# Human ghrelin modifies lipid metabolism of the common frog *Rana temporaria*

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A b s t r a c t . The functions of ghrelin, a novel weight-regulatory peptide, have not been intensively investigated in amphibians. The aim of this experiment was to study the effects of exogenous, mammalian ghrelin on the carbohydrate and lipid metabolism of two Palearctic Anurans: the common toad (*Bufo bufo*) and the common frog (*Rana temporaria*). Twenty-eight common toads and 20 common frogs were injected daily with purified human Ser3-acylated ghrelin at 10 $\mu$g kg$^{-1}$ d$^{-1}$ or with 0.9 % saline into the dorsal lymph sac for four days. Exogenous ghrelin decreased the plasma total cholesterol (Chol) and LDL-Chol concentrations and increased the HDL-LDL ratios of the common frogs. The liver lipase activities decreased but the fat body lipase activities increased due to the ghrelin treatment. Ghrelin did not influence the measured variables in the common toad. In addition to stimulating growth hormone and prolactin secretion in amphibians, ghrelin also influences their lipid metabolism.

**Key words**: amphibians, *Bufo bufo*, carbohydrate metabolism, weight-regulatory hormones

## Introduction

Ghrelin is a novel weight-regulatory peptide characterized from teleost fish (U n n i a p p a n et al. 2002) to mammals (K o j i m a et al. 1999). In 2001, amphibian ghrelin was identified from the bullfrog *Rana catesbeiana* (K a i y a et al. 2001). In fact, the bullfrog has three forms of ghrelin, each consisting of 27–28 amino acids. They share 29 % sequence identity with mammalian peptides, the N-terminal amino acids 1 and 4–7 being highly conserved. Similar to mammals (D a t e et al. 2000), bullfrog ghrelin is synthesized mainly in the stomach, while lower levels of gene expression are located e.g. in the small intestine, pancreas, heart and testes (K a i y a et al. 2001). In the European green frog (*R. esculenta*), a ghrelin-like immunoreactivity has been detected in the stomach mucosa and in several locations of the brain (G a l a s et al. 2002).

Mammalian ghrelin is a lipogenic hormone which induces weight gain based on accretion of fat mass without changes in longitudinal skeletal growth or in lean muscle mass (M u c c i o l i et al. 2002). The increased fat mass results from ghrelin-induced stimulation of appetite, decreased energy expenditure and increased respiratory quotient indicating a shift from fat to carbohydrate utilization. Blood ghrelin concentrations correlate inversely with body adiposity (A r i y a s u et al. 2001), increase by fasting and reduce by re-feeding (T s c h ö p et al. 2000). Ghrelin may play a role in the process of adipogenesis in rodents as it has been shown to stimulate the differentiation of preadipocytes (C h o i et al. 2003). Furthermore, ghrelin has been demonstrated to antagonize isoproterenol-induced lipolysis *in vitro*.

Ghrelin stimulates growth hormone (GH) secretion in mammals (K o j i m a et al. 1999). In birds mammalian ghrelin increases GH release (A h m e d & H a r v e y 2002)

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but decreases appetite (F u r u s e et al. 2001), which is also strongly suppressed by bullfrog ghrelin (S a i t o et al. 2002). The appetite-stimulatory effect of ghrelin has been documented in teleost fish treated with fish ghrelin (U n n i a p p a n et al. 2002). Mammalian ghrelin increases their GH and prolactin secretion and decreases water intake (R i l e y et al. 2002, K o z a k a et al. 2003). Ghrelin also induces fat accumulation in liver and muscle of teleost fish (R i l e y et al. 2005). Bullfrog ghrelins have been shown to stimulate GH and prolactin secretion of the bullfrog *in vitro* (K a i y a et al. 2001). The other functions of ghrelin in amphibian physiology have not yet been investigated. The aim of this experiment was to study the effects of exogenous, mammalian ghrelin on the carbohydrate and lipid metabolism of two Anuran amphibians with a wide Palearctic distribution: the common toad (*Bufo bufo*; Bufonidae) and the common frog (*R. temporaria*; Ranidae).

## Material and Methods

For this study, 28 common toads and 20 common frogs were collected by hand in Northern Karelia, Finland (62º30´N; 30ºE) during the summer and autumn 2002. The Animal Care and Use Committee of the University of Joensuu approved all procedures. The animals were housed in groups of 2–4 individuals in terrariums (45 × 31 × 35 cm) equipped with sand, plant material and water pools in 12L:12D (lights on at 08.00 am) at 20 ± 1 ˚C. Before the beginning of the experiment they were fed with earthworms (*Lumbricus* spp.) every other day, but during the study period they were fasted. On Oct 18th 2002 three days before the beginning of the study, the toads were weighed, and the animals of different body mass (BM) were randomly assigned either to the control group (n = 14; 4 mature and 4 immature females, 5 mature and 1 immature males) or to the ghrelin-treated group (n = 14; 7 mature and 2 immature females, 5 mature males).

Between Oct 21st–24th 2002 the ghrelin group received purified human Ser3-acylated ghrelin (Alpha Diagnostics Intl. Inc., San Antonio, TX, USA) diluted in 0.9 % saline into the dorsal lymph sac at 10 µg $kg^{-1} d^{-1}$ (average volume injected 90 µl). A mammalian ghrelin was chosen as there does not exist a commercial amphibian ghrelin, and the N-terminal amino acids 1 and 4–7 are identical in human and bullfrog peptides (K o j i m a et al. 1999, K a i y a et al. 2001). The injections were given daily between 08.00–09.00 am and the control group received equivolume injections of saline. A similar study procedure was repeated for the control (n = 10; 5 mature and 3 immature females, 2 mature males) and the ghrelin-treated (n = 10; 4 mature and 2 immature females, 4 mature males) common frogs between Oct 28th–31st 2002. As their BMs were generally lower than those of the toads, the volume injected to lymph sacs was approximately 65 µl.

During the study, the BMs of the animals were recorded daily before the injections were given. On Oct 24th (the toads) and on Oct 31st (the frogs), the animals were given their last ghrelin dose between 09:00–12:00 am in order to keep the time interval between the injection and sampling constant for each individual. Within the next hour, they were euthanized with diethyl ether. Their BMs and lengths were measured. Blood samples were obtained with cardiac punctures with sterile needles and syringes into test tubes containing 5 % EDTA and centrifuged at 4000 g for 20 min (+23 ºC) to obtain plasma. The sex and maturity of the animals were determined intraabdominally. The livers, kidneys, fat bodies and gonads were dissected and weighed and all the samples were frozen in liquid nitrogen and stored at –40 ˚C.

The different enzyme activities were determined spectrophotometrically. The liver, kidney and fat body (i.e. white adipose tissue, WAT) samples were weighed and homogenized in cold citrate buffer in pH 6.5 for the glucose-6-phosphatase (G6Pase; liver and kidney) and

in cold 0.85 % NaCl for the lipase measurements (liver and WAT). The activity of G6Pase was measured using glucose-6-phosphate as substrate in the presence of EDTA after an incubation time of 30 minutes at 25 °C (H e r s & v a n H o o f 1966). The lipase activity was measured according to the method of S e l i g m a n & N a c h l a s (1962) using 2-naphthyl laurate without taurocholate as substrate. The glycogen concentrations in the livers were measured spectrophotometrically according to the method of L o et al. (1970).

Plasma total cholesterol (Chol) was determined with the Cholesterol enzymatic endpoint method of the Randox Laboratories Ltd. (Crumlin, UK). Plasma low-density-lipoprotein (LDL) Chol and high-density-lipoprotein (HDL) Chol levels were measured with the Direct LDL- and HDL-cholesterol reagents of the Randox Laboratories Ltd. Plasma triacylglycerol and glucose levels were measured with the Triglycerides GPO-PAP and Glucose liquid reagent hexokinase methods (Randox Laboratories Ltd.). Also the Total protein biuret method, Ammonia enzymatic UV-method and Uric acid enzymatic colorimetric method were purchased from the Randox Laboratories Ltd. For the actual measurements the Technicon RA-XT™ Analyser (Swords, Dublin, Ireland) was used.

Comparisons between the study groups were performed with the Student's t-test or with the Mann-Whitney U test for parametric and nonparametric data, respectively. The $p < 0.05$ level was considered to be statistically significant. The results are expressed as mean ± SE.

## Results and Discussion

Peripheral ghrelin affected the variables of fat metabolism of the common frogs, but the BMs, organ weights and the parameters of glucose and nitrogen metabolism of the frogs and toads remained unaffected after the acute ghrelin treatment (Table 1). The relative mass of the fat body of the frogs did not respond to ghrelin, but the treatment affected their plasma Chol profile. As the total Chol and LDL-Chol concentrations of the frogs decreased with a simultaneous increase in the ratio of HDL- to LDL-Chol, these changes could be considered beneficial from the human point of view. In a previous study (N i e m i n e n & M u s t o n e n 2004), peripheral ghrelin increased the plasma HDL-Chol concentrations of tundra voles *Microtus oeconomus* without affecting their total Chol or LDL-Chol levels.

In addition to the plasma Chol status of the common frogs, ghrelin also affected their tissue lipase activities estimating the overall lipolytic activity within a tissue. Opposite changes were observed in the liver and WAT with decreased and increased activity levels, respectively. The decrease in the lipolytic activity of the liver could indicate decreased lipid turnover caused by the ghrelin administrations. Ghrelin has previously induced a lipogenic pattern of gene expression in rodent liver by increasing the transcript levels of lipogenic enzymes and by decreasing those of the rate-limiting enzyme of lipid oxidation (B a r a z z o n i et al. 2005). The suppression of the liver lipase activities of the frogs fits to these earlier results. In contrast, increased lipase activities have been previously measured from the livers of ghrelin-treated tundra voles (N i e m i n e n & M u s t o n e n 2004). The higher lipase activities in the WAT of the ghrelin-treated frogs may indicate ghrelin-induced stimulation of lipolysis in adipose tissue increasing the availability of fatty acids as metabolic fuel for other tissues.

In contrast to the common frogs, the measured physiological variables of the common toads did not respond to the exogenous ghrelin treatment. The reasons for this difference remain obscure, but it could have been caused by the different nutritional statuses of the animals. During the acclimation period to laboratory conditions it was observed that the food intake of the common toads greatly surpassed the appetite of the frogs, which could

**Table 1.** Measured variables of the common frogs and toads according to the ghrelin treatment (mean ± SE). BM=body mass, P=plasma, Chol=cholesterol, HDL=high-density-lipoprotein, LDL=low-density-lipoprotein, L=liver, K=kidney, G6Pase=glucose-6-phosphatase, WAT=white adipose tissue.

| | Frog control | Frog ghrelin | Toad control | Toad ghrelin |
|---|---|---|---|---|
| BM g beginning of study | 25.0 ± 2.4 | 28.1 ± 2.8 | 25.8 ± 3.9 | 26.9 ± 3.9 |
| BM change g | -0.27 ± 0.24 | -0.57 ± 0.29 | -1.06 ± 0.57 | -0.47 ± 0.50 |
| BM g end of study | 24.7 ± 2.6 | 27.6 ± 2.6 | 24.8 ± 3.6 | 26.4 ± 3.9 |
| Body length cm | 7.0 ± 0.3 | 6.7 ± 0.7 | 6.5 ± 0.3 | 6.5 ± 0.3 |
| Fat body mg | 37 ± 6 | 32 ± 5 | 272 ± 105 | 271 ± 138 |
| Fat body $BM^{-1}$ % | 0.16 ± 0.04 | 0.12 ± 0.02 | 0.86 ± 0.25 | 0.71 ± 0.26 |
| Gonad g | 1.91 ± 0.63 | 1.67 ± 0.62 | 0.91 ± 0.40 | 1.24 ± 0.65 |
| Gonad $BM^{-1}$ % | 7.0 ± 1.8 | 5.3 ± 1.7 | 3.1 ± 1.1 | 3.0 ± 1.2 |
| Liver mg | 435 ± 54 | 446 ± 51 | 692 ± 134 | 839 ± 162 |
| Liver $BM^{-1}$ % | 1.84 ± 0.02 | 1.64 ± 0.02 | 2.63 ± 0.17 | 2.95 ± 0.27 |
| P glucose mmol $l^{-1}$ | 4.02 ± 0.57 | 4.11 ± 0.43 | 2.13 ± 0.30 | 1.90 ± 0.19 |
| P uric acid $\mu$mol $l^{-1}$ | 125.2 ± 39.7 | 79.8 ± 18.6 | 55.6 ± 10.2 | 42.2 ± 5.7 |
| P ammonia $\mu$mol $l^{-1}$ | 156.8 ± 20.5 | 149.7 ± 7.2 | 36.0 ± 2.1 | 34.8 ± 3.1 |
| P total protein g $l^{-1}$ | 43.3 ± 4.5 | 34.2 ± 3.7 | 26.6 ± 3.3 | 22.5 ± 2.9 |
| P total Chol mmol $l^{-1}$ | 3.23 ± 0.78 | 1.27 ± 0.22* | 1.85 ± 0.42 | 1.38 ± 0.23 |
| P HDL-Chol mmol $l^{-1}$ | 0.34 ± 0.09 | 0.22 ± 0.03 | 0.39 ± 0.09 | 0.31 ± 0.03 |
| P LDL-Chol mmol $l^{-1}$ | 1.19 ± 0.29 | 0.48 ± 0.06* | 0.73 ± 0.18 | 0.55 ± 0.08 |
| P HDL-Chol LDL-$Chol^{-1}$ | 0.29 ± 0.03 | 0.44 ± 0.03* | 0.57 ± 0.05 | 0.65 ± 0.07 |
| P HDL-Chol total $Chol^{-1}$ | 0.12 ± 0.01 | 0.30 ± 0.12 | 0.23 ± 0.02 | 0.24 ± 0.03 |
| P triacylglycerols mmol $l^{-1}$ | 0.21 ± 0.05 | 0.15 ± 0.04 | 0.38 ± 0.10 | 0.16 ± 0.05 |
| L glycogen $\mu$g $mg^{-1}$ | 36.1 ± 11.6 | 40.5 ± 9.0 | 22.0 ± 6.3 | 25.0 ± 6.2 |
| L G6Pase $\mu$g P $mg^{-1}$ $h^{-1}$ | 6.3 ± 0.9 | 4.8 ± 0.5 | 34.8 ± 3.3 | 30.7 ± 3.2 |
| K G6Pase $\mu$g P $mg^{-1}$ $h^{-1}$ | 3.41 ± 0.17 | 3.22 ± 0.18 | 5.65 ± 0.67 | 5.10 ± 0.33 |
| L lipase $\mu$g 2-naphthol $mg^{-1}$ $h^{-1}$ | 7.46 ± 0.67 | 5.17 ± 0.67* | 17.22 ± 1.63 | 19.26 ± 2.66 |
| WAT lipase $\mu$g 2-naphthol $mg^{-1}$ $h^{-1}$ | 6.11 ± 1.21 | 9.59 ± 0.68* | 10.66 ± 2.10 | 13.21 ± 2.02 |

* t-test, Mann-Whitney U test $p < 0.05$ between the control and the ghrelin-treated frogs

have affected their responses to ghrelin, a peptide of nutritional scarcity in mammals. The different nutritional status was clearly reflected in the relative masses of the fat bodies, which were approximately 0.14 % in the frogs but several fold higher in the toads.

Investigations about the functions of ghrelin in amphibians have been scarce. By now it is known that ghrelin can affect the release of GH and prolactin in the bullfrog (K a i y a et al. 2001). The present study adds evidence that ghrelin is able to influence the lipid metabolism of Anurans, as it suppressed the lipid mobilization in the liver of the common frog. However, unlike observed previously in mammals (N i e m i n e n & M u s t o n e n 2004), a simultaneous increase in the WAT lipase activity could be detected. The common frog does not seem to experience a ghrelin-induced shift from lipid to carbohydrate utilization unlike rodents (M u c c i o l i et al. 2002).

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