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Characterization of *ghrelin* mRNA expression in fasting *Larimichthys crocea* juveniles

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Abstract

Larimichthys crocea is a marine fish species cultured in China. Short-term starvation is often applied to improve the quality of cultured *L. crocea*, and the expression of *ghrelin* in tissues of stomach, muscle, brain, intestines, liver, and kidney, involved in starvation response, under starvation conditions were studied to understand the effect of starvation on the expression of *ghrelin* in *L. crocea* juveniles. The *ghrelin* expression was tissue-specific, and expression was significantly higher in the stomach compared to other tissues (P<0.01). Additionally, *ghrelin* expression in different tissues changed along with prolongation of fasting. In the stomach, *ghrelin* expression levels increased gradually at the beginning of the fast, and then declined after eight days of fasting. Gene expression in the brain and intestines increased at the beginning of the fast, and then decreased with longer fasting time. Interestingly, *ghrelin* expression declined at the beginning of the fast, then increased with longer fasting in the kidneys and muscles. These results suggest that *ghrelin* is involved in starvation response in *L. crocea* juveniles. This study provids insights into *ghrelin* function and an important reference for the development of reasonable feeding strategies for *L. crocea* juveniles.

Key words: *Larimichthys crocea*, starvation, *ghrelin*, gene expression

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1 Introduction

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) that is involved in promoting growth hormone release (Kojima et al., 1999; Arvat et al., 2000; Fox et al., 2009), controlling appetite (Wren et al., 2000; Karapanagiotou et al., 2009), and regulating energy metabolism (Diéguez et al., 2010; Pusztai et al., 2008; Wren and Bloom, 2007; Heijboer et al., 2006). In fish, *ghrelin* was first cloned in *Carassius auratus*, followed by *Oreochromis niloticus* (Kaiya et al., 2003c), *Ictalurus punctatus* (Kaiya et al., 2005), *Acanthopagrus schlegeli* (Yeung et al., 2006), *Danio rerio* (Olsson et al., 2008) and *Gadus morhua* (Xu and Volkoff, 2009). Fish grown in culture conditions change their feeding patterns based on growth stage, photoperiod, culture density, food distribution or culture environment (Volkoff and Peter, 2006). Fish experiencing starvation stress show changes in physiology and gene expression. Starvation leads to changes in fish *ghrelin* expression. For example, *D. rerio* (Amole and Unniappan, 2009), goldfish (Unniappan et al., 2004), *Ctenopharyngodon idellus* (Feng et al., 2013) and *Schizothorax davidi* (Zhou et al., 2014) deprived of food have increased *ghrelin* expression in the intestine, and *Salmo salar* (Murashita et al., 2009) and *O. mossambicus* (Peddu et al., 2009) under starvation conditions have significantly up-regulated *ghrelin* in the stomach.

Larimichthys crocea is a marine fish endemic to the South China Sea, East China Sea and Yellow Sea (Feng, 1979), and is one of the major species cultured in China. This fish is widely cultured throughout the coastal areas of Zhejiang Province and Fujian Province, and the farming is a rapidly growing industry. As a result of long-term, high-intensity stocking and unscientific breeding management, the quality of *L. crocea* has decreased significantly. This has resulted in a decrease in market value of *L. crocea*, affecting the healthy development of the aquaculture industry in this species. Currently, there are active efforts to improve the quality of *L. crocea*, such as regulating the nutrient composition of the feed (Xing et al., 2016; Zhang et al., 2016a; Li et al., 2013), changing culture patterns (Wang et al., 2017), and breeding improved varieties (Wang et al., 2012; Ye et al., 2014). In addition to these, a popular strategy has been induced to short-term starvation stress to reduce the amount of fat in the abdominal cavity, which improves the quality of the fish for human consumption by decreasing the fattiness (Ginés et al., 2002; Thakur et al., 2003). This method has been widely used in some fish culture systems and resulted in improvement of the quality of cultured fish. Without exception, this method of short-term starvation stress improves the quality of *L. crocea*.

As mentioned above, starvation will lead to changes of *ghrelin* expression in fish. However, the effect of starvation stress on the expression of *ghrelin*, which regulates growth and feeding of juvenile *L. crocea,* has not been reported, although starvation stress alters the muscle fat composition and serum parameters of adult

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L. crocea (Zhang et al., 2016b). To this end, the *ghrelin* mRNA expression was studied in the muscle, liver, intestine, stomach, kidney and brain of *L. crocea* juveniles after placing them in fasting condition for 0 d, 4 d, 8 d, 12 d, 16 d, and 20 d. The changes of the gene expression according to fasting times were analyzed to develop a reasonable feeding strategy for culturing juvenile *L. crocea*.

2 Materials and methods

2.1 *Fish and experimental design*

The experiment was carried out on the Xishan Island in Zhoushan (Zhejiang, China), and 540 healthy *L. crocea* juveniles with an initial average individual body weight of (40.59 ± 1.79) g were obtained from the Dengbu Aquatic Company located in Zhoushan. Fish were randomly divided into six groups, with three parallel groups per condition, and acclimated to laboratory condition in 18 cylindrical fiberglass tanks (0.5 m^3) with 30 fish in each tank. Fish were acclimated for seven days prior to the experiment under flow-through sand-filtered seawater conditions. During acclimation, the fish were fed twice daily at 08:00 am and 04:00 pm to satiation with an artificial formulated diet. The seawater conditions during acclimatization were as follows: pH, 7.7–8.5; salinity, 27.0–29.0; temperature, 25–27°C; dissolved oxygen concentration>5.5 mg/L. The filth at the bottom of the tank was removed by siphoning, and about 70% of the water was exchanged after half an hour of feeding.

Following the acclimation period, the six groups were treated with fasting for 0 d (control group), 4 d, 8 d, 12 d, 16 d, and 20 d, and the control group continued to be fed twice daily. Once fish were fasted to the assigned length of time, 9 fish from each group (three fish from each parallel experiment) were sacrificed at 7:00 am. At the time of sampling, the animals were netted and anesthetized using 100 mg/L of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma Aldrich Co., USA), and the back muscle, liver, intestine, stomach, kidney and brain tissue of each fish were collected and stored in RNAlater at –80°C. The control group was sampled, at the same time the target treatment groups were sampled.

2.2 *RNA isolation and cDNA synthesis*

Total RNA from each tissue was extracted from tissues using the TransZol Up kit (TransGen Biotech, China) following the manufacturers' protocol. Final RNA concentrations were determined by optical density reading at 260 nm using a NanoDrop 2000c spectrophotometer (NanoDrop Technologies Inc., USA). To ensure that RNA samples were of high quality, only RNA samples with the absorbance ratio of 1.8 to 2.0 for 260 nm and 280 nm were used.

First-strand cDNA was synthesized from 1 μg total RNA using a TransScript All-in-One First-Strand cDNA Synthesis SuperMix (one-step gDNA removal) kit (TransGen Biotech, China) following the manufacturer's protocol.

2.3 *Sequence analysis*

cDNA sequence of *ghrelin* was obtained from GenBank (NM_001303331.1). The cDNA sequence similarity analysis was performed by the BLAST from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Protein structure was predicted by SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignment of amino acid sequences was performed using the ClustalX program (http://www.ebi.ac.uk/clustaw/). Phylogenetic tree was reconstructed by MEGA software version 5.0 using the neighbor-joining method.

2.4 *Quantitative real-time PCR analyses*

Primers for *ghrelin* and a house-keeping gene *β-actin* were designed based on GenBank sequences (NM_001303331.1 and XM_019257255.1) and synthesized by Genewiz Biotechnology Co., Ltd (China). Primer sequences are shown in Table 1. Real-time qPCR was conducted as follows: 10 μL of 2×TransStart Tip Green qPCR SuperMix, 0.4 μL (10 μmol/L) of forward and 0.4 μL (10 μmol/L) of reverse primer, 0.4 μL of Passive Reference DyeI, 6.8 μL of ddH_2O, and 2 μL of cDNA with a final reaction volume of 20 μL. Reactions were conducted in 96-well plates and samples were run in triplicate. PCR reaction was carried out on a Stepone Real-Time PCR System (ABI, American) using the following thermal cycling program: 94°C for 30 s, 40 cycles of 94°C for 5 s, 60°C for 30 s.

Real time qPCR measurements were displayed as threshold cycle values and used to calculate ΔCT. Gene expression levels were measured as a relative to *β-actin* expression levels, and $2^{-\Delta\Delta CT}$ was used to determine the relative level of *ghrelin* mRNA expression.

2.5 *Statistical analysis*

The results of the fasting experiments are represented as mean±SEM (n=9). Data were analyzed using SPSS 19.0 (IBM Corp., USA). One-way ANOVA was used to examine the differences among the values obtained from the fasting experiment, followed by post hoc analysis using Tukey's multiple range tests. Differences were considered significant when P<0.05.

3 Results

3.1 *cDNA sequence analysis of ghrelin*

A *ghrelin* cDNA fragment of 327 bp was obtained from GenBank. The alignment of the *ghrelin* amino-acid sequence of *L. crocea* and other fish are shown in Fig. 1. The amino-acid sequence of *ghrelin* of *L. crocea* showed low identity with the other fish (40%–80%). The construction of a phylogenetic tree was based on *ghrelin* amino-acid sequences of *L. crocea* and other vertebrate. Phylogenetic tree analysis indicated that *L. crocea* was in the same subgroup with other fishes and had the closest phylogenetic relationship with *Epinephelus coioides*, and *Siniperca chuatsi* (Fig. 2).

3.2 *The ghrelin expression in different tissues*

The *ghrelin* mRNA expression was detected in the six tissues obtained from the experiment groups, which included stomach, kidney, liver, brain, intestine, and muscle. Expression levels of all tissues are expressed relative to the brain (Fig. 3). It is showed

Table 1. List of PCR primers used in this study

Gene	Primer name	Sequence (5′–3′)	Product length/bp
ghrelin	Ghrelin_F	CTTCCTCAGCCCTTCACAAA	198
	Ghrelin_R	AGACGCTGAATGATCTCCTG	
β-actin	*β*-actin_F	CCAACTCATTGGCATGGCTT	134
	β-actin_R	GATGCAACTGCAGAACCCTG	

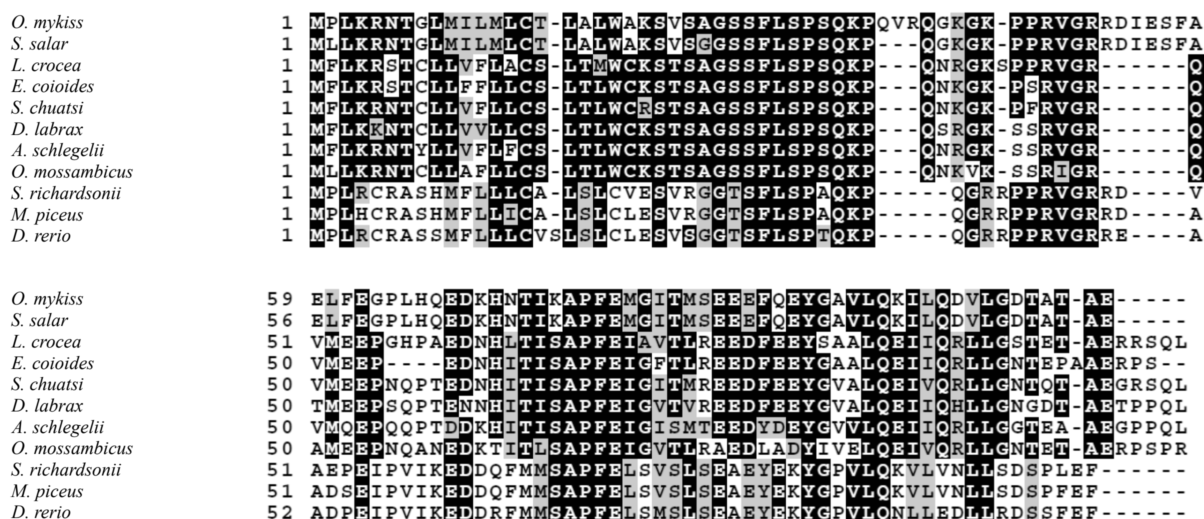

Fig. 1. Multiple alignment of *ghrelin* amino acid sequences. The comparison includes *ghrelin* sequences from the teleost species *O. mykiss* (BAD02980.1), *S. salar* (NP_001133057.1), *L. crocea* (NP_001290260.1), *E. coioides* (AJS13600.1), *S. chuatsi* (ALB25888.1), *D. labrax* (ABG49130.1), *A. schlegelii* (AAV65509.1), *Oncorhynchus mossambicus* (BAC55160.1), *S. richardsonii* (AWB11399.1), *M. piceus* (AIZ50369.1), *D. rerio* (ACJ76436.1). Identical amino acids are highlighted in black, strongly similar amino acids are printed in white letters with dark gray underline.

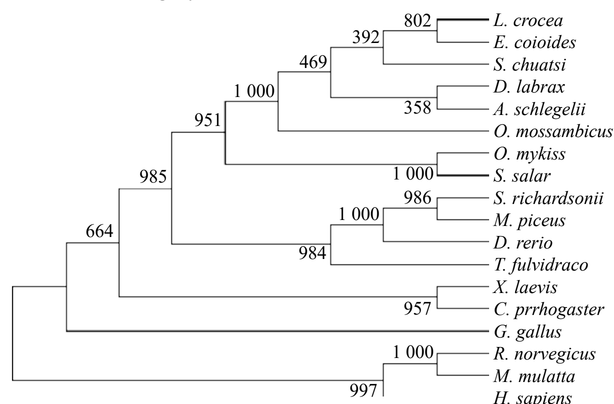

Fig. 2. Phylogenetic trees analysis based on *ghrelin* amino sequences. GenBank Accession numbers of sequences used are *R. norvegicus* (BAA89370.1), *M. mulatta* (AAQ74381.1), *H. sapiens* (ADM33790.1), *G. gallus* (AAP57945.1), *X. laevis* (BAL70270.1), *C. pyrrhogaster* (BAM29300.1), *S. richardsonii* (AWB11399.1), *M. piceus* (AIZ50369.1), *D. rerio* (ACJ76436.1), *T. fulvidraco* (ALK82256.1), *Oncorhynchus mykiss* (BAD02980.1), *S. salar* (NP_001133057.1), *O. mossambicus* (BAC55160.1), *L. crocea* (NP_001290260.1), *E. coioides* (AJS13600.1), *S. chuatsi* (ALB25888.1), *D. labrax* (ABG49130.1), and *A. schlegelii* (AAV65509.1).

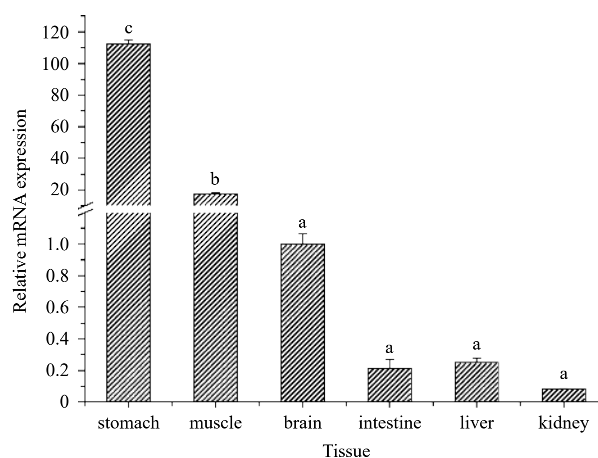

Fig. 3. Expression of *ghrelin* mRNA in different tissues of *L. crocea* juvelines. Expression levels of all tissues are expressed relative to the brain. Data are shown as mean±SEM (n=9). Significant differences are indicated with different lowercase letters above the vertical bars (P<0.05).

that *ghrelin* expression was highest in the stomach followed by muscle, and lowest expression was found in the kidney. Also, the expression levels of the kidney, liver, brain, and intestine were not different significantly.

3.3 *Changes of ghrelin mRNA expression in fasting L. crocea juveniles*

The *ghrelin* expression levels were different in each tissue, and that fasting induced significant and tissue-specific changes in *ghrelin* expression in the different fasting-treated groups (P<0.05). In addition to this, gene expression changed with increased time of fasting. In the stomach, *ghrelin* levels first increased then decreased with the prolongation of fasting, reaching highest expression level at 8 d of fasting (Fig. 4a). In contrast, muscle (Fig. 4b) and kidney (Fig. 4f) showed down-regulation of *ghrelin* at the beginning of fasting and an up-regulation with prolonged fasting. The lowest *ghrelin* expressions in the two tissues were observed at 12 d of fasting. In the kidney, *ghrelin* expression in fasting individuals was consistently lower than the control. Those in the brain (Fig. 4c) and intestine (Fig. 4d) showed increased *ghrelin* expression with highest levels reaching at 4 d of fasting, followed by a decrease in expression. Interestingly, *ghrelin* levels increased again with longer fasting. Lastly, the expression patterns of *ghrelin* in the liver showed fluctuating with prolonged fasting time, where highest expression was observed at 12 d of fasting, and lowest expression level was observed at 8 d of fasting (Fig. 4e).

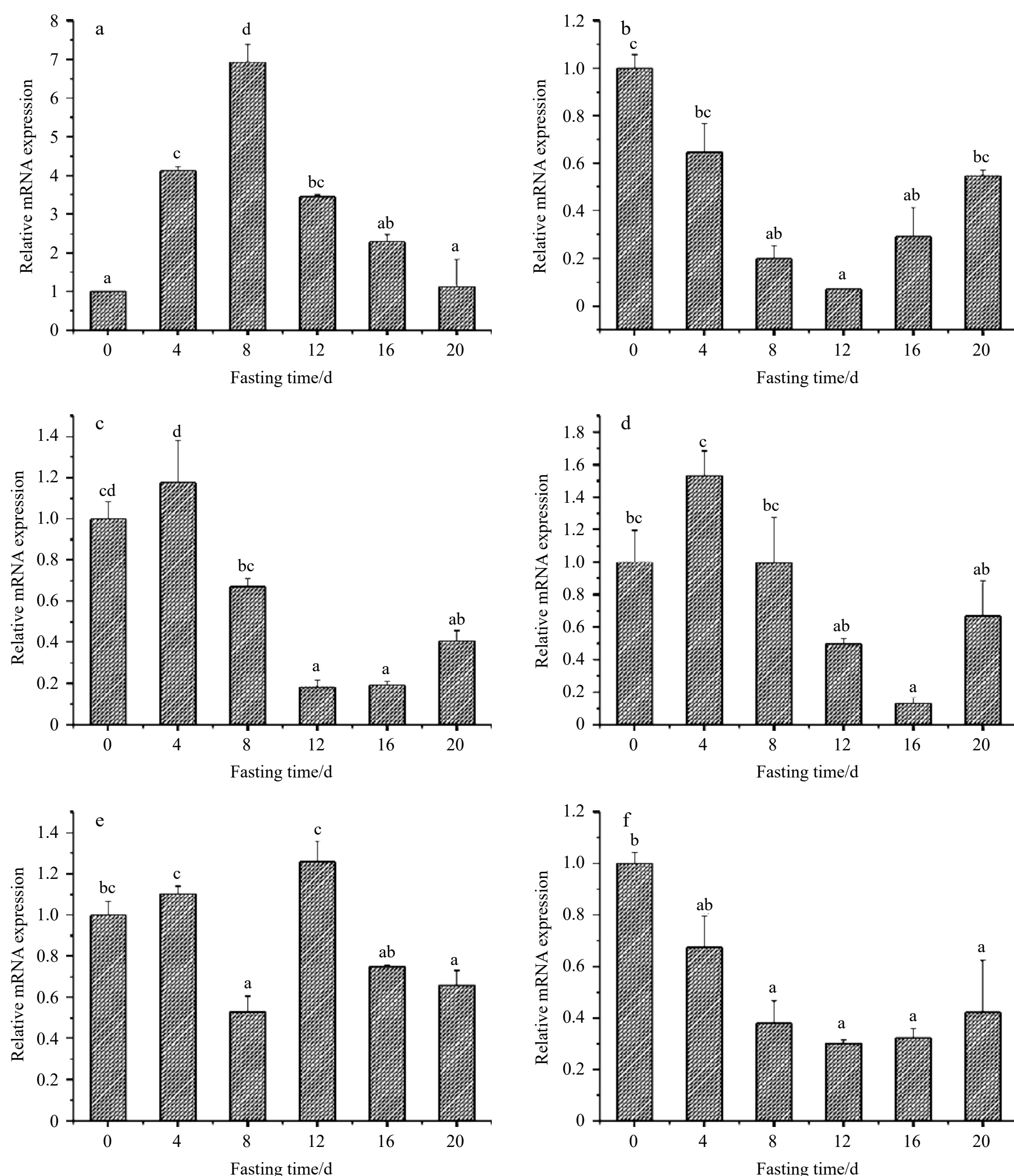

Fig. 4. Relative expression level of *ghrelin* in tissues of *L. crocea* juveniles treated with different lengths of fasting for stomach (a), muscle (b), brain (c), intestine (d), liver (e), and kidney (f). Expression levels were normalized as the average expression levels of the treatment group where the expression levels the control group (0 d) were set as 1. Data are shown as mean±SEM (n=9). Significant differences are indicated with different lowercase letters above the vertical bars (P<0.05).

4 Discussion

In order to investigate the effects of fasting on the mRNA expression of *ghrelin* in *L. crocea*, this study extracted the complete cDNA sequence of *ghrelin* from GenBank at first. And then, an analysis of amino acid sequence of *ghrelin* was carried out. The results showed that the *L. crocea ghrelin* presented most similarities with that in *E. coioides*. Following the sequence analysis, this study examined the expressions of *ghrelin* mRNA in muscle, liver, intestine, stomach, kidney and brain of *L. crocea* juveniles using real-time qPCR. The *ghrelin* was expressed in all six tissues, which was different from studies focusing on other fish species. In *Cyprinus carpio*, *ghrelin* expression is highest in the intestine and lowest in the spleen and brain, and *ghrelin* expression is non-detectable in sputum, liver, kidney and muscle tissues (Kono et al., 2008). This indicates that the tissues-specific expression pattern of *ghrelin* depends on the species. This study also found that *ghrelin* expression was highest in the stomach, and similar results were observed in *A. schlegeli* (Ma et al., 2009), *Oncorhynchus mykiss* (Kaiya et al., 2003a), *I. punctatus* (Kaiya et al., 2005), *Anguilla japonica* (Kaiya et al., 2003b), *G. morhua* (Xu and Volkoff, 2009), and *O. niloticus* (Parhar et al., 2003). The second highest expression of *ghrelin* was found in the muscle, which is quite different from goldfish (Pusztai et al., 2008) and *Pygocentrus nattereri* (Volkoff, 2015), but consistent with *O. hornorum* (Gao et al., 2010), suggesting that there are some similar patterns in relative expression levels of *ghrelin* in different fish species.

This study found that *ghrelin* expression was highest in the stomach, followed by muscle, brain, intestine, liver and kidney.

Cui (2013) reported that in adult *L. crocea*, *ghrelin* expression is highest in the stomach, followed by intestine, brain, liver and muscle. The most notable difference between adult and juvenile expression patterns is the expression level in muscle. This may be due to the rapid growth of *L. crocea* juveniles, as *ghrelin* expression can induce growth. *L. crocea* adults grow slowly, which may underlie the reduced *ghrelin* expression level in the adult muscle. Also, the function of Ghrelin may be different between the juveniles and adults, thus resulting in the difference in gene expression.

As an important regulator of food intake, *ghrelin* can enhance the sense of hunger before feeding and induce feeding behavior (Hosoda et al., 2000). According to this theory, it is speculated that the expression level of *ghrelin* in *L. crocea* increases before feeding or after a short-term starvation. This study found that *ghrelin* expression levels in the stomach and intestine were significantly increased after 4 d of fasting, suggesting that *ghrelin* may regulate feeding of *L. crocea* juveniles. Similar results have been reported in other animals. For example, *Dicentrarchus labrax* that was starved for 5 weeks had significantly higher *ghrelin* expression in the stomach (Terova et al., 2008), *P. nattereri* (Volkoff, 2015) and goldfish (Feng et al., 2013) fasted for 7 d had significantly higher *ghrelin* levels in the intestine. This study found that *ghrelin* expression in the stomach gradually decreased after 8 d of fasting. In the intestine, *ghrelin* levels initially increased and then gradually declined, reaching the lowest level at 16 d of fasting. This may have been caused by the change in physiology between short-term and long-term fastings, as long-term fasting leads to burning more proteins as energy source to maintain basic physiological activities, thereby inhibiting the synthesis unnecessary proteins. Therefore, the decrease in *ghrelin* expression may be a result of the reduced metabolic activity and energy expenditure of long-term fasting individuals (Kono et al., 2008).

The expression level of *ghrelin* in the brain increased at the beginning of fasting, then decreased, and lastly slowly increased as animals experienced prolonged fasting, and the results were similar to those in *O. mossambicus* (Riley et al., 2008), where expression of *ghrelin* mRNA was elevated in fasted *O. mossambicus* on Day 3 and reduced on Day 5, and then elevated again on Day 7. The reason for this change is unclear. The fluctuation result from the reduction in plasma Ghrelin content caused by starvation stress was speculated, therefore *ghrelin* mRNA expression increases under the regulation of the body itself to restore plasma Ghrelin to induce food intake. However, prolonged fasting may lead to a decline in energy used for body growth, leading to reduced production of growth hormones by reducing *ghrelin* expression. Since Ghrelin has multiple functions in the body, the long-term low level of *ghrelin* expression leads to the continuous decline of Ghrelin content in the hypothalamus, thus affecting the normal physiological function (Jönsson, 2013). This may further lead to promoting *ghrelin* expression through self-regulation.

The *ghrelin* mRNA expression has been detected in the muscle, liver and kidney of *P. nattereri* (Riley et al., 2008), *A. japonica* (Kaiya et al., 2003b), and *O. mossambicus* (Kaiya et al., 2003c). Interestingly, changes in *ghrelin* expression levels have not been reported in the liver, kidney and muscle under starvation conditions. This study found that *ghrelin* expression in these tissues changed as animals experienced short and long term fastings. However, the reasons for the changes in *ghrelin* expression at different fasting time points need to be further studied in the context of *ghrelin* function in these three tissues.

Taken together, this study found that *ghrelin* mRNA levels in *L. crocea* juveniles were higher in the stomach and intestine at 4–8 d of fasting, suggesting that refeeding at this time would effectively improve the ingestion rate of *L. crocea* juveniles. Certainly, the inference needs to be verified by a recovery feeding experiment.

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