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Research Article

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Neuropeptide Y receptor Y8b (*npy8br*) regulates feeding and digestion in Japanese medaka (*Oryzias latipes*) larvae: evidence from gene knockout

Xiaodan JIA[1,2*], Ke LU[1,2*], Xufang LIANG[1,2✉]

[1]*College of Fisheries, Chinese Perch Research Center, Huazhong Agricultural University, Wuhan 430070, China*
[2]*Engineering Research Center of Green Development for Conventional Aquatic Biological Industry in the Yangtze River Economic Belt, Ministry of Education, Wuhan 430070, China*

Abstract: Neuropeptide Y receptor Y8 (NPY8R) is a fish-specific receptor with two subtypes, NPY8AR and NPY8BR. Changes in expression levels during physiological processes or in vivo regulation after ventricular injection suggest that NPY8BR plays an important role in feeding regulation; this has been found in only a few fish, at present. In order to better understand the physiological function of *npy8br*, especially in digestion, we used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology to generate *npy8br*$^{-/-}$ Japanese medaka (*Oryzias latipes*). We found that the deletion of *npy8br* in medaka larvae affected their feeding and digestion ability, ultimately affecting their growth. Specifically, *npy8br* deficiency in medaka larvae resulted in decreased feed intake and decreased expression levels of orexigenic genes (*npy* and *agrp*). *npy8br*$^{-/-}$ medaka larvae fed for 10 d (10th day of feeding) still had incompletely digested brine shrimp (*Artemia nauplii*) in the digestive tract 8 h after feeding, the messenger RNA (mRNA) expression levels of digestion-related genes (*amy*, *lpl*, *ctra*, and *ctrb*) were significantly decreased, and the activity of amylase, trypsin, and lipase also significantly decreased. The deletion of *npy8br* in medaka larvae inhibited the growth and significantly decreased the expression of growth-related genes (*gh* and *igf1*). Hematoxylin and eosin (H&E) sections of intestinal tissue showed that *npy8br*$^{-/-}$ medaka larvae had damaged intestine, thinned intestinal wall, and shortened intestinal villi. So far, this is the first *npy8br* gene knockout model established in fish and the first demonstration that *npy8br* plays an important role in digestion.

Key words: Neuropeptide Y receptor Y8b (*npy8br*); Japanese medaka (*Oryzias latipes*); Knockout; Feeding; Digestion

1 Introduction

The main activities of fish larvae are feeding and avoiding being eaten, so their sensory organs, feeding organs, digestive organs, and motility are of primary importance (Rønnestad et al., 2013). Appetite and feeding behaviors involve complex regulation between the brain and the central and peripheral nervous systems (Kulczykowska and Sánchez Vázquez, 2010; Assan et al., 2021). At present, a variety of appetite regulators have been identified in fish, including agouti-related protein (*agrp*) (Song et al., 2003; Wan et al., 2012), cholecystokinin (*cck*) (Volkoff, 2006), cocaine and amphetamine-regulated transcript (*cart*) (Wan et al., 2012), pro-opiomelanocorticoid (*pomc*) (Volkoff and Peter, 2006), neuropeptide Y (*npy*) (Assan et al., 2021), and melanocortin 4 receptor (*mc4r*) (Wan et al., 2012). The stomach and intestine are two important digestive organs in fish, which can produce proteolytic enzymes, carbohydrate hydrolases, lipase, and other digestive enzymes. Because pepsin is present in the functional stomach, which is not fully developed in the larval stage of gastric fish, pepsin has a limited effect on protein digestion in the early larval stage (Galaviz et al., 2012; Salze et al., 2012; Mir et al., 2018). Therefore, proteolytic enzymes from the pancreas and gut, including trypsin and chymotrypsin, are essential for agastric fish and fish larvae before the development of a functional stomach (Sveinsdóttir et al., 2006; Cara et al., 2007; Rønnestad

✉ Xufang LIANG, xufang_liang@hotmail.com
* The two authors contributed equally to this work
Xufang LIANG, https://orcid.org/0000-0002-6010-3066

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et al., 2013; Solovyev et al., 2023). Amylase, especially α-amylase, is widely distributed in fish and is mainly responsible for the hydrolysis of α-1,4-glycosidic bonds in starch and glycogen (Douglas et al., 2000). Studies have shown that the amylase activity of omnivorous fish is higher than that of carnivorous fish (Al-Tameemi et al., 2010; Solovyev et al., 2014). Lipoprotein lipase is a member of the lipase family and is a key enzyme involved in lipid and lipoprotein metabolism (Hide et al., 1992; Wong and Schotz, 2002; Feng and Liang, 2022).

NPY is composed of 36 highly conserved amino acids and forms the NPY family, together with peptide YY and pancreatic peptide (PP) (Cerdá-Reverter et al., 2000; Conlon, 2002; Liang et al., 2007). Over the past few decades, much research has been conducted on NPY and its receptors. NPY is widely distributed throughout the brain and is mediated by different NPY receptors, thereby regulating various physiological functions such as feeding (Kamijo et al., 2011; Zhou et al., 2013; Loh et al., 2015), anxiety (Tschenett et al., 2003; Shiozaki et al., 2020), and circadian rhythm (Huhman et al., 1996; Harrington et al., 2007; Singh et al., 2017). Five receptor subtypes—NPY receptor Y1 (NPY1R), NPY2R, NPY4R, NPY5R, and NPY6R—have been identified in mammals (Wraith et al., 2000). *npy3r* was first proposed by pharmacology-related studies of human and mouse tissues but has not been cloned or identified in any vertebrate (Larhammar et al., 2001; Pedragosa-Badia et al., 2013). In addition to the five known receptors, *npy7r* and *npy8br* were also identified in teleost fishes, while *npy7r* may have been lost in mammals (Larhammar and Salaneck, 2004; Zhou et al., 2013). In particular, *npy8r* has two subtypes, namely *npy8ar* and *npy8br*, which have been reported in some fish (Wang et al., 2014; Zhang et al., 2021). It has been reported that *npy8br* messenger RNA (mRNA) expression levels were significantly increased after lateral ventricular injection of NPY in grass carp, suggesting that *npy8br* may play an important role in feeding regulation (Zhou et al., 2013). A cloning analysis of orange-spotted grouper (*Epinephelus coioides*) showed that *npy8br* may be involved in the brain–gut axis regulation of feeding, while *npy8ar* is not involved (Wang et al., 2014). Similarly, the injection of small interfering RNA (siRNA)-*npy8ar* and siRNA-*npy8br* into the ventricles of mandarin fish (*Siniperca chuatsi*) revealed that *npy8ar* could not play a role in the regulation of food intake, while *npy8br* was a negative feedback factor in the regulation of appetite (Zhang et al., 2021).

Current studies on *npy8br* are based on changes in expression levels during physiological processes or in vivo regulation after ventricular injection. In order to better understand the function of *npy8br* in appetite regulation and discover more potential roles in the body, knockout experiments on this gene are needed. Japanese medaka (*Oryzias latipes*) is an important model organism that has the advantages of transparent embryos, fast growth rate, and easy cultivation. In this study, we used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology to knockout the *npy8br* gene in Japanese medaka to further explore the physiological function of the *npy8br* gene.

2 Materials and methods

2.1 Animals

Wild type (WT) Japanese medaka HdrR (orange-red strain) were used in this study to generate *npy8br* mutants. Medaka were cultured in the circulating water system of the Mandarin Fish Research Center of Huazhong Agricultural University (Wuhan, China). All fish were placed in a standard light cycle of 26 °C, 14 h/10 h light/dark. The medaka larvae were fed with brine shrimp (*Artemia nauplii*) twice a day at 9:00 am and 6:00 pm.

2.2 Sequence analysis of *npy8br*

The National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) was used to search NPY8BR amino acid sequences of Japanese medaka (*O. latipes*), Mandarin fish (*S. chuatsi*), largemouth bass (*Micropterus salmoides*), Nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), Japanese flounder (*Paralichthys olivaceus*), barramundi perch (*Lates calcarifer*), torafugu (*Takifugu rubripes*), grass carp (*Ctenopharyngodon idella*), European seabass (*Dicentrarchus labrax*), European eel (*Anguilla anguilla*), and goldfish (*Carassius auratus*). Multiple amino acid alignments of NPY8BR among medaka and other fish species were performed, and then the protein genealogies were assessed by the neighbor-joining method using Mega 11 (Auckland, New Zealand). The

reliability of the analysis was assessed by 1000 bootstrap replicates.

2.3 Tissue expression analysis of *npy8br* gene in medaka

Adult WT medaka fish were randomly selected, and brain, gill, heart, eye, kidney, liver, gonad, and intestinal tissues were dissected after anesthesia with tricaine methanesulfonate (MS-222, Aladdin, China). The tissues were immediately placed in liquid nitrogen and then stored in a −80 °C refrigerator for subsequent tissue expression analysis.

2.4 Generating *npy8br*$^{-/-}$ mutants by CRISPR/Cas9 technology

The single-guide RNAs (sgRNAs) of the medaka *npy8br* (ENSORLG00000014260) gene were designed using the Consensus Constrained TOPology prediction (CCTOP) server (https://cctop.cos.uni-heidelberg.de). The sequences of sgRNAs and polymerase chain reaction (PCR) primers are shown in Table 1. sgRNAs were cloned into pMD-19T vector and were synthesized using the Transcript Aid T7 High Yield Transcription kit (Thermo Fisher Scientific, USA). The compounds of sgRNAs (50 ng/μL) and Cas9 protein (NEB, USA) were co-injected into one- or two-cell stage WT embryos. The F0 medaka were outcrossed with WT to generate F1 medaka. The F1 heterozygous fish with the same mutation were incrossed to obtain F2 homozygous fish, and all experiments were conducted with F3 homozygous specimens. All experiments were performed using the *npy8br* Δ695 mutants, named *npy8br*$^{-/-}$.

Table 1 Primers used for clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)

Gene	Sequence (5'→3')
npy8br guide RNA1	CCTATGGAGAGAGATACCTTGGG
npy8br guide RNA2	AGGTAAGCACGCTGTACGAGAGG
npy8br test-F	ATCATGGAGCCACAGCACAA
npy8br test-R	GTCATGGCCACAGGTTGGAA

2.5 *npy8br*$^{-/-}$ medaka larvae growth and digestive capacity

Both WT and *npy8br*$^{-/-}$ medaka began feeding at 6 d post-hatching (dph), and the growth and digestion data were collected until the 15th day of feeding (15 d). The 1 d (1st day of feeding), 5 d (5th day of feeding), 10 d (10th day of feeding), and 15 d WT and *npy8br*$^{-/-}$ medaka were randomly selected and fixed with fixative solution for 24 h. Then, pictures were taken with a stereo microscope (SZX10, Olympus, Japan) and the total length of the medaka larvae (n=30) was measured and counted using Image Analysis software. The WT and *npy8br*$^{-/-}$ medaka fed for 1, 5, and 10 d were randomly selected and lightly anesthetized with MS-222 after 1 h of feeding. The larvae were photographed immediately and their food intake was recorded; they were then quickly transferred to fresh water to resume normal swimming. The digestibility of the larval medaka (n=30) was then evaluated by photographing them after 4 h and 8 h of feeding.

2.6 *npy8br*$^{-/-}$ medaka larvae gene expression

Medaka larvae at 15 d were randomly selected and fed for 2 h. Six fish were mixed in each tube and placed in liquid nitrogen, and were then stored in a −80 °C refrigerator. Total RNA was extracted by TRIzol Reagent (TaKaRa Japan), and then 1 μg RNA was reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (Vazyme, China). Subsequently, the expression level of each gene was determined using SYBR (Vazyme, China) and cDNA as a template with gene-specific primers, according to the manufacturer's instructions (Table 2) (Chisada et al., 2014; Feng and Liang, 2022). The mRNA expression levels of the target genes were quantified relative to the expression of *β-actin* using the optimized comparative C_T ($2^{-\Delta\Delta C_T}$) value method (Livak and Schmittgen, 2001).

2.7 Digestive enzyme analysis

Fifteen medaka larvae were mixed into a tube, according to the ratio of sample weight/volume= 1 g/9 mL, adding nine times the volume of normal saline. After mechanical homogenization under ice water bath conditions, the mixture was centrifuged at 4 °C for 10 min. The supernatant was taken to measure the activity of protein concentration (catalog No. A054-2, Nanjing Jiancheng, China), amylase (catalog No. DFMA-1-Y, Suzhou Comin, China), trypsin (catalog No. YPT-1-W, Suzhou Comin), lipase (catalog No. A054-2-1, Nanjing Jiancheng), aspartate aminotransferase (AST) (catalog No. C010-2-1, Nanjing Jiancheng), and alanine aminotransferase (ALT) (catalog

Table 2 Primer sequences for the quantitative real-time polymerase chain reaction (PCR)

Gene	Gene ID	Sequence of primer (5'→3')	Length (bp)
β-actin	100049433	F: TTTATGCCAGCAACGACT R: CGACGAAAGCCTACTCCC	129
npy8br	101167799	F: CGACTGGAACCACGATGTGA R: TCGATCCCTTGGTGACCTCT	233
amy	101163630	F: ATCTCGGAGGTGAGCCCATT R: CGTCAGCCATGAATCCCCAA	162
lpl	101160309	F: TCCACCTGTTCATCGACT R: AGCTTGTTGCAGCGGTTC	124
ctra	101170410	F: ACCGGTTACTCTCGGATTGTG R: GTTGGAGTTGCGGTCATGTTC	350
ctrb	105356289	F: GAACACAACAAGGGCGCTG R: CCAGAGGTGACGCAGGTTGA	298
gh	101171460	F: AGAGCAGATCCATCAGAGCCA R: TCAGAGAAAAGTCTCTGGGCG	188
igf1	101168167	F: CCCCAGCTGTTTCCTGTTGAA R: CAGCAGTAGTGAGAGGGTGT	286
agrp	101171147	F: GCATCCCTCACCAGCAGTC R: GCCTATTTGGCGGCAGTAAC	110
cck	101165324	F: TCCTTCTGAAGTTGCTCTT R: CCGTGAATCTCCATCCTC	124
pomc	101168338	F: TTGCTGGCTGTTGGTGGTTCT R: AGGTCTGGGCTTTCAGGTTTGA	147
npy	100144351	F: GCCTTGGAGCCTTAACAGAGG R: TCTCAGGACTGGACCTCTTCC	153

F: forward; R: reverse; *npy8br*: neuropeptide Y receptor Y8b; *amy*: amylase; *lpl*: lipoprotein lipase; *ctra*: chymotrypsin A; *ctrb:* chymotrypsin B; *gh*: growth hormone; *igf1*: insulin-like growth factor 1; *agrp*: agouti-related protein; *cck*: cholecystokinin; *pomc*: pro-opiomelanocortin; *npy*: neuropeptide Y.

No. C009-2-1, Nanjing Jiancheng), according to the manufacturer's instructions.

2.8 Intestinal histological analysis

The medaka larvae fed for 15 d were randomly selected and fixed in paraformaldehyde for 24 h. The larvae were dehydrated and embedded in paraffin. Paraffin sections were made using a microtome and were stained with hematoxylin and eosin (H&E). The sections were observed using an optical microscope (MshOt, China) and the intestinal wall thickness and villi height were measured using Case Viewer software (Servicebio, China).

2.9 Statistical analysis

SPSS 25.0 software was used for the statistical analysis. All data were expressed as mean±standard error of the mean (SEM) and analyzed with an independent-samples *t*-test. GraphPad Prism 8.0.2 was used to create a data analysis diagram. $P<0.05$ was considered statistically significant.

3 Results

3.1 Biological evolutionary analysis

Fish NPY8BR protein genealogies were assessed by the neighbor-joining method using Mega 11. A biological evolutionary analysis showed that the Japanese medaka, Nile tilapia, and other Perciformes clustered together; the other cyprinids (zebrafish, grass carp, goldfish) were sorted into another group (Fig. 1).

3.2 Tissue expression analysis of *npy8br* in medaka

The differential expression of the medaka *npy8br* gene in different tissues was determined by quantitative real-time reverse transcription PCR (qRT-PCR) (Fig. 2). The *npy8br* mRNA was primarily expressed

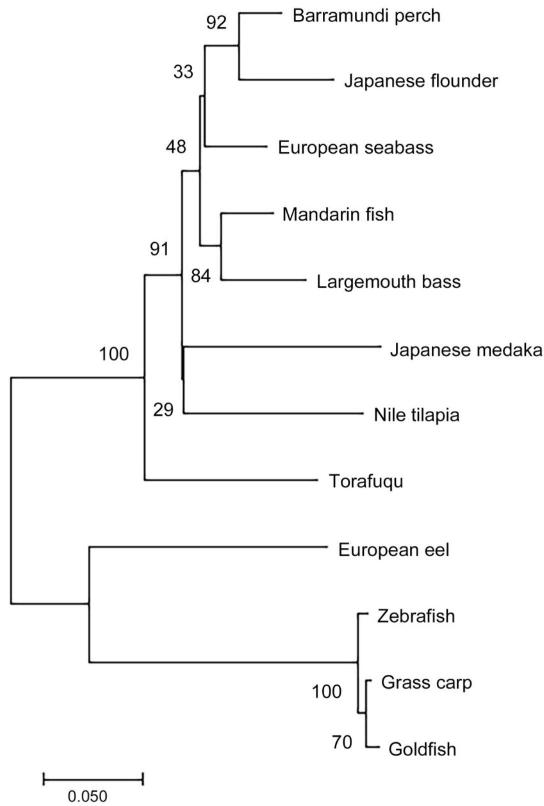

Fig. 1 Biological evolutionary analysis of neuropeptide Y receptor Y8b (NPY8BR). The tree was constructed by neighbor-joining method with the Mega 11 software. Selected bootstrap values from 1000 traditional trees are shown in each internal branch of the phylogenetic tree nodes. The sequences are Japanese medaka (XP_020561804.1), mandarin fish (XP_044053231.1), largemouth bass (XP_038560701.1), Nile tilapia (XP_025752935.1), zebrafish (NP_571511.1), Japanese flounder (XP_019949193.1), barramundi perch (XP_018523805.1), torafugu (NP_001098074.1), grass carp (XP_051760258.1), European seabass (XP_051279398.1), European eel (XP_035291642.1), and goldfish (XP_026124554.1).

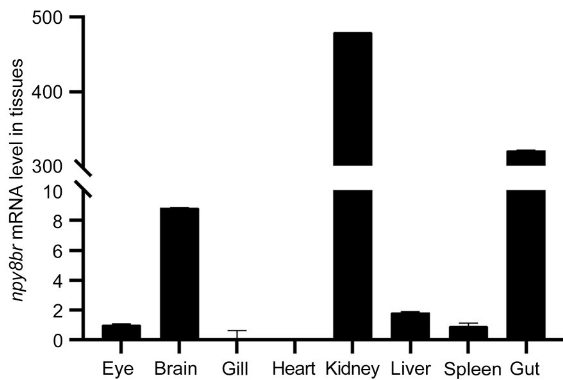

Fig. 2 Tissue expression analysis of neuropeptide Y receptor Y8b (*npy8br*) in medaka. The results are expressed as mean±standard error of the mean (SEM) (*n*=6).

in the kidney and gut, and low mRNA expression levels were detected in the brain, liver, eye, spleen, gill, and heart tissues.

3.3 Generation of the *npy8br* mutant medaka

To understand the role of *npy8br* in digestive function, we used the CRISPR/Cas9 technique to generate two *npy8br* mutants. The *npy8br* mutant lines with 695-bp deletion (*npy8br* Δ695) and 469-bp deletion (*npy8br* Δ469) were obtained (Figs. 3a and 3b). The deletions led to a frameshift and premature stop codon in the 7-tm_1 domain (Fig. 3c). The mRNA level of *npy8br* was significantly reduced in *npy8br*$^{-/-}$ medaka (Fig. 3d). No obvious developmental defects were observed between WT and *npy8br*$^{-/-}$ medaka.

3.4 Effects of *npy8br* knockout on feed intake and digestion of medaka larvae

The feeding and digestive capacities of WT and *npy8br*$^{-/-}$ medaka larvae were assessed by taking photographs of food intake at 1 h after feeding from 6 dph and digestibility at 4 h and 8 h after feeding. The results showed that the food intake of *npy8br*$^{-/-}$ medaka larvae was lower than that of WT on the first day of feeding, and there were significant differences between WT and *npy8br*$^{-/-}$ medaka larvae fed for 1, 5, 10, and 15 d (Fig. 4a). The photographic statistics showed that there was incomplete digestion of brine shrimp in the digestive tracts of WT and *npy8br*$^{-/-}$ medaka larvae at 1 and 5 d after feeding for 4 and 8 h (Figs. 4b and 4c). At 10 d, incomplete digestion of brine shrimp could still be observed in the intestinal tract of *npy8br*$^{-/-}$ medaka larvae after 8 h of feeding (Fig. 4d).

3.5 Effects of *npy8br* knockout on the growth of medaka larvae

Medaka larvae began feeding at 6 dph, and the growth data at 1, 5, 10, and 15 d were collected (Fig. 5). The results showed that there was reduced growth compared to WT within 5–15 d of feeding.

3.6 Effects of *npy8br* knockout on mRNA expression of medaka larvae

Based on the results of growth, feeding, and digestion, we explored whether *npy8br* knockout would affect the expression pattern of appetite genes (*agrp*, *cck*, *pomc*, and *npy*), digestive genes (*amy*, *lpl*, *ctra*,

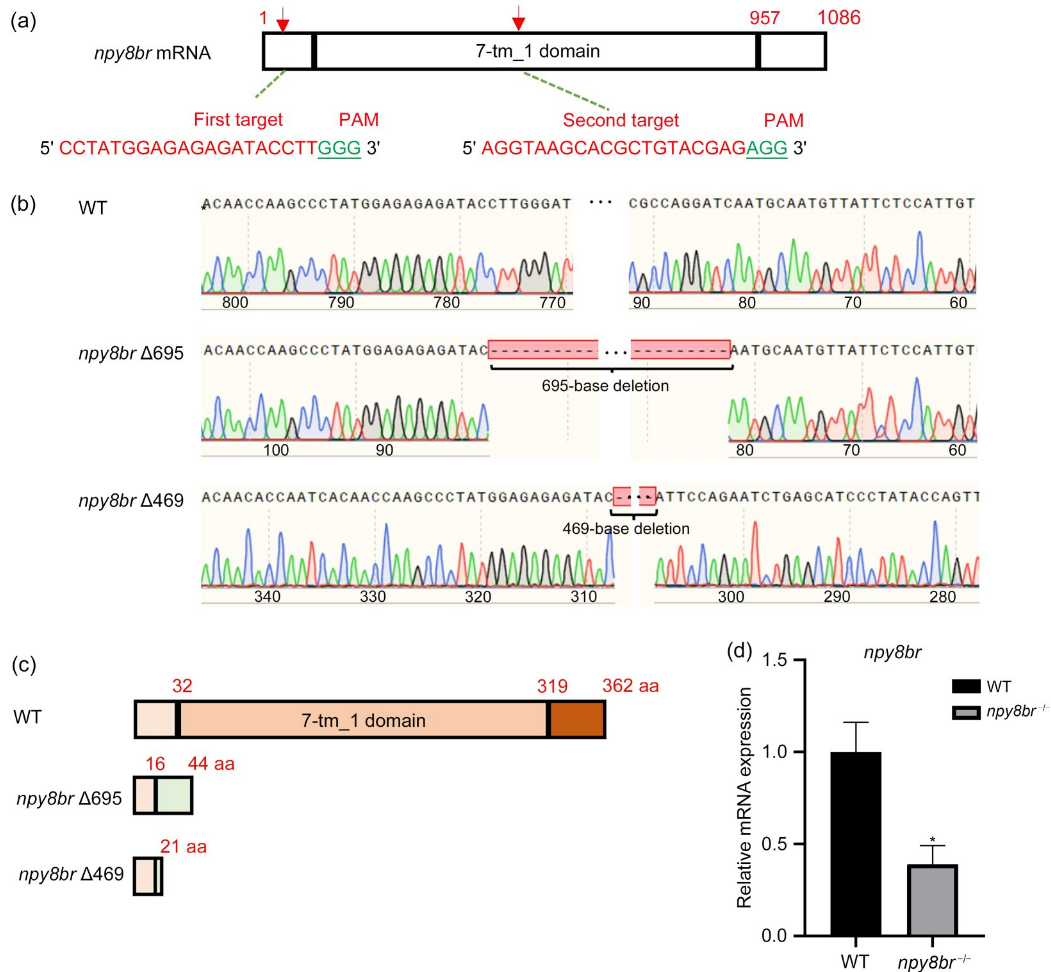

Fig. 3 Establishment of neuropeptide Y receptor Y8b (*npy8br*)-knockout medaka. (a) *npy8br* gene and target sites of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). The numbers indicate the base positions starting at the adenine nucleotide of the initiation codon. The red arrows indicate the location of the single-guide RNA (sgRNA), and the blue arrows indicate the primer location to detect the *npy8br* mutation. (b) DNA sequencing showing the *npy8br* mutant lines (*npy8br* Δ695 and *npy8br* Δ469). (c) Comparison of *npy8br* Δ695 and *npy8br* Δ469 mutants with wild-type (WT) NPY8BR protein structure. (d) Messenger RNA (mRNA) expression level of *npy8br*$^{-/-}$ medaka. The results are expressed as mean±standard error of the mean (SEM) (*n*=6). * *P*<0.05, representing that significant differences exist between WT and *npy8br*$^{-/-}$ medaka.

and *ctrb*), and growth hormone (GH)/insulin-like growth factor (IGF) growth axis-related genes (*gh* and *igf1*). Firstly, we detected the expression levels of appetite genes. We found that there was no difference in the expression levels of anorexia factors (*cck* and *pomc*) between WT and *npy8br*$^{-/-}$ medaka larvae (Figs. 6b and 6c), while *npy8br* knockout medaka showed a significant decrease in the expression levels of appetitive genes (*agrp* and *npy*) (Figs. 6a and 6d). The mRNA levels of digestive enzyme genes (*amy*, *lpl*, *ctra*, and *ctrb*) were significantly reduced in *npy8br*$^{-/-}$ medaka larvae (Figs. 6e–6h). Finally, the qRT-PCR analyses showed that the transcriptional levels of *gh* and *igf1* genes were significantly reduced in *npy8br*$^{-/-}$ medaka larvae (Figs. 6i and 6j).

3.7 Effects of *npy8br* knockout on digestive enzyme activity of medaka larvae

The effects of *npy8br* gene knockout medaka larvae on digestive enzyme activity are shown in Fig. 7. Compared with WT, *npy8br* knockout medaka larvae significantly reduced the activity of α-amylase, trypsin, and lipase (Figs. 7a–7c). However, AST or ALT activity was not significantly different between WT and *npy8br*$^{-/-}$ medaka larvae (Figs. 7d and 7e).

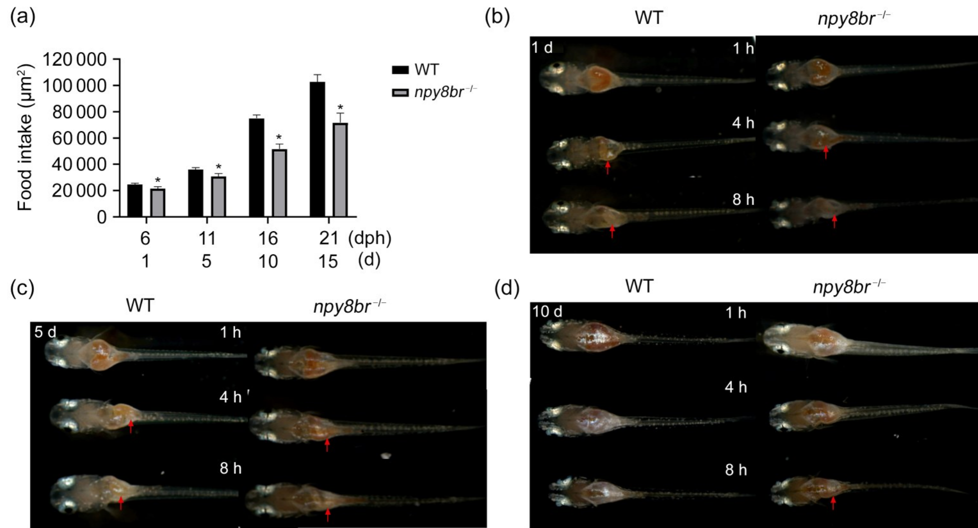

Fig. 4 Feeding and digestion of neuropeptide Y receptor Y8b (*npy8br*) knockout medaka larvae. (a) Food intake statistics for 15 d of feeding. (b) Digestion of medaka larvae at 1 d. (c) Digestion of medaka larvae at 5 d. (d) Digestion of medaka larvae at 10 d. The red arrows indicate the incomplete digestion of brine shrimp. The results are expressed as mean±standard error of the mean (SEM) (*n*=30). * ***P*<0.05, representing that significant differences exist between wild type (WT) and *npy8br*$^{-/-}$ medaka larvae. dph: days post-hatching.**

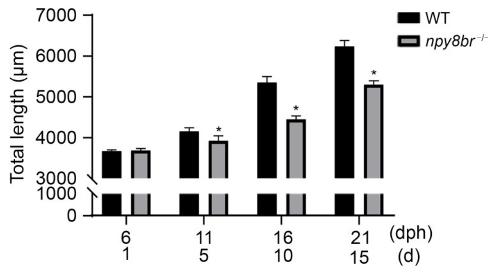

Fig. 5 Growth of neuropeptide Y receptor Y8b (*npy8br*) knockout medaka larvae. The growth data from 1 to 15 d of feeding were counted. The results are expressed as mean±standard error of the mean (SEM) (*n*=30). * ***P*<0.05, representing that significant differences exist between wild type (WT) and *npy8br*$^{-/-}$ medaka larvae. dph: days post-hatching.**

3.8 Effects of *npy8br* knockout on intestinal tissue of medaka larvae

The observation of the intestinal tissues of the medaka larvae showed that the intestinal tissues of *npy8br*$^{-/-}$ medaka were damaged, with thinner intestinal walls, shorter intestinal villi, and incomplete intestinal villi compared with WT (Fig. 8).

4 Discussion

The NPY receptor family underwent two whole-genome duplications early in vertebrate evolution, resulting in seven receptor subtypes, named NPY1R, NPY2R, NPY4R, NPY5R, NPY6R, NPY7R, and NPY8R. In particular, NPY8R is divided into two subtypes, NPY8AR and NPY8BR (Larsson et al., 2009; Sundström et al., 2013; Xu et al., 2015). In orange-spotted grouper, *npy8br* is expressed not only in the tissues of the central nervous system but also in the eye, heart, thymus, stomach, and intestine of the peripheral system (Wang et al., 2014). In allotetraploid common carp, *npy8br* is expressed in sperm, brain, and gill (Zou et al., 2022). In Japanese medaka, *npy8br* is highly expressed in kidney, gut, and brain, suggesting potential functions of *npy8br* in immunity, appetite regulation, and digestion. It has been shown that *npy8br* plays an important role in regulating food intake in some fish (Sundström et al., 2013; Zhou et al., 2013; Wang et al., 2014; Zhang et al., 2021). In order to further explore the biological function of *npy8br* in teleost fish—especially its role in digestion—we used the CRISPR/Cas9 system to establish two *npy8br* mutants of Japanese medaka; the *npy8br* mutant lines with 695-bp deletion (*npy8br* Δ695) and 495-bp deletion (*npy8br* Δ495) were obtained. The base deletion results in a frameshift and premature stop codon in the 7-tm_1 domain. Meanwhile, the mRNA expression level of *npy8br* was significantly decreased in *npy8br*$^{-/-}$ medaka. No obvious developmental defects were observed between WT and *npy8br*$^{-/-}$ medaka.

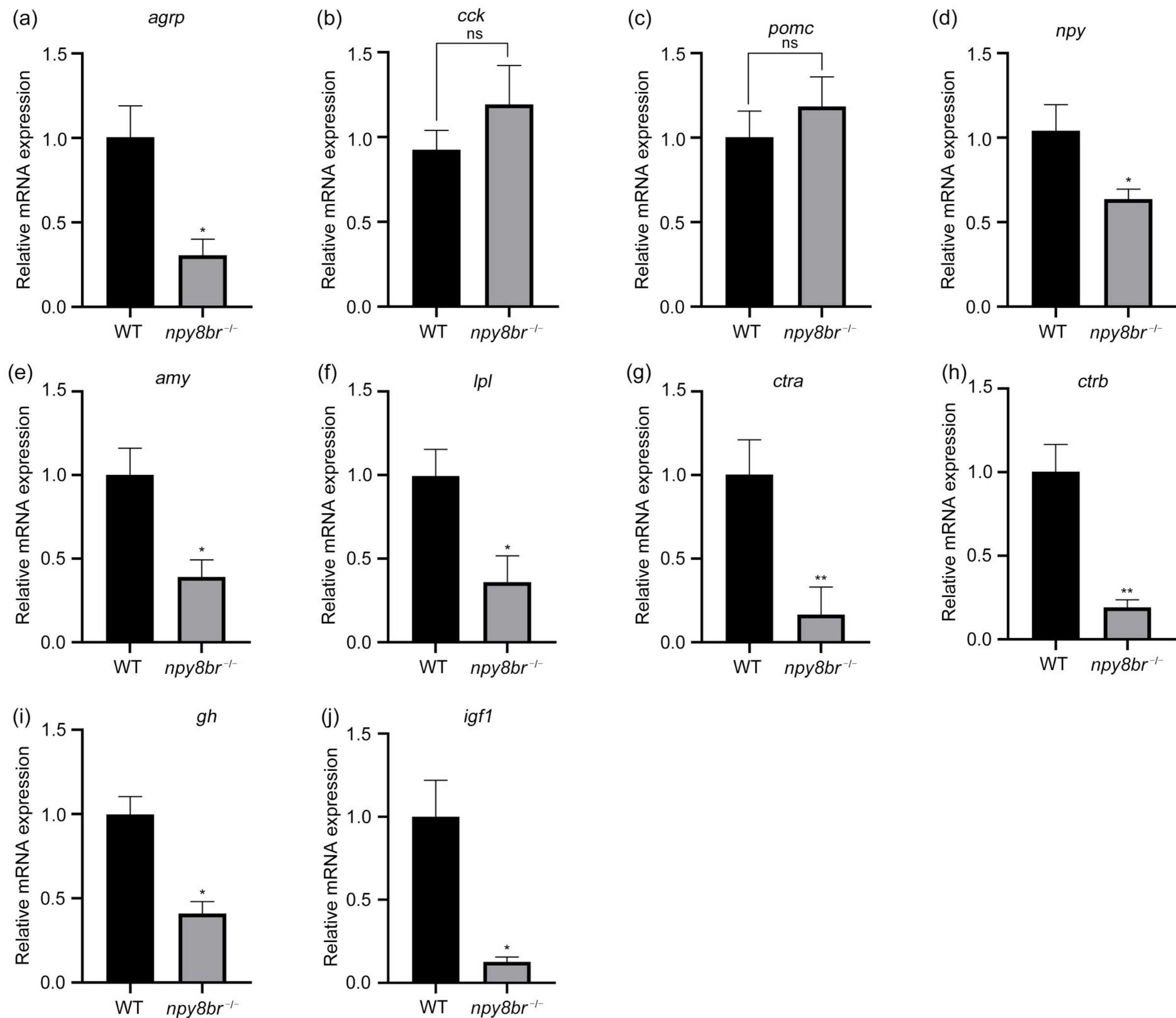

Fig. 6 Gene messenger RNA (mRNA) expression levels. Quantitative analyses of agouti-relate protein (*agrp*) (a), cholecystokinin (*cck*) (b), pro-opiomelanocorticoid (*pomc*) (c), neuropeptide Y (*npy*) (d), amylase (*amy*) (e), lipoprotein lipase (*lpl*) (f), chymotrypsin A (*ctra*) (g), chymotrypsin B (*ctrb*) (h), growth hormone (*gh*) (i), and insulin-like growth factor1 (*igf1*) (j) gene mRNA expression levels. Data were presented as mean±standard error of the mean (SEM) (*n*=6). * *P*<0.05, ** *P*<0.01, vs. WT. ns: not significant; WT: wild type.

These results indicated that the *npy8br* mutant in medaka was established successfully.

In this study, compared with WT, the food intake of *npy8br*$^{-/-}$ medaka larvae on the first day of first feeding was significantly reduced, which suggested that *npy8br* may be involved in regulating the feeding of medaka larvae. Feeding activity involves complex regulation of the central and peripheral nervous systems of the brain (Kulczykowska and Sánchez Vázquez, 2010; Chisada et al., 2014; Assan et al., 2021). We detected the expression levels of orexigenic genes using qRT-PCR, and the results showed that the expression levels of orexigenic genes (*npy* and *agrp*) were significantly decreased, while the expression levels of anorexia factors (*cck* and *pomc*) were not significantly different between WT and *npy8br*$^{-/-}$ medaka. It has been reported that *npy8br* mRNA expression was significantly increased after intracerebroventricular injection of NPY in grass carp (Zhou et al., 2013). Based on the tissue expression and ligand-binding characteristics of *npy8br* in orange-spotted grouper, it is speculated that *npy8br* may be involved in the regulation of food intake through the brain–gut axis (Wang et al., 2014). Our results are consistent with previous reports. Since *npy* is an important factor involved in feeding regulation, *npy8br* may have a higher affinity with *npy* and play a role in feeding regulation.

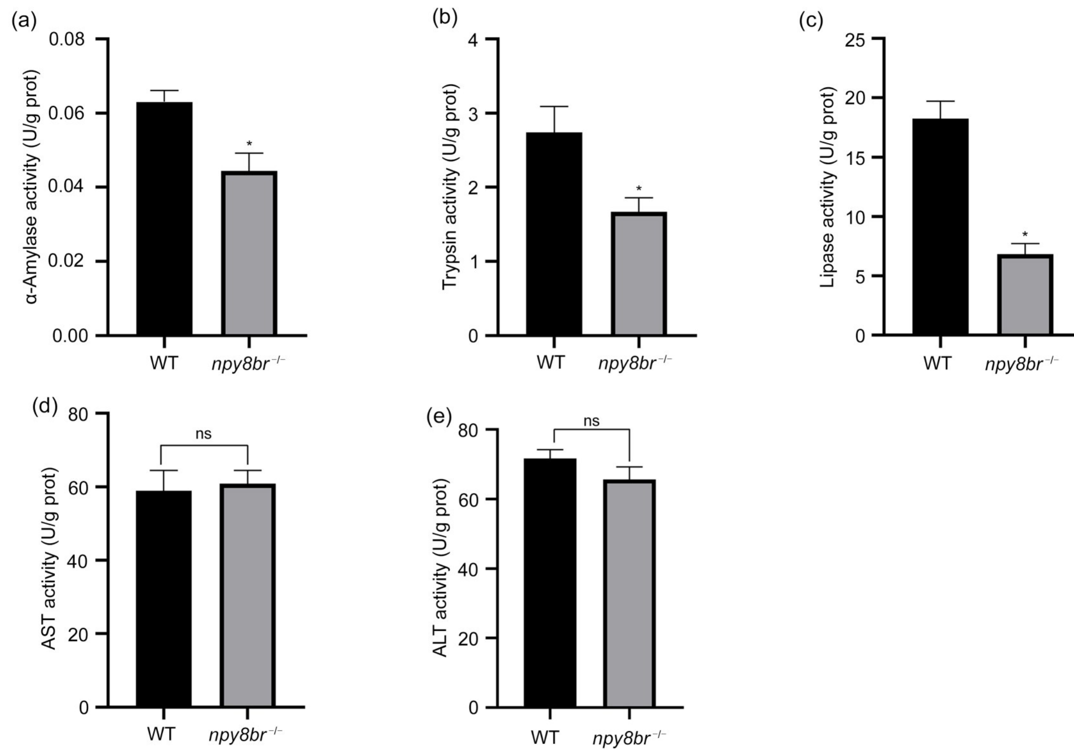

Fig. 7 Digestive enzyme activity of neuropeptide Y receptor Y8b (*npy8br*)$^{-/-}$ medaka larvae: α-amylase (a), trypsin (b), lipase (c), aspartate aminotransferase (AST) (d), and alanine aminotransferase (ALT) (e). Data were presented as mean±standard error of the mean (SEM) (*n*=6). * *P*<0.05, vs. WT. ns: not significant; prot: protein; WT: wild type.

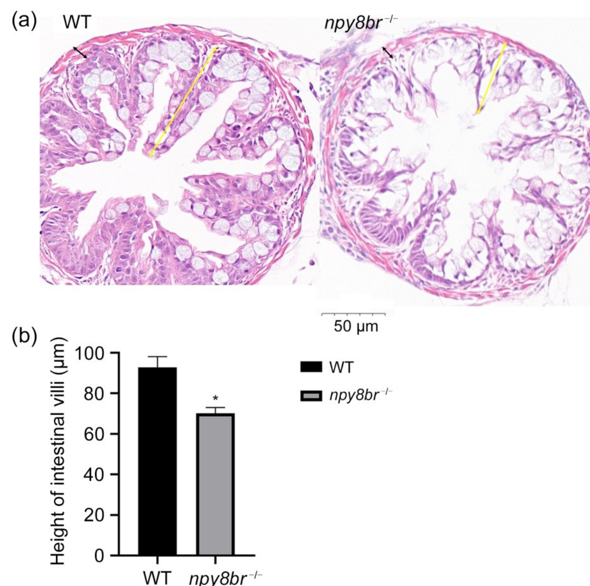

Fig. 8 Intestinal tissue of neuropeptide Y receptor Y8b (*npy8br*)$^{-/-}$ medaka larvae. (a) Hematoxylin and eosin (H&E) sections of the intestine in medaka larvae. (b) The height of the intestinal villi. The black double arrow indicates intestinal wall thickness and the yellow double arrow indicates villi height. Data were presented as mean± standard error of the mean (SEM) (*n*=3). * *P*<0.05, vs. WT (wild type).

Individual growth is the result of the deposition of various nutrients through a series of physiological activities, and growth data are a direct indicator of physiological functions such as the feeding and digestion of fish (Persson and de Roos, 2006). Growth data showed that there was reduced growth in *npy8br*$^{-/-}$ medaka compared to WT within 5–15 d of feeding. Studies have shown that *gh* induces the secretion of *igf1*. The GH/IGF1 axis is considered to be the core of the growth endocrine axis of fish and is involved in their growth and development (Canosa et al., 2007; Tu et al., 2015; Ndandala et al., 2022). In the present study, the expression of *gh* and *igf1* mRNA was significantly lower in *npy8br*$^{-/-}$ medaka larvae than in WT. Therefore, *npy8br* may eventually affect medaka larval growth by affecting the normal function of the GH/IGF1 axis.

After feeding, fish need amylase, protease, and lipase to be present in the digestive tract to break down the food into small molecules that can be absorbed (Rønnestad et al., 2013). In this study, we found that incompletely digested brine shrimp could still be observed in the digestive tract of *npy8br*$^{-/-}$ medaka larvae at 10 d after feeding for 8 h. Therefore, we

speculate that *npy8br* knockout may affect the digestive capacity of medaka larvae. In order to verify our speculation, we first detected the expression levels of *amy*, *lpl*, *ctra*, and *ctrb*, and the results showed that the mRNA expression levels of *amy*, *lpl*, *ctra*, and *ctrb* were significantly lower in *npy8br*$^{-/-}$ medaka larvae than in WT. In addition, the digestive enzyme activity of *npy8br*$^{-/-}$ medaka larvae was tested and the results showed that amylase, trypsin, and lipase activity was significantly decreased, while AST or ALT activity was not significantly different between WT and *npy8br*$^{-/-}$ medaka. Amylase is the main enzyme involved in the digestion of carbohydrates, and the activity of amylase is higher in herbivorous and omnivorous fish than in carnivorous fish (Hidalgo et al., 1999). In this study, *npy8br* knockout significantly reduced *amy* gene expression and the enzyme activity of amylase. Lipids play an important role in fish metabolism, providing energy for growth and development. Lipase and *lpl* are the key enzymes involved in lipid metabolism (Hide et al., 1992; Wong and Schotz, 2002; Feng and Liang, 2022). In this study, *npy8br* knockout medaka larvae significantly reduced the expression of the *lpl* gene and the activity of lipase, suggesting that *npy8br* may play a role in lipid digestion. Trypsin and chymotrypsin are widely present in the hepatopancreas and intestines of fish and play important roles in protein digestion (Zhou et al., 2011). When the gastric glands of fish are not yet formed, the extracellular digestion of food by larvae is mainly dependent on tryptases. Therefore, trypsin and chymotrypsin play important roles in the digestion and absorption of nutrients in fish larvae in the early stages (Sveinsdóttir et al., 2006; Cara et al., 2007; Rønnestad et al., 2013; Solovyev et al., 2023). Our study showed that *npy8br* knockout medaka significantly reduced the protein digestibility of fish larvae. AST and ALT are the two most important aminotransferases involved in amino acid metabolism in fish liver (Zhao et al., 2012). There was no significant difference in AST or ALT activity between WT and *npy8br*$^{-/-}$ medaka larvae in this study, suggesting that *npy8br* may not cause liver damage. Finally, we observed the intestinal tissue and found that the intestinal development of *npy8br*$^{-/-}$ medaka was impaired—the intestinal wall was thinner and the height of the intestinal villi was reduced. The integrity of vertebrate intestinal structure is an important basis for body health and normal development (Zhang et al., 2013; Ullah et al., 2022). Studies have shown that decreased intestinal villus height can affect the digestibility of fish larvae (de Souza et al., 2020). This may be the reason for the decreased activity of digestive enzymes in *npy8br*$^{-/-}$ medaka larvae.

5 Conclusions

In summary, *npy8br* gene mutant lines were successfully established in Japanese medaka. We found that *npy8br* deficiency can affect the feeding and digestive abilities of medaka larvae. So far, this is the first *npy8br* gene knockout model established in fish and the first demonstration that *npy8br* plays an important role in digestion.

Data availability statement

All data generated or analyzed during this study can be made available by the corresponding author upon reasonable request.

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Author contributions

Xiaodan JIA designed and performed the experiment, analyzed experimental data, and wrote the manuscript. Ke LU performed the establishment of animal models, designed and performed the experiment, and edited the manuscript. Xufang LIANG designed the experiment and checked the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Xiaodan JIA, Ke LU, and Xufang LIANG declare that they have no conflicts of interest.

All experimental procedures involving chickens were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (approval number: HZAUFI-2020-0028).

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