JFB	jfb_12954	B	Dispatch: March 7, 2016	Journal: JFB	CE: Praveena
Journal Name	Manuscript No.		Author Received:	No of pages: 17	TS: suresh

Journal of Fish Biology (2016) **0**, 0–0
doi:10.1111/jfb.12954, available online at wileyonlinelibrary.com

Growth hormone receptors in the atherinid *Odontesthes bonariensis*: characterization and expression profile after fasting–refeeding and growth hormone administration

P. E. Botta, I. Simó, A. A. Sciara and S. E. Arranz*

Instituto de Biología Molecular y Celular de Rosario, CONICET – Área Biología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Argentina

(Received 9 December 2014, Accepted 16 February 2016)

In order to improve the understanding of pejerrey *Odontesthes bonariensis*, growth hormone (Gh)-insulin-like growth factor-1(Igf1) axis, *O. bonariensis* growth hormone receptor type 1 (*ghr1*) and type 2 (*ghr2*) mRNA sequences were obtained. Both transcripts were ubiquitously expressed except in kidney, encephalon and anterior intestine. Alternative transcripts of both receptors were found in muscle. Interestingly, two different *ghr2* transcripts with alternative polyadenylation (APA) sites located in the long 3′ untranslated region (UTR-APA) were also found in liver. Hepatic *ghr1*, *ghr2* and insulin-like growth factor type 1 (*igf1*) transcript levels were examined under two different metabolic conditions. In the first experimental condition, fish were fasted for 2 weeks and then re-fed for another 2 weeks. Despite *igf1* mRNA relative expression did not show significant differences under the experimental period of time examined, both *ghr* transcripts decreased their expression levels after the fasting period and returned to their control levels after re-feeding. In the second treatment, recombinant *O. bonariensis* growth hormone (r-pjGh) was orally administered once a week. After 4 weeks of treatment, liver *igf1, ghr1* and *ghr2* mRNA relative expression increased (13, 4·5 and 2·1 fold, $P < 0{\cdot}05$) compared to control values. These results add novel information to the growth hormone-insulin-like growth factor system in teleosts.

Key words: alternative mRNA; compensatory growth; ghr; pejerrey.

INTRODUCTION

The growth hormone (Gh) plays a major role in vertebrate growth regulation. This hormone binds the growth hormone receptor (Ghr) to regulate Igf1 synthesis and secretion in target tissues (Moriyama *et al.*, 2000). Despite the relevance of this receptor as a mediator of Gh action, the presence of ghr isoforms (Reindl & Sheridan, 2012; Fuentes *et al.*, 2013) and their tissue-specific expression are still poorly understood in teleosts.

Serum levels of free Gh are modulated by a growth hormone-binding protein (Ghbp). In addition, the action of Gh can be further regulated by tuning levels of Ghr expression (Le Roith *et al.*, 2001). The growth hormone receptor is codified by the *ghr* gene

*Author to whom correspondence should be addressed. Tel.: +54 341 4237070 ext. 649; email: arranz@ibr-conicet.gov.ar

and ubiquitously expressed in mammals and birds. A number of Ghr mRNA variants have been described, where most of them show different 5′ UTR. These differences have been proposed to regulate protein synthesis in different tissues or under different physiological conditions (Leung *et al.*, 1987; Edens & Talamantes, 1998). The growth hormone-binding protein (Ghbp) has the same sequence as the Ghr but lacks most of the intracellular region (Baumann, 2002). Interestingly, like Ghr, Ghbp is also codified by the *ghr* gene. Two different mechanisms regulate Ghbp synthesis, depending on the species (Le Roith *et al.*, 2001). One mechanism produces Ghbp by alternative splicing whereas the other involves proteolytic cleavage of the full-length receptor. Although mammals and birds present one *ghr* gene, two different genes (*ghr1* and *ghr2*) encoding different Ghr isoforms were found in almost all fishes analysed (Calduch-Giner *et al.*, 2001; Lee *et al.*, 2001; Calduch-Giner, 2003; Tse *et al.*, 2003; Fukada *et al.*, 2004; Fukamachi, 2005; Di Prinzio *et al.*, 2010; Walock *et al.*, 2014). These genes are orthologous to the mammalian *ghr* and have been most likely acquired through a fish-specific genome duplication event (Fukamachi & Meyer, 2007). Both types of fish Ghr present around 40% identity between each other (Fukamachi & Meyer, 2007), and differ essentially in the cysteine and tyrosine number of the extracellular and intracellular regions. Both genes are ubiquitously expressed, with transcript levels that vary with nutritional status (Tse *et al.*, 2003; Saera-Vila *et al.*, 2005; Small *et al.*, 2006; Breves *et al.*, 2014). Relative variations in ghrs transcript levels were sometimes measured by RT-qPCR without considering the probable existence of alternative transcripts encoding Ghr isoforms or Ghbp. The presence of a soluble Ghbp has been demonstrated in the blood of rainbow trout *Oncorhynchus mykiss* (Walbaun 1792), goldfish *Carassius auratus* (L. 1758) and Atlantic salmon *Salmo salar* L. 1758 (Sohm *et al.*, 1998; Zhang & Marchant, 1999; Einarsdóttir *et al.*, 2014). Which of the *ghr* genes give rise to Ghbp and the mechanism of synthesis, however, remain to be clarified.

The study of growth-related factors can be used to predict whether a fish is using its energy to maintain homeostasis or for somatic growth (Beckman, 2011). Accordingly, several studies have shown a decrease in hepatic Ghr expression during fasting, suggesting that a reduction in hepatic Ghr contributes to a Gh resistance state (Uchida *et al.*, 2003; Pierce *et al.*, 2005; Small *et al.*, 2006). On the other hand, few studies have analysed hepatic *ghr* transcription levels under Gh administration. Furthermore, conflicting results have been reported in ghr mRNA levels under Gh treatment (Gahr *et al.*, 2008, 2009; Shved *et al.*, 2011; Breves *et al.*, 2014) possibly due to the presence of truncated Ghr (Ghrt) variants that result in opposite physiological effects (Fuentes *et al.*, 2012).

Pejerrey *Odontesthes bonariensis* (Valenciennes 1835) is a South American euryhaline fish considered to be the largest atheriniform in the world (Dyer & Chernoff, 1996). *Odontesthes bonariensis* presents a high quality flesh and commercial value. Its slow growth rate in captivity, however, is a drawback for its production (Somoza *et al.*, 2008). In order to obtain a more comprehensive understanding of how growth is controlled, Gh and Igf-1 mRNA sequences from *O. bonariensis* have been characterized (Sciara *et al.*, 2006, 2008), and a biologically active recombinant *O. bonariensis* Gh (r-pjGh) has been obtained. Fish that were orally administered r-pjGh (2 μg g^{-1} of body mass) produced a 30% increase in mean body mass and stimulated liver igf-1 mRNA expression after 1 month of treatment (Sciara *et al.*, 2011). Higher r-pjGH doses, however, negatively affected somatic growth

The present study was performed to gain insight into the Gh/Igf system and Ghrs regulation under various different physiological conditions, considering the possible existence of ghr mRNA variants that would negatively regulate Gh action.

MATERIALS AND METHODS

ANIMAL CARE, EXPERIMENTAL DESIGN AND SAMPLING

Adult tissue sampling

Odontesthes bonariensis were obtained from IIB-INTECH aquatic facilities (Chascomús, Buenos Aires, Argentina). Adult *O. bonariensis* were maintained in 20 000 l tanks at 18–20° C under natural photoperiod (32° S). Fish were selected, anaesthetized in 0·1 g l^{-1} of benzocaine solution and killed by decapitation for tissue collection and analysis. Samples were collected and stored in RNAlater solution (Qiagen; www.qiagen.com) at −20° C until analysis. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina.

Fasting and re-feeding growth assessment

Sixty *O. bonariensis* juveniles of 0·21 ± 0·03 g (mean ± S.D.) were distributed among six 100 l tanks. Temperature was maintained at 22° C, range ± 1° C and water salinity at 15. Fish were exposed to a constant photoperiod (14L:10D) and acclimatized for 2 weeks before the beginning of experiments. Specimens were fed two times a day with commercially available food and once a day with *Artemia* sp. After this period, one group continued to be fed (control group, three tanks) while the other was fasted for 2 weeks and then re-fed for another 2 weeks (fasting and re-feeding group, three tanks). Body mass (M_B) and total length (L_T) of all specimens were measured at week 0, 2 and 4. Fulton condition factor was calculated for each sample ($K = M_B\, 100\, L_T^{-3}$).

Fasting and pjGH administration for gene expression determination

In order to evaluate growth hormone oral administration and fasting effects on liver growth hormone receptor transcript levels, 72 juveniles of 14·5 ± 1·5 g (mean ± S.D.) were distributed among three 100 l tanks. Temperature was maintained at 19° C, range ±1° C and water salinity at 15. Fish were exposed to a constant photoperiod (14L:10D) and acclimatized for 2 weeks before the beginning of experiments. Specimens were fed three times a day with commercially available food. After this period, one group continued to be fed (control group) while the other was fasted for 2 weeks and then re-fed for another 2 weeks (fasting and re-feeding group). The last group was subjected to oral administration of 2 µg r-pjGh g^{-1} of body mass once a week, applying the protocol previously used by Sciara *et al.* (2011). Three samples were taken during experiments, the first one on day zero, the second one on the 17th day (after the fasting period had finished) and the last one on the 31st day. In each sample, six fish were randomly selected from each treatment and sacrificed by overdose of benzocaine (100 mg l^{-1}). Liver samples were collected rapidly and stored in RNAlater solution (Qiagen) at −20° C for further analysis.

RNA PURIFICATION AND CDNA SYNTHESIS

Total RNA for each tissue sample was extracted using Trizol Reagent (Invitrogen; www.thermofisher.com) following the manufacturer's instructions. RNA concentration and purity were determined by absorbance measurements at 260 and 280 nm, and integrity tested by gel-electrophoresis. Polyadenylated RNA was isolated from liver total RNA using a PolyATract kit (Promega; www.promega.com). Liver total RNA samples were treated with DNase-I (RQ1 DNase, Promega), and reverse-transcribed using the Transcriptor First Strand

cDNA Synthesis Kit (Roche Life Science; https://lifescience.roche.com/). Reactions lacking reverse transcriptase were included as negative controls.

CLONING AND SEQUENCING OF GHRS

Reactions lacking reverse transcriptase were included as negative controls. The synthesized cDNAs were used as templates for PCR amplification using gene degenerate primers (dGHR1F/R, dGHR2F/R; Table SI, Supporting Information) designed considering the full-length sequences alignment of known *ghr* sequences from other teleosts. PCR amplifications were carried out by a routine procedure.

Rapid amplification of the cDNA 5′ and 3′ ends was performed with liver mRNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences; www.roche-applied sciences.com) and nested reactions. Primer pairs (Table SI, Supporting Information) used for 3′ RACE were (5′–3′): ghr1 (forward, PjRACE3extGH and PjRace3intGHR1; reverse, Qt_2 and Q_0) and ghr2 (forward, PjRace3ext and pjRACE 3 int; reverse, Qt_2 and Q_0). Primer pairs (Table SI, Supporting Information) used for 5′ RACE were (5′–3′): ghr1 complementary DNA for RACE 5′ was synthesized with specific primers for *ghr1* and *ghr2* (RACE 5 ext ghr1 and pjRACE 5 ext). Both cDNAs were purified with CENTRICOM 100 (Merck Millipore; www.merckmillipore.com) and treated with terminal deoxynucleotidyl transferase (Promega) and dATP. The first PCR was run with specific forward primers for each receptor (pjRACE 5 ext and pjRACE 5 ext ghr1), and reverse primers Q_0 and Qt_2. Nested PCRs were then performed with 1 μl of a 1/20 dilution of the first PCR, forward primers (RACE 5 int ghr1 and pjRACE 5 int) and reverse primer R_2. Resulting PCR products were cloned in pGEM-T easy vector (Promega) and sequenced. Once assembled, the sequence data encoding the full-length cDNA of *ghr1* and *ghr2* were deposited in the GenBank database under accession numbers KF055461 (*ghr1* transcript), KF055459 (*ghr2* short transcript) and KF055460 (*ghr2* long transcript). Messenger RNA region corresponding to the complete ORF was also amplified for each receptor using specific primers GHR1ORF-F/R and GHR2ORF-F/R (Table SI, Supporting Information) and subsequently sequenced.

NORTHERN BLOT

Total RNA from muscle, liver and adipose tissue were obtained from 10 specimens. Samples (40 μg) were pooled for each tissue, concentrated by ethanol precipitation (Sambrook & Russell, 2001) and solubilized in sterilized distilled water ($V_f = 5$ μl). RNA integrity was checked by agarose gel electrophoresis. Probes for *ghr1* and *ghr2* were synthesized by PCR using cDNA fragments corresponding to the extracellular region of each *ghr* transcript (RE*ghr1*F/RE*ghr1*R and RE*ghr2*F/RE*ghr2*R primer pairs). Northern blot detection was performed using standard procedures (Sambrook & Russell, 2001).

SEQUENCE ANALYSIS, DATABASE HOMOLOGY SEARCH AND SEQUENCE ALIGNMENT

Sequences were assembled using the Ape-A plasmid Editor v2.0.45 (Wayne Davis; http://biologylabs.utah.edu/jorgensen/wayned/ape/). Database homology search was performed using the BLASTN and BLASTP programmes (Altschul *et al.*, 1997). Phylogenetic analysis and alignments were carried out with Mega 5, version 5.1 (Tamura *et al.*, 2011), and Clustal X software version 2.0 (Larkin *et al.*, 2007). Proteins encoded by sequenced cDNAs were tested for the presence of signal peptides at SignalP 3.0 Server (Bendtsen *et al.*, 2004; www.cbs.dtu.dk/services/SignalP) and OCTOPUS server (Viklund *et al.*, 2008; http://octopus.cbr.su.se/). N-linked glycosylation sites were predicted at NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc), and putative phosphorylation sites with NetPhos 2.0 Server (Blom *et al.*, 1998; www.cbs.dtu.dk/ser-vices/NetPhos). Putative transmembrane regions were predicted with Protscale Server (Wilkins *et al.*, 1999; www.expasy.ch/tools/protscale.html) and TMHMM Server v.2.0 (Krogh *et al.*, 2001; www.cbs.dtu.dk/services/TMHMM).

TISSUE DISTRIBUTION

Samples of liver, white and red muscle, anterior and posterior intestine, spleen, male and female gonads, heart, skin, brain and adipose tissue were obtained from adult fish. Furthermore, brain tissue was divided into three types of samples according to Roberts (2001): anterior encephalon (diencephalon and telencephalon), medium encephalon (which includes hypothalamic area and optic tectum) and posterior encephalon (includes cerebellum and spinal cord). Total RNA was extracted and cDNA synthesized as described above. PCRs were performed using 2 U Taq Platinum DNA Polymerase High Fidelity (Invitrogen, www.thermofisher.com/ar). Specific *ghr1* and *ghr2* transcripts were detected using qpcrpjGHR1F3/qpcrpjGH-R1-R3 and pjGHR2ri-F/pcrpjGHR2-R2. Primers hybridizing to different exons were used to amplify the intracellular region of each *ghr*, in order to avoid both *ghbp* putative transcript amplification and genomic contamination. PCR amplified fragments were resolved by electrophoresis in 1·5% (w:v) agarose gels. Another PCR protocol with the same steps but applying 50 cycles was performed in selected samples when *ghr1* or *ghr2* transcripts were undetected.

REAL-TIME PCR

Expression levels of *ghr1* and *ghr2* were determined in *O. bonariensis* liver tissue ($n = 6$) by RT-qPCR (SYBR Green I, Invitrogen) using eukaryote elongation factor 1 alfa (*eef1a*; GenBank accession number KM273019) as reference gene for normalization. Reference and target gene expression was calculated by the relative quantification method with PCR efficiency correction (Pfaffl *et al.*, 2002). Target gene data were normalized to e*ef1a* and relative quantification was achieved by comparing the normalized data from treatment samples with corresponding controls. Relative gene expression ratios between groups are reported as a fold-change from controls. To avoid the use of interplay calibrator, all samples for each treatment and control group were analysed for each gene in the same plate. cDNA was synthesized from every tissue as previously described. PCRs were performed in triplicates on a Stratagene Mx-3005P qPCR system (Thermo Scientific, www.thermoscientific.com), and threshold cycle numbers were averaged. For each particular gene, PCR efficiency was the same (94–104·5%) for all serial dilutions of cDNA tested (Pfaffl, 2001). Agarose gel electrophoresis revealed a single band of expected size, and all amplified products showed a single melting peak on RT-PCR. Each gene was analysed separately using specific primers for *ghr1* and *ghr2* corresponding to the intracellular coding region (qpcrpjGHR1 F/R, qIGF1 F/R; Table SI, Supporting Information); *igf1* (qpcrpjGHR2 F/R, Sciara *et al.*, 2011) and e*ef1a* (qEF1 F/R; Table SI, Supporting Information). PCR was run in 10 µl final volume containing FastStart Universal SYBR Green Master Mix (Roche Life Science).

STATISTICAL ANALYSIS

Statistical analysis of growth variables (M_B, L_T and K) were performed using a nested ANOVA test (Statistica 8.0, http://statistica.software.informer.com/8.0/). Statistical analysis of qPCR results was performed using the REST software (Pfaffl, 2001; rest.gene-quantification.info) considering treatment as variable. Statistically significant differences with respect to the control group were analysed by the pair-wise fixed reallocation randomization test (Pfaffl *et al.*, 2002). Significance levels of $P \leq 0{\cdot}05$ were chosen to determine differences between samplings or treatments. Analytical variations are presented as mean ± S.D.

RESULTS

GHR SEQUENCE ANALYSIS AND PHYLOGENY

Odontesthes bonariensis ghr1 and *ghr2* cDNAs sequences were obtained from liver. *Ghr1* cDNA has 3460 nucleotides (nt) and comprised an open reading frame (ORF)

	GHR *H. sapiens*	SLR *O. latipes*	GHR1 *O. bonariensis*	GHR1 *E. coioides*	GHR1 *O. niloticus*	GHR1 *S. aurata*	GHR *O. latipes*	GHR2 *O. bonariensis*	GHR2 *E. coioides*	GHR2 *O. niloticus*	GHR2 *S. aurata*	
GHR *H. sapiens*	***	31·7	32·4	32·7	30·5	32·8	34·0	31·7	32·8	32·3	31·3	GHR *H. sapiens*
SLR *O. latipes*		***	72·9	73·1	67·2	71·9	39·7	40·5	41·9	39·4	40·9	SLR *O. latipes*
GHR1 *O. bonariensis*			***	80·4	74·1	78·3	40·7	40·0	42·3	40·3	41·8	GHR1 *O. bonariensis*
GHR1 *E. coioides*				***	76·9	85·6	40·9	42·2	44·4	42·0	43·0	GHR1 *E. coioides*
GHR1 *O. niloticus*					***	74·0	37·2	38·7	38·7	38·5	37·4	GHR1 *O. niloticus*
GHR1 *S. aurata*						***	40·6	41·2	44·0	42·0	43·3	GHR1 *S. aurata*
GHR *O. latipes*							***	64·2	62·5	58·2	61·1	GHR *O. latipes*
GHR2 *O. bonariensis*								***	72·0	66·7	70·8	GHR2 *O. bonariensis*
GHR2 *E. coioides*									***	71·7	80·3	GHR2 *E. coioides*
GHR2 *O. niloticus*										***	71·1	GHR2 *O. niloticus*
GHR2 *S. aurata*											***	GHR2 *S. aurata*

FIG. 1. Sequence identity of *Odontesthes bonariensis* Ghr1 and Ghr2 compared with *Homo sapiens* and other acanthopterygians: *Oryzias latipes*, *Epinephelus coioides*, *Oreochromis niloticus* and *Sparus aurata*. Data analysis was completed using the MegAlign programme and alignments performed with the Clustal W algorithm.

of 1928 nt, a 5′ untranslated region (UTR) of 26 nt and a 3′ UTR of 1506 nt (GenBank accession number KF055461). No proximal polyadenylation signal was found in the sequence upstream of the poly (A) tail. The ORF codes for a 581 amino acid (aa) protein, with a predicted signal peptide of 22 aa in the amino terminal region, an extracellular domain of 234 aa containing five potential N-glycosylation sites, seven cysteine residues and the characteristic Ghr ligand-binding motif FGEFS. The transmembrane domain comprises a hydrophobic motif of 23 aa in length; the cytoplasmic region includes a 302 aa domain with nine tyrosine residues and two conserved motifs box 1 and box 2.

Two different *ghr*2 transcripts of 3259 and 3560 nt were sequenced. Both of them have the same 5′ UTR (208 nt) and an open reading frame sequence of 1746 nt, but differ in 300 nt of their 3′ UTR sequences. The longest 3′ UTR has 1606 nt in length (GenBank accession number KF055460), and the shortest 1305 nt long (GenBank accession number KF055459). The *ghr2* open reading frame codes for a 581 aa protein. The N-terminal region has a putative 22 aa signal peptide, an extracellular region of 221 aa with four potential glycosylation sites, five cysteine residues and the GHR characteristic binding site FGEFS. A transmembrane domain of 23 hydrophobic aa and the cytoplasmic region (315 aa) are also part of this protein. As in Ghr1, the intracellular region has two conserved domains, box1 and box2, but only five tyrosines.

Odontesthes bonariensis Ghr1 (pjGhr1) and Ghr2 (pjGhr2) sequences have 38% identity between them (Fig. 1). DNA alignment analysis revealed that pjGhr1 displays high identity when compared to sequences from other acanthopterygians (67–85%; Fig. 1). A phylogenetic analysis shows that pjGhr1 and pjGhr2 group in the corresponding Ghr1 and Ghr2 fish clades. Both Ghr clades are more related to each other than to the GHRs of tetrapods, which are included as an out group.

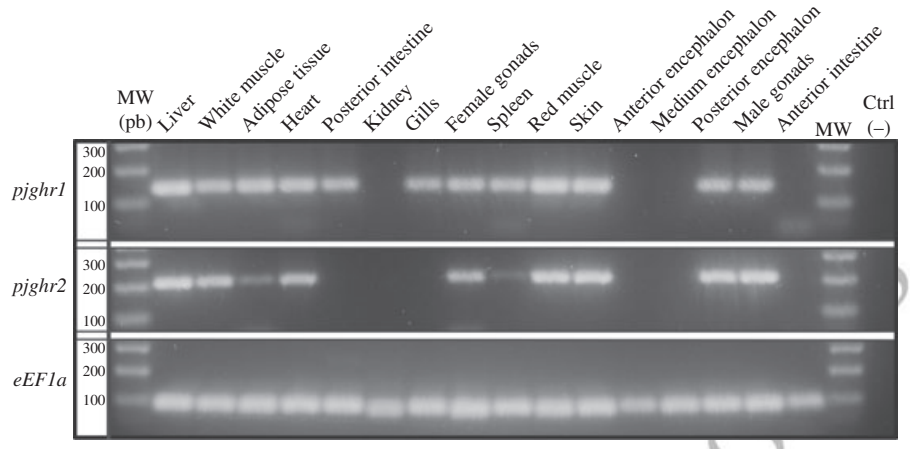


FIG. 2. Tissue distribution of *ghr* transcripts in adult *Odontesthes bonariensis* determined by RT-PCR analysis. *eEf1a* transcripts were amplified as internal controls in each cDNA preparation.

TISSUE DISTRIBUTION OF *PJGHR1* AND *PJGHR2* TRANSCRIPTS

RT-PCR assays were carried out in order to determine the tissue distribution of *ghr* transcripts in adult *O. bonariensis* (Fig. 2). A second PCR assay was performed using a 50 cycle programme in samples where *ghr1* or *ghr2* transcripts were undetected (Fig. 3). Altogether, both transcripts were detected in liver, white and red muscle, adipose tissue, heart, male and female gonads, spleen, skin, gills, posterior intestine and posterior encephalon. In kidney, only *ghr1* transcripts were detected, while only *ghr2* transcripts were detected in anterior and medium encephalon. Also, neither *ghr1* nor *ghr2* transcripts could be detected in the anterior intestine. A summary of the results is shown in Fig. 3.

ODONTESTHES BONARIENSIS GHR VARIANTS

In order to determine the presence of *ghr1* and *ghr2* mRNA variants in relevant metabolic tissues, northern blot assays were performed using total RNA from liver, muscle and adipose tissue. Probes that hybridized to the extracellular region of each transcript were used to detect putative *ghbp* transcripts or truncated anchored forms of Ghrs. A *ghr1* transcript of 5500 nt was found in the three tissues analysed [Fig. 4(a)]. Moreover, a *ghr1* transcript of 1100 nt appeared only in muscle. A *ghr2* transcript with a length between 2604 and 3638 nt was detected in all tissues studied. A muscle-specific *ghr2* transcript of 6000 nt was also found [Fig. 4(b)].

EFFECTS OF FASTING–REFEEDING ON GROWTH

At the end of the fasting period (week 2), the body mass of control group significantly differed from fasted fish ($0{\cdot}40 \pm 0{\cdot}03$ g control *v.* $0{\cdot}23 \pm 0{\cdot}01$ g fasted; $P < 0{\cdot}001$) [Fig. 5(a)]. Similarly, the L_T of control fish significantly differed from fasted fish ($43{\cdot}2 \pm 1{\cdot}0$ mm control group *v.* $39{\cdot}1 \pm 0{\cdot}7$ mm fasted group; $P < 0{\cdot}001$) [Fig. 5(b)]. After re-feeding, M_B and L_T did not significantly differ between groups (Fig. 5). *K* was

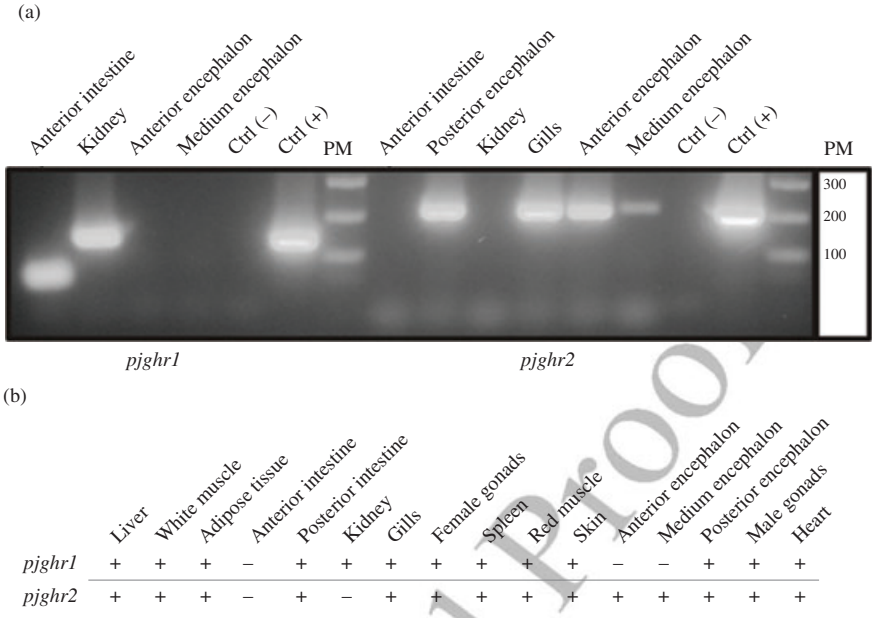


FIG. 3. Tissue distribution of *ghr* transcripts in adult *Odontesthes bonariensis*. (a) PCR amplification performed with 50 cycles in those tissues where *ghr1* and *ghr2* transcripts had not been detected in the first assay. (b) Summary of *ghr* transcript distribution in *O. bonariensis.*

significantly reduced on fasted group compared to controls ($K = 4{\cdot}7 \pm 0{\cdot}1$ control *v.* $K = 3{\cdot}9 \pm 0{\cdot}2$ fasted; $P < 0{\cdot}001$) and did not show statistical difference after re-feeding ($K = 3{\cdot}9 \pm 0\ 0{\cdot}2$ fasted group *v.* $K = 4{\cdot}7 \pm 0{\cdot}1$ control group; $P < 0{\cdot}001$). These results indicate a complete restoration of M_B and L_T after re-feeding.

EFFECTS OF FASTING–REFEEDING AND GH ADMINISTRATION ON LIVER *GHR* TRANSCRIPT EXPRESSION

A 2 week fasting treatment resulted in a decrease (3·5 times, $P < 0{\cdot}05$) in both *ghr* transcript levels compared with control fish [Fig. 6(a)]. After the re-feeding, transcript levels of both *ghr*s did not differ compared with the control group. *Igf1* transcript level nor did they differ from controls throughout the experiment. Growth hormone treatment increased *igf1* mRNA expression in *O. bonariensis* liver by four-fold and 13 fold at 2 and 4 weeks respectively [$P < 0{\cdot}05$; Fig. 6(b)]. Consistent with increased *igf1*, both *ghr1* and *ghr2* mRNA levels increased 4·5 and 2·1 fold ($P < 0{\cdot}05$) in liver after 4 weeks of Gh treatment. *ghr2* transcript levels, however, did not change while *ghr1* transcript levels decreased 3·5 fold ($P < 0{\cdot}05$) compared with controls after the first 2 weeks of Gh treatment [Fig. 6(b)]. The complete biological assay was repeated with identical results.

DISCUSSION

Odontesthes bonariensis Ghr1 and *Ghr2* coding sequences showed high homology with growth hormone receptor sequences of other fishes. Many fish Ghr coding

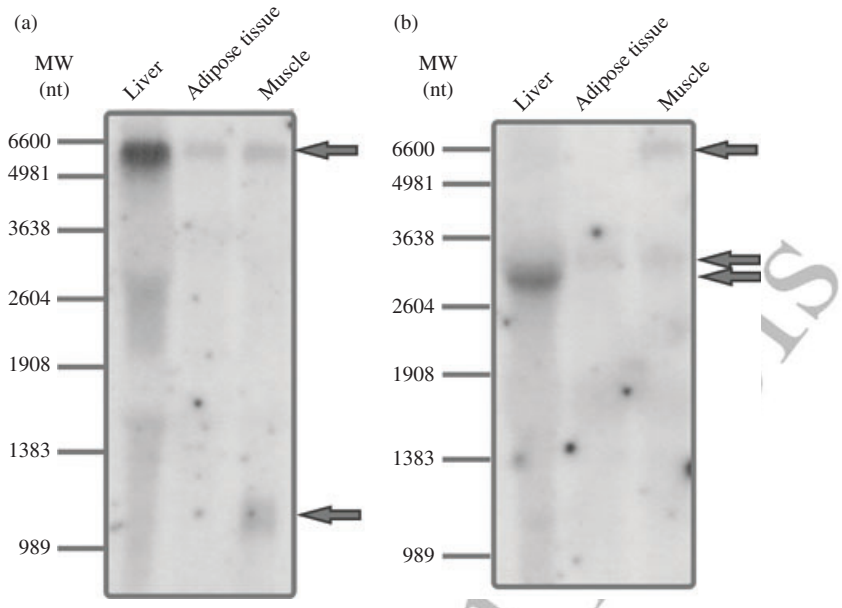


FIG. 4. Alternative transcript detection by northern blot assays of (a) ghr1 and (b) ghr2. Forty micrograms of total RNA from each tissue were separated on an agarose gel, transferred to a nylon membrane and incubated with radioactive probes.

sequences have been characterized, but sequence and length information of their respective 3′ UTRs are scarce. This is particularly relevant since northern blot and ghr mRNA sequence analyses in *O. bonariensis* showed that ghr1 and ghr2 transcripts have 6 and 3·5 kb in length in all tissues analysed, and exhibited long 3′ UTRs (3·5 kb for pjghr1 and 1·3–1·6 kb for pjghr2). These results are consistent with findings in other species like the Japanese eel *Anguilla japonica* Temminck & Schlegel 1847 and Nile tilapia *Oreochromis niloticus* (L. 1758) that showed similar length ghr1 and ghr2 transcripts using northern blot analysis (Ozaki *et al.*, 2006; Ma *et al.*, 2007).

In addition, two liver alternative *pjghr2* transcripts were sequenced that codify for the same protein, but differ in their 3′ UTRs as a result of alternative polyadenylation (APA). APA has only recently gained attention as a major player influencing the dynamics of gene regulation (Di Giammartino *et al.*, 2011), and has the potential to affect expression quantitatively since 3′ UTRs often harbour micro-RNA (miRNA)-binding sites (Li *et al.*, 2012; Lin *et al.*, 2012) and other regulatory sequences, such as AU-rich elements (AREs) (Fabian *et al.*, 2010) related to mRNA stability. APA can also regulate protein localization, especially in membrane bound proteins, and affect protein surface expression (Bercovitz & Meyer, 2015). The results of this study strongly suggest that long and alternative 3′ UTR isoform abundance could be regulating *ghrs* expression in fishes.

In tetrapods such as rat *Rattus norvegicus*, mouse *Mus musculus* and chicken *Gallus gallus*, alternative *ghr* transcripts 1200 nt in length have been found (Smith *et al.*, 1988; Tiong & Herington, 1991; Lau *et al.*, 2007). These organisms synthesize a blood-circulating growth hormone-binding protein (Ghbp). The presence of a Ghbp has been demonstrated in *O. mykiss*, *C. auratus* and the fine flounder *Paralichthys*

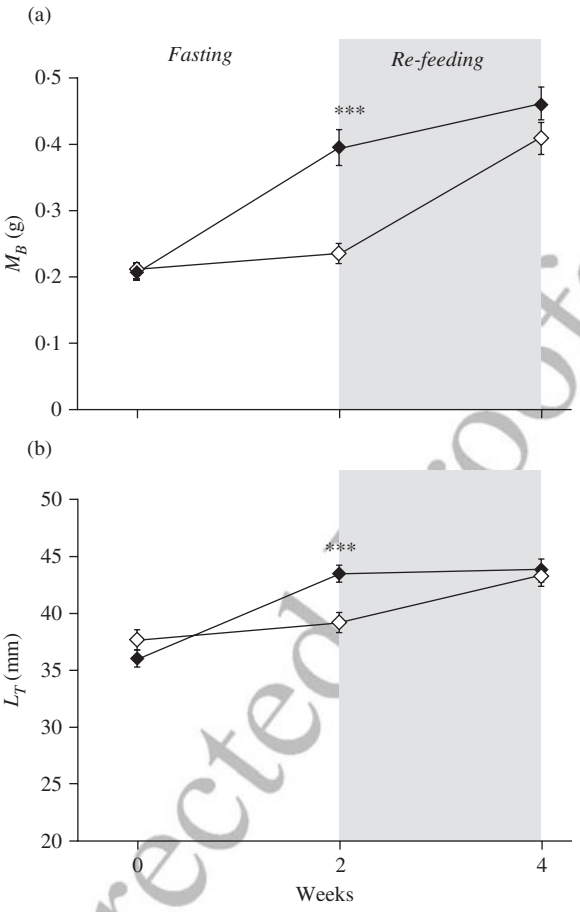


FIG. 5. Response of (a) mean body mass (M_B) and total length (L_T) of *Odontesthes bonariensis* juveniles during fasting and re-feeding regime (◇, treatment; ◆, control). Fish were measured at weeks 2 and 4 of treatment. Statistically significant differences between control and treated groups were analysed by nested ANOVA test (***$P < 0{\cdot}001$; $n = 9$–12 animals per group).

adspersus (Steindachner 1867) by Gh-binding protein assays, ligand and western blotting techniques (Zhang & Marchant, 1999; Fuentes *et al.*, 2012; Einarsdóttir *et al.*, 2014). Its mechanism of synthesis, however, has not been determined yet. Moreover, there is no clear evidence of alternative transcripts that could synthesize a soluble Ghbp in fishes. A membrane-anchored truncated Ghr isoform lacking most of the intracellular domain and acting as a dominant-negative inhibitor of receptor signalling, has been also described in mammals (Ross *et al*, 1997) and flatfishes (Calduch-Giner *et al.*, 2001; Nakao *et al.*, 2004; Hildahl *et al.*, 2007). In *P. adspersus*, Ghrt was detected in muscle cell membranes by western blot analyses and its abundance correlated with a low JAK2/STAT5 signalling pathway activation and Igf1 expression (Fuentes *et al.*, 2012), but the *ghr* gene from which it was produced has not been determined yet. An alternative transcript for *ghrb* that codifies a truncated isoform has been described in zebrafish *Danio rerio* (Hamilton 1822). The resulting isoform lacks the intracellular

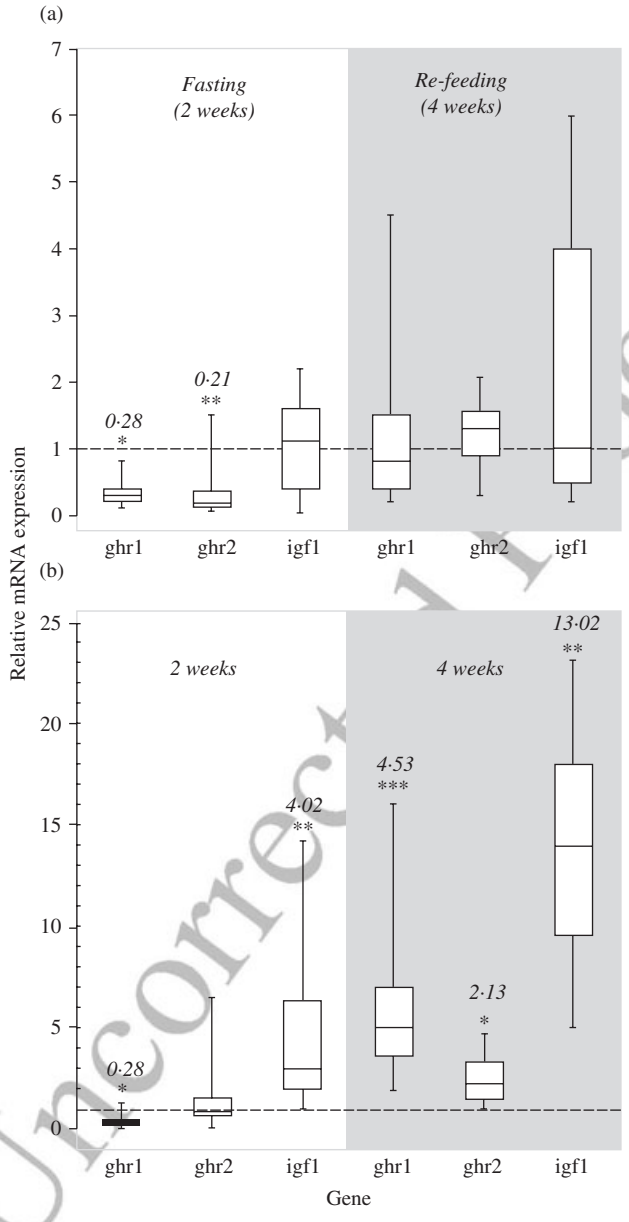


FIG. 6. Liver relative gene expression of *ghr1*, *ghr2* and *igf1 mRNA* in juvenile *Odontesthes bonariensis* after (a) fasting and re-feeding regimen and (b) r-pjGh administration (2 µg r-pjGh per g of body mass, once a week). Samples ($n = 6$) were taken at weeks 2 and 4 of treatment. Data in control fish were used as arbitrary reference values at each sampling time in the normalization procedure (values >1 or <1 indicate increase or decrease with respect to reference values). Lower and upper boundaries of the box indicate the 25th and 75th percentile. Whisker plots above and below the box indicate the 90th and 10th percentiles. Median is represented by a continuous line inside the box and median value is indicated above the box in statistically significant samples. Statistically significant differences compared with the control group were analysed by pair-wise fixed reallocation randomization test (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$)

region necessary for the activation of signal transducers and activators of transcription (STAT) family transcription factors (Di Prinzio *et al*, 2010). The current northern blot results showed an alternative ghr1 transcript in muscle with similar length to the mammalian transcript that codes for the soluble Ghrbp isoform, suggesting that *O. bonariensis* Ghbp could be produced from *ghr1* gene in a tissue-specific manner. The pjghr1 alternative transcript, however, should be sequenced to determine if it codifies a soluble or a membrane-anchored protein.

Numerous studies have indicated that transcription of both receptors is ubiquitous in fishes (Edens & Talamantes, 1998; Saera-Vila *et al.*, 2005; Di Prinzio *et al.*, 2010; Gao *et al.*, 2011). In adult *O. bonariensis*, both transcripts were detected in almost every tissue analysed except the anterior intestine. Furthermore, *ghr1* was not detected in anterior or medium brain, and *ghr2* in kidney. This could suggest specific functions of each receptor in these tissues. Head kidney serves as a major lymphoid organ in fishes, in addition to the thymus and spleen (Press & Evensen, 1999). Recent evidence suggests a role for the GH and IGF-axis in the fish immune system. Moreover, Gh was reported to regulate Tnf-α expression in head kidney (Shved *et al.*, 2011). In this context, Ghr1 could be mediating Gh action related to the immune response. On the other hand, recent studies have demonstrated that neuron generation takes place in *O. bonariensis* forebrain (Strobl-Mazzulla *et al.*, 2010), suggesting that Ghr2 could be related to cell proliferation and differentiation in this tissue.

Fish subjected to 2 week fasting showed a decreased level of *ghr1* and *ghr2* transcripts in liver that returned to control levels after refeeding. Similar observations were also reported in other fishes (Fukada *et al.*, 2004; Saera-Vila *et al.*, 2005; Small *et al.*, 2006; Fox *et al.*, 2010; Walock *et al.*, 2014). It has been proposed that this reduction in *ghr* transcripts could diminish hepatocyte sensitivity to growth hormone and cause a decrease in *igf1* transcript number (Duan & Plisetskaya, 1993; Picha *et al.*, 2008; Peterson & Waldbieser, 2009; Fox *et al.*, 2010). Interestingly, the present results showed that expression levels of the *O. bonariensis* Gh receptors were modified before a noticeable descent of *igf1* transcript level occurs.

Odontesthes bonariensis is highly sensitive to handling and crowding, which results in elevated cortisol levels (Tsuzuki *et al.*, 2001). In order to avoid stress, oral administration of homologous recombinant growth hormone, instead of intraperitoneal injection, has been then preferred in this species. A methodology for oral administration of r-pjGh has been achieved by properly mixing it with food and developing feeding schedule (four doses once a week). By this means, a significant increase in liver *igf1* expression level was achieved after 1 month of treatment (Sciara *et al.*, 2011). The current results showed that there is a concordant relation between growth, *igf1* and *ghrs* expression levels after 4 week treatment with exogenous Gh. Similar results were observed in other Gh-treated fishes (Biga *et al.*, 2004; Gahr *et al.*, 2008; Biga & Meyer, 2009; Shved *et al.*, 2011). Interestingly, after the first 2 weeks of r-pjGh administration, *O. bonariensis* exhibited up-regulation of *igf1* mRNA levels, but decreased or unchanged ghr1 and ghr2 mRNA levels, respectively. Few studies have analysed the acute response to GH administration on *ghr*s expression in fish liver. In *O. niloticus*, after two doses of 2 μg Gh/g of body mass in less than 24 h, a down-regulation in liver *ghr1* transcription was produced 6 h after the last administration (Shved *et al.*, 2011). Since down-regulation of *ghr* expression is associated with metabolic adjustment during fasting, changes in Gh level could produce a similar energy imbalance between

available food energy and the energy required for growth that could down-regulate *ghr1* expression in liver.

In summary, the sequences of *ghr* types 1 and 2 of an atherinid are reported for the first time. Alternative transcripts of both receptors were found in muscle, the truncated form of muscle *ghr1* an interesting candidate for the Ghbp. Two different *ghr2* transcripts were also found by APA-UTR in liver. The presence of long and alternative 3′ UTRs in fish ghr transcripts strongly suggests a new control mechanism of *ghrs* expression. *Ghr* hepatic transcription was examined under two different physiological situations. Growth hormone treatment produces an acute negative feedback response on *ghr1* expression in liver of growing animals, which opens up new questions about *ghr1* and *ghr2* expression regulation and function, while fasting reduces both *ghr1* and *ghr2* expression in liver. Altogether, these results add novel information to current knowledge on growth hormone receptor sequences and growth regulation in fishes.

The authors thank G. Somoza, T. Chalde and E. Scharrig for their outstanding technical assistance. They also thank the Departamento de Estadística, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, for their assistance in the statistical analysis, and D. Krapf for English language editing. This work was supported by grants from ANPCyT and SECTeI (PID 2008-019) awarded to S.E.A.

Supporting Information

Supporting Information may be found in the online version of this paper:

Table SI. Primer sequences used in this study

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped blast and psi-blast: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Baumann, G. (2002). Growth hormone binding protein. The soluble growth hormone receptor. *Minerva Endocrinology* **27,** 265–276.

Beckman, B. R. (2011). Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. *General and Comparative Endocrinology* **170,** 233–252.

Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). Improved prediction of signal peptides: Signalp 3.0. *Journal of Molecular Biology* **340,** 783–795.

Bercovitz, B. D. & Meyer, C. (2015). Alternative 39 UTRs act as scaffolds to regulate membrane protein localization. *Nature* **522,** 363–367.

Biga, P. R. (2004). Growth hormone differentially regulates muscle myostatin1 and -2 and increases circulating cortisol in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **138,** 32–41.

Biga, P. R. & Meyer, J. (2009). Growth hormone differentially regulates growth and growth-related gene expression in closely related fish species. *Comparative Biochemistry and Physiology A* **154,** 465–473.

Biga et al. (2004).

Blom et al. (1998).

Breves, J. P., Tipsmark, C. K., Stough, B. A., Seale, A. P., Flack, B. R., Moorman, B. P., Lerner, D. T. & Gordon, G. E. (2014). Nutritional status and growth hormone regulate insulin-like growth factor binding protein (igfbp) transcripts in Mozambique tilapia. *General and Comparative Endocrinology* **207,** 66–73.

Calduch-Giner, J. (2003). Molecular cloning and characterization of gilthead sea bream (*Sparus aurata*) growth hormone receptor (GHR). Assessment of alternative splicing. *Comparative Biochemistry and Physiology B* **136,** 1–13.

Calduch-Giner, J., Duval, H., Chesnel, F., Boeuf, G., Perez-Sanchez, J. & Boujard, D. (2001). Fish growth hormone receptor: molecular characterization of two membrane-anchored forms. *Endocrinology* **142,** 3269–3273.

Di Giammartino, D. C., Nishida, K. & Manley, J. L. (2011). Mechanisms and consequences of alternative polyadenylation. *Molecular Cell* **43,** 853–866.

Di Prinzio, C. M., Botta, P. E., Barriga, E. H., Ríos, E. A., Reyes, A. E. & Arranz, S. E. (2010). Growth hormone receptors in zebrafish (*mol cellanio rerio*): adult and embryonic expression patterns. *Gene Expression Patterns* **10,** 214–225.

Duan, C. & Plisetskaya, E. M. (1993). Nutritional regulation of insulin-like growth factor-I mRNA expression in salmon tissues. *Journal of Endocrinology* **139,** 243–252.

Dyer, B. S. & Chernoff, B. (1996). Phylogenetic relationships among atheriniform fishes (Teleostei: Atherinomorpha). *Zoological Journal of the Linnean Society* **117,** 1–69.

Edens, A. & Talamantes, F. (1998). Alternative processing of growth hormone receptor transcripts. *Endocrine Reviews* **19,** 559–582.

Einarsdóttir, I. E., Gong, N., Jönsson, E., Sundh, H., Hasselberg-Frank, T. O., Nilsen, S. O., Stefansson, S. O., Sundell, K. & Björnsson, B. T. (2014). Plasma growth hormone-binding protein levels in Atlantic salmon *Salmo salar* during smoltification and seawater transfer. *Journal of Fish Biology* **85,** 1279–1296.

Fabian, M. R., Sonenberg, N. & Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNA. *Annual Review of Biochemistry* **79,** 351–379.

Fox, B. K., Breves, J. P., Davis, L. K., Pierce, A. L., Hirano, T. & Grau, E. G. (2010). Tissue-specific regulation of the growth hormone/insulin-like growth factor axis during fasting and re-feeding: importance of muscle expression of IGF-I and IGF-II mRNA in the tilapia. *General and Comparative Endocrinology* **166,** 573–580.

Fuentes, E. N., Einarsdottir, I. E., Valdes, J. A., Alvarez, M., Molina, A. & Bjornsson, B. T. (2012). Inherent growth hormone resistance in the skeletal muscle of the fine flounder is modulated by nutritional status and is characterized by high contents of truncated GHR, impairment in the JAK2/STAT5 signaling pathway, and low IGF-I expression. *Endocrinology* **153,** 283–294.

Fuentes, E. N., Valdés, J. A., Molina, A. & Björnsson, B. T. (2013). Regulation of skeletal muscle growth in fish by the growth hormone - insulin-like growth factor system. *General and Comparative Endocrinology* **192,** 136–148.

Fukada, H., Ozaki, Y., Pierce, A., Adachi, S., Yamauchi, K., Hara, A., Swanson, P. & Dickhoff, W. W. (2004). Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting. *General and Comparative Endocrinology* **139,** 61–71.

Fukamachi, S. (2005). Medaka receptors for somatolactin and growth hormone: phylogenetic paradox among fish growth hormone receptors. *Genetics* **171,** 1875–1883.

Fukamachi, S. & Meyer, A. (2007). Evolution of receptors for growth hormone and somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon orthologues. *Journal of Molecular Evolution* **65,** 359–372.

Gahr, S. A., Vallejo, R. L., Weber, G. M., Shepherd, B. S., Silverstein, J. T. & Rexroad, C. E. 3rd. (2008). Effects of short-term growth hormone treatment on liver and muscle transcriptomes in rainbow trout (*Oncorhynchus mykiss*). *Physiological Genomics* **32,** 380–392.

AQ7 Gahr et al. (2009).

Gao, F. Y., Lu, M. X., Ye, X., Huang, Z. H., Wang, H., Zhu, H. P. & Yang, L. P. (2011). Identification and expression analysis of two growth hormone receptors in zanzibar tilapia (*Oreochromis hornorum*). *Fish Physiology and Biochemistry* **37,** 553–565.

Hildahl et al. (2007).

Hildahl, J., Power, D. M., Björnsson, B. T. & Einarsdóttir, I. E. (2008). Involvement of growth hormone-insulin-like growth factor I system in cranial remodeling during halibut metamorphosis as indicated by tissue- and stage-specific receptor gene expression and the presence of growth hormone receptor protein. *Cell and Tissue Research* **332,** 211–225.

AQ8

Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* **305,** 567–580.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. & Higgins, D. G. (2007). Clustal w and clustal X version 2.0. *Bioinformatics* **23,** 2947–2948.

Lau, J. S., Yip, C. W., Law, K. M. & Leung, F. C. (2007). Cloning and characterization of chicken growth hormone binding protein (cGHBP). *Domestic Animal Endocrinology* **33,** 107–121.

Le Roith, D., Bondy, C., Yakar, S., Liu, J. L. & Butler, A. (2001). The somatomedin hypothesis: 2001. *Endocrine Reviews* **22,** 53–74.

Lee, L. T., Nong, G., Chan, Y. H., Tse, D. L. & Cheng, C. H. (2001). Molecular cloning of a teleost growth hormone receptor and its functional interaction with human growth hormone. *Gene* **270,** 121–129.

Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J. & Wood, W. I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* **330,** 537–543.

Li, H. M., Wang, C. M., Li, Q. Z. & Gao, X. J. (2012). Mir-15a decreases bovine mammary epithelial cell viability and lactation and regulates growth hormone receptor expression. *Molecules* **17,** 12037–12048.

Lin, S., Li, H., Mu, H., Luo, W., Li, Y., Jia, X., Wang, S., Nie, Q. & Zhang, X. (2012). Let-7b regulates the expression of the growth hormone receptor gene in deletion-type dwarf chickens. *BMC Genomics* **13,** 306–316.

Ma, X., Liu, X., Zhang, Z., Zhu, P., Ye, W. & Lin, L. (2007). Two growth hormone receptors in Nile tilapia (*Oreochromis niloticus*): molecular characterization, tissue distribution and expression profiles in the gonad during the reproductive cycle. *Comparative Biochemistry and Physiology B* **147,** 325–339.

Moriyama, S., Ayson, F. G. & Kawauchi, H. (2000). Growth regulation by insulin-like growth factor-I in fish. *Bioscience Biotechnology and Biochemistry* **64,** 1553–1562.

Nakao, N., Higashimoto, Y., Ohkubo, T., Yoshizato, H., Nakai, N., Nakashima, K. & Tanaka, M. (2004). Characterization of structure and expression of the growth hormone receptor gene of the Japanese flounder (*Paralichthys olivaceus*). *Journal of Endocrinology* **182,** 157–164.

Ozaki, Y., Fukada, H., Kazeto, Y., Adachi, S., Hara, A. & Yamauchi, K. (2006). Molecular cloning and characterization of growth hormone receptor and its homologue in the Japanese eel (*Anguilla japonica*). *Comparative Biochemistry and Physiology B* **143,** 422–431.

Peterson, B. C. & Waldbieser, G. C. (2009). Effects of fasting on IGF-I, IGF-II, and IGF-binding protein mRNA concentrations in channel catfish (*Ictalurus punctatus*). *Domestic Animal Endocrinology* **37,** 74–83.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29,** 2002–2007.

Pfaffl, M. W., Horgan, G. W. & Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30,** 36–46.

Picha, M. E., Turano, M. J., Tipsmark, C. K. & Borski, R. J. (2008). Regulation of endocrine and paracrine sources of igfs and gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). *Journal of Endocrinology* **199,** 81–94.

Pierce, A. L., Fukada, H. & Dickhoff, W. W. (2005). Metabolic hormones modulate the effect of growth hormone (GH) on insulin-like growth factor-I (IGF-I) mRNA level in primary culture of salmon hepatocytes. *Journal of Endocrinology* **184,** 341–349.

Press, C. M. L. & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology* **9,** 309–318.

Reindl, K. M. & Sheridan, M. A. (2012). Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comparative Biochemistry and Physiology A* **163,** 231–245.

Roberts (2001).

AQ9 Ross, J. (1995). mRNA stability in mammalian cells. *Microbiological Reviews* **59,** 423–450.

Ross, R. J., Esposito, N., Shen, X. Y., Von Laue, S., Chew, S. L., Dobson, P. R., Postel-Vinay, M. C. & Finidori, J. (1997). Short isoform of the human growth hormone receptor functions

as a dominant negative inhibitor of the full-length receptor and generates large amounts of binding protein. *Molecular Endocrinology* **11,** 265–273.

Saera-Vila, A., Calduch-Giner, J.-A. & Pérez-Sánchez, J. (2005). Duplication of growth hormone receptor (GHR) in fish genome: gene organization and transcriptional regulation of ghr type I and II in gilthead sea bream (*Sparus aurata*). *General and Comparative Endocrinology* **142,** 193–203.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sciara, A. A., Rubiolo, J. A., Somoza, G. M. & Arranz, S. E. (2006). Molecular cloning, expression and immunological characterization of pejerrey (*Odontesthes bonariensis*) growth hormone. *Comparative Biochemistry and Physiology C* **142,** 284–292.

Sciara, A. A., Somoza, G. M. & Arranz, S. E. (2008). Insulin-like growth factor-I of pejerrey, *Odontesthes bonariensis*: cDNA characterization, tissue distribution and expression profiles after growth hormone administration. *Journal of Experimental Zoology A* **309A**, 407–418.

Sciara, A. A., Somoza, G. M. & Arranz, S. E. (2011). Muscular hypertrophy and growth promoting effects in juvenile pejerrey (*Odontesthes bonariensis*) after oral administration of recombinant homologous growth hormone obtained by a highly efficient refolding process. *Aquaculture Research* **42,** 844–857.

Shved, N., Berishvili, G., Mazel, P., Baroiller, J. F. & Eppler, E. (2011). Growth hormone (GH) treatment acts on the endocrine and autocrine/paracrine GH/IGF-axis and on TNF-alpha expression in bony fish pituitary and immune organs. *Fish and Shellfish Immunology* **31,** 944–952.

Small, B. C., Murdock, C. A., Waldbieser, G. C. & Peterson, B. C. (2006). Reduction in channel catfish hepatic growth hormone receptor expression in response to food deprivation and exogenous cortisol. *Domestic Animal Endocrinology* **31,** 340–356.

Smith, W. C., Linzer, D. I. & Talamantes, F. (1988). Detection of two growth hormone receptor mRNAs and primary translation products in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 9576–9579.

Sohm, F., Manfroid, I., Pezet, A., Rentier-Delrue, F., Rand-Weaver, M., Kelly, P. A., Boeuf, G., Postel-Vinay, M. C., de Luze, A. & Edery, M. (1998). Identification and modulation of a growth hormone-binding protein in rainbow trout (*Oncorhynchus mykiss*) plasma during seawater adaptation. *General and Comparative Endocrinology* **111,** 216–224.

Somoza, G. M., Miranda, L. A., Berasain, G. E., Colautti, D., Remes Lenicov, M. & Strüssmann, C. A. (2008). Historical aspects, current status and prospects of pejerrey aquaculture in South America. *Aquaculture Research* **39,** 784–793.

Strobl-Mazzulla, P. H., Nuñez, A., Pellegrini, E., Gueguen, M.-M., Kah, O. & Somoza, G. M. (2010). Progenitor radial cells and neurogenesis in pejerrey fish forebrain. *Brain, Behavior and Evolution* **76,** 20–31.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). Mega5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28,** 2731–2739.

Tiong, T. S. & Herington, A. C. (1991). Identification of a novel growth hormone binding protein mRNA in rat liver. *Biochemical and Biophysical Research Communications* **180,** 489–495.

Tse, D. L., Tse, M. C., Chan, C. B., Deng, L., Zhang, W. M., Lin, H. R. & Cheng, C. H. (2003). Seabream growth hormone receptor: molecular cloning and functional studies of the full-length cDNA, and tissue expression of two alternatively spliced forms. *Biochimica et Biophysica Acta* **1625,** 64–76.

Tsuzuki, M. Y., Ogawa, K., Strüssmann, C. A., Maita, M. & Takashima, F. (2001). Physiological responses during stress and subsequent recovery at different salinities in adult pejerrey *Odontesthes bonariensis*. *Aquaculture* **200,** 349–362.

Uchida, K., Kajimura, S., Riley, L. G., Hirano, T., Aida, K. & Grau, E. G. (2003). Effects of fasting on growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology A* **134,** 429–439.

Viklund, H., Bernsel, A., Skwark, M. & Elofsson, A. (2008). Spoctopus: a combined predictor of signal peptides and membrane protein topology. *Bioinformatics* **24,** 2928–2929.

Walock, C. N., Kittilson, J. D. & Sheridan, M. A. (2014). Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state. *Gene* **533,** 286–294.

Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D. & Hochstrasser, D. F. (1999). Protein identification and analysis tools in the expasy server. *Methods in Molecular Biology* **112,** 531–552.

Zhang, Y. & Marchant, T. A. (1999). Identification of serum gh-binding proteins in the goldfish (*Carassius auratus*) and comparison with mammalian GH-binding proteins. *Journal of Endocrinology* **161,** 255–262.

QUERIES TO BE ANSWERED BY AUTHOR

Queries from the Copyeditor:

AQ1. Please confirm that given names (red) and surnames/family names (green) have been identified correctly

AQ2. Please check for the redundancy of the sentence "Reactions lacking reverse transcriptase...".

AQ3. We have changed the citation of Table I to Table SI throughout the article. Kindly check and confirm if this is appropriate.

AQ4. Please provide significance for "***" given in the art work of Fig. 1.

AQ5. The fundRefNames “ANPCyT and SECTeI” are not found in the fundRef database. Please check if you have the correct funder information.

AQ6. Reference “Biga (2004)” is not cited in the text. Please indicate where it should be cited; or delete from the reference list.

AQ7. Please provide complete details for Gahr et al. (2009), Blom et al. (1998), Roberts (2001), Biga et al. (2004) and Hildahl et al. (2007).

AQ8. Reference “Hildahl et al. (2008)” is not cited in the text. Please indicate where it should be cited; or delete from the reference list.

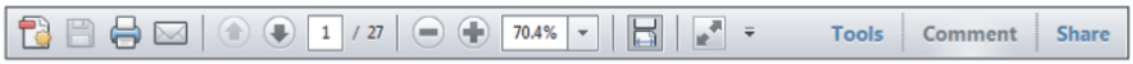
AQ9. Reference “Ross (1995)” is not cited in the text. Please indicate where it should be cited; or delete from the reference list.

WILEY

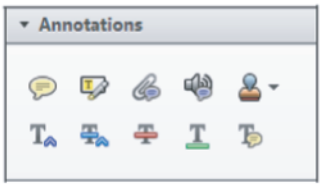
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

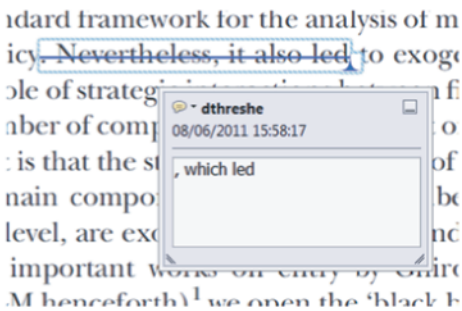


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

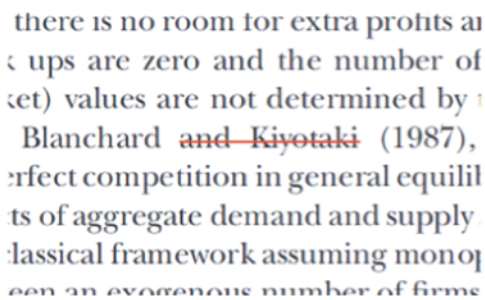


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

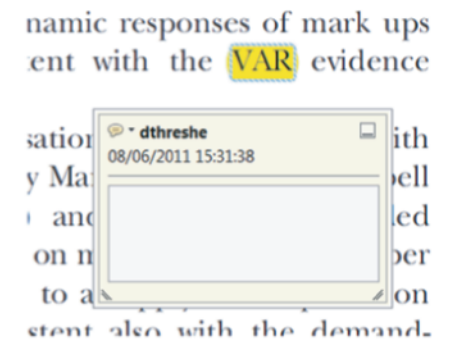


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.



4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

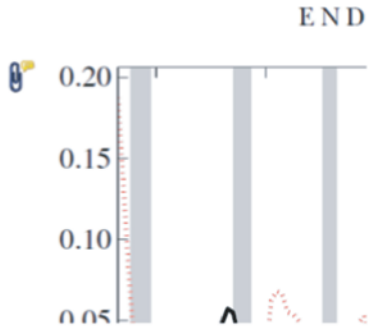
- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

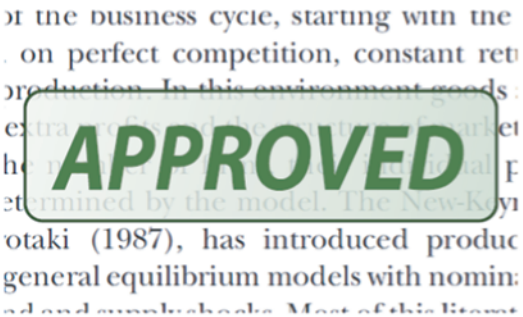


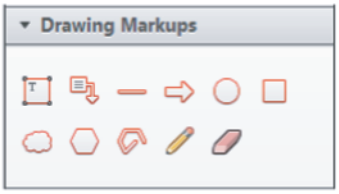
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



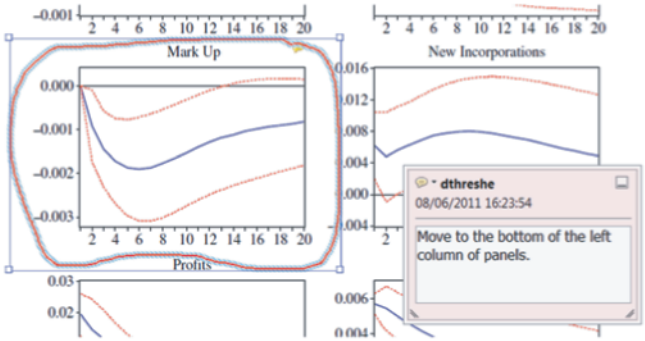


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: