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Influence of water salinity on genes implicated in somatic growth, lipid metabolism and food intake in Pejerrey (*Odontesthes bonariensis*)

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ABSTRACT

Pejerrey, *Odontesthes bonariensis*, is an euryhaline fish of commercial importance in Argentina. This work aimed to determine if water salinity affects the expression of genes involved in somatic growth (*gh*; *ghr-I*; *ghr-II*; *igf-I*), lipid metabolism (*Δ6-desaturase*) and food intake (*nucb2/nesfatin-1*). First, we identified the full-length cDNA sequences of Δ6-desaturase (involved in lipid metabolism) and nesfatin-1 (an anorexigen). Then, pejerrey juveniles were reared during 8 weeks in three different water salinity conditions: 2.5 g/L (S2.5), 15 g/L (S15) and 30 g/L (S30) of NaCl. Brain, pituitary, liver and muscle samples were collected in order to analyze mRNA expression. The expression of *gh* and *ghr-II* mRNAs increased in the pituitary of fish reared at S2.5 and S30 compared with the S15 group. The expression of *ghr-I* was higher in the liver of S30 group compared to S2.5 and S15. I*gf-I* mRNA expression in liver increased with the increment of water salinity, while it decreased in the muscle of S15 and S30 groups. *Δ6-desaturase* expression increased in S2.5 group compared to S15 in both liver and muscle. S30 caused a decrease in the *Δ6-desaturase* expression in liver compared to S15. The S30 treatment produced an increase in *nucb2/nesfatin-1* mRNA expression in the brain and liver compared to S2.5 and S15. The changes in gene expression observed could help pejerrey perform better during salinity challenges. The S30 condition would likely promote pejerrey somatic growth in the long term.

1. Introduction

Pejerrey, *Odontesthes bonariensis* (Valenciennes, 1835), is an inland water fish from Argentina (Miranda et al., 2006) that belongs to the Atherinopsidae family. It is highly appreciated for its value in sport fishing and for the quality of its flesh that has taste, smell, texture and even chemical characteristics similar to those of expensive marine species, making it an ideal candidate for aquaculture (Somoza et al., 2008). Although it is certainly known that pejerrey survives a wide range of water salinity (Gómez et al., 2007; Ringuelet et al., 1967), no studies were conducted to determine how this environmental factor affects some adaptive parameters such as growth, lipid metabolism and food intake. Water salinity is an important factor for fish aquaculture, since it can directly modify fish growth (Boeuf and Payan, 2001). Some studies propose that the energy cost of osmoregulation is lower where the gradients between blood and water are minimal, and that these energy savings are substantial enough to promote growth. As an example, osmoregulation appears to use a high proportion of the available energy, ranging from 20 to 50% of the total energy expenditure depending on the water salinity (Bushnell and Brill, 1992; Nordlie, 1978; Nordlie and Leffler, 1975; Rao, 1968). Few studies have demonstrated the effect of water salinity on the expression of the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis genes in fish. Growth hormone (GH) is secreted by the pituitary, and is involved in many physiological functions in fish, most of them associated with somatic growth (Biga and Meyer, 2009; Mommsen, 2001; Pérez-Sánchez and Le

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, ecosapentaenoic acid; FO, fish oil; HUFA, highly unsaturated fatty acid; LNA, linolenic acid; LA, Linoleic acid; ORF, open reading frame; PUFA, poly unsaturated fatty acid; RACE, rapid amplification of cDNA ends; SfO, sunflower oil; S2.5, water salinity of 2.5 g/L; S15, water salinity of 15 g/L; S30, water salinity of 30 g/L

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Bail, 1999; Reinecke et al., 2005) and stress resistance (Ababutain, 2011; Deane and Woo, 2009; Meier et al., 2009; Sakamoto and McCormick, 2006). The GHR-I and GHR-II receptors mediate GH actions, and are highly expressed in fish liver (Maestro et al., 1998; Ozaki et al., 2006; Rhee et al., 2012). In response to GH binding to its receptors, the liver releases insulin-like growth factors I and II (IGF-I and IGF-II), which act in an endocrine manner, promoting somatic growth in different tissues (Duan, 1997; Wood et al., 2005). Since osmoregulation affects growth and energy expenditure, it is expected that salinity changes modulate the expression of genes involved in the regulation of somatic growth. The GH-IGF axis is also involved in stress response. Thus, changes in water salinity could modify the expression of genes in the stress axis without generating changes in somatic growth (Boeuf and Payan, 2001).

Fatty acid composition of cellular membranes is important in osmolarity regulation. Changes in environmental factors, including salinity, affect membrane lipid composition (Fonseca-Madrigal et al., 2012; Li et al., 2008; Sarker et al., 2011). Unsaturation level of fatty acids important for determining the volume occupied by phospholipids; the more unsaturations a fatty acid has, the greater the volume it occupies. This affects the cellular membrane fluidity (Bourre et al., 1993; Brenner, 1984; Mead, 1984; Sakamoto and Murata, 2002) and thus, ion exchange. Results from our group showed that pejerrey can survive and grow well on diets in which sunflower oil partially or totally replaces fish oil as the source of lipids (manuscript under review). This finding indicates that pejerrey could convert low unsaturated fatty acids into polyunsaturated fatty acids (PUFA) within n-6 or n-3 fatty acid series. In vertebrates, the rate-limiting enzyme involved in the biosynthetic pathway of PUFAs from both n-6 and n-3 series, is the fatty acyl Δ6-desaturase, which catalyzes the first step of the desaturation/elongation process in both pathways, converting linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6) into 18:3n-6 and 18:4n-3, respectively. At least in humans and mice, this membrane-bound enzyme is also involved in the synthesis of docosahexaenoic acid (DHA; 22:6n-3) from the eicosapentaenoic acid (EPA; 20:5n-3), but this remains unclear in fish (Vagner and Santigosa, 2011; Yang et al., 2013).

Food intake is an important aspect to take into consideration when an environmental condition is tested, since it could modify growth and other related-parameters, such as the condition factor. Nesfatin-1 is a peptidyl orphan ligand with hormone-like actions, produced by the N-terminal cleavage of its precursor nucleobindin-2 (NUCB2), encoded in the *nucb2* gene (Oh-I et al., 2006; Mohan and Unniappan, 2013; Ramesh et al., 2017). In rodents, a wide distribution of *nucb2/nesfatin-1* with prominent expression in hypothalamic nuclei, several brainstem areas, autonomic centers (Goebel et al., 2011), gut (Mohan et al., 2014; Ramesh et al., 2015; Stengel et al., 2010) and pancreas (Gonzalez et al., 2011; Mohan and Unniappan, 2012) was found. In fish, *nucb2/nesfatin-1* is abundant in many tissues, including the brain, pituitary, liver, gonads and gastrointestinal tract (Gonzalez et al., 2010; Lin et al., 2014). Nesfatin-1 reduces food intake after central or peripheral administration in mammals (García-Galiano et al., 2010; Goebel et al., 2011; Gonzalez et al., 2012; Mortazavi et al., 2015; Oh-I et al., 2006; Shimizu et al., 2009; Sugino et al., 2004; Tang-Christensen et al., 2004) and fish (Gonzalez et al., 2010; Kerbel and Unniappan, 2012), which supports an anorexigenic role for this peptide.

The aim of this work was to determine how water salinity affects the expression of genes involved in growth (*gh*, *ghr-II*, *ghr-II*, *igf-I*), fatty acid metabolism (*Δ6-desaturase*) and food intake regulation (*nucb2/nesfatin-1*) in pejerrey. To achieve these, we first characterized the Δ6-desaturase and nesfatin-1 cDNA sequences in pejerrey. Then, an experiment, in which juvenile pejerrey were exposed to three different water salinity conditions was carried out to determine the expression of above-mentioned genes. Results of this work could help understand the molecular changes that occur in fish in response to variations in water salinity and contribute to our knowledge, and approaches in pejerrey aquaculture.

2. Materials and methods

2.1. Fish management and experimental design

The experiment was carried out with one-year juvenile pejerrey that were bred at a salinity of 15 g/L until the commencement of the experiment. Fish were divided into three experimental groups kept in tanks with the following water salinity concentrations: 2.5 g/L (S2.5); 15 g/L (S15; control) and 30 g/L (S30). Each treatment was carried out in duplicate in 300 L tanks with 5 fish per tank. Since all fish were initially maintained in the same salinity condition (15 g/L), and in order to change that condition in the least stressful manner possible, water salinity was gradually decreased or increased during 7 days to achieve the desired concentration. After that, fish were kept under experimental conditions during 8 weeks. One third of the total water of each tank was replaced once a week for all treatments and controls. During the assay, fish were fed at 3% of total body mass, three times per day with a commercial diet (Shulet ®). Every 15 days, standard length, total length and body weight of all fish were measured. At the end of the experiment, fish were anesthetized using benzocaine 0.05% (weight/volume, W/V), sacrificed by decapitation and brain, pituitary, liver and muscle were collected in order to perform the gene expression analysis. Besides, liver, gonads and adipose tissue of all fish were weighed to calculate the hepatosomatic (Liver weight (g)/Fish weight (g) × 100), Gonadosomatic (Gonad weight (g)/Fish weight (g) × 100) and adipose (Adipose tissue weight (g)/Fish weight (g) × 100) indexes.

2.2. Sequencing of Δ6-desaturase and NUCB2 cDNAs from pejerrey

For the sequencing of pejerrey Δ6-desaturase and nesfatin-1, we used samples of brain and gut collected from fish reared at acclimation conditions (S15). Total RNA from brain and gut of an adult male pejerrey was extracted using TRIzol® reagent (Invitrogen, Ltd., USA). The mRNA was reverse-transcribed using Gene Racer kit (Invitrogen, USA) according to manufacturer's protocol. To obtain the Δ6-desaturase sequence, an internal region of the transcript was amplified and sequenced from brain cDNA using primers saFAD-F1 and saFAD-R1 designed on the *Sparus aurata* FAD sequence (**AY055749.1**). Then, following Gene Racer kit protocol and using gene specific primers pjFAD-F1, pjFAD-R1 and those provided in the kit (GeneRacer™ 5′ Primer 1, GeneRacer™ 3′ Primer1, GeneRacer™ 5′ Nested Primer 1 and GeneRacer™ 3′ Nested Primer 1), 5′ and 3′ ends were amplified. To obtain the nesfatin-1 sequence, an internal region (800 bp) of the pejerrey transcript was amplified using primers gfNUCB2-F1 and gfNUCB2-R1 designed on goldfish *nucb2* sequence (**HM065567.1**). Then, another PCR was conducted using a reverse primer designed on the pejerrey partial sequence obtained (pjNUCB2-R1) and a forward primer (lcNUCB2-F1) designed on *Larimichthys crocea nucb2* sequence (**XM_010734428.2**). With this approach, the 5′ end was amplified. Sequences of all primers are detailed in Table 1. PCR amplifications were performed using TAQ DNA polymerase (Invitrogen, USA) with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of a denaturation step at 94 °C for 30 s, annealing at specific Tm (Table 1) for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 3 min. The Δ6-desaturase PCR fragments were purified from agarose gels using the kit QIAEX®II (QIAGEN Inc., Canada) and were cloned into the pGEM-T Easy vector (Promega Corporation, USA). *E. coli* DH5α was transformed in order to increase the number of copies of each fragment and purified vectors were sequenced using the sequencing service of University of San Martín (San Martín, Buenos Aires province, Argentina). The nesfatin-1 PCR fragments were purified from agarose gels using the kit QIAEX®II (QIAGEN Inc., Canada) and sequenced by the sequencing service of the National Research Council of Canada (Saskatoon, SK, Canada).

Table 1
Primers used for sequencing and quantifying gene expression by RT-qPCR.

Gene	GenBank accession number	Primer sequence (5′ to 3′)	Tm (°C)
Primers used for sequencing			
Δ6-desaturase	**ADD50000.1**	saFAD-F1: GTTTACACCTGGGAGGAGG saFAD-R1: CGATTTGAAAGTTGAGGTG	57
Δ6-desaturase	**KY695233**	pjFAD6-F1: TACGAGAGCAGGCAGAGAAAGAG pjFAD6-R1: GCAGCAGGATGTGACCCAGATG	58
GeneRacer™		5′ Primer[1]: CGACTGGAGCACGAGGACACTGA 3′ Primer[1]: GCTGTCAACGATACGCTACGTAACG	58
GeneRacer™		5′ Nested Primer[1]: GGACACTGACATGGACTGAAGGAGTA 3′ Nested Primer[1]: CGCTACGTAACGGCATGACAGTG	58
nucb2/nesfatin-1	**HM065567.1**	gfNUCB2-F1: GAGAAAAGCTCCACAACACAG gfNUCB2-R1: GTGCTCATTTCTGCGCATTAG	55
nucb2/nesfatin-1	**KKF17838.1**	lcNUCB2-F1: TAAAATGTGTTGG AGCCGG pjNUCB2-R1: ACTGCTATGTCATTTCCTCC	58
Primers used for RT-qPCR analysis			
Δ6-desaturase	**KY695233**	pjFAD6-F2-Q: CCTTCGCTACCTCTCCTGTTACG pjFAD6-R2-Q: TCAGCCAGTCTTTGTGCCTCTC	60
nucb2/nesfatin-1	**KY695234**	pjNUCB2-F1: CAAGCAAGACCTCGAGGGAG pjNUCB2-R1: TCTCTCGTCCTCATCCAGGG	60
Growth hormone	**AY187284.2**	GH-f1: AGAAGTAGCATTAGCGTAG GH-r1: AACACTTTATTTTACACACG	60
Growth hormone receptor-I	**KF055461.1**	GHRI-f1: CTTTGGAGAGAGGTGAAACGGTTG GHRI-r1: CTTGGGCGTAGAAGTCTGTGTTG	60
Growth hormone receptor-II	**KF055460.1**	GHRII-f1: GACGGACCTGGACACGGACTG GHRII-r1: ACGGGAGAATGGAGCGGTGAC	60
Insulin-like growth factor-I	**EU257205.1**	IGFI-f1: CTGCGCAATGGAACAAAG IGFI-r1: GAATGAGCGCTAGACATCC	60
Insulin-like growth factor-II		IGFII-f1: AGCAGAGAACAGCCGAAT IGFII-r1: GTAGAGCGTGAGAGCCAG	60
β-actin	**EF044319.1**	pjBETA-F1: GCTGTCCCTGTACGCCTCTGG pjBETA-R1: GCTCGGCTGTGGTGGTGAAGC	60
Elongation factor	**KM273019.1**	EF-F: AGAAATCCGTCGTGGATACG EF-R: TGATGACCTGAGCGTTGAAG	60

F, Forward primer; R, Reverse primer. Tm, melting temperature.

2.3. Sequence analysis

Assembling, translation and sequence analysis were made using Lasergene Software (DNASTAR, Inc.). DNA sequence and translated protein sequence were identified using BLAST software (http://blast.ncbi.nlm.nih.gov/). The alignments were made using ClustalO (Goujon et al., 2010; McWilliam et al., 2013; Sievers et al., 2011). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).

2.4. RNA extraction and quantitative real-time PCR (RT-qPCR) analysis

Tissues were disrupted using a PRO 200 homogenizer (PRO Scientific Inc., USA) and total RNA was extracted using the Ambion TRIzol Reagent (Life Technologies, USA) following the protocol provided by the manufacturer. Total RNA (1 μg) was reversely transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, USA). Expression of target genes was measured by real-time quantitative PCR with Fast Start Universal SYBR Green Supermix, (Roche Diagnostics, USA) on a Thermal Cycler StepOne Plus (Life Technologies Corporation, USA), using *β-actin* (primers pjBETA-F1 and pjBETA-R1) and *elongation factor-1* (primers EF-F and EF-R) as reference genes. All primers sequences used for gene expression analysis are shown in Table 1. Each sample was run in duplicate and PCR reaction, without the addition of template, was used as negative control. The qPCR profiles contained an initial activation step at 95 °C for 5 min, followed by 35 cycles: 30 s at 95 °C, 30 s at 60 °C and 30 s at 73 °C. After the amplification, a melt curve of 0.5 °C increments from 65 °C to 95 °C was performed, enabling confirmation of amplification of single products. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method (where ΔΔCt = ΔCt sample − ΔCt reference). The efficiency of amplification ranged 95–100% for all genes studied. The expression level in each group was normalized to the control, and was presented as a fold of change (increase/decrease) (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Data were analyzed by one-way ANOVA, followed by post hoc Student-Newman-Keuls (SNK) test at a significance level of $P < 0.05$. Data that failed to pass homogeneity tests were log-transformed and re-tested. All tests were performed using Infostat Version 2008 (di Rienzo et al., 2013) software. As no differences between the two tanks of each treatment were found, the total number of fish ($n = 10$) per treatment were used for the statistics.

3. Results

3.1. Sequencing of Δ6-desaturase from pejerrey

A cDNA fragment of *Δ6-desaturase* of 1889 bp was amplified and sequenced from pejerrey brain. This sequence includes an open reading frame (ORF) with the complete coding region (441 amino acids) of pejerrey Δ6-desaturase, which contains characteristic structural features of fatty acid desaturases (Supplementary data Fig. S1). It includes two transmembrane regions, the HPGG-heme binding motif, three histidine boxes (HDxGH, HFQHH, QIEHH) and the cytochrome *b*5-like domain. Multiple alignment (Supplementary data Fig. S2) showed that the predicted amino acid sequence of pejerrey Δ6-desaturase has high percentage of similarity with other fish Δ6-desaturases, such as *Menidia estor* (89.5%), *Rachycentron canadum* (81.63%), *Oreochromis niloticus* (75.51%), *Salmo salar* (75.51%) and *Scophthalmus maximus* (78.46%). This sequence also shows 64.63% of identity with *Homo sapiens* Δ6-desaturase predicted sequence. Percentages of identity among these desaturases and others from different organisms showed that pejerrey

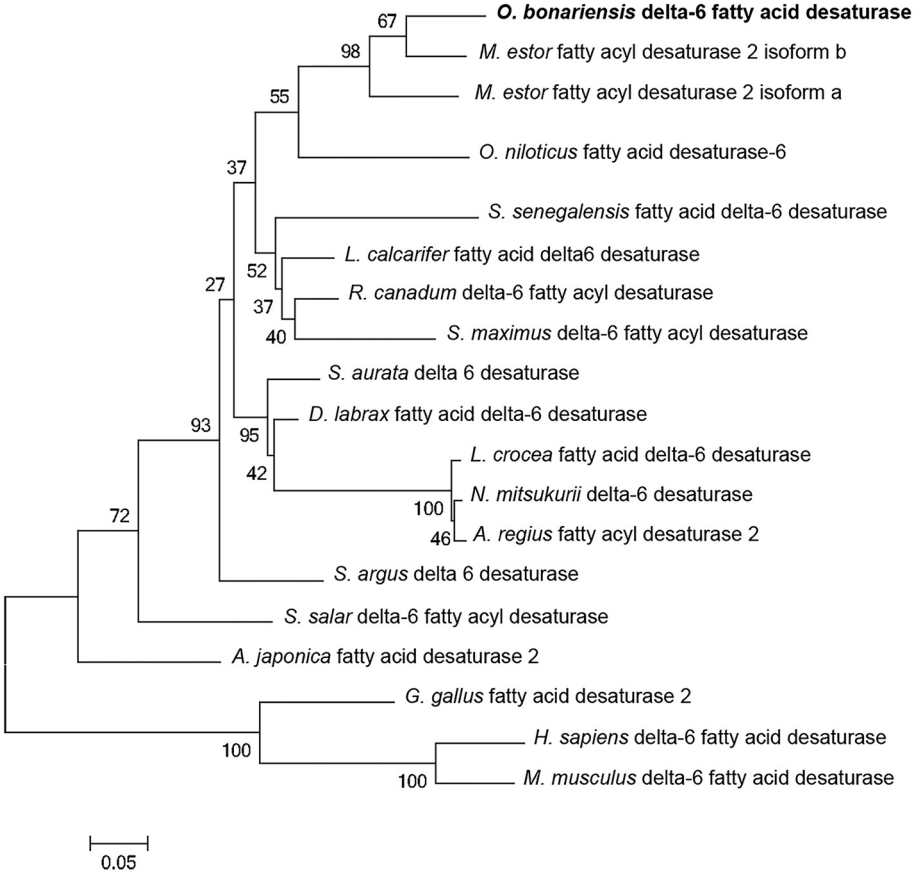

Fig. 1. Phylogenetic tree constructed by the Neighbor-joining method with MEGA6 showing the phylogenetic relationships of pejerrey Δ6-desaturase. The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 500 bootstrap iterations. *Menidia estor* isoform a (**614461325**); *Menidia estor* isoform b (**614461327**); *Oreochromis niloticus* (**543174590**); *Solea senegalensis* (**353332433**); *Lates calcarifer* (**308125307**); *Rachycentron canadum* (**215276658**); *Scophthalmus maximus* (**44921596**); *Sparus aurata* (**290576383**); *Dicentrarchus labrax* (**187438554**); *Larimichthys crocea* (**740529938**); *Nibea mitsukurii* (**261036339**); *Argyrosomus regius* (**459585655**); *Scatophagus argus* (**558554668**); *Salmo salar* (**288683293**); *Anguilla japonica* (**620689734**); *Gallus gallus* (**238028975**); *Homo sapiens* (**4406527**); *Mus musculus* (**4406525**).

desaturase shares between 75% and 89% identity with teleost fish sequences, while the identity percentage is lower (68% to 65%) when delta-6-desaturase from pejerrey is compared with the predicted sequences of birds, amphibians and mammals (Supplementary data Table S1). The phylogenetic tree (Fig. 1) clustered the pejerrey Δ6-desaturase deduced sequence with the a and b isoforms from *Menidia estor* fatty acid desaturase 2 gene. This cluster was also shared with *Oreochromis niloticus* fatty acid desaturase 6, while mammalian deduced sequences (human and mouse) were selected as outgroups.

3.2. Sequencing and characterization of nesfatin-1 in pejerrey

A cDNA fragment of 1170 bp was amplified and sequenced. This partial sequence corresponds to pejerrey *nucb2* gene as suggested by sequence analysis. As it is shown in Fig. S3A (Supplementary data), this sequence has an ORF that codes for the complete nesfatin-1 amino acid sequence (81 aa). The nefatin-1 acidic sequence was aligned with others from fish, birds and mammals (Supplementary data Fig. S3B), showing a high percentage of identity with those from other fish such as *Larimichthys crocea* (96.43%) and *Scleropages formosus* (91.67%) (Supplementary data Table S2). Also, the bioactive core of nesfatin-1 (Supplementary data Fig. S3A) has 100% identity with those from other fish, except with the bioactive core of the isoform b from *Danio rerio* in which the percent is 93.33%. The percent identity between the bioactive core of pejerrey nesfatin-1 and that of birds and mammals is lower, ranging from 70% to 57%. The phylogenetic tree (Fig. 2) showed that sequences from fish split in a separate cluster from those of birds and mammals. This analysis clustered the pejerrey nesfatin-1 sequence near those from the cyprinodontiforms order, such as *Xiphophorus maculatus*, *Poecilia reticulata*, *Poecilia formosa*, *Poecilia latipinna*, *Fundulus heteroditus*, *Cyprinodon variegatus* and *Nothobranchius furzeri*.

3.3. Fish growth parameters

As shown in Table 2, changes in water salinity did not induce changes in body weight, standard length, total length, adipose index and condition factor of pejerrey after 8 weeks of treatment. Meanwhile, the hepatosomatic index was significantly lower in those fish that were kept in the highest salinity condition (S30) during 8 weeks, while the gonadosomatic index was significant higher in those fish reared at S30 compared with those reared at S15, with intermediate values for the S2.5 group.

3.4. Water salinity increased the expression of genes of the gh-igf axis

The expression of *gh* significantly increased in the pituitary of fish reared at S2.5 and S30 compared with the S15 group (Fig. 3A). In the liver, the expression of *ghr-I* was significantly higher in S30 group compared with S2.5 and S15 groups (Fig. 3B). The expression of *ghr-II* was significantly higher in the liver of fish reared at S2.5 and S30 compared to S15 (Fig. 3C). In the brain (Fig. 3D), no differences in *igf-I* expression were detected among treatments. *Igf-I* expression increased significantly with higher water salinity in juvenile pejerrey liver (Fig. 3E). The expression of *igf-I* decreased significantly in muscle of juvenile pejerrey in S15 and S30 groups (Fig. 3F).

3.5. Water salinity modified Δ6-desaturase gene expression

No effects for water salinity on *Δ6-desaturase* gene expression were observed in the brain of juvenile pejerrey (Fig. 4A). However, low salinity (S2.5) caused a three-fold increase ($p < 0.05$) in the expression of *Δ6-desaturase* compared to the control treatment (S15) in liver and muscle (Fig. 4B and C). Furthermore, the highest salinity condition (S30) caused a 50% decrease ($p < 0.05$) in the *Δ6-desaturase* expression levels in liver compared to the controls (Fig. 4B). In muscle, *Δ6-*

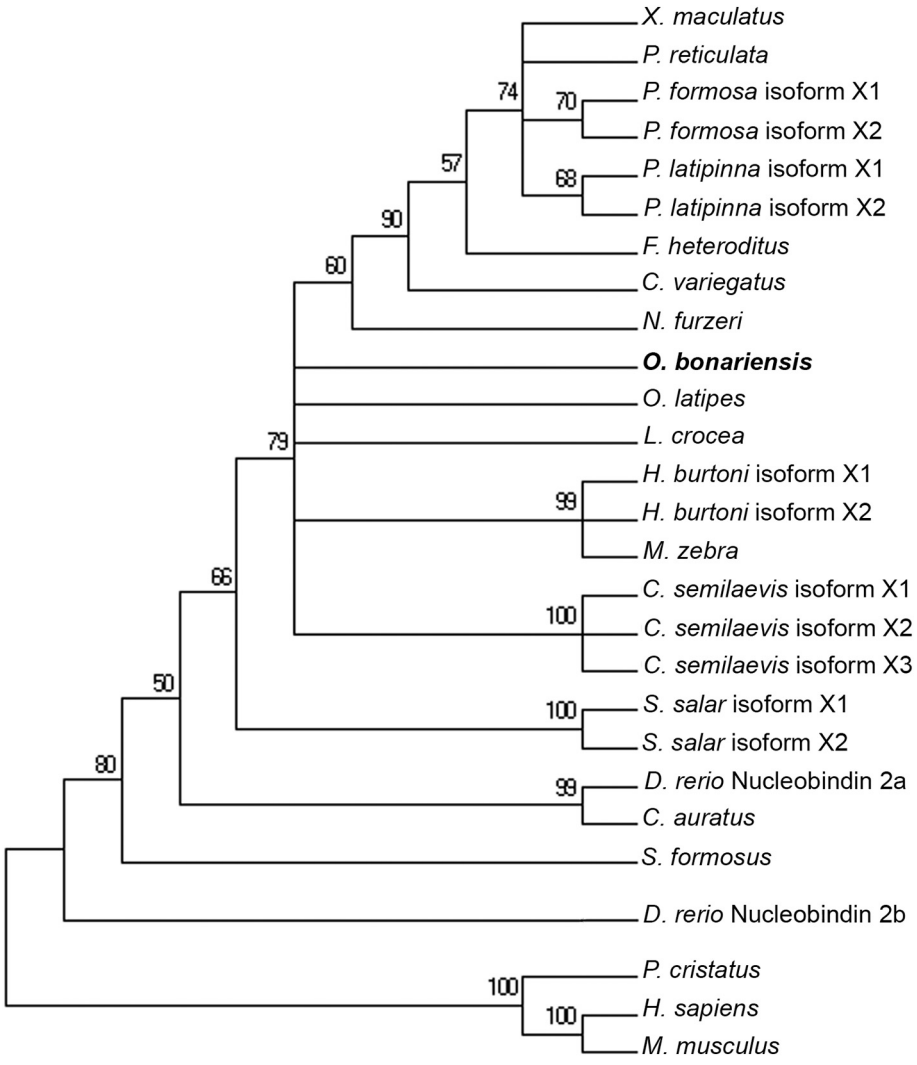

Fig. 2. Phylogenetic tree constructed by the Neighbor-joining method with MEGA6 showing the phylogenetic relationships of pejerrey nesfatin-1. The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. *Nothobranchius furzeri* (PREDICTED, **XP_015808604.1**); *Cyprinodon variegatus* (PREDICTED, **XP_015231506.1**); *Xiphophorus maculatus* (PREDICTED, **XP_005805632.1**); *Poecilia reticulata* (PREDICTED, **XP_008403447.1**); *Fundulus heteroclitus* (PREDICTED, **XP_012706149.1**); *Poecilia formosa* isoform X1 (PREDICTED, **XP_007554116.1**); *Poecilia formosa* isoform X2 (PREDICTED, **XP_016527953.1**); *Poecilia latipinna* isoform X1 (PREDICTED, **XP_014905457.1**); *Poecilia latipinna* isoform X2 (PREDICTED, **XP_014905458.1**); *Cynoglossus semilaevis* isoform X1 (PREDICTED, **XP_008308284.1**); *Cynoglossus semilaevis* isoform X2 (PREDICTED, **XP_008308285.1**); *Cynoglossus semilaevis* isoform X3 (PREDICTED, **XP_008308286.1**); *Maylandia zebra* (PREDICTED, **XP_004543259.1**); *Haplochromis burtoni* isoform X1 (PREDICTED, **XP_005931497.1**); *Haplochromis burtoni* isoform X2 (PREDICTED, **XP_005931498.1**); *Oryzias latipes* (PREDICTED, **XP_004067067.1**); *Salmo salar* isoform X1 (PREDICTED, **XP_014031425.1**); *Salmo salar* isoform X2 (PREDICTED, **XP_014031426.1**); *Larimichthys crocea* (**KKF17838.1**); *Danio rerio* isoform A (**AAH65437.1**); *Danio rerio* isoform B (**AAH47852.1**); *Carassius auratus* (**ADK94363.1**); *Scleropages formosus* (**KPP73951.1**); *Podiceps cristatus* (**KFZ66430.1**); *Homo sapiens* (**NP_005004.1**); *Mus musculus* (**NP_001123951.1**).

Table 2
Data on growth performance and somatic indexes of fish treated with different water salinities during 8 weeks.

	S2.5	S15	S30
Initial body weight (g)	22 ± 5	20 ± 2	21 ± 2
Final body weight (g)	21 ± 4	20 ± 2	19 ± 2
Initial standard length (cm)	11.4 ± 0.8	11.4 ± 0.5	11.4 ± 0.4
Final standard length (cm)	11.5 ± 0.8	11.0 ± 0.5	11.3 ± 0.4
Final K	0.82 ± 0.05	0.84 ± 0.04	0.79 ± 0.05
Hepatosomatic index	1.0 ± 0.1 a	0.98 ± 0.08 a	0.68 ± 0.06 b
Gonadosomatic index	0.5 ± 0.2 ab	0.27 ± 0.05 a	0.9 ± 0.2 b
Adipose index	0.6 ± 0.2	0.51 ± 0.06	0.4 ± 0.1

Each value is the mean ± SEM of 10 fish. All data were analyzed by one way ANOVA followed by post hoc Student-Newman-Keuls (SNK) test at a significance level of $P < 0.05$. Condition Factor "K": (weight/Total length^3) × 100. Hepatosomatic index: (Liver weight (g)/Fish weight (g)) × 100. Gonadosomatic index: (Gonad weight (g)/Fish weight (g)) × 100. Adipose index: (Adipose tissue weight (g)/Fish weight (g)) × 100.

desaturase expression levels remained unaltered in fish exposed to a high salinity compared to the control group (Fig. 4C).

3.6. Increased water salinity induced nucb2/nesfatin-1 expression

Effects of water salinity on *nucb2/nesfatin-1* mRNA expression are shown in Fig. 5A and B. The highest salinity (S30) caused a significant increase in *nucb2/nesfatin-1* mRNA expression in the brain and liver of juvenile pejerrey compared to other treatments. No differences in *nucb2/nesfatin-1* expression in the brain and liver were detected between S2.5 and S15 treatments in pejerrey juveniles.

4. Discussion

4.1. Sequencing and characterization of Δ6-desaturase and nesfatin-1 from pejerrey

In this work, we report the sequences of pejerrey *Δ6-desaturase* and *nesfatin-1*, and how these genes, together with gh-igf, are modulated by salinity. We found an 1889-bp ORF corresponding to pejerrey cDNA, having all the features of Δ6-desaturases previously reported, and which are conserved in all members of this enzyme family (López Alonso et al., 2003; Los and Murata, 1998). This cDNA sequence was translated into peptide sequence and the phylogenetic analysis shows that the deduced protein sequence has been clustered with the a and b isoforms of fatty acid desaturase 2 gene from the atherinid fish *Menidia estor*. The isoform a has a Δ4-desaturase activity while the isoform b, which is the isoform with the highest percent of identity with the pejerrey sequence, has both Δ5 and Δ6-desaturase activities (Fonseca-Madrigal et al., 2014). Also, as we expected, the phylogenetic analysis located the pejerrey cDNA sequence close to other sequences from teleost fishes including the perciforms (*Oreochromis niloticus*, **AGV52807.1**; *Lates calcarifer*, **ACS91458.1**) and salmonids (*Salmo salar*, **NP_001165752.1**), but far from sequences of birds (*Gallus gallus*, **NP_001153900.1**) and mammals (*Mus musculus*, **Q9Z0R9.1**; *Homo*

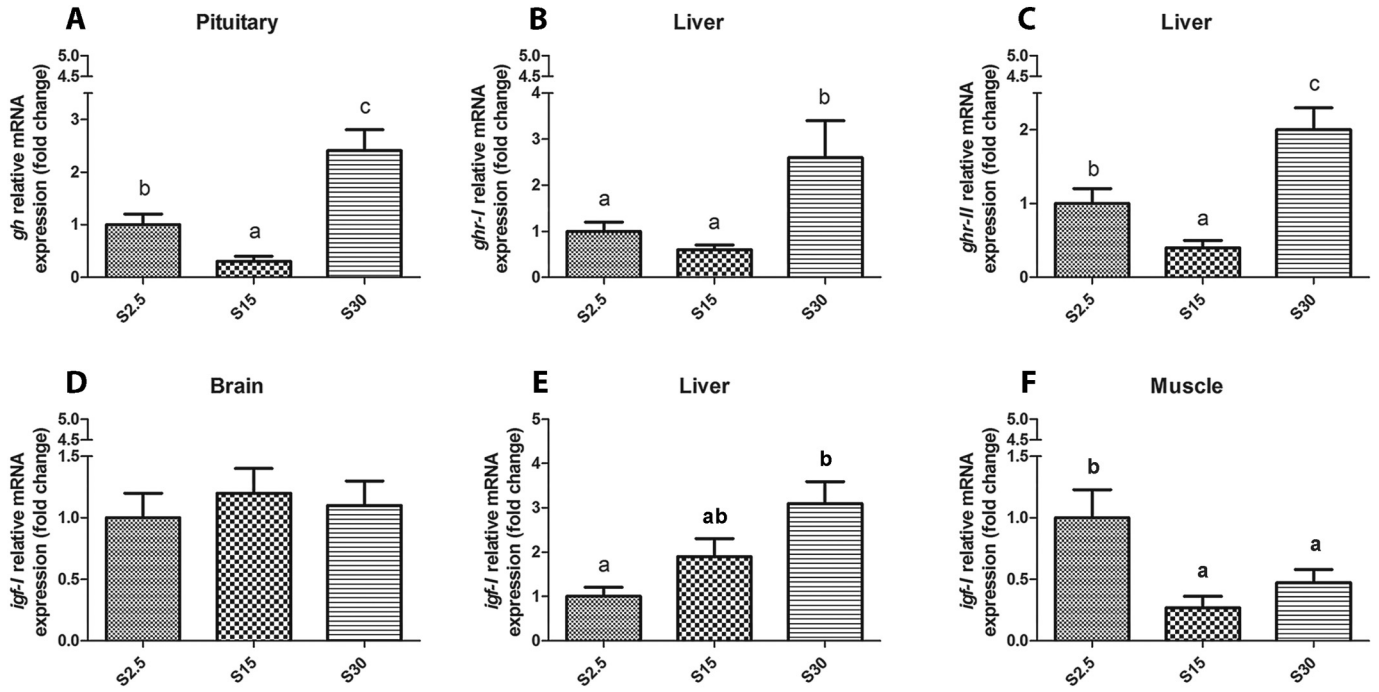

Fig. 3. Influence of water salinity on *gh* mRNA expression in pituitary (A), *ghr-I*, *ghr-II* mRNA expression in liver (B, C) and *igf-I* mRNA expression in brain (D), liver (E) and muscle (F), of pejerrey juvenile ($n = 10$). Bars represent media +/− standard error of 10 replicates. Different letters indicate significant differences among treatments ($p < 0.05$). S2.5, S15 and S30 refer to water salinity of 2.5, 15 and 30 g of NaCl per liter of water, respectively.

sapiens, **O95864.1**). These results indicate that the cDNA sequence identified here belongs to fatty acid desaturases family, specifically to those that present Δ6-desaturase activity. Therefore, this sequence has the potential to code for a functional protein with Δ6-desaturase activity, although functional assays should be performed. In addition, we obtained a partial cDNA sequence of *nucb2* from pejerrey, which codifies the complete amino acid sequence of the anorexic peptide nesfatin-1. The high percentage of identity between the pejerrey nesfatin-1 and perciforms sequences is usual for pejerrey sequences (Sciara et al., 2006; Strobl-Mazzulla et al., 2005). Moreover, the pejerrey nesfatin-1 sequence was located near to the cyprinodontiforms sequences in the phylogenetic tree. This is in agreement with the fact that cyprinodontiforms and atheriniforms are closely related (Hernandez et al., 2008). The presence of duplicated NUCB2 genes in teleost fish presumably arose by an independent tetraploidization known as 3R (third round of genome duplication) (Christoffels et al., 2004; Dehal and Boore, 2005), causing paralogous versions of genes. This is observed in a wider phylogenetic analysis of *nucb2* sequences, where several teleosts display strong clustering for two paralogous *nucb2* genes, named *nucb2* A and *nucb2* B (Gonzalez et al., 2010; Hatef et al., 2015). As most of the sequences used for the phylogenetic analysis presented in our work are predicted sequences, we cannot be certain on which subtype of *nucb2* they belong to. Nevertheless, this analysis shows pejerrey sequence clustered near to others that are known to belong to the *nucb2* A subgroup, such as *Danio rerio* or *Carassius auratus*. This analysis also shows the *Danio rerio nucb2* B sequence separately from those that belong to other fish, which are presumably from the *nucb2* A subgroup.

4.2. Fish growth parameters and index

During the experiment, no changes in body weight and length of pejerrey juveniles were observed. As the main objective of this work was to determine the effect of water salinity on the expression of mRNAs involved in growth, metabolism and food intake, the period of exposure was 8 weeks. Since pejerrey juveniles have low growth rates (Miranda et al., 2006; Somoza et al., 2008), this period was long enough to observe changes in mRNA expression, but perhaps not long enough to reveal differences in somatic growth. The hepatosomatic index decreased in those fish maintained at S30. A decrease of the hepatosomatic index with the increase of water salinity was previously reported in rainbow trout fed with a high carbohydrate diet (Krogdahl et al., 2004). Since water salinity affects fish metabolic processes in which the hepatopancreas plays a key role, we hypothesize that an increase in the PUFAs and HUFAs metabolism with the increase in the

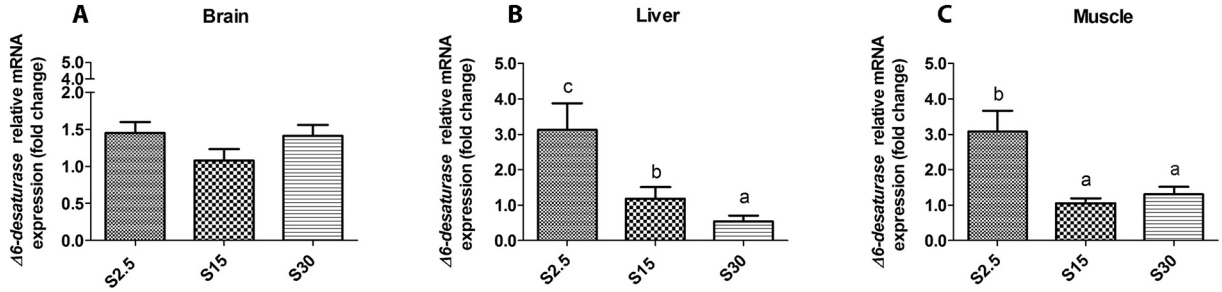

Fig. 4. Influence of water salinity on *Δ6-desaturase* mRNA expression in brain (A), liver (B) and muscle (C) of pejerrey juvenile ($n = 10$). Bars represent media +/− standard error of 10 replicates. Different letters indicate significant differences among treatments ($p < 0.05$). S2.5, S15 and S30 refer to water salinity of 2.5, 15 and 30 g of NaCl per liter of water, respectively.

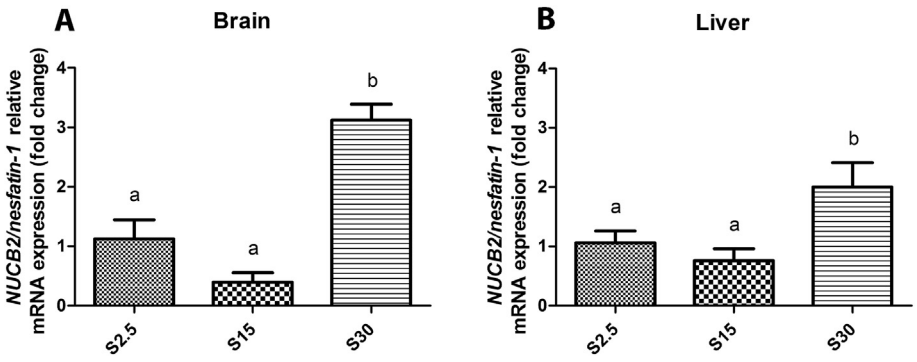

Fig. 5. Influence of water salinity on *NUCB2/nesfatin-1* mRNA expression in brain (A) and liver (B) of pejerrey juvenile ($n = 10$). Bars represent media +/− standard error of 10 replicates. Different letters indicate significant differences among treatments ($p < 0.05$). S2.5, S15 and S30 refer to water salinity of 2.5, 15 and 30 g of NaCl per liter of water, respectively.

water salinity to hold the fish growth could led to a decrease in liver lipid deposits, decreasing the weight of this organ (Vargas-Chacoff et al., 2015). The gonadosomatic index was high in fish reared in S30 compared to those reared at S15. This may indicate a gonadal maturation triggered by the increase in the water salinity from 15 g/L to 30 g/L. We did not find differences between the S2.5 and S30, but the standard error was high enough in these treatments to mask any possible significant effect.

4.3. Influence of water salinity on the gh-igf axis gene expression

In the pituitary and liver, the *gh*, *ghr-I and ghr-II* mRNA expression shows an upregulation with the increase of water salinity from S2.5 and S15 to S30. In the cases of the *gh* and *ghr-II*, their mRNA expression is also high in S2.5 compared with the S15. As S15 is the condition at which the juveniles were reared before the beginning of the experiment, the increase in *gh*, *ghr-II* mRNA expression could be a result of a stress response. In rainbow trout, it has been reported that the plasmatic GH levels are high when fish are under chronical stress (Pickering et al., 1991). In tilapia, which grows better in sea water, the transfer from sea water to fresh water generates an elevation on plasmatic GH (Riley et al., 2003). Additionally, as GH is involved in the regulation of energy metabolism, this upregulation found in pejerrey juveniles could be due to a mobilization of energy stores that are required for osmoregulation. The highest mRNA expression levels of *gh*, *ghr-I* and *ghr-II* was produced by the S30 treatment, which could be explained by the key role that GH has in the hyperosmolar tolerance in fish (Ababutain, 2011; Deane and Woo, 2009; Reindl and Sheridan, 2012; Reinecke, 2010). The action of GH seems to be related with branchial osmoregulatory function, including the activity and distribution of chloride cells (Evans et al., 1999; McCormick, 1995; Olson, 2002), and associated with these cells are several ion transport mechanisms, including the sodium pump enzyme (Deane and Woo, 2009). The osmolarity adaptive effects of GH seem to be mediated by those of IGF-I since it has been shown that IGF-I decreased plasma osmolality and increased gill Na +, K +-ATPase activity (Feidantsis et al., 2013; Inoue et al., 2003; McCormick, 1995; Meier et al., 2009). Also, IGF-I induces the expression of genes involved in osmotic regulation in Atlantic salmon (Yada et al., 2012). Additionally, it was demonstrated by Mancera and McCormick (1998) that the application of both GH and IGF-I had a significantly higher effect than either hormone alone. As in the pituitary and liver, the increase of water salinity from S15 to S30 produced higher expression of genes in the *gh-igf* axis than those caused by the decrease from S15 to S2.5, it is possible to hypothesize that this axis is not only mediating the osmoregulatory response, but it also is influencing the somatic growth in response to an increase in water salinity. There are examples of increasing growth with an increase in water salinity (Boeuf and Payan, 2001), but whether this would be the case in pejerrey remains unknown. The morphometric data do not show differences in somatic growth among groups. The changes in the mRNA expression of the GH-IGF axis genes might result in an increase in somatic growth during a long-term exposure, which warrants future consideration to test such extended time dependent effects.

4.4. Influence of water salinity on the Δ6-desaturase gene expression

Another factor that seems to be related with water osmolarity is the unsaturated fatty acid levels (Sui et al., 2007). In this research, we found that water salinity influences the expression of *Δ6-desaturase* in both liver and muscle, suggesting that PUFAs and HUFAs are regulated by water salinity in these tissues. More specifically, our studies showed a decrease in the expression levels of *Δ6-desaturase* with the increase in water salinity, which could generate a decrease in the unsaturation levels of the membrane lipids. Supporting this, Sarker et al. (2011) found that EPA and DHA were higher in the liver of red sea bream reared at 15 and 20 ppt of NaCl than those reared at 33 ppt. Additionally, in that work, the authors detected transcripts of *Δ6-desaturase* by RT-PCR in the liver of fish reared at 15 ppt while the same transcript was not detected when the fish were reared at 33 ppt (in both cases fish were fed with diets that replace fish oil with vegetable oil). We conclude that a decrease in the expression of *Δ6-desaturase* in response to an increase of water salinity might decrease the amount of PUFAs present in cell membranes, decreasing its fluidity and therefore water loss. Interestingly, we did not find any difference in the *Δ6-desaturase* expression among treatments in brain, the tissue with the highest levels of PUFAs (Mourente, 2003; Mourente and Tocher, 1992; Tocher and Harvie, 1988). As mentioned previously, the presence of PUFA in neural tissues is associated with the maintenance of cell membrane properties which is important for nerve conduction and consequently for the proper function of this tissue (Khodayari et al., 2015; Zangiabadi et al., 2007). Therefore, brain needs to maintain the membrane properties constant, regardless osmolarity challenges. This could explain the absence of modulation of *Δ6-desaturase* gene expression found in pejerrey brain.

4.5. Influence of water salinity on the nucb2/nesfatin-1 gene expression

Several reports have demonstrated that changes in water osmolarity not only affect growth rates, but also stress levels and food intake (Boeuf and Payan, 2001; Deane and Woo, 2009; Likongwe et al., 1996; Rhee et al., 2012; Tsuzuki et al., 2001). In this context, the upregulation of *nucb2/nesfatin-1* mRNA that we found in brain and liver with the salinity of 30 g/L could be associated with those changes in food intake. As nesfatin-1 is an anorexigenic peptide, our results suggest an anorexigenic effect of the increase in water salinity. This could be associated with changes in energy metabolism and distribution, as we discussed before, mediated by the GH/IGF axis. Nesfatin-1 is a multifunctional peptide with effects on many organs (Mohan and Unniappan, 2013; Ramesh et al., 2017). This result, together with the decreased hepatosomatic index and the highest gh mRNA expression found in S30, could be explained by a lipolytic action mediated by the GH in response to a high-water salinity concentration. We did not observe differences

among groups in the adipose index, but it is widely known that fish use the liver for lipid storage (Sheridan, 1988).

5. Conclusion

In summary, we report the sequences of Δ6-desaturase and nesfatin-1 from pejerrey. Both sequences are similar to those found in other teleosts and possess functional features reflective of biological actions of these peptides in pejerrey. Results of water salinity trials showed an effect of this parameter on the expression of genes in the *gh-igf* axis, the *Δ6-desaturase* and *nucb2/nesfatin-1*. These results seem to indicate a coordinated response to water salinity variations, mediated by the gh-igf axis, which results in changes in the expression of genes involved in lipid metabolism and food intake regulation. Bearing in mind that fish were reared at S15 before the commencement of the experiment, the increase in the expression of *gh* or *igf-I* when fish were keep at S2.5 or S30 can be attributed to a stress response. But on the other hand, the expression pattern of other members of the *gh-igf* axis, such as *ghr-I*, *ghr-II* and *igf-I*, may indicate an effect of S30 condition that could lead to somatic growth in the long term.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2017.05.005.

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