J Comp Physiol B (2016) 186:727–738
DOI 10.1007/s00360-016-0986-0

ORIGINAL PAPER

Periprandial changes and effects of short- and long-term fasting on ghrelin, GOAT, and ghrelin receptors in goldfish (*Carassius auratus*)

A. M. Blanco[1] · M. Gómez-Boronat[1] · I. Redondo[1] · A. I. Valenciano[1] · M. J. Delgado[1]

Received: 2 February 2016 / Revised: 26 March 2016 / Accepted: 29 March 2016 / Published online: 9 April 2016

Abstract The periprandial profile and effects of short- (7 days) and long-term (30 days) fasting on the ghrelinergic system were studied in goldfish (*Carassius auratus*). Plasma levels of acyl-ghrelin, desacyl-ghrelin, and ghrelin *O*-acyl transferase (GOAT) were analyzed by enzymoimmunoassays, and expression of *preproghrelin*, *goat* and *growth hormone secretagogue* receptors (*ghs-r*) was quantified by real-time PCR. Circulating levels of acyl-ghrelin and GOAT rise preprandially, supporting the role of acyl-ghrelin as a meal initiator in this teleost. Consistently, *preproghrelin* and *ghs-r1a1* expression increases 1 h before feeding time in intestinal bulb, suggesting that this receptor subtype might be involved in the preprandial action of ghrelin in this tissue. Significant postfeeding variations are detected for *preproghrelin* in telencephalon, *goat* in telencephalon and hypothalamus, *ghs-r1a1* in vagal lobe, *ghs-r1a2* and *ghs-r2a1* in hypothalamus and *ghs-r2a2* in telencephalon and vagal lobe, especially in unfed fish. Short- and long-term fasting significantly increase *preproghrelin* expression in telencephalon and gut. *Goat* expression is upregulated by short-term fasting in telencephalon and hypothalamus, and by both short- and long-term fasting in gut. Expression of *ghs-r* increases by fasting in telencephalon, while an upregulation of type 2, but not type 1, receptors is observed in vagal lobe. In intestinal bulb, *ghs-r1a2* transcripts increase after both short- and long-term fasting. These results show a high dependence of the ghrelinergic system on feeding and nutritional status in fish, and demonstrate for the first time a differential implication of the various components of this system suggesting different roles for the four ghrelinergic receptor subtypes.

Keywords Feeding · Preproghrelin · Ghrelinergic system · Starvation · Fish · Teleost · Food deprivation

Introduction

Ghrelin is a peptide hormone identified for the first time in rat stomach by Kojima et al. (1999) as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R), also named ghrelin receptor. Although discovered as a 28-amino acid peptide, ghrelin is shorter in many species, including goldfish, in which the peptide has been characterized as 11 molecular forms consisting of 14-, 17-, 18- and 19-amino acid residues (Miura et al. 2009). At present, the gastrointestinal tract is known to be the main site for the synthesis of this hormone in vertebrates (Kaiya et al. 2008), although it has been also detected in other peripheral and central locations (Cowley et al. 2003). In fish, the highest levels of ghrelin mRNA occur in the stomach or the foregut, depending on the species (Breves et al. 2009; Kaiya et al. 2003; Unniappan et al. 2002), with low levels detected in other peripheral tissues and also in the brain (Amole and Unniappan 2009; Unniappan et al. 2002). The first physiological role described for ghrelin as a ligand for the GHS-R was the stimulation of growth hormone release from pituitary (Kojima et al. 1999). Later, many other functions have been attributed to this peptide, including stimulation of food intake, carbohydrate utilization and adiposity, and regulation of insulin secretion, among others (Delporte 2013; Heppner et al. 2011; Lim et al. 2011; Sato et al. 2012).

Communicated by G. Heldmaier.

✉ M. J. Delgado
mjdelgad@bio.ucm.es

[1] Department of Animal Physiology II, Faculty of Biology, Complutense University, 28040 Madrid, Spain

Ghrelin is the only biologically active peptide known to undergo a post-translational acyl modification, mainly the attachment of a medium-chain fatty acid, typically octanoate, to the third serine residue of the N-terminal region of the peptide. This acylation is catalyzed by a member of the membrane-bound *O*-acyltransferase family, named ghrelin *O*-acyltransferase (GOAT or mBOAT4) (Gutierrez et al. 2008; Yang et al. 2008). Since this modification is essential for the peptide to bind the GHS-R, desacyl-ghrelin (desacyl-GRL) has been considered until recently as the degradation product of ghrelin without biological activity. However, recent studies have shown that desacyl-GRL can exert a variety of biological functions in vertebrates binding a different type of receptor (Gauna et al. 2007; Lear et al. 2010; Matsuda et al. 2006a), acting in many cases as a functional antagonist of acyl-ghrelin (acyl-GRL) (Delhanty et al. 2012).

Ghrelin receptor was discovered in humans and pigs in 1996 (Howard et al. 1996) and belongs to the G protein-coupled receptor superfamily. In contrast to mammals and other tetrapod vertebrates with only one GHS-R receptor gene, two paralog genes (GHS-R1 and GHS-R2) have been identified in teleosts derived from the ancestral genome duplication that this group of fish underwent. The goldfish (*C. auratus*) has experienced an additional tetraploidization (Larhammar and Risinger 1994), and then eight subtypes of GHS-Rs are present in this species (GHS-R1a1, GHS-R1b1, GHS-R1a2, GHS-R1b2, GHS-R2a1, GHS-R2b1, GHS-R2a2 and GHS-R2b2). Receptors named with "a" correspond to the fully functional isoforms of the receptor, and they have been recently characterized in goldfish (Kaiya et al. 2010, 2013). The amount of *ghs-r1a1* and *r1a2* transcripts is 2- to 50-fold higher than *ghs-r2a*, and they show a wide distribution with high levels in the brain, intestine, liver, and testis, whereas *ghs-r2a1* and *r2a2* expression is almost restricted to the brain, body kidney, ovary and testis (Kaiya et al. 2010). Recent own results report a wide distribution of *ghs-r1a* immunoreactivity in many encephalic areas (including the telencephalon, some hypothalamic nuclei, the pineal gland and the cerebellum) and in the gastrointestinal tract, with the most abundant signal in the mucosal epithelium (Sánchez-Bretaño et al. 2015).

Feeding and the nutritional state are the most important factors regulating ghrelin levels. In humans and rodents, an increase in ghrelin secretion and *preproghrelin* expression has been reported prior to a meal (Müller et al. 2015), as well as an upregulation of the system under negative energy-balance conditions such as fasting (González et al. 2008; Korhonen and Saarela 2005; Zhao et al. 2008). In fish, results are not so consistent. Circulating ghrelin was described to decrease after feeding in goldfish, although no preprandial rise was found neither in this teleost (Unniappan et al. 2004) nor in Mozambique tilapia (*Oreochromis mossambicus*, Peddu et al. 2009). The periprandial *preproghrelin* expression in brain and gut varies depending on the species (goldfish, Unniappan et al. 2004; Mozabique tilapia, Peddu et al. 2009; zebrafish, Hatef et al. 2015). The effects of food deprivation on ghrelin secretion and *preproghrelin* expression have been studied in several fish species, such as goldfish (Matsuda et al. 2006b; Unniappan et al. 2004), zebrafish (*Danio rerio*, Amole and Unniappan 2009; Eom et al. 2013; Koven and Schulte 2012), tilapia (Fox et al. 2009; Riley et al. 2008), Atlantic salmon (*Salmo salar*, Hevrøy et al. 2011), sea bass (*Dicentrarchus labrax*, Terova et al. 2008), striped bass (*Morone saxatilis*, Picha et al. 2009), grass carp (*Ctenopharyngodon idelluss*, Feng et al. 2013), rainbow trout (*Oncorhynchus mykiss,* Jönsson et al. 2007; Pankhurst et al. 2008), and *Schizothorax davidi* (Zhou et al. 2014). Similarly, controversial results are reported depending on the species, tissue and duration of the starvation.

While diverse data are available concerning ghrelin regulation by feeding and nutritional status in fish, very few studies have investigated this subject in other components of the ghrelinergic system. In fact, despite the relevance of the acylation process of ghrelin for the bioactivity of this hormone, only one recent study in zebrafish have shown that GOAT mRNAs increase in brain of unfed fish after subjective feeding time, and are upregulated by fasting in both brain and gut (Hatef et al. 2015). Concerning GHS-R, the only study exploring the periprandial profile of ghrelin receptors was carried out in tilapia and reported a significant elevation in preprandial brain expression of *ghs-r1a* and a significant reduction of *ghs-r1b* at 3-h postfeeding (Peddu et al. 2009). The effects of different fasting periods on *ghs-r* expression have been reported in some fish species, including tilapia (Riley et al. 2008), Atlantic salmon (Hevrøy et al. 2011), zebrafish (Eom et al. 2014) and goldfish (Kaiya et al. 2010).

Overall, the knowledge of the regulation of the ghrelinergic system by feeding and food deprivation in fish is controversial, probably due to the existence of partial results and the lack of a complete analysis of all the components of the system. Furthermore, to our knowledge, no studies to date have investigated the relevance of the acylation process of ghrelin in appetite regulation. Therefore, this study aimed to investigate the periprandial profiles and possible effects of short- and long-term fasting on all the elements of the ghrelinergic system in goldfish, including circulating acyl-ghrelin, desacyl-ghrelin, GOAT, and expression of *preproghrelin*, *goat* and the four subtypes of *ghs-r*.

Table 1 Primers used for qPCR assays

Gene	GenBank accession number	Primer sequence (5′–3′)	Amplicon size (bp)
preproghrelin	AF454389.1	F: TTCATGATGAGTGCTCC R: GTCAGAATTCAAGTGGC	124
goat	KT726983	F: ATTGCTGTTCTTCAGTGCCG R: TGTACAAGTGCCAGACGGTT	119
ghs-r1a1	AB504275	F: TAAATGTTGAGCAGCCCTTCGCGC R: TGCTCGAATCCCCAGTATGT	187
ghs-r1a2	AB504276	F: ACAGGTTGTATAAGTTGAGCG R: TGCTCGAATCCCCAGTATGT	180
ghs-r2a1	AB504277	F: CCAAGTCATCCGAGGCCAAT R: AAAAGCTCTCGGCGTTCTTC	216
ghs-r2a2	AB504278	F: CCAAGTCATCCGAGGCCAAT R: GCTGTATTCATTCCAAACCGAAACA	233
β-actin	AB039726.2	F: CAGGGAGTGATGGTTGGCAT R: AACACGCAGCTCGTTGTAGA	168
ef-1α	AB056104	F: CCCTGGCCACAGAGATTTCA R: CAGCCTCGAACTCACCAACA	101

F forward, *R* reverse

Methods

Animals

Goldfish (*C. auratus*) with a body weight of 20–30 g were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria ($n = 7$–8/aquarium) with filtered fresh water at 21 ± 2 °C and continuous aeration, and maintained under a 12 h light:12 h darkness (12L:12D) photoperiod (lights on at 0800 hours). Food from a commercial flake diet (1 % body weight, Sera Pond, Heinserberg, Germany) was offered daily at 10:00 hours (fasting experiment) or at 12:00 hours (periprandial experiment) for at least 2 weeks before the experiments. All procedures were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14), and were in accordance with the Guidelines of the European Union Council (2010/63/EU) for the use of research animals.

Experimental designs

Periprandial changes of ghrelinergic system in goldfish

Seven groups of fish ($n = 7$/group) were maintained in aquaria under the same conditions as during the acclimation period. On the day of the experiment, 21 fish from three aquaria (7 fish per sampling time) were sampled at −3, −1 and 0 h before scheduled feeding time. At scheduled feeding time, two of the remaining four aquaria were fed while food was withheld from the other two aquaria. Fish from both fed and unfed aquaria were killed at +1 and +3 h after scheduled feeding time. In each sampling point, fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, Madrid, Spain) and killed by decapitation. Telencephalon, hypothalamus, vagal lobe, intestinal bulb and liver were collected, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Blood samples were collected by caudal venous puncture using a 25-gauge needle, and centrifuged at 5000*g* for 10 min at 4 °C to obtain the plasma, which was stored at −20 °C until assays were conducted.

Effects of short- and long-term fasting on ghrelinergic system in goldfish

Four groups of fish ($n = 8$/group) were established as follows: (1) control (7-day normal feeding), (2) 7-day fasting, (3) control (30-day normal feeding), and (4) 30-day fasting. Following acclimation for at least 2 weeks, fish of the experimental groups were starved for 7 days (group ii) or 30 days (group iv), while the fish of the control groups (groups i and iii) were fed as throughout the acclimation period. At the end of each experimental period, control and fasted fish were killed at 12:00 hours, and tissues were collected as above described.

Quantification of plasma hormones levels

Plasma levels of acyl-GRL, desacyl-GRL and GOAT were quantified using fish ELISA kits (Catalog # MBS034979, MBS025500 and MBS063620, respectively, Mybiosource, San Diego, USA). ELISAs were conducted in duplicate

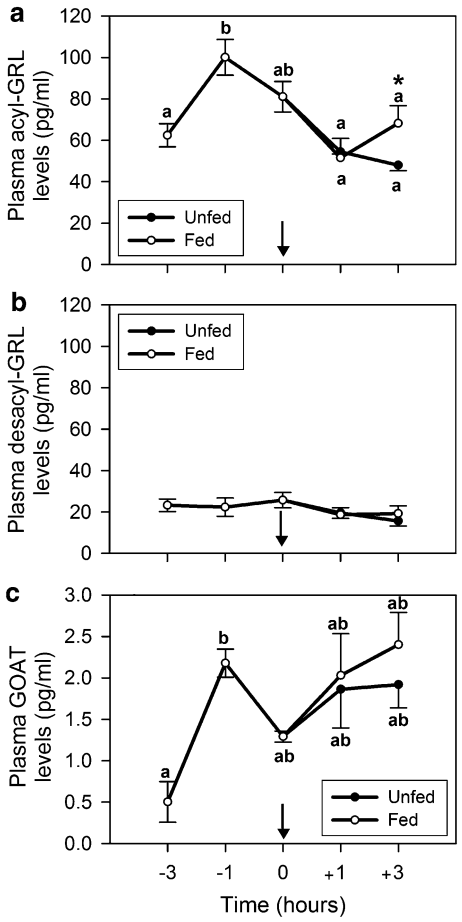

Fig. 1 Periprandial changes in plasma acyl-ghrelin (**a**), desacyl-ghrelin (**b**) and GOAT (**c**) levels before scheduled feeding time (−3 and −1 h), at feeding time (0 h) and after scheduled feeding time (+1 and +3 h) in goldfish. Data are expressed as mean ± SEM ($n = 7$). *Arrows* denote feeding time. *Different letters* indicate significant differences among the different time points in fed (*black dots*) or unfed (*white dots*) groups ($p < 0.05$), while *stars* indicate significant differences ($p < 0.05$) between groups at the same time point

according to the manufacturer's instructions and the amount of immunoreactive material was determined by using a cubic or quadratic regression curve-fit. Sensitivity limit of assays is 5.0 pg/ml for acyl-GRL and desacyl-GRL, and 0.1 ng/ml for GOAT, and the detection range is 31.2–1000 pg/ml for acyl-GRL and desacyl-GRL, and 0.5–16 ng/ml for GOAT.

Quantification of mRNA expression

Total RNA from central and peripheral tissues was isolated using TRI Reagent (Sigma-Aldrich, Madrid, Spain) and treated with RQ1 RNase-Free DNase (Promega, Madison, USA) according to the manufacturer's instructions. RNA purity was validated performing cDNA synthesis of serial dilutions of a known RNA concentration to be tested by quantitative PCR. Then, an aliquot of 0.3–1 µg of total RNA was reverse transcribed into cDNA in a 25 µl reaction volume using random primers, RNase inhibitor and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). The reverse transcription reaction conditions consisted of an initial step at 25 °C for 10 min, an extension at 42 °C for 50 min and a denaturalization step at 70 °C for 15 min.

Real-time or quantitative PCRs (qPCRs) were performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, USA), considering the abundance of goldfish *β-actin* and *elongation factor 1α* (*ef-1α*) as reference genes. The specific primers sequences used for target genes and reference genes (Sigma-Aldrich, Madrid, Spain) are shown in Table 1. Genes were amplified in qPCR runs using a 96-well plate loaded with 1 µl (*β-actin, ef-1α, goat* and *ghs-r* in brain) or 2 µl (*preproghrelin* and *ghs-r* in intestinal bulb) of cDNA and 0.5 µM (*β-actin, ef-1α, goat* and *ghs-r*) or 0.4 µM (*preproghrelin*) of each forward and reverse primer in a final volume of 10 µl. Each PCR run included a standard curve for the corresponding gene made of two replicates of four serial dilution points and water controls in order to ensure that the reagents were not contaminated. The qPCR cycling conditions consisted of an initial denaturation at 95 °C for 30 s and 40 cycles of a two-step amplification program (95 °C for 5 s and 60 °C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5 °C/5 s from 65 to 95 °C) at the end of each run to confirm the specificity of the amplification reaction. All runs were performed using a CFX96™ Real-time System (Bio-Rad Laboratories, Hercules, USA). The PCR products were checked by electrophoresis on a 1 % agarose gel. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to determine the relative mRNA expression.

Statistics

Statistical differences in circulating hormone levels and amount of mRNA transcripts were assessed using the Student's *t* test (for the fasting experiments), and one-way ANOVA followed by Student–Newman–Keuls multiple comparison (for the periprandial experiment), after data were checked for variances homogeneity. Significance was assigned when $p < 0.05$. All analyses were carried out using SigmaPlot 12.0 statistics package. Figures were created using SigmaPlot 12.0 statistics package.

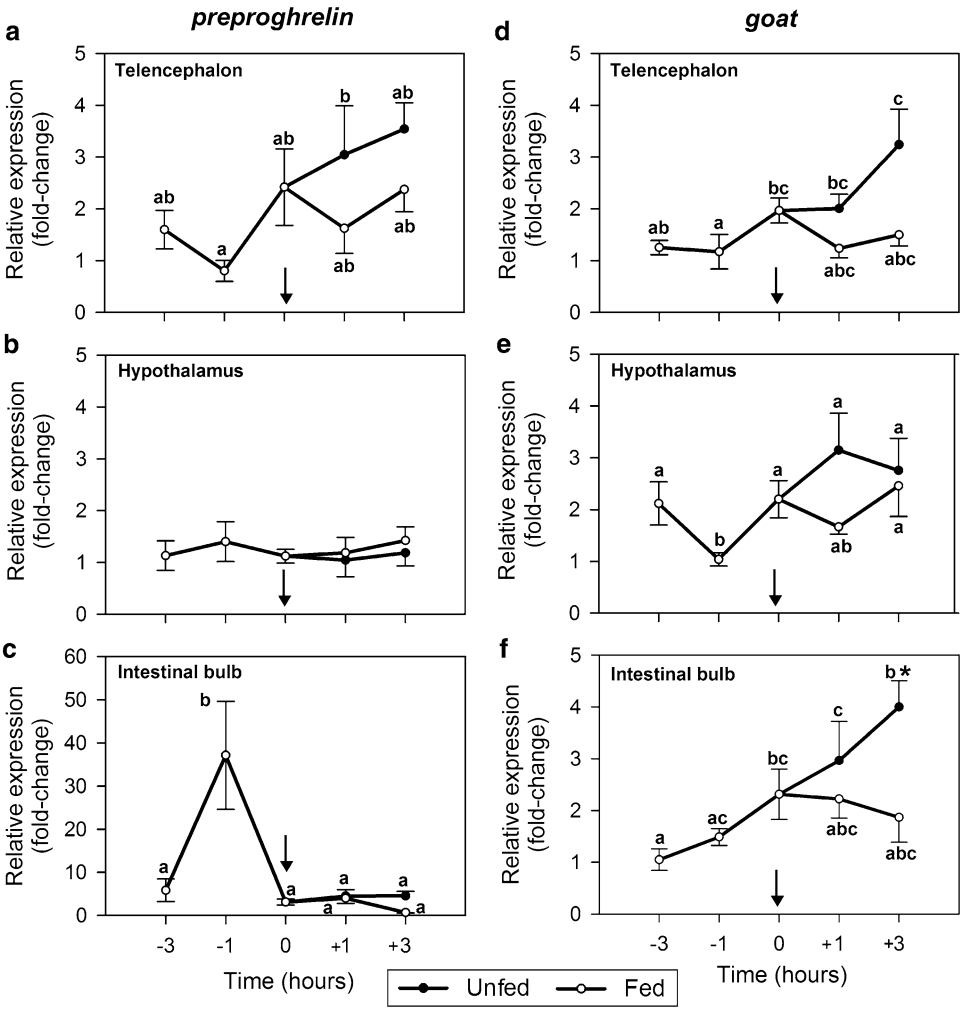

Fig. 2 Periprandial changes in *preproghrelin* (*left panel*) and *goat* (*right panel*) mRNA expression in telencephalon (**a**, **d**), hypothalamus (**b**, **e**) and intestinal bulb (**c**, **f**) before scheduled feeding time (−3 and −1 h), at feeding time (0 h) and after scheduled feeding time (+1 and +3 h) in goldfish. Data are expressed as mean ± SEM ($n = 6$–8) relative to the lowest average expression. *Arrows* denote feeding time. *Different letters* indicate significant differences ($p < 0.05$) among the different time points in fed (*black dots*) or unfed (*white dots*) groups, while *stars* indicate significant differences ($p < 0.05$) between groups at the same time point

Results

Periprandial profiles of circulating acyl-ghrelin, desacyl-ghrelin and GOAT, and of mRNAs encoding *preproghrelin*, *goat* and *ghs-r*

The periprandial variations in acyl-GRL, desacyl-GRL and GOAT plasma levels are shown in Fig. 1. Acyl-GRL levels (Fig. 1a) significantly increased ($p < 0.05$) from −3 to −1 h before scheduled feeding and decreased after scheduled feeding in both the fish that were fed at the regular feeding time and in unfed fish. Circulating levels of desacyl-GRL were around threefold lower than detected levels of acyl-GRL and were unaltered periprandially (Fig. 1b). There was a significant preprandial increase (around fivefold higher, $p < 0.05$) in GOAT plasma levels at −1 h compared to −3 h before feeding; feeding did not result in any significant differences in postprandial GOAT levels in none of the experimental groups (Fig. 1c).

The periprandial expression profiles of *preproghrelin* and *goat* in brain and intestinal bulb are shown in Fig. 2. No significant preprandial variations were detected for *preproghrelin* in either telencephalon or hypothalamus (Fig. 2a, b, respectively). However, the mRNA levels of *preproghrelin* were significantly higher at 1 h postfeeding in the telencephalon of unfed fish compared to time −1 h. A significant ($p < 0.05$) preprandial increase in *preproghrelin* mRNA levels was observed in the intestinal bulb at −1 h before scheduled feeding compared to −3 and 0 h values. No significant differences are observed in postprandial transcript levels in intestinal bulb from fed and unfed fish (Fig. 2c). Expression levels of *goat* in hypothalamus were significantly lower ($p < 0.05$) 1 h before scheduled feeding compared to the rest of periprandial values, which remained unaltered in both fed and unfed fish (Fig. 2e). The periprandial profiles of *goat* mRNA were very similar in telencephalon and intestinal bulb (Fig. 2d, f, respectively), with an important increase in transcript levels at +3 h when compared to the preprandial time points. The amount of

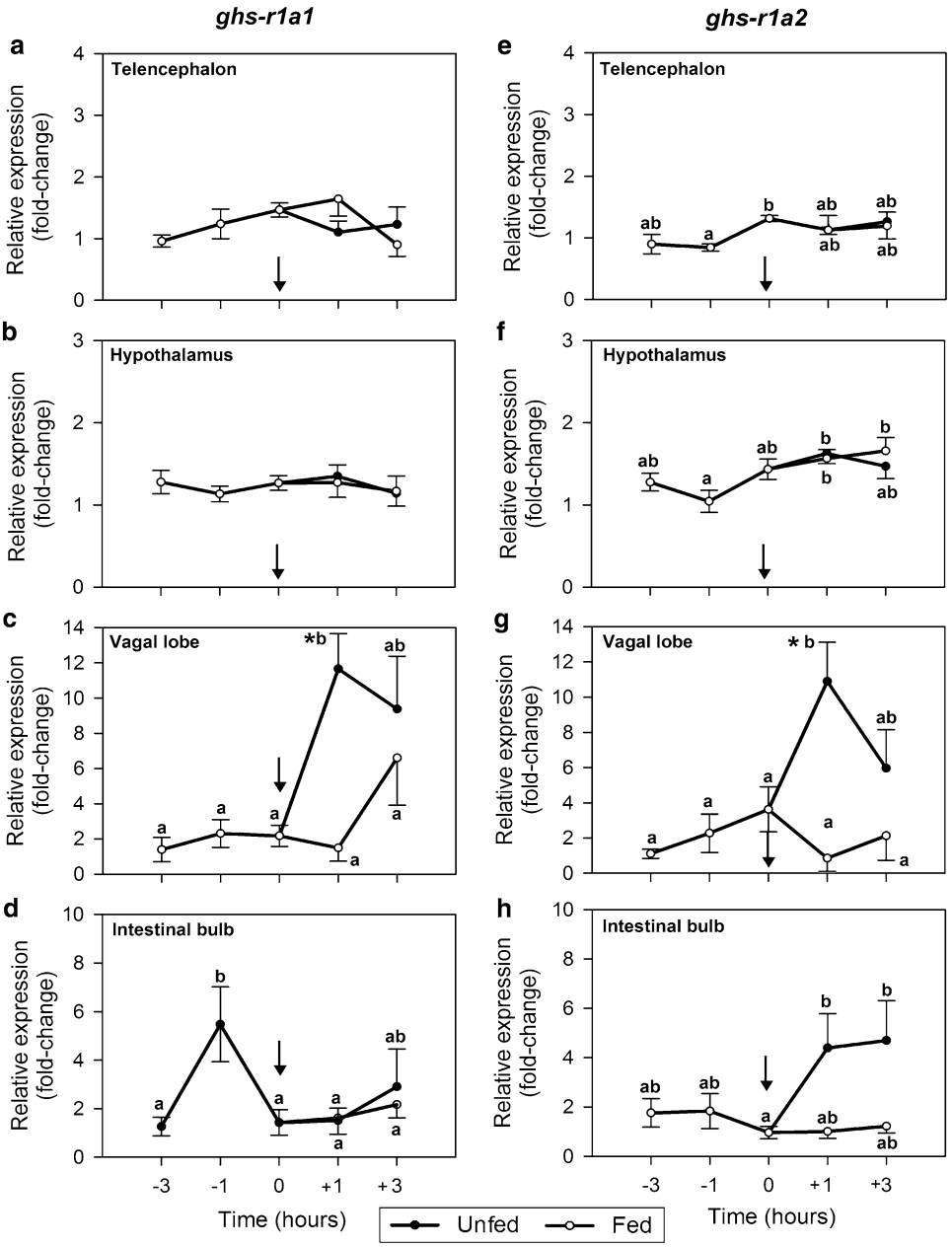

Fig. 3 Periprandial changes in *ghs-r1a1* (*left panel*) and *ghs-r1a2* (*right panel*) mRNA expression in telencephalon (**a**, **e**), hypothalamus (**b**, **f**), vagal lobe (**c**, **g**) and intestinal bulb (**d**, **h**) before scheduled feeding time (−3 and −1 h), at feeding time (0 h) and after scheduled feeding time (+1 and +3 h) in goldfish. Data are expressed as mean ± SEM ($n = 6$–8) relative to the lowest average expression. *Arrows* denote feeding time. *Different letters* indicate significant differences ($p < 0.05$) among the different time points in fed (*black dots*) or unfed (*white dots*) groups, while *stars* indicate significant differences between groups at the same time point ($p < 0.05$)

goat transcripts found in the intestinal bulb after scheduled feeding time was significantly higher ($p < 0.05$) in unfed than in fed fish.

The periprandial changes in *ghs-r* mRNA expression are shown in Figs. 3 and 4. The expression levels of *ghs-r1a2*, *ghs-r2a1* and *ghs-r2a2* in goldfish hypothalamus were significantly higher ($p < 0.05$) at +3 h postfeeding compared to −1 h values in unfed fish (Figs. 3f, 4b, e). However, expression of *ghs-r1a1* remained unaltered periprandially in hypothalamus (Fig. 3b) and in telencephalon (Fig. 3a). In this tissue, *ghs-r1a2* mRNA levels showed a significant increase ($p < 0.05$) at feeding time compared to levels at −1 h (Fig. 3e), while an important increase in *ghs-r2a2* mRNA levels was detected in unfed fish at +3 h after scheduled feeding (Fig. 4d). The four subtypes of ghrelin receptors showed a similar periprandial profile in vagal lobe (Figs. 3c, g, 4c, f) with statistical differences for *ghs-r1a1*, *ghs-r1a2* and *ghs-r2a2* but not for *ghs-r2a1*. These profiles were characterized by unaltered mRNA levels preprandially and an important increase ($p < 0.05$) at +1 h in unfed fish compared to both unfed and fed fish at the rest of the time points. In intestinal bulb, a significant ($p < 0.05$) preprandial increase in *ghs-r1a1* mRNA levels was observed at −1 h before scheduled feeding compared to times −3 and 0 h values. No postprandial differences were detected when compared transcripts levels in fed and unfed

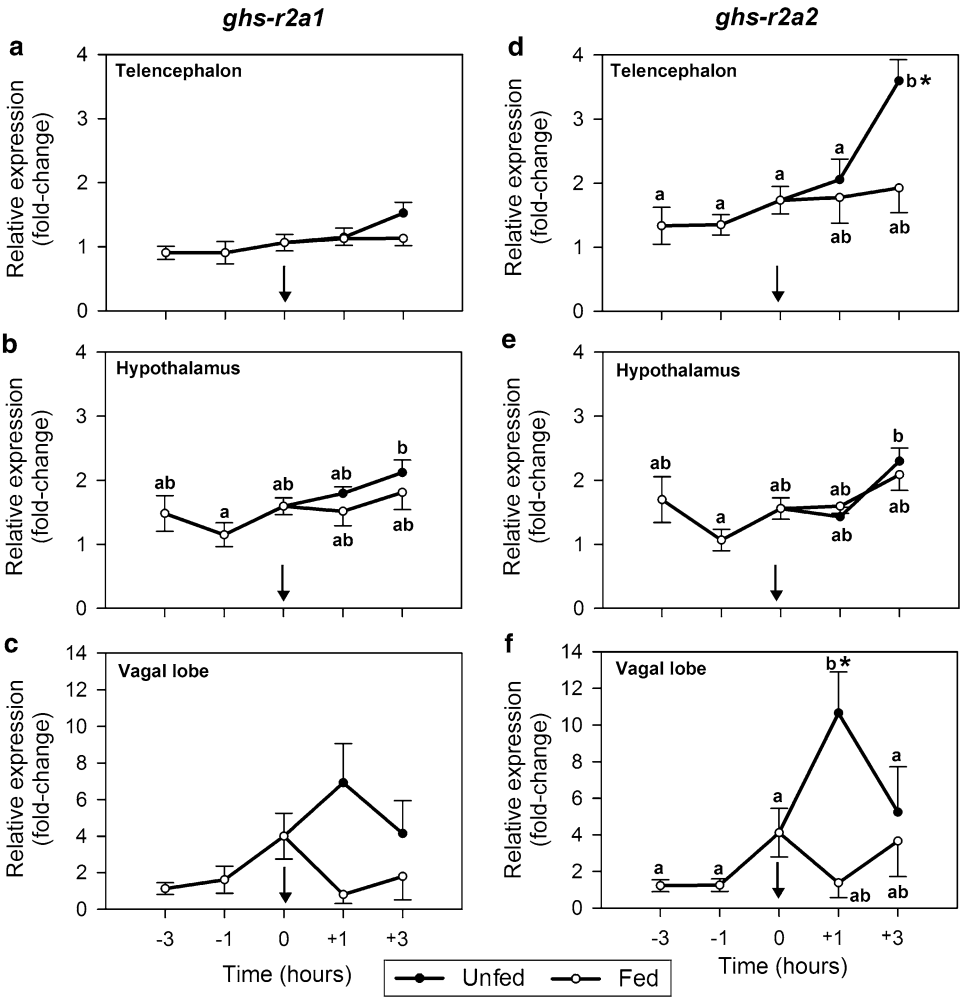

Fig. 4 Periprandial changes in *ghs-r2a1* (*left panel*) and *ghs-r2a2* (*right panel*) mRNA expression in telencephalon (**a**, **d**), hypothalamus (**b**, **e**) and vagal lobe (**c**, **f**) before scheduled feeding time (−3 and −1 h), at feeding time (0 h) and after scheduled feeding time (+1 and +3 h) in goldfish. Data are expressed as mean ± SEM ($n = 6$–8) relative to the lowest average expression. *Arrows* denote feeding time. *Different letters* indicate significant differences ($p < 0.05$) among the different time points in fed (*black dots*) or unfed (*white dots*) groups, while *stars* indicate significant differences between groups ($p < 0.05$) at the same time point

fish at +1 and +3 h (Fig. 3d). Expression of *ghs-r1a2* was unaltered preprandially in intestinal bulb, but did exhibit a significant elevation ($p < 0.05$) at +1 and +3 h in fish that were not fed at the scheduled feeding when compared to time 0 (Fig. 3h). There were no periprandial changes in the *ghs-r1a1* and *ghs-r1a2* mRNA expression in liver (data not shown).

Effects of short- and long-term fasting on the ghrelinergic system

Figure 5 shows the circulating levels of acyl-GRL, desacyl-GRL and GOAT in goldfish after a starvation period of 7 and 30 days. None of the studied fasting periods resulted in significant differences with respect to the control group regarding the circulating levels of any of the parameters analyzed.

Short- and long-term food deprivation were found to significantly ($p < 0.05$) upregulate *preproghrelin* and *goat* expression in intestinal bulb, as it can be observed in Fig. 6c, f, respectively. The magnitude of these increases was around six times compared to the control for *preproghrelin* and two times for *goat*. Ghrelin precursor mRNA levels were also increased by both fasting periods in telencephalon ($p < 0.05$, Fig. 6a), but were not modified in hypothalamus (Fig. 6b). By contrast, expression of *goat* was significantly increased by long-term (but not short-term) food deprivation in telencephalon and hypothalamus ($p < 0.05$, Fig. 6d, e).

The effects of starvation on the expression of the different receptor subtypes in central and peripheral locations of goldfish are shown in Fig. 7. In brain, we found that a 7-day fasting led to a significant increase ($p < 0.05$) in *ghs-r1a1* and *ghs-r1a2* expression in telencephalon, while a 30-day fasting significantly enhanced ($p < 0.05$) *ghs-r1a1*, *ghs-r2a1* and *ghs-r2a2* expression (Fig. 7a). Expression of *ghs-r* type *2*, but not type *1*, was also found to be significantly upregulated by food deprivation in vagal lobe (Fig. 7c). There were no significant differences in the expression of none of the ghrelin receptor subtypes in hypothalamus after a short- or long-term fasting (Fig. 7b). In peripheral locations, a long-term fasting significantly increased ($p < 0.05$)

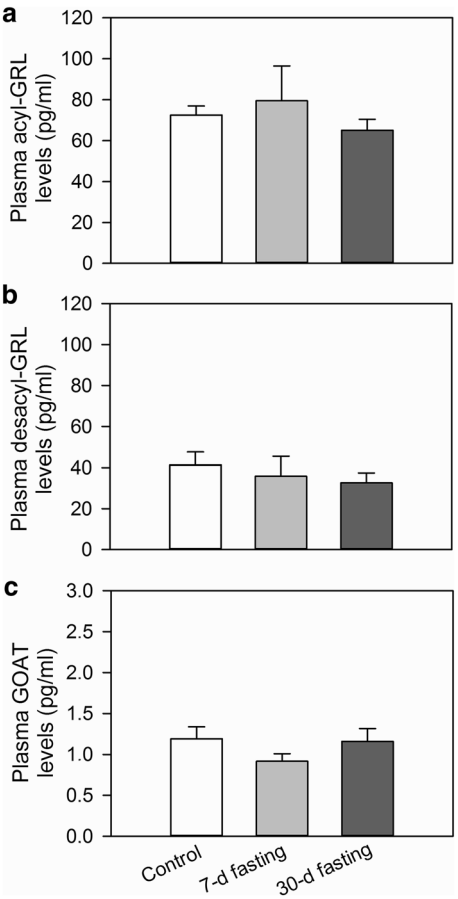

Fig. 5 Effects of short-term (7 days, *light grey columns*) and long-term (30 days, *dark grey columns*) fasting on plasma acyl-ghrelin (**a**), desacyl-ghrelin (**b**) and GOAT (**c**) levels in goldfish. Data are expressed as mean + SEM ($n = 6$–8). *Control bar* corresponds to data from control groups in both 7- and 30-day fasting experiments

ghs-r1a2 expression in intestinal bulb (Fig. 7d). No effects on *ghs-r* expression were observed in liver in response to fasting (data not shown).

Discussion

Present study reports the periprandial and starvation-induced changes in all the main components of the ghrelinergic system in central and peripheral locations in a teleost. Results from the periprandial study show that circulating levels of acyl-GRL, but not desacyl-GRL, rise shortly before feeding and fall after eating, indicating a specific involvement of acyl-ghrelin as a meal initiator signal in fish. Supporting this observation, circulating levels of GOAT also increase preprandially, probably in response to the need of an increase in ghrelin acylation. This periprandial circulating profile observed for plasma acyl-GRL is similar to the one described in mammals (Ariyasu et al. 2001; Cummings et al. 2001), and is consistent with an evolutionary conserved physiological role of ghrelin in initiating individual meals in vertebrates. In fish, very few studies are available on this subject, and it has been shown that ghrelin circulating levels decrease after feeding in goldfish (Unniappan et al. 2004), although no preprandial rise was detected either in this species or in Mozambique tilapia (Peddu et al. 2009). However, it must be taken into consideration that circulating ghrelin consists of more than 90 % of desacyl-GRL and less than 10 % of acyl-GRL, at least in humans (Delporte 2013), and circulating total ghrelin was measured in these two studies. Then, desacyl-GRL might be masking the possible variations in acyl-GRL levels, which can be only detected when quantifying separately acyl and desacyl forms. Until recently, acyl-GRL has been considered the only active form of ghrelin, while desacyl-GRL has been postulated as the degradation product of the hormone without biological activity. Several functions have been attributed to desacyl-GRL, including inhibition of glucose release (Gauna et al. 2005), and induction of neuronal precursor cells proliferation (Sato et al. 2006), but it seems that acyl-GRL is the only form of ghrelin implicated in food intake stimulation in mammals (Inhoff et al. 2009). Consistent with this, the injection of desacyl-GRL did not affect food intake in goldfish and even inhibited the orexigenic activity induced by acyl-GRL (Matsuda et al. 2006b), in agreement with a possible antagonist activity of desacyl-GRL (Delhanty et al. 2012). The changes of acyl-GRL in response to feeding found in the present study in goldfish support the hypothesis that acyl-GRL is the only form of ghrelin that plays a role in food intake regulation in vertebrates.

Food intake also modifies gene expression of the ghrelinergic system in goldfish, as observed by periprandial profiles for the different ghrelin-related genes. Consistent with the role of ghrelin in meal initiation, we observe that ghrelin precursor expression significantly rises 1 h before scheduled feeding time in intestinal bulb. This is in accordance with the reported increase in stomach *preproghrelin* mRNA levels 1 h before feeding in tilapia (Peddu et al. 2009), but it is in discordance with the lack of preprandial increase in *preproghrelin* expression in the whole gut of goldfish (Unniappan et al. 2004) and zebrafish (Hatef et al. 2015). Considering that the stomach is the main source of ghrelin in vertebrates, the periprandial variations in expression could only be detected when analyzing this part of the whole gastrointestinal tract separately. Such possibility could explain the reported differences in periprandial profile in different species, and even in the same species when analyzing mRNA expression in the whole gut (Unniappan et al. 2004; Hatef et al. 2015) versus the intestinal bulb (current study). Furthermore, the GHS-R1a1 receptor subtype seems to be the main receptor involved in

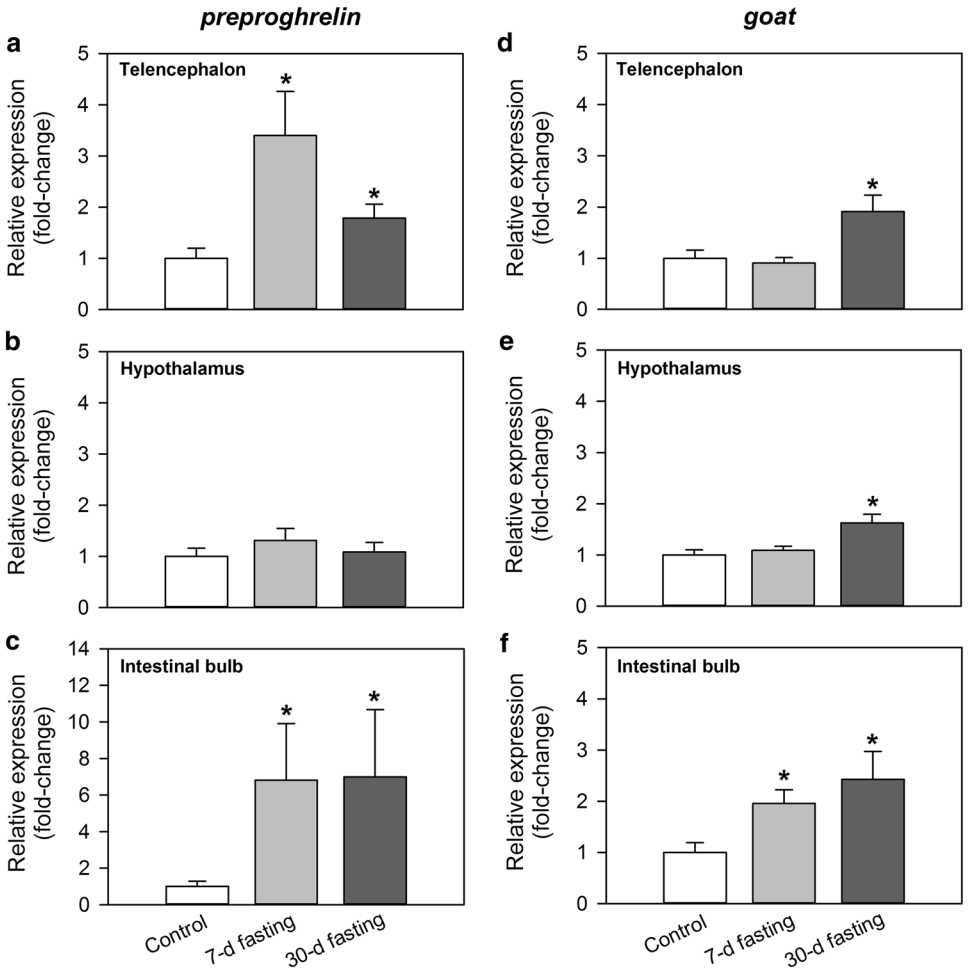

Fig. 6 Effects of short-term (7 days, *light grey columns*) and long-term (30 days, *dark grey columns*) fasting on *preproghrelin* (*left panel*) and *goat* (*right panel*) mRNA expression in telencephalon (**a**, **d**), hypothalamus (**b**, **e**) and intestinal bulb (**c**, **f**) in goldfish. Data are expressed as mean + SEM ($n = 6$–8). *Control bar* corresponds to data from control groups in both 7- and 30-day fasting experiments. *Stars* indicate significant differences ($p < 0.05$) respect to the control

the preprandial action of ghrelin in the intestinal bulb, as this gene shows a similar expression profile to ghrelin with an important preprandial increase. Thus, this observation suggests the implication of GHS-R1a1 in the responses of ghrelin to regular feeding in the gastrointestinal tract. By contrast, GHS-R1a2 might act under food scarcity conditions, as it is the only receptor subtype whose expression is significantly increased postprandially in the intestinal bulb of unfed fish. This hypothesis is supported by an upregulation of *ghs-r1a2*, but not *ghs-r1a1,* after food deprivation in goldfish intestine bulb.

An interesting finding of the present study is the differential effect of food intake on the ghrelinergic system expression in different brain areas. For instance, *preproghrelin* expression was unaltered periprandially in hypothalamus, in accordance with previous reports in the goldfish hypothalamus (Unniappan et al. 2004). However, a significant increase in mRNA levels was detected postprandially in telencephalon of fish that missed the scheduled feeding. Similarly, expression of *goat* was interestingly affected by feeding in telencephalon, but not in hypothalamus. The expression profile observed for *goat* in telencephalon was very similar to the *goat* expression profile in the brain of zebrafish (Hatef et al. 2015), the only report to date on periprandial changes in *goat* expression in vertebrates. Together, present results in telencephalon suggest that feeding induces changes in the functional activation rate of ghrelin in this tissue, suggesting the telencephalon to be a key regulator of feeding responses in the goldfish brain. Another major finding in the present study concerns the different periprandial expression profiles of ghrelin receptors in fed versus unfed fish, with significant increases of *ghs-r* transcripts in the vagal lobe when the scheduled meal is missing. These results point out for the first time the vagal lobe, together with the telencephalon, as a key target for ghrelin in the central response to a meal ingestion.

Neither short- nor long-term fasting modifies circulating levels of acyl-GRL, desacyl-GRL and GOAT in goldfish. Previous studies reported an upregulation of plasma ghrelin in response to very short periods of food deprivation, i.e., 24 and 48 h in rats (Ariyasu et al. 2002), 24 h in rainbow trout (Pankhurst et al. 2008), 48 h in Atlantic salmon (Hevrøy et al. 2011) and 72 h in goldfish (Unniappan et al.

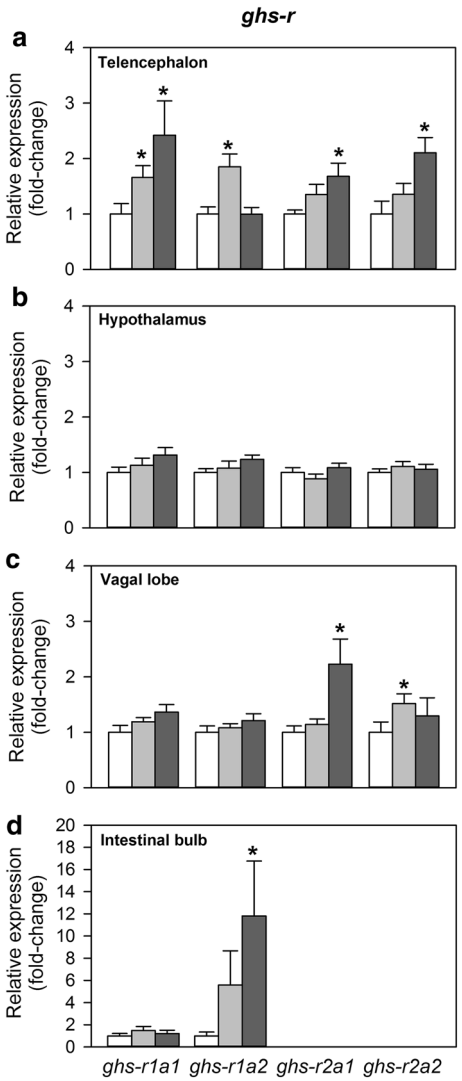

Fig. 7 Effects of short-term (7 days, *light grey columns*) and long-term (30 days, *dark grey columns*) fasting on *ghs-r1a1*, *ghs-r1a2*, *ghs-r2a1* and *ghs-r2a2* mRNA expression in telencephalon (**a**), hypothalamus (**b**), vagal lobe (**c**) and intestinal bulb (**d**) in goldfish. Data are expressed as mean + SEM ($n = 6$–8). *Control bar* corresponds to data from control groups in both 7- and 30-day fasting experiments. *Stars* indicate significant differences ($p < 0.05$) respect to the control

2004). However, fasting periods of a duration longer than 4 days have been generally described to produce no effects on circulating ghrelin levels in several fish species (Hevrøy et al. 2011; Fox et al. 2009; Riley et al. 2008; Unniappan et al. 2004). Only two studies go against this hypothesis. On one hand, a significant increase in plasma ghrelin levels was reported in tilapia starved for 14 and 28 days (Fox et al. 2009). On the other, Jönsson et al. (2007) described a reduction in hormone circulating levels after fasting periods of 7, 14 and 21 days in rainbow trout. While some species differences exist, general observations seem to indicate that circulating ghrelin is a short-time regulator of energy homeostasis, in accordance with our present results.

Our results indicate that the effect of fasting on the expression of the ghrelinergic system in goldfish depends on the tissue. In peripheral tissues, both a 7-day and a 30-day fasting leads to an increase in *preproghrelin* and *goat* expression in goldfish intestinal bulb, as expected for an appetite-enhancing hormone. Stimulation of ghrelin precursor expression in gut induced by fasting is evolutionary conserved, from fish (Amole and Unniappan 2009; Feng et al. 2013; Matsuda et al. 2006a; Terova et al. 2008; Unniappan et al. 2004; Zhou et al. 2014) to mammals (González et al. 2008; Zhao et al. 2008). However, the possible modulation of *goat* by food deprivation is less studied. Our results demonstrate that long-term fasting leads to high levels of mRNAs encoding this enzyme in intestinal bulb, in agreement with a recent report testing short-term (3- and 7-day) food deprivation in zebrafish (Hatef et al. 2015). In mammals, the scarce available data are inconsistent: *goat* expression in stomach is upregulated by 1-day (Gahete et al. 2010) and 21-day (González et al. 2008) fasting, but it is downregulated upon food deprivation periods of 12, 24 and 36 h (Kirchner et al. 2009). Given this context of scarce and variable observations among species, further studies are needed to determine the relevance of GOAT as a target for the regulation of the ghrelinergic system by food availability in vertebrates. Regarding GHS-R, only one recent study in zebrafish has shown that fasting elevates the expression of *ghs-r1a* and *ghs-r2a* in anterior intestine (Eom et al. 2014). Our results on *ghs-r1a2* expression are consistent with this previous observation, suggesting a specific involvement of this receptor subtype in the ghrelin-mediated fasting regulation in goldfish.

In the brain, the mRNAs encoding *preproghrelin* and *goat*, as well as all receptor subtypes, are increased by fasting in telencephalon, reinforcing our suggestion that the telencephalon is a key brain area involved in the responses to food deprivation in goldfish. *Goat*, but not *preproghrelin*, expression was also upregulated by a long-term fasting in hypothalamus. Studies on the effects of fasting on *goat* expression are very scarce, but it seems that food deprivation, regardless of its duration, upregulates the expression of this enzyme in the brain of both mammals (Gahete et al. 2010) and fish (Hatef et al. 2015). However, data regarding the effects of food deprivation on brain *preproghrelin* expression are more controversial, with different observations depending on species, duration of fasting period, and the brain area analyzed (Unniappan et al. 2004; Matsuda et al. 2006a; Eom et al. 2013; Feng et al. 2013). Many factors may justify such different results, and before obtaining physiological conclusions, it should be borne in mind that most of studies quantify mRNA expression, but it is unknown whether the changes observed at the transcription

level extend to translation and post-translational modifications. Thus, further studies at protein level are needed to better understand the possible different effects of fasting on *preproghrelin* expression depending on the species, duration and tissue.

The effects of food deprivation on ghrelinergic receptors in vagal lobe depend on its duration. Thus, a short-term fasting is observed to produce an increase in *ghs-r2a1* expression, whereas a long-term fasting leads to higher levels of *ghs-r2a2* transcripts. This data indicates an implication of GHS-R type 2, but not type 1, in the physiological responses to food deprivation in vagal lobe of goldfish, and even that the two subtypes of GHS-R2 are differentially involved as duration of starvation increases. The lack of effect on *ghs-r* type *1* expression in vagal lobe disagrees with previous observations on a decrease in *ghs-r1a1* in the vagal lobe after 7 days of fasting (Kaiya et al. 2010). The highly different body condition (weight and reproductive stage) of fish used in both studies (3–10 g versus 20–30 g) might justify such different results.

In summary, present novel data demonstrate the different involvement of the components of the ghrelinergic system (ghrelin, the enzyme responsible for its acylation and the four subtypes of ghrelin receptors) in the responses to feeding in a teleost. We found that the telencephalon, vagal lobe and intestinal bulb are key targets for the responses of the ghrelinergic system to food availability, and we suggest that GHS-R1a1 could be involved in regular ghrelin responses to feeding, while GHS-R1a2 might act in situations of food scarcity. The periprandial study reinforces the hypothesis that ghrelin in its acylated form is implicated in the ghrelin-induced responses to feeding, and it could be acting as a meal initiator signal in goldfish.

Compliance with ethical standards

Funding This study was funded by the Spanish Ministerio de Economía y Competitividad (AGL2013-46448-C3-2-R) to M.J. Delgado. A.M. Blanco and M. Gómez-Boronat are predoctoral fellows from the Spanish Ministerio de Educación y Ciencia and the Spanish Ministerio de Economía y Competitividad, respectively.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Reference

Amole N, Unniappan S (2009) Fasting induces preproghrelin mRNA expression in the brain and gut of zebrafish, *Danio rerio*. Gen Comp Endocrinol 161:133–137

Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K (2002) Delayed short-term secretory regulation of ghrelin in obese animals:evidenced by a specific RIA for the active form of ghrelin. Endocrinology 143:3341–3350

Arisayu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K (2001) Stomach is the major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 86:4753–4758

Breves JP, Veillette PA, Specker JL (2009) Ghrelin in the summer flounder: immunolocalization to the gastric glands and action on plasma cortisol levels. Comp Biochem Physiol A Mol Integr Physiol 152:268–272

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS (2001) A preprandial rise in ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719

Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ et al (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661

Delhanty PJD, Neggers SJ, van der Lely AJ (2012) Ghrelin: the differences between acyl- and des-acyl-ghrelin. Eur J Endocrinol 167:601–608

Delporte C (2013) Structure and physiological actions of ghrelin. Scientifica 2013:1–25

Eom J, Hong M, Cone RD, Song Y (2013) Zebrafish ghrelin is expressed in pancreatic endocrine cells and regulated by metabolic state. Biochem Biophys Res Commun 439:115–120

Eom J, Hong A, Kang YH, Yoo HJ, Chang EJ, Kang SW, Yoon SY, Kim SY, Song Y (2014) Molecular cloning, regulation, and functional analysis of two GHS-R genes in zebrafish. Exp Cell Res 326:10–21

Feng K, Zhang GR, Wei KJ, Xiong BX (2013) Molecular cloning, tissue distribution, and ontogenetic expression of ghrelin and regulation of expression by fasting and refeeding in the grass carp (*Ctenopharyngodon idellus*). J Exp Zool Part Ecol Genet Physiol 319:202–212

Fox BK, Breves JP, Hirano T, Grau EG (2009) Effects of short- and long-term fasting on plasma and stomach ghrelin, and the growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. Domest Anim Endocrinol 37:1–11

Gahete MD, Córdoba-Chacón J, Salvatori R, Castaño JP, Kineman RD, Luque RM (2010) Metabolic regulation of ghrelin O-acyl transferase (GOAT) expression in the mouse hypothalamus, pituitary, and stomach. Mol Cell Endocrinol 317:154–160

Gauna C, Delhanty PJ, Hofland LJ, Janssen JA, Broglio F, Ross RJ, Ghigo E, van der Lely AJ (2005) Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. J Clin Endocrinol Metab 90:1055–1060

Gauna C, Kiewiet RM, Janssen JAMJL, van de Zande B, Delhanty PJD, Ghigo E, Hofland LJ, Themmen APN, van der Lely AJ (2007) Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions. Am J Physiol Endocrinol Metab 293:E697–E704

González CR, Vázquez MJ, López M, Diéguez C (2008) Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa. J Mol Endocrinol 41:415–421

Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE (2008) Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci USA 105:6320–6325

Hatef A, Yufa R, Unniappan S (2015) Ghrelin *O*-acyl transferase in zebrafish is an evolutionarily conserved peptide upregulated during calorie restriction. Zebrafish 12:327–338

Heppner KM, Tong J, Kirchner H, Nass R, Tschöp MH (2011) The ghrelin *O*-acyltransferase–ghrelin system: a novel regulator

of glucose metabolism. Curr Opin Endocrinol Diabetes Obes 18:50–55

Hevrøy EM, Azpeleta C, Shimizu M, Lanzén A, Kaiya H, Espe M, Olsvik PA (2011) Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. Fish Physiol Biochem 37:217–232

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M et al (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974–977

Inhoff T, Wiedenmann B, Klapp BF, Mönnikes H, Kobelt P (2009) Is desacyl ghrelin a modulator of food intake?. Peptides 30:991–994

Jönsson E, Forsman A, Einarsdottir IE, Kaiya H, Ruohonen K, Björnsson BT (2007) Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. Comp Biochem Physiol A Mol Integr Physiol 147:1116–1124

Kaiya H, Kojima M, Hosoda H, Moriyama S, Takahashi A, Kawauchi H, Kangawa K (2003) Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. Endocrinology 144:5215–5226

Kaiya H, Miyazato M, Kangawa K, Peter RE, Unniappan S (2008) Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp Biochem Physiol A Mol Integr Physiol 149:109–128

Kaiya H, Miura T, Matsuda K, Miyazato M, Kangawa K (2010) Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, *Carassius auratus*. Mol Cell Endocrinol 327:25–39

Kaiya H, Kangawa K, Miyazato M (2013) Ghrelin receptors in non-mammalian vertebrates. Front Endocrinol 4:81

Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschöp MH (2009) GOAT links dietary lipids with the endocrine control of energy balance. Nat Med 15:741–745

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660

Korhonen T, Saarela S (2005) Role of adiposity hormones in the mouse during fasting and winter-acclimatization. Comp Biochem Physiol A Mol Integr Physiol 140:217–223

Koven W, Schulte P (2012) The effect of fasting and refeeding on mRNA expression of PepT1 and gastrointestinal hormones regulating digestion and food intake in zebrafish (*Danio rerio*). Fish Physiol Biochem 38:1565–1575

Larhammar D, Risinger C (1994) Molecular aspects of tetraploidy in the common carp *Cyprinus carpio*. Mol Phylogenet Evol 3:59–68

Lear PV, Iglesias MJ, Feijóo-Bandín S, Rodríguez-Penas D, Mosquera-Leal A, García-Rúa V, Gualillo O et al (2010) Des-acyl-ghrelin has specific binding sites and different metabolic effects from ghrelin in cardiomyocytes. Endocrinology 151:3286–3298

Lim CT, Kola B, Korbonits M (2011) The ghrelin/GOAT/GHS-R system and energy metabolism. Rev Endocr Metab Disord 12:173–186

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408

Matsuda K, Miura T, Kaiya H, Maruyama K, Uchiyama M, Kangawa K, Shioda S (2006a) Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, *Carassius auratus*. Peptides 27:1335–1340

Matsuda K, Miura T, Kaiya H, Maruyama K, Shimakura SI, Uchiyama M, Kangawa K, Shioda S (2006b) Regulation of food intake by acyl and des-acyl-ghrelins in the goldfish. Peptides 27:2321–2325

Miura T, Maruyama K, Kaiya H, Miyazato M, Kangawa K, Uchiyama M, Shioda S, Matsuda K (2009) Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*. Peptides 30:758–765

Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, Batterham RL et al (2015) Ghrelin. Mol Metab 4:437–460

Pankhurst NW, King HR, Ludke SL (2008) Relationship between stress, feeding and plasma ghrelin levels in rainbow trout, *Oncorhynchus mykiss*. Mar Freshw Behav Physiol 41:53–64

Peddu SC, Breves JP, Kaiya H, Grau EG, Riley LG Jr (2009) Pre- and postprandial effects on ghrelin signaling in the brain and on the GH/IGF-I axis in the Mozambique tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol 161:412–418

Picha ME, Strom CN, Riley LG, Walker AA, Won ET, Johnstone WM, Borski RJ (2009) Plasma ghrelin and growth hormone regulation in response to metabolic state in hybrid striped bass: Effects of feeding, ghrelin and insulin-like growth factor-I on in vivo and in vitro GH secretion. Gen Comp Endocrinol 161:365–372

Riley LG, Fox BK, Breves JP, Kaiya H, Dorough CP, Hirano T, Grau EG (2008) Absence of effects of short-term fasting on plasma ghrelin and brain expression of ghrelin receptors in the tilapia, *Oreochromis mossambicus*. Zool Sci 25:821–827

Sánchez-Bretaño A, Blanco AM, Unniappan S, Kah O, Gueguen M-M, Bertucci JI, Alonso-Gómez AL, Valenciano AI, Isorna E, Delgado MJ (2015) *In situ* localization and rhythmic expression of ghrelin and *ghs-r1* ghrelin receptor in the brain and gastrointestinal tract of goldfish (*Carassius auratus*). PLoS One 10:e0141043

Sato M, Nakahara K, Goto S, Kaiya H, Miyazato M, Date Y, Nakazato M, Kangawa K, Murakami N (2006) Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord. Biochem Biophys Res Commun 350:598–603

Sato T, Nakamura Y, Shiimura Y, Ohgusu H, Kangawa K, Kojima M (2012) Structure, regulation and function of ghrelin. J Biochem 151:119–128

Terova G, Rimoldi S, Bernardini G, Gornati R, Saroglia M (2008) Sea bass ghrelin: molecular cloning and mRNA quantification during fasting and refeeding. Gen Comp Endocrinol 155:341–351

Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K, Peter RE (2002) Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology 143:4143–4146

Unniappan S, Canosa LF, Peter RE (2004) Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 79:100–108

Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132:387–396

Zhao Z, Sakata I, Okubo Y, Koike K, Kangawa K, Sakai T (2008) Gastric leptin, but not estrogen and somatostatin, contributes to the elevation of ghrelin mRNA expression level in fasted rats. J Endocrinol 196:529–538

Zhou C, Zhang X, Liu T, Wei R, Yuan D, Wang T, Lin F et al (2014) *Schizothorax davidi* ghrelin: cDNA cloning, tissue distribution and indication for its stimulatory character in food intake. Gene 534:72–77