Long-term fasting in the anadromous Arctic charr is associated with down-regulation of metabolic enzyme activity and up-regulation of leptin A1 and SOCS expression in the liver.

Short title: Arctic charr leptin and liver metabolism

Even Hjalmar Jørgensen[1†], Mads Martinsen[1], Vidar Strøm[1], Kristin Elisa Ruud Hansen[1], Chandra Sekhar Ravuri[1], Ningping Gong[2], Malcolm Jobling[1].

[1]*Faculty of Biosciences, Fisheries and Economy, Department of Arctic and Marine Biology, University of Tromsø, N-9037 Tromsø, Norway*
[2]*Fish Endocrinology Laboratory, Department of Zoology/Zoophysiology, University of Gothenburg, Box. 463, S-40530 Göteborg, Sweden.*

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[†]**Corresponding author:**

Dr. Even H Jørgensen

1Faculty of Biosciences, Fisheries and Economy, Department of Arctic and Marine Biology, University of Tromsø, N-9037 Tromsø, Norway
Tel: (47) 77 64 67 91; Fax: (47) 77 64 60 20; e-mail: even.jorgensen@uit.no

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Abstract

The life-strategy of the anadromous Arctic charr (*Salvelinus alpinus*) includes several months of voluntary fasting during overwintering in fresh water leading to emaciation prior to seawater migration in spring. In this study we compared changes in condition, substrate utilization and liver metabolism between captive anadromous charr subjected to food-deprivation during late winter and spring, and conspecifics fed in excess. In March, 9 out of the 10 sampled fed fish had not eaten, indicating that they were a voluntary anorexic state. In June, the fed fish were eating and all had higher body mass (BM), condition factor (CF) and adiposity than in March. In fasted fish there were only small decreases in BM, CF and adiposity between March and May, but all these parameters decreased markedly from May to June. The fasted fish were fat- and glycogen-depleted in June, had suppressed activity of hepatic enzymes involved in lipid metabolism (G6PDH and HOAD) and seemed to rely on protein-derived glucose as a major energy source. This was associated with up-regulated liver gene expression of *leptin A1*, *leptin A2*, *SOCS1*, *SOCS2* and *SOCS3*, and reduced *IGF-I* expression. In an *in vitro* study with liver slices it was shown that recombinant rainbow trout leptin stimulated *SOCS1* and *SOCS3* expression, but not *SOCS2*, *IGF-I* or genes of enzymes involved in lipid (*G6PDH*) and amino acid (*AspAT*) metabolism. It is concluded that liver leptin interacts with SOCS in a paracrine fashion to suppress lipolytic pathways and depress metabolism when fat stores are depleted.

1. Introduction

The anadromous life-strategy of Arctic charr (*Salvelinus alpinus*) is characterized by substantial seasonal changes in food intake, growth and adiposity. Most of the annual growth and energy accumulation occurs during a short summer seawater residence, whereas overwintering in fresh water is characterized by anorexia and depletion of energy reserves (Dutil, 1986; Boivin and Power, 1990; Jørgensen et al., 1997; Jobling et al., 1998). Captive offspring of anadromous Arctic charr also exhibit pronounced seasonal changes in food intake and growth when held at constant temperature and given food in excess (Tveiten et al., 1998), showing that the seasonal cycle of food intake and growth is under physiological control. In captive offspring of anadromous charr feeding commences in May (Tveiten et al., 1996; Bottengård and Jørgensen, 2008; Aarseth et al., 2010). In wild anadromous charr, appetite seems to return in early May (Aas-Hansen et al., 2005), whereas seaward migration takes place 3 to 4 weeks later (Aas-Hansen et al., 2005; Jensen et al., 2012). During the pre-migratory period the fish apparently find little food, and their condition factor continues to decrease until the time of downstream migration, when it may be as low as 0.70 (Nilssen et al., 1997; Aas-Hansen et al., 2005). Hence, the anadromous life-strategy of Arctic charr incorporates a prolonged, voluntary anorexia with emaciation, indicating high tolerance to food deprivation. As such, the anadromous charr represents an interesting model for investigation of mechanisms associated with long-term regulation of appetite and energy homeostasis.

Since the first identification of a gene encoding for a cDNA homologue to mammalian leptin (Lep) in pufferfish (*Takifugu rubripes*) (Kurokawa et al., 2005), *Lep* genes have been identified in many fish species, including the Arctic charr (Frøiland et al., 2010). In contrast to mammals, in which Lep is produced mainly in adipose tissue (Masuzaki et al., 1995), Lep is produced in numerous tissues in fish, including the liver (Wong et al., 2007; Kurokawa and

Murashita, 2009; Rønnestad et al., 2010; Tinoco et al., 2012; Trombley et al., 2012). Hepatic *LepA1* and *LepA2* expression increased towards the end of a 7 week period of feed restriction in Atlantic salmon (*Salmo salar*) (Trombley et al., 2012) and during the latter part of a 75 days period of feed deprivation in Arctic charr (Frøiland et al., 2012). Increased liver *LepA1* expression was observed without any change in plasma Lep concentrations (Frøiland et al., 2012; Kling et al., 2012), indicating a paracrine role for Lep in nutrient processing and regulation of endogenous energy reserves in the liver. A paracrine role for Lep in the fish liver is also supported by the presence of the *Lep receptor* (*LepR*) gene in this organ in several fish species (Liu et al., 2010; Rønnestad et al., 2010; Tinoco et al., 2012; Trombley et al., 2012).

We have studied the metabolic responses of the liver to long-term fasting in the Arctic charr, and have investigated whether Lep and the suppressor of cytokine stimulation (SOCS) may play a role in regulating these responses. Examination of *SOCS* gene expression was included in the study because SOCS is known to modulate the signalling of hormones regulating energy homeostasis such as growth hormone (GH) and Lep in mammals (Fujimoto and Naka, 2003).

2. Material and Methods

2.1. Experiment 1. Fish, experimental set-up and sampling

The experiment was carried out at Tromsø Aquaculture Research Station (TARS), Tromsø, Norway (69^{o}N), with hatchery reared offspring of anadromous Arctic charr derived from a broodstock captured in Lake Vårfluesjøen, Svalbard (79^{o}N) in 1990. The eggs hatched in winter 2008 and juveniles were held in fresh water at 6^{o}C under continuous light until July 2009, and thereafter at ambient water temperature and natural light (transparent roof) conditions until the start of the experiment. On March 8 and 9, 2010, a total of 360 individually tagged (Floy FTF-69

fingerling tags; MGF, Seattle) fish with an average body mass of 146 g were taken from a stock tank, anaesthetised in Benzocaine (60 ppm) and randomly distributed among 4, 300 litre circular tanks. The tanks were subjected to simulated natural light conditions (69^{o}N) and were supplied with fresh water at ca. 5^{o}C (ranging between 4.7 and 6.3^{o}C). The fish were provided with commercial dry-pellet feed (Nutra Parr; Skretting, Stavanger, Norway) in excess until March 24, after which the fish in two tanks were feed-deprived until the end of the experiment in June (FU fish). The fish in the other two tanks continued to be fed in excess throughout the experiment (FF fish). The experiment was terminated on June 17, when the condition factor [(Mass/Length^{3}) x 100] of the fasted fish was approaching that (0.70) of wild, anadromous Arctic charr when they descend to the sea.

On March 25, May 6 and June 17, a total of 5 randomly selected fish were quickly netted from each tank and killed with an overdose of Benzocaine (120 ppm). Blood was collected from the caudal vein using 2-ml, lithium-heparinized (30 USP units) vacutainers and held on ice until plasma was separated by centrifugation (6000 × *g*, for 10 min). Plasma was stored at -80^{o}C until analyses of cortisol and glucose concentrations were carried out. Thereafter, body mass (BM; g) and fork length (FL; cm) of the fish were measured before each fish was dissected. Liver weights were noted and three small pieces were removed. One piece was put in a tube with RNAlater (Ambion®, Life Technologies), and stored at -20^{o}C until analysis of *LepA1, LepA2, IGF-I* and *SOCS 1-3* gene expressions, and the other two were wrapped in aluminium foil and frozen at -80^{o}C for later analysis of glycogen and enzyme activities. The fish were then examined for feed in the stomach, sex and maturity status, before the body was wrapped in aluminium foil and stored at – 80 ^{o}C for later analysis of proximate chemical composition. After the sampling in March and May the remainder of the fish in each tank were anaesthetized in benzocaine for body mass and length measurement.

For the FF fish, 1 out of the 10 fish sampled in March had eaten and in May, 4 fish had eaten, whereas 6 had empty stomachs. In June, 7 out of the 10 sampled fish had well-filled stomachs. The FF fish were intended to simulate the seasonal feeding cycle of wild, anadromous Arctic charr, so the fish that was recorded as eating in March, and those that were anorexic in June, were excluded from the analyses. The FF fish sampled in May were also excluded, due to the low number of feeding fish and the heterogeneity of feeding behaviour within the group.

The experiment was approved by the Norwegian Committee on Ethics in Animal Experimentation (id. 2442).

2.2. Plasma cortisol and glucose, and liver glycogen and enzyme analyses

Plasma cortisol concentration was determined by radioimmunoassay (RIA), using an established protocol (Schultz, 1985) validated for Arctic charr (Tveiten et al., 2010). The detection limit for the assay was 3.0 ng $\cdot$ ml^{-1}. Plasma glucose concentration, given as nM, was measured spectrophotometrically using a commercial enzymatic kit (Randox HL 1611; Crumlin, UK). Liver glycogen concentration was determined by glucose measurements, using the same kit as for plasma glucose measurements, before and after hydrolysis of glycogen with amyloglucosidase (Keppler and Decker, 1974) and expressed as µmol $\cdot$ g protein^{-1}. Liver enzyme activities were measured as described in Aas-Hansen et al. (2005) and expressed as µmol $\cdot$ min^{-1} $\cdot$ g protein^{-1}. Enzyme activities determined were alanine aminotransferase (AlaAT) (EC 2.6.1.2), aspartate aminotransferase (AspAT) (EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49), 3-hydroxyacylcoenzyme A dehydrogenase (HOAD) (EC 1.1.1.35), lactate dehydrogenase (LDH) (EC 1.1.1.27), phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32), and pyruvate kinase (PK) (EC 2.7.1.40).

153 *2.3. Whole body proximate composition analysis*

154 Whole fish were homogenized by passing them three times through a mincer (Sirman TC22F,
155 Marsango, Italy). Duplicate samples of ca. 10 g homogenate were weighed (± 0.1 g), dried for 24
156 h at 105^{o}C and then re-weighed to calculate water content. Fat extraction was carried out as
157 described by Johansen et al. (2002), using a Behrotest extraction system (Behr Labor-technik,
158 Düsseldorf, Germany) and ether (Merck, Darmstadt, Germany) as the solvent. Following fat
159 extraction, the extraction thimbles with tissue samples were dried and weighed. Fat content was
160 calculated as weight loss during extraction, and fat concentration was calculated in relation to
161 sample wet weight and expressed as a percentage of body mass. Finally, samples were ashed at
162 500^{o}C for 12 h to estimate the percentage of ash in the sample. Protein was estimated as the
163 difference between the dry sample mass and the combined masses of fat and ash, under the
164 assumption of negligible carbohydrate content. The full dataset for these results and calculations
165 are given in Supporting information (Table 2).
166
167 *2.4. Experiment 2. In vitro study of liver leptin treatment.*

168 The *in vitro* study was performed in January 2013, with liver slices obtained from one Arctic
169 charr (345 gram) of the same strain as those used in Experiment 1. The fish were not eating at the
170 time of sampling, but had a higher condition factor (1.06) than the fasted fish used in Experiment
171 1. The fish was killed by a sharp blow to the head and then decapitated. Thereafter the fish was
172 dissected and the liver was perfused with ice-cold PBS via the hepatic portal vein until it turned
173 white. The liver was then excised and placed in ice-cold L15 medium. A liver piece of ca. 0.7 x
174 0.7 x 0.5 cm. was then taken and sliced (250 µm thickness) with a Leica VT1200 (Leica
175 Microsystems GmbH, Germany).

Nine liver slices were incubated individually under gentle shaking for 24 h in a 24-well plate at 5°C. Three wells contained 0.5 ml L15 medium with recombinant rainbow trout Lep dissolved in 5 µl PBS, resulting in a Lep concentration in the medium of 50 nM (PBS + Lep). The choice of concentration was based on results from treatment of grass carp (*Ctenopharyngodon idellus*) hepatocytes with recombinant grass carp leptin (Lu et al., 2012). The recombinant rainbow trout leptin with proved bioactivity was produced by the method described by Murashita et al (2008). The purity of produced leptin was around 60% determined by SDS-PAGE. Three wells contained 0.5 ml L15 medium added 0.5 µl PBS (PBS) and the final 3 wells contained 0.5 ml L15 medium alone (Untreated). After 24 h the slices were put in RNAlater and stored at -20°C until analysis of *SOCS1, SOCS2, SOCS3, G6PDH, AlaAT and IGF-I* gene expressions. The choice of metabolic enzymes were done in order to investigate representatives of lipid metabolism (G6PDH) and gluconeogenesis (AspAT) which decreased and remained unchanged with fasting, respectively. Liver slice viability was estimated by lactate dehydrogenase (LDH; EC 1.1.1.27) leakage, using a Cytotoxicity Detection Kit (LDH) from Roche (Roche Diagnostics GmbH, 68298 Mannerheim, Germany) and a Spetramax plus spectrophotometer (Molecular devices, LLC, US) set at 490 nm.

2.5. Real-time quantitative PCR of liver LepA1, LepA2, IGF-I, SOCS1, 2 and 3, AspAT and G6PDH mRNA expression.

Reverse transcription of total RNA was performed using QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) with 1000 ng of RNA per 20 µl cDNA reaction according to the manufacturer's instructions. This kit includes an initial step of gDNA removal, and a -RT test on a selection of samples showed that this was effective. Quantitative real-time PCR (qPCR) was performed, using the comparative C_T method (Applied Biosystems, Warrington, UK), on an ABI

7500 Fast Real-Time PCR System. For real-time estimations, cDNA corresponding to 25 ng RNA was amplified for 40 cycles in a 20 µl PCR mix (Fast SYBR® Green Master Mix (Applied Biosystems, Fast City, CA 94404) containing a final concentration of 500 nM of each primer (PrimerDesign Ltd, The Mill Yard, Southampton, United Kingdom, SO16 0AJ;Table 1). Cycling conditions: 95˚C for 20 sec, 40 cycles at 95˚C for 3 sec, and 60˚C for 30 sec including melt curve analysis. Duplicate PCR analyses were performed on each cDNA sample, the absence of gDNA was confirmed by performing a no reverse transcriptase control for every RNA sample, and absence of contamination was confirmed by including a no template control in every run. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative amount of target gene, normalizing against the average expression of the two reference genes *EF1α* and *β-Actin* in Experiment 1 and against *EF1α* in Experiment 2. The primer sequences used for RTqPCR analyses are listed in Table 1.

2.6. Data treatment and statistics

All data from Experiment 1 are presented as means ± standard error of the mean (s.e.m.). *LepA1* and *LepA2* expression are given as relative values in comparison with the mean liver *LepA1* expression in FF fish in March. *SOCS* genes expression are given relative to the mean *SOCS1* expression in FF fish in March, and *IGF-I* gene expression relative to the mean liver *IGF-I* expression in FF fish in March. Only values normalized against EF1α are given for the *in vitro* data. Condition factor (CF) and hepatosomatic index (HSI) were calculated as $[(BM \cdot FL^{-3}) \cdot 100]$ and $[(\text{liver mass} \cdot 100) \cdot BM^{-1}]$, respectively. The weights of body fat and protein present in the fish were calculated by combining the body fat and protein data for the fish sampled in March, May and June, with those relating to changes in BM over time for the fish sampled in May (only FU fish) and June. All data were log-transformed before statistical analyses, with 1 being added

to the condition factor, liver HOAD, PEPCK, PK enzyme activity and liver *Lep*, *IGF-I* and *SOCS* expression data in order to achieve positive log-numbers. A two-way Analysis of Variance test was used to reveal possible effects of time and treatment (replicates pooled) on BM, CF, percentage body fat and protein, HSI, plasma cortisol and glucose concentrations, liver glycogen concentration and enzyme activities and liver *LepA1*, *LepA2*, *SOCS1-3* and *IGF-I* gene expressions. A post-hoc (Tukey HSD) test was used to reveal where significant differences occurred. Possible effects of Lep on the mRNA expression of liver *SOCS1-3*, *AspAT*, *G6PDH* and *IGF-I* genes in the *in vitro* study were tested using a non-parametric Kruskal-Wallis test. Results were considered significant when probability level was lower than, or equal to, 0.05.

3. Results

3.1. Experiment 1.

BM did not differ between treatments in March (Fig. 1A), but in June FF fish had a much higher BM than FU fish ($p < 0.001$). A similar pattern was seen for CF, which did not differ between the two treatment groups in March, but was much higher ($p < 0.001$) in FF than in FU fish in June (Fig. 1B). The FF fish sampled in June had increased markedly in BM ($p < 0.01$) and CF ($p < 0.01$) from March to June (Supporting information, Table 1). There was no change in BM between March and May in FU fish sampled in March, while those sampled in June had only a minor, insignificant decline in BM from March to June (from 155.4 ± 9.3 g to 138.1 ± 7.5 g). The latter fish decreased in CF ($p < 0.001$) from 0.88 ± 0.01 to 0.75 ± 0.01 (Supporting information, Table 1).

There were no differences in the percentage of body protein between FF and FU fish sampled in March and June. The percentage of total body protein did not differ between FU fish

sampled in March and May, but was slightly lower ($p < 0.05$) in June than in March and May (Fig. 1C). There were no differences in the percentage of total body protein in FF fish sampled in March and June. The percentage of total body fat did not differ significantly between FF and FU fish in March (Fig. 1D). In FU fish the percentage of total body fat did not differ significantly between March and May, but was much lower ($p < 0.01$) in June than in May. In FF fish the proportion of body fat was lower in March (4.5 ± 0.5 %) than in June (10.4 ± 1.5 %) and was higher in FF fish than in FU fish (1.2 ± 0.4 %) in June ($p < 0.001$). In mass terms, there was an almost total depletion of body fat in FU fish during the course of the experiment, and a greater loss of protein between May and June than between March and May (Fig. 2 B). In FF fish both total body fat and protein content were much higher in June than in March (Fig. 2A).

Plasma cortisol concentration did not differ between FF and FU fish in March and June (Fig. 1E), but was higher in FU fish in May than in March ($p < 0.05$). The same trend was seen for plasma glucose concentrations (Fig. 1F), which also showed a transient increase in FU fish from March to May ($p < 0.05$).

Liver *LepA1* mRNA expression did not differ between FF and FU fish in March (Fig. 3A) and for FF fish there was no change in expression between March and June. In FU fish there was lower expression in March than in May ($p < 0.05$) and a higher expression June than in May ($p < 0.01$). In June, *LepA1* expression was markedly lower in FF fish than in FU fish ($p < 0.05$). The same general pattern was seen for *LepA2* expression (Fig. 3B), which was higher in FU fish than in FF fish in June ($p < 0.01$). Liver *IGF-I* mRNA expression did not differ between FF and FU fish in March. There was no difference in *IGF-I* expression for FU fish sampled in March and May (Fig. 3C), but a decrease from May to June ($p < 0.05$). There was no difference in *IGF-I* expression in FF fish sampled in March and June.

Liver *SOCS* gene expressions are shown in Fig. 4. There were no differences between FF and FU fish in *SOCS1*, *SOCS2* and *SOCS3* in March. In FF fish *SOCS1* mRNA expression was higher in March than in June ($p < 0.05$). There were no differences in *SOCS1* expression in FU fish sampled at different times, resulting in a tendency toward a higher *SOCS1* expression in FU fish than in FF fish in June ($p = 0.062$). For *SOCS2*, expression was higher in FF fish sampled in March than in June ($p < 0.001$), whereas the opposite was seen in FU fish ($p < 0.001$). This resulted in a higher *SOCS2* expression in FU fish than in FF fish in June ($p < 0.001$). *SOCS3* expression was higher in March than June in both FF ($p < 0.01$) and in FU fish ($p < 0.05$), with a higher expression in FU than in FF fish in June ($p < 0.05$).

Hepatosomatic index (HSI) did not differ between FF and FU fish in March, after which there was a decrease in FU fish ($p < 0.05$), and an increase in FF fish ($p < 0.001$), resulting in a higher ($p < 0.001$) HSI in FF than in FU fish in June (Table 2). Liver glycogen concentration (Table 2) did not differ between FF and FU fish in March, but was higher ($p < 0.05$) in FF fish in June than in March and lower in FU fish in June than in both March and May ($p < 0.001$). This resulted in a marked difference in liver glycogen concentrations between FF (28. 2 ± 4.7 µmol · g protein^{-1}) and FU fish (1.8 ± 0.5 µmol · g protein^{-1}) in June ($p < 0.001$).

There were no differences in any enzyme activities between FF and FU fish in March, nor between March and May in FU fish (Table 2). In FF fish, G6PDH ($p < 0.001$), LDH ($p < 0.01$), HOAD ($p < 0.001$) and PEPCK ($p < 0.05$) activities were higher in June than in March. In FU fish, G6PDH ($p < 0.01$), LDH ($p < 0.01$) and HOAD ($p < 0.01$) activities were lower in June than in March. In June, there were higher Asp-AT, G6PDH, LDH, HOAD, PEPCK and PK activities in FF than in FU fish.

3.2. Experiment 2.

There were significantly higher *SOCS1* ($p < 0.05$) and *SOCS3* ($p < 0.05$) gene expressions in Lep treated liver slices (PBS + Lep) than in untreated and sham (PBS) treated slices, but no effects of Lep were seen for *SOCS2*, *G6PDH*, *AspAT* and *IGF-I* gene expressions (Fig 5).

4. Discussion

The present study was designed to simulate the overwintering fast in wild, anadromous Arctic charr and the emaciation experienced by these fish during the latter part of their overwintering in fresh water. Since there were similar CFs in the fish used in the present study (Fig. 1B) as in those sampled in the study on wild anadromous charr by Aas-Hansen et al. (2005) in which CF decreased from 0.84 in April to 0.77 in June, we consider this objective to have been achieved. In our study, only one fish sampled in March had food in its stomach, despite access to food, indicating that the fish were in a voluntary, anorexic state. Some FF fish had consumed food when sampled on May 6 (see Material and Methods), which agrees with previous findings of a return of appetite in May in both captive, anadromous Arctic charr (Tveiten et al., 1996; Bottengård and Jørgensen, 2008; Aarseth et al., 2010) and in their wild conspecifics (Aas-Hansen et al., 2005).

4.1. Metabolic responses to fasting

In FU fish fasting resulted in a decrease in BM, CF, body fat and protein, particularly between the samplings in May and June, whereas there were increases in BM, CF, body fat and protein in FF fish between March and June (Figs. 1 A,B,D; 2). The low percentage (1.2 ± 0.4 %) and low absolute amount (Fig. 2) of body fat in FU fish in June indicates that stored fat was severely depleted at this time. In a previous study plasma triglyceride concentration was also found to

decrease in anadromous Arctic charr deprived of food during late spring/early summer, concomitant with an increase in plasma glucose concentration (Frøiland et al., 2012). Thus it seems that the energy needed during the latter part of overwintering, and migration, must be taken from the body protein store in the anadromous Arctic charr. This conclusion is supported by the reduction in body protein observed in the FU fish from May to June (Fig. 2).

Prolonged fasting in fish is generally associated with reduced plasma glucose, HSI, liver glycogen and liver metabolic capacity (Navarro et al., 1992; Foster and Moon, 1991; Bastrop et al., 1992; Navarro and Gutierrez, 1995). The lack of any large change in these parameters in FU fish between March and May (Fig. 1 F; Table 2), indicates that physiological responses may differ between voluntarily fasting fish and fish subjected to enforced feed deprivation. The reduction in liver glycogen and liver enzyme activities from May to June in FU fish (Table 2) support the notion that these fish were voluntarily anorexic until May, and that they subsequently experienced starvation stress in the absence of food. The reduced metabolic capacity of the liver, together with the depletion of glycogen in FU fish from May onwards indicates that they responded by entering a state of metabolic depression, i.e. reduced metabolism under adverse environmental conditions (Guppy and Withers, 1999). Nevertheless, FU fish had plasma glucose concentrations similar to those of FF fish (Fig 1 F). This glucose was probably derived from protein, as evidenced from the decrease in the body protein store from May to June (Fig. 2 B) and the maintained activity of enzymes involved in gluconeogenesis and amino acid catabolism (PEPCK and AlaAT; Table 2). In support of this, FU fish had plasma cortisol concentrations similar to those of FF fish at the end of the experiment in June (Fig. 1E), in accordance with the accepted gluconeogenetic action of this hormone (Mommsen et al., 1999). The maintenance of a high *de novo* glucose synthesis in the liver, despite depleted liver glycogen levels, reflects the importance of this substrate during a long-term fast (Polakof et al., 2012).

343

344 *4.2. A role of leptin in liver metabolism?*

345 The changes in liver *LepA1* and *LepA2* expression during the course of the experiment resembled
346 those previously reported for anadromous Arctic charr (Frøiland et al., 2012); a decrease in fed
347 fish from March to July and higher *LepA1* and *LepA2* expression in FU than in FF fish at the end
348 of the fasting period in June. An increase in liver *Lep* expression with fasting/feed restriction has
349 also been reported in Atlantic salmon (Rønnestad et al., 2010; Trombley et al., 2012), whereas a
350 reduced liver *Lep* expression with fasting has been reported in goldfish (*Carassius auratus*)
351 (Tinoco et al., 2012) and striped bass (*Morone saxatilis*) (Won et al., 2012), and no change with
352 prolonged fasting in common carp (*Cyprinus carpio*) (Huising et al., 2006). The reason for these
353 differences is not known but they could relate to the degree of emaciation of the fish used in the
354 different experiments, and interspecies differences in metabolic responses to an imposed fast.

355 In the present study the activity of enzymes involved in lipid metabolism (G6PDH and
356 HOAD) and glycolysis (PK) appeared to be inversely related to liver *LepA1* and *LepA2*
357 expression in FU fish. In addition, the highest activity of these enzymes was recorded
358 concurrently with low liver *LepA1* and *LepA2* expression in FF fish in June (Fig. 3 A,B; Table 2).
359 No such interrelationships were seen for enzymes involved in amino acid metabolism and
360 gluconeogenesis; LDH activity decreased in FU fish and increased in FF fish, but there were no
361 changes in liver AlaAT and AspAT activities with time (Table 2). These results suggest that Lep
362 may be involved in the suppression of lipolytic pathways, but *in vitro* Lep treatment of liver
363 slices did not affect the gene expression of *G6PDH* (Fig. 4 D). This indicates that Lep was not
364 directly responsible for the changes in liver enzyme activity.

365

366 *4.3. A role of SOCS in liver metabolism?*

In mammals, SOCS1, 2 and 3 are involved in attenuating growth hormone (GH) and Lep signalling by downregulating post-receptor JAK/STAT induced gene transcription (Fujimoto and Naka, 2003). *SOCS1-3* genes have been identified in several fish species (Jin et al., 2007; Xiao et al., 2010; Wang and Secombes, 2008; Studzinski et al., 2009) and their expression may be induced by cytokines in fish as in mammals (Wang and Secombes, 2008). In the present study *SOCS* gene expression was affected by the feeding regime (Fig 4). The higher expression of the *SOCS* genes in FU fish than in FF fish in June coincided with a reduced expression of *IGF-I* in FU fish (Fig. 3 C). This situation closely resembles the reduced *IGF-I* expression seen in fasted salmonid fish, as a result of downregulated hepatic GH signalling (Björnsson, 1997), and that in homozygotic GH transgenic zebrafish (*Danio rerio*), in which *SOCS1* and *SOCS3* seemed to be responsible for the downregulation of GH signalling in the liver (Studzinski et al., 2009). In common with the elevated plasma GH concentration in fasting salmonids (Björnsson, 1997), GH transgenic zebrafish overexpress GH in comparison with their hemizygotic counterparts (Figueiredo et al., 2007).

It is interesting to note that liver IGF-I expression in the FU fish used in our study only decreased during the latter part of the enforced fasting period (i.e. from May onwards), despite the fact that the fish most likely had been fasting voluntarily for months when sampled in May. This resembles the situation seen in seasonal mammals, which differ in their responses when undergoing seasonal winter weight loss and when they are subjected to feed deprivation during summer (Adam and Mercer, 2004). The reduction in liver *IGF-I* expression with fasting seen in the present study (Fig. 3 C) was probably not a direct result of the increase in *Lep* expression since *in vitro* Lep treatment did not affect liver IGF-I expression (Fig. 5 F), but more likely related to the elevated *SOCS1* and *SOCS3* expression (Fig. 4 A,C). This would comply with the

results obtained with GH-transgenic zebrafish, which indicated that reduced GH signalling was caused by SOCS1 and SOCS3 (Studzinski et al., 2009).

The present study showed that Lep stimulated hepatic *SOCS1* and *SOCS3* gene expression *in vitro* (Fig. 5 A,C). This result provides evidence for a link between the higher liver *LepA1* and *LepA2* expression in FU fish than in FF fish in June (Fig. 3 A,B) and the corresponding difference in liver *SOCS1* and *SOCS3* gene expression in FU and FF fish (Fig. 4 A, C). The higher liver *SOCS2* expression in FU fish than in FF fish in June was probably not caused by Lep, which did not affect *SOCS2* expression in the *in vitro* experiment (Fig. 5B). Another candidate could be cortisol, which was recently shown to stimulate *SOCS2* expression in rainbow trout hepatocytes *in vitro* (Philip et al., 2012). However, we did not find differences in plasma cortisol concentrations between FU and FF fish in June (Table 2), even though the FU fish may have been suffering from starvation stress during the latter part of the fasting period.

In summary, the present study has revealed a metabolic suppression in anadromous Arctic charr subjected to fasting after they had entered their natural summer feeding period. Upregulated *Lep* and *SOCS* genes and downregulated *IGF-I* expression in the liver are suggested to be a part of the mechanisms that suppress lipolytic pathways and depress metabolism when fat stores are depleted. We have demonstrated that Lep stimulated hepatic *SOCS1* and *SOCS3* expression *in vitro*, and this stimulation may be responsible for downregulation of GH signalling and, consequently, reduced *IGF-I* expression in the liver. Further studies are needed to elucidate how Lep and SOCS interact to regulate intermediary metabolism in fish that display seasonal feeding cycles.

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Table legends

Table 1. Nucleotide sequences used in the qPCR analyses and accession numbers of the gene sequences they were constructed from.

Table 2. Mean ± s.e.m. hepatosomatic index (HSI, %), liver glycogen concentrations (μmol · g protein^{-1}) and enzyme activities (μmol · min^{-1} · g protein^{-1}) in fed and fasted Arctic charr. n denotes the number of fish analyzed and different letters indicate values that are significantly different.

Figure legends

Figure 1. Temporal changes in mean (± s.e.m.) body mass (A), condition factor (B), body protein (C) and fat (D) concentrations, and plasma cortisol (E) and glucose (F) concentrations in fed and fasted anadromous Arctic charr. Different letters denote significant differences between groups.

Figure 2. Temporal change in weights of body protein (bottom column, P) and fat (top column, F) of the fed (FF) (A) and fasted (FU) (B) fish sampled in June. Body fat and protein mass in March and May were calculated from the BM in March and May of the fish sampled in June, and the data for percentage of body fat and protein of the fish sampled in March and May (see Appendix, Table 2 for more information).

Figure 3. Temporal changes in mean (± s.e.m.) liver *leptinA1* (A), *leptinA2* (B) and *IGF-I* (C) gene expressions in fed and fasted anadromous Arctic charr. Different letters denote significant differences between groups.

Figure 4. Temporal changes in *SOCS1* (A), *SOCS2* (B) and *SOCS3* (C) gene expressions in liver of fed and fasted Arctic charr. Different letters denote significant differences between groups.

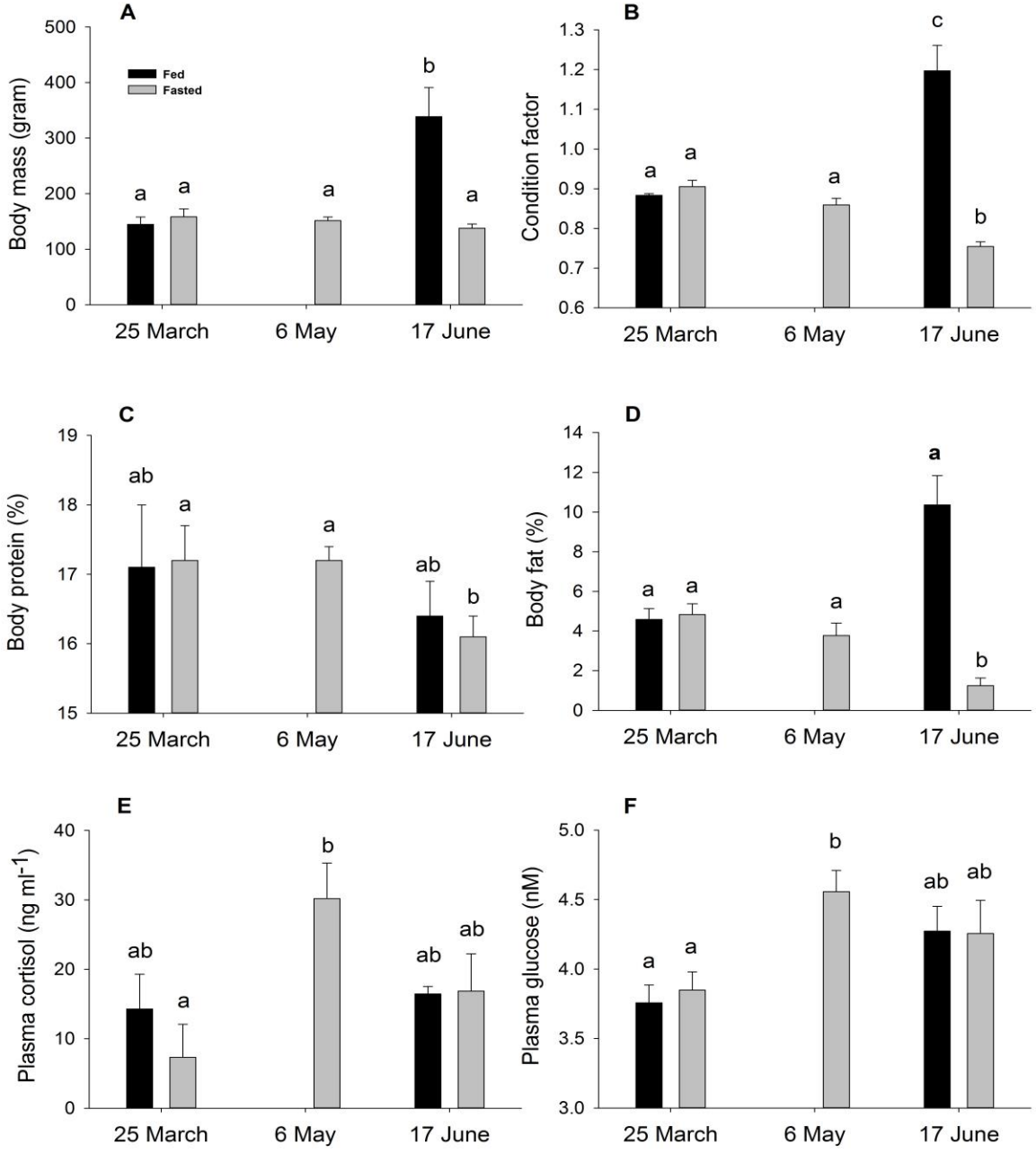
Figure 5. *In vitro* effects of recombinant trout leptin on *SOCS1*, *SOCS2*, *SOCS3*, *G6PDH*, *AspAT* and *IGF-I* mRNA expressions in liver slices from Arctic charr. Each column represents one liver slice. Asterisks denote those genes for which the expression was significantly different from that in the leptin treated group.

Table 1.

Gene symbol	Gene Name	Accession number	Primer Sequences
LepA1	*Leptin A1*	JQ615967	F: TCC TAG ACT GGG CAG ACC T R: GCC TGG GCA GCG TGA TAT
LepA2	*Leptin A2*	AB490667	F: TGG CAC TAA ACA GAC TCA AGG R: CTC AGT GAT GAT CTA TGT CAG TAA C
β-Actin	*Beta Actin*	AF503211	F: GCC CCC CTG AAC CCC R: GAA GGT CTC AAA CAT AAT CTG GGT C
EF1α	*Elongation Factor*	AF498320	F: AGG CAT TGA CAA GAG AAC CATT R: TGA TAC CAC GCT CCC TCT C
SOCS1	*Suppressor of cytokine signalling 1*	NM_001146166	F: TCA GCG TAC GCA TCG TCT AT R: CGG TCA GGC TTT TCT TAG AGG
SOCS2	*Suppressor of cytokine signalling 2*	AM748722.1	F: TCG GAT GAC TTT TGG CCT AC R: CCG TTC TTC TCT CGT TTT CG
SOCS3	*Suppressor of cytokine signalling 3*	AM748723	F: ACC TCT GAC GAA GCA CAC AT R: GCT GTA ATG GTT GAA TGG ATA GGA
IGF-1	*Insulin-like growth factor 1*	M95183	F: TGG ACA CGC TGC AGT TTG TGT GT R: CAC TCG TCC ACA ATA CCA CGG T
AspAT	*Aspartate aminotransferase, cytoplasmic*	NM_001141739	F: TCC TTT CCT CAT CTC CTT CAT TAC R:TTT CCT TGT GTC CTT CCC TCT
G6PDH	*Glucose-6-phosphate dehydrogenase*	EF551311.1	F: CAC TAC CTG GGC AAG GAG AT R: TTG CTC ATC ATC TTG GCG TA

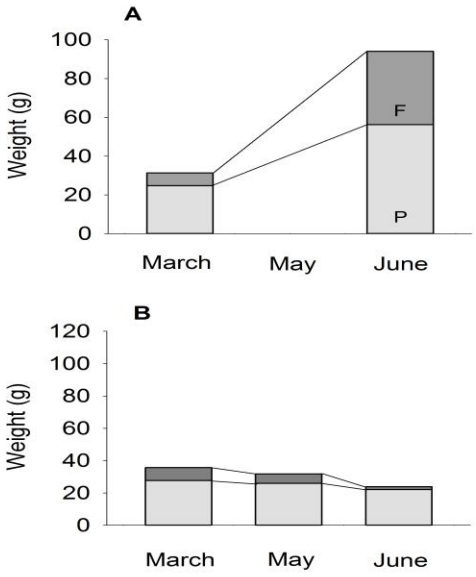
Table 2

	March 25		May 6	June 17	
Liver	Fed (n=9)	Fasted (n=10)	Fasted (n=10)	Fed (n=7)	Fasted (n=10)
HSI	1.2 ± 0.06^{a}	1.2 ± 0.07^{a}	1.1 ± 0.02^{ab}	2.0 ± 0.12^{c}	1.0 ± 0.04^{b}
Glycogen	12.6 ± 1.6^{a}	15.0 ± 2.2^{a}	11.5 ± 1.2^{a}	28.2 ± 4.7^{b}	1.8 ± 0.5^{c}
Ala-AT	20.0 ± 1.6	21.6 ± 2.0	21.8 ± 1.6	20.7 ± 2.1	18.6 ± 1.5
Asp-AT	12.5 ± 1,3^{ab}	10.3 ± 0.6^{ac}	10.1 ± 0.7^{ac}	16.4 ± 2.3^{b}	8.3 ± 0.7^{c}
G6PDH	4.1 ± 0.3^{a}	5.2 ± 0.4^{a}	3.9 ± 0.3^{a}	15.0 ± 2.3^{b}	2.5 ± 0.3^{c}
LDH	45.5 ± 2.5^{a}	51.4 ± 4.1^{a}	49.6 ± 1.4^{a}	68.2 ± 6.9^{b}	35.3 ± 2.0^{c}
HOAD	0.47 ± 0.02^{a}	0.48 ± 0.02^{a}	0.45 ± 0.01^{a}	0.76 ± 0.1^{b}	0.33 ± 0.03^{c}
PEPCK	0.18 ± 0.02^{a}	0.19 ± 0.02^{ab}	0.19 ± 0.02^{ab}	0.27 ±0.04^{b}	0.16 ± 0.02^{a}
PK	0.70 ± 0.06^{ab}	0.78 ± 0.07^{ab}	0.91 ± 0.05^{a}	1.13 ± 0.16^{a}	0.61 ± 0.08^{b}



689

690 Fig. 1



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693 Fig. 2

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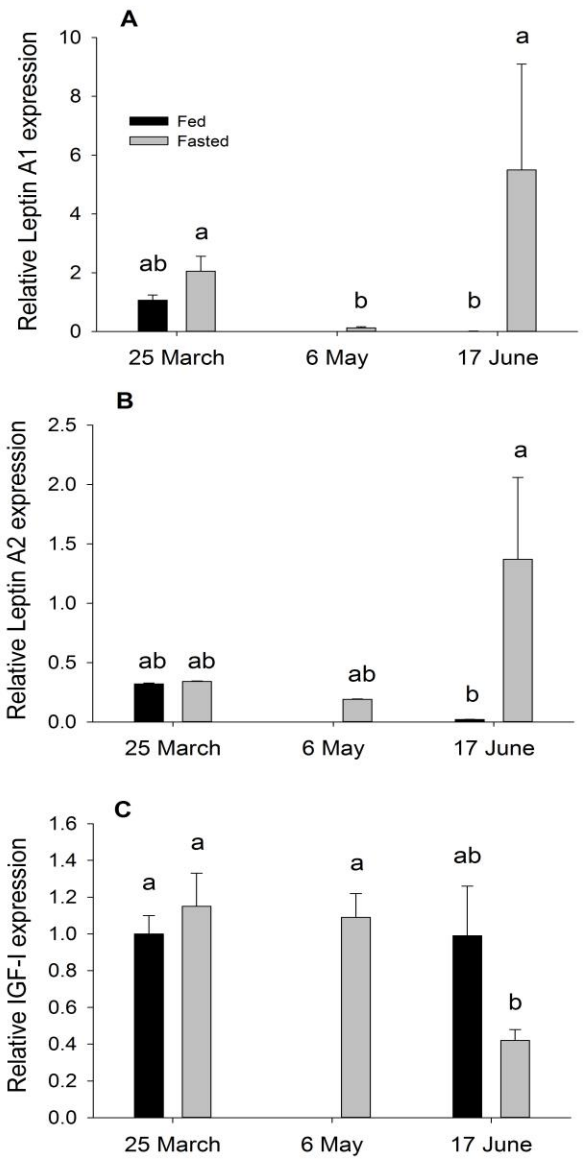

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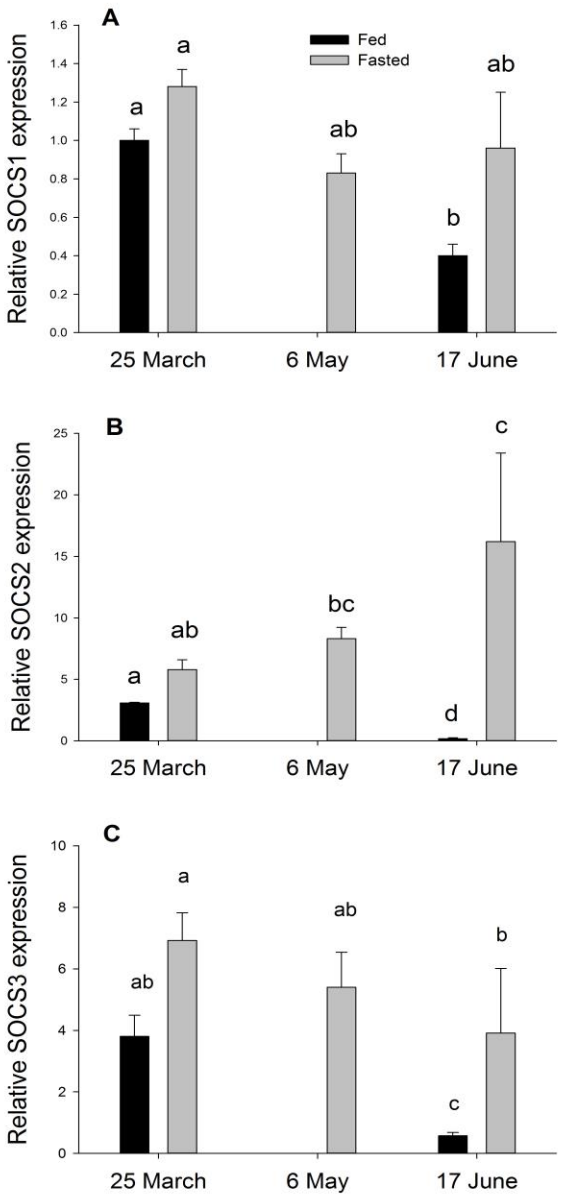

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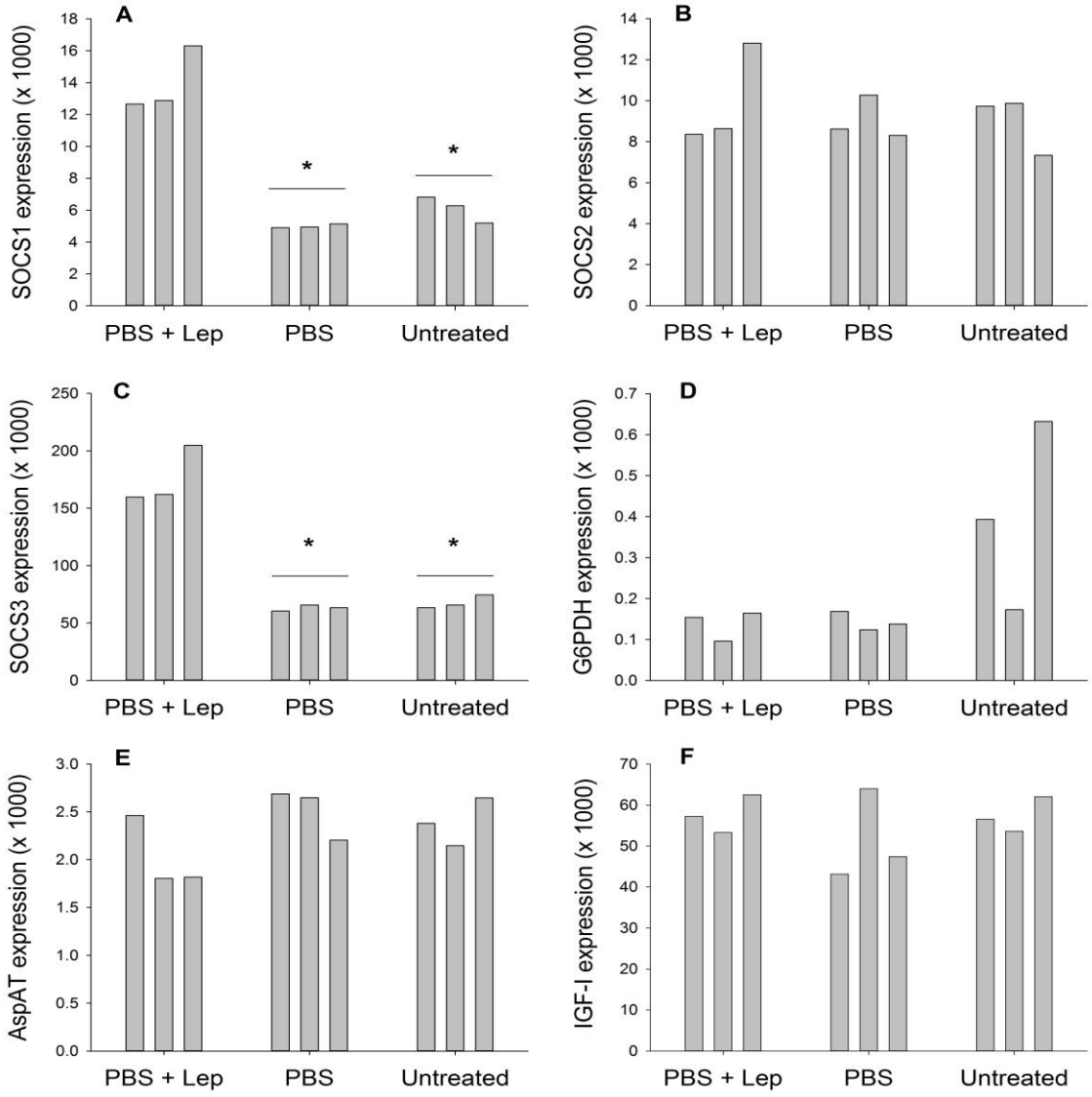
702 Fig. 4



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704 Fig. 5

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