data, using Shapiro-Wilk test, and homogeneity of variance, using Leven´s test, implemented in the program Statgraphics Centurion XVI. Significant values were considered when $P < 0.01$.

3- Results

3.1. Prolactin (prl)

The partial *prl* cDNA fragment from *A. regius* isolated in this study was 498 base pairs (bp) long (Figure 1). The nucleotide sequence showed 91 % sequence identity with *S. aurata prl* (GenBank acc. no.AF060541), 90 % with *R. sarba prl* (GenBank acc. no. DQ202396), and 89 % with *A. schlegelii* (GenBank acc. no. EU165342). This cDNA encoded for 166 amino acids (aa). BLAST comparisons showed that our Prl aa sequence belongs to the growth hormone peptides superfamily, sharing 90 % sequence identity with Prl of *L. crocea* (GenBank acc. no. KKF32453), 89 % with *S. aurata* (GenBank acc. no. CAD52820), and 87 % with *R. sarba* (GenBank acc. no.ABB17072). Phylogenetic analysis showed the clustering of most of perciformes *prl* precursors in a single clade, in which *A. regius prl* precursor was also found (Figure 2). mRNA levels of *prl* presented an inverse relationship with respect to environmental salinity, with the maximum levels at the lowest salinity (5 ‰) and the minimum values in the highest salinity (55 ‰) (Figure 3).

*3.2. Growth hormone (*gh*)*

PCR cloning for *A. regius* growth hormone cDNA resulted in an amplicon of 552 bp (Figure 4). Sequencing resulted in a *gh* precursor whose nucleotide sequence shared 97 % identity with *S. ocellatus gh* (GenBank acc. no. AF065165), 93 % with *Sineperca kneri* (GenBank acc. no. AY155227) and *S. aurata* (GenBank acc. no. U01301), and 90

% with *Epinephelus coioides* (GenBank acc. no. AY038606). This nucleotide sequence encoded for 184 aa, covering most of the *gh* ORF, as noted upon comparing the primary protein sequence with other Gh protein sequences published in the GenBank database. This comparison showed that our Gh protein sequence shared 98 % identity with *N. coibor* (GenBank acc. no. ACI95760), *S. ocellatus* (UniProtKB/Swiss-Prot Q9IB11), *Siniperca chuatsi* (GenBank acc. no. ABM67063), and 97 % with *S. aurata* (GenBank acc. no. AAB19750). Our sequence was missing 8 aa from the C-terminal end and 20 aa from the N-terminus, in comparison to Gh proteins full primary aa sequences in other perciforms.

Phylogenetic analysis for the *gh* nucleotide sequence obtained showed the grouping of all *gh* nucleotide precursors of the family Sciaenidae in a single monophyletic group, forming a sub-clade that is tightly related to the other subclade containing members of the family of Sparidae (Figure 5).

gh mRNA enhanced in the group maintained in 12 ‰ and 55 ‰ salinities in comparison to other groups. Nonetheless, the group maintained in extremely high salinity presented a 3-fold increase in *gh* expression than the one maintained under seawater condition (Figure 6).

3.3. Insulin-like growth factor 1 (igf1)

Cloning of *igf1* cDNA by PCR resulted in two PCR products with different sizes, one was 594 bp long and the other was 679 bp long (Figure 7). Both of them included the full open reading frame (ORF) for the *igf1* and a part of the 3’ and 5’ untranslated areas (UTRs). The shorter transcript shared 100 % sequence identity with the isoform *igf1_ea2*

from other sciaenid fish, *S. ocellatus*, but only 86 % and 83 % with isoforms *igf1_ea3* and *igf1_ea4*, respectively. Hence, we termed this shorter transcript *igf1_ea2*.The longest one showed 100 % nucleotide sequence identity with *S. ocellatus igf1_ea4,* 93 % with *igf1_ea3*, and 87 % with *igf1_ea2.* Therefore, the longest transcript was named *igf1_ea4.* This isoform is found to code for a 186 aa protein, while *igf1_ea2* coded for a 159 aa protein.

On terms of phylogeny, both isoforms, that are always the result of alternative splicing at the same *igf1* precursor, were found to belong to a monophyletic sub-clade including all sciaenid *igf1* genes. The other sub-clade includes perciformes *igf1* precursors (Figure 8).

The expression patterns for total *igf1* and for *igf1_ea4* isoform only were similar, showing their maximum levels in the nearly isosmotic salinity (12 ‰) group, and the minimum in individuals under hyposmotic condition (5 ‰) or seawater (38 ‰) (Figure 9).

3.4. β-actin (actb)

The isolated form of *A. regius actb* was 1,113 bp long (Figure 10), encoding for 371 aa. The nucleotide sequence showed 99 % sequence identity with *L. crocea actb* (GenBank acc. no. GU584189) and *S. ocellatus* (GenBank acc. no. KC795558), 98 % with *P. major* (GenBank acc. no. JN226150), and 98 % with *S. aurata* (GenBank acc. no. AF384096). BLAST comparisons showed the completeness of the ORF from the 5’ end, but it lacked about 4 aa from the N-terminus. Primary protein structure showed 100 % sequence identity with *actb* of *S. aurata* (GenBank acc. no. AEW67142), *S. ocellatus*

294 (GenBank acc. no. AGO64768), and *L. crocea* (GenBank acc. no. ACB98723). As in *prl*,
295 *gh* and both *igf1* isoforms, the cloned *actb* could be located in a sub-clade joining with the
296 large yellow croaker, *L. crocea*, the only sciaenid species other than *A. regius* which have
297 a cloned *actb* sequence (Figure 11).
298

4- Discussion

4.1. Structure and phylogenetic results of the obtained precursors

For the first time in the Mediterranean meagre *A. regius*, partial precursors for *prl, gh, igf1,* and *actb* were cloned and sequenced. To our knowledge, the precursor of *prl* provided in this study is the first to be cloned in the family Sciaenidae in general. Despite being *prl* precursor in the same clade that combined *prl1* form of all vertebrates, including *prl*177 and *prl188* from *Oreochromis niloticus*, that were first described by Specker et al. (1985), we did not isolate any further forms of *prl*. Moreover, the obtained precursor was phylogenetically distant from *prl2* isoform that was previously detected in the brain and the eye, but not in the pituitary, of the non-mammalian vertebrates by Huang et al. (2009). However, *gh* precursor showed much more taxon-specific pattern with *gh* from all representative species belonging to family Sciaenidae placed in a single cluster, Sparidae in the second, Otophysi in the third, and Salmonidae in the fourth. For *igf1*, two forms were identified, *igf1_ea2* and *igf1_ea4*, showing similar patterns to that of *gh*, with all *igf1* forms identified in Sciaenidae belonging to the same clade. In general, there is a phylogenetic ambiguity in what concerns *igf1* genomic copies, although it is commonly accepted that such copies share a high degree of similarity in their coding regions (Moghadam et al. 2007). Alternative splicing for *igf1* precursor was described in different fish and non-fish models, but duplication in the genomic DNA of *igf1* in sciaenids or in other perciformes cannot be clearly judged since the difference in these duplicate copies is mainly distinctive in the 3′ and 5′ UTRs of *igf1* genes, not in the ORFs (Zou et al. 2009).

322 The cloned *actb* precursor clustered in a single clade with its counterparts in other fish
323 species that previously showed the least *actb* expression changes upon different
324 treatments, such like *L. crocea* and *E. coioides* (Zhang et al. 2004; Chen et al. 2015).
325
326 *4.2.* Regulation of hormonal transcripts by different environmental salinities
327
328 For the first time, Mediterranean meagre *A. regius prl, gh, igf1* and *actb* cDNAs
329 could be cloned, sequenced and tested, using quantitative real time PCR, under different
330 environmental salinity regimes. This study aimed directly to pursue the state expression
331 of *prolactin* and main elements in the Gh/Igf1 axis in different environmental salinities to
332 which fish are normally subjected to in their native habitats (hyposmotic, isosmotic, and
333 hyperosmotic) (Cardenas 2010, FAO 2005-2015). This study can aid future works that
334 aim to assess growth and survival of this species under different salinities. Our results
335 indicated a classical inverse response of *prl* to environmental salinity, together with about
336 2 to 4-fold upregulation for *igf1* in the nearly isosmotic salinity in comparison to seawater,
337 which may be in line with the known roles of Igf1 in stimulating salinity tolerance promoting
338 growth (Takei and McCormcik 2013).
339 Molecular endocrinological alterations due to salinity acclimation is a completely
340 dark zone in *A. regius*. However, some clues to the way Prl and the Gh/Igf1 axis response
341 to environmental salinity alterations can be obtained from similar teleost species,
342 especially the ones that succeed under different environmental salinities. *prl* inverse
343 correlation with environmental salinities is well considered as an essential adjustment
344 mechanism in many euryhaline teleost species, including *A. regius* as we found in the

current work. This pattern and the potent roles in inducing proper osmoregulation in response to hyposmotic salinities is very conserved in many fish species, despite being very phylogenetically-diverse (McCormick 2001; Sakamoto and McCormick 2006). *prl* can be regulated by the changes in environmental salinity due to the osmosensitivity of pituitary prolactotrophs (Manzon 2002;; Fuentes et al. 2010. Kültz, 2013). Minute changes in the extracellular osmolality, together with the presence of some autocrine modulator proteins like Prl_{177} and Prl_{188}, directly triggered Prl production in the European eel, *S. aurata*, and the tilapias (Suzuki et al. 1991; Uchida et al. 2004; Mohammed-Geba et al. 2015; Yamaguchi et al. 2016). Moreover, it is noteworthy to mention that both plasma Prl levels and pituitary *prl* expression patterns corresponded in their upregulation in response to hyposmotic salinity in different perciform species, like *O. mossambicus* and *S. aurata* (Riley et al. 2003; Laiz-Carrión et al 2009; Vargas-Chacoff et al. 2009). Hence, the *prl* upregulation we found in *A. regius* under low salinity can directly contribute to the species survival in environments like estuaries and coastal lagoons that are known to be within the native range of life of this species, as mentioned before.

gh and *igf1* expression levels enhanced under isosmotic conditions. Regarding *igf1*, both primer sets, designed for the *igf1_ea4 and igf1_ea2* shared region and for the unique sequence of *igf1_ea4* mRNA, showed a 2 to 4-fold upregulation of *igf1* in the nearly isosmotic salinity than in the normal seawater salinity. On the other hand, *igf1* gene active transcription in relation to growth is known, signifying that the more production of such hormone the better growth the organism exhibits (Wood et al. 2005). This can explain the low energy expenditure and the high growth rates noted for *A. regius* when reared under isosmotic salinity (Muñoz et al. 2008). *igf1* upregulation in nearly isosmotic

368 salinity and a corresponding enhanced growth and metabolism was previously reported
369 in other euryhaline perciformes experiencing isosmotic environments, such like *Sparus*
370 *sarba* and *Mylio macrocephalus* (Woo and Kelly 1995; Deane and Woo 2004, 2005). The
371 abundance of Igf1 receptors in teleost muscles contribute directly to the enhancement of
372 growth by this hormone, both *in vivo* and *in vitro* (Kwasek et al. 2015; Vélez et al. 2016).
373 Therefore, the upregulation in *A. regius igf1* under isosmotic salinity found in the current
374 study, could participate in the growth enhancement noted before in this species reared
375 under similar environments (Muñoz et al. 2008).

376 Interestingly, *gh* expression enhanced under the hyperosmotic environment
377 tested herein (55 ‰), while observed *igf1* increase was not statistically significant in the
378 same environment in comparison to normal seawater control group. This *gh* upregulation
379 agrees with the hyposmotic role of this axis in other teleost species, as mentioned before
380 in the introduction section. Gh stimulates general cell proliferation in gills, including the
381 mitochondria-rich cells (Gonzalez 2012). Despite the role of Gh in promoting acclimation
382 to hypersaline environments is almost constitutive in salmonids, non-salmonid fishes
383 exhibit contradictory Gh responses and under such salinities (Mancera and McCormick
384 1998; Deane and Woo 2009). Our results for *gh* upregulation in the hyperosmotic
385 environment agreed with what was found before in another estuarine teleost, *S. aurata*,
386 that was subjected to the same environmental salinity 55 ‰ (Mohammed-Geba et al.
387 2015). *S. aurata* is known to have very similar life pattern to that of *A. regius,* including
388 the transition between open sea and estuaries. This can suggest then a common role for
389 Gh, in the estuarine perciformes, in aiding acclimation to extremely hyperosmotic

environments, possibly through modifying osmotic adjustments required for fish survival under such environments.

4.3. Conclusions

The Mediterranean meagre *A. regius* proved to be a robust fish species, tolerating a wide range on environmental salinities (5-55 ‰). *A. regius prl* exhibited the classic inverse response to changes in environmental salinity, indicating that in this species Prl performed its conserved hyperosmoregulatory role. *igf1* peaked in the nearly-isosmotic salinity, correspondent with enhanced growth in other estuarine perciformes. *gh* seemed to play a role in the survival of *A. regius* individuals in the extremely hypersaline environment, due to its specific upregulation in such environment, plausibly in order to supply metabolic energy for acclimation under this extreme salinity. Finally, and since the best growth of *A. regius* was noted in the isosmotic salinity, Prl and the Gh/Igf1 axis are likely to participate in such good performance through stimulating fish acclimation to this salinity.

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419

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Legends to Figures

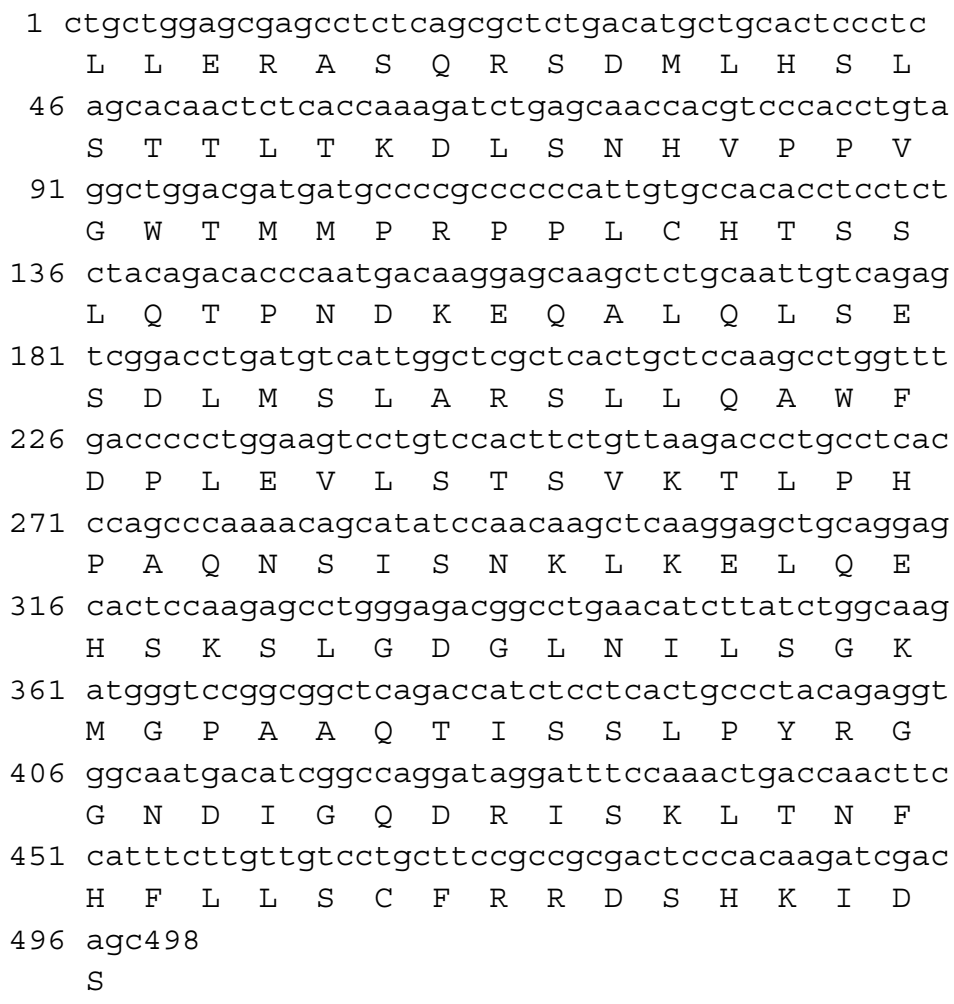


Fig.1. Nucleotide and predicted amino acid sequence of *A. regius prl* precursor.

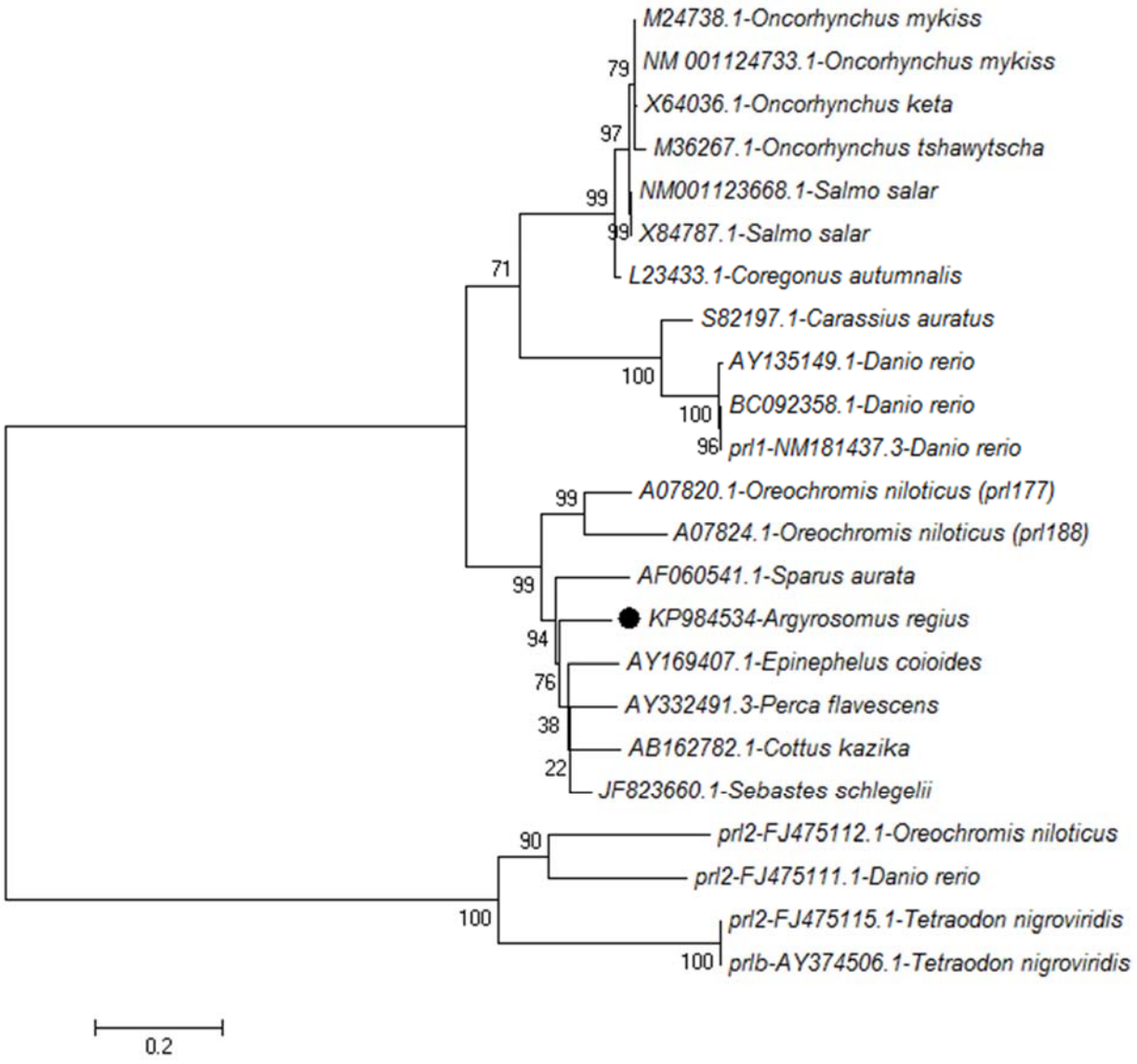
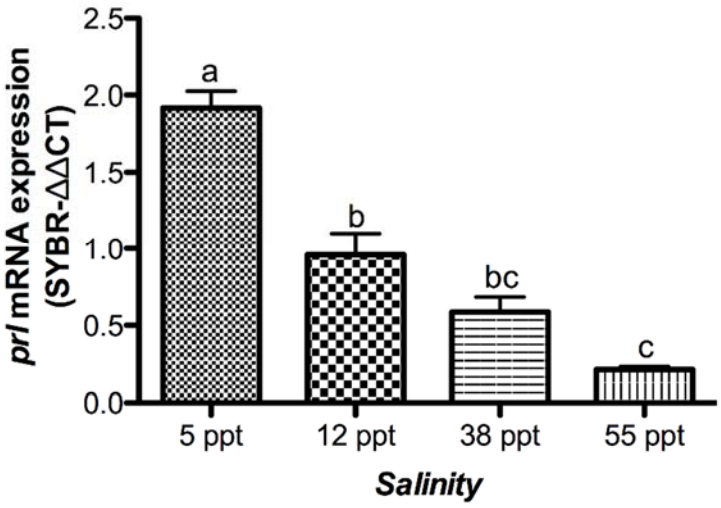


Fig. 2. Neighbor-joining phylogenetic tree of *prl* nucleotide sequences in *A. regius*. 1,000 bootstraps were used to ensure the efficacy of the test. Species and accession numbers are shown in the tree. The position of *A. regius prl* precursor is marked by a black circle.



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603

604 **Fig. 3.** mRNA expression patterns for *prl* in *A. regius* juveniles acclimated to different
605 environmental salinities (5, 12, 38, and 55 ‰) during 21 days. Data are represented as
606 mean ± standard error of the mean (SEM; n = 8). Different letters indicate significant
607 differences among experimental groups (one way ANOVA and Tukey-HSD post-hoc, *P*<
608 *0.01*).
609

```
  1 ctgtcggtggtgtctctgggtgtgtcctctcagccaatcacagac
    L  S  V  V  S  L  G  V  S  S  Q  P  I  T  D
 46 gtccagcgtctgttctccatcgctgtgagcagagttcaacacctc
    V  Q  R  L  F  S  I  A  V  S  R  V  Q  H  L
 91 cacctgctcgctcagagactcttctctgactttgagagctctctg
    H  L  L  A  Q  R  L  F  S  D  F  E  S  S  L
136 cagacggaggaacagcgtcaactcaacaaaatcttcctgcaggat
    Q  T  E  E  Q  R  Q  L  N  K  I  F  L  Q  D
181 ttctgcaactctgattacatcatcagtccgatcgacaagcacgag
    F  C  N  S  D  Y  I  I  S  P  I  D  K  H  E
226 acgcaacgcagctcagttctgaagctgctgtccatctcctatcga
    T  Q  R  S  S  V  L  K  L  L  S  I  S  Y  R
271 ttggtcgagtcttgggagttccccagtcgttctctgtctggaggt
    L  V  E  S  W  E  F  P  S  R  S  L  S  G  G
316 tctgctccaaggaaccagatttcacccaaactttctgagctgaag
    S  A  P  R  N  Q  I  S  P  K  L  S  E  L  K
361 acggggatcctgctgctgatcagggccaatcaggatggagcagaa
    T  G  I  L  LL  I  R  A  N  Q  D  G  A  E
406 atctttcctgatagctccgccctccagctggctccgtatgggaac
    I  F  P  D  S  S  A  L  Q  L  A  P  Y  G  N
451 tattatcaaagtctgagcggcgacgagtcgctgagacgaacctac
    Y  Y  Q  S  L  S  G  D  E  S  L  R  R  T  Y
496 gaactgctcgcctgcttcaagaaagacatgcacaaggtggagacc
    E  L  L  A  C  F  K  K  D  M  H  K  V  E  T
541 tacctgacg549
    Y  L  T
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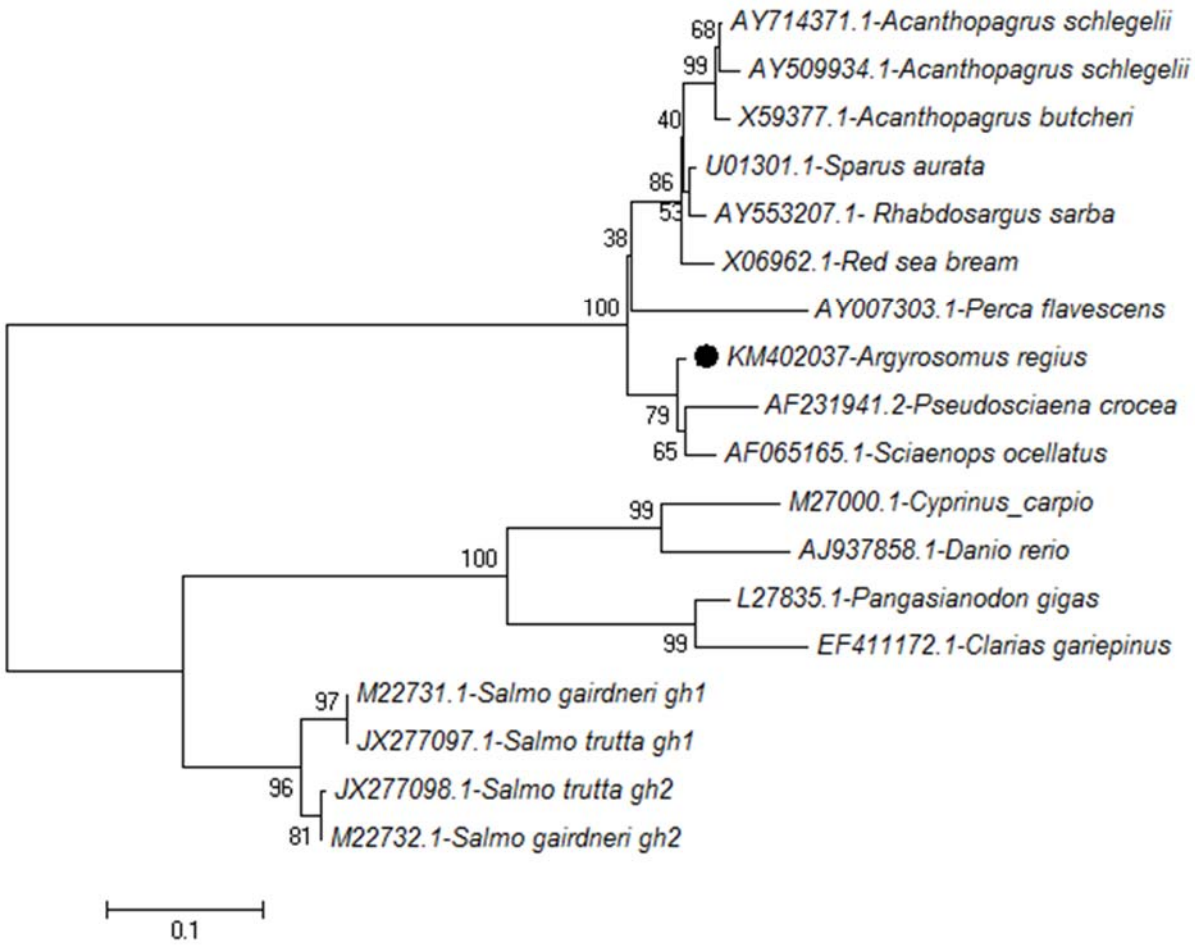
Fig. 4. Nucleotide and predicted amino acid sequence of *A. regius gh* precursor.



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615

616 **Fig. 5.** Neighbor-joining phylogenetic tree of *gh* nucleotide sequences in *A. regius*. 1,000
617 bootstraps were used to ensure the efficacy of the test. Species and accession numbers
618 are shown in the tree. The position of *A. regius* GH precursor is marked by the black
619 circle.

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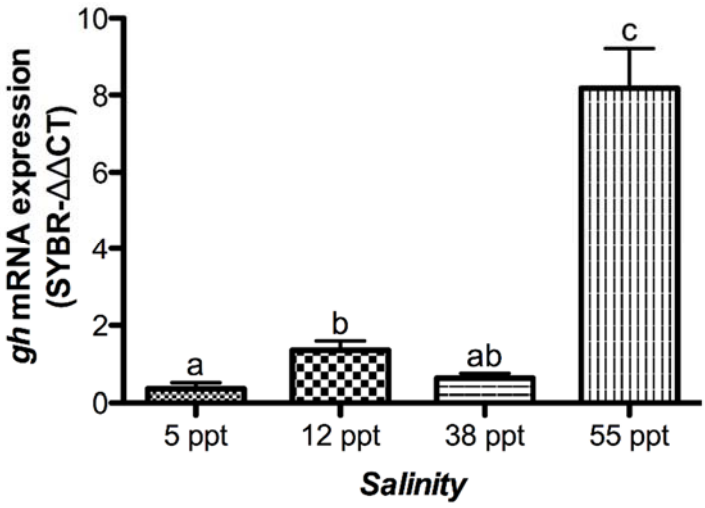

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622

623 **Fig. 6.** mRNA expression patterns for *gh* in *A. regius* juveniles acclimated to different
624 environmental salinities (5, 12, 38, and 55 ‰) during 21 days. Data are represented as
625 mean ± SEM (*n* = 8). Different letters indicate significant differences among experimental
626 groups (one way ANOVA and Tukey-HSD post-hoc, *P< 0.01*).

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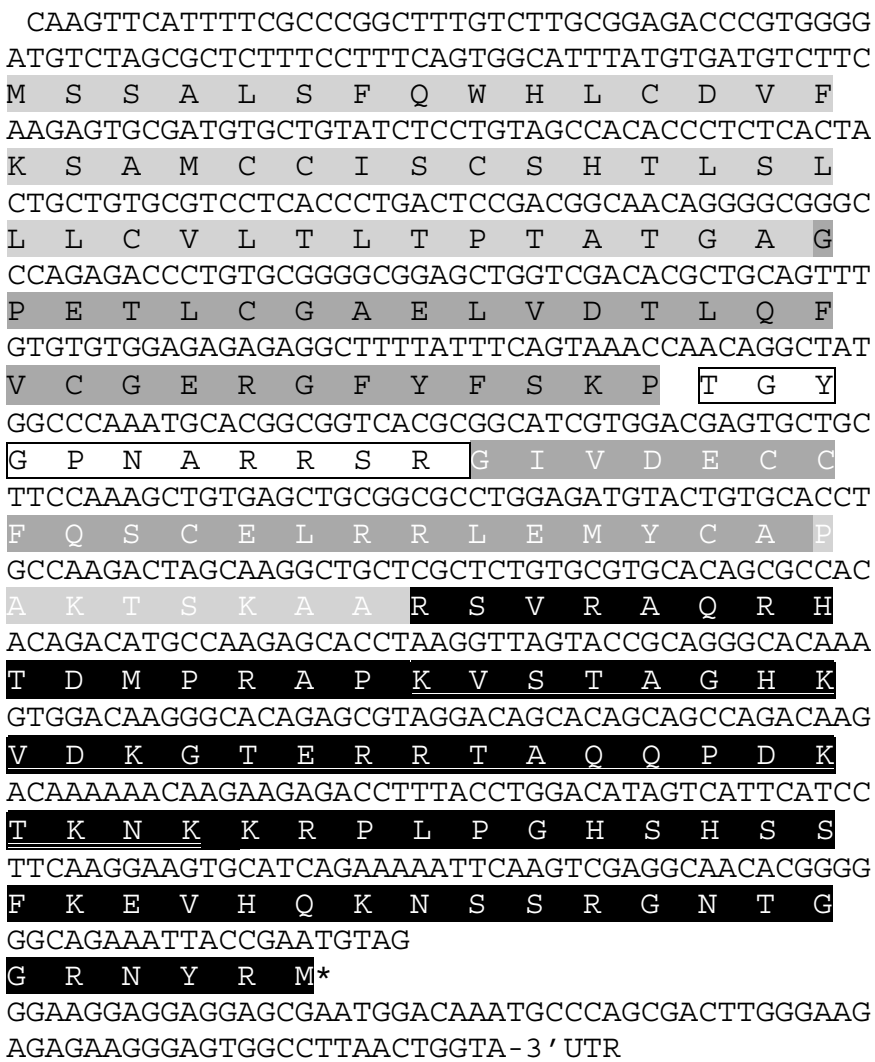

629

630 **Fig. 7.** Nucleotide and predicted amino acid sequence of *A. regius igf1* precursor. As the
631 key adopted by Tiago et al. (2008) for visual description of *S.aurata* Igf1 primary protein
632 structure, Light gray box and black letters indicate the signal peptide; dark gray box and
633 black letters indicate the B domain; white box indicates the A domain; dark gray box and
634 white letters indicate the C domain; light gray box and white letters indicate the D domain;
635 and black box and white letters indicate the E domain. In the E domain, alternatively
636 spliced region present in *igf1_ea4* transcript but absent in *igf1_ea2* is double underlined
637 by white color.

638

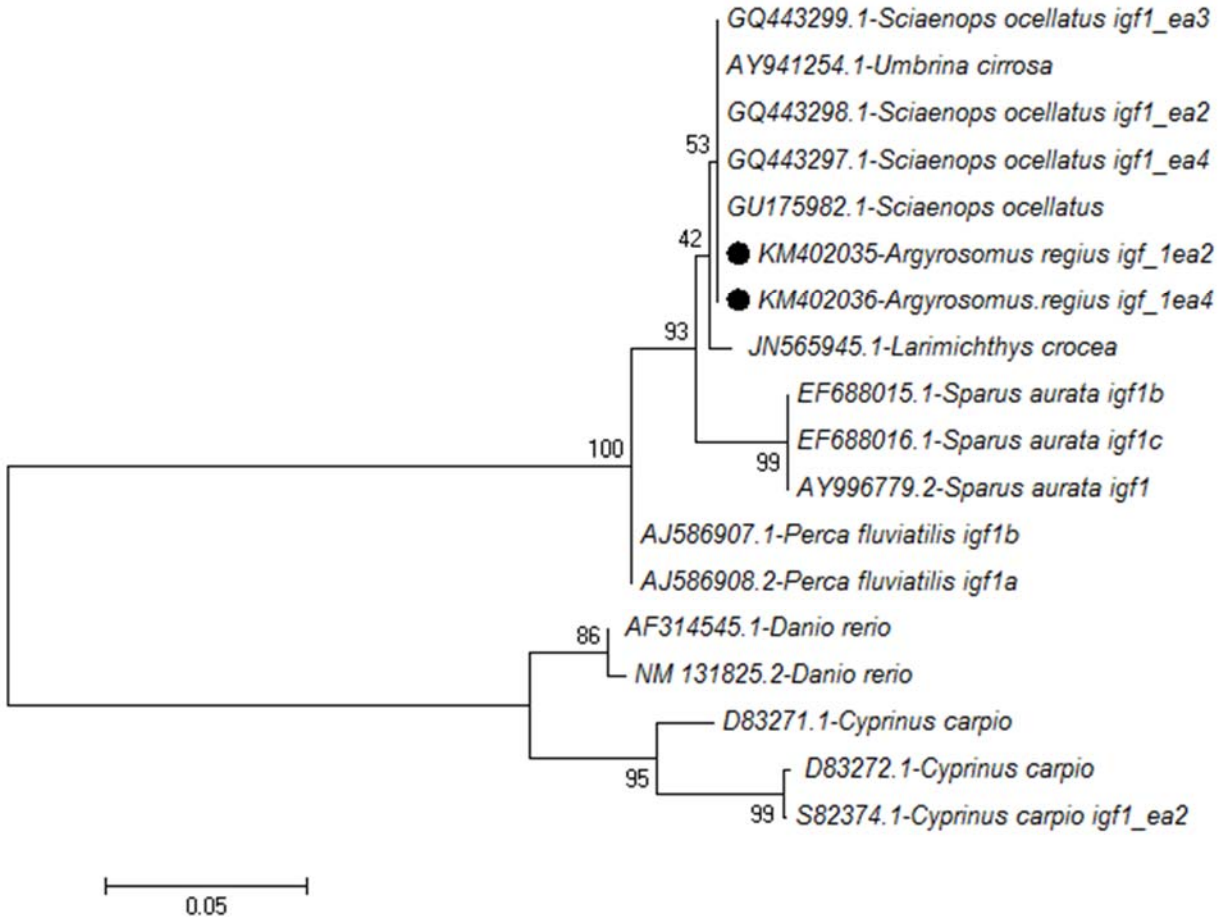

Fig. 8. Neighbor-joining phylogenetic tree of *igf1* nucleotide sequences in *A. regius*. 1,000 bootstraps were used to ensure the efficacy of the test. Species and accession numbers are shown in the tree. The position of *A. regius igf1* precursor is marked by the black circle.

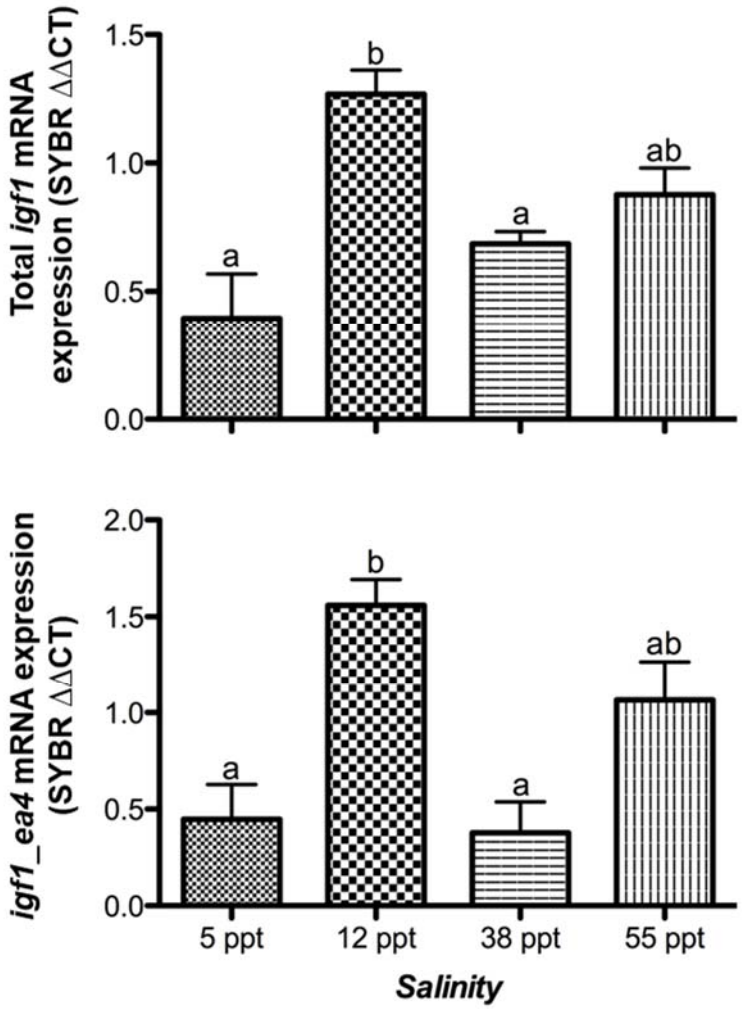

Fig. 9. mRNA expression patterns for total *igf1* transcripts (above, using primers designed from the shared region in both *igf1* isoforms found) and *igf1_ea4* only (using qPCR primers designed from the unique zone in isoform *igf1_ea4*) in *A. regius* juveniles acclimated to different environmental salinities (5, 12, 38, and 55 ‰) during 21 days. Data are represented as mean ± SEM (n = 8). Different letters indicate significant differences among experimental groups (one way ANOVA and Tukey-HSD post-hoc, $P< 0.01$).

```
   1 ATGGATGAGGAAATCGCCGCACTGGTTGTTGACAACGGATCCGGT
     M  D  E  E  I  A  A  L  V  V  D  N  G  S  G
  46 ATGTGCAAAGCCGGATTCGCCGGAGACGACGCCCCTCGTGCTGTC
     M  C  K  A  G  F  A  G  D  D  A  P  R  A  V
  91 TTCCCATCCATCGTCGGTCGCCCCAGGCATCAGGGTGTGATGGTT
     F  P  S  I  V  G  R  P  R  H  Q  G  V  M  V
 136 GGTATGGGCCAGAAGGACAGCTACGTTGGTGATGAAGCCCAGAGC
     G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S
 181 AAGAGAGGTATCCTGACCCTGAAGTACCCCATCGAGCACGGTATT
     K  R  G  I  L  T  L  K  Y  P  I  E  H  G  I
 226 GTGACCAACTGGGATGACATGGAGAAGATCTGGCATCACACCTTC
     V  T  N  W  D  D  M  E  K  I  W  H  H  T  F
 271 TACAACGAGCTCAGAGTTGCCCCTGAGGAGCACCCCGTCCTGCTC
     Y  N  E  L  R  V  A  P  E  E  H  P  V  L  L
 316 ACAGAGGCCCCCCTGAACCCCAAAGCCAACAGGGAGAAGATGACC
     T  E  A  P  L  N  P  K  A  N  R  E  K  M  T
 361 CAGATCATGTTCGAGACCTTCAACACCCCCGCCATGTACGTTGCC
     Q  I  M  F  E  T  F  N  T  P  A  M  Y  V  A
 406 ATCCAGGCTGTGCTGTCCCTGTATGCCTCTGGTCGTACCACTGGT
     I  Q  A  V  L  S  L  Y  A  S  G  R  T  T  G
 451 ATCGTCATGGACTCCGGTGATGGTGTGACCCACACAGTGCCCATC
     I  V  M  D  S  G  D  G  V  T  H  T  V  P  I
 496 TACGAGGGTTACGCCCTGCCCCACGCCATCCTGCGTCTGGACTTG
     Y  E  G  Y  A  L  P  H  A  I  L  R  L  D  L
 541 GCCGGCCGCGACCTCACAGACTACCTCATGAAGATCCTGACAGAG
     A  G  R  D  L  T  D  Y  L  M  K  I  L  T  E
 586 CGTGGCTACTCCTTCACCACCACAGCCGAGAGGGAAATCGTGCGT
     R  G  Y  S  F  T  TT  A  E  R  E  I  V  R
 631 GACATCAAGGAGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAG
     D  I  K  E  K  L  C  Y  V  A  L  D  F  E  Q
 676 GAGATGGGCACTGCTGCCTCCTCCTCCTCCCTGGAGAAGAGCTAT
     E  M  G  T  A  A  S  SSS  L  E  K  S  Y
 721 GAGCTGCCCGACGGACAGGTCATCACCATCGGCAATGAGAGGTTC
     E  L  P  D  G  Q  V  I  T  I  G  N  E  R  F
 766 CGTTGCCCAGAGGCCCTCTTCCAGCCTTCCTTCCTCGGTATGGAA
     R  C  P  E  A  L  F  Q  P  S  F  L  G  M  E
 811 TCTTGCGGAATCCACGAGACCACCTACAACAGCATCATGAAGTGC
     S  C  G  I  H  E  T  T  Y  N  S  I  M  K  C
 856 GACGTCGACATCCGTAAGGACCTGTACGCCAACACCGTGCTGTCT
     D  V  D  I  R  K  D  L  Y  A  N  T  V  L  S
 901 GGAGGTACCACCATGTACCCCGGCATCGCTGACAGGATGCAGAAG
     G  G  T  T  M  Y  P  G  I  A  D  R  M  Q  K
 946 GAGATCACAGCCCTGGCCCCATCCACCATGAAGATCAAGATCATT
     E  I  T  A  L  A  P  S  T  M  K  I  K  I  I
 991 GCCCCACCTGAGCGTAAATACTCTGTCTGGATCGGAGGCTCCATC
     A  P  P  E  R  K  Y  S  V  W  I  G  G  S  I
1036 CTGGCCTCTCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAG
     L  A  S  L  S  T  F  Q  Q  M  W  I  S  K  Q
1081 GAGTACGATGAGTCCGGCCCCTCCATCGTTCAC 1113
     E  Y  D  E  S  G  P  S  I  V  H
```

Fig. 10. Nucleotide and predicted amino acid sequence of *A. regius actb* precursor.

657

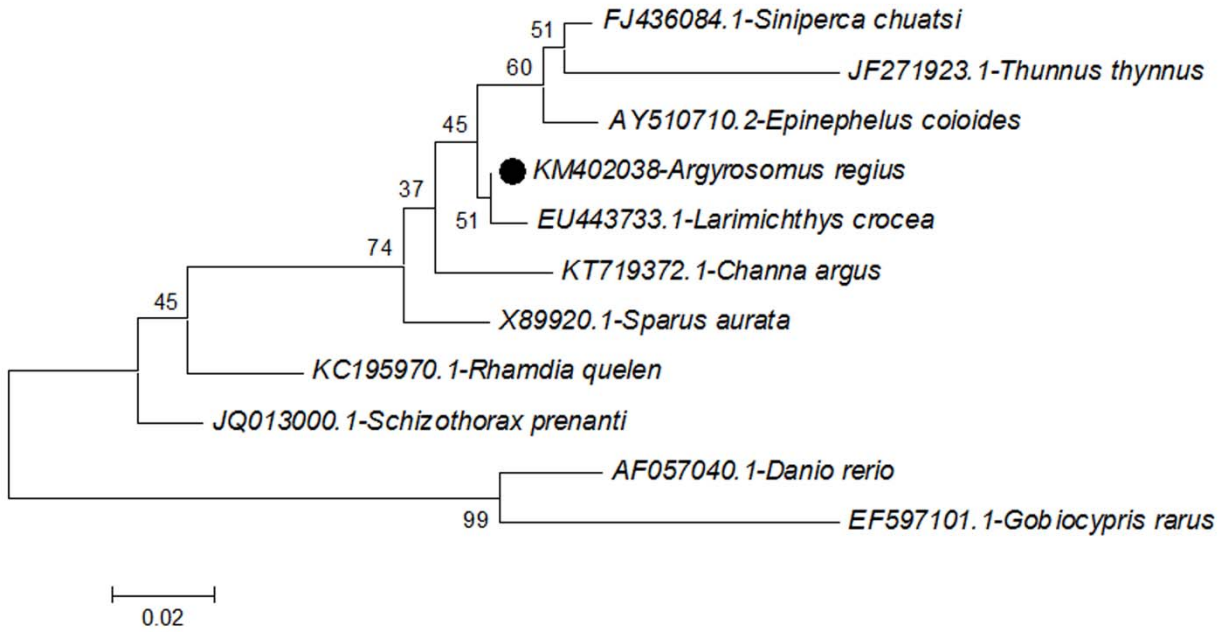

Fig. 11. Neighbor-joining phylogenetic tree of *actb* nucleotide sequences in *A. regius*. 1,000 bootstraps were used to ensure the efficacy of the test. Species and accession numbers are shown in the tree. The position of *A. regius actb* precursor is marked by the black circle.

665 ***Tables***

666

667 **Table 1:** Sequences of primers used for cloning of the open reading frames (ORFs) of *A.*
668 *regius prl*, *gh, igf1,* and *actb.*

669

Gene	Orientation	Sequences (5'→3')	Location in *A. regius*
prl	Sense	GCTGTCGGTGVTGWCTYTGG	1
	Antisense	CCGTCAGGTAGGTCTCCACC	552
gh	Sense	CTGCTBGABCGAGCCTCTCAG	1
	Antisense	GCTGTCRATCTTGTGVGAGTC	552
igf1	Sense	CAAGTTCATTTTCGCCGGGC	1
	Antisense	TACCAGKTAAGGCCACTCCC	679
actb	Sense	ATGGAWGABGAAATCGC	1
	Antisense	GTGRACGATGGAKGGGCC	1113

670

671

672 **Table 2**. PCR steps used for (1) cloning of *prl*, *gh, igf1* and *actb* intermediate fragments
673 and (2) qPCR amplifications.
674

		(1)	
Step		***Temperature***	***Time***
Initial denaturation		95 ºC	10 min
40 cycles	**Denaturation**	95 ºC	30 s
	Annealing	55 ºC	30 s
	Extension	72 ºC	60 s
Final Extension		72 ºC	10 min
		(2)	
Initial denaturation		95 ºC	10 min
40 cycles	**Denaturation**	95 ºC	15 s
	Annealing & Extension	60 ºC	30 s

675
676

677 **Table 3**. Orientation, sequences, positions, GenBank accession numbers of the genes cloned and used, amplicon sizes,
678 amplification efficiencies and regression coefficients of the primers used for qPCR amplification.
679

Primers	Orientation	Sequence 5'→3'	Position	Acc. no.	Amplicon Size (bp)	E	r^2
prl	Sense Antisense	TGGTTTGACCCCCTGGAA GAGTGCTCCTGCAGCTCCTT	220-320	KP984534	101	0.96	0.996
gh	Sense Antisense	GGAGTTCCCCAGTCGTTCTC CCCTGATCAGCAGCAGGAT	287-387	KM402037	101	0.99	0.99
igf1 TOTAL (igf1_ea2, igf1_ea4)	Sense Antisense	CCTTCAAGGAAGTGCATCAGA CATTTGTCCATTCGCTCCTC	457-556	KM402035 KM402036	100	1	0.99
igf1_ea4	Sense Antisense	GTTAGTACCGCAGGGCACAA CGTGTTGCCTCGACTTGAAT	430-582	KM402036	153	0.99	0.99
actb	Sense Antisense	ATCCACCATGAAGATCAAGA GCTGGAAGGTGGACAGAGAG	966-1061	KM402038	108	1	0.99

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