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Short-term, but not long-term feed restriction causes differential expression of leptins in Atlantic salmon

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ABSTRACT

Atlantic salmon was used to investigate the effect of long- and short-term dietary ration on the tissue expression levels of leptins. Compared to *ad libitum* fed fish (0.8–3 kg), 6 months of dietary restriction (60%) resulted in significantly lower body mass and adiposity, but did not produce a clear effect on the expression levels of either *lepa1* or *lepa2*. For visceral adipose tissue, however, the long-term data indicated that season appeared to influence the levels of *lepa1* expression of *ad libitum* fed fish, but not feed-restricted fish. By comparing the total levels of leptin mRNA expression to the tissue lipid contents, we found that only white muscle *lepa1* showed the positive relation reported in mammals. The existence of a postprandial leptin response in Atlantic salmon parr was determined in fed and unfed parr over a 24 h period. In contrast to other animals, *lepa1* peaked in the unfed fish, initially in the white muscle at 6 h, and subsequently in belly flap, liver and visceral adipose tissue at 9 h. Only *lepa2* in the visceral adipose tissue of fed fish showed a similar 9 h peak, but at an order of magnitude lower than *lepa1* in the unfed fish. These data reveal that short-term feed restriction causes a latent (6–9 h) upregulation of *lepa*-type genes in the fatty tissues of Atlantic salmon, a finding that contrasts the mammalian response.

1. Introduction

The role of leptin, a 16 kDa product of the obese (*OB*) gene, has been extensively studied in mammals as a link between appetite, adiposity and energy homeostasis [49]. The early experiments revealed that the absence of leptin causes extreme obesity in rodents [7,21,39]. However, emerging evidence indicates that the link between adiposity and its hypothalamic regulation is more complex than originally envisaged, since the ablation of leptin does not cause obesity in other mammals, and leptin is now known to have pleiotropic effects associated with the regulation of several hypothalamic–pituitary–endocrine organ axes, blood pressure, reproduction, osteogenesis, hematopoesis, immunity, angiogenesis and tumorigenesis [5,22,25,34]. In fish, leptin was first characterised in torafugu (*Takifugu rubripes*) [31]. Subsequently one or more forms of leptin [18,19,23,29,30,33,37,47,48], as well as the cognate receptor (*lepr*) [29] have been identified in a number of teleosts including adult [41], embryonic and larval stages of Atlantic salmon (*Salmo salar*) [35]. The studies in adult salmon suggest that the major site of leptin expression in fish is the liver, whereas in mammals it is the adipose tissue [36,49]. Due to the presence of multiple leptin genes in teleosts [9,19,23,29,41], the physiological roles played by leptins in this class of vertebrate may be more divergent than in mammals.

Leptin is considered an anorexigenic hormone in mammals [17,49] and amphibians [10]. This function may be conserved in teleosts, since exogenous administration of leptin has been found to attenuate food intake in goldfish (*Carassius auratus*) [11] and rainbow trout (*Oncorhynchus mykiss*) [37]. Similarly a study on common carp (*Cyprinus carpio*) found an increase in the expression of leptins shortly after a meal [23] indicating that a conserved postprandial response could exist between teleosts and mammals. However, leptin expression has also been shown to be related to the pineal–melatonin axis in other species [27,50,51], and feeding rhythm is known to be endogenously generated by an internal timing mechanism or biological clock in both mammals [40,43] and fish [13,14,28].

The aim of the present study was to determine the effect of long-term and short-term rationing on the mRNA expression levels of leptins in Atlantic salmon. Recently cloned paralogues of Atlantic salmon leptins (*lepa1* and *lepa2*) and a leptin receptor (*lepr*) [41] were used to examine the tissue expression dynamics in fish subjected to different feeding rates during winter and early spring. To determine whether leptins are associated with the short-term energy status, fish were trained to consume a single meal per day over a two-week period, after which the endocrine response in

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the presence or absence of a meal over a 24 h period was examined.

2. Materials and methods

2.1. Fish material and experimental design

Atlantic salmon from the AquaGen strain were used in the present long-term study. The study was conducted at the Institute of Marine Research at Matre (80 km north of Bergen, Norway) from 5th October 2006 until 21st March 2007.

On the 5th of October, a total of 3200 fish with average body mass of 849 ± 70 g were randomly distributed into 4 sea cages (5 m^3). The water temperature during the trial decreased from 14 °C (October) to 6.5 °C (March) (Fig. 1) and the salinity varied between 18‰ and 25‰. Two groups were established: a control group (FF) which was fed *ad libitum* (100% ration) a commercial Biomar Classic diet (Biomar, Bergen, Norway, Lipid content: 28–32%) seven days a week, and a restricted fed group (60% ration, RF) which was fed four days a week (Monday, Tuesday, Thursday and Friday). Feeding levels for both groups were adjusted to fish size and temperature in accordance with established protocols (Austereng et al., 1987). On the day of sampling, fish were not fed.

For the determination of the post-prandial expression kinetics of *lepa1* and *lepa2* in the absence or presence of a meal, salmon parr (44.7 ± 2.1 g) from the AquaGen strain were maintained indoors with a continuous flow of fresh water at 8 °C in circular tanks and a light regime of 12 h light 12 h dark. The parr were distributed into two tanks, and received a single meal a day (09:00–09:30) for 14 days (hand fed dry pellets to satiation, EWOS, Bergen, Norway, lipid: 28%). At the start of the 24 h trial, parr in one tank continued to receive food (FG = fed group) until sampling started, whereas food was withheld from the other tank for 24 h prior to sampling (UG = unfed group). Inspection of the stomach, gallbladder and gut showed that the gut was fully cleared during this time. All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals [3].

2.2. Sampling

In the long-term trial, twenty fish from each group were caught by dip nets in the cages after crowding and immediately anaesthetized (metomidate 10 mg l^{-1}) at each sampling event on October 5th, November 15th, December 13th, January 10th and 24th, February 7th and March 21st. Fork length (nearest mm) and body mass (nearest g) were recorded. The sex of each fish was noted, however no qualitative effect on leptin expression or gonad formation was noted. For analysis of lipid content and dry matter, the left fillet and all visceral organs were dissected out and weighed at each sampling. Lipid content and dry matter was only analysed from the October 5th and January 10th sampling. Tissue samples were flash-frozen in liquid N_2 and stored at −80 °C until subsequent RNA extraction. For the short-term study, six fish were sampled from each tank, following anaesthetisation (metomidate 10 mg l^{-1}). Both FG and UG post smolts were sampled at 0, 0.5, 1.5, 3, 6, 9, 12 and 24 h. Tissue samples were collected as described above.

2.3. Lipid and dry matter

Total lipid in visceral organs and muscle was extracted with chloroform: methanol (2:1) according to Folch's procedure [15], evaporated to dryness at 100 °C and reconstituted in dioxane. Total lipid content was determined by spectrophotometry (505 nm) using triolein as standard. Dry matter in visceral organs and muscle was measured by drying at 105 °C for 18 h, and the dry matter content (DM%) was calculated by the formula: (dry mass × 100)/wet mass.

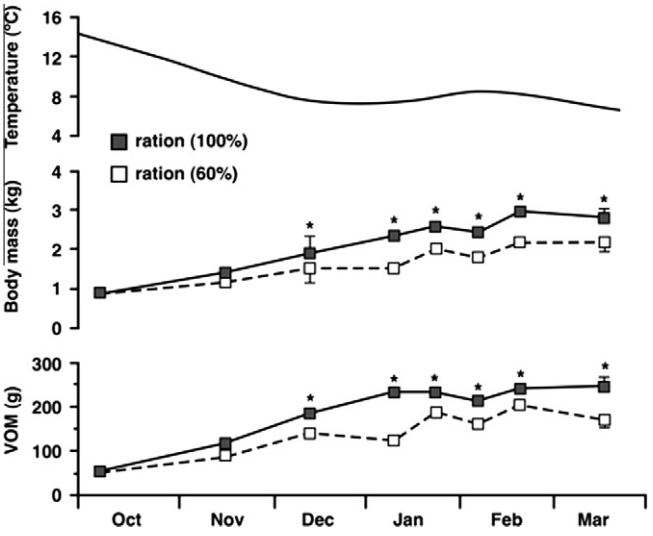

Fig. 1. Changes in temperature, body mass and visceral organ mass (VOM) throughout the trial. Data are given as mean ± standard error of the mean (SEM) with $N = 20$ fish per treatment. * Indicates a significant difference ($p < 0.05$) to the 60% ration group.

2.4. Real-time quantitative PCR (qPCR)

Total RNA for quantification of mRNA levels was extracted from tissues using Tri Reagent® (Sigma–Aldrich®, Steinheim, Germany). Total RNA was quantified spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and the integrity checked on 1% agarose/formaldehyde gels. RNA was DNase treated (TURBO DNA-free™ kit, Ambion, Austin, TX, USA) and cDNA reverse-transcribed using 5 μg total RNA and oligo (dT) primers in conjunction with the Superscript III First-Strand Synthesis System kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) following the manufacturers guidelines.

Expression levels of *lepa1*, *lepa2*, and *lepr*, were normalised to levels of beta actin (*actβ*, accession No. AF012125) and elongation factor-1a (*ef1α* accession No. AF321836) mRNA levels were measured by quantitative PCR (qPCR), using the ABI prism 7000 sequencer detection platform v.3.1 (Applied Biosystems Inc. Foster City, CA, USA) as described previously [41]. Primers and FAM MGB-labelled probes used are listed in Table 1. For *lepa1*, reactions were carried out using 12.5 μl TaqMan® Universal PCR Master Mix containing AmpErase® uracil N-glycosylase (ABI), 5 μl cDNA, 100 nM probe and 100 nM primers in a total volume of 25 μl. The real-time PCR protocol employed consists of 50 °C for 2 min, 95 °C for 10 min and then 50 cycles of 95 °C for 15 s and 60 °C for 60 s. *lepa2* and *lepr* reactions were carried out using 12.5 μl SYBRgreen PCR Master Mix containing AmpliTaq Gold® DNA Polymerase (ABI), 10 μl cDNA, 100 nM primers in a total volume of 25 μl. The real time PCR protocol employed was identical to that of *lepa1*, with the exception of only running 45 cycles. The specificity of single target amplification of *lepa1*, *lepa2*, and *lepr* was confirmed by separating the qPCR products by agarose gel (1.2%) electrophoresis followed by cloning and sequencing of the purified fragments [41]. qPCR of the reference transcripts *actβ* and *ef1α* was as previously described [38]. Omission of reverse transcriptase in the RT reaction showed that interference from residual DNA in the extracted RNA was negligible.

For each assay, triplicate 2-fold cDNA dilution series made from total RNA (range: 39 ng–1.25 μg) from the different tissues investigated were used to determine amplification efficiencies (E) calculated as the slope from the plot of log RNA concentration versus cycle threshold (Ct) values using the following formula: $E = 10^{(-1/}$