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Fasting Reduces Plasma Leptin- and Ghrelin-Immunoreactive Peptide Concentrations of the Burbot (*Lota lota*) at 2°C But Not at 10°C

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ABSTRACT—The effects of fasting at two water temperatures (2 and 10°C) on plasma leptin- and ghrelin-immunoreactive peptide concentrations and energy metabolism were investigated in the burbot (*Lota lota*), a freshwater gadoid, which lives in cold waters and tolerates long periods of fasting. Burbot were assigned to fed and fasted groups at 2 and 10°C. Leptin- and ghrelin-immunoreactive peptides were identified in burbot plasma. Fasting at 2°C reduced the plasma leptin- and ghrelin-immunoreactive peptide concentrations and reduced the relative liver weights and the liver and muscle glycogen concentrations. The concentrations of the leptin- and ghrelin-immunoreactivities correlated positively with each other. At 10°C there were decreased plasma thyroxine levels in both sexes, and slightly lower plasma testosterone concentrations in males. The results support previous studies in stating that immunoreactivities resembling mammalian leptin and ghrelin can be detected in burbot plasma and liver, but the specific functions of these peptides will have to be determined in future studies.

Key words: fasting, ghrelin, leptin, *Lota lota*, temperature

INTRODUCTION

The burbot (*Lota lota*) is a stenothermal freshwater gadoid with a Holarctic distribution (Muus, 1968; Bernard *et al*., 1993). It feeds on zooplankton, invertebrates and fish depending on the season and the stage of its life history and survives several weeks without feeding (Pääkkönen, 2000). The species is rarely observed at temperatures higher than 12°C. It is also well adapted to darkness and able to catch pray successfully in lake-bottom conditions.

Leptin is an adipocyte-derived hormone distributed in many phyla (Zhang *et al*., 1994). In mammals, exogenous leptin reduces feeding and body mass (BM) (Pelleymounter *et al*., 1995), and the plasma leptin concentrations correlate with body adiposity (Maffei *et al*., 1995). Low temperature suppresses leptin gene expression in rodents via the sympathetic nervous system (Trayhurn *et al*., 1995; Li *et al*., 1997). Leptin has been discovered also in reptiles and birds (Ashwell *et al*., 1999; Niewiarowski *et al*., 2000). In fish, leptin concentrations are reduced by fasting; and brain, but not blood leptin levels correlate with body fat content (Johnson *et al*., 2000). Exogenous human leptin has no influence on the BM or lipid and carbohydrate metabolism of the immature Coho salmon (*Oncorhynchus kisutch*; Baker *et al*., 2000).

The actions of leptin are antagonized by ghrelin (Shintani *et al*., 2001) secreted by the stomach and the hypothalamus (Kojima *et al*., 1999; Date *et al*., 2000). Circulating ghrelin concentrations correlate negatively with body adiposity in humans (Tschöp *et al*., 2001). Exogenous ghrelin increases feeding and BM, and inhibits lipolysis in rodents (Tschöp *et al*., 2000). Recently, ghrelin has been identified from the bullfrog (*Rana catesbeiana*) with 29% sequence identity to mammalian ghrelins (Kaiya *et al*., 2001). Ghrelin cDNA has been also identified and characterized from some teleosts. Goldfish (*Carassius auratus*) ghrelin has 47% similarity with the amino acid sequence of human ghrelin (Unniappan *et al*., 2002). In the Japanese eel (*Anguilla japonica*), the overall similarity is the same, but the first seven amino acids are 100% identical to mammalian ghrelins and eel ghrelin has the ability to stimulate growth hormone (GH) and prolactin release from the pituitary (Kaiya *et al*., 2003). The same effect can be induced *in vitro* in the tilapia (*Oreochromis mossambicus*) with rat ghrelin (Riley *et al*., 2002). This suggests that ghrelin peptide and its function in GH secretion are evolutionarily quite conserved. Yet the functions of leptin and ghrelin in weight regulation vary in different vertebrates demonstrated by previous observations on leptin- and ghrelin-immunoreactivities in burbot plasma with increasing levels after reproduction at a time of decreased

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BM and liver adiposity (Mustonen *et al.*, 2002a, b).

The aim of this study was to investigate the possible functions of endogenous leptin and ghrelin in the weight regulation of fish. As the burbot is well adapted to low water temperatures and prolonged fasting (Pääkkönen, 2000), it is an attractive model to study the effects of fasting and temperature on these peptides. Several variables of energy metabolism were also studied.

MATERIALS AND METHODS

Burbot (n=43, 30 males and 13 females) were caught with nets from Lakes Haukivesi and Pyhäselkä, Eastern Finland (62°30'N) during the spawning season of the species in February 2001. During the experiment all the fish were post-spawn. The fish were randomly divided into four groups consisting of 9–12 individuals. They were placed into two 750 L tanks (1.1×1.1×0.62 m) with water flow of 1.5 l min^{-1}. The fasting and the control groups of a particular temperature were kept in the same tank separated by a net. Tanks were fitted with lids to maintain darkness and simulate conditions below the ice cover. The procedures conformed to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and were approved by the Animal Care and Use Committee of the University of Joensuu.

Two groups of fish were acclimated to +2°C (close to the natural lake-bottom conditions throughout the year) and two groups to

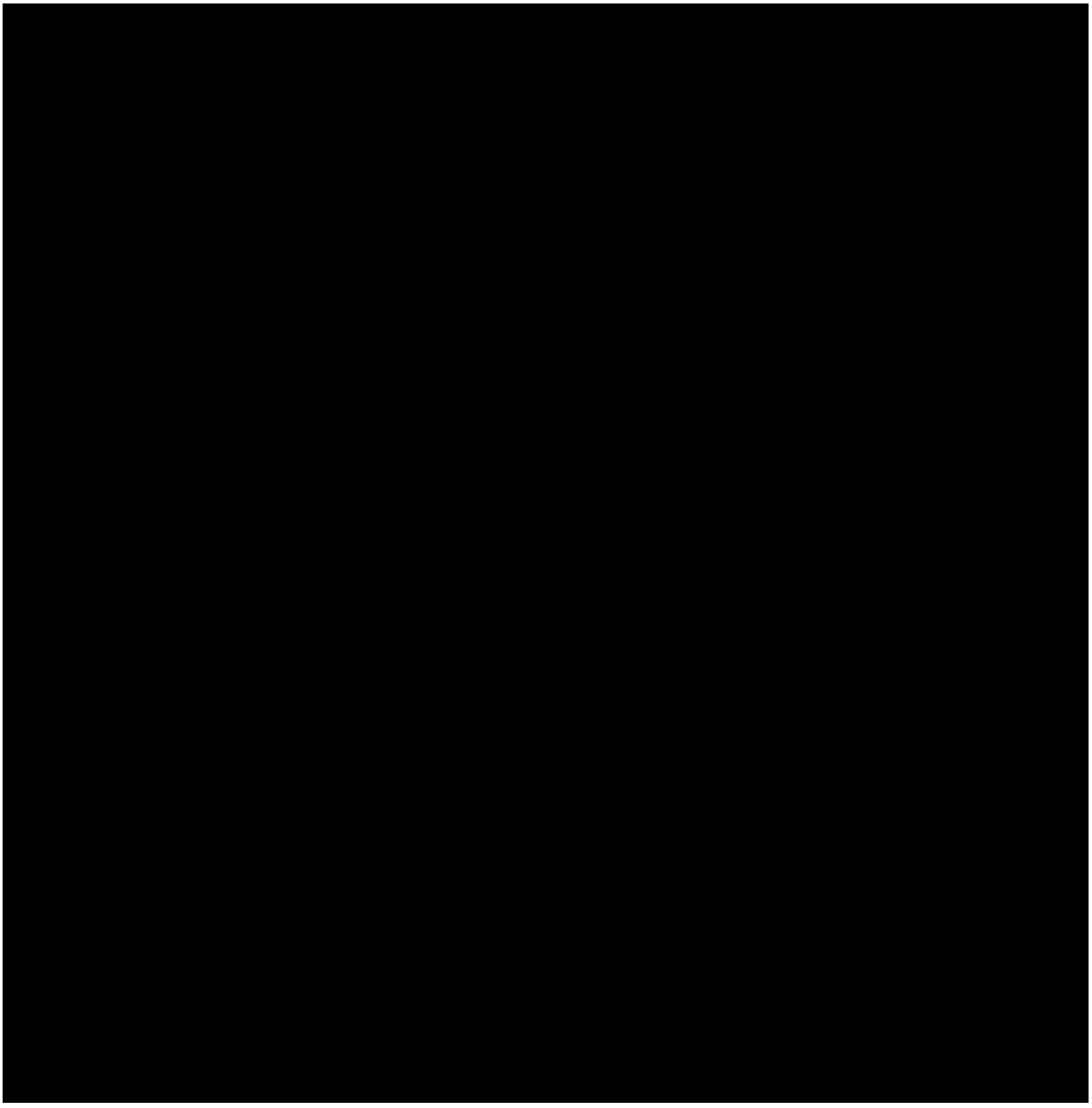

Fig. 1. Standard curves for leptin (A–B) and ghrelin (C) and the corresponding dose-response curves of serial dilutions of burbot plasma (A, C) or liver homogenate (b, plasma leptin: 50, 100 and 200 μl; liver leptin: 25, 50, 100 and 200 μl; ghrelin: 25, 50, 100 and 200 μl), B=sample or standard binding, B_0 = maximun binding.

+10°C (the temperature above which adult burbots are seldom encountered). The fish were weighed at the beginning and end of the study period. One group at each temperature was fed with fresh vendace (*Coregonus albula*, 98.7 kcal energy, 19.1 g protein, 2.4 g fat 100 g^{-1}) cut into pieces of 2–3 cm every other day to satiation, the other two groups of fish were fasted for two weeks. A two-week period was considered to be long enough for the metabolic responses to fasting to appear. The abbreviations for these study groups are the following: C2 (control burbots at + 2°C; n=12, 9 males and 3 females), F2 (fasting burbots at + 2°C; n=12, 9 males and 3 females), C10 (control burbots at + 10°C; n=9, 6 males and 3 females) and F10 (fasting burbots at + 10°C; n=10, 6 males and 4 females).

The relative food intake (kcal kg BM^{-1} d^{-1}) of the fed study groups was recorded every other day 24 hr after feeding. After two weeks, the fish were euthanized by a blow on the head. They were weighed and their total length was measured. Blood samples were obtained from the ventral aorta with aseptic needles into test tubes containing EDTA and centrifuged at 1000 *g* to obtain plasma. The presence of feed in the stomach and intestine was verified in the control groups. The livers and trunk white muscle samples from the dorsal muscles were dissected and immediately frozen in liquid nitrogen and stored at –40°C.

The different enzyme activities were determined spectrophotometrically. The liver and muscle samples were weighed and homogenized. The homogenization was carried out in cold citrate buffer (21.008 g citric acid, 200 ml 1 M NaOH, H_2O ad 1000 ml; 0.1 M NaOH until pH 6.5) for the glucose-6-phosphatase (G-6-Pase) (pH 6.5) and the glycogen phosphorylase measurements (pH 6.1). The activity of G-6-Pase was measured using glucose-6-phosphate as substrate in the presence of EDTA after an incubation time of 30 minutes at 25°C (Hers and van Hoof, 1966). The glycogen phosphorylase activity was measured in the presence of glucose-1-phosphate, glycogen, sodium fluoride and AMP (Hers and van Hoof, 1966). The homogenization was carried out in cold 0.85% sodium chloride for the lipase esterase measurement. The lipase esterase activities were measured according to the method of Seligman and Nachlas (1962) using 2-naphthyl-laurate without taurocholate as substrate. The glycogen concentrations were measured spectrophotometrically according to the method of Lo *et al.* (1970).

Measurement of the plasma leptin concentrations was carried out with the Multi-species Leptin RIA kit (intraassay variation 2.8–3.6% CV, interassay variation 6.5–8.7% CV; Linco Research Inc., St. Charles, MO, USA) and plasma ghrelin using the Ghrelin (Human) RIA kit (<5%, <14%; Phoenix Pharmaceuticals Inc., Belmont, CA, USA). For the measurement of the liver leptin concentrations, 200 µg liver was homogenized in 1 ml leptin assay buffer (0.05 M phosphosaline pH 7.4 containing 0.025 M EDTA, 0.1% sodium azide, 1% RIA grade bovine albumin and 0.05% Triton X-100). These assays were validated such that serial dilutions of burbot plasma or liver homogenate showed linear changes in B B_0^{-1} values that were parallel with the standard curves produced with human standards (Fig. 1A–C) described also in a previous study (Mustonen *et al.*, 2002a, b). Plasma testosterone, estradiol and thyroxine (T4) concentrations were determined with the Spectria [^{125}I] Coated Tube Radioimmunoassay kits of Orion Diagnostica (Espoo, Finland). Plasma thyroid stimulating hormone (TSH) was determined using the immunoradiometry method (Spectria TSH [^{125}I] Coated Tube Immunoradiometric assay, Orion Diagnostica).

The Fulton's Condition Factor (FCF) was calculated by the formula: 100×, (weight (g) $length^3$ $(cm)^{-1}$) (Ricker, 1975). The multiple comparisons were performed with the SPSS program using the one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan's test. Comparisons between fed and fasted fish or between fish kept at different temperatures were performed with the Student's t-test for two independent samples. The normality of distribution and the homogeneity of variances were tested with the Kolmogorov-Smirnov test and the Levene test. For nonparametric data (normality of distribution not accomplished after standard transformations), the Mann-Whitney U test was performed. Correlations were calculated using the Spearman's correlation coefficient (r_s). The p value less than 0.05 was considered to be statistically significant. The results are presented as mean±SE.

RESULTS

Water temperature did not affect the BMs, FCFs, liver weights, liver or muscle glycogen contents, liver G-6-Pase or lipase esterase activities, muscle phosphorylase activities, plasma ghrelin-immunoreactive peptide, estradiol or TSH levels, or plasma or liver leptin-immunoreactive peptide levels of the burbots (Table I). The water temperature had no effect on the cumulative food intake of the fish, either (C2: 108±32; C10: 170±44 g (kg BM)$^{-1}$ during the whole study). The liver glycogen phosphorylase activities of the F2 group were lower than those of the F10 group (ANOVA, $p<0.048$). The T4 concentrations of the burbots decreased with increasing temperature (*t*-test, $p<0.05$; Table I). In the plasma testosterone concentrations of the male burbots there was a nonsignificant trend towards lower levels at 10°C (Mann Whitney U test, $p<0.056$). The liver leptin-immunoreactive peptide levels were slightly lower at 2°C than at 10°C (*t*-test, $p<0.056$).

The fed animals gained some weight (24±10.9 g, 4% of BM) at +2°C, while the fasted animals lost about 10±4.7 g (2% of BM) during the study period. At +10°C the BM of both the fed (–20±27.9 g, 4% of BM) and fasted animals (–60±39.2 g, 12% of BM) decreased (Table I). The relative liver weights (*t*-test, $p<0.028$) and the liver (*t*-test, $p<0.045$) and muscle glycogen contents (Mann-Whitney U test, $p<0.015$) also decreased due to fasting. Fasting also reduced the FCFs at +10°C (*t*-test, $p<0.049$) but not at +2°C (Table I). The liver glycogen phosphorylase activities increased due to fasting at +10°C (*t*-test, $p<0.045$) but not at +2°C. Fasting did not affect the liver G-6-Pase or lipase esterase activities, muscle phosphorylase activities, plasma testosterone, estradiol or T4 levels.

The liver leptin-immunoreactivity concentrations were the same in the fed and fasted fish, but plasma leptin-immunoreactive peptide concentrations were lower in the fasted group compared to the fed group at 2°C (*t*-test, $p<0.003$) but did not reach significance at 10°C. When all data of both temperatures were analyzed together, the difference between the plasma leptin-immunoreactive peptide levels between the fed and fasted groups was significant (*t*-test, $p<0.04$; Table I). Water temperature *per se* had no effect on the leptin-immunoreactive peptide levels, but when all the animals from both temperatures and feeding regimes were analyzed together, the plasma leptin-immunoreactive peptide concentrations were higher in the female burbots (2.92±0.22 vs. 2.34±0.11 ng ml^{-1}; *t*-test, $p<0.011$). Also the mean relative liver weight was higher in the females (*t*-test, $p<0.016$). There was no sexual dimorphism in the other variables.

The plasma ghrelin-immunoreactive peptide levels were

Table 1. Body mass, length, liver weight, concentrations of hormones and liver (L) or muscle (M) enzyme activities and glycogen content of the burbots at 2°C or at 10°C, mean±SE.

	Controls 2°C	Fasters 2°C	Controls 10°C	Fasters 10°C
BM	578±67	542±70	498±52	511±55
change in BM in two weeks (g/%)	+24±11 (4)	–10±5 (2)	–20±28 (4)	–60±39 (12)
FCF (g $(cm^3)^{-1}$)*100	0.53 ± 0.03^{AB}	0.50 ± 0.02^{AB}	0.54 ± 0.02^{B}	0.49 ± 0.01^{A}
Length (cm)	47.3±1.3	46.8±1.4	44.8±1.3	46.4±0.7
Liver weight/body mass (%)	4.8±0.5	3.8±0.3*	5.2±0.6	4.3±0.4*
Plasma leptin-immunoreactive peptide (ng ml^{-1})	2.87 ± 0.11^{B}	$2.23\pm0.16^{*A}$	2.58 ± 0.25^{AB}	$2.37\pm0.30^{*A}$
Liver leptin-immunoreactive peptide (ng g^{-1})	7.16±0.38	7.60±0.87	8.62±0.96	8.88±0.42
Ghrelin-immunoreactive peptide (ng ml^{-1})	0.36 ± 0.08^{B}	$0.16\pm0.04^{A*}$	0.43 ± 0.01^{B}	$0.30\pm0.06^{AB*}$
T4 (nmol l^{-1})	12.38 ± 1.18^{B}	7.87 ± 1.73^{AB}	$7.39\pm1.78^{AB\dagger}$	$5.97\pm1.99^{A\dagger}$
TSH (mIU l^{-1})	0.14±0.05	0.14±0.04	0.12±0.05	0.06±0.04
Testosterone (nmol l^{-1}) (only males)	0.46±0.17	1.09±0.62	$0.16\pm0.09^{\dagger}$	$0.16\pm0.08^{\dagger}$
Estradiol (nmol l^{-1}) (only females)	21.9±1.4	19.0±4.1	22.6±2.7	17.8±2.4
L glycogen (μg mg $liver^{-1}$)	7.96±1.09	6.78±0.92	15.61± 5.30	8.22±2.10
L glycogen/liver/BM (μg $liver^{-1}$ g BM^{-1})	0.41±0.08	0.28±0.05*	0.81±0.25	0.35±0.09*
M glycogen (μg mg $tissue^{-1}$)	0.80±0.06	0.72±0.15*	1.31±0.54	0.44±0.05*
L phosphorylase (μg P mg $tissue^{-1}$ hr^{-1})	10.37 ± 1.58^{AB}	9.32 ± 1.41^{A}	9.26 ± 1.27^{A}	12.19 ± 0.60^{B}
M phosphorylase (μg P mg $tissue^{-1}$ hr^{-1})	13.35±1.31	12.26±1.31	11.62±1.50	13.73±1.81
L G6Pase (μg P mg $tissue^{-1}$ hr^{-1})	16.83±1.67	20.50±1.19	19.90±2.04	18.77±0.88
L Lipase esterase/liver/BM (μg 2-naphthol $liver^{-1}$ g BM^{-1} hr^{-1})	4.34±0.48	4.43±0.22	4.09±0.32	4.89±0.66

The means with dissimilar superscripts differ at p<0.05 (ANOVA).
*=The fasting groups together differ from the control groups together (*t*-test, $p<0.05$, Mann-Whitney U-test, $p<0.05$).
†=The groups at 10°C together differ from the groups at 2°C together (*t*-test, Mann-Whitney U test, $p<0.05$).

lower in the fasted group compared to the fed group at 2°C (*t*-test, $p<0.04$). When all the fasted animals were compared to all the fed animals, the difference was also significant (*t*-test, $p<0.05$; Table 1). Water temperature had no effect on the ghrelin-immunoreactive peptide concentrations. When all the material was analyzed together, the ghrelin-immunoreactive peptide levels correlated significantly with the plasma leptin-immunoreactive peptide concentrations ($r_s=0.363$, $p<0.05$). The plasma T4 concentrations correlated significantly with the leptin-immunoreactive peptide levels, when all the material was analyzed together ($r_s=0.382$, $p<0.05$). This was not due to fasting as the correlation was significant also in the control groups ($r_s=0.458$, $p<0.05$). The leptin-immunoreactive peptide levels also correlated with the plasma TSH levels ($r_s=0.427$, $p<0.05$).

DISCUSSION

In humans and rodents leptin concentrations correlate positively with body adiposity, are reduced by fasting and increase in re-feeding, but ghrelin concentrations react in the opposite manner. The situation is different for the burbot as previously suggested by Mustonen *et al.* (2002a) with increased plasma leptin- and ghrelin-immunoreactivities after spawning, energetically the most expensive annual challenge of the species. The detected peptides have similarity to leptin and ghrelin, but are not necessarily exactly the same molecules with the same functions that mammalian leptin and ghrelin have.

The recorded values for leptin- and ghrelin-immunoreactive peptides in this study confirm previous observations in burbots during the reproductive period (Mustonen *et al.*, 2002a, b). The plasma leptin-immunoreactivity concentrations varied between 1.7 and 2.0 ng ml^{-1} before and during reproduction but increased to approximately 2.8 ng ml^{-1} after spawning. This is similar to the average leptin-immunoreactivity values (2.2–2.8 ng ml^{-1}) of the present study. Furthermore, the average value of the F2 group — the experimental group with conditions most similar to the natural environment of the species during reproduction — was 2.2 ng ml^{-1}. This suggests that instead of body fat reserves, seasonal phenomena such as reproduction, could have a more pronounced influence on the regulation of leptin secretion in the burbot.

The lower leptin-immunoreactive peptide levels seen in the fasted burbots at 2°C have been previously observed in fasting humans (Kolaczynski *et al.*, 1996) and fish (Johnson *et al.*, 2000). The female burbots had significantly higher leptin-immunoreactivity levels than the males (see also Mustonen *et al.*, 2002a). This phenomenon has been described in various mammalian species with higher plasma leptin levels in females than in males (Maffei *et al.*, 1995; Considine

et al., 1996; Casabiell *et al*., 1998). In this respect, the detected leptin-immunoreactivity could have similar functions to mammalian leptin. The burbots still had quite high circulating estradiol levels at the time of the experiment — approximately four weeks after spawning. Thus estradiol may increase leptin-immunoreactive peptide concentrations in female fish as observed in women (Casabiell *et al*., 1998).

In mice, cold temperature suppresses the leptin gene expression probably via the sympathetic nervous system (Trayhurn *et al*., 1995). This did not happen in the poikilothermic fish. The connection observed between the plasma leptin-immunoreactivity and thyroid hormone levels in the burbots has, however, been previously observed *in vitro* also in mice (Yoshida *et al*., 1997). Leptin-immunoreactive peptide could also be measured in burbot liver supporting the previous results of Mustonen *et al.* (2002b) with similar average leptin-immunoreactivity concentrations (8–11 ng g^{-1} liver) to the values of this study. Previously leptin protein expression has been observed in the chicken (*Gallus domesticus*) liver, presumably due to its crucial role in avian lipogenesis (Taouis *et al*., 1998). As the liver is the principal fat store of the burbot (Karhapää, 1978), this could indicate that the liver is a main source of leptin in this species. This hypothesis will, however, need confirmation in future studies.

Previously Shepherd *et al.* (2000) have suggested that a ghrelin-like peptide could be expressed in teleost fish. This has been confirmed by later studies in the goldfish and the Japanese eel (Unniappan *et al*., 2002; Kaiya *et al*., 2003). The results of this study support previous findings that a peptide with similarities to mammalian ghrelin can be detected in burbot plasma with values on the same scale as in a previous study (0.35–0.76 ng ml^{-1}; Mustonen *et al*., 2002a). The circulating concentrations of this ghrelin-immunoreactive peptide were influenced by fasting but, unlike in mammals (Tschöp *et al*., 2000), the fasted fish had lower ghrelin-immunoreactive peptide concentrations than the fed fish. Nor did the positive correlation between the leptin- and ghrelin-immunoreactive peptide levels (see also Mustonen *et al*., 2002a) support the theory of these peptides being antagonistic to each other in teleosts (for mammals see Shintani *et al*., 2001). Although exogenous ghrelin increases the feeding of rodents (Tschöp *et al*., 2000) and goldfish when administered intracerebroventricularly (Unniappan *et al*., 2002), the effect of intracerebroventricular ghrelin in neonatal chicks is the opposite: it inhibits food intake in a dose-dependent manner (Furuse *et al*., 2001).

The differences in the leptin- and ghrelin-immunoreactive peptide levels between the fasted and the fed groups were more pronounced at 2°C than at 10°C. At the same time, fed burbots gained weight at 2°C but lost weight at 10°C. The colder temperature is consistent with the natural environment of the burbot. This indicates more effective assimilation of nutrients at the colder temperature, or alternatively, an increase in the basal metabolic rate at 10°C as often happens in poikilotherms.

The other physiological changes seen in the fasting burbots were mostly expected. The fasting burbots lost BM at a higher rate at 10°C than at 2°C. In concert with this, the FCFs were lower in the fasted burbots at 10°C. The BM of the fed fish decreased at 10°C, as well, but the decrease was much more pronounced in the fasting group. In nature, temperatures of 10°C cannot be encountered below the ice cover at the time of spawning, so the slighter BM loss at 2°C could represent the natural rate of weight loss of postspawn burbot. The observed decrease in relative liver weight and liver glycogen content is, of course, predictable in fasting. Also the muscle glycogen stores were depleted in the fasted groups. The liver glycogen phosphorylase activities, however, were higher in the fasted group at 10°C. This could simply indicate a higher metabolic demand associated with the higher ambient temperature. Glycogen phosphorylase is the first enzyme in the pathway of glycogen degradation (Harris, 1986). Fasting thus seems to increase the liver carbohydrate turnover rate more at warmer water temperatures.

The T4 levels were slightly lower in fasted fish indicating a trend towards reduced metabolic output in the fasting state as seen previously in other fish species (Matty, 1985). It has been observed previously in the rainbow trout (*O. mykiss*) that T4 levels increase significantly in transition from 18°C to 11°C (Matty, 1985). The burbots reacted similarly. The T4 levels were significantly higher in the fish kept at 2°C compared to the groups at 10°C. The profundal lifestyle of the burbot makes it an effective ambush predator at low-temperature conditions (Pääkkönen, 2000). Predation in the cold could explain the extra metabolic output provided by T4 at 2°C. Another hormonal variable affected by temperature in this study was the decreasing trend in the plasma testosterone concentrations of the male burbots at 10°C. The burbots spawn under the ice cover in the mid-winter in cold water (Muus, 1968). Although the fish were sampled after spawning, the testosterone levels remained higher in the males kept in cold water. It is thus possible that a warmer environment enhances the post-spawning decrease in sex steroid production in male burbots. As 2°C represents the natural temperature at this time of the year, the slower decline in sex hormone levels is probably encountered in nature.

The physiological changes seen after a two-week fast were not very dramatic indicating that the burbot is quite well adapted to food deprivation. For instance, the hepatic lipase esterase activity did not change, suggesting that the basal rate of lipolysis was sufficient to provide the burbot with adequate energy during the two-week fast. This is also supported by the unaffected liver G-6-Pase activities indicating that a two-week fast does not require increased gluconeogenesis in the burbot.

In summary, fasting decreased the plasma leptin- and ghrelin-immunoreactive peptide levels of the burbot at 2°C but not at 10°C. The functions of these peptides have some similarity to mammalian models, but the presumably differ-

ent structures of these peptides can partly explain the observed differences seen in the response of these peptides to fasting. Fasting reduced the liver and muscle glycogen stores. Yet the decrease in BM was more rapid at 10°C. Increasing temperature decreased the plasma T4 levels. As the burbot forages effectively at cold temperatures (Pääkkönen, 2000), it requires a relatively high metabolic output at 2°C.

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