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RESEARCH PAPER

Identification, expression analysis, and functional characterization of *ghrelin* and its receptors in spotted sea bass (*Lateolabrax maculatus*)

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Abstract

Ghrelin (Ghrl), an appetite-inducing peptide hormone secreted by the stomach, is the endogenous ligand for the growth hormone secretagogue receptor (Ghs-r). In this study, we identified the *preproghrelin* gene and its receptors in spotted sea bass (*Lateolabrax maculatus*). The *ghrl* gene consisted of an open reading frame (ORF) of 324 nucleotides encoding 107 amino acids, and the premature protein contained a 20-amino-acid mature peptide. Through a syntenic analysis, we also validated the annotation of *growth hormone secretagogue receptor 1a* (*ghs-r1a*) and *growth hormone secretagogue receptor 1a-like* (*ghs-r1a-like*), which contained seven-transmembrane structures, in spotted sea bass. The ORF of *ghs-r1a* consisted of 1152 bp that encoded a 383-amino-acid protein, and *ghs-r1a-like* contained an ORF of 2631 bp and produced a protein with 876 amino acids. A phylogenetic analysis showed that spotted sea bass *ghrl* and its receptors clustered with those of other fishes and were more distantly related to those of other vertebrates. In situ hybridization revealed that *ghrl* was highly expressed in the stomach and localized in the mucosa and submucosa. The expression of these genes varied during short-term starvation in a time-dependent manner. In vitro studies showed that after incubation with Ghrl for 3 h enhanced the expression of *motilin* (*mln*), *gastrin* (*gas*) and *cholecystokinin* (*cck*), but this effect vanished after 6 h of incubation. In summary, Ghrl and its receptors might play important roles in the regulation of food intake in spotted sea bass.

Keywords *Ghrl* · *Ghs-r* · In vitro experiment · Localization · Spotted sea bass · Short-term starvation

Introduction

Ghrelin (Ghrl) was first isolated from the stomachs of rats and humans and was identified as an endogenous ligand for the growth hormone secretagogue receptor (Ghs-r), which is a G-protein-coupled receptor with seven transmembrane domains (Kojima et al. 1999). Ghrl is encoded by the *preproghrelin* gene, which also encodes the 23-amino-acid peptide obestatin, a peptide that can inhibit food intake and counteract the effect of Ghrl (Zhang et al. 2005). In addition to mammals, Ghrl has also been identified in some fishes, including goldfish (*Carassius auratus*) (Unniappan et al. 2002), eel (*Anguilla anguilla*) (Kaiya et al. 2003b), channel catfish (*Ietalurus punetaus*) (Kaiya et al. 2005), and sea bream (*Sparus auratus*) (Yeung et al. 2006). Ghrl is found in two major forms, *n*-octanoyl-modified Ghrl and des-acyl Ghrl, which exhibit an *n*-octanoyl modification on serine-3 and constitute the main source of Ghrl activity (Sato et al. 2011). *N*-octanoyl-modified Ghrl functions by inducing the release of growth hormone (Takaya et al. 2000) and plays roles in energy expenditure (Huda et al. 2009) and food uptake (Druce et al. 2005). The GHS-R was first identified as the specific receptor for GHRL in mammals (Howard et al. 1996) and has since been identified in several fish species, including zebrafish (*Danio rerio*) (Olsson et al. 2008), rainbow trout (*Oncorhynchus mykiss*) (Kaiya et al. 2009), goldfish (*Carassius auratus*) (Kaiya et al. 2010) and Atlantic salmon (*Salmo salar*) (Hevrøy et al. 2011). In general, at least two paralogs of Ghs-r exist (Petersenn 2002), and these include Ghs-r1a and Ghs-r1b. Ghs-r1a is active and derives from regular splicing, whereas Ghs-r1b is poorly

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understood and derives from alternative splicing (Howard et al. 1996). Another paralog named Ghs-r1a-like has only been found in fishes (Kaiya et al. 2013, 2014). Multiple Ghs-r types generally exist in Atlantic salmon (Hevrøy et al. 2011), black sea bream *(Acanthopagrus schlegelii*) (Chan and Cheng 2004), and zebrafish (Olsson et al. 2008).

Motilin (Mln), gastrin (Gas) and cholecystokinin (Cck) are gastrointestinal hormones that play vital roles in promoting or inhibiting food intake (Gué and Buéno 1996). Mln, a 22-amino-acid peptide, functions in regulating interdigestive motility, gallbladder contractions, and enzyme secretion in the stomach and pancreas (Layer et al. 1988). Gas is one of the major gastrointestinal hormones and has multiple physiological activities. The most studied activity of Gas is the stimulation of gastric acid secretion while other well-known activities include the activation of gastric movement and the stimulation of pancreatic enzyme secretion (Kato et al. 1983). Cck, a gastrointestinal peptide hormone, has been implicated in the internal mechanism responsible for the termination of eating and the physiological state of satiety (McCaleb and Myers 1980).

Regulating food intake and the energy balance is essential for ensuring that an organism has sufficient energy to maintain normal growth, survive periods of insufficient food supply, and perform the activities necessary for survival. However, the roles of the Ghrl/Ghs-r system in fish and the associated regulatory mechanisms remain largely unknown. Spotted sea bass (*Lateolab*rax *maculatus*) is a popular mariculture fish species in China due to its tastiness and high nutritional value (Wang et al. 2017). To achieve higher economic profits, aquaculture operators control their feed costs by exposing the fish to short-term starvation every few weeks. Ghrl can promote growth hormone secretion and has important functions in regulating food intake and energy balance. The study of *ghrl* mRNA expression changes during short-term starvation is helpful for understanding its potential function in the regulation of food intake. In this study, we identified Ghrl and its receptors in spotted sea bass, determined their locations, validated the annotation of *ghs-r1a* and *ghs-r1a-like* and examined the variation in their expression after short-term starvation. To evaluate the functions of Ghrl, we measured the mRNA expression levels of *mln*, *gas* and *cck* after the in vitro stimulation of cultured stomach fragments with Ghrl. We also explored the application of Ghrl as a food-promoting agent and a growth-promoting agent in the development of the fishery economy.

Results

Cloning and sequence analysis of *ghrl* and its receptors

The analysis revealed that the *ghrl* gene of spotted sea bass (MH046053) encodes a Ghrl precursor. In addition, the *preproghrelin* open reading frame (ORF) consists of 324 nucleotides that encode 107 amino acids, including a 26-amino-acid signal peptide and a 20-amino-acid mature peptide with an acyl-modified Ser at the third position. The alternative splicing of *preproghrelin* yielded another peptide named "obestatin", which is composed of 23 amino acids (Supplementary Fig. S1A). As shown in Supplementary Fig. S1B, the predicted Ghrl amino acid sequence is highly conserved among teleost species. The first seven amino acids (GSSFLSP) of the mature Ghrl were identical to those in most other species. The mature Ghrl peptide of spotted sea bass shows high sequence homology to the Ghrl peptides of other fish species, including 56.25% homology to the damselfish (*Stegastes partitus*) Ghrl peptide and 53.13% homology to the Ghrl peptide of large yellow croaker (*Larimichthys crocea*), but low homology to those of mammalian and poultry species, including 31.25%, 31.25% and 37.50% homology to the human (*Homo sapiens*), mouse (*Mus musculus*), and chicken (*Gallus gallus*) Ghrl peptides, respectively. The analysis also showed that obestatin is highly conserved among fish and vertebrate species, and sea bass obestatin showed 73.91%, 65.22%, 52.17%, 43.48%, 30.43% and 34.78% homology to the obestatin peptides of large yellow croaker (*Larimichthys crocea*), damselfish (*Stegastes partitus*), zebrafish (*Danio rerio*), chicken (*Gallus gallus*), human (*Homo sapiens*), and mouse (*Mus musculus*), respectively.

The *ghs-r1a* gene of spotted sea bass (MH046055) has an ORF of 1152 bp, and the predicted protein contains 383 amino acids with seven transmembrane structures (Supplementary Fig. S2). The amino acid sequence of Ghs-r1a displays high sequence homology with the corresponding sequences of other fish species, including 88.57% homology to Ghs-r1a of orange-spotted grouper (*Epinephelus coioides*), 91.69% homology to Ghs-r1a of large yellow croaker (*Larimichthys crocea*), 61.30% homology to Ghs-r1a of zebrafish (*Danio rerio*), 60.52% homology to Ghs-r2a of zebrafish (*Danio rerio*), but low homology with those of other vertebrate species, e.g., 58.96% homology to chicken (*Gallus gallus*) Ghs-r1a, 54.29% homology to human (*Homo sapiens*) Ghs-r1a, 41.30% homology to human (*Homo sapiens*) Ghs-r1b, and 53.77% homology to rat (*Rattus norvegicus*) Ghs-r1a (Supplementary Fig. S3). The *ghs-r1a-like* gene of spotted sea bass (MH046056) includes an ORF of 2631 bp that encodes an 876-amino-acid protein. However, this protein contains eight transmembrane domains (Supplementary Fig. S4) and displays low homology to the corresponding sequences of other species (Supplementary Fig. S5).

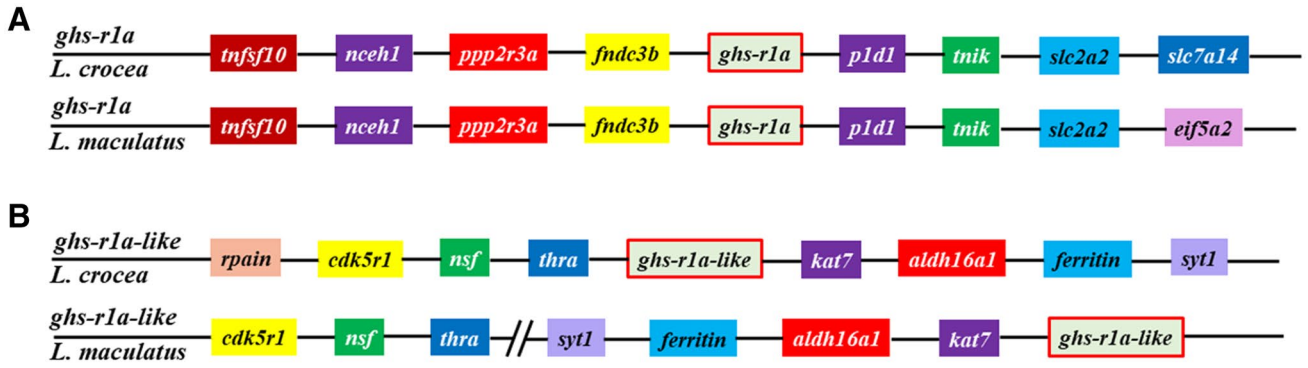

Fig. 1 Syntenic analysis of spotted sea bass *ghs-r* genes with those of *Larimichthys crocea*. **a** *ghs-r1a*. **b** *ghs-r1a-like*. These syntenies were generated using the information obtained from the genome browser Genomicus

Syntenic and phylogenetic analysis

A syntenic analysis was performed to provide additional support for the annotation of the *ghrl* receptors. As shown in Fig. 1, the spotted sea bass *ghs-r* genes shared similar neighboring genes to those of large yellow croaker, although both spotted sea bass and large yellow croaker had unique genes, which were potentially required after their divergence. The two *ghs-r* genes were derived from the initial whole-genome duplication event (WGD). Overall, the syntenic analysis validated the annotation of the *ghs-r* genes.

To analyze the evolutionary relationships of Ghrl and its receptors, a phylogenetic tree was constructed based on the amino acid sequences of these genes in several species (Fig. 2). The results revealed that Ghrl appears to be conserved. The Ghrl of higher vertebrates (human, mouse, monkey, and chicken) and teleosts were grouped into respective lineages in the phylogenetic tree, and the Ghrl of spotted sea bass clustered with its teleost counterparts as expected. Additionally, the clustering pattern provides evidence that spotted sea bass Ghrl shares its highest homology with the Ghrl peptides of European sea bass (*Dicentrarchus labrax*) and large yellow croaker (*Larimichthys crocea*). The variants of the *ghrl* receptor in spotted sea bass were clearly divided into two lineages and share high homology with those of other fishes.

Tissue expression of *ghrl* and its receptors

As shown in Fig. 3a, the expression of *ghrl* was detected in multiple tissues, including pituitary and muscle tissues, and high expression levels were found in the stomach. Both of its receptors were detected in the liver, where high expression levels of *ghs-r1a-like* were detected (Fig. 3c). The expression of *ghs-r1a* was also detected in the gonad and pituitary gland, but its expression in the skin, fin and muscle was very low (Fig. 3b).

In situ hybridization (ISH) of *ghrl* in spotted sea bass

The localization of *ghrl* mRNA expression in the stomach was tested by ISH. The sense probe was used as a control probe. The sea bass stomach consists of the mucosa (MUC), submucosa (SUB), muscularis (MUSC) and serosa (SER). The MUC is composed of the simple columnar epithelium (SCE), gastric gland (GG), lamina propria (LP) and mucosal

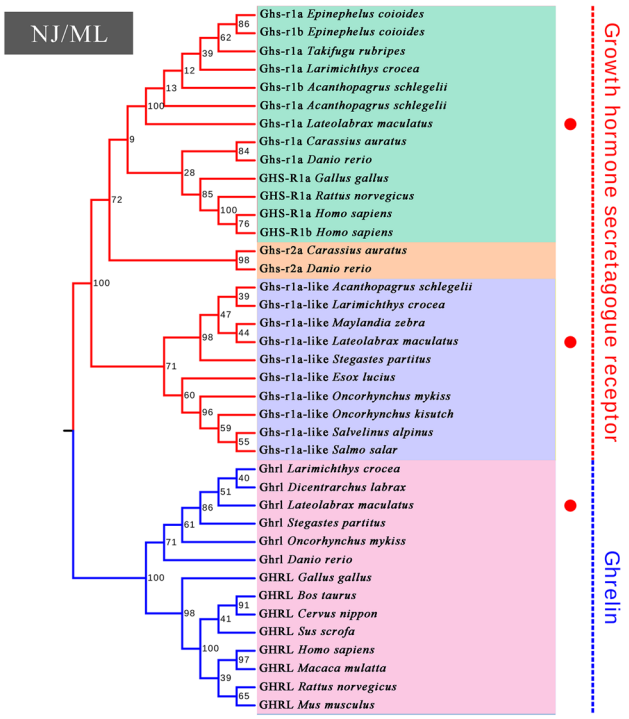

Fig. 2 Phylogenetic tree of Ghrl and its receptors in spotted sea bass. The phylogenetic trees were constructed using MEGA 6 software based on the maximum likelihood method (The phylogenetic tree constructed by the NJ method has the same clustering effect as the ML method). The red dots indicate the genes encoding Ghrl and its receptors in spotted sea bass

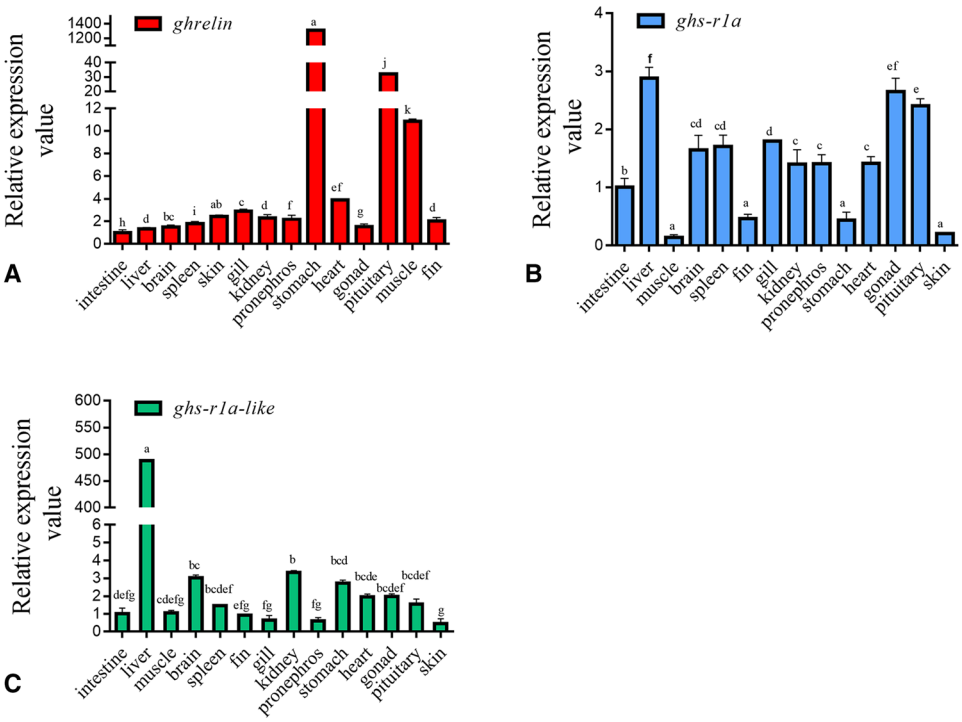

Fig. 3 Relative expression levels of the *ghrl* (**a**) and *ghs-r* (**b**, **c**) genes in different tissues of spotted sea bass. The fold changes relative to the control are indicated on the *y*-axis, and the tissues are displayed on the *x*-axis. The expression levels were quantified by real-time PCR and normalized to the 18S rRNA level

muscularis (MM). The hybridization signal of *ghrl* mRNA in the MUC and SUB was more intense than that of the sense probe (Fig. 4).

Changes in gene expression induced by short-term fasting in spotted sea bass stomach

To understand the potential functions of Ghrl and its receptors in the stomach, which is a digestive glandular organ, the expression patterns of the genes in spotted sea bass after different fasting times were examined. Fishes sampled at 0 h served as the control. The expression levels of *ghrl* and its receptors declined gradually after 0 h reaching a minimum at 6 h. The highest expression of *ghrl* and *ghs-r1a-like*, which was detected at the 12-h time point, was significantly higher than the corresponding expression level in the control group ($P < 0.05$), whereas the expression of *ghs-r1a* in the treated group was consistent with that in the control group. The expression of *ghs-r1a* and *ghs-r1a-like* increased continuously from 12 to 72 h (Fig. 5).

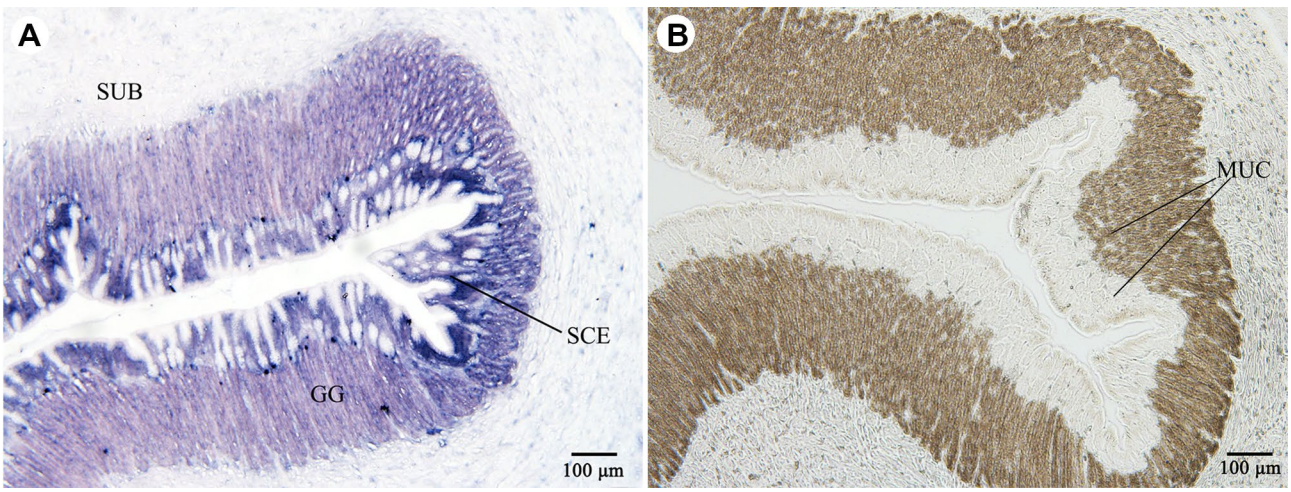

Fig. 4 In situ hybridization-based analysis of the tissue localization of the *ghrl* gene in the spotted sea bass stomach. Adjacent sections hybridized with the anti-sense (**a**) and sense (**b**) probes are shown. Scale bar: 100 μm

In vitro actions of spotted sea bass Ghrl on the expression of *motilin, gastrin* and *cholecystokinin* mRNAs in stomach fragments

To further evaluate the effects of Ghrl on spotted sea bass, an in vitro analysis of the effects of Ghrl on the mRNA expression of *mln*, *gas* and *cck* in stomach fragments was performed. The sequences of *ghrl*, *mln*, *gas* and *cck* were identified from genomic and transcriptomic databases of spotted sea bass. The PCR products of these genes in the stomach were confirmed by sequencing.

As shown in Fig. 6, the expression of *mln*, *gas* and *cck* exhibited pronounced increases after incubation with Ghrl at a concentration of 10^{-6} mol/L for 3 h. However, the expression of *mln* and *cck* did not show significant differences after incubation with Ghrl at concentrations of 10^{-7} or 10^{-8} mol/L for 3 h. These results indicated that *mln*, *gas* and *cck* expression exhibited sensitivity to Ghrl in a dose-independent manner. The effects of Ghrl peptide treatment on *mln*, *gas* and *cck* mRNA expression were all disappeared after 6 and 12 h of incubation.

Discussion

In the present study, we identified *ghrl* and its receptors, determined their expression patterns and regulation in the stomach, identified the localization of *ghrl* mRNA by ISH and investigated the effects of the in vitro administration of Ghrl on *mln*, *gas* and *cck* gene expression. Our findings provide evidence supporting the notion that the Ghrl/Ghs-r system acts as regulator of *mln*, *gas* and *cck* in spotted sea bass.

We analyzed the sequences and structures of Ghrl and its receptors. The C-terminal portion but not the N-terminal end of the mature Ghrl peptide showed variability. The lack of variation at the N-terminal end can be attributed to the fact that this is the biologically active segment of Ghrl. The first seven amino acids at the N-terminal end of mature Ghrl were found to be highly conserved and include a serine residue at the third position, which is the site of fatty acylation and constitutes an essential modification for the binding of Ghrl to its receptor (Muccioli et al. 2001). In this study, the N-terminal amino acid sequence of sea bass Ghrl (GSSFLSP) showed 100% identity to the corresponding sequences in human, mouse, chicken, European sea bass, bicolor damselfish, and large yellow croaker, but less identity to zebrafish Ghrl (GTSFLSP) (Amole and Unniappan 2009). The high conservation of the biologically active fragment during evolution not only indicates that this peptide is important for organisms but also that its biological function might be well conserved.

The Ghrl receptors in teleosts, which include Ghs-r1a, Ghs-r1b and Ghs-r1a-like, are more complex and diverse than those in mammals. In this study, we identified two receptors in spotted sea bass and, to ensure consistent terminology with the orthologous genes of other teleosts, we validated the annotation of *ghs-r1a* and *ghs-r1a-like* in sea bass through a syntenic analysis with the large yellow croaker genome. These two receptor isoforms are considered to have been derived by a WGD event in teleosts, but a loss of duplication has been observed in some branches. Previous studies have shown that only a small number of fish classified as Percomorpha within the superorder Acanthopterygii have *ghs-r1a-like.* These include Perciformes species such as spiny bream (*Acanthochromis polyacanthus*), damselfish (*Stegastes partitus*) and large yellow croaker (*Larimichthys*

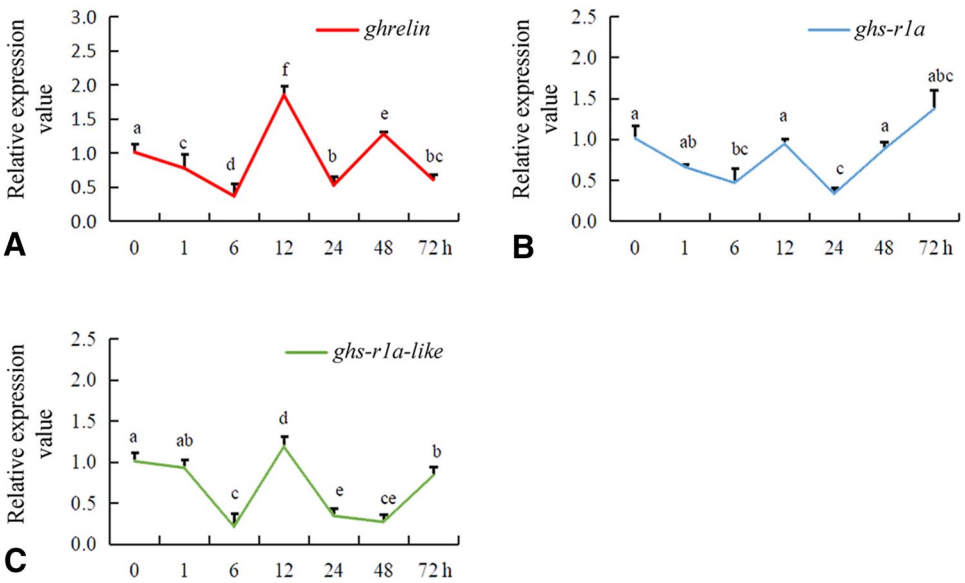

Fig. 5 Effect of different fasting times on the expression of *ghrl* with the genes encoding its receptors in the stomach. The relative expression levels are shown in the y-axis, and the fasting duration is shown in the x-axis. Significant differences in each gene are noted by different letters ($P < 0.05$)

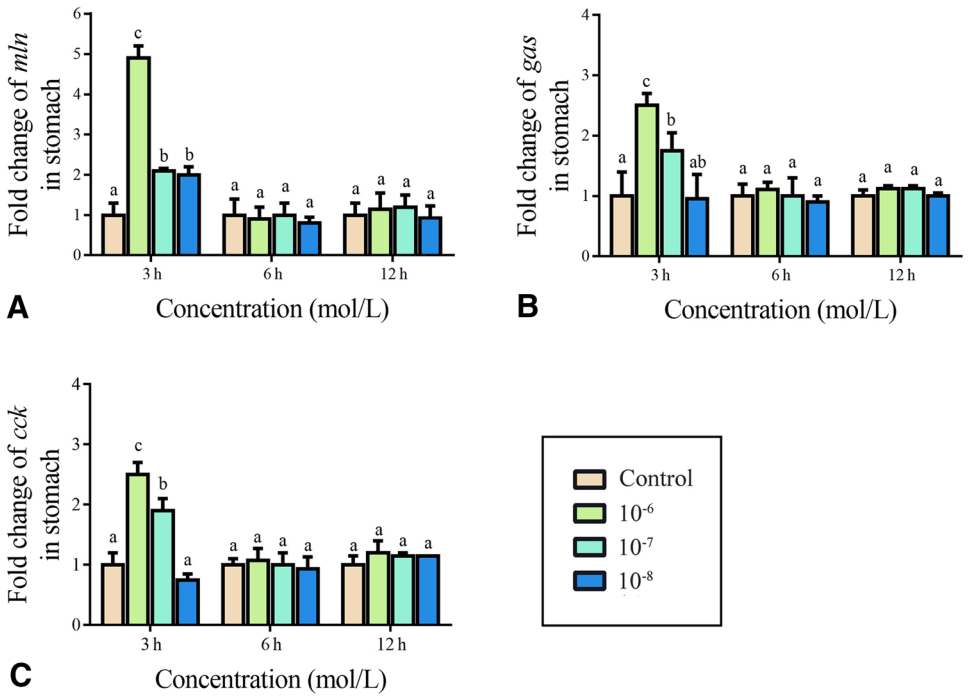

Fig. 6 Effect of Ghrl peptide treatment on *mln* (**a**), *gas* (**b**) and *cck* (**c**) mRNA expression in the spotted sea bass stomach. The results are presented as the means ± SEMs and expressed as fold changes relative to the mRNA expression in the control group incubated without Ghrl peptide. Significant differences for each concentration are denoted by different letters ($P < 0.05$)

crocea), and Salmoniformes species such as rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), coho salmon (*Oncorhynchus kisutch*), and Atlantic salmon (*Salmo salar*) (Kaiya et al. 2014).

The *ghrl* mRNA is mainly expressed in the stomach which is consistent with previous studies of other fish species such as rainbow trout (Kaiya et al. 2003a, b), channel catfish (Kaiya et al. 2005) and Atlantic cod (Xu and Volkoff 2009) and indicates that the main source of *ghrl* is the stomach. In addition, intensely positive signals of *ghrl* mRNA were observed in the MUC and SUB of the stomach, whereas no signal was found in the MUSC or SER. These findings are consistent with those for rainbow trout, where *ghrl* mRNA expression has been observed in both open- and closed-type cells throughout the stomach MUC (Sakata et al. 2004). The *ghrl* mRNA has also been detected in pituitary gland and muscle. Furthermore, Ghrl can potentially stimulate growth hormone release from primary pituitary cells and an increase in body weight (Xu and Volkoff 2009), protect skeletal muscle from atrophy (Porporato et al. 2013; Sheriff et al. 2012) and promote myoblast differentiation (Filigheddu et al. 2007). The gene expression profiles of the two *ghs-r* forms showed that both were widely distributed in the liver where particularly high expression levels of *ghs-r1a-like* were detected. These findings indicate that both forms likely contribute to the functions of *ghrl* in lipogenesis and glucogenesis (Barazzoni et al. 2005). Ghs-r1a is also distributed in the pituitary gland, which is consistent with the significant increases in plasma growth hormone and pituitary *gh* mRNA expression observed in channel catfish after the administration of *ghrl* (Kaiya et al. 2005). Similar observations have been reported for seabream (Chan and Cheng 2004), chickens (Tanaka et al. 2003) and rats (Katayama et al. 2000).

In this study, the stomach, a digestive glandular organ, was selected to determine the gene expression patterns of *ghrl* and its receptors in spotted sea bass after different fasting times. The expression level of *ghrl* under starvation varies widely and is affected by the germline, genetic background, and fasting duration (Beck et al. 2003; Wren et al. 2000). Accordingly, fasting has been reported to upregulate, downregulate or have no effect on *ghrl* mRNA levels in the gastrointestinal tracts of different fish species (Jönsson et al. 2007; Murashita et al. 2009; Unniappan et al. 2004; Volkoff 2015; Xu and Volkoff 2009). After fasting for 7 days, no significant changes from baseline in the expression of *ghrl* were observed in the stomach of Nile tilapia (Parhar et al. 2003), whereas *ghrl* expression in the intestines of grass carp peak after 7 days of fasting (Feng et al. 2013). In Atlantic salmon, the *ghrl* expression levels were significantly higher after 2 rather than 14 days of fasting (Hevrøy et al. 2011). Taken together, these observations support the hypothesis that in many fish species, increases in Ghrl levels occur mainly at the early stage of fasting and are not always associated with changes in *ghrl* mRNA expression in the gastrointestinal tract. In this study, the expression levels of *ghrl* and its receptors declined gradually after the onset of fasting with the lowest expression levels observed at 6 h. This result suggested that lipid and glucose metabolites are mainly consumed

within 6 h after fasting, which results in a decrease in the binding of *ghrl* with its receptors. The highest expression levels of *ghrl* and *ghs-r1a-like* were detected after starvation for 12 h, but the expression of *ghs-r1a* was consistent with that of the control group. These results suggest that in spotted sea bass, Ghrl mainly interacts with the receptor Ghs-r1a-like and acts as a signal of feeding initiation after fasting for 12 h to promote food intake. Moreover, fasting for 24 h decreased the expression level of *ghrl* but gradually increased that of its receptors. The spatial differences in the expression of the two *ghrl* receptors suggested functional divergence because duplicated genes might attain new functions through neofunctionalization and subfunctionalization after WGD. In our study, the functional differentiation of the two paralogs might have been caused by changes in sequence and structure. Ghs-r1a-like shows significant differences in its amino acid sequence and structure from Ghs-r1a and exhibits a unique structural feature; specifically, the second extracellular loop (ECL2) located between TMD IV and V is much longer in Ghs-r1a-like than in Ghs-r1a (Kaiya et al. 2013). The specific functional difference between the two paralogs of spotted sea bass *ghrl* receptors remains to be investigated. To further study the functions of Ghrl in the gastrointestinal tract, we tested the effects of Ghrl on the expression of gastrointestinal-related genes (*mln*, *gas* and *cck*) in gastric fragments through in vitro incubation experiments. The results showed that incubation for 3 h with Ghrl, particularly at a concentration of 10^{-6} mol/L, increased the expression of *mln* and *gas*, but no significant differences from the control expression levels were observed at 6 or 12 h. As a hormone, Ghrl, is thought to stimulate gastrointestinal motility in a manner similar to that observed with gastrointestinal peptides, such as Mln and Gas (Inui et al. 2004). Ghrl and Mln share a number of common features; specifically, these proteins are structurally similar, both are synthesized in the upper gastrointestinal tract and both exert prokinetic effects on gastrointestinal motility (Ohno et al. 2010). In rats, the intravenous administration of Ghrl reportedly stimulates gastric acid secretion and gastric movement by activating the vagal nerve (de la Cour et al. 2004), and gastrin and insulin secretion (Lee et al. 2002). As a result, expression levels of *gas* and *mln* were increased in response to Ghrl. In contrast, Ghrl and Cck are gastrointestinal hormones that regulate feeding and are transmitted through activation of the vagal afferent nerve: Ghrl triggers starvation signals, whereas Cck induces satiety signals (Date et al. 2005). Cck has been found to significantly and directly stimulate Ghrl release from the peptic cells of gastric fundic glands (Rehfeld 2006; Shrestha et al. 2009). In this study, Ghrl incubation for 3 h stimulated the expression of *cck*, which is consistent with the previous finding that a single bolus injection of *cck* in rats significantly increase *ghrl* release by approximately fourfold (Murakami et al. 2002).

In conclusion, we identified and characterized the Ghrl/Ghs-r system in spotted sea bass and analyzed its roles in stimulating food intake in this species. Using ISH, we found that *ghrl* was highly expressed in the stomach and localized in the MUC and SUB. Both the *ghs-r1a* and *ghs-r1a-like* genes were highly expressed in the liver. Short-term starvation for 6 h decreased the expression levels of *ghrl* and its receptors. After fasting for 12 h, the results showed that Ghrl mainly interacted with Ghs-r1a-like as a signal of feeding initiation to promote food intake. In addition, the Ghrl peptide stimulated the expression of *mln*, *gas* and *cck*, with expressions levels reaching a maximum after 3 h in vitro incubation. In summary, this study provides new evidence regarding the functions of the Ghrl/Ghs-r system in regulating food intake in spotted sea bass.

Materials and methods

Animal preparation and short-term starvation

Spotted sea bass (weighing 100.0 ± 5.0 g) were obtained from the Shuangying aquatic breeding factory in Lijin, Shandong. Before the experiment, 100 healthy spotted sea bass were acclimatized in an indoor pool for 2 weeks. The temperature is maintained at 19–21 °C. The fish were fed twice per day until satiety at 9:00 a.m. and 5:00 p.m., and 1/3 of the water was exchanged every day. Fish sampling was conducted after 0, 1, 6, 12, 24, 48 and 72 h of short-term starvation following a meal, and 0 h was used as the control time point. We sampled three fish randomly after each of the different fasting durations. Each fish was anesthetized with MS-222 (200 mg/L).

Cloning and sequence analysis of *ghrl* and its receptors

The *ghrl* and its receptors were identified by searching the whole genome sequence database (PRJNA408177) and the transcriptomic database (PRJNA407434) using TBLASTN (1e−5) (Zhang et al. 2017). The available amino acid sequences of *ghrl* and its receptors in humans and zebrafish retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) were used as queries. The amino acid sequences were predicted using the online tool ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and further verified by Smart-BLAST against the NCBI nonredundant (NR) protein sequence database. SignalP 4.1 Server (https://www.cbs.dtu.dk/services/SignalP/) and NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) were used to predict the signal peptide and the neuropeptide prohormone cleavage sites, respectively.

Potential transmembrane helices in the encoded amino acid sequences were predicted using TMHMM Server v2.0 (https://www.cbs.dtu.dk/services/TMHMM/). To obtain additional information for gene identification and orthology, a syntenic analysis was conducted by comparing the syntenic regions that harbor genes in spotted sea bass with those in other species based on genome information from the Ensembl genome and NCBI databases. The sequences of Ghrl and its receptors in spotted sea bass and several representative vertebrates were used for the phylogenetic analysis. Multiple protein sequence alignments were constructed using DNAMAN 6.0 with the default parameters. The phylogenetic tree was built with MEGA 6 (Tamura et al. 2013).

Quantitative real-time PCR

The gonads, intestine, liver, brain, spleen, skin, gill, kidney, pronephros, stomach, heart, pituitary gland, muscle and fin were collected and stored at − 80 °C. Total RNA from the samples was extracted using RNAiso Plus reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. The concentration and integrity of the extracted RNAs were examined using the Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing) and electrophoresis. Complementary DNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara, Otsu, Japan) with gDNA Eraser (Perfect Real Time) (RR047A Takara) following the manufacturer's instructions. All cDNA products were diluted to 250 ng/μl for quantitative real-time PCR.

The StepOnePlus Real-Time PCR system (Applied Biosystems, USA) was used to detect the mRNA expression of *ghrl* and its receptors in various tissues of spotted sea bass including stomach tissue at each fasting time point. In addition, we analyzed the mRNA expression of gastrointestinal feeding-related genes (*mln*, *gas*, and *cck*) after static incubation for different times (3, 6, and 12 h) and with different concentrations (10^{-6}, 10^{-7}, and 10^{-8} mol/L). The specific primers used for quantitative real-time RT-PCR are listed in Table 1. Based on our previous gene expression study of spotted sea bass, 18S ribosomal RNA (18S) was used as the internal positive control for qRT-PCR normalization (Wang et al. 2018). Triplicate RNA samples were used for gene expression profile analysis. Each 20-μl qRT-PCR contained 10 μl of SYBR® FAST qPCR Master Mix (2X), 0.4 μl of ROX, 2 μl of template cDNA, 0.4 μl of each primer and 6.8 μl of nuclease-free water. The entire PCR process consisted of 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 56 °C for 15 s and a final extension at 72 °C for 2 min. For the gene qPCR assay, the melting curve analysis showed a single peak, which confirmed the specificity of the PCRs. To analyze the expression of the genes in spotted sea bass tissues, the Ct values of each gene in various tissues were measured and 18S rRNA was used as the reference for the normalization of the relative expression of the genes. The comparative $2^{-\Delta\Delta CT}$ method was used to analyze the relative mRNA expression levels.

Table 1 Primers used for quantitative RT-PCR of hormone genes and their receptors

Primer name	Primer sequence 5′–3′
ghrl-F	ACACCTGTTTGCTGGTCTTTC
ghrl-R	ATGTGATGTGGTTGGCCTCTG
ghs-r1a-F	CTACTTGGCGATCTGCTTCC
ghs-r1a-R	CACTGCGTAATGCGTCATCT
ghs-r1a-like-F	TTTTGGATGCTGAGATGGGA
ghs-r1a-like-R	ACTGGATAAGGTGCGGAGGT
mln-F	TGCTGATGAAGGAGCGAGAA
mln-R	TCCACCATGTTCCACCTGAG
gas-F	TGCTAAGAGGGAGAAACTG
gas-R	TATCTCGCGTTCATCGTC
cck-F	TGCCAACTACAACCAACCT
cck-R	GCGTCGTCCAAAGTCCAT
18S-F	GGGTCCGAAGCGTTTACT
18S-R	TCACCTCTAGCGGCACAA

Table 2 Probe primers for in situ hybridization

Primer name	Primer sequence 5′–3′
ghrl probe-F	CGCATTTAGGTGACA CTATAGAAGCGA CCTGTTTGCTGG TCTTT
ghrl probe-R	CCGTAATACGACTCA CTATAGGGAGAC ATCAAAGTTGTG ATGGTCTC

ISH of *ghrl* and its receptors in spotted sea bass

Sea bass stomach samples were fixed overnight at room temperature in buffered 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut in sections of 7 μm thickness. The primers used for probe preparation are listed in Table 2. Specific nonisotopic antisense and sense riboprobes were synthesized using the DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. DIG-labeled ISH was performed as described previously (Zhou et al. 2019), and the sections were examined by light microscopy.

In vitro actions of spotted sea bass Ghrl on the expression of *mtn*, *gas* and *cck* mRNAs in stomach fragments

Spotted sea bass Ghrl was synthesized by GL Biochem, Shanghai, China. The peptide was purified by HPLC to > 97%, dissolved to the required concentration in DMSO (cell-culture grade), and diluted in culture media for in vitro experiments.

The fish were anesthetized with MS-222 (200 mg/L) before sampling. The gastric tissue was washed three times with phosphate-buffered saline (PBS), cut into small pieces (1 mm^3) and placed in a 24-well plate containing 1 ml of culture medium 199 (M199), which contained 100 U/ml penicillin, 100 mg/ml streptomycin and 20% FBS. After preincubation at 28 °C for 6 h, the medium was replaced with fresh culture medium containing Ghrl (10^{-6}, 10^{-7}, or 10^{-8} mol/L). After incubation for 3, 6 and 12 h, gastric fragments were collected and stored at − 80 °C for subsequent RNA extraction and real-time PCR.

Statistical analysis

The values are presented as the means ± SEMs ($n = 3$). The data were analyzed by one-way ANOVA and Duncan's method for multiple comparisons using SPSS 17.0 software. Differences were considered significant at $P < 0.05$.

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Author contributions HW and YL designed this research study. PY wrote the article. Yangyang Zhou et al. helped with the experiments. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Animal and human rights statement: All animal experiments were conducted in accordance with established guidelines and approved by the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201).

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