1 **Duplication of Neuropeptide Y and Peptide YY in Nile tilapia**
2 ***Oreochromis niloticus* and their roles in food intake regulation**

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Abstract

In vertebrates, the neuropeptide Y (NPY) family peptides have been recognized as key players in food intake regulation. NPY centrally promotes feeding, while peptide YY (PYY) and pancreatic polypeptide (PP) mediate satiety. The teleost tetraploidization is well-known to generate duplicates of both NPY and PYY; however, the functional diversification between the duplicate genes, especially in the regulation of food intake, remains unknown. In this study, we identified the two duplicates of NPY and PYY in Nile tilapia (*Oreochromis niloticus*). Both *NPYa* and *NPYb* were primarily expressed in the central nervous system (CNS), but the mRNA levels of *NPYb* were markedly lower than those of *NPYa*. Hypothalamic mRNA expression of *NPYa*, but not *NPYb*, decreased after feeding and increased after 7-days of fasting. However, both *NPYa* and *NPYb* caused a significant increase in food intake after an intracranial injection of 50 ng/g body weight dose. *PYYb*, one of the duplicates of PYY, had an extremely high expression in the foregut and midgut, whereas another form of duplicate *PYYa* showed only moderate expression in the CNS. Both hypothalamic *PYYa* and foregut *PYYb* mRNA expression increased after feeding and decreased after 7-days of fasting. Furthermore, the intracranial injection of *PYYb* decreased food intake, but *PYYa* had no significant effect. Our results suggested that although the mature peptides of *NPYa* and *NPYb* can both stimulate food intake, *NPYa* is the main endogenous functional NPY for feeding regulation. A functional division has been identified in the duplicates of PYY, which deems *PYYb* as a gut-derived anorexigenic peptide and *PYYa* as a CNS-specific PYY in Nile tilapia.

Key words

Neuropeptide Y; Peptide YY; Food intake; Nile tilapia

1. Introduction

Feeding regulation is a complex phenomenon involving elaborate interactions between the brain and peripheral signals. The NPY family peptides, which comprise NPY from the central nervous system, and the gut-derived hormones PYY and PP, are

crucial in this aspect. NPY is a 36-amino acid peptide widely distributed in the central and peripheral nervous system and is known for its orexigenic effect. In rodents, a central injection of NPY stimulates feeding behavior, food intake, and body weight gain (Clark et al., 1984; Stanley et al., 1986) and the effects are mediated by Y1 and Y5 receptors (Kanatani et al., 2000; Nguyen et al., 2012; Schaffhauser et al., 1997). PYY, which has a structure similar to that of NPY, is predominantly produced in the intestine L-cells in response to food ingestion (Adrian et al., 1985) and is best known as an anorectic peptide (Batterham et al., 2002; Persaud and Bewick, 2014). In mammals, PYY₁₋₃₆ is a substrate for the dipeptidyl peptidase 4 (DPP-4), which cleaves the N-terminal dipeptide to produce PYY₃₋₃₆, which is the major circulating form of PYY (Grandt et al., 1994). Peripheral administration of both PYY₁₋₃₆ and PYY₃₋₃₆ reduce food intake and weight gain in rodents (Chelikani et al., 2007; Unniappan et al., 2006), and the effects are mediated by the Y2 receptor (Abbott et al., 2005). In addition to inhibiting food intake, PYY also inhibits gut motility and secretion (Cox, 2007). PP is produced in pancreatic PP cells (Ekblad and Sundler, 2002), and its secretion is stimulated postprandially in proportion to caloric load (Katsuura et al., 2002). PP acts primarily on peripheral and central Y4 and Y5 receptors to influence biliary and exocrine pancreatic function, gastric acid secretion, and gastrointestinal motility (Cummings and Overduin, 2007).

The NPY family is more complex in teleost fishes than in mammals. It is proposed that the evolution of NPY family peptides involves the duplication of a single ancestral gene in an early vertebrate, before the origin of gnathostomes, resulting in NPY and PYY (Larhammar, 1996). The third round of tetraploidization (3R) in the ray-finned fishes (*Actinopterygii*) results in the duplicates of NPY and PYY; therefore, four members of NPY family peptides exist in this lineage, namely, NPYa, NPYb, PYYa, and PYYb (Sundstrom et al., 2008). Moreover, differential losses have occurred in teleost fishes such that zebrafish (*Danio rerio*) seems to lack NPYb and medaka (*Oryzias latipes*) lacks PYYb (Sundstrom et al., 2005; Sundstrom et al., 2008). A recent study indicated that PP arose before the divergence of lobe-finned fishes (*Sarcopterygii*) and ray-finned fishes, and has been lost in the

ray-finned fishes lineage (Larhammar and Bergqvist, 2013). In teleosts, *NPYa* is predominantly expressed in the brain (Kehoe and Volkoff, 2007; Kurokawa and Suzuki, 2002; Murashita et al., 2009a; Zhou et al., 2013), and the synthetic mature peptide of *NPYa* can increase food intake by central or peripheral administration (Aldegunde and Mancebo, 2006; Kiris et al., 2007; Narnaware et al., 2000; Yokobori et al., 2012). In addition, fasting increases *NPYa* expression (Narnaware et al., 2000; Yokobori et al., 2012), while feeding decreases its expression (Narnaware et al., 2000) in the hypothalamus. On the other side, teleost *PYYb* is mainly expressed in the gut, especially in foregut (Chen et al., 2013a; Kurokawa and Suzuki, 2002; Murashita et al., 2006), whereas *PYYa* is mostly expressed in the brain (Chen et al., 2013b; Gonzalez and Unniappan, 2010). Recent studies in goldfish (*Carassius auratus*) and grass carp (*Ctenopharyngodon idellus*) have provided evidence for the anorexigenic actions of *PYYa* in teleosts. In these studies, brain *PYYa* expression was increased after feeding, and both central and peripheral administration of the synthetic mature peptide of *PYYa* reduced food intake (Chen et al., 2013b; Gonzalez and Unniappan, 2010). However, little is known about the effects of *NPYb* and *PYYb* on food intake in teleost fishes.

Gene duplication may lead to nonfunctionalization, subfunctionalization or neofunctionalization (Xue and Fu, 2009). Whether the duplications of *NPY* and *PYY* in teleost fishes have resulted in such division needs to be research. We present here the identification of *NPYa*, *NPYb*, *PYYa*, and *PYYb* in Nile tilapia *Oreochromis niloticus*, a commercially significant fish species worldwide. We also discuss their tissue distributions, differential expression patterns after feeding and fasting, and direct effects on food intake while emphasizing the differential or coordinating roles of the duplicates of *NPY* and *PYY* on food intake regulation in teleosts.

2. Materials and methods

2.1. Animals

Juvenile male Nile tilapia with a body weight (BW) range of 25-35 g were

purchased from the tilapia breeding farm of Guangdong (Panyu, Guangdong, China). The fish were cultured in 180-L tanks at a density of 12 fish per tank at 27-30 °C under a 12 h: 12 h light regime. The fish were fed once daily at 8:30 am every morning with commercial extruded feed (Tongwei, Foshan, China) until satiety. The body weight of the fish and the feeding conditions in the present study were the same as mentioned above unless otherwise stated. All fish were acclimatized to the aquarium conditions for at least 2 weeks before the experiment. The fish were anesthetized in 0.05 % MS222 (Sigma) before intracranial injection and dissection of tissues for total RNA extraction. All the animal experiments were carried out in accordance with the guidelines and approval from the Sun Yat-Sen University Animal Care and Use Committee.

2.2. Total RNA extraction and first-strand cDNA preparation

Total RNA from different tissues was isolated by TRIzol/chloroform extraction using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was reconstituted in DEPC-treated distilled water followed by quantification using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). The RNA integrity was checked by agarose gel electrophoresis, and the purity was determined from the A260/280 ratio and all samples used for cDNA synthesis had an A260/280 ratio between 1.8 and 2.0. After the removal of residual contaminating DNA with RNase-free DNase I (New England Biolabs, USA) according to the manufacturer's instructions, 1 µg of total RNA isolated from samples of each tissue was reverse-transcribed into the first-strand cDNA with random hexamers using the M-MLV First Strand Kit (Invitrogen). The resulting cDNAs were stored at -80 °C.

2.3. Quantitative real-time PCR (qPCR)

qPCR was carried out on the LightCycler[®] 480 Real-Time PCR System (Roche, Switzerland), and was performed in duplicate in LightCycler[®] 480 Multiwell Plate 384 (Roche, Switzerland) in a total volume of 10 µL containing 1 µL of 10-fold diluted cDNA, 0.3 µL of each of forward and reverse primer (final concentration of

300 nM each), 5 µL of SYBR[®] Green Realtime PCR Master Mix (Toyobo, Japan), and 3.4 µL of nuclease-free water. The program for the real-time thermal cycler was set as follows: 95 °C for 1 min, followed by 40 cycles of amplification with denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, extension at 72 °C for 30 s and then fluorescent signal collection at 82 °C for 15 s. Melting curves were generated by final denaturation at 95 °C for 10 s, annealing from 65 °C to 95 °C with an increment of 0.5 °C per 5 s. Nile tilapia 18s rRNA was used as the reference gene. The standard curve for each gene was generated through a 10-fold series dilution of plasmids containing the target or reference genes. The primer sequences used in these experiments, their sequence accession numbers, PCR efficiencies, and the amplicon size are shown in table 1. A single melting peak was detected by melting temperature analyses for the target and reference genes, and each transcript exhibited a PCR efficiency near 100 % (Supplementary figure 1). The relative mRNA expression of NPY family peptides was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.4. Identification of NPY family peptides of Nile tilapia and their sequence analyses

Gene-specific primers (Supplementary table 1) were designed according to the predicted sequences of Nile tilapia NPY family peptides in Ensemble database (<http://www.ensembl.org>) for PCR amplification of their open reading frame (ORF). PCR products were cloned into a pCR2.1 vector (Invitrogen) and sequenced. Then the ORF sequences were mapped to different chromosomes by NCBI Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview>). The chromosomal locations of NPY family peptide genes and *Hox*, *MPP*, *DLX*, *OSBPL* gene clusters were proposed by searching on the scaffolds carrying NPY family peptide genes in the tilapia genome database on Ensemble release 81. The deduced mature peptides were aligned with the proposed ancestral gnathostome NPY and PYY (Larhammar, 1996) by Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). A neighbor-joining phylogenetic tree was constructed using Mega 6.06; standard settings and 1000 bootstrap replicates were applied. Precursor sequences of NPY family peptides from the following species were

used for phylogenetic tree construction: human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus laevis*), zebrafish (*Danio rerio*), tiger puffer (*Takifugu rubripes*), medaka (*Oryzias latipes*), grass carp (*Ctenopharyngodon idellus*), seabass (*Dicentrarchus labrax*), stickleback (*Gasterosteus aculeatus*), and Nile tilapia. A list of genes with corresponding accession numbers is provided in supplementary table 2, and the Nile tilapia NPY family peptide sequences are shown in bold.

2.5. Tissue distribution of NPY family peptides mRNA in Nile tilapia

For tissue distribution analysis, total RNA was extracted from the tissue samples of the brain (without hypothalamus), hypothalamus, spinal cord, pituitary, thymus, head-kidney, gill, spleen, kidney, liver, heart, stomach, foregut, midgut, hindgut, muscle, adipose tissue, and testes of three adult male Nile tilapia (BW 100-120 g). The target tissues were removed and snap-frozen in liquid nitrogen immediately, followed by storage at -80 °C until RNA extraction. After RNA extraction and reverse transcription, the first-strand cDNA samples were used as templates for qPCR assay as described previously.

2.6. Postprandial changes in the mRNA expression of Nile tilapia NPY family peptides

To examine the feeding-induced changes in NPY family peptides mRNA expression, eight groups of weight-matched male Nile tilapia (n = 12/ group) were fed daily at a scheduled time (8:30 am) until satiety and allowed to acclimate to the tank conditions and feeding time for 2 weeks. The hypothalamus and foregut were collected 1 h prior to feeding (-1 h), upon commencement of feeding (0 h), 1 h after feeding (1 h), 3 h after feeding (3 h), and 6 h after feeding (6 h). In 0 h group, the fish were fed at the scheduled feeding time and were withdrawn out of the tank immediately, so they did not actually take feed. Three groups of fish remained unfed and were sampled at 1 h, 3 h, and 6 h after the scheduled feeding time and served as the unfed control groups. Extraction of total RNA, cDNA synthesis, and qPCR

analysis were conducted as described previously.

2.7. Fasting induced changes in the mRNA expression of Nile tilapia NPY family peptides

To determine whether fasting changes the mRNA expression of Nile tilapia NPY family peptides, mRNA expression was tested under fasting conditions over a 7-day period. Three groups of weight-matched male Nile tilapia (n = 12/ group) were fed daily at a scheduled feeding time and allowed to acclimate to tank conditions for 2 weeks before the study. Upon commencement of the study, one group of fish was fed daily for 7 days while the remaining two groups were not fed. At the end of the 7-day fasting period, one of the unfed groups was re-fed. The hypothalamus and foregut were collected 2 h after the scheduled feeding time. Tissue extraction and relative gene expression data analysis were conducted as described previously.

2.8. Effects of intracranial (IC) injections of NPY family peptides on food intake of Nile tilapia

The putative mature peptides of Nile tilapia NPYa, NPYb, PYYa, and PYYb were chemically synthesized for intracranial injection (IC injections) by ChinaPeptides Co. Ltd. (Shanghai, China) with C terminal amidation and the purity $\geq 95\%$. The reports for the synthesis of NPYa, NPYb, PYYa, and PYYb are shown in the supplementary documents (supplemental Fig. 6). The peptides were lyophilized and stored at $-80\text{ }^{\circ}\text{C}$ until use. Before experiments, each peptide was freshly dissolved in fish physiological saline (125.3 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 5.6 mM D-glucose, 5.0 mM Tris, pH 7.4).

This study examined the influence of central (intracranial) administration of NPY family peptides on food intake. IC injections were achieved using a 50 μl -Hamilton syringe with 26 G 1/2-inch needles inserted through the top of the skull. The depth of penetration was limited by a collar fitted on the needle. Previous trial injections with 10 μl of methylene blue and subsequent postmortem examination indicated that 90 % of injections correctly reached the cranial cavity. For details on the operations of IC

injection, see supplementary figure 2.

To reduce the effects of stress on the animals, Nile tilapia were routinely handled over a period of two weeks and acclimated to a scheduled feeding time (9: 30 am). On the day of injection, the fish were given IC injections of fish physiological saline or 50 ng/g BW NPY family peptide dissolved in saline, a dose that was selected based on our pilot studies, at 0.5 μ L/g BW. For each treatment, three tanks of male Nile tilapia weighing approximately 30 g (8 fish/tank) were used. After injection, the fish were returned to their tanks and fed a 5 % of body weight ration per fish after a 30 min recovery period. Fish were allowed to feed for 1 h, and uneaten pellets were withdrawn from each container, dried and weighed, allowing calculation of the total weight of food consumed.

2.9. Statistical analysis

All statistical analyses were conducted using SPSS version 19.0. All data are reported as the means $\pm$ SEM. The significance of the results obtained was analyzed using the Student's unpaired two-tailed t-test or one-way ANOVA with the Dunnett's T3 test for multiple comparisons. $P < 0.05$ was considered significant.

3. Results

3.1 Identification and analysis of Nile tilapia NPY family peptides

The ORF of *NPYb*, *PYYa*, and *PYYb* were successfully isolated from the brain of Nile tilapia. The ORF of Nile tilapia *NPYb* was 279 bp, which encoded a precursor containing a putative signal peptide of 20 amino acids and a mature peptide of 36 amino acids. Both *PYYa* and *PYYb* had a 294 bp ORF encoding a precursor of 97 amino acids that contained a putative signal peptide of 28 amino acids and a mature peptide of 36 amino acids (Supplementary figure 3). The Nile tilapia NPY that was previously cloned by our laboratory (Wang et al., 2011) was renamed NPYa according to our phylogenetic and chromosomal location analysis. The mature peptides of Nile tilapia NPYa and NPYb had three and eight residues that differed from the proposed

ancestral NPY, respectively, and showed 81 % similarity with each other (Fig. 1). The mature peptides of PYYa and PYYb differed from the proposed ancestral PYY at one and nine positions, respectively, and showed 75 % similarity with each other (Fig. 1).

The Nile tilapia *NPYa* and *NPYb* were mapped to LG22 and LG11, respectively, while *PYYa* and *PYYb* were mapped to LG4 and LG8-24, respectively, in the NCBI Map Viewer (Table 2).

HoxA, *MPP6*, and *OSBPL3* were found to be located next to *NPYa* and *NPYb* on the scaffold GL831202 and GL831286, respectively, while *MPP2*, *DLX3b*, and *DLX4* were found neighboring to *PYYa* and *PYYb* on the scaffolds GL831179 and GL831136, respectively, in the Ensemble database (Fig. 2).

The phylogenetic tree clearly separated the two NPY paralogs in teleosts into two groups, and Nile tilapia *NPYa* and *NPYb* could be clearly identified as orthologs to the previously described *NPYa* and *NPYb* in teleosts. Although the *PYYa* in Nile tilapia had high homology with other teleost fishes, the teleost *PYYa* did not cluster in a separate clade in our phylogenetic analysis. In addition, the teleost *PYYb* was clustered into an independent branch from the PYY of other vertebrates, and Nile tilapia *PYYb* was included in this clade (Supplementary figure 4).

3.2 Tissue distribution of NPY family peptides in Nile tilapia

qPCR analysis showed that both *NPYa* and *NPYb* mRNA were abundantly expressed in the brain and hypothalamus; however, the expression level of *NPYb* was markedly lower than that of *NPYa* (Fig. 3A). In addition, *NPYa* had a substantial expression in the spinal cord, but a weaker expression in the kidney and testes.

PYYb mRNA expression was detected in the gut, at apparently high levels in the foregut and midgut and low level in the hindgut (Fig. 3B). Low *PYYb* mRNA expression was found in the brain, hypothalamus, and the spinal cord. *PYYa* mRNA was mainly expressed in the brain and spinal cord at levels similar to that of *PYYb*.

3.3 Postprandial changes of NPY family peptides mRNA expression in Nile tilapia

Within the hypothalamus, *NPYa* mRNA expression significantly decreased in the

fed animals 1 h post-feeding compared with the unfed control (Fig. 4A). However, there were no significant differences in the *NPYb* hypothalamic mRNA expression after feeding (Fig. 4B). The level of *PYYa* mRNA expression increased after feeding, whereas no significant changes were observed until 6 h post-feeding compared with unfed controls (Fig. 4C). *PYYb* mRNA expression in the fed animals 1 h post-feeding significantly increased compared with the unfed control (Fig. 4D). Moreover, no significant changes were detected among unfed groups (one-way ANOVA with Dunnett's T3 test).

Within the foregut, *PYYb* mRNA expression was significantly increased in the fed fish 1 h post-feeding compared with the unfed control (Fig. 4E). Interestingly, the level of *PYYb* mRNA expression in the 0 h group was significantly higher than other unfed groups.

3.4 Fasting induced changes in the mRNA expression of NPY family peptides of Nile tilapia

Within the hypothalamus, fasting induced a significant increase in *NPYa* mRNA expression and a decrease in *PYYa* mRNA expression (Fig. 5A, C). When the 7-day fasted fish were re-fed, both *NPYa* and *PYYa* mRNA levels returned levels similar to the fed control groups. There were no significant differences in either *NPYb* or *PYYb* hypothalamic mRNA expressions among fed, fasted, and re-fed fish (Fig. 5B, D).

Within the foregut, *PYYb* mRNA expression significantly decreased in the fasted fish compared with fed fish, and re-feeding reversed the decrease (Fig. 5E). Although the *PYYb* mRNA expression in the re-fed group was lower than that of fed group, there was no statistically significant difference.

3.5 Effect of IC injection of NPY family peptides on food intake of Nile tilapia

During 1 h after IC injection of 50 ng/g BW *NPYa* and *NPYb*, the food intake of Nile tilapia increased by 36.3 % and 43.3 %, respectively, compared with that of the saline-injected group (Fig. 6A). IC injections of *PYYb* at 50 ng/g BW reduced food intake in Nile tilapia by 43.8 % compared with the saline-injected group (Fig. 6B). In

addition, a single IC injection of 50 ng/g BW synthetic PYYa had no significant effect on food intake during the 1 h observation period.

4. Discussion

We show here that four members of NPY family peptides are present in Nile tilapia and that they consist of duplicates of both NPY and PYY. Among teleosts, three species have previously been reported to possess these duplicates, namely the two pufferfish *Tetraodon nigroviridis* and *Takifugu rubripes*, and the stickleback (Sundstrom et al., 2008). Previous studies in mammals and teleosts indicate that the NPY and PYY genes are neighboring to *Hox* clusters and *MPP*, *DLX*, *OSBPL* genes (Soderberg et al., 2000). Consistently, Nile tilapia *NPYa* and *NPYb* genes were both co-localized with *HoxA*, *MPP6*, and *OSBPL3* genes, and both Nile tilapia *PYYa* and *PYYb* genes were neighboring to *MPP2*, *DLX3b*, and *DLX4* genes in our results. Together with their close relationship obtained from our phylogenetic analysis, these results confirmed that the two kinds of identified NPY and PYY, in Nile tilapia, are paralogs originating from an ancestral gene. Moreover, the four NPY family peptide genes were mapped to different chromosomes/linkage groups, suggesting that the two duplicates of NPY and PYY in Nile tilapia arose from genome duplication as proposed by Sundstrom et al. (2008).

Expression divergence is the first step in the functional divergence between duplicate genes (Li et al., 2005). *NPYb*, which is the teleost specific duplicate of NPY, had a markedly lower CNS mRNA expression than that of *NPYa* in Nile tilapia. Similarly, a higher brain expression level of *NPYa* than of *NPYb* was detected in flounder (*Paralichthys olivaceus*) (Kurokawa and Suzuki, 2002). Moreover, in flounder, *NPYb* had been misidentified as PYY (Sundstrom et al., 2008). The duplicates of PYY showed entirely different expression patterns in Nile tilapia, with high *PYYb* mRNA expression levels in the foregut and midgut and *PYYa* mainly expressed in the CNS at low level. Consistent with our result, *PYYb* mRNA has been found to be abundantly expressed in the digestive tract of grass carp, flounder, and yellowtail (*Seriola quinqueradiata*) (Chen et al., 2013a; Kurokawa and Suzuki, 2002;

Murashita et al., 2006), indicating that PYYb possibly exists as a kind of gut derived hormone in teleosts. The central expression of *PYYa* and *PYYb* in Nile tilapia and other fish species, including grass carp, goldfish, flounder, and yellowtail (Chen et al., 2013a; Chen et al., 2013b; Gonzalez and Unniappan, 2010; Kurokawa and Suzuki, 2002; Murashita et al., 2006), suggesting that both genes may act as neurotransmitters in the CNS.

To determine whether the duplicates of NPY and PYY produced by genome duplication in teleost fishes participate in food intake regulation, we analyzed the feeding and fasting induced expressions. Previous studies have shown that *NPY* mRNA expression is affected by feeding conditions with fasting increasing its expression (Brady et al., 1990; Kamijo et al., 2011; Narnaware et al., 2000; Yokobori et al., 2012) and feeding decreasing its expression (Narnaware et al., 2000; Persaud and Bewick, 2014; Tian et al., 2015). In the present study, Nile tilapia on an entrained feeding schedule had a significant decrease in hypothalamic *NPYa* expression 1 h post-feeding, whereas 7-days of fasting increased its expression, which was reversed by re-feeding, consistent with the feature of orexigenic peptides. However, *NPYb* mRNA expression was not affected by feeding conditions in our study. Similar results have been found in tiger puffer, where feeding conditions affected the mRNA levels of *NPYa* in the hypothalamus, but not *NPYb* (Kamijo et al., 2011). Considering the extremely low expression level of *NPYb*, one can speculate that it is *NPYa* but not *NPYb* that acts as the main endogenous NPY in teleosts. On the other hand, compared with NPY, there is limited information about the effects of PYY on feeding regulation in fish. We present here that both *PYYa* and *PYYb* mRNA expressions were stimulated by feeding, suggesting a functional role of the two duplicates in feeding regulation. Consistent with our results, previous studies have shown that meal stimulates the brain *PYYa* mRNA expression in goldfish and grass carp (Chen et al., 2013b; Gonzalez and Unniappan, 2010), and *PYYb* brain mRNA expression is increased 1 h post-feeding in Mexican blind cavefish (*Astyanax fasciatus mexicanus*) (Wall and Volkoff, 2013). *PYYa* hypothalamic mRNA expression was decreased after 7-day starvation and this reduction was reversed by re-feeding, further supporting the role of

PYYa in feeding regulation in Nile tilapia. However, a 7-day fasting period had no significant effect on *PYYb* hypothalamic mRNA expression in Nile tilapia, which is consistent with what was observed in red-bellied piranha (*Pygocentrus nattereri*) and Mexican blind cavefish (Volkoff, 2014; Wall and Volkoff, 2013). These results suggest that this peptide is less affected by long-term fasting in the CNS.

Nile tilapia *PYYb* was mainly expressed in the gut, and the intestine plays a dominant role in satiation (Cummings and Overduin, 2007); therefore, the fasting- and feeding-induced *PYYb* mRNA expression were also detected in this tissue. Our results showed that foregut *PYYb* mRNA expression was stimulated by feeding and suppressed by fasting, which is in agreement with the results of previous reports in other fish species (Chen et al., 2013a; Volkoff, 2014). Conversely, gut *PYYb* mRNA expression was significantly lower after feeding, but increased after 3-day fast in yellowtail (Murashita et al., 2006; Murashita et al., 2007). The cause for such discrepancy is still unknown. However, these postprandial and fasting induced changes in *PYYb* mRNA expression in the gut suggest that gut-derived *PYYb* may participate in the regulation of food intake, similar to PYY in mammals. Oddly, *PYYb* mRNA expression increased at the scheduled feeding time compared with unfed groups in our result. A previous study in dogs has demonstrated that postprandial secretion of PYY is biphasic, which is initially stimulated by atropine-sensitive neural projections from the proximal gut, followed by direct nutrient stimulation in the distal gut (Lin and Taylor, 2004). It is speculated that the increased *PYYb* mRNA expression at 0 h may depend on the neural pathway, and the increased mRNA expression at 1 h post-feeding is stimulated by the chyme in the foregut. Future studies on the regulation of PYY release in the gut by the neural regulatory system in teleosts will help to solve this puzzle.

It is noteworthy that feeding conditions induced variation in the mRNA expression of NPY family peptides might be region-specific as proposed by Volkoff (2014). For example, a significant increase in hypothalamic NPY mRNA expression of zebrafish fasted for 7 days has been detected but this effect is not seen when using whole brain (Yokobori et al., 2012). In addition, tiger puffer *NPYa* mRNA levels in the

hypothalamus, but not in the telencephalon, are increased after one week of fasting (Kamijo et al., 2011). Since hypothalamus is the most important center that controls appetite and energy balance, we chose hypothalamus as the target tissue to detect the effects of feeding and fasting on NPY family peptides expression in the present study. Moreover, the fasting periods sufficient to cause significant changes in the mRNA expression of NPY family peptides in different species can be different (Kehoe and Volkoff, 2007). Six-day fasting has no effect on *NPYa* and *PYYa* mRNA expression in the brain of salmon (Murashita et al., 2009a; Murashita et al., 2009b), whereas 3-days of fasting induces a significant increase in *NPYa* and *PYYa* mRNA expression in goldfish (Gonzalez and Unniappan, 2010; Narnaware et al., 2000). In our preliminary study, 3-days of fasting did not significantly affect hypothalamic *NPYa*, *NPYb*, *PYYa*, or *PYYb* mRNA expression (Supplementary figure 5), suggesting that more than 3 days of starvation is required to reveal such detectable changes in Nile tilapia. The differences in expression patterns of NPY family peptides among fish species can be attributed to the differences in their diet and digestive physiology as proposed by Kehoe and Volkoff (2007).

Our *in vivo* studies provide the first direct evidence for the orexigenic effect of NPYb and the anorexigenic effect of PYYb in teleost fishes. NPY is the most potent orexigenic peptide, and its effect on increasing food intake is well known (Aldegunde and Mancebo, 2006; Kiris et al., 2007; Narnaware et al., 2000; Yokobori et al., 2012; Zhou et al., 2013). However, there is little direct evidence for the involvement of NPYb in appetite regulation. In the present study, IC injection of NPYb promoted food intake to an extent similar to NPYa, suggesting that the mature peptide of NPYb is a potential new feeding stimulatory agent in teleosts. Previous studies have shown that both peripheral and central administration of PYYa can inhibit food intake in teleosts (Chen et al., 2013b; Gonzalez and Unniappan, 2010), whereas no related study has been reported for PYYb. The feeding inhibitory effect of PYYb in our results indicates that PYYb is an anorexigenic peptide in teleosts. However, central administration of PYYa had no significant effect on food intake in the present study. For technical reasons, the present studies use IC injection but not ICV or specific

nucleus injection. There is a possibility that in our experiment, PYYa could not be transported to the target site where its specific receptor was expressed. Future studies on the effects of PYYa in specific regions of the brain will help to unravel the role of PYYa in Nile tilapia.

In conclusion, our results show that Nile tilapia has two duplicates of NPY (NPYa, NPYb) and PYY (PYYa, PYYb). Both NPYa and NPYb are orexigenic peptides. Since the *NPYb* expression level was markedly low, NPYa may serve as the main endogenous NPY in feeding regulation. Moreover, *PYYb* may function as a gut-derived anorexigenic peptide like mammalian PYY, while *PYYa* may act as a CNS-specific PYY in Nile tilapia. Our results, for the first time, provide direct evidence for the appetite-stimulating effect of NPYb and appetite-inhibiting effect of PYYb in teleost fishes. Further research on the receptors of NPY family peptides will help to better understand the mechanisms of these peptides on food intake regulation in teleosts.

Author contribution

Peipei Yan planned and performed the experiments, analyzed the data and drafted the manuscript. Jirong Jia and Guokun Yang contributed to the tissue collection and intracranial injection experiments. Dongfang Wang helped with the tissue distribution studies. Caiyun Sun revised the manuscript. Wensheng Li, corresponding author, took charge of research design, supervised the study and critically revised the manuscript.

Funding

This work was supported by the China Agriculture Research System (CARS-49), the National Science Foundation of China (31272639 and 31472259), the Guangdong Provincial Science and Technology Program (2015A020216006), Guangdong Oceanic & Fishery Program (A201601C02), the Developmental Funds for Strategic Emerging Industries of Shen Zhen (NYSW20140401010064), the Modern Agriculture Talents Support Program (2016-2020) and the Science and Technology Planning Project of Guangzhou (201607020014) to Dr. Wensheng Li.

Acknowledgments

We are thankful to Guangdong tilapia breeding farm for providing the fish for the study.

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Table and Figure legends

Table 1

Primers used for qPCR analyses of NPY family peptide mRNAs and 18s rRNA in Nile tilapia.

Table 2

Mapping of Nile tilapia NPYa, NPYb, PYYa, and PYYb in their Linkage groups.

Fig. 1 Alignment of the Nile tilapia NPY family peptides and the proposed ancestral gnathostome NPY (An NPY) and PYY (An PYY). Amino acid differences are shadowed.

Fig. 2 Chromosomal location of Nile tilapia NPY family peptides. The relative positions of NPY family peptide genes and *Hox*, *MPP*, *DLX*, *OSBPL* that have been demonstrated to be adjacent to the NPY family peptide genes were proposed according to the tilapia genome database on Ensemble release 81.

Fig. 3 Tissue distribution of the duplicates of NPY (A) and PYY (B) in Nile tilapia. Br, brain; Hy, hypothalamus; Pi, pituitary; Sc, spinal cord; Th, thymus; Hk, head-kidney; Gi, gill; Sp, spleen; Ki, kidney; Li, liver; He, heart; St, stomach; Fg, foregut; Mg, midgut; Hg, hindgut; Mu, muscle; Ad, adipose; Te, testes. The data are presented as the means $\pm$ SEM (n = 3 males).

Fig. 4 Postprandial changes of hypothalamic NPYa (A), NPYb (B), PYYa (C), PYYb (D), and foregut PYYb (E) mRNA expression in Nile tilapia. Fish were fed daily at a fixed time of day (0 h) for 2 weeks. On the experimental day, fish in each group were killed each at -1 h, 0 h and 1, 3, 6 h after feeding. Three groups remained unfed and were sampled at 1 h, 3 h, and 6 h and served as the unfed control. For each transcript, the mRNA expression was normalized to 18s rRNA and represented as the percentage of the -1 h group. Data are expressed as the mean $\pm$ SEM (n=8). *P < 0.05, **P < 0.01 compared with unfed group at the same time point by Student's t test.

644

645 **Fig. 5 Fasting induced hypothalamic NPYa (A), NPYb (B), PYYa (C), PYYb (D), and**
646 **foregut PYYb (E) mRNA expression in Nile tilapia.** Fish were either fed once daily or
647 fasted for 7 days, and an additional unfed group was re-fed at the end of 7-day fast. The
648 hypothalamus and foregut were collected 2 h after the scheduled feeding time. For each
649 transcript, the mRNA expression was normalized to 18s rRNA and represented as the
650 percentage of the fed group (100 %). Data are expressed as the mean $\pm$ SEM (n=10-12). *P
651 < 0.05 , **P < 0.01 by Student's t test.

652

653 **Fig. 6 Effects of intracranial injections of the mature peptides of NPYa and NPYb (A),**
654 **PYYa and PYYb (B) on food intake in Nile tilapia.** Food intake was measured over 1 h
655 after injection of synthetic NPYa, NPYb, PYYa, or PYYb (50 ng/g BW). Data are expressed
656 as the mean food intake $\pm$ SE (n = 8). *P < 0.05 compared with the saline injected group by
657 Student's t test.

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Table 1

Gene		Sequence (5' - 3')	Product size (bp)	Efficiency (%)
NPYa	F	ACAAGACAGAGGTATGGGAAGA	130	99.6
	R	GGCAGCATCACCACATTG		
NPYb	F	GTGTTCCAACGCTGTGATGT	193	96.9
	R	TCGTTTCCCATACCTCTGTC		
PYYa	F	ACACTGGCTGATGCCTACC	135	95.4
	R	TGAACGCTTTCCATACCTC		
PYYb	F	GCCGTCAGGCACTATGTCAACC	184	93.7
	R	GTGTTGGTAGTGCGGGATTGTG		
18s rRNA	F	AGAAACGGCTACCACATCC	222	99.9
	R	CACCAGACTTGCCCTCCA		

Table 2

Genes	Linkage group (LG)
NPYa	LG 22
NPYb	LG 11
PYYa	LG 4
PYYb	LG 8-24