Insulin-like growth factor I (IGF-1) regulates prolactin, growth hormone and IGF-1 receptor expression in the pituitary gland of the gilthead sea bream *Sparus aurata*

Khaled Mohammed-Geba^{1,2,3*}, J.A. Martos-Sitcha^{1,2}, A. Galal-Khallaf^{1,2,3}, J.M. Mancera², G. Martínez-Rodríguez^{1a}

(1) Departamento de Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucía, Consejo Superior Investigaciones Científicas (ICMAN-CSIC), E-11510 Puerto Real (Cádiz), Spain.

(2) Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Campus de Excelencia Internacional del Mar (CEIMAR), Universidad de Cádiz, E-11510 Puerto Real (Cádiz), Spain.

(3) Genetic Engineering and Molecular Biology Division, Department of Zoology, Faculty of Sciences, Menoufia University, Shebin El- Kom, Menoufia, Egypt.

*Address for correspondence: Dr. Khaled Mohammed-Geba, Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas (CSIC), E-11510 Puerto Real (Cádiz), Spain. Tel.: +34 956 832612, fax : +34 956 834701, e-mail: khaled.mohammed@icman.csic.es

(a) ORCID: 0000-0003-3379-580X

Abstract

The role of insulin-like growth factor 1 (IGF-1) on regulation of growth hormone (GH) and prolactin (PRL) as well as the possible involvement of IGF-1 receptor subtype a (IGF-1Ra) mRNA was assessed in juvenile specimens of *Sparus aurata*. IGF-1Ra was successfully cloned and active receptor domains were localized in its mRNA precursor. Also, phylogenetic analysis of the protein sequence indicated a closer proximity to IGF-1Ra isoform found in zebrafish and other teleosts, than to the isoform IGF-1Rb. The most abundant presence of IGF-1Ra mRNA was detected in white muscle, whereas head kidney showed the lowest gene expression among 24 different studied tissues. Pituitaries of juvenile specimens of *S. aurata* were incubated *in vitro* with different doses of IGF-1 (0, 1, 100, and 1000 ng mL⁻¹) during a period of 10 hours. Total RNA with a high-quality could be obtained from these pituitaries. PRL mRNA expression significantly increased with increasing IGF-1 doses. Similarly, IGF-1Ra mRNA increased its expression in response to IGF-1. However, GH mRNA levels decreased in a dose-dependent manner after IGF-1 treatment. The contradictory responses of GH and PRL expressions to IGF-1 in our experiment are possibly mediated by IGF-1Ra presence on the somatotrophs and prolactotrophs. The increase in IGF-1Ra mRNA levels may be related to the proper activation of the PI3 K/Akt signal transduction pathways which are normally involved in GH and PRL regulation.

Key words: GH, *in vitro*, IGF-1, IGF-1Ra, pituitary, PRL.

1. Introduction

In teleosts, similarly to other vertebrates, the key controller of somatic growth is the growth hormone (Gh)/Insulin-like growth factor 1 (IGF-1) axis (Le Roith et al., 2001). The classical model of work of this axis includes: i) Gh secretion from the pituitary gland, ii) binding to Gh receptors in liver and other body organs, and iii) hepatic, chiefly, production of IGF-1 and stimulation of body growth through the Gh and IGF-1 receptors distributed all over the animal body (Le Roith et al., 2001; Sakamoto and McCormick, 2006). Two types of Insulin-like growth factors receptors (IGFRs) have been characterized in vertebrates: i) the tyrosine-kinase type, IGF-R1, and ii) the mannose-6-phosphate IGF-R2. Tyrosine kinase receptors binding by IGF-1 induces a conformational change and autophosphorylation of IGF-1R. This allows the receptors to modulate intracellular processes via phosphorylation of specific proteins (Dantzer and Swanson, 2012). In fish, IGF-1R subtypes a and b were characterized in zebrafish, common carp, salmonids, Japanese flounder and few other teleosts (Maures et al., 2002; Reindl and Sheridan, 2012).

The role of IGF-1 signaling systems, including IGF-1 receptors and binding proteins, in the growth of mammals and fishes is generally accepted. However, the differential roles of its components and their interactions with other systems in the body are still to be explored more (Dai et al., 2015). The difference in the growth patterns and the components of the IGF-1 signaling system themselves between mammals and fishes point to more variable functions of the system between these two animal taxa. Fishes, for example, grow continuously, possess more copies of IGF-1Rs and binding proteins, and lack the activity of the mannose-6-phosphate/IGF-2 receptor in the adult stages (Maures et al., 2002). From the few studies conducted on the biological roles of IGF-1Rs in teleosts, it is known that their interaction with IGF-1 aid embryonic development, osmoregulation, muscular cells growth and differentiation, and gonadal differentiation and maturation (Reinecke et al., 2005; Xu et al., 2014; Mei et al., 2015). The existence of IGF-1R has been demonstrated in adenohypophyseal GH and prolactin (PRL) producing cells of hybrid striped bass (*Morone saxatilis* X *M. chrysops*) (Fruchtman et al., 2002a,b), supporting the GH down-regulation and PRL up-regulation reported after IGF-1 treatment in this hybrid, as well as in *Oreochromis mossambicus* (Fruchtman et al., 2002a,b; Kajimura et al., 2002).

Hence, we believe that more studies are necessary in each fish species to reveal these components and their specific roles in relation to specific biological processes. Also, out of salmonids and cyprinids, very few fish orders were subjected to the effort of investigating the receptors and binding proteins of IGF-1, so more light need to be shed on this system and its diverse roles on higher classes of bony fishes. Gilthead sea bream (*Sparus aurata*) is the targeted fish model by this study. It is an economically important, euryhaline species that is

being used in our research group as a model organism for osmoregulatory studies. Acclimation to different environmental salinities induced several osmoregulatory, endocrine and metabolic adjustments (Mancera et al. 1993; Mancera et al., 2002; Laiz-Carrión et al. 2005a,b, 2009; Vargas-Chacoff et al., 2009a,b, Mohammed-Geba et al., 2015). We aimed with this work to clone the tyrosine-kinase receptor of the IGF-1 and to study the transcriptional responses of this receptor and of major hormonal transcripts in pituitary, these of GH and the PRL, that represent key hormonal controllers of fish osmoregulatory systems in general (Mancera and McCormick, 2007) and *S. aurata* in particular (e.g., Pérez-Sánchez, et al., 1994; Mancera et al., 2002), to gradually increasing concentrations of IGF-1. Very few studies were carried out reporting this regulation especially at the molecular level of IGF-1, mainly Eppler et al., 2007; Fruchtman et al. 2002a,b; and Mohammed-Geba et al., 2015.

2. Materials and methods

2.1. Animals

Juvenile specimens of *S. aurata* ($n = 40$, 120 ± 10 g body mass) were provided by Servicio Central de Investigación de Cultivos Marinos, SCI-CM (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) where they were kept in four 400-L tanks containing seawater (38 ‰ salinity, 1,049 mOsm kg⁻¹ H₂O osmolality, 21-22 °C temperature, 3 kg m⁻³ density) in a flow through water system for 10 days. 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. The fishes were fasted for 1 day before sacrificing them. All experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

2.2. Chemicals

Pre-incubation and incubation media were prepared for *in vitro* experimentation with *S. aurata* pituitary glands according to Fuentes et al. (2010). All reagents were prepared one day before the experiment and continuously aerated with 95 % O₂-5 % CO₂ just before starting fish samples dissection. A stock solution of Hepes-buffered Krebs's Ringer bicarbonate buffer (KRB) served as a pre-incubation medium, consisting of 145 mmol NaCl, 5.0 mmol NaHCO₃, 3.7 mmol KCl, 1.2 mmol KH₂PO₄, 1.8 mmol CaCl₂, 1.2 mmol MgSO₄, 10 mmol HEPES (pH 7.8) and 5 mmol glucose. The solution was sterilized by filtration through a 0.22 µm filter. The mix was gased with 95 % O₂-5 % CO₂, and the pH was adjusted whenever necessary by either 10 N NaOH or HCl at 7.8. An incubation solution was also prepared, adding to the Hepes-buffered KRB 10 µL mL⁻¹ of vitamins (MEM Vitamin Solution 100x, Sigma-Aldrich, M6895), 20 µL mL⁻¹ essential amino acids (MEM Amino Acids Solution 50x, Sigma-Aldrich, M5550), 10 µL mL⁻¹ nonessential amino acids (100x, Gibco Life Technologies, 11140-035), 20 µL mL⁻¹ L-glutamine (200 mM, Gibco Life Technologies, 25030-024), 10 µL mL⁻¹ antibiotics solution (penicillin-streptomycin, Gibco Life Technologies, 15140-122) and Fungizone (Gibco Life Technologies, 15290-026). Osmolality of both pre-incubation and incubation media (375 ± 10 mOsm kg⁻¹) was adjusted by modifying the Na⁺ and Cl⁻ content and measuring the osmolality in 10 µL of the medium with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA), according to plasma osmolality values reported previously for unstressed *S. aurata* SW-acclimated specimens of similar size (Laiz-Carrión et al., 2005a).

2.3. Experimental protocol

Juvenile specimens of *Sparus aurata* ($n = 40$) were anesthetized (2 mL L⁻¹ of 2-phenoxyethanol, Sigma-Aldrich), decapitated and hypophysectomized. The pituitaries were

immediately rinsed in 60 μ L of the freshly prepared pre-incubation medium to clean out blood, then directly transferred to other new 60 μ L of pre-incubation medium at 21 °C in a sterile, low-coating capacity, cell culture-specific 96 well-plate (Costar, USA) for 2 hours. Serial dilutions were also prepared, using the incubation medium, from *rhIGF-1* (recombinant human IGF-1, Sigma-Aldrich, Cat. no. I3769): 1000 ng mL⁻¹, 100 ng mL⁻¹, and 10 ng mL⁻¹. The pre-incubation medium was then replaced by the incubation solution containing 6 μ L of each IGF-1 dose (so as to reach a final in-well concentrations of 100, 10, 1 ng mL⁻¹), or without IGF-1 for the control (0 ng) group. Following Fuentes et al. (2010), the incubation media was completely changed every 2 hours for a period of 10 hours. In a preliminary incubation trial with a subsequent RNA extraction we showed that this setup and timing was correct to assure the best incubation period with the highest RNA integrity.

2.4. Cloning of *Sparus aurata* IGF-1Ra

2.4.1. Cloning of IGF-1Ra partial sequence

All kits were used according to manufacturers' instructions. Total RNA was extracted from the pituitary gland of a juvenile *S. aurata* with the NucleoSpin® RNA XS kit (Macherey-Nagel). RNA quality was checked in the Bioanalyzer 2100 system (Agilent Technologies, Life Sciences). A RIN (RNA Integrity Number) value >8 and RNA quantity >10 ng μ L⁻¹ were considered acceptable conditions for continuing to synthesize the cDNA. cDNA was synthesized with the SuperScript™ III Reverse Transcriptase (Invitrogen, LifeTechnologies), using a final quantity of 100 ng of total RNA per reaction.

Primers for IGF-1Ra (Table 1) were designed from the most conserved blocks in the alignments performed using published sequences in other teleost fish, mainly *Epinephelus coioides* (AY772254.1), *Oreochromis mossambicus* (AF493795.1), *Paralichthys olivaceus* (AB065098.1) and *Scophthalmus maximus* (AJ224993.1). For amplifying the target gene fragment by PCR, 2 U of BIOTAQ™ DNA polymerase (Bioline) were used in each reaction, applying an annealing temperature of 60 °C and extension step for 2 min in each cycle. Fresh PCR product was directly cloned into the pCR®4-TOPO cloning vector (Invitrogen, Life Technologies) and sequenced in the *Unidad de Genómica*, University of Córdoba. The resulting sequences were analyzed using BLAST (Basic Local Alignment Sequence Tool, www.ncbi.nlm.nih.gov/blast) to affirm their identity with other IGF-1Ra nucleotide sequences.

2.4.2. Screening of a brain cDNA library

In order to isolate the full length IGF-1Ra cDNA, a gilthead sea bream brain and pituitary cDNA library constructed in lambda ZAP (Stratagene, Agilent Technologies Life Sciences) (Martos-Sitcha et al., 2013), was screened, using the partial cDNA fragment obtained for IGF-1Ra as a probe. Positive clones with adequate sizes were sequenced in a sequencing service (*Unidad de Genómica*, University of Córdoba, Spain). All procedures carried out in this protocol were as previously described by Mohammed-Geba et al. (2015).

2.4.3. IGF-1Ra cloning by 5' RACE

About 900 base pairs were missing from the 5' end of the precursor obtained by screening the cDNA library, according to BLAST comparisons. Hence, we proceeded to complete the mRNA sequence by 5' Rapid Amplification of cDNA Ends (RACE), using the 5' RACE kit, Version 2.0 from Invitrogen (LifeTechnologies). All gene-specific primers used were derived from the 5'-end of the partial IGF-1Ra fragment previously obtained (Table 1). Two rounds of PCR, with a nested reaction using the first PCR product as template, were run with the same conditions described above. The PCR product was directly cloned into the pCR®4-TOPO vector (Invitrogen, LifeTechnologies), sequenced and analyzed as above in section 2.4.1.

2.4.4. Assembly and phylogenetic analysis of IGF-1Ra

After having the 5'-RACE product and the expression library IGF-1Ra clone, the sequences were assembled according to the 100 % identities between their overlapping ends using the algorithm *merger* (<http://pro.genomics.purdue.edu/emboss/>). The putative protein sequence was compared using BLAST algorithm in GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi#63169284>). Sequences for IGF-1R in different vertebrate classes were retrieved from GenBank, aligned together using MEGA 6 (Tamura et al., 2013), and a Neighbor-Joining tree was drawn to exhibit the relation between our sequence and other IGF-1R receptor sequences.

2.5. Optimizing qPCR primers for IGF-1Ra

IGF-1Ra qPCR primers (Table 2) were designed using the software primer3 (<http://frodo.wi.mit.edu/primer3/>) based on the full cDNA sequences published in GenBank (<http://www.ncbi.nlm.nih.gov/nuccore>) for *S. aurata* IGF-1Ra (KJ591052). Total RNA (500 ng) was reverse-transcribed in a 20-μL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences). To optimize the qPCR conditions, several primer concentrations (100-500 nM) and a temperature gradient (from 55 to 60 °C) were used. Different cDNA template concentrations estimated from total input of RNA were applied in triplicates (in serial 1/10 dilutions from 10 ng/reaction to 1 pg/reaction, as 10 ng, 1 ng, 100 pg, 10 pg and 1 pg of input RNA) to assess the assay linearity and amplification efficiency (Table 2). For IGF-1Ra and all other genes under the coverage of this study, all qPCR optimization trials and steps were performed using PerfeCTa™ SYBR®Green FastMix™ (Quanta BioSciences), in the PCR cycler Mastercycler®ep Realplex² (Eppendorf) operated with Realplex 2.2 software (Eppendorf). Finally, although the assay was linear in the tested range of concentrations, 1 ng of total RNA from each sample was used for qPCR estimation.

209 *2.6. Tissue distribution of IGF-1Ra*

210 Total RNA was purified from brain, eye, pituitary gland, skin, operculum, gills, heart,
211 white muscles, gas bladder, perivisceral fat, testis, ovary, mouth epithelium, oesophagus,
212 stomach, anterior intestine, middle intestine, pyloric caeca, spleen, liver, gall bladder, rectum,
213 head kidney and distal kidney (n = 3/tissue) as previously described by Martos-Sitcha et al.
214 (2014). 50 ng of the total RNA were reverse transcribed as above, and 10 ng of mRNA from
215 each tissue were used for qPCR estimation of IGF-1Ra distribution using the $\Delta\Delta C_T$ method
216 (Livak and Schmittgen, 2001) using β -actin (acc. no. X89920.1) as an internal reference gene.

217
218 *2.7. Total RNA extraction and quantitative reverse transcription polymerase chain reaction*
219 *(qPCR)*

220 Specific primers used for use in qPCR are shown in Table 2. Total RNA extraction, RNA
221 quality and quantity, as well as reverse transcription reaction and relative gene quantification by
222 qPCR were all performed as mentioned above.

223
224 *2.8. Statistics*

225 Statistical analyses were performed using one way analysis of variance (ANOVA),
226 followed by LSD Post-Hoc test. Significance was defined at $P < 0.01$.

3- Results

3.1. Cloning, sequencing and gene analysis of *Sparus aurata* IGF-1Ra sequence

The cDNA from IGF-1Ra was submitted to GenBank with the accession number KJ591052. It consists of 4,718 nucleotides encoding a 1,419 amino acid protein (Figure 1) covering the entire open reading frame (ORF) for the IGF-1Ra. The short 5'-UTR contains only 99 nucleotides, while the 3'-UTR encompasses 368 nucleotides. However, neither a putative polyadenylation signal nor a poly-A tail could be found, what may indicate the incompleteness of the cloned 3'-UTR of the IGF-1Ra. The protein showed 87 % - 62 % sequence identity with other published protein sequences for IGF-1Ra in different vertebrate classes. IGF-1R sequence analysis was carried out using the model from Ullrich et al. (1986) for human IGF-1R and several algorithms and databases like CDD (Marchler-Bauer et al., 2011), SignalP 4.0 (Peterson et al., 2011), and the NetNGlyc 1.0 (Figure 1). The primary structure of IGF-1R showed a 35 amino acid signal peptide rich in polar amino acids, especially serine and threonine (22 %). The mode of signal peptide separation from the mature peptide seems to be the same as in human IGF-1R since both have the last glycine residue, on which the signal peptidase cleaves, to expose the terminal IGF-1R α subunit glutamic acid residue. Sixteen putative N-Glycosylation locations could be localized in the sequence, all belong the motif *Asn-Xaa-Ser/Thr*. The tetrapeptide Arg⁷³⁷- Arg⁷³⁸- Arg⁷³⁹- Arg⁷⁴⁰ was found and it is the cleavage site for the α/β proreceptor peptide, hence the β subunit begins in the Asp⁷⁴¹ position. The α subunit is characterized by comparatively higher number of glycosylation sites (9) and cysteine, with the latter more concentrated between Leu¹⁵⁹ and Tyr³⁵⁶. The β subunit is characterized by a zone of 24 hydrophobic amino acids that form together the transmembrane domain. A stretch of basic amino acids flanking the carboxy terminus of that hydrophobic zone seems to be conserved at different evolutionary levels since they are found in both human and *Sparus aurata* IGF-1Ra. In the latter case, these are represented by Lys⁹⁶²-Lys⁹⁶³-Arg⁹⁶⁴, characterizing the motif that facilitates the attachment of the β subunit to the cell membrane. The cytoplasmic domain of the β subunit, downstream to the hydrophobic residues zone, contains 5 N-glycosylation sites. The tyrosine kinase features are located in the cytoplasmic domain of the β subunit. The catalytic domain of Insulin Receptor-like Protein Tyrosine Kinases was found between Trp⁹⁹⁴-Asp¹²⁶⁸. Binding of the ligand, IGF-1 in this case, to its extracellular α subunit activates the transmembrane part of this domain, leading then to the autophosphorylation, and downstream kinase activities which initiate signaling cascades and biological functions (Lammers et al., 1989). The extracellular receptor L domains, responsible for ligand binding, were located between Asn⁵⁵-Met⁷¹ and Cys³⁵⁴-Met⁴⁶⁹.

The phylogenetic tree constructed using Neighbor-joining method showed a close proximity of the obtained sequence with IGF-1R of the flounders *Paralichthys olivaceus* and *P.*

adspersus. All of them belonged to the clade containing IGF-1Ra of the zebrafish, in contrast to other *P. olivaceus* and *P. adspersus* IGF-1R sequences which are closer to zebrafish IGF-1Rb (Figure 2).

3.2. Tissue expression of IGF-1Ra mRNA

Figure 3 shows IGF-1Ra tissue distribution. Its mRNA was ubiquitously expressed in all the tissues examined, being mainly found in the white muscles, followed by the pituitary gland and heart. After these, come in a descending order of IGF-1Ra mRNA levels: heart, gall bladder, brain, oesophagus, gills, gas bladder, skin, distal kidney, mouth pit, eye, perivisceral fat, operculum, testis, liver, stomach, ovary, rectum, middle intestine, spleen, anterior intestine and pyloric caeca. All the expression found in these organs were statistically non-significantly different. Finally, the organ that showed the least values of receptor expression was head kidney, which differed significantly from all other organs.

3.3. Expression patterns of target genes

Incubation of *S. aurata* full pituitary glands for 10 hours resulted in high total RNA quality (RIN>8) in all tested samples. IGF-1Ra expression showed a dose-dependent increase, with the maximum levels of its mRNA found at 100 ng mL⁻¹ IGF-1 dose, and the minimum in the control (0 ng) group (Fig. 4A). Similarly, PRL expression revealed a increased gradually with raising the IGF-1 dose, reaching significant maximum with the highest IGF-1 dose (Fig. 4B). In addition, GH decreased when IGF-1 doses increased, getting a strongly significantly (P<0.001) drop in response to the 100 ng mL⁻¹ IGF-1 dose (Fig. 4C).

290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320

```

1  GCTTTAATTTTATTTCTATTTTCGTCCAGCCTGTTTTCTTTCTCTTTTGGATTATTATCTTTTTTTTTTTTTTTTTT
91  ATGTTAAGGAACATGAGATCTGACACACTGATGGGTAGCCGACCTGTTTTGGGGTCTGATGCTTCTGTCTTCACTATCTGCATCTGG
1  M L R N M R S D T L M G S P T L F W G L M L S V F T I C I W
181  TCCACAAATGGAGAAATCTGTGGTCCGAGCATCGACATCCGAAATGAGATCAGTGAGTTCAAGCGGCTGGAGAACTGCACGGTGGTGGAG
321  S T N G E I C G P S I D I R N E I S E F K R L E N C T V V E
271  GGCTACCTGCAGATCCTCTCATCAACGACAGCAACCAACGCTCCACCAAGAGCTTTCGGCTCCCTCAGCTTCCCAAGCTGACCGTGC
61  G Y L Q I L L I N D K T N N V H Q E L F R S L S F P K L T V
361  ATCACCAGTACTGCTGCTATTTTCGCGTCTCGGGCTGGACAGCCTCAGCATGCTTTTCCCAACTTGAGCGTCATCCGCGGGCGCAAT
91  I T D Y L L L F R V S G L D S L S M L F P N L S V I R G R N
451  CTCTTCTACAACTACGCCCTGGTGTATCTACGAGATGACAGCCTGAAGGACATCGGCCTGTACAACCTGAGGAACATCACCAGGGGAGCC
121  L F Y N Y A L V I Y E M T S L K D I G L Y N L R N I T R G A
541  ATGCGAATTGAAAAGAACCCAGAGCTCTGTACTCTGGACTCAGTGGAGTGGTCCCTTATTATGGATGCTGGGACGAACAATGTTATCAAT
151  M R I E K N P E L C Y L D S V D W S L I M D A G T N N V I N
631  GGGAATAAGAGCAAGGAGTGTGGCAATGTCTGCCGGGCATGAGGAGATAACCTCTTGTGAGAGTACGTCTTTCAACGACAAG
181  G N K K A K E C G N V C P G I M E D N P L C Q S T S F N D K
721  TACGACTACCGTCTGTGGACCTCCAAACAGTGGCCAGAAAGTGTGCCCGGACCACTGCAAGCTCGCCTGCACGGACAAGGGGAGTGTTC
211  Y D Y R C W T S N Q C Q K V C P D H C K L A C T D K G E C C
811  CACAGCCAGTGCCTGGGCGAGCTGCACCGAGCCCAACACGACATGGCCTGCTCCGCTGCCTCCACTACCACCAAGGGTCCGCTGCGTA
241  H S Q C L G S C T E P N N D M A C S A C L H Y H H E G R C V
901  CCAGACTGTCCCCGGACACCTACAAGTTCGAGGGCTGGCGCTGCATCACCATGGACCTGTGCTCCCAAGCGCACCTGCCCATAGACATG
271  P D C P P D T Y K F E G W R C I T M D L C S Q A H L P I D M
991  CACTTTGTTCATCCACAGGGGAGAGTGCATGCCGAATGCCCTCCGGCTTCACACGCAACGAGACTAATCGCATGTTATGCACTGCCCTGC
301  H F V I H R G E C M P E C P S G F T R N E T N R M L C S A C
1081  AACGGCCCGTGGGATAAGCCCTGCACCTCCTCGTGCATCGACTCTGTGGATGCCGCTCAGTCTCTAAAGGACTGCACCATCATCGAAGGC
331  N G P C D K P C T S S V I D S V D A A Q S L K D C T I I E G
1171  AATCTGGACATCAACATCCGCGCGGAAATAACATCGAGTCTGAGAGCTTCATGGGATTCATCCAGAGAGTATGGGCTATGTTG
361  N L D I N I R R G N N I E S E L E S F M G L I Q R V M G Y V
1261  AGGATCCGACACTCCACGCACTTGGCTCGCTGCTTCTCTCAAGAGCCTGCGTTACATCGATGGGAATGAACATCATGGACAAATGTAC
391  R I R H S H A L G S L S F L K S L R Y I D G N E L M D K M Y
1351  TCGTTCTCCGCCATCGACAACGACCTGCAGTATCTTTGGGATGGAACGACGACAACCTGACAGTTCTGAACGGAGCTCTCTCTCTT
421  S F S A I D N Q H L Q Y L W D W N Q H N L T V L N G R L F F

```

[illegible]

Fig. 1. Nucleotide and predicted amino acid sequences of IGF-1Ra. Cysteine (C) residues are highlighted in grey. Black letters in bold and italics over grey background: signal peptide. White letters in bold and italics over black background: transmembrane domain. Underlined letters: putative N-Glycosylation locations. Stop codon motif TGA was referred to by the asterisk symbol (*).

398
399

400

401 **Fig. 2.** Neighbor-joining phylogenetic tree of vertebrate IGF-1R primary protein sequences.
 402 1,000 bootstraps were used to assess the efficacy of the test. Species and accession numbers
 403 are shown in the tree. Location of *S. aurata* IGF-1R sequence is marked by a violet rhomboid.

Fig. 3. mRNA expression patterns in different tissues of *Sparus aurata*. Data (n = 3) are represented as mean $\pm$ SEM. Different letters (a,b,c) refer to a significant difference from the previous point(s) ($P < 0.01$).

410

411

412

413 **Fig. 4.** mRNA expression patterns for (A) IGF-1Ra, (B) PRL, and (C) GH in pituitaries of *Sparus*
 414 *aurata* incubated for 10 hours in grading concentration of recombinant human IGF-1 (rhIGF-1).
 415 Data (n = 8) are represented as mean $\pm$ SEM. Different letters indicate significant differences
 416 among experimental groups (one way ANOVA, $P < 0.01$).

417

418
419 **4- Discussion**

420

421 In this study, the full-length cDNA sequence of insulin-like growth factor I receptor type
422 a (IGF-1Ra) in the teleost species *S. aurata* was characterized, obtaining new tools to study its
423 physiological roles after different internal or external challenges. In addition, the functionality of
424 the obtained precursor was confirmed through the presence of the main domains characterizing
425 an active receptor, mainly the ones concerning receptor activation and ligand binding.
426 Furthermore, the constructed phylogenetic tree could group all non-fish vertebrates in one
427 clade, and all bony fishes in the other. The IGF-1R precursor from *S. aurata* belongs to the
428 same subclade to which IGF-1Ra from the zebrafish *Danio rerio* belongs. Its mRNA maximum
429 abundance was found in white muscles, which are well-known to be the main IGF-1 binding
430 site. The second organ in order of IGF-1R mRNA abundance was the pituitary gland, which
431 indicates the special importance of this receptor there, and further confirms the activity of the
432 obtained receptor. Although the IGF-1 we used is a recombinant human protein, several studies
433 in fish found that human IGF-1 can successfully bind fish IGF-1R, stimulating both signal
434 transduction pathways for IGF-1 in a fish-phosphatidylinositol 3-kinase (PI 3-K) and mitogen-
435 activated protein kinase (MAPK), and trigger normal IGF-1 actions in fish (Upton et al., 1998;
436 Kajimura et al., 2002; Fuentes et al., 2011). The doses applied fall around the physiological
437 limits for IGF-1 presence in plasma of *S. aurata*, that are about 70-80 ng mL⁻¹, according to the
438 results previously recorded for this species by Gómez-Requeni et al. (2004) and Sánchez-
439 Gurmaches et al. (2013). The *in vitro* model used in this work resulted in a total RNA of a high
440 quality after 10 h of incubation (Fuentes et al., 2010; present results).

441 Tissue distribution of IGF-1R we cloned was similar between *S. aurata* and several
442 other, phylogenetically-related teleosts (Mauress et al., 2002; Nakao et al., 2002; Escobar et al.,
443 2011), suggesting some conserved functions in the organs that express this receptor in teleosts.
444 The excessive presence of IGF-1Ra mRNA in muscles as we found in juvenile *S. aurata* is
445 common in fishes even from very different taxa (Elies et al., 1996; Greene and Chen, 1999).
446 This is a known process in fish in general since fishes, in contrast to mammals, exhibit more
447 active muscular IGF-1 binding, replaces the active mammalian insulin binding in muscles. This
448 probably points to more important roles of IGF-1 in muscular growth and differentiation in fishes
449 than in mammals (Dai et al., 2015). Also, presence of IGF-1Ra in the pituitary gland plays
450 common regulatory roles over the production of its hormones, mainly PRL, GH and ACTH,
451 controlling then the key axes of osmoregulation, growth, reproduction, and stress in the pituitary
452 (Fruchtman et al., 2002; Leatherland et al., 2010). IGF-1 binding in the hearts of fishes
453 excessively surpasses insulin binding, in contrast to what happens in the endothermic
454 mammals. This suggested some more essential roles for IGF-1 over insulin in teleosts' hearts in
455 what concerns protein synthesis, carbohydrates influxes, growth and metabolism (Gutiérrez et
456 al., 1995; Gallardo et al., 2001). Hepatic and gastrointestinal presence of IGF-1Ra in the

gastrointestinal tract, with its related organs, supports the active roles of IGF-1 in promoting protein and carbohydrate uptake, as well as enhancing metabolism and growth (Shamblott et al., 1995). Also, presence of IGF-1Ra in the brain and other nervous tissues could be related to the mitogenic and neurogenic roles of IGF-1 (Otteson et al., 2002).

In rat anterior pituitary cells, IGF-1 receptors were located mainly in the GH producing cells rather than in any in other cell type (Eppler et al., 2007). In the hybrid striped bass (*Morone saxatilis* x *M. chrysops*), Fruchtmann et al. (2002a,b) found specific, high affinity IGF-1 receptors in the GH producing cells. Binding of IGF-1 to these receptors significantly depressed GH release through both PI 3-K and MAPK pathways in a dose-dependent manner. Furthermore, Kajimura et al. (2002) found the same GH down-regulation in pituitaries of *O. niloticus* in response to IGF-1 treatment. Our results showed that incubation of *S. aurata* pituitaries with increasing concentrations of IGF-1 significantly decreased GH mRNA expression, which is a common feedback effect for GH production and secretion in fish (Canosa et al., 2007; Reindl and Sheridan, 2012), and consequently for the regulation of the somatotrophic axis. In addition, another completely reversed effect of IGF-1 on PRL cells has been also reported. In the hybrid striped bass, IGF-1 stimulates PRL release, only via the MAPK cascade, in a dose-dependent manner (Fruchtmann et al., 2002a,b). In *O. niloticus*, IGF-1 treatment up-regulated PRL expression in *in vitro* studies with pituitaries (Kajimura et al., 2002). Our results agree with this observation and indicated that PRL mRNA levels enhanced with increasing IGF-1 dose, until reaching a significant level in the highest dose applied (100 ng mL⁻¹). The concerted action between IGF-1, PRL and Gh may allow animals to shift from physiological states influenced by Gh, as skeletal growth, to other states regulated by PRL, as reproduction and osmoregulation, as pointed out by Seale et al. (2013).

Our results indicated that IGF-1 significantly enhanced IGF-1Ra mRNA expression about 2 folds for the 10 ng mL⁻¹ dose and 3 folds for the 100 ng mL⁻¹ addition. This similarity between IGF-1Ra mRNA levels and increasing doses of IGF-1 is an interesting phenomenon, and its physiological significance is not yet well established. Several studies relate IGF-1 binding to the IGF-1Ra to the activation of the PI3 K/Akt pathway and the roles of this pathway in enhancing cell survival, proliferation, and proper functioning of both IGF-1 and IGF-1Ra. Li et al. (2013) detected that IGF-1R rich-cells exhibit high proliferation activities and a significantly enhanced Akt protein phosphorylation, which contributes positively to the activation of the PI3 K/Akt pathway. These findings may explain then the strong GH inhibition by IGF-1 in a dose-dependent manner. Furthermore, Romanelli et al. (2007) incubated progenitor glial cells *in vitro* with IGF-1 in the culture medium for 24 h and 30 min then withdrawn IGF-1. This resulted in a sustained Akt phosphorylation in the group maintained with IGF-1 for 24 h but decreased phosphorylation in the group incubated for only 30 min. Although IGF-1 stimulation provokes IGF-1 receptor internalization and recycling for sustaining Akt phosphorylation (Romanelli et al., 2007; Reindl and Sheridan, 2012), some correspondent phenomena need more study. For example, IGF-1 did not alter IGF-1R protein levels but changed the receptor's surface

496 availability through internalization, followed by a recycling process for the internalized receptor
497 to recover the surface levels rapidly (within 120 min), the matter assisting positively Akt
498 phosphorylation (Ness et al., 2002; Romanelli et al., 2014). These results may suggest an
499 available IGF-1Ra mRNA for these rapid responses and turnover processes. In this way, the
500 enhanced IGF-1Ra expression observed in pituitary after IGF-1 incubation supports this idea.
501 More importantly, the responses of pituitary IGF-1Ra, PRL and GH to increased levels of IGF-I
502 can provide some clue for the control of mechanisms normally mediated by each of these
503 hormones in supporting the success of *S. aurata*, and fishes in general, in their environments.
504 First, the expression of IGF-1R in tissues is responsive to environmental factors, including
505 nutritional state and temperature (Gabillard et al., 2003; Dai et al., 2015). Second, it is now
506 currently accepted that PRL in fish is intimately related to osmoregulation in low-salinity waters.
507 However, growth hormone role in osmoregulation in non-salmonid teleosts is still under debate,
508 with some reports referring to more active IGF-1 stimulation upon fish movement to hypersaline
509 waters and consequent activation of osmoregulatory processes (McCormick 2001; Mancera and
510 McCormick, 2007; Tipsmark et al. 2008; Mohammed-Geba et al., 2015). Differential regulation of
511 growth hormone and prolactin is very well known in migratory fish during their migration or
512 spawning periods (Mancera and McCormick., 1998; Persson et al., 1998; Sakamoto and
513 McCormick., 2006; Makino et al., 2007). *S. aurata* is a well-known euryhaline teleost moving
514 from open seawater to estuarine waters continuously during its different life stages (Arias,
515 1976), hence it must keep a state of osmoregulatory preparedness for supporting such
516 environmental transitions. So, again, an available IGF-1Ra mRNA for the processes of rapid
517 recovery of receptor protein surface levels may be necessary especially in cases that
518 necessitate the production of more osmoregulation-promoting hormone, as the case of PRL
519 increase we found, or switching from a growth promoting role to other different one more
520 importantly related to the osmoregulatory process, as the case of GH down-regulation we found
521 in response to IGF-1 treatment. In any case, it seems that the classic IGF-1 feedback over GH
522 production extends in fish far beyond regular feedback process to some kind of switch between
523 regular physiological processes to other ones need more adjustments, like osmoregulation.

524 In conclusion, we successfully cloned the whole open reading frame from IGF-1Ra
525 receptor in *S. aurata*. The mRNA of the active receptor showed excessive presence in white
526 muscles and pituitary gland, and it responded in a dose-dependent manner to the increased
527 IGF-1 concentrations *in vitro*. This mode of response seems to be related to the activation of the
528 signal transduction pathways essential for regulating GH and PRL production in the pituitary
529 gland of the gilthead sea bream *S. aurata*.

530

531 ***Acknowledgment***

532

533 The authors would like to appreciate their thanks to Prof. Juan Fuentes (Centre of
534 Marine Sciences, University of Algarve, Faro, Portugal) for his generous and comprehensive
535 guidance during the fulfillment of this work. This work has been done in the Campus of
536 Excellence on the Sea (CEI-MAR).

537

538 **References**

539

540 Arias A (1976) Sobre la biología de la dorada, *Sparus aurata*, L., de los esteros de la provincia
541 de Cádiz. Investigación Pesquera 40, 201-222.

542 Canosa LF, Chang JP, Peter RE (2007) Neuroendocrine control of growth hormone in fish. Gen
543 Comp Endocrinol 151: 1–26.

544 Dai X, Zhang W, Zhuo Z, He J, Yin Z (2015) Neuroendocrine regulation of somatic growth in
545 fishes. Science China Life Sciences 58(2) 137-147.

546 Dantzer B, Swanson EM (2012) Mediation of vertebrate life histories via insulin-like growth
547 factor-1. Biol Rev Camb Philos Soc 87: 414-29.

548 Elies G, Groigno L, Wolff J, Boeuf G, Boujard D (1996) Characterization of the insulin-like
549 growth factor type 1 receptor messenger in two teleost species. Mol Cell Endocrinol
550 124: 131-40.

551 Eppler E, Jevdjovic T, Maake C, Reinecke M (2007) Insulin-like growth factor I (IGF-1) and its
552 receptor (IGF-1R) in the rat anterior pituitary. Eur J Neurosci 25: 191-200.

553 Escobar S, Fuentes EN, Poblete E, Valdés JA, Safian D, Reyes AE, Álvarez M, Molina A.
554 (2011) Molecular cloning of IGF-1 and IGF-1 receptor and their expression pattern in
555 the Chilean flounder (*Paralichthys adspersus*). Comp Biochem Physiol 159: 140-147.

556 Fruchtmann S, Gift B, Howes B, Borski R (2001) Insulin-like growth factor-I augments prolactin
557 and inhibits growth hormone release through distinct as well as overlapping cellular
558 signaling pathways. Comp Biochem Physiol 129: 237-42.

559 Fruchtmann S, McVey DC, Borski RJ (2002) Characterization of pituitary IGF-1 receptors:
560 modulation of prolactin and growth hormone. Am J Physiol 283: R468-76.

561 Fuentes EN, Björnsson BT, Valdés JA, Einarsdottir IE, Lorca B, Alvarez M, Molina A (2011)
562 IGF-1/PI3K/Akt and IGF-1/MAPK/ERK pathways in vivo in skeletal muscle are regulated
563 by nutrition and contribute to somatic growth in the fine flounder. Am J Physiol 300:
564 R1532-42.

565 Fuentes J, Brinca L, Guerreiro PM, Power DM (2010) PRL and GH synthesis and release from
566 the sea bream (*Sparus auratus* L.) pituitary gland in vitro in response to osmotic
567 challenge. Gen Comp Endocrinol 168: 95-102.

568 Gabillard JC, Weil C, Rescan PY, Navarro I, Gutiérrez J, Le Bail PY (2003) Effects of
569 environmental temperature on IGF1, IGF2, and IGF type I receptor expression in
570 rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 133: 233-42.

571 Gallardo MA, Castejon C, Navarro I, Blasco J, Gutiérrez J, and Sánchez J (2001) L-leucine and
572 L-alanine uptake by trout (*Salmo trutta*) cardiomyocytes: the effect of IGF-I and insulin.
573 Fish Physiol Biochem 25: 239-248.

574 Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF,
575 Kaushik S, Pérez-Sánchez J (2004). Protein growth performance, amino acid utilisation

576 and somatotropic axis responsiveness to fish meal replacement by plant protein
577 sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232: 493–510.

578 Greene MW, Chen TT (1999) Characterization of teleost insulin receptor family members. II.
579 Developmental expression of insulin-like growth factor type I receptor messenger RNAs
580 in rainbow trout. Gen Comp Endocrinol 115: 270-81.

581 Gutiérrez J, Párrizas M, Maestro MA, Navarro I, Plisetskaya EM. (1995) Insulin and IGF-I
582 binding and tyrosine kinase activity in fish heart. J Endocrinol 146(1): 35-44.

583 Kajimura S, Uchida K, Yada T, Hirano T, Aida K, Gordon Grau E (2002). Effects of insulin-like
584 growth factors (IGF-1 and -II) on growth hormone and prolactin release and gene
585 expression in euryhaline tilapia, *Oreochromis mossambicus*. Gen Comp Endocrinol 127:
586 223-31.

587 Laiz-Carrión R, Fuentes J, Redruello B, Guzmán JM, Martín del Río MP, Power D, Mancera JM
588 (2009) Expression of pituitary prolactin, growth hormone and somatolactin is modified in
589 response to different stressors (salinity, crowding and food-deprivation) in gilthead sea
590 bream *Sparus auratus*. Gen Comp Endocrinol 162:293-300.

591 Laiz-Carrión R, Guerreiro PM, Fuentes J, Canario AV, Martín Del Río MP, Mancera JM (2005a)
592 Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus*
593 *auratus*. J Exp Zool 303A:563-76.

594 Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Soengas JL, Mancera
595 JM (2005b) Growth performance of gilthead sea bream *Sparus aurata* in different
596 osmotic conditions: Implications for osmoregulation and energy metabolism.
597 Aquaculture 250: 849-861.

598 Lammers R, Gray A, Schlessinger J, Ullrich A (1989) Differential signaling potential of insulin-
599 and IGF-1-receptor cytoplasmic domains. EMBO J 8: 1369-75.

600 Le Roith D, Bondy C, Yakar S, Liu JL, Butler A (2001) The somatomedin hypothesis: 2001.
601 Endocr Rev 22: 53-74.

602 Leatherland JF, Li M, Barkataki S (2010) Stressors, glucocorticoids and ovarian function in
603 teleosts. J Fish Biol 76(1), 86-111.

604 Li Q, You C, Liu L, Rao Z, Sima X, Zhou L, Xu J (2013) Craniopharyngioma cell growth is
605 promoted by growth hormone (GH) and is inhibited by tamoxifen: involvement of growth
606 hormone receptor (GHR) and IGF-1 receptor (IGF-1R). J Clin Neurosci 20: 153-7.

607 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time
608 quantitative PCR and the 2- $\Delta\Delta$ CT method. Methods 25:402-8.

609 Makino K, Onuma TA, Kitahashi T, Ando H, Ban M, Urano A (2007) Expression of hormone
610 genes and osmoregulation in homing chum salmon: a minireview. Gen Comp
611 Endocrinol 152: 304-9.

612 Mancera JM, Fernández-Llebrez P, Pérez-Fígares JM (1993) Osmoregulatory responses during
613 abrupt salinity changes in the euryhaline gilthead sea bream (*Sparus aurata* L.). Comp
614 Biochem Physiol 106: 245-250.

615 Mancera JM, Laiz-Carrión R, Martín del Río MP (2002). Osmoregulatory action of PRL, GH,
616 and cortisol in the gilthead seabream (*Sparus aurata* L.). Gen Comp Endocrinol 129:
617 95-103.

618 Mancera JM, McCormick SD (1998) Osmoregulatory actions of the GH/IGF axis in non-
619 salmonid teleosts. Comp Biochem Physiol 121(1), 43-48.

620 Mancera JM, McCormick SD (2007). Role of prolactin, growth hormone, insulin-like growth
621 factor I and cortisol in teleost osmoregulation. In: Kapoor BG, Baldisserotto B, Mancera
622 Romero JM (eds) Fish osmoregulation. Science Publishers, Enfield, pp 497–515.

623 Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH,
624 Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki
625 CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS,
626 Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH (2011) CDD: a
627 Conserved Domain Database for the functional annotation of proteins. Nucleic Acids
628 Res. 39: D225-9.

629 Martos-Sitcha JA, Fuentes J, Mancera JM, Martínez-Rodríguez G (2014) Variations in the
630 expression of vasotocin and isotocin receptor genes in the gilthead sea bream *Sparus*
631 *aurata* during different osmotic challenges. Gen. Comp. Endocrinol. 197: 5-17.

632 Martos-Sitcha JA, Wunderink YS, Gozdowska M, Kulczykowska E, Mancera JM, Martínez-
633 Rodríguez G (2013) Vasotocinergic and isotocinergic systems in the gilthead sea bream
634 (*Sparus aurata*): an osmoregulatory story. Comp. Biochem Physiol 166: 571-81.

635 Maures T, Chan SJ, Xu B, Sun H, Ding J, Duan C (2002) Structural, biochemical, and
636 expression analysis of two distinct insulin-like growth factor I receptors and their ligands
637 in zebrafish. Endocrinol 143: 1858-71.

638 McCormick SD (2001) Endocrine control of osmoregulation in teleost fish. Am Zool 41:781–794.

639 Mei J, Yan W, Fang J, Yuan G, Chen N, He Y (2014) Identification of a gonad-expression
640 differential gene insulin-like growth factor-1 receptor (Igf1r) in the swamp eel
641 (*Monopterus albus*). Fish Physiol Biochem 40: 1181-90.

642 Mohammed-Geba K, Mancera JM, Martínez-Rodríguez G (2015) Acclimation to different
643 environmental salinities induces molecular endocrine changes in the GH/IGF-1 axis of
644 juvenile gilthead sea bream (*Sparus aurata* L.). J Comp Physiol B 185(1): 87-101.

645 Nakao N, Tanaka M, Higashimoto Y, Nakashima K (2002) Molecular cloning, identification and
646 characterization of four distinct receptor subtypes for insulin and IGF-I in Japanese
647 flounder, *Paralichthys olivaceus*. J Endocrinol 173: 365-375.

648 Ness JK, Wood TL (2002) Insulin-like growth factor I, but not neurotrophin-3, sustains Akt
649 activation and provides long-term protection of immature oligodendrocytes from
650 glutamate-mediated apoptosis. Mol Cell Neurosci 20: 476-88.

651 NetNGlyc software: N-Glycosylation sites. NetNGlyc 1.0 Server:
652 <http://www.cbs.dtu.dk/services/NetNGlyc/>

653 Otteson DC, Cirenza PF, Hitchcock PF (2002) Persistent neurogenesis in the teleost retina:
 654 evidence for regulation by the growth-hormone/insulin-like growth factor-I axis. *Mech*
 655 *Dev* 117: 137-149.

656 Pérez-Sánchez J, Marti-Palanca H, Le Bail P-Y (1994) Seasonal changes in circulating growth
 657 hormone (GH), hepatic GH-binding and plasma insulin-like growth factor-I
 658 immunoreactivity in a marine fish, gilthead sea bream, *Sparus aurata*. *Fish Physiol*
 659 *Biochem* 13: 199-208.

660 Persson P, Sundell K, Björnsson BT, Lundqvist H (1998) Calcium metabolism and
 661 osmoregulation during sexual maturation of river running Atlantic salmon. *J Fish Biol* 52:
 662 334-349.

663 Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal
 664 peptides from transmembrane regions. *Nature Methods* 8: 785-786.

665 Reindl KM, Sheridan MA (2012) Peripheral regulation of the growth hormone-insulin-like growth
 666 factor system in fish and other vertebrates. *Comp Biochem Physiol* 163: 231-45.

667 Reinecke M, Björnsson BT, Dickhoff WW, McCormick SD, Navarro I, Power DM, Gutiérrez J
 668 (2005) Growth hormone and insulin-like growth factors in fish: where we are and where
 669 to go. *Gen Comp Endocrinol* 142: 20-4.

670 Romanelli RJ, LeBeau AP, Fulmer CG, Lazzarino DA, Hochberg A, Wood TL (2007) Insulin-like
 671 growth factor type-I receptor internalization and recycling mediate the sustained
 672 phosphorylation of Akt. *J. Biol Chem.* 282: 2251324.

673 Sakamoto T, McCormick SD (2006) Prolactin and growth hormone in fish osmoregulation. *Gen*
 674 *Comp Endocrinol.* 147(1): 24-30.

675 Sánchez-Gurmaches J, Cruz-García L, Ibarz A, Fernández-Borrás J, Blasco J, Gutiérrez J,
 676 Navarro I (2013) Insulin, IGF-1, and muscle MAPK pathway responses after sustained
 677 exercise and their contribution to growth and lipid metabolism regulation in gilthead sea
 678 bream. *Domest Anim Endocrinol* 45:145-53.

679 Seale AP, Yamaguchi Y, Johnstone WM 3rd, Borski RJ, Lerner DT, Gordon Grau E (2013).
 680 Endocrine regulation of prolactin cell function and modulation of osmoreception in the
 681 Mozambique tilapia. *Gen Comp Endocrinol* 192: 191-203.

682 Shambloot MJ, Cheng CM, Bolt D, Chen TT (1995) Appearance of insulin-like growth factor
 683 mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth
 684 hormone. *Proc Natl Acad Sci U S A* 92(15): 6943-6946.

685 Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular
 686 evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30(12): 2725-2729.

687 Tipsmark CK, Luckenbach JA, Madsen SS, Kiillerich P, Borski RJ (2008) Osmoregulation and
 688 expression of ion transport proteins and putative claudins in the gill of Southern flounder
 689 (*Paralichthys lethostigma*). *Comp Biochem Physiol* 150A: 265–273.

690 Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T,
 691 Kathuria S, Chen E (1986) Insulin-like growth factor I receptor primary structure:

692 comparison with insulin receptor suggests structural determinants that define functional
693 specificity. EMBO J 5(10): 2503-12.

694 Upton Z, Yandell CA, Degger BG, Chan SJ, Moriyama S, Francis GL, Ballard FJ (1998)
695 Evolution of insulin-like growth factor-I (IGF-1) action: in vitro characterization of
696 vertebrate IGF-1 proteins. Comp Biochem Physiol 121: 35-41.

697 Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM,
698 Martínez-Rodríguez G (2009a). Gene and protein expression for prolactin, growth
699 hormone and somatolactin in *Sparus aurata*: seasonal variations. Comp Biochem
700 Physiol 153: 130-135.

701 Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM,
702 Martínez-Rodríguez G (2009b). Pituitary gene and protein expression under
703 experimental variation on salinity and temperature in gilthead sea bream *Sparus aurata*,
704 Comp Biochem Physiol 154B: 303-308.

705 Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM,
706 Martínez-Rodríguez G (2009b). Pituitary gene and protein expression under
707 experimental variation on salinity and temperature in gilthead sea bream *Sparus aurata*.
708 Comp Biochem Physiol 154B: 303-308.

709 Xu Y, Zang K, Liu X, Shi B, Li C, Shi X (2015) Insulin-like growth factors I and II in starry
710 flounder (*Platichthys stellatus*): molecular cloning and differential expression during
711 embryonic development. Fish Physiol Biochem 41: 139-52.

712

713

714 Table 1. Sequences of primers designed for molecular identification of IGF-1Ra cDNA
 715 sequence.

716

Primer	Orientation	Sequence 5'→3'	Location in <i>S. aurata</i>
Intermediate cDNA fragment	Sense	GGCGCTGCATCACCATGGACC	939
	Antisense	GCTGTCCCTGAGAGACCACG	3317
5'-RACE	cDNA-synthesizer	ACTGAGCGGCATCCACAGA	1142
	5'- RACE 1	GCACGGGCCGTTGCAGGCA	1093
	5'- RACE 2	ATGTCTATGGGCAGGTGCG	989

717

718

Table 2. Sequences, positions, amplicon sizes, amplification efficiencies and regression coefficients of primers used for qPCR study.

Primer	Orientation	Sequence 5' → 3'	Position	Acc. no.	Amplicon size (bp)	E	r ²
IGF-1Ra	Sense	ACACGCAACGAGACTAATCG	1042	KJ591052	106	0.99	0.99
	Antisense	TTAGAGACTGAGCGGCATCC	1147				
GH	Sense	CGTCTCTTCTCAGCCGAT	90	U01301.1	131	0.99	0.99
	Antisense	GCTGGTCCTCCGTCTGC	220				
PRL	Sense	TGACATCGGCGAGGACAACATT	540	AF060541.1	111	1	0.99
	Antisense	CGGCAGCGGAGGACTTTCAG	650				
β-actin	Sense	TCTTCCAGCCATCCTTCCTCG	782	X89920.1	108	1	0.99
	Antisense	TGTTGGCATAACAGGTCCTTACGG	889				

719