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Effects of postprandial starvation on mRNA expression of endocrine-, amino acid and peptide transporter-, and metabolic enzyme-related genes in zebrafish (*Danio rerio*)

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Abstract The goal of this study was to systematically evaluate the molecular activities of endocrine-, amino acid and peptide transporters-, and metabolic enzyme-related genes in 35-day-old mixed-sex zebrafish (*Danio rerio*) after feeding. Zebrafish with initial body weights ranging from 9 to 11 mg were fasted for 384 h in a controlled indoor environment. Fish were sampled at 0, 3, 6, 12, 24, 48, 96, 192, and 384 h after fed. Overall, the present study results show that the regulatory mechanism that insulin-like growth factor I negative feedback regulated growth hormone is conserved in zebrafish, as it is in mammals, but that regulation of growth hormone receptors is highly intricate. Leptin and cholecystokinin are time-dependent negative feedback signals, and neuropeptide Y may be an important positive neuropeptide for food intake in zebrafish. The amino acid/carnitine transporters $B^{0,+}$ ($ATB^{0,+}$) and broad neutral (0) amino acid transporter 1(B^0AT1) mRNA levels measured in our study suggest that protein may be utilized during 24–96 h of fasting in zebrafish. Glutamine synthetase mRNA levels were downregulated, and glutamate dehydrogenase, alanine aminotransferase, aspartate transaminase, and trypsin mRNA levels were upregulated after long-time fasting in this study. The mRNA expression levels of fatty acid synthetase decreased significantly ($P < 0.05$), whereas those of lipoprotein lipase rapidly increased after 96 h of fasting. Fasting activated the expression of glucose synthesis genes when fasting for short periods of time; when fasting is prolonged, the mRNA levels of glucose breakdown enzymes and pentose phosphate shunt genes decreased.

Keywords Postprandial starvation · Transporters · Metabolic enzymes · Zebrafish

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Introduction

The zebrafish (*Danio rerio*) is an established vertebrate model organism that has been used frequently in biomedical, developmental, molecular, and genetic studies. Zebrafish has also been proposed as a model organism for nutrition and growth studies in fishes (Watts et al. 2012; Ribas and Piferrer 2013; Ulloa et al. 2011).

Postprandial period is most studied as the physiological response of nutrients ingestion and provides an integrated measure of the energy expended on all of

the activities involved in the processing of a meal (McCue 2006; Secor 2009). Following a meal, a series of physiological changes occurs in animals as they digest, absorb, and assimilate ingested nutrients (Carter et al. 2007). In zebrafish, Seiliez et al. (2013) investigated the effect of a single meal and the effect of an increase in the dietary carbohydrate/protein ratio on the postprandial expression of several hepatic and muscle metabolic-related genes and proteins.

Starvation represents an extreme condition in the nutritional continuum. Aquaculture offers an abundance of data from controlled studies that can be used to examine the starvation biology of fishes (Bar and Volkoff 2012). Most studies focused on the physiological consequences of the imbalance between energy intake and expenditure. However, some researchers have considered a continuous series of metabolic phases composed of a short initial period of adaptation followed by a second phase characterized by nutrient oxidation during fasting. At this latter point, fasting affects hormone secretion and nutrient metabolism (Hatch 2012; Lignot and LeMaho 2012). The few existing studies of starvation in zebrafish focused mainly on the effects of long-term food deprivation and/or refeeding on hepatic, brain, and skeletal muscle transcriptomes (Drew et al. 2008) and on mRNA expression of the peptide transporter PepT1 and gastrointestinal hormones (Koven and Schulte 2012).

Although many researchers have described the changes that occur in the body during fasting in many fish species, a systematic evaluation of molecular events has not been conducted for zebrafish, especially in fed-to-starvation transition. To better understand the dynamic changes that occur at the level of transcription during fasting, 35-day-old mixed-sex zebrafish were deprived of food for 384 h and sampled at different time points, and the expressions of endocrine-, amino acid and peptide transporter-, and metabolic enzyme-related genes were measured at each time point. The molecular responses of following genes were concurrently measured in the present study: appetite-related genes [neuropeptide Y (NPY), leptin, ghrelin, and cholecystokinin (CCK)], growth axis gene [growth hormone (GH), growth hormone receptor A (GHRA), growth hormone receptor B (GHRB), and insulin-like growth factor-I (IGF-I)], three representative amino acid transporters [the neutral amino acid transporter (ASCT2), amino acid/carnitine transporters $B^{0,+}$ ($ATB^{0,+}$), and broad neutral (0) amino acid transporter 1(B^0AT1)], peptide transporters [oligopeptide transporter 1 (PepT1) and oligopeptide transporter 2 (PepT2)], and key metabolic enzyme-related genes including lipid metabolic enzyme [fatty acid synthetase (FAS) and lipoprotein lipase (LPL)], glucose breakdown [pyruvate kinase (PK) and glucokinase (GK)], glucose synthesis [fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK)], pentose phosphate shunt [glucose-6-phosphate dehydrogenase (G6PDH)], and nitrogen metabolic enzyme [glutamine synthetase (GS), glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), aspartate transaminase (AST), and trypsin].

Materials and methods

Experimental animals

Wild-type zebrafish were selected from a group provided by the Key Laboratory of Marine Drugs, Ocean University of China, Qingdao, Shandong Province. They were maintained in four 5-L tanks (30 fish each tank) at 28 °C under a 14-h light:10-h dark cycles. Fish water consisted reverse osmosis water supplemented with a commercially available salt solution, and that was continuously running through a gravel-activated charcoal filter in a recirculation system. Mean values for pH and dissolved oxygen concentration were 7.1 and 6.2 mg L^{-1}, respectively. Zebrafish were fed ad libitum twice daily (am 9:00 and pm 4:00) with a sufficient amount of brine shrimp (*Artemia*) which were hatched for 12 h. All experiments were conducted at Ocean University of China following approved protocols.

Experimental procedure

Thirty-five-day-old mixed-sex zebrafish (weights ranging from 9 to 11 mg) were used in this fasting study. After being satiated fed ad libitum with brine shrimp, fish were deprived of food for 384 h (a small amount of fasting fish was died at 432 h in this experiment) and sampled at 0, 3, 6, 12, 24, 48, 96, 192, and 384 h. Care has been taken to minimize stress, and at each sampling time point, two fish randomly selected from each tank were swum out from the overflow to another small tank that filled with 2–4 °C

chilled water to be euthanized (Association 2013). These fish were placed in 2-mL microcentrifuge tubes, immediately frozen in liquid nitrogen for 24 h, and then placed in RNAlater (Ambion® Life Technologies, Carlsbad, CA, USA) at 4 °C for 24 h; samples were stored at −150 °C until RNA analysis. The gastric evacuation time of these zebrafish was about 5.5 h, and the intestinal evacuation time was approximately 12 h. In the present study, the period from 0 to 12 h after food administration was defined as the postprandial period, and the sample time points were designated as P0, P3, P6, and P12. The starvation period lasted from 24 to 384 h after food administration, and the time points were abbreviated as F24, F48, F96, F192, and F384. The initial level (P0) was used as the control.

The qRT-PCR (real-time fluorogenic quantitative PCR) protocol adhered to the guidelines (Bustin et al. 2009). Fish was homogenized in liquid nitrogen, and total RNA was prepared with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Using a PrimeScript® RT reagent kit, cDNA was synthesized from each RNA sample (2 μg) following the manufacturer's recommendations. qRT-PCR was conducted on a QIAGEN Rotor-Gene Q 6000 Real-Time PCR System with SYBR® Premix Ex Taq™. Elongation factor 1 alpha 1 (EF1), β-actin, 18 s ribosomal RNA, glucose-6-phosphate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase were evaluated for use as reference genes by ranking the expression levels according to their stability. EF1 was used as the gene for normalization. Table 1 lists the qRT-PCR primer pairs used in this analysis. All primers were synthesized by BGI (Beijing, China), and all kits were provided by Takara Biotech (Dalian, China). When different isoforms of a gene were known in zebrafish (as for PEPCK), gene expression analysis was performed on each isoform.

Real-time PCR was carried out with a 2-μL cDNA sample, 10 μL SYBR® Premix Ex Taq™, 0.4 μL PCR forward/reverse primers (10 μM), and 7.2 μL nuclease-free water. The thermocycling conditions for the reaction were as follows: 95 °C for 5 min, followed by 40 cycles consisting of 95 °C for 15 s, 58/59 °C for 15 s, and 72 °C for 20 s. After the final PCR cycle, melting curves were systematically monitored (increasing the temperature of the reaction mixtures up to 95 °C, by 0.1 °C/s, starting at 72 °C for 5 s) to ensure that only one fragment was amplified. Samples without cDNA were run for each reaction as negative controls. The reaction was carried out with three duplicates of each sample. Values for the threshold (C_T) were determined using Light-Cycler software. Data (normalized C_T values) from the treated and control tissue templates were compared, and the $2^{-\Delta\Delta CT}$ method was used as the relative quantification calculation method (Livak and Schmittgen 2001).

Statistical analysis

Firstly, each parameter was tested for each sample fish, and each sample fish was carried out with three duplicates. Eight average values of each parameter were obtained from eight fish in the same group ($n = 8$). Finally, the averages were analyzed by one-way ANOVA and Tukey's multiple range tests using SPSS 17.0 for Windows (SPSS Inc, Chicago, Illinois, USA). Data were expressed as means with their standard deviation (SD) in figures. Differences were considered to be significant at $P < 0.05$.

Results

Changes in relative mRNA expression of NPY, ghrelin, CCK, and leptin of zebrafish during postprandial starvation

Figure 1 shows the changes in relative mRNA expression of the appetite-related genes NPY, ghrelin, CCK, and leptin in zebrafish during postprandial starvation. Compared with the initial level, NPY expression was significantly lower at P6 ($P < 0.05$) and then significantly increased from 12 h of fasting onwards ($P < 0.05$). Ghrelin expression was upregulated and increased to 11.31 times and 6.38 times the P0 level after fasting for 48 and 384 h, respectively. Compared with the initial values, CCK expression significantly increased during fasting for 3–6 h and then decreased significantly from F24 to F192 ($P < 0.05$); however, expression increased to its initial level when fish were fasted for 384 h ($P > 0.05$). Leptin expression peaked at P12, then returned to initial levels at F96 h, and then decreased significantly from F192 to F384 ($P < 0.05$).

Table 1 Nucleotide sequences of primers and cycling conditions used for PCR amplification

Gene	Accession no.[a]	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Tm (°C)	Amplicon size (bp)
Neuropeptide Y (NPY)	NM_131074	CGCGTTTCTCTTGTTCGTC	ACCTTTTCCCATACCTCTGC	58	161
Ghrelin	EU908735	GCTCCTGTGTGTTTCTCTTTCC	TCTCTTCTGCCCACTCTTGG	59	114
Cholecystokinin (CCK)	XM_001346104	GTTCAGTCTAATGTCGGCTCC	TAGTTCGGTTAGGCTGCTGC	59	129
Leptin	XM_009293460	CATCATCGTCAGAATCAGGG	CTTGGATGGGTTTGTCAGC	59	110
Growth hormone (GH)	AJ937858	AGGTCTTATGCCTGAGGAACG	AAGGTCTGGCTGGGAAACTC	59	177
Growth hormone receptor A (GHRA)	NM_001083578	AAGCCAGACTTCTACCACGAG	GCTCTCCCTGTTCAGAATGGT	59	241
Growth hormone receptor B (GHRB)	NM_001111081	CTAATACACCAACAGCCCCAC	CACTGGAGAAGGCTTGAGAAC	58	220
Insulin-like growth factor I (IGF-I)	NM_131825	ACACAGGGGGCAGAAACTAT	AAGATGGGGCTTAAACGTCC	58	196
Neutral amino acid transporter (ASCT2)	XM_003200016.1	ACACGGAAGAACCCCTACAC	CAGCGACGCACTGGAAAC	58	208
Broad neutral (0) amino acid transporter 1 (B^0AT1)	NM_199736	GCCAGAGGGGTTCATCAATC	ACAAGCCAAGGCAGAAGAGC	58	239
Amino acid/carnitine transporters $B^{0,+}$ ($ATB^{0,+}$)	BC117616	GGTCTGATGGCTCTGTCGT	CTTAGAGAGAGCGTCAGGGT	59	219
Oligopeptide transporter 1 (PePT1)	NM_198064	TGTTCCTCTACATCCCTCTGC	AGGCTGCCAAAACCATACC	58	258
Oligopeptide transporter 2 (PePT2)	NM_001039828	AGGACATCCAAGCCAACAAC	AAGCAGAAACTCCACCCAC	58	236
Trypsin	NM_131708	CTGATGTGCCTGAATGCTCC	TAACCCCAGGACACGATACC	59	182
Glutamine synthetase (GS)	NM_181559	TGTGAAGACTTCGGTGTGGT	TGCGGATGTGATAGTTGTGC	59	175
Alanine aminotransferase (ALT)	NM_001142774	ATCATCAACCCAGGAAACCC	GGACCCATCTCAAACAGCA	59	179
Aspartate transaminase (AST)	NM_213057	GTGTTCATCATACCGCACTTC	GACTCCCAGATTCACCTTCTTC	58	188
Glutamate dehydrogenase (GDH)	NM_199545	CCATCCCTATTGTACCCACTG	TTGACGTAAGCCGCTGTTC	58	172
Fatty acid synthetase (FAS)	XM_682295.5	ATGGAGTTTTCAGGGCGAG	GGGAATAATATGCGGTGGC	58	175
Lipoprotein lipase (LPL)	BC064296	GAATACACGGCGAGAAGGA	CAGTTTGCGAATGTGGAAGG	58	188
Glucokinase (GK)	BC122359	TGAGGATGAAGAGCGAGGC	AGAGAAGGTGAATCCCAGCG	58	178
Pyruvate kinase (PK)	NM_201289.1	CAAAGGACACTTCCCTGTAGAG	GGACAACGAGGACGATAACG	58	249
Phosphoenolpyruvate carboxykinase 1 (PEPCK)	NM_214751	GTGAACTGAACCGAGACCTG	AGCACTTGAGAGCAAACGAT	59	192
Fructose-1,6-bisphosphatase 1 (FBPase)	NM_199942	CATCTGTATGGGATTGCTGG	TTACCCCGTCTATCTGGCTC	59	173
Glucose-6-phosphate dehydrogenase (G6PDH)	XM_692728.6	GCCTCCCTTCAGCACATAGA	ATGGGGATGCCCTCGTATT	59	170
Elongation factor 1 alpha 1 (EF1)	XM_005173785	TGTCCTCAAGCCTGGTATGG	TGGGTCGTTCTTGCTGTCTC	58/59	190

[a] Accession numbers are from www.ncbi.nlm.nih.gov

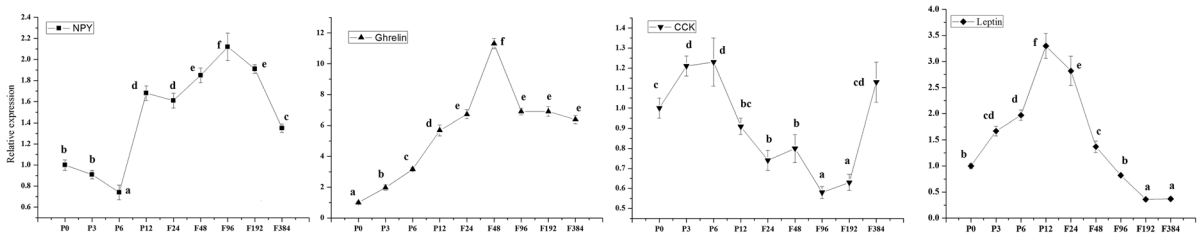

Fig. 1 Changes in relative mRNA expression of NPY, ghrelin, CCK, and leptin of zebrafish during postprandial starvation. Data shown as mean ± SD ($n = 8$). Means with *different letters* in the same figure are significantly different. *Error bars* represent SD

Changes in relative mRNA expression of growth axis genes of zebrafish during postprandial starvation

Figure 2 shows the changes in relative mRNA expression of growth axis genes of zebrafish during postprandial starvation. mRNA expression of both GH and GHRA increased significantly during fasting ($P < 0.05$). After fasting for 384 h, GH mRNA expression was 6.88 times greater than that at P0 ($P < 0.05$). The peak GHRA mRNA expression occurred at F96 and was 3.03 times greater than that of the initial level ($P < 0.05$). GHRB mRNA expression decreased significantly after feeding for 3–6 h and then increased significantly from after

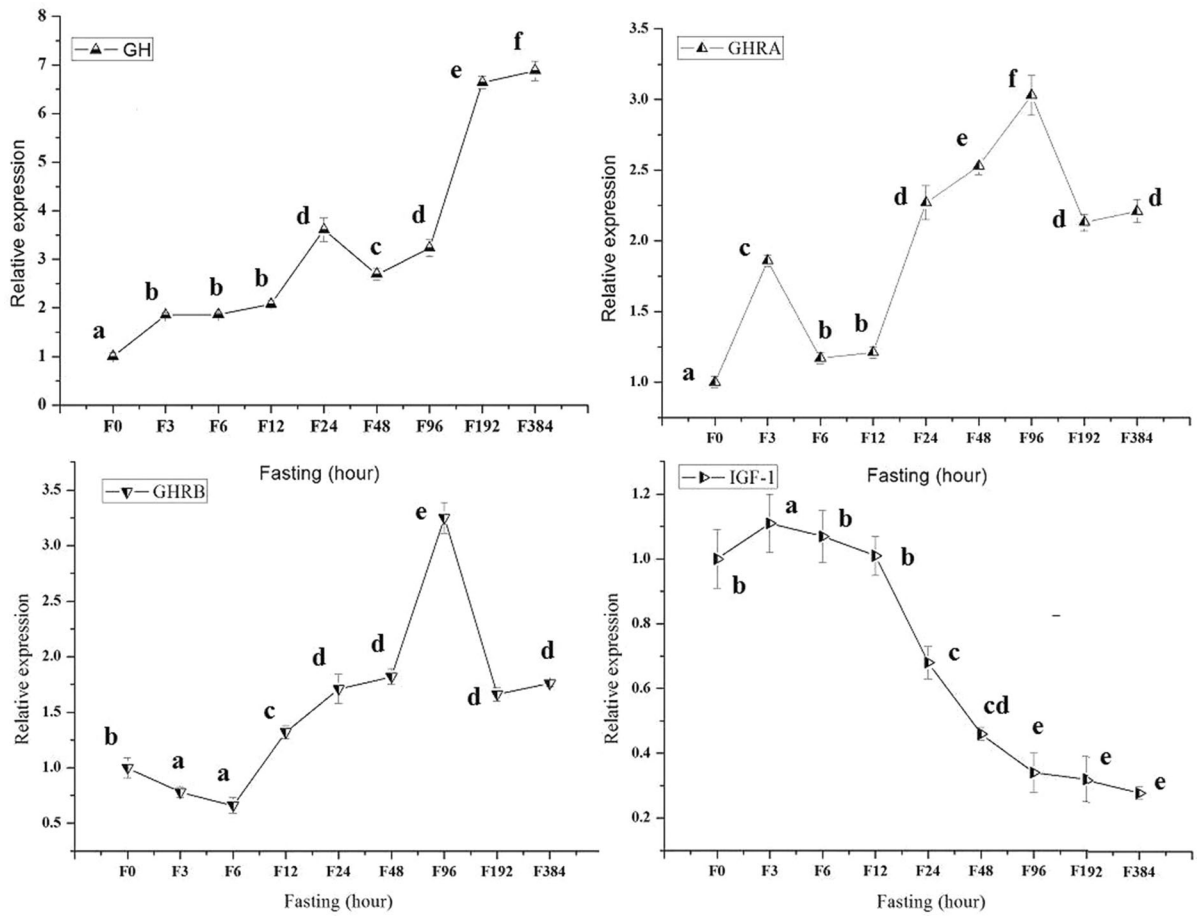

Fig. 2 Changes in relative mRNA expression of growth axis genes of zebrafish during postprandial starvation. Data shown as mean ± SD ($n = 8$). Means with *different letters* in the same figure are significantly different. *Error bars* represent SD

feeding 12 h onwards ($P < 0.05$). Compared with the initial values, fasting for 3 h led to increased expression of IGF-I ($P < 0.05$), but expression did not change significantly from P6 to P12. After 96 h of fasting, the IGF-I mRNA level had decreased by 64 %, and this level was maintained at F192 and F384 h ($P < 0.05$).

Changes in relative mRNA expression of amino acid and peptide transporters of zebrafish during postprandial starvation

Figure 3 shows the changes in relative mRNA expression of these transporters throughout the experiment. The mRNA expression of ASCT2 significantly increased after fasting for 3–6 h and then began to decrease after 12 h. $ATB^{0,+}$ and B^0AT1 mRNA expression decreased at first, then increased, and finally decreased again. Expression levels of two peptide transporters also were measured in this experiment. mRNA expression of PepT1 decreased significantly throughout the experiment ($P < 0.05$). Expression of PepT2 decreased significantly at P6 ($P < 0.05$), increased between 12 and 192 h ($P < 0.05$), and then decreased again ($P < 0.05$).

Changes in relative mRNA expression of metabolic enzyme-related genes of zebrafish during postprandial starvation

Figure 4 shows the changes in relative mRNA expression of these genes in zebrafish during postprandial starvation. Faster, more generalized responses were found in the lipid and carbohydrate metabolic enzymes. There was a significant decrease in the mRNA expression level of FAS during fasting ($P < 0.05$) and a rapid increase in the mRNA expression level of LPL after 96 h of fasting.

The mRNA expression levels of GK were 1.36, 7.56, 23.72, 3.55, 1.49, and 1.30 times the original level ($P < 0.05$) following fasting for 3, 6, 12, 24, 48, and 96 h, respectively. However, the GK mRNA level returned to the baseline level by 192 h and then decreased significantly after fasting for 384 h ($P < 0.05$). The PK mRNA level showed variation similar to that of GK in this experiment. The expression of the FBPase was found to be elevated, and G6PDH was significantly decreased from 3 to 384 h after a meal. The expression of PEPCK was significantly decreased 3–6 h after feeding and then significantly elevated after 24 h ($P < 0.05$).

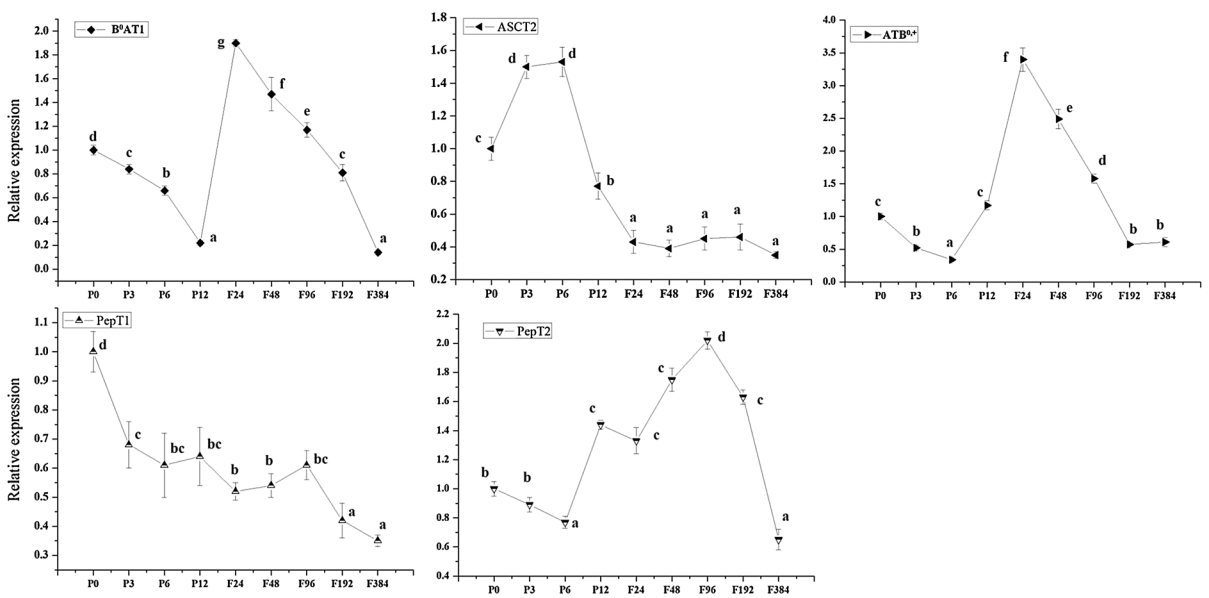

Fig. 3 Changes in relative mRNA expression of amino acid and peptide transporters of zebrafish during postprandial starvation. Data shown as mean ± SD ($n = 8$). Means with *different letters* in the same figure are significantly different. *Error bars* represent SD

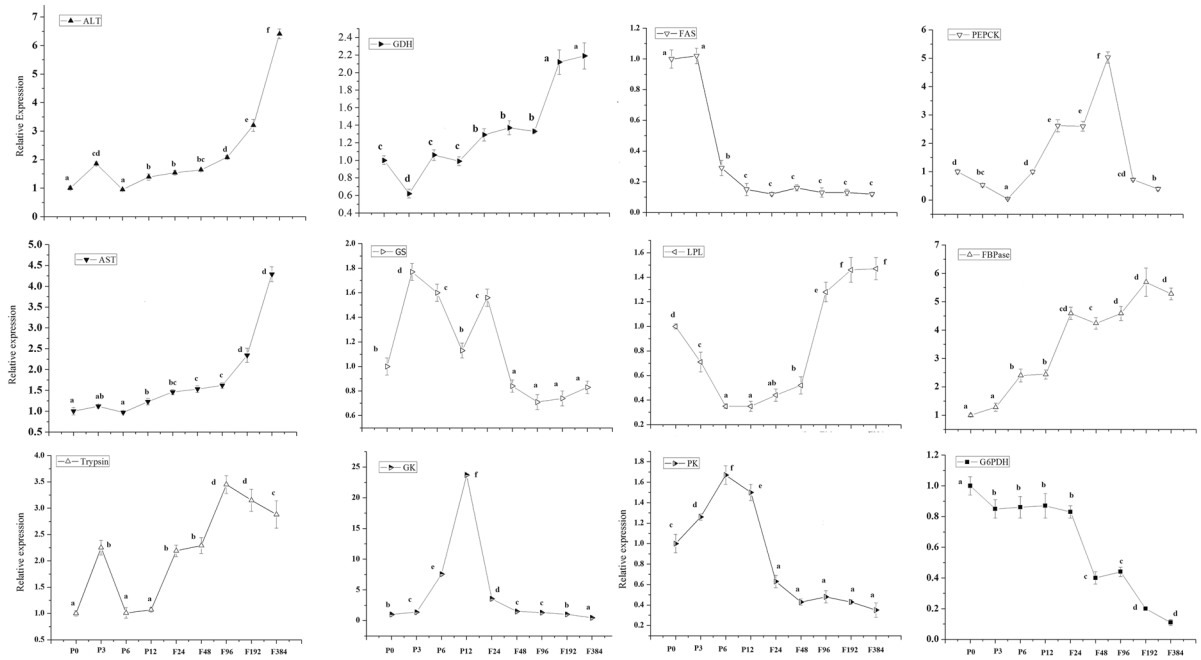

Fig. 4 Changes in relative mRNA expression of metabolic enzyme-related genes of zebrafish. Data shown as mean ± SD ($n = 8$). Means with *different letters* in the same figure are significantly different. *Error bars* represent SD

Fasting for 3, 6, and 24 h led to an increase in the expression of GS mRNA, but GS mRNA levels were downregulated when fasting for 48–384 h. GDH mRNA levels was significantly suppressed by 3 h after feeding ($P < 0.05$), then returned to initial level at P6 and P12, and finally increased significantly thereafter ($P < 0.05$). The trypsin mRNA level was significantly higher at P3 compared with P0 ($P < 0.05$), but it returned to the initial value at P6 and P12 and then increased significantly thereafter ($P < 0.05$). A similar pattern was also observed for ALT mRNA level in this experiment. No significant variation was observed for AST mRNA levels 3–6 h after feeding; nonetheless, fasting 12–384 h upregulated the expression of AST.

Discussion

The present study focused on fed-to-starvation transition molecular responses in the whole body of zebrafish. The progressive decrease in stomach somatic index suggests that a period of 5–8 h is needed to complete the gastric emptying. Consequently, the transit of digested food through the intestine extended until 12 h as suggested by intestine somatic index. We defined 0–12 h after feeding was postprandial and 24–384 h after feeding was starvation.

The GH/IGF-I axis controls somatic growth in all vertebrates, including fish. Numerous environmental factors, including nutritional state, photoperiod, stress, and temperature, have dramatic effects on the expression and activity of peripheral components of the GH/IGF system, and the biological actions of GH are mediated by the transmembrane GHRs (Holzenberger et al. 2005; Reindl and Sheridan 2012). GH controls the expression of GHRs and IGFs (Fuentes et al. 2013). However, in adult zebrafish, the expression of IGF-I but not IGF-II mRNAs is regulated by GH (Maures et al. 2002). In several studies of other fish species, the plasma GH level was found to be significantly elevated in the first weeks of starvation (Riley et al. 2008; Picha et al. 2009; Kling et al. 2012); moreover, GHR gene expression did not change during fasting for 15 days (Deng et al. 2004) but declined following longer-term fasting (Schneider et al. 2011; Fukada et al. 2004; Fox et al. 2006). Fasting reduces the gene expression and plasma concentrations of IGF-I rapidly, and levels remain low throughout the starvation period (Davis and Gaylord 2011; Fox et al. 2009). In the present study

of zebrafish, both GH and GHRA mRNA expression increased significantly during this experiment. GHRB mRNA expression significantly decreased 3 h post-feeding, together with significantly increased 12 h post-feeding. During the postprandial state, the mRNA expressions of IGF-I increased at P3, then returned to initial values after feeding 6–12 h, and decreased notably during starvation (from F24 to F384). These results for GH and IGF-I but not GHR expression in zebrafish were similar to data from other studies and show that the regulatory mechanism that IGF-I negative feedback regulated GH is conserved in zebrafish, as it is in mammals, but that regulation of GHRs is highly intricate. In zebrafish, the GH family contains five ligands, including GH, the somatolactin family (SLα and SLβ), the prolactin family (PRL1 and PRL2), and four cognate receptors, including two GHRs and two PRL receptors (PRLR1 and PRLR2); however, GH can only interact with the GHRs, and PRLs can only interact with the PRL receptors (PRLRs). The zebrafish SLs were found to be ineffective at interacting with the zebrafish GHRs and PRLRs (Chen et al. 2011). Phylogenetic studies have shown that the function of GHRs together with SL receptors (SLRs) in *Oryzias latipes* (Fukamachi et al. 2005) and *Oncorhynchus masou* (Fukada et al. 2005) may be affected by different physiological conditions, and this phenomenon should be explored in zebrafish.

Ghrelin plays an integral role not only as a GH-releasing hormone but also in the regulation of appetite, energy metabolism, cardiovascular performance, and immune responses in a variety of vertebrates (Nakazato et al. 2001; Kaiya et al. 2008). Nutritional status is intimately linked to a variety of peripheral and central signals that maintain energetic homeostasis (Canosa et al. 2007). Thus, ghrelin represents a key link between nutritional status in the stomach and the neuroendocrine response from the brain (Volkoff and Peter 2006; Kaiya et al. 2008). Ghrelin mRNA expression and ghrelin secretion reportedly change under different nutritional regimes. For example, after a meal, ghrelin mRNA expression in the hypothalamus and gut was found to decrease, and serum ghrelin levels also decreased in the goldfish, *Carassius auratus* (Canosa et al. 2005; Unniappan et al. 2004). Ghrelin levels are generally high during fasting in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Pankhurst et al. 2008; Hevrøy et al. 2011), although no change in ghrelin concentration was found in studies of tilapia (*Oreochromis niloticus*) (Riley et al. 2008), Atlantic cod (*Gadus morhua*) (Xu and Volkoff 2009), and rainbow trout (Jönsson et al. 2007). However, in our study, ghrelin expression was upregulated throughout the experiment. These varying results indicate that ghrelin may not act as a meal-initiated signal in all fish. Food intake in fish is regulated by the coordination of multiple, redundant neuroendocrine pathways (Volkoff et al. 2005). Further studies of the interactions between ghrelin and other appetite regulatory peptides in fish are warranted.

Leptin is a protein synthesized and secreted by adipose tissue that has been suggested as having a role in long-term regulation appetite and suppressing food intake, thereby inducing weight loss in both rodents and humans (Klok et al. 2007; Stieg et al. 2015). In fish, leptin also plays an important role in sexual maturation (Trombley and Schmitz 2013), immune system, appetite regulation, growth, and feed intake (Francis et al. 2014). Leptin concentration falls during starvation, and totally leptin-deficient *ob/ob* mice have neuroendocrine abnormalities similar to those of starvation (Ahima et al. 1996). In the present study, leptin significantly raised 3–48 h post-feeding (peaked at P12, 3.30 times of the initial values) and significantly fell until 192 h after feeding (36 % of the initial values). These data suggest that leptin is a longtime negative feedback signal for integrating stimulation of food intake in zebrafish.

NPY is the most abundant peptide in the brain, and it is widely expressed in the central and peripheral nervous systems (White 1993). In mammals, NPY is the most effective stimulator of appetite and food intake, and hypothalamic NPY-expressing cells represent a critical site of integration of peripheral hormonal signals with regulation of energy homeostasis (Kalra et al. 1999). To date, studies of NPY in fish have mainly involved molecular characterization [e.g., in *Ctenopharyngodon idellus* (Zhou et al. 2013), *D. rerio* (Winata et al. 2013), *S. salar* (Murashita et al. 2009), *Siniperca chuatsi* (Liang et al. 2007), *C. auratus* (Pontet et al. 1989), *Dicentrarchus labrax* (Cerdá-Reverter et al. 2000b, Cerdá-Reverter et al. 2000a), and *Takifugu rubripes* (Kamijo et al. 2011)], and a high level of NPY gene expression in these fish was detected in the brain. In the present study, NPY gene expression was significantly lower at P6

compared with P0, but it increased significantly from 12 h of fasting onwards. These results differ from those for Atlantic salmon (*Salmo salar* L.) (no significant change after 6 days of fasting) (Murashita et al. 2009), but are similar to those for zebrafish (in the hypothalamus, fasted 7 days) (Yokobori et al. 2012), goldfish (Narnaware and Peter 2001), salmon (*Oncorhynchus* sp.) (Silverstein and Plisetskaya 2000), and bullfrog (*Rana catesbeiana*) (Shimizu et al. 2013). These data suggest that a time-dependent increase in NPY mRNA levels occurs in most fish in response to long fasting, and NPY may be an important neuropeptide for integrating stimulation of food intake.

CCK, a peptide hormone found in the small intestine, regulates pancreatic enzyme secretion and pancreatic growth, intestinal motility, satiety signaling, and the inhibition of gastric acid secretion (Rehfeld 2004). In mammals, CCK inhibits food intake by inducing satiety and reducing meal size, inhibits gastric emptying by reducing gut motility, induces gastrointestinal vasodilation, gallbladder contraction, and intestinal peristalsis, and stimulates gastric acid and pancreatic secretion (Liddle 1997). In *D. labrax*, researchers found that orally administered CCK induced an anorexigenic effect on both total food and single macronutrient intake and that this effect was counteracted by the CCK antagonist proglumide (Rubio et al. 2008). In *C. idellus*, fasting decreased CCK mRNA expression levels in the brain and intestine, whereas refeeding resulted in increased expression; similar results were reported for *Tautogolabrus adspersus* (Hayes and Volkoff 2014; Babichuk and Volkoff 2013); these results suggest that CCK mRNA expression may play a role in feed intake regulation in this species (Feng et al. 2012). In contrast, fasting had no effect on the brain expression of CCK in *Astyanax fasciatus mexicanus* (Wall and Volkoff 2013). In our study, CCK expression significantly increased after fasting for 3–6 h but then decreased significantly from F24 to F192. However, it increased to its initial level by 384 h. This result indicates that CCK negative feedback regulated food intake when fasting for short periods of time in the zebrafish.

Amino acid transporters play an important role in intestinal absorption of amino acids, and they are in an ideal location to relay nutritional information as well as nutrients themselves to the cell interior. They also are able to modulate signaling downstream of intracellular amino acid receptors by regulating intracellular amino acid concentrations through processes of coupled transport (Hundal and Taylor 2009). These transporters mediate amino acid exchanges between extracellular and intracellular fluid compartments and deliver substrates to intracellular amino acid sensors (Taylor 2014). Most EAAs are neutral and are absorbed apically through B^0AT1 and ASCT2, and the cationic EAA lysine is absorbed apically through $ATB^{0,+}$ (Poncet and Taylor 2013). Many studies have focused on changes in the amino acid concentration dynamics of fish during fasting (García-Garrido et al. 2013; George-Zamora et al. 2011; Blasco et al. 1991). In general, fasting was found to decrease amino acid levels, but studies of amino acid transporters are very limited in fish. In the present study, compared with the initial level, B^0AT1 expression decreased significantly from 3 to 12 h post-feeding, increased from 24 to 96 h, and then decreased again. $ATB^{0,+}$ showed similar changes. mRNA expression of ASCT2 significantly increased after feeding for 3–6 h and then began to decrease from 12 h onwards. In zebrafish, glutamic acid was the most abundant amino acid throughout development (13–14 % of total amino acids), followed by aspartic acid, lysine, leucine, and valine (Gómez-Requeni et al. 2010). The gastric evacuation time of 35-day-old zebrafish was about 5.5 h in this experiment, and the significant increase in mRNA expression of ASCT2 after fasting for 3–6 h may indicate that neutral amino acids were quickly and easily absorbed through the ASCT2 transporter after feeding. The intestinal evacuation time in zebrafish is approximately 12 h, and $ATB^{0,+}$ and B^0AT1 significantly increased after fasting for 24–96 h. Many studies have shown that protein is used as the energy source in the initial stages of fasting (Barclay et al. 1983; Loughna and Goldspink 1984; Machado et al. 1988). Little is known about which nutrients are used first as the energy source in zebrafish under starvation conditions. The $ATB^{0,+}$ and B^0AT1 mRNA levels measured in our study suggest that protein may be utilized during 24–96 h of fasting in zebrafish.

Peptide transporters also play key roles during the absorption of amino acids and oligopeptides. The peptide transporters expressed in the brush border membrane of the intestinal and renal epithelial cells are responsible for the uptake of small peptides consisting of two or three amino acids (Ganapathy et al. 1997). The PepT1 predominantly present in the

intestine and PepT2 predominantly present in the proximal tubule of the kidney function in the absorption of peptides arising from the digestion of dietary proteins and in the reabsorption of peptides present in the kidney and brain, respectively (Ganapathy et al. 1998). PepT1 mRNA expression in zebrafish in our study decreased markedly during food deprivation. This observation is consistent with a previous report for zebrafish (Koven and Schulte 2012), European sea bass (*D. labrax*) (Corà 2011; Terova et al. 2009), and Atlantic salmon (Rønnestad et al. 2010). However, these findings differ from data from rats and broilers, which showed increased expression and activity of PepT1 during food deprivation or restriction (Gilbert et al. 2008). In the present study, PepT2 expression was significantly lower at P6 compared with P0; it then increased from 12 to 192 h and finally decreased again. Compared with PepT1, data on the regulation of PepT2 are sparse, especially in regard to in vivo study conditions. No reports about the effect of fasting/starvation on mRNA expression of PepT2 in fish exist at present. Increasing the expression and activity of PepT1 transporters would be a suitable strategy to compensate for a period of deficient nutrient intake. In many fish, protein is used as the energy source in the initial stages of fasting. Conversely, glucose or carbohydrates are exhausted first during fasting in humans and other mammals. No data in the existing literature support the existence of a rise in protein utilization in fasting humans undergoing long-term starvation (Lignot and LeMaho 2012). We hypothesize that decreased PepT1 transporter mRNA expression in zebrafish reflected a decline in absorption and assimilation efficiency of oligopeptides. When PepT2 expression increased from 12 to 192 h, it increased oligopeptide reabsorption. In this way, more amino acids were used to maintain life under starvation conditions.

Fasting induces profound metabolic perturbations in the body and induces changes in expression of genes encoding enzymes involved in nitrogen, lipid, and carbohydrate metabolism. During fasting, there is increased competition between nutrient anabolism and catabolism to maintain life and functional integrity. Protein is usually used as the energy source in the initial stages of fasting in fish; however, data at the transcriptional level in nitrogen metabolism in fish during fasting are scarce. ALT and AST are the most important aminotransferases in the teleostean fish. GDH catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate (Li et al. 2014). GS is an enzyme that plays an essential role in the metabolism of nitrogen in fish, as it catalyzes the condensation of glutamate and ammonia to form glutamine (Dhanasiri et al. 2012). Trypsin is produced in the pancreas as the inactive proenzyme trypsinogen, and it cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine (except when either is followed by proline) (Rodriguez et al. 2007). We tested the mRNA expression of above genes in the present study. The ALT mRNA level was significantly higher at P3 compared with P0, but it returned to the initial value at P6 and then increased significantly thereafter. No significant variation was observed for AST mRNA levels 3–6 h after feeding; nonetheless, fasting 12–384 h upregulated the expression of AST. Our results were supported by other species fish data. Fasting increased ALT activity in *Sparus aurata* (Anemaet et al. 2008). In *Lates calcarifer*, expression of ALT was significantly upregulated within 0.5 h of feeding and had returned to normal levels by 8 h; the change in expression of the AST was significant upregulation until 4 h after feeding (Wade et al. 2014). In *L. calcarifer*, the GDH was only very small after a feeding event, and in most cases, this change was not significant. In contrast to our study, GDH mRNA levels was significantly suppressed by 3 h after feeding, then returned to initial level at P6 and P12, and finally increased significantly thereafter. Data from rats have shown increased GS activity and mRNA levels in response to starvation (Arola et al. 1981; Ardawi, 1990; Mezzarobba et al. 2003; Kong et al. 2000; Labow et al. 1999). Our results support the notion that GS mRNA expression can be upregulated by a 24-h period of starvation, but GS mRNA levels were downregulated during fasting for 48–384 h. Trypsin mRNA levels increased significantly during fasting for 24–384 h in our experiment.

During fasting, more protein would be used as fuel. In addition, increased competition between proteolytic and proteogenesis occurs to maintain life under long-term starvation. Therefore, GS mRNA levels were downregulated, and ALT, AST, GDH, and trypsin mRNA levels were upregulated after longtime fasting, and the expression of these genes showed significant difference comparing with the initial level at post-feeding 12–24 h in this study, and this point of time was earlier than that time of lipid

and carbohydrates metabolic enzyme genes. These results further confirm the premise that zebrafish used protein as an energy source during the long fasting period.

LPL is as a gatekeeper for fatty acid uptake into organs (Greenwood 1984), and a large body of evidence from animal models, including fish, suggests that the level of LPL expression in a given tissue is the rate-limiting process for the uptake of triacylglycerol (TAG)-derived fatty acids (Weil et al. 2013; Preiss-Landl et al. 2002). FAS is a key enzyme in the opposite process of de novo lipogenesis, as it converts acetyl CoA and malonyl CoA into the final end product, palmitate, which is subsequently esterified into TAG and stored in adipose tissue (Wakil 1989). In several studies, fasting was shown to induce LPL activities and increase mRNA expression (Liang et al. 2002; Tian et al. 2013), thereby resulting in reduced FAS mRNA expression levels and activity (Gosmain et al. 2005; Tian et al. 2013; Palou et al. 2010). In our study, the mRNA levels of FAS decreased significantly during fasting for 6–384 h, which suggests that FAS is an important enzyme related to energy metabolism and that the expression of FAS may be regulated by nutrient quantities at a pre-translational level. In contrast, the mRNA levels of LPL decreased significantly during fasting for 3–48 h and then increased significantly during fasting for 96–384 h. We speculate that after short periods of fasting (<48 h), the amino acids may be rapidly consumed or mobilized; with prolonged fasting (>96 h), however, lipids will be used as an energy source.

GK and PK regulate carbohydrate metabolism by acting as glucose sensor, triggering shifts in metabolism or cell function in response to rising or falling levels of glucose, such as those that occur after a meal or when fasting (Magnuson and Matschinsky 2004). Hepatic PK activity in rainbow trout and gilthead sea bream (*S. aurata*) was found to significantly decrease with starvation (Enes et al. 2009). However, no difference in PK gene expression between food deprived and fed fish was observed in rainbow trout liver (Kirchner et al. 2003; Panserat et al. 2001). Food deprivation decreased GK mRNA expression in rainbow trout (Soengas et al. 2006) and gilthead sea bream (Metón et al. 2004). In the present study, both GK and PK mRNA expressions were regulated by nutritional status (Fig. 4).

In mammals, the expression of FBPase and PEPCK is downregulated by insulin in response to feeding and upregulated in response to starvation (Hanson and Reshef 1997). However, in previous study of zebrafish, the PEPCK expression was remained unchanged at 6 h after feeding and significantly decreased at 24 h after feeding; there was no effect on FBPase expression (Seiliez et al. 2013), which is opposed to their regulation in mammals. In this study, zebrafish rapid downregulated PEPCK expression after feeding 6 h and upregulated after feeding 24 h, and FBPase mRNA level was upregulated after feeding 6 h.

G6PDH plays the key role in regulating carbon flow through the pentose phosphate pathway (Kletzien et al. 1994) and catalyzes the first step of one pathway for producing pentose, a precursor of nucleic acids and of all nucleotide coenzymes (Luzzatto and Battistuzzi 1985). In the present study, the mRNA expression of G6PDH in the fasting zebrafish decreased at post-feeding 3 h, remained constant from post-feeding 3–24 h, and finally decreased by 89 % from the baseline at F384. In contrast, the expression of the G6PDH gene was significantly increased from 2 to 8 h after feeding, and the downregulation did not occur until 24 h after feeding in the barramundi (Wade et al. 2014). We speculate that the difference result is attributed to the different species of fish or the stress during sampling.

As glucose is an essential energy source for a number of tissues, it is particularly important that glucose levels are maintained throughout starvation (Romijn et al. 1990). Our results on carbohydrate metabolic genes suggest that fasting activates the expression of glucose synthesis genes when fasting for short periods of time; when fasting is prolonged, there are lower levels of glucose available to maintain life and functional integrity, and the mRNA levels of glucose breakdown enzymes and pentose phosphate shunt genes decreased.

In summary, although a large number of gene candidates remain untested, 25 genes involved that endocrine-, amino acid and peptide transporter-, and metabolic enzyme-related were detected in 35-day-old mixed-sex zebrafish during fasting for 384 h. The results suggest that the regulatory mechanism that IGF-I negative feedback regulated GH is conserved in zebrafish, as it is in mammals, but that regulation of GHRs is highly intricate. Ghrelin may not act as a meal-initiated signal, NPY may be an important neuropeptide for integrating stimulation of food

intake, and CCK negative feedback appears to regulate food intake when fasting for short periods of time in the zebrafish. The mRNA expressions of amino acid and peptide transporters and metabolic enzymes observed in this study suggest that protein may be utilized first as an energy source during 24–96 h fasting in zebrafish.

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