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Phoenixin participated in regulation of food intake and growth in spotted scat, *Scatophagus argus*

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ABSTRACT

Phoenixin (Pnx) is an endogenous peptide known to be involved in reproduction and food intake in rats, with two active isoforms, phoenixin-14 (Pnx-14) and phoenixin-20 (Pnx-20). However, little is known about the functions of Pnx in teleost. Here, *pnx* was cloned and was detected in all tissues of both male and female in spotted scat (*Scatophagus argus*), including growth axis, hypothalamus, pituitary, and liver. Real-time PCR analysis showed that *pnx* in the hypothalamus increased significantly after 2 d and 7 d fasting, while reduced significantly after re-feeding ($P < 0.05$). When pituitary and liver fragments were cultured *in vitro* with Pnx-14 and Pnx-20 (10 nM and 100 nM) for 6 h, the expression of *ghrhr* (growth hormone-releasing hormone receptor) and *gh* (growth hormone) in the pituitary, and *ghr1* (growth hormone receptor 1) in the liver increased significantly, except *ghr2* (growth hormone receptor 2) incubated with 10 nM and 100 nM Pnx-20 and *ghr1* incubated with 10 nM Pnx-20. Similarly, the expression of *ghrhr* and *gh* in the pituitary, as well as *ghr1* and *ghr2* in the liver, increased significantly after injecting *S. argus* with Pnx-14 and Pnx-20 (10 ng/g and 100 ng/g body weight). These results indicate that Pnx is likely to be involved in the regulation of food intake, and also regulates the growth of *S. argus* by increasing *ghrhr* and *gh* expression in the pituitary, *ghr1* and *ghr2* in the liver, and *ghr1* directly in the liver.

1. Introduction

Phoenixin (Pnx), a novel endogenous neuropeptide, mainly exists as two active isoforms, phoenixin-14 (Pnx-14) and phoenixin-20 (Pnx-20) (Yosten et al., 2013). Pnx is a highly conserved peptide across different species including human, rat, mouse, porcine, canine and *Xenopus* (Yosten et al., 2013). These peptides have similar biological activities and share a common C-terminal fourteen amino acids segment that is essential for biological activity. Pnx is a recently identified peptide initially implicated in reproduction (Yosten et al., 2013; Treen et al., 2016; Stein et al., 2016). However, it is not seemingly limit to one function and was additionally detected in the modulation of anxiety (Jiang et al., 2015a; Hofmann et al., 2017), memory (Jiang et al., 2015b), the induction of pruritus (Cowan et al., 2015) and food intake (Schalla et al., 2017).

Interestingly, Pnx immunoreactivity was detected in various peripheral tissues, including heart, thymus, stomach, and spleen, with the highest expression in hypothalamus in rats (Yosten et al., 2013). Moreover, Pnx immunoreactivity was identified in multiple food intake areas in the hypothalamus and co-expressed with the anorectic factor Nesfatin-1 in rats (Yosten et al., 2013; Schwartz et al., 2000; Pałasz et al., 2015). In addition, Pnx was shown to exert a food intake-

Abbreviations: Pnx, phoenixin; *ghrhr*, growth hormone-releasing hormone receptor; *gh*, growth hormone; *ghr*, growth hormone receptor; Igf, insulin-like growth factor; Ghrh, growth hormone-releasing hormone; PVN, paraventricular nucleus; Arc, arcuate nucleus; VMH, ventromedial and lateral hypothalamus; bw, body weight; icv, intracerebroventricularly; JAK-STAT, just another kinase; ERK, extracellular regulated protein kinases; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B

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stimulatory effect following icv (intracerebroventricularly) injection (Schalla et al., 2017). These results indicate Pnx involvement in the regulation of food intake. Though food intake is closely related to growth, the role of Pnx in mammalian and fish growth has not yet been reported.

As in other vertebrates, many of the growth-promoting actions are regulated by the growth hormone/insulin-like growth factor (Gh/Igf) axis in fish (Duan, 1997). Gh plays an important role in regulating normal somatic growth and developmental processes in fish (Björnsson et al., 2002). Growth hormone-releasing hormone receptor (Ghrhr) is a specific receptor for the growth hormone-releasing hormone (Ghrh), constitute important components of the hypothalamus-pituitary growth axis in fish which controls the synthesis and secretion of *gh* from the anterior pituitary somatotrophs (Liang et al., 2015; Qian et al., 2012; Lee et al., 2007). Gh actions are triggered by binding of the hormone to Gh receptors 1 and 2 (Ghr1 and Ghr2, respectively) (Costa et al., 2016). However, the physiological role of Pnx in growth regulation is unclear. Therefore, we here investigate if Pnx is involved in the regulation of growth in fish.

Spotted scat, *Scatophagus argus*, a member of Perciformes, is a euryhaline subtropical fish widely distributed in Indian-Pacific waters, including the Guangdong, Taiwan and Guangxi coasts in China. *S. argus* is an important aquaculture fish with high economic value, with females growing two times faster and larger than males (Sivan and Radhakrishnan, 2011; Yang et al., 2017). Previous study from our lab showed that when under 2 years old, the faster growth rate in the females was related to the higher daily food consumption, food conversion, and enzymatic activities of protease and amylase (Wu et al., 2013). Consistent with the growth difference, the *gh* mRNA levels in the female pituitary were higher than in males (Deng et al., 2014). However, it was unclear whether the high daily food consumption and high *gh* expression in female *S. argus* are related to Pnx. Therefore, in the present study, we report the cloning, functional characterization, tissue expression and growth regulation of Pnx from *S. argus*, with the aim to understand if Pnx is involved in the growth regulation of *S. argus*, provide further insights into Pnx function in vertebrates.

2. Materials and methods

2.1. Experimental fish

The *S. argus* (body weight, 250–300 g) used for cloning, tissue distribution studies and incubation with Pnx-14 and Pnx-20 was purchased from Dongfeng Market (Zhanjiang, Guangdong, China). The fasting and re-feeding fish (body weight, 50–60 g) and adult fish (body weight, 225.5 ± 25.2 g) injected intraperitoneally with Pnx-14 and Pnx-20 were obtained from Zhuhai Yucheng Fry Cultivation Base (Zhuhai, Guangdong, China).

2.2. Chemical reagents

Pnx-14 (1 mg/ml) and Pnx-20 (1 mg/ml) were synthetized from GL Biochem Ltd. (Shanghai, China). Pnx-14 and Pnx-20 were dissolved in Gibco M199 media (Mediateah, Manassas, VA, USA) at a stock concentration of 1 nM. 2× PCR MIX purchased from Dongsheng Biotechnology Company Ltd. (Guangzhou, China).

2.3. cDNA cloning and tissue distribution of pnx

Total RNA was extracted from adult *S. argus* hypothalamus using TRIZOL Reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) using 1 μg of total RNA. Two pairs of *pnx* specific nested primers were designed according to sequenced contigs from the *S. argus* transcriptome. The primers were used to amplify *pnx* cDNA fragments containing the corresponding open reading frames. All primers used in this study are listed in Table 1. PCR was carried out under the following conditions: 94 °C (30 s), 60 °C (30 s), and 72 °C (60 s) for 32 cycles, with a final extension at 72 °C for 10 min using a PCR Thermal Cycler (BIO-RAD-C1000, USA). The PCR products were separated by electrophoresis on 1.5% agarose gel, and the gel was stained with ethidium bromide (EB). Specific fragments were extracted and extracted and cloned into pMD19-T vector (Takara, Japan), and confirmed by sequencing (Sangon Biotech).

Female (n = 3) and male (n = 3) adult fish were anesthetized with 100 mg/l tricaine methane sulfonate (MS 222, Sigma, Saint Louis, MO) and dissected. Intestine, gill, heart, spleen, kidney, testis, ovary, hypothalamus, muscle, pituitary and liver tissues were collected, frozen with liquid nitrogen and stored at −80 °C. Total RNA (1 μg) from each tissue was reversed transcribed into cDNA as described above. Tissue distribution was analyzed by Reverse transcription PCR (RT-PCR). *β-actin* was used as an internal control. *pnx* and *β-actin* primers for tissue distribution are listed in Table 1. 2× PCR MIX was used as an enzyme. The amplification regime consisted of 35 cycles of 15 s at 95 °C, 55 °C for 15 s, and 72 °C for 30 s; followed by further amplification at 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel and visualized with EB.

2.4. Fasting and refeeding experiments

The *S. argus* were divided into five groups randomly (n = 8) and put inside indoor cages (50 cm × 50 cm × 50 cm) placed in an indoor cement pond (5 m × 5 m × 1.5 m) with a continuous supply of water at a temperature of 26–28 °C. The fish were fed with a commercial diet

Table 1
Primers for RT-PCR and qPCR in *S. argus*.

Gene	Primer	Sequence	Purpose
pnx	*pnx-f1*	ACATTTGGAGGTGAGAGCGT	Cloning
pnx	*pnx-r1*	CATCAGCATCAGTGTCAGGAG	Cloning
pnx	*pnx-f2*	GTGAGAGCGTGACATCCGAT	Cloning
pnx	*pnx-r2*	AGTTAGTGAAGGGTGAGCCG	Cloning
pnx	qpnx-F1	CGCTGCATTTTACCCCATA	qPCR and RT-PCR
pnx	qpnx-R1	GTCACTGGCCATCATTTGC	qPCR and RT-PCR
ghrhr	qghrhr-F1	TGTTCTTCAGGAGGTTACG	qPCR
ghrhr	qghrhr-R1	CAGTGGTTGGTGTCATCA	qPCR
gh	qgh-F2	ACAAGCACGAGACACAAC	qPCR
gh	qgh-R2	AGCAGAACCTCCAGACAG	qPCR
ghr1	qghr1-F	GCCAGGAATGTGAGGTAA	qPCR
ghr1	qghr1-R	GGAGAAGAAGTGATTGTTGT	qPCR
ghr2	qghr2-F	CACTCACTCCACTCTTCAG	qPCR
ghr2	qghr2-R	CTTCTTCCTCCACATCTTCA	qPCR
β-actin	Actin-F	GAGAGGTTCCGTTGCCCAGAG	qPCR
β-actin	Actin-R	CAGACAGCACAGTGTTGGCGT	qPCR

Fig. 1. Nucleotide and deduced amino acid sequence of Pnx in *S. argus*. Putative transmembrane helix region is boxed. The domain in shadow is the DUF4538 domain. N-linked glycosylation site is in italic. Casein kinase II phosphorylation site is bold. N-myristoylation sites have a wavy underline. The dotted line shows Microbodies C-terminal targeting signal. N-terminal domain of DnaB helicase is double underline. Single underlines show initiation codon and stop codon. * - stop codon.