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# Stimulatory effect of ghrelin on food intake in bullfrog larvae

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ABSTRACT

Ghrelin is a potent orexigenic peptide implicated in appetite regulation in rodents. However, except for teleost fish, the involvement of ghrelin in the regulation of feeding in non-mammalian vertebrates has not been well studied. Anuran amphibian larvae feed and grow during the pre- and prometamorphic stages, but, thereafter they stop feeding as the metamorphic climax approaches. Therefore, orexigenic factors seem to play important roles in growing larvae. In the present study, we examined the effect of intraperitoneal (IP) or intracerebroventricular (ICV) administration of synthetic bullfrog ghrelin (*n*-octanoylated 28-amino acid form) on food intake in larvae at the prometamorphic stages. Cumulative food intake was significantly increased by IP (8 and 16 pmol/g body weight (BW)) or ICV (0.5 and 1 pmol/g BW) administration of ghrelin during a 15-min observation period. The orexigenic action of ghrelin at 8 pmol/g BW (IP) or at 0.5 pmol/g BW (ICV) was blocked by treatment with a growth hormone secretagogue-receptor antagonist, [D-Lys$^3$]GHRP-6 at 80 pmol/g BW (IP) or at 5 pmol/g BW (ICV). We then investigated the effect of feeding status on expression levels of the ghrelin transcript in the hypothalamus and gastrointestinal tract. Ghrelin mRNA levels in both were decreased 15 and 60 min after feeding. These results indicate that ghrelin acts as an orexigenic factor in bullfrog larvae.



## 1. Introduction

Ghrelin was first isolated from rat and human stomachs as an endogenous ligand for the growth hormone secretagogue (GHS)-receptor type-1a (GHS-R1a) [33]. In general, ghrelin is a 28-amino acid peptide with an *n*-octanoylated serine residue at the third N-terminal position. Ghrelin is now considered to be a multifunctional peptide that plays an important role in energy preservation by regulating food intake, body weight and glucose homeostasis in mammals [7,47,54]. Ghrelin has also been isolated and characterized from sub-mammalian vertebrates, and its primary structure has been determined in non-mammalian species including the chicken (*Gallus gallus*) [26], red-eared turtle (*Trachemys scripta elegans*) [23], bullfrog (*Rana catesbeiana*) [18], Japanese eel (*Anguilla japonica*) [21], zebrafish (*Danio rerio*) [1], sea bream (*Acanthopagrus schlegeli*) [57], goldfish (*Carassius auratus*) [40], rainbow trout (*Oncorhynchus mykiss*) [19], tilapia (*Oreochromis mossambicus*) [20], channel catfish (*Ictalurus punctatus*) [25], sharks (*Sphyrna lewini* and *Carcharhinus melanopterus*) [29] and stingray (*Dasyatis akajei*) [16]. In the bullfrog, three molecular forms of ghrelin have been purified and identified from the stomach: *n*-octanoylated and *n*-decanoylated ghrelins with 27- or 28-amino acid-residues [18]. Recently, GHS-R1a has been characterized in this and other anuran species, and it has been indicated that bullfrog ghrelin and a GHS-R agonist, GHRP-6, could interact with frog GHS-R1as in vitro [15,18]. However, there is limited information about the control of feeding by ghrelin in anuran amphibians. In a goldfish model, it has been shown that intraperitoneal (IP) and intracerebroventricular (ICV) injections of goldfish ghrelin with an *n*-octanoic acid modification stimulate food intake as well as the release of GH and gonadotropins from the pituitary gland [40,53].

In the larvae of anuran amphibians, it is known that as metamorphosis progresses, the oral and digestive organs are reconstructed [11], resulting in a decline of feeding behavior [34]. Some previous reports have indicated that corticotropin-releasing factor (CRF) reduces feeding or foraging behavior in anurans such as the African clawed toad (*Xenopus laevis*), the Western spadefoot toad (*Spea hammondii*) and bullfrog [4–6,43]. In addition, our recent study has clearly demonstrated that CRF suppresses food intake in bullfrog larvae, suggesting that CRF acts on the central nervous system to suppress food intake during the period of reconstruction of the digestive system [37]. We have also indicated that neuropeptide Y (NPY) stimulates food consumption in bullfrog larvae during pre- and pro-metamorphosis [48]. Therefore, it is likely that anuran

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larvae can feed and grow until the metamorphic climax, and that NPY acts as an orexigenic factor in appetite regulation during these periods. However, there has been no information about the role of ghrelin in the bullfrog larva. The bullfrog has often been used as an animal model in studies on the function of regulatory peptide [3,17,18,31,36,39,43–46,55]. As yet, however, there is no direct evidence that ghrelin affects food consumption in bullfrog larvae.

The present study was conducted to clarify whether ghrelin enhances food intake in bullfrog larvae at the prometamorphic stages, since the larvae grow and gain fat body mass during these stages [30,55]. In this species, ghrelin mRNA is expressed in the gastrointestinal tracts [18]. In the present study, we examined (1) the effect of IP and ICV administration of synthetic bullfrog ghrelin on food intake in bullfrog larvae, and (2) the effect of feeding status on expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract of the larvae.

## 2. Materials and methods

### 2.1. Animals

Prometamorphic bullfrog (*R. catesbeiana*) larvae weighing 5–7 g were collected from ponds in the suburbs of Toyama City, Japan. Two hundred fifty larvae at the prometamorphic stages (XI–XIX) were used. The developmental stages of the larvae were determined according to Taylor and Kollros [49]. The animals were kept for 1–2 weeks under controlled light/dark conditions (12L/12D) with the water temperature maintained at 24–28 °C. The larvae were fed every day at noon with a powder diet (Itosui Co., Tokyo, Japan) until used in experiments. Animal experiments were conducted in accordance with the Invasive Alien Species Act of Japan and the University of Toyama's guidelines for the care and use of alien and laboratory animals.

### 2.2. Chemicals

Synthetic bullfrog ghrelin with 28 amino acid-residues with *n*-octanoyl nodification, GLT(C8:0)FLSPADMQKIAERQSQNKLRHGNMN, was synthesized at ASBIO Pharma Co. Ltd. (Gunma, Japan) [17,18]. Bullfrog ghrelin was dissolved in 0.6% NaCl and 0.02% $Na_2CO_3$ (saline) at a concentration of 1.0 mM and then stored at −80 °C until use. In order to examine whether the action of ghrelin is mediated by ghrelin receptor signaling, a growth hormone secretagogue (GHS)-receptor antagonist, [D-Lys$^3$]GHRP-6 (HDWDKWDFK-$NH_2$; Bachem AG, Bubendorf, Switzerland), was tested. [D-Lys$^3$]GHRP-6 was also dissolved in saline at a concentration of 50 mM and then stored at −80 °C until use.

### 2.3. Measurement of food intake in larvae

Details of the methods used for evaluating food consumption in the larvae have been reported elsewhere [39,48]. Two types of powder diet colored green and red, respectively (containing 32–47% proteins, 4–5% dietary fat, 3% dietary fibers, 14–17% minerals, 10–12% water and other components), were obtained from Itosui Co., Tokyo, Japan. First, the test larvae including stages XI–XIX were fed the green-colored diet and kept under laboratory conditions. Then, after a 24-h fast, an adequate amount of the red-colored food was made available at 3% of BW. After 15 min, each animal was decapitated and the gastrointestinal tract was removed. The weight of the red-colored gastrointestinal contents absorbed intestinal juice by tissue paper was measured, and expressed as mg food taken per g BW during the 15-min period. The experiments were conducted around noon.

### 2.4. Effect of IP and ICV administration of ghrelin on food intake in bullfrog larvae

Each larva that had been fed the green-colored diet was fasted for 24 h. For IP administration, larvae were injected with 20 μl of 4, 8 or 16 pmol/g BW of bullfrog ghrelin. Larvae in the control group were given injections of the same volume of saline. For ICV administration, each animal was placed in a stereotaxic apparatus under anesthesia with MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich, St. Louis, MO, USA). A small area (approximately 1 $mm^2$ square) of the parietal skull was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan), and then 0.1 μl/g BW (0.5–0.7 μl) of bullfrog ghrelin dissolved in saline at a concentration of 0.25, 0.5 or 1 pmol/g BW was injected into the third ventricle of the brain using a 10-μl Hamilton syringe with 0.1-μl scale. The gap in the parietal skull was then filled with a surgical bonding agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site and volume was confirmed after the experiment by examining whether Evans blue dye was present in the ventricle without leakage. Control larvae in each experiment were injected with the same volume of saline in the same way as for the experimental group. Each larva that had received the IP or ICV injection was placed individually in a small experimental tank (diameter 11 cm) containing 700 ml of tap water. After recovery from anesthesia, each larva was supplied with the red-colored food equivalent to 3% of its BW. After 15 min, the weight of the intestinal contents was measured as described earlier.

### 2.5. Effect of IP and ICV injection of [D-Lys$^3$]GHRP-6 on the orexigenic action of ghrelin

In order to examine the effect of IP and ICV injection of [D-Lys$^3$]GHRP-6, a GHS receptor antagonist, on the peripheral and central actions of ghrelin, 20 μl of [D-Lys$^3$]GHRP-6 at 80 pmol/g BW in addition to bullfrog ghrelin at 8 pmol/g BW was injected into the abdominal cavity, or 0.5–0.7 μl of [D-Lys$^3$]GHRP-6 at 5 pmol/g BW in addition to bullfrog ghrelin at 0.5 pmol/g BW was injected into the third ventricle of the brain of larvae, as described earlier. The IP- and ICV-injected doses of [D-Lys$^3$]GHRP-6 had been determined in preliminary experiments using [D-Lys$^3$]GHRP-6 at 80 and 160 pmol/g BW (IP) or 5 and 10 pmol/g BW (ICV). Larvae in the control group were given injections of the same volume of saline. The feeding experiment was performed according to the procedures described in the above section.

### 2.6. Effect of feeding status on expression of ghrelin mRNA in the hypothalamus and gastrointestinal tract

The larvae were kept individually under controlled light/dark conditions (12L/12D) in a tank filled with tap water, and fed daily at 3% of their BW. Before feeding, or 15 and 60 min after feeding, the larvae were anesthetised with MS-222 and each hypothalamus and gastrointestinal tract including stomach and intestine was removed. Each part was weighed, placed immediately in liquid nitrogen, and stored at −80 °C until use. Total RNA was extracted from each part with TriPure Isolation reagent (a solution containing phenol and guanidinium thiocyanate; Roche Diagnostics Inc., Basel, Switzerland). For amplification and quantitation of the cDNA fragments encoding ghrelin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the two-step reverse-transcription polymerase chain reaction (RT-PCR) method was carried out. First-stand cDNAs were synthesized from 1 μg of total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time, Takara, Tokyo, Japan) and the Oligo$(dT)_{15}$ primer. The reaction mixtures consisted of 250 nM each primer and template (100 ng total RNA equivalent) in 1× master mix. Reverse