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Molecular Cloning and Function Characterization in Feeding of Neuropeptide Y in Gibel Carp, *Carassius auratus gibelio*

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Authors' Contribution
LL, XD and CZ supervised the study. DY, ZZ , CZ and HL fed the fish, collected the samples and performed the laboratory work. LL, CZ, BL and HY analyzed the data and drafted the article.

Key words
Gibel carp, *neuropeptide Y*, Cloning, Fasting, Feeding.

ABSTRACT

To elucidate the mechanism of neuropeptide *Y* (*npy*) gene in feeding in gibel carp, we cloned and identified the *npy* cDNA sequence. Furthermore, we examined the *npy* mRNA distribution in 21 tissues and investigated the effect of its expression at different nutritional levels. Herein, we found that gibel carp *npy* has an Open Reading Frame (ORF) containing 291 bp. Moreover, the expression of *npy* was detected in all tested tissues, with the hypothalamus showing the highest expression in gibel carp. The *npy* mRNA expression in the hypothalamus significantly decreased 1 and 3 h after feeding ($P < 0.01$). Furthermore, its expression in the hypothalamus was significantly increased after fasting for 7 days and declined 2 days after refeeding (at 11-day and 14 -day; $P < 0.01$), suggesting that *npy* is an orexigenic peptide in gibel carp. In conclusion, these results indicated that *npy* is a conserved peptide within vertebrates and might exert orexigenic role in gibel carp.

INTRODUCTION

Feeding is a complicated behavior involving food ingestion and appetitive behavior, which reflects the motivation for food intake (Keen-Rhinehart *et al*., 2013; Simpson and Balsam, 2016). Furthermore, it is an important process in mammals (Valassi *et al*., 2008) and fish (Volkoff, 2016). Feeding is ultimately regulated by receiving and transducing endocrine signals from the central nervous system and periphery tissues (Begg and Woods, 2013; Keen-Rhinehart *et al*., 2013; Volkoff, 2016; Woods and Begg, 2016). To date, many appetite-regulating peptides have been identified in fish including cocaine, leptin (Zhang *et al*., 1994), ghrelin (GRLN) (Zhou *et al*., 2016), peptide YY (PYY) (Holzer *et al*., 2012), neuropeptide Y (NPY) (Wei *et al*., 2014; Volkoff, 2016). Although lots of research on food intake in fish has progressed in the last few years, very little attention has been paid to the endocrine regulation mechanism of food intake. How to strengthen the production performance of fish through appetite regulation is still a hot research area.

Since neuropeptide Y (NPY) was first extracted from a mammalian brain in 1982 (Tatemoto *et al*., 1982), many studies have found that the *npy* mRNA is expressed in the central nervous system, peripheral nervous system and peripheral tissues of mammals and teleosts, with the highest expression in the brain (Chen and Li, 2005; Kehoe and Volkoff, 2007; Malmström, 2001; Murashita *et al*., 2009). NPY is a highly conserved neuroendocrine peptide (Hoyle, 1999), which is involved in the regulation of multiple physiological functions, such as circadian rhythms (Thorsell and Mathé, 2017), body temperature (Bouali *et al*., 1995), food intake (Wei *et al*., 2014), blood pressure (Dahlöf *et al*., 1985) and sexual behavior (Clark *et al*., 1985; Inaba *et al*., 2016). In general, it is a multifunctional complicated hormone that governs various physiological processes in several animals.

In recent years, several researchers have reported that NPY and its related peptides are important in feeding in mammals and fish (Xu *et al*., 2015; Zhang *et al*., 2017). In *Schizothorax prenanti*, the *npy* expression level in fed

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fish was lower than in fasting fish (Wei *et al*., 2014). A similar result was found in other fish, such as *Cyprinus carpio* (Tang *et al*., 2014), *Danio rerio* (Dalmolin *et al*., 2015) and *Seriola quinqueradiata* (Hosomi *et al*., 2014), suggesting that NPY plays an orexigenic role in teleost fish. However, the *npy* expression level was elevated after feeding in Atlantic salmon (Valen *et al*., 2011). Differences in these studies could be due to fish species, age, size, and also to the physiological differences among different fish species (Chen *et al*., 2016; Fuentes *et al*., 2012; Volkoff *et al*., 2017). Therefore, further study is needed to validate the molecular mechanisms and biological function of NPY in feeding in fish.

Gibel carp (*Carassius auratus gibelio*), one of the major farmed fish species in China, has been subjected to major breeding efforts with high yield. So far, in gibel carp, most studies on the appetite and feeding modes have emphasized the effectiveness of the diet (Yin *et al*., 2017), but only little attention was given to the molecular regulation mechanism of food intake. Research on the biological function of NPY in feeding in gibel carp has not been described.

Here, we identified the gibel carp *npy* mRNA sequence and analyzed its expression patterns in different tissues. Furthermore, we analyzed the change in *npy* mRNA expression level between postprandial and fasting status using real-time quantitative PCR (RT-qPCR). This study provides the foundation for further investigation into the molecular mechanisms regulating food intake in gibel carp.

MATERIALS AND METHODS

Ethics statement

Based on the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004), all the research that involved animals was approved by the Institutional Animal Care and Use Committee in the College of Animal Science, Southwest University, Chongqing, China. The feeding conditions, including dissolved oxygen and temperature were under strict control.

Fish

All the gibel carp were purchased from a local nursery of fish in Chongqing, China, and then transported to the laboratory in the Aquaculture Department at Southwest University. Before the experiments, the fish were in a circulating aquarium system at 23 ± 2°C for 14 days and fed a commercial diet (6% fat and 40% crude protein, Haida, Guangzhou, China) once a day (17:00).

According to an overview about regulation of food intake in fish (Volkoff, 2016), *npy* is highly expressed in the brain. Therefore, for the cloning study, brain samples were collected from six fish with a mean body weight of 293.5 ± 17.56 g(1:1 gender ratio). For the tissue distribution study, 21 tissues were collected from six fish with a mean body weight of 316.33 ± 11.7 g (1:1 gender ratio; for more information about the 21 tissues see section 2.3).

To conduct the postprandial research, seven groups of weight-matched fish with a mean body weight of 308.7.36 ± 17.5 g (n = 15/group) were allowed to acclimate to the tank conditions (110 × 44 × 44 cm^3). In terms of the feeding activity and behavior, no difference was observed between the tanks. The fish were sampled before the feeding (−3 h and -1 h), at commencement of feeding (0 h) and after feeding (+1 h and +3 h). For the unfed control groups, two unfed groups were sampled at +1 and +3 h. Six individuals were sampled for every sampling point and group.

For the long fasting study, fish with a mean body weight of 458.33 ± 20.1 g (n = 30 × 6, three tanks of unfed fish and three tanks of fed fish) were sampled. After 2 weeks of feeding, three groups' fish were endured fasting, while other three groups' fish were fed as before. The fasted group's fish were sampled severally on day 1, 3, 5 and 7. Six fish in the fed group were sampled at every sampling point. The fasted group was fed again on day 9 on a regular basis, and six fish were sampled on day 9, 11 and 14.

Tissue collection and RNA extraction

Before being sacrificed, fish were anesthetized with tricaine methanesulfonate (MS-222, 0.02%). Then, 21 tissues (telencephalon, hypothalamus, mesencephalon, cerebellum, myelencephalon, liver, spleen, heart, eye, skin, foregut, gill, white muscle, hindgut, midgut, red muscle, trunk kidney and gonad) were removed immediately and stored in liquid nitrogen. RNA was extracted from these tissues with TRIzol A^+ reagent, according to the manufacturer's instructions (TaKaRa, Dalian, China). The concentration of all the RNA samples was determined by a photometer (Thermo Scientific, Dreieich, Germany) at 280 nm and 260 nm. Until further use, the total RNA was stored at –80°C.

Molecular cloning

RNA (around 2 µg) was used for first strand synthesis of cDNA with a Prime Script RT reagent Kit (TaKaRa, Dalian, China). The primers for *npy* fragment amplification were designed based on *npy* sequences of other fish species found in the NCBI GenBank.

SMART RACE cDNA Construction Kit (Clontech, USA) was used to synthesize the entire brain's first-strand

cDNA with 3′ or 5′ adaptors for cDNA ends (RACE) PCR rapid amplification. 3′ and 5′ RACE PCR were conducted to obtain the sequence of the full-length gibel carp *npy*. The RACE PCR products were obtained from the nested RACE PCR for *npy*'s 3′ and 5′ RACE. Based on the *npy* fragment, the primers for the gibel carp *npy* gene were designed. The PCR conditions were: 5 cycles of 94°C for 30 s and 72°C for 180 s; 5 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 180 s; 20 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 180 s. The PCR product was separated by agarose gel electrophoresis (1.5%). Next, the target bands were extracted by a Universal DNA Purification Kit (TIANGEN, Beijing, China). Subsequently, the PCR fragments were ligated to a pMD®19-T plasmid (TaKaRa, Dalian, China). The ligated plasmid was transformed into *Escherichia coli* DH5α. Colonies with inserts were cultured in LB medium at 37°C overnight. OMEGA Mini Plasmid Kit (TaKaRa, Dalian, China) was used to extract the plasmids from the colonies, which were then sequenced by an automated sequencing study.

Structural analysis

Multiple sequence alignments were generated using the clustalx1.83. The cleavage site of the signal peptide was estimated using the SignalP Ver. 4.0 program (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree based on the amino acid sequences was constructed by the neighbor-joining method of the Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) (Thompson *et al*., 1994) and MEGA 5.1 programs (http://www.megasoftware.net/index.html) (Kumar *et al*., 2004).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was used to evaluate the tissue distribution, postprandial variations and fasting effect on *npy* mRNA. According to the sequence information gained from cloning (Table I), gene-specific primers were designed for qPCR. SYBR Green (TaKaRa) was used to conduct the qPCR on *npy* with QuantStudio™ 3 Real-Time PCR (ABI) (TaKaRa). All the real-time qPCR reactions were carried out in triplicate. Every PCR reaction (total volume, 25 µL) included 9.5 µL of RNase-free H_2O, 2.5 µL of cDNA, 0.5 µL of each primer and 12.5 µL of 2×SYBR *Premix Ex* Taq™. To analyze the samples, the $2^{-\Delta\Delta Ct}$ method was used to determine the mRNA abundance. The amplification reaction conditions were: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. In this research, *18S* and *β-actin* were used as housekeeping control genes.

Statistical analysis

The normality test was performed before the Student's *t*-test or one-way analysis of variance with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA), to ensure that the *t*-test is suitable for data comparison. Data were expressed as means ± SEM. $P < 0.05$ was considered statistically significant.

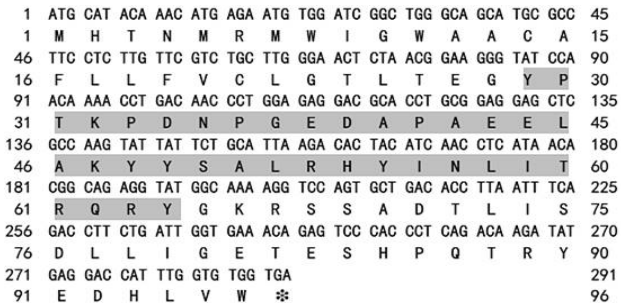

Fig. 1. The cDNA coding sequence of *Carassius auratus gibelio npy*. The shaded boldface letters stand for the mature peptide. * Indicates the stop codon.

Table I.- Primers used for sequences.

Primer name	Sequence (5′→3′)	Application
NPY-1F	ACCCACCGAGCAAGAAGT	NPY cloning
NPY-1R	TCACTATCCTGACGATGGCT	NPY cloning
NPY3′-F1	TGTCAGCACTGGACCTTTTGCCATACCT	NPY 3′RACE outer
NPY3′-F2	ACCCTTCCGTTAGAGTTCCCAAGCAGAC	NPY 3′RACE inner
NPY5′-F1	CTGCTTGGGAACTCTAACGGAAGGGTAT	NPY 5′RACE outer
NPY5′-F2	ATAACACGGCAGAGGTATGGCAAAAGGT	NPY 5′RACE inner
NPY-F	CCTTCCTCTTGTTCGTCTGCT	Real time PCR
NPY-R	TGCCATACCTCTGCCGTGT	Real time PCR
β-Actin-F	CGAGCTGTCTTCCCATCCA	Real time PCR
β-Actin-R	TCACCAACGTAGCTGTCTTTCTG	Real time PCR
18s F	ACCACCCACAGAATCGAGAAA	Real time PCR
18s R	GCCTGCGGCTTAATTTGACT	Real time PCR

RESULTS

Sequence analysis of the gibel carp npy *gene*

The complete Open Reading Frame (ORF) of the gibel carp *npy* mRNA is 291 bp, encoding 96 amino acids including a 28-amino-acid signal peptide and a 36-amino-acid mature peptide (Fig. 1; GenBank accession No. MF805788).

Gibel carp *NPY* has a lower similarity to mammalian *NPY* (74% with *Mus musculus*) than to cyprinid *NPY* (98% with *Carassius auratus* and 99% with *Schizothorax prenanti*; Fig. 2). The phylogenetic analysis revealed that the gibel carp *NPY* was grouped with the *Cyprinid* fish cluster (Fig. 3).

Fig. 2. Amino acid sequence alignment of *NPY*. The shadowed regions are conserved in the listed peptides. * Indicates identical amino acids.

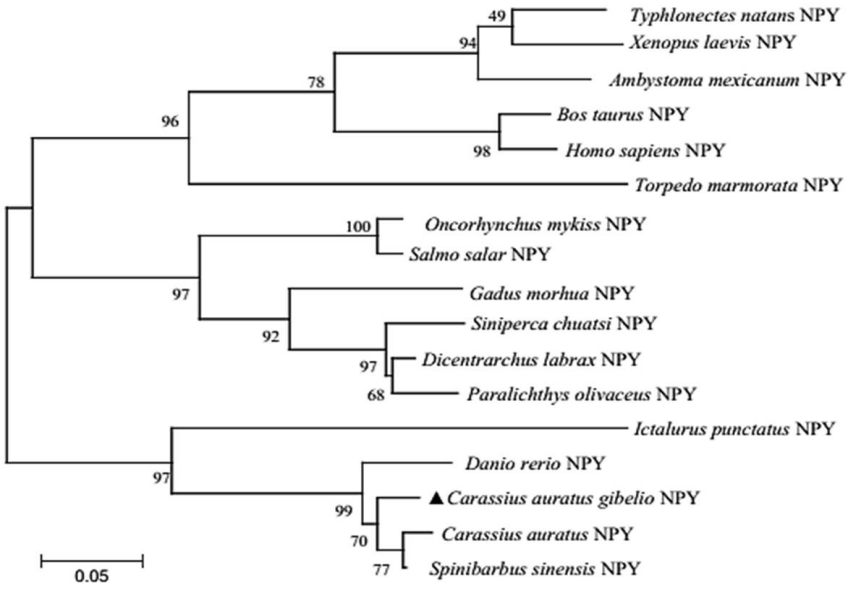

Fig. 3. The phylogenetic tree of vertebrate *NPY*. Scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained per 1000 replicates. GenBank accession numbers: *Typhlonectes natans* (AAD48033), *Xenopus laevis* (NP_001081300), *Ambystoma mexicanum* (AAT66407), *Bos taurus* (AAR37328), *Homo sapiens* (AAA59944), *Torpedo marmorata* (AAA49281), *Oncorhynchus mykiss* (AAB25269), *Salmo salar* (BAH24101), *Gadus morhua* (AAX19943), *Siniperca chuatsi* (ABS83815), *Dicentrarchus labrax* (CAB64932), *Paralichthys olivaceus* (BAB62409), *Ictalurus punctatus* (AAF71617), *Danio rerio* (NP_571149), *Carassius auratus* (AAA49186), *Spinibarbus sinensis* (ABE73783).

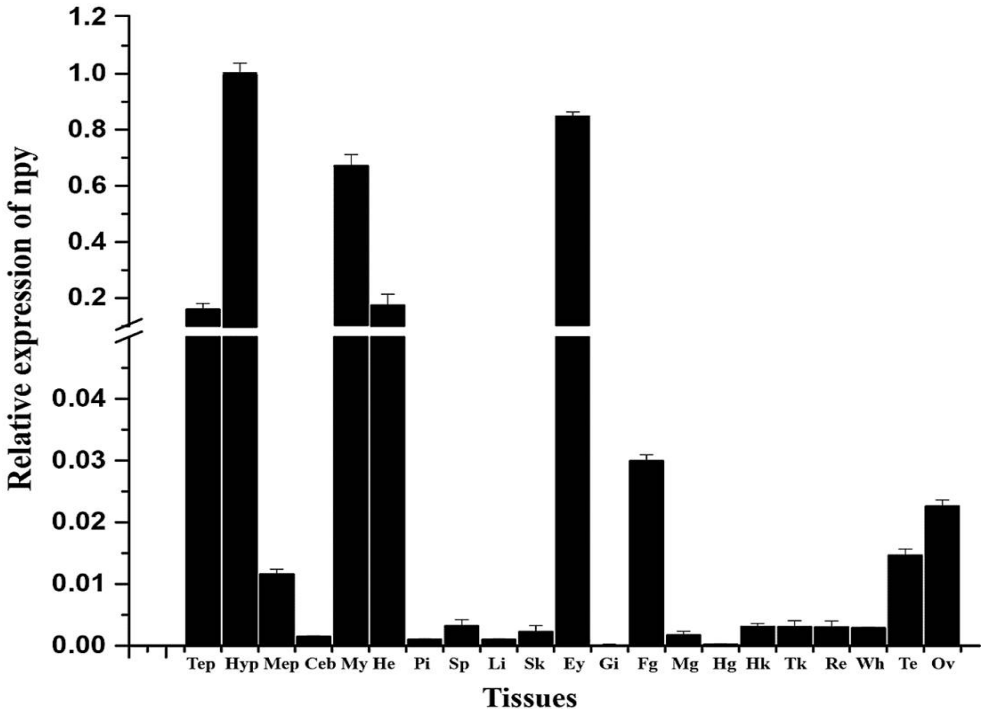

Fig. 4. Relative expression of *npy* mRNA in tissues of gibel carp. Error bars represent standard error of the mean. Tep, telencephalon; Hyp, hypothalamus; Mep, mesencephalon; Ceb, cerebellum; Myp, myelencephalon; Pi, pituitary; Hk, head kidney; Tk, trunk kidney; Li, liver; Fg, foregut; Mg, midgut; Hg, hindgut; He, heart; Sp, spleen; Gi, gill; Te, testis; Ov, ovary. Re, red muscle; Wh, white muscle; Sk, skin; Ey, eye.

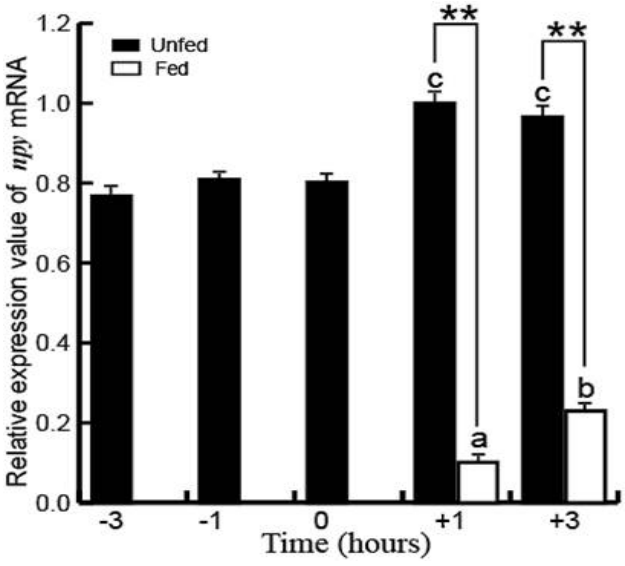

Fig. 5. Postprandial changes in *npy* mRNA expression in the hypothalamus of gibel carp. The preprandial (-3, -1 and 0 h) and postprandial groups (+3 and +1 h) that differed significantly are indicated by different letters above the bars. Asterisks represent significant differences between the fed and unfed groups at the same time point. $^{**}P < 0.01$.

Tissue distribution of npy *in gibel carp*

We investigated the expression level of *npy* mRNA in 21 tissues of gibel carp. As shown in Figure 4, the highest expression was found in the hypothalamus, eye and myelencephalon. The lowest expression of *npy* mRNA was observed in the liver, heart, gill, pituitary and hindgut.

The effect of food on npy *mRNA expression in gibel carp*

As shown in Figure 5, after 1 and 3 h of feeding, the *npy* mRNA expression in the hypothalamus was significantly lower in the fed group compared with the unfed group ($P < 0.01$). The *npy* mRNA expression of unfed group at +1h and +3h was significantly higher than the *npy* mRNA expression at -3h,-1h and 0h ($P < 0.05$).

The effect of long fasting on npy *mRNA expression in gibel carp*

As shown in Figure 6, on day 7, a significant change was detected between the fed group and the fasting group ($P < 0.01$). After refeeding, the expression of *npy* in the refed groups at 9, 11 and 14 days was significantly lower than that in the fed groups ($P < 0.01$). There was no significant change in *npy* mRNA expression among the fed groups ($P > 0.05$; Fig. 6).

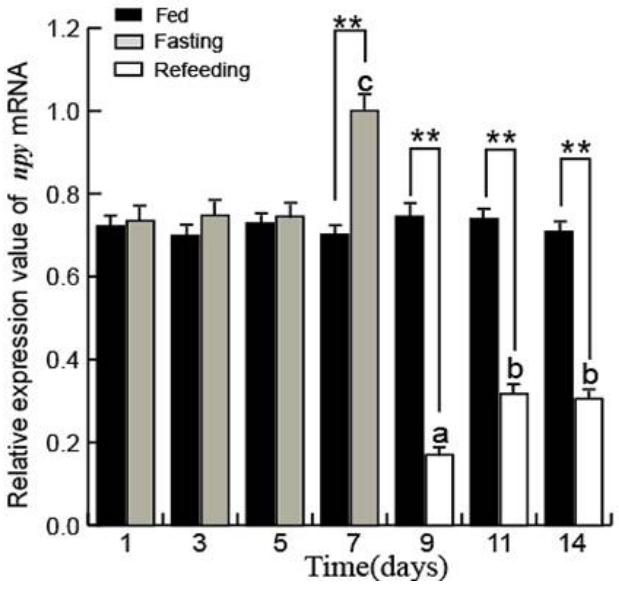

Fig. 6. Effects of fasting and refeeding on *npy* mRNA expression in the hypothalamus of gibel carp. Bars with different letters represent significant differences between experimental groups. Asterisks represent significant differences between the groups at the same time point. $^{**}P < 0.01$.

DISCUSSION

In our research, we isolated from gibel carp a 291-bp ORF of *npy*, encoding 96 amino acids. Using multiple sequences alignment and phylogeny evolution analysis, we found that the sequence of this gene is highly homologous with other species, especially cyprinid fish. The results indicated that the gibel carp *npy* gene is highly conserved within teleost species.

To explore the gene's expression profile in gibel carp, *npy* mRNA was investigated in peripheral and central tissues by qPCR. The results showed that *npy* mRNA was highly expressed in the brain, especially in the hypothalamus, which is consistent with reports on other fish species, such as *S. prenanti* (Wei *et al*., 2014) and Atlantic salmon (Murashita *et al*., 2009). The hypothalamic neuronal circuitry is critical for the regulation of feeding behavior in vertebrates (Berthoud, 2002; Carr *et al*., 2002; Nuria and Thrur, 2001; Saper *et al*., 2002; Volkoff *et al*., 2005). Thus, it can be inferred from the results that *npy* may play a role in feeding regulation. Interestingly, we also found a high expression level in the eye. Similar result was also reported in the *Sparus aurata* L. (Pirone *et al*., 2008). We speculated that *npy* may be involved in modulating neurotransmitter release. However, research on *npy* function in the eye is still inadequate and remains to be explored. In addition, *npy* was detected in the heart, foregut, ovary and testis. These results indicate that *npy* may be involved in a wide range of biological functions in gibel carp such as cardiac function (Xiang, 1994), gastrointestinal processes (Kehoe and Volkoff, 2007; MacDonald and Volkoff, 2009; Montpetit *et al*., 2005) and sexual behavior (Gaikwad *et al*., 2005; Peng *et al*., 1994).

Previous studies have demonstrated that *npy* is an important appetite-regulating hormone in mammals (Clark *et al*., 1984) and fish (Wei *et al*., 2014). The obtained data of the current study showed that 1 and 3 h after feeding, the *npy* mRNA expression in the hypothalamus was significantly lower in the fed group compared with the unfed group ($P < 0.01$). This result is consistent with previous studies on other fish. In goldfish, the brain *npy* mRNA levels sharply decreased at +1h and +3h after feeding (Narnaware *et al*., 2000). In *S. prenanti*, the expression level of *npy* in fed fish was significantly lower than that in unfed fish at 0.5, 1.5, 3 and 9 h after feeding (Wei *et al*., 2014). The *npy* mRNA expression of fasted fish at day 7 was significantly higher compared with the fed group ($P < 0.01$). On days 9, 11 and 14, the *npy* mRNA expression was significantly lower in the refed group than in the fed group (Fig. 6; $P < 0.01$). These results are consistent with previous research on other species. In zebrafish, the *npy* mRNA expression in the hypothalamus was significantly increased after 7 days of fasting (Yokobori *et al*., 2012). In Chinook salmon and coho salmon, *npy* mRNA expression in the hypothalamus increased after fasting for 2–3 weeks (Silverstein *et al*., 1998). In Brazilian flounder, the *npy* mRNA expression in the brain after 2 weeks of fasting was higher than in fed fish (Campos *et al*., 2010). In summary, these results indicated that *npy* has an orexigenic role in gibel carp. Nevertheless, fasting did not affect the *npy* mRNA expression in Atlantic salmon (Murashita *et al*., 2009), suggesting that not all fish are alike in the endocrine regulation of feeding.

CONCLUSION

In our study, the *npy* gene was first identified from gibel carp. We found that it is highly expressed in the brain, especially in the hypothalamus. Furthermore, fasting caused its mRNA expression to change in gibel carp, suggesting that *npy* may have orexigenic activity in gibel carp. Our findings offer an essential foundation for future research on the regulating mechanisms and functions that underlie the role of *npy* in feeding in teleosts.

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Statement of conflict of interest

The authors declare no conflict of interest.

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