Hypoxemia-induced leptin secretion: a mechanism for the control of food intake in diseased fish

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Abstract
Leptin is a potent anorexigen, but little is known about the physiological conditions under which this cytokine regulates food intake in fish. In this study, we characterized the relationships between food intake, O₂-carrying capacity, liver leptin-A1 (lep-a1) gene expression, and plasma leptin-A1 in rainbow trout infected with a pathogenic hemoflagellate, Cryptobia salmositica. As lep gene expression is hypoxia-sensitive and Cryptobia-infected fish are anemic, we hypothesized that Cryptobia-induced anorexia is mediated by leptin. A 14-week time course experiment revealed that Cryptobia-infected fish experience a transient 75% reduction in food intake, a sharp initial drop in hematocrit and hemoglobin levels followed by a partial recovery, a transient 17-fold increase in lep-a1 gene expression, and a sustained increase in plasma leptin-A1 levels. In the hypothalamus, peak anorexia was associated with decreases in mRNA levels of neuropeptide Y (npy) and cocaine- and amphetamine-regulated transcript (cart), and increases in agouti-related protein (agrp) and pro-opiomelanocortin A2 (pomc). In contrast, in non-infected fish pair-fed to infected animals, lep-a1 gene expression and plasma levels did not differ from those of non-infected satiated fish. Pair-fed fish were also characterized by increases in hypothalamic npy and agrp, no changes in pomc-a2, and a reduction in cart mRNA expression. Finally, peak infection was characterized by a significant positive correlation between O₂-carrying capacity and food intake. These findings show that hypoxemia, and not feed restriction, stimulates leptin-A1 secretion in Cryptobia-infected rainbow trout and suggest that leptin contributes to anorexia by inhibiting hypothalamic npy and stimulating pomc-a2.

Key Words
- leptin
- appetite
- anemia
- fish disease
- parasites

Introduction
Leptin is recognized as playing an important role in the homeostatic control of feeding and energy expenditure in vertebrates (Ahima & Flier 2000). In mammals, leptin is primarily produced by adipocytes and its circulating levels increase with overfeeding and decrease with fasting (Ahima et al. 1996, Walder et al. 1997). While leptin is a potent anorexigenic signal and stimulator of energy use, it does not suppress feeding and weight gain in obese individuals because of mechanisms that promote leptin resistance and energy conservation (Myers et al. 2008). Instead, the primary role of leptin in mammals is to communicate a state of energy deficiency to the brain, i.e. low leptin levels induce overfeeding and suppress energy expenditure (Flier 1998, Ahima 2008). In contrast, very
little is known about the physiological roles of leptin in other vertebrate taxa. Although the tertiary structure of leptin is highly conserved, the low degree of primary sequence conservation among orthologs has delayed the characterization of leptin in non-mammalian vertebrates (Huising et al. 2006a). The recent cloning of fish leptins (see Londraville et al. (2014) for review), the recognition that several fish species express multiple leptin orthologs which may have led to the subfunctionalization of leptins in fish (Gorissen et al. 2009, Kurokawa & Murashita 2009, Angotzi et al. 2013), and the development of research tools for leptins in fish (e.g. Kling et al. (2009)) offer new opportunities to determine the functional roles of this important metabolic signal in aquatic poikilotherms.

To date, fish studies on the regulation of lep expression and on the role of leptin in feeding suggest some conserved actions between teleosts and mammals but also fundamental differences. For example, although the liver is a major site of lep expression in fish, much lower levels have been detected in visceral adipose tissue (Huising et al. 2006b, Murashita et al. 2008, Ronnestad et al. 2010, Kobayashi et al. 2011, Won et al. 2012). As observed in mammals and in the African clawed frog (Xenopus laevis; Crespi & Denver 2006), it is generally recognized that leptin is anorexigenic in fish. Although heterologous leptins have no effect on feeding in some species (Baker et al. 2000, Silverstein & Plisetskaya 2000), feeding is reduced by i.p. and i.c.v. injections of murine and human leptin in goldfish (Carassius auratus; Volkoff et al. 2003, De Pedro et al. 2006), by i.p. injections of human leptin in striped bass (Morone saxatilis; Won et al. 2012), and by i.c.v. injection of human leptin in rainbow trout (Oncorhynchus mykiss; Aguilar et al. 2010). The i.p. injection of recombinant native leptin also reduces food intake in rainbow trout and affects the expression of hypothalamic appetite-regulating genes (Murashita et al. 2008). In contrast, in fish, a clear relationship between energy balance and lep expression has yet to be established. While prolonged fasting has no effect on the expression of liver lep in some species (Huising et al. 2006b, Gorissen et al. 2009, Kobayashi et al. 2011), it decreases (Gorissen et al. 2009, Won et al. 2012) or increases (Kling et al. 2009, Ronnestad et al. 2010, Fuentes et al. 2012) hepatic lep mRNA levels or leptin plasma levels in others. Thus, while leptin has anorectic actions, the physiological conditions under which it plays a role in the regulation of food intake in fish are still poorly understood.

Anorexia is a characteristic response to a variety of different viral, bacterial, and parasitic infections (Laviano et al. 2008, Bernier 2010). Among the different parasites known to affect feeding in fish, the effects of the pathogenic hemoflagellate, Cryptobia salmositica (order Parabodonomida, class Kinetoplastea), on food intake in rainbow trout have been well characterized (Woo 2003). While the onset of anorexia in Cryptobia-infected fish coincides with a significant rise in parasitemia and a decrease in hematocrit, the return of appetite is associated with the establishment of an immune response that significantly reduces both the parasitemia and anemia (Li & Woo 1991, Thomas & Woo 1992, Chin et al. 2004). During acute infection, Cryptobia-infected fish are also characterized by a reduced aerobic scope (Kumaraguru et al. 1995) and an increased susceptibility to hypoxia (Woo & Wehnert 1986). As leptin is a hypoxia-sensitive gene in mammals (Ambrosini et al. 2002) and fish (Chu et al. 2010), and its expression is stimulated by hypoxia-inducible factor 1 (HIF1) in response to a deficiency in O2, we hypothesized that leptin contributes to the regulation of food intake in Cryptobia-infected rainbow trout.

In this study, to identify potential mechanisms mediating Cryptobia-induced anorexia, we first characterized the relationships between food intake, hepatic lep-A1 expression, plasma leptin-A1 and the expression of key appetite-regulating genes in the hypothalamus of rainbow trout infected with C. salmositica. To differentiate between the effects of fasting from those of hypoxemia on the regulation food intake, we compared the effects of Cryptobia infection with those of restricted feeding on leptin signaling and the hypothalamic expression of appetite-regulating genes. Finally, we also examined the effects of Cryptobia infection on the O2-carrying capacity of rainbow trout and the relationship between O2-carrying capacity and food intake.

**Materials and methods**

**Experimental animals**

Rainbow trout of either sex were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and housed in the Hagen Aqualab, University of Guelph (Guelph, ON, Canada). Fish were maintained in 800 l cylindrical tanks before experimental use and kept on a 12-h light:12-h darkness photoperiod cycle. All tanks were supplied with aerated and u.v.-treated well water at 12 °C. Fish were fed daily ad libitum with commercial trout feed (4 PT Regular; Martin Mills, Elmira, ON, Canada). All procedures were carried out in accordance with the Canadian Council for Animal Care guidelines.
and approved by the University of Guelph’s Animal Care Committee.

Experimental design

**Experiment 1: effects of Cryptobia infection on food intake, hematocrit, hemoglobin, liver lep-a1 expression, plasma leptin-A1, and the hypothalamic expression of appetite-regulating genes**

A total of 216 fish (145.7 ± 1.3 g) were anesthetized in a buffered (NaHCO3, 0.2 g/l) solution of tricaine methanesulfonate (0.1 g/l; MS-222; Syndel, Vancouver, BC, Canada), weighed, randomly assigned to one of 24 125 l tanks (n=9 per tank), and acclimated to these conditions for at least 4 weeks. During this time and over the course of the experiment, fish were hand fed at 0900 h daily to satiation, i.e. the fish were fed until they showed no movement toward the feed. Duplicate tanks of fish were randomly assigned one of two treatments: i) an i.p. injection of PBS (150 μl; control treatment) or ii) an i.p. injection of the parasite C. salmositica (TP4) at a dose of 100 000 parasites in 150 μl of PBS (parasite treatment). The animals received i.p. injections under MS-222 anesthesia as mentioned earlier. Fish were terminally sampled at 1, 2, 3, 4, 8, and 14 weeks post-injection (wpi). On the day of sampling, fish were fed to satiation with trout feed labeled with X-ray-dense markers. Ninety minutes after feeding, all fish within a tank of fish were randomly assigned one of three treatments: i) an i.p. injection of PBS (150 μl) and satiation feeding (control treatment), ii) an i.p. injection of the parasite C. salmositica (TP4; 100 000 parasites in 150 μl PBS) and satiation feeding (parasite treatment), or iii) an i.p. injection of PBS (150 μl) and pair feeding to the mean food intake consumed by the pathogen (TP4)-injected fish the day before (pair-fed treatment). All fish were killed at the time of maximal anorexia. This was considered as the point at which mean food intake of the pathogen-injected fish had reached a minimum and stayed at this level over a 3-day period. On the day of sampling, fish were fed to satiation with labeled trout feed. Ninety minutes after feeding, all fish within a tank were terminally anesthetized as above and blood was obtained via caudal puncture. The blood samples were used immediately to determine parasitemia, hematocrit, and hemoglobin concentration (n=16 per treatment). The remaining blood was centrifuged as above to recover plasma for the analysis of leptin-A1 levels (n=16 per treatment), and the liver was sampled to quantify lep-a1 mRNA levels (n=12 per treatment, i.e. six per tank). Given the role of pro-inflammatory cytokines such as interleukin 1β (IL1β) in mediating anorexia–cachexia in mammals (Laviano et al. 2008), both the head kidney and spleen were recovered to measure il1b gene expression (n=12 per treatment, i.e. six per tank). As in Experiment 1, the hypothalamus was isolated to assess the expression of key hypothalamic appetite-regulating genes (n=8 per treatment, i.e. four per tank), and to quantify the mRNA levels of il1b. Individual food intake was quantified from all fish using X-radiography.

**Experiment 2: effects of Cryptobia infection or restricted feeding on food intake, plasma leptin-A1, and on the expression of lep-a1, interleukin 1β, and hypothalamic appetite-regulating genes**

A total of 48 fish (206.0 ± 3.2 g) were used in this experiment. All fish were anesthetized as mentioned earlier, weighed, randomly assigned to one of six 125 l tanks (n=8 per tank), and acclimated to these conditions for at least 4 weeks. During this time, and over the course of the experiment, all fish were fed daily at 0900 h. Duplicate tanks of fish were randomly assigned one of three treatments: i) an i.p. injection of PBS (150 μl) and satiation feeding (control treatment), ii) an i.p. injection of the parasite C. salmositica (TP4; 100 000 parasites in 150 μl PBS) and satiation feeding (parasite treatment), or iii) an i.p. injection of PBS (150 μl) and pair feeding to the mean food intake consumed by the pathogen (TP4)-injected fish the day before (pair-fed treatment). All fish were killed at the time of maximal anorexia. This was considered as the point at which mean food intake of the pathogen-injected fish had reached a minimum and stayed at this level over a 3-day period. On the day of sampling, fish were fed to satiation with labeled trout feed. Ninety minutes after feeding, all fish within a tank were terminally anesthetized as above and blood was obtained via caudal puncture. The blood samples were used immediately to determine parasitemia, hematocrit, and hemoglobin concentration (n=16 per treatment). The remaining blood was centrifuged as above to recover plasma for the analysis of leptin-A1 levels (n=16 per treatment), and the liver was sampled to quantify lep-a1 mRNA levels (n=12 per treatment, i.e. six per tank). Given the role of pro-inflammatory cytokines such as interleukin 1β (IL1β) in mediating anorexia–cachexia in mammals (Laviano et al. 2008), both the head kidney and spleen were recovered to measure il1b gene expression (n=12 per treatment, i.e. six per tank). As in Experiment 1, the hypothalamus was isolated to assess the expression of key hypothalamic appetite-regulating genes (n=8 per treatment, i.e. four per tank), and to quantify the mRNA levels of il1b. Individual food intake was quantified from all fish using X-radiography.

**Experiment 3: effects of Cryptobia infection on O2-carrying capacity**

A total of 33 fish (198.8 ± 4.8 g) were used in this experiment. Each fish was anesthetized as above, weighed, and randomly assigned to one of three 125 l tanks (n=11 per tank). Fish were acclimated and fed according to Experiment 1. At the onset of the study, each tank of fish was randomly assigned one of three treatments: i) an i.p. injection of PBS (150 μl; control
Experiment 4: validation of Cryptobia vaccine

In order to confirm that the attenuated vaccine strain of Cryptobia salmonisitica was generating protective immunity, 24 fish (141.5 ± 8.5 g) were anesthetized as mentioned above, weighed, and randomly assigned to one of three 125 l tanks (n = 8 per tank). Fish were acclimated and fed according to Experiment 1. At the onset of the study, each tank of fish was randomly assigned one of three treatments: i) an i.p. injection of PBS (150 µl; control treatment), ii) an i.p. injection of an attenuated form of Cryptobia salmonisitica (vaccine TV4; 100 000 parasites in 150 µl PBS; vaccine treatment), or iii) an i.p. injection of the parasite Cryptobia salmonisitica (TP4; 100 000 parasites in 150 µl PBS; parasite treatment). The attenuated vaccine strain of Cryptobia salmonisitica which does not cause disease (e.g. Woo & Li (1990) and Beamish et al. (1996)) was used in Experiment 3. It was serially cultured in Minimum Essential Medium at 10 °C over a 62-week period before inoculation into the fish. All fish were terminally sampled at 3 wpi. On the day of sampling, fish were fed to satiation with labeled trout feed. Ninety minutes after feeding, all fish within a tank were rapidly and terminally anesthetized as above and blood samples were obtained via caudal puncture. The blood samples were used immediately to determine parasitemia, hematocrit, hemoglobin concentration (n = 11 per treatment), and O2-carrying capacity (n = 8 per treatment). Individual food intake was quantified from all fish using X-radiography.

Parasitemia determination

Whereas the absence of parasites in non-infected groups was confirmed using the wet mount technique (Woo 1979), parasitemia in the parasite-injected treatments was quantified using a Neubauer hemocytometer (Hauser Scientific, Horsham, PA, USA). Briefly, the collected blood was vortexed before and after dilution with PBS (21-fold dilution proved to be sufficient in all cases), dispensed to occupy each well of the hemocytometer, and the number of parasites counted.

Assessment of hematological parameters and O2-carrying capacity

Whole blood was collected in heparinized capillary tubes to determine hematocrit. The tubes were sealed, centrifuged at 13 400 g for 3 min, and used to determine the ratio of erythrocytes in whole blood samples. Whole blood samples were also assayed immediately for hemoglobin concentration using a microplate assay. Briefly, a hemoglobin standard (15 g/dl; Pointe Scientific, Detroit, MI, USA) was sequentially diluted using Drabkin’s solution (Sigma–Aldrich) to construct a standard curve. Blood samples were also diluted 1:250 using Drabkin’s solution and allowed to incubate for 20 min at room temperature. The optical density of samples and standards were then read at 540 nm on a SpectraMAX 190 microplate reader using SOFTmax Software 4.6 (Molecular Devices, Menlo Park, CA, USA). Individual O2-carrying capacity was measured on whole blood using the modified method of Tucker (1967). The blood samples were allowed to equilibrate for 30 min in a tonometer supplied with 100% water-saturated air, kept at 32 °C, and constantly agitated at 182 r.p.m. Following tonometry, 50 µl blood samples were injected into a temperature-controlled (32 °C) tucker chamber, fitted with a Clark-type O2 electrode, and the change in O2 partial pressure was recorded using Labview Software (National Instruments, Austin, TX, USA).

Food intake quantification

On sampling days, fish were fed a labeled feed that could be detected using X-radiography. The regular diet (4 PT Regular, Martin Mills) was ground to a fine powder and re-pelleted with 450-µm hardened cast carbon steel spheres (Draiswerke, Mahwah, NJ, USA) at a ratio of 5% by mass of dry powdered feed. Fish were X-rayed using an ACU-RAY HFJ portable X-ray unit (50 kV peak; 1.05 mA·s at 90 cm; Sterne, Brampton, ON, Canada). Radiographs were developed, the individual spheres present in the gastrointestinal tract tallied and the amount of food consumed was determined using a calibration curve. Preliminary experiments showed that re-pelleting and diet labeling did not affect palatability.
Quantification of gene expression

Total RNA was extracted using TRIzol Reagent (Life Technologies) and the concentrations quantified using u.v. spectrophotometry at 260 nm (Nanodrop 8000; Nanodrop Products, Wilmington, DE, USA). A random subset of samples from all treatment groups and sampling times were run on agarose gels to check for RNA integrity and genomic DNA contamination. One microgram of total RNA was treated with DNase I (DNase I amplification grade, Life Technologies) and reverse transcribed to cDNA using SuperScript II RNase H⁻ reverse transcriptase (Life Technologies). Each cDNA sample was amplified using an ABI Prism 7000 sequence detection system (Applied Biosystems). Each reaction contained 10 μl of primer (0.4 μM), 0.8 μl of SYBR Green PCR Master Mix (Applied Biosystems), 5 μl cDNA template diluted in 1:5 and 2.5 μl each of forward and reverse primers (0.4 μM) respectively. Default cycling conditions were used: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. This protocol was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency, standard curves were constructed for each target using serial dilutions of cDNA samples. Using the threshold cycle of each unknown, the relative dilution of a target using serial dilutions of cDNA samples. Using the efficiency, standard curves were constructed for each product. To account for differences in amplification efficiencies, each sample was normalized to the expression level of the housekeeping gene, elongation factor 1α (ef1α). Note that the expression of ef1α did not differ between any of the treatments (P > 0.05). All samples were assayed in triplicate and only one target was assayed per well. Finally, non-reverse transcribed RNA and water controls were run to ensure that no genomic DNA was being amplified and the reagents were not contaminated.

Primer pairs for qRT-PCR were designed using Primer Express 3.0 (Applied Biosystems) based on rainbow trout sequences for each target gene (Table 1). Gene expression data is reported as fold change relative to the control treatment.

Quantification of plasma leptin-A1 levels

Plasma leptin-A1 concentrations were determined using RIA according to the methods of Kling et al. (2009). Briefly, a high antigenicity 14 amino-acid peptide (tLep(110–123)) corresponding to the residues 110–123 of rainbow trout leptin-A1 (accession no. AB354909) was synthesized (GenScript, Piscataway, NJ, USA). This peptide shares 71 and 21% sequence identity of rainbow trout leptin-A2 (accession no. JX123129) and leptin-B1 (accession no. JX131306) respectively (Angotzi et al. 2013). tLep(110–123) was subsequently used to immunize rabbits and produce affinity purified polyclonal antibodies (GenScript) and iodinated by the chloramine-T method to produce tracer. The incubation mixture for the standard curve consisted of 0.2 ml standard (tLep(110–123)) and 0.05 ml anti-tLep(110–123) polyclonal antibody (1:1000 dilution). After 24 h at 4 °C, 0.05 ml 125I-labeled tLep(110–123) (~ 10 000 c.p.m.) was added to the mixture and incubated for another 24 h at 4 °C. Bound antigen was precipitated with 0.1 ml pansorbin cells (0.25%; EMD Millipore, Billerica, MA, USA) for 5 h at 4 °C and thereafter centrifuged at 2000 g for 1 h at

### Table 1 Nucleotide sequences of rainbow trout primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>agrp</td>
<td>CR376289</td>
<td>90.8</td>
</tr>
<tr>
<td>cart</td>
<td>CA380644</td>
<td>91.7</td>
</tr>
<tr>
<td>ef1α</td>
<td>AF498320</td>
<td>95.5</td>
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<tr>
<td>il1β</td>
<td>AJ278242</td>
<td>85.7</td>
</tr>
<tr>
<td>lep-a1</td>
<td>AB354909</td>
<td>95.1</td>
</tr>
<tr>
<td>npy</td>
<td>AF203902</td>
<td>99.8</td>
</tr>
<tr>
<td>pomc-a2</td>
<td>TC89514*</td>
<td>87.4</td>
</tr>
</tbody>
</table>

*Expressed sequence tag (EST) contiguous from the rainbow trout gene index (www.tigr.org).
5 °C. The supernatants were removed and precipitates counted on a WIZARD2 gamma counter (Perkin Elmer, Waltham, MA, USA). In the incubation mixture of unknown samples, the 0.2 ml of standard ligand was replaced with rainbow trout plasma. All measurements were made in duplicate. The lowest detectable level of the leptin-A1 RIA was 250 pM. The dilution curve of immunoreactive leptin in rainbow trout plasma was parallel to the standard curve of tLept(110–123) (Supplementary Fig. 1B, see section on supplementary data given at the end of this article). No cross-reactivity was detected between anti-tLep(110–123) polyclonal antibody and recombinant rainbow trout growth hormone or insulin-like growth factor 1 (0.3–100 ng/ml; GroPep Bioreagents, Adelaide, SA, Australia). However, given the 71% sequence identity between residues the 110–123 of rainbow trout leptin-A1 and leptin-A2, we cannot exclude the possibility that our assay is also measuring leptin-A2. All samples were measured in a single assay and the intra-assay coefficient of variation was 5.9% (n = 8).

Western blot analysis of full-length recombinant leptin-A in Arctic char (Salvelinus alpinus; generously provided by Dr M M Vijayan (University of Calgary, Calgary, AB, Canada)) using the anti-tLep(110–123) polyclonal antibody revealed specific binding at the expected molecular mass of 16 kDa (Supplementary Fig. 1B). Leptin-A1 in Arctic char (accession no. BAH83335) shares 71% sequence identity with tLept(110–123).

Statistical analysis

All data are presented as mean ± S.E.M. For a given parameter in Experiments 1 and 2, differences between duplicate tanks were assessed by Student’s t-test. Since no significant difference was observed between duplicate tanks, further statistical analysis was performed on combined data. In Experiment 1, differences in parasitemia between sampling times were assessed by a one-way ANOVA and by pairwise Tukey’s post hoc test. A two-way ANOVA followed by a Holm–Sidak test for multiple comparisons was used to determine the effects of treatment and time on food intake, hematocrit, hemoglobin, liver leptin-A1 expression, and plasma leptin-A1 levels. Differences between treatments in hypothalamic gene expression data were determined by Student’s t-test. In Experiments 2 and 3, differences between treatments were assessed by a one-way ANOVA followed by a Tukey’s post hoc test for all pairwise comparisons. In Experiment 4, a two-way ANOVA followed by a Holm–Sidak test for multiple comparisons was used to determine the effects of treatment and time on parasitemia. Data that did not meet the assumption of normality were log-transformed before analysis. The correlation between O2-carrying capacity and food intake in Experiment 3 was analyzed using Pearson’s Product Moment Correlation. All analyses were performed using SigmaStat 3.0 (SPSS). The significance level for all statistical tests was P < 0.05.

Results

Experiment 1: effects of Cryptobia infection on food intake, hematocrit, hemoglobin, liver lep-a1 expression, plasma leptin-A1, and the hypothalamic expression of appetite-regulating genes

C. salmositica infection caused severe disease in the pathogen-injected fish. Although C. salmositica infection was undetectable in the control fish, the number of blood parasites increased exponentially post-injection and peaked at ~11 × 10⁶ parasites/ml at 3 wpi in Cryptobia-infected fish. Parasitemia declined rapidly thereafter and remained at ~ 0.5 × 10⁶ parasites/ml between 8 and 14 wpi (Fig. 1A). Hematocrit values in the parasite-injected fish fell to 50% when compared with those in the control fish at 3 and 4 wpi (Fig. 1B). With the decline in parasitemia, hematocrit values partially recovered in the pathogen-infected fish, but were still 29% lower than that in controls by the end of the experiment. Similarly, peak infection with C. salmositica was associated with a 50% drop in hemoglobin concentrations and a partial recovery by 14 wpi that paralleled the changes in hematocrit (Fig. 1C).

Relative to the control fish, food intake in the parasite treatment remained relatively unchanged during the first 2 wpi, quickly dropped by 75% at 3 wpi, and then gradually recovered back to control levels over the following 11 weeks (Fig. 1D). In general, fish infected with C. salmositica were characterized by an inverse relationship between food intake and liver lep-a1 gene expression. Liver lep-a1 gene expression in the parasite treatment increased 6.5- and 16.7-fold over control levels at 3 and 4 wpi, respectively, decreasing thereafter but remaining four times higher than control levels at 14 wpi (Fig. 1E). Plasma leptin-A1 in the parasite treatment also increased 1.6- and 1.7-fold over control levels at 3 and 4 wpi, respectively, and remained elevated through 14 wpi (Fig. 1F).

At 4 wpi, relative to the control treatment, fish infected with C. salmositica were characterized by significant changes in the hypothalamic expression of several appetite-regulating genes (Fig. 2). Interestingly, while the
reduction in food intake was associated with a decrease in npy mRNA levels; it was also characterized by an increase in the transcript levels of another orexigenic gene, agp. Similarly, although C. salmositica infection resulted in a greater than twofold increase in pomic-a2 expression, it was also associated with a reduction in the mRNA levels of the anorexigenic gene, cart.

**Experiment 2: effects of Cryptobia infection or restricted feeding on food intake, plasma leptin-A1, and on the expression of lep-a1, il1b, and hypothalamic appetite-regulating genes**

At the time of maximal anorexia, 25 days post-injection, numbers of blood parasite in the pathogen-injected fish were $4.53 \pm 1.14 \times 10^6$ parasites/ml. In contrast, parasites were not detected in either the control or pair-fed fish. The parasite-injected fish ate 77% less than the controls and the diet of the pair-fed fish was matched to that of the parasite treatment (Fig. 3A). In contrast, while the hematocrit and hemoglobin concentration of the pathogen-injected fish were 62 and 75% lower than in the controls, respectively, there were no differences in either hematocrit or hemoglobin between the pair-fed and control fish (Supplementary Fig. 2A and B, see section on supplementary data given at the end of this article). Similarly, although the reduction in food intake in the parasite treatment was associated with an 8.7-fold increase in liver lep-a1 mRNA levels and a 1.8-fold increase in plasma leptin-A1, both lep-a1 gene expression and plasma levels did not differ between the pair-fed and control treatments (Fig. 3B and C). Finally, relative to the control and pair-fed fish, the il1b mRNA levels in the parasite treatment were markedly reduced in the head kidney and spleen (Fig. 3D and E) and unchanged in the hypothalamus (Fig. 3F).

Despite both having significantly reduced levels of food intake, the parasite and pair-fed fish were characterized by opposite changes in hypothalamic npy gene expression (Fig. 4). While parasite-induced appetite suppression was associated with lower npy mRNA levels, reduced feed availability in the pair-fed fish increased the
expression of this transcript. In contrast, relative to satiated controls, both parasite-infected and pair-fed fish had increased 
agrp
gene expression. As observed in Experiment 2, 

*Fig. 2*
Effects of an i.p. injection of PBS (control treatment) or 

Cryptobia salmositica
(100 000 parasites in PBS; parasite treatment) on the gene expression of hypothalamic neuropeptide Y (npy), agouti-related protein (agrp), pro-opiomelanocortin A2 (pomc-a2), and cocaine- and amphetamine-regulated transcript (cart). Fish were sampled at 4 weeks post-injection. The expression data are reported as the ratio of the gene of interest:elongation factor 1α (ef1α) mRNA levels and expressed relative to the value of the control treatment. *Difference between treatments for a given gene as determined by Student’s t-test. The significance level for all statistical tests was P<0.05 (n=8). Values are mean +/− S.E.M.*

expression but pair feeding had no effect. Finally, the parasite and pair-fed treatments were both characterized with a decrease in hypothalamic 
cart
gene expression, but the reduction only reached significance in the pair-fed fish.

**Experiment 3: effects of Cryptobia infection on **

*O₂*-carrying capacity

At the time of acute disease, i.e. at 3 wpi, the parasitemia of parasite-infected fish was 3.4 times higher than that in vaccine-injected fish (Fig. 5A). Relative to the PBS-injected control fish, both vaccine- and parasite-infected fish had reduced hematocrit (Fig. 5B) and hemoglobin concentration (Fig. 5C), but the effects were significantly more pronounced in the parasite treatment than in the vaccine treatment. Although food intake in the parasite-infected fish was reduced by 60%, it was not significantly affected in the vaccinated fish (Fig. 5D). The *O₂*-carrying capacity of the vaccine- and parasite-infected fish was respectively 34 and 61% lower than that in the controls (Fig. 5E). Overall, among the three treatments, there was a significant positive linear correlation (*R²=0.61, P<0.0001*) between *O₂*-carrying capacity and food intake (Fig. 5F).

**Experiment 4: validation of Cryptobia vaccine**

Parasites were detectable in the blood of both the vaccine- and parasite-injected fish at 2 and 3 wpi, but the parasite-injected fish had consistently higher parasite numbers (Supplementary Fig. 3A, see section on supplementary data given at the end of this article). Following challenge with the pathogenic 

C. salmositica
at 5 wpi, previously vaccinated fish had similar parasite numbers to naïve fish at 2 weeks post-challenge and significantly lower parasitemia than naïve fish at 4 weeks post-challenge (Supplementary Fig. 3B).

**Discussion**

This study provides original evidence that the appetite-suppressing effects of 

C. salmositica
infection in rainbow trout are associated with marked increases in hepatic 
lep-a1
mRNA expression and plasma leptin-A1 levels. During infection, maximum anorexia and 
lep-a1
mRNA expression correspond with the lowest hematocrit values, and at peak parasitemia there is a strong linear relationship between *O₂*-carrying capacity and food intake. Using fish pair fed to the parasite treatment, we also demonstrate that the elevated plasma leptin-A1 levels of infected fish are not due to a reduction in food intake, and are specifically associated with a reduction in 
npy
and an increase in 
pomc-a2
hypothalamic mRNA levels. Together, these results suggest that the expression of hepatic leptin in rainbow trout is stimulated by hypoxemic conditions and that the appetite-suppressing effects of 

C. salmositica
infection are at least partly mediated by leptin.

The parasitemia and hematocrit changes observed in this study are consistent with the known progression of 

C. salmositica
infection in salmonids (Woo & Wehnert 1986, Beamish et al. 1996, Chin et al. 2004). The number of blood parasites peaked during acute infection at 3–4 wpi and subsequently declined in correspondence with a large increase in the production of 

Cryptobia
-agglutinating antibodies (Sitja-Bobadilla & Woo 1994, Chin et al. 2004). The anemic condition is caused by the direct lytic action of a 

Cryptobia
-secreted metalloprotease along with complement-mediated destruction of antibody-coated erythrocytes and necrosis of hematopoietic tissues (Thomas & Woo 1988, Zuo & Woo 2000, Bamanrokh & Woo 2001). Herein, we show that the marked reduction in hemoglobin levels at peak parasitemia results in a
Leptin and food intake in hypoxemic trout

Figure 3

Effects of an i.p. injection of PBS (control treatment), Cryptobia salmositica (100,000 parasites in PBS; parasite treatment), or PBS and restricted feeding (pair-fed treatment) on (A) food intake (n = 16), (B) liver leptin-A1 (lep-a1) gene expression (n = 12), (C) plasma leptin-A1 levels (n = 16), (D) head kidney, (E) spleen, and (F) hypothalamus interleukin 1β (il1β) gene expression (n = 12). Whereas the control and parasite treatments were fed to satiation, the pair-fed treatment was fed the mean food intake consumed by the parasite treatment the day before. All fish were terminally sampled at maximal anorexia, 25 days post-parasite injection. Parasites were not detected in the control and pair-fed treatments. The gene expression data is reported as the ratio of the gene of interest: elongation factor 1α (ef1α) mRNA levels and expressed relative to the value of the control treatment. Treatments that do not share a common letter are significantly different from each other as determined by a one-way ANOVA and pairwise Tukey’s post hoc test. The significance level for all statistical tests was P < 0.05. Values are mean ± S.E.M.

significant decrease in O₂-carrying capacity. Relative to parasite-injected fish, the smaller reduction in hemoglobin and O₂-carrying capacity in the vaccinated fish concurs with the reduced ability of attenuated C. salmositica strains to produce the hemolytic metalloprotease (Zuo & Woo 1997, Woo 2003). Overall, the hypoxemic state is consistent with the increased hypoxia susceptibility of Cryptobia-infected rainbow trout (Woo & Wehnert 1986) and the reduced aerobic scope and swimming capacity associated with this disease (Kumaraguru et al. 1995).

Anorexia is also a distinctive clinical sign of C. salmositica infection (Thomas & Woo 1992, Beamish et al. 1996, Chin et al. 2004). In this study, peak anorexia coincided with peak parasitemia as well as the lowest hematocrit and hemoglobin values. This relationship between anorexia, parasitemia and anemia has been previously reported in C. salmositica-infected fish (Woo 1979, Chin et al. 2004) and in mammals infected with the causative agent of sleeping sickness, Trypanosoma brucei (order Trypanosomatida, class Kinetoplastea) (Dumas & Bisser 1999, Darsaud et al. 2003). While it is well established that chronic exposure to environmental hypoxia can reduce food intake in both hypoxia-sensitive and -tolerant fish species (e.g. Chabot & Dutil (1999), Pichavant et al. (2001), Bernier & Craig (2005) and Bernier et al. (2012)), previous studies have not directly examined the impact of hypoxemia on food intake. Herein, we show that acute infection with C. salmositica is characterized by a positive linear relationship between individual food intake and O₂-carrying capacity.

The marked reduction in O₂-carrying capacity during peak C. salmositica infection also corresponded with a sharp increase in hepatic lep-a1 mRNA expression and a significant increase in plasma leptin-A1 levels. These results are consistent with the observation that lep is a hypoxia-responsive gene and that its expression is stimulated by HIF1 in response to a reduction in O₂ availability in both mammals (Ambrosini et al. 2002) and fish (Chu et al. 2010). While adipose tissue hypoxia (Hosogai et al. 2007, Wang et al. 2008) and exposure to hypobaric hypoxia (Chen et al. 2007, Simler et al. 2007) are associated with increases in lep mRNA expression and plasma leptin levels in mammals, chronic hypoxia also

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increases hepatic lep mRNA expression in fish (Chu et al. 2010, Bernier et al. 2012, Yu et al. 2012). Given the anorexigenic effects of leptin in rainbow trout (Murashita et al. 2008, Aguilar et al. 2010), the inverse relationship between hepatic lep-a1 mRNA expression and food intake in C. salmositica-infected fish and the increase in plasma leptin-A1 levels during acute infection suggest that leptin contributes to the regulation of food intake at peak parasitemia.

In contrast, the return to control food intake despite elevated plasma leptin-A1 levels during the chronic stage of C. salmositica infection, suggest a gradual desensitization to the anorectic actions of leptin. While a state of leptin resistance has not been previously described in fish, several mechanisms are known to reduce leptin signaling and promote leptin tolerance in obese individuals with chronically elevated plasma leptin levels (Myers et al. 2008, Schneeberger et al. 2013). In common carp, chronic hypoxia leads to a gradual reduction in hypothalamic leptin receptor (lepr) mRNA expression (Bernier et al. 2012). Similarly, changes in lepr expression or signaling within the hypothalamic feeding circuits of C. salmositica infected rainbow trout may be important for understanding the discrepancy between circulating leptin levels and appetite regulation. Interestingly, the high plasma leptin-A1 levels of the C. salmositica infected fish between 4 and 14 wpi were sustained despite a significant decrease in hepatic lep-a1 mRNA expression. In chronically fasted rainbow trout, elevated plasma leptin levels are associated with reduced leptin-binding protein levels (Gong et al. 2013). Similarly, a reduction in plasma leptin-binding protein levels during the chronic stage of C. salmositica infection may serve to maintain high plasma leptin levels and explain the discrepancy between hepatic lep-a1 mRNA and plasma leptin levels. Alternatively, the clearance of leptin from the blood may slow down during chronic C. salmositica infection thereby increasing the half-life of plasma leptin.

Using fish pair fed to the parasite treatment, our results demonstrate that the marked increases in hepatic lep-a1 mRNA expression and plasma leptin-A1 levels of C. salmositica-infected rainbow trout are not due to a reduction in nutrient availability. While these results are consistent with previous studies, which failed to observe an effect of fasting on lep gene expression in some fish species (Huisings et al. 2006b, Gorissen et al. 2009, Kobayashi et al. 2011, Tinoco et al. 2012), they also contrast with others where a positive relationship between fasting and hepatic lep mRNA expression has been observed. In rainbow trout (Kling et al. 2009), Atlantic salmon (Ronnestad et al. 2010, Trombley et al. 2012) and fine flounder (Paralichthys adspersus; Fuentes et al. 2012), fasting and restricted feeding have been associated with elevated plasma leptin levels. However, in those fish species where feed restriction can cause an increase in plasma leptin levels, the response is only observed after a minimum of 1 week of complete fast or several months of rationed feeding. In contrast, the reduction in food intake in C. salmositica-infected rainbow trout is transient and at peak anorexia the parasite-infected fish still consumes ~25% of the ration in the control treatment.

The overall changes in the expression pattern of the hypothalamic appetite-regulating genes within the parasite-infected and pair-fed fish at peak anorexia also support a role for leptin in the regulation of food intake during C. salmositica infection. Leptin inhibits food intake in mammals by inhibiting the hypothalamic expression of the orexigenic signals, npy and agrp, and by stimulating the expression of the anorexigenic signals α-melanocyte-stimulating hormone (α-MSH; a product of pomc) and cart (Ahima & Flier 2000). In fish, the appetite-suppressing effects of native leptins have been associated with reductions in npy (Murashita et al. 2008, Li et al. 2010),...
Figure 5
Effects of an i.p. injection of PBS (control treatment), an attenuated form of Cryptobia salmositica (100 000 parasites in PBS; vaccine treatment) or C. salmositica (100 000 parasites in PBS; parasite treatment) on (A) parasitemia (n=11), (B) hematocrit (n=11), (C) hemoglobin concentration (n=11), (D) food intake (n=11), (E) blood O2-carrying capacity (n=8), and (F) the relationship between blood O2-carrying capacity, and individual food intake (n=8). All fish were terminally sampled 3 weeks post-injection. Parasites were not detected in the control treatment. Treatments that do not share a common letter are significantly different from each other as determined by Student’s t-test (parasitemia) or by a one-way ANOVA and pairwise Tukey’s post hoc test. The relationship between O2-carrying capacity and food intake was analyzed using Pearson’s product moment correlation test (R² = 0.61, P<0.0001). The significance level for all statistical tests was P<0.05 (n=8-11). Values are mean ± S.E.M.

increases in pomc (Murashita et al. 2008, 2011), and no change in agrp or cart (Murashita et al. 2011) hypothalamic mRNA expression. In vitro, human leptin also decreases hypothalamic npy mRNA levels, but does not have any direct effect on pomc or cart expression in rainbow trout (Aguilar et al. 2010). In contrast, feed deprivation in fish is generally associated with increases in the expression of npy and agrp, decreases in cart mRNA levels, and no change in pomc hypothalamic expression (Leder & Silverstein 2006, Volkoff et al. 2009, Cerda-Reverter et al. 2011). Therefore, in this study, the reduction in npy and increase in pomc-a2 hypothalamic expression during peak anorexia in the parasite-infected rainbow fish are consistent with the known effects of recombinant native leptins in fish. Similarly, the parallel increases in agrp and decreases in cart mRNA levels during peak anorexia in the parasite and pair-fed treatments are consistent with the known effects of feed restriction on these transcripts and support the earlier observation that leptin may not affect the hypothalamic expression of these genes. Peak anorexia in C. salmositica-infected rainbow trout is also associated with a marked increase in the expression of brain preoptic area (POA) corticotropin-releasing factor (crf; Madison et al. 2013), a potent anorexigenic signal in fish (Bernier 2006, Ortega et al. 2013). The fact that the anorexigenic action of α-MSH is mediated by the CRF-signaling pathway in goldfish (Matsuda et al. 2008) and mice (Kawashima et al. 2008), suggest that POA CRF neurons may act as a downstream mediator of hypothalamic POMC neuron signaling and contribute to the regulation of food intake in Cryptobia-infected rainbow trout.

In addition to leptin, several other factors are known to contribute to anorexia (Carlton et al. 2012). Key among the signals that contribute to the regulation of food intake during infection are the pro-inflammatory cytokines IL1β, IL6, and tumor necrosis factor alpha (TNFα; Buchanan & Johnson 2007). Produced both peripherally and centrally by cells of the innate immune system, these cytokines inhibit food intake in mammals through multiple pathways including the stimulation of leptin release (Sarraf et al. 1997). While the role of cytokines in the regulation of food intake in fish is largely unknown, peripheral injection of the pro-inflammatory cytokine-inducing bacterial endotoxin, lipopolysaccharide, induces
anorexia in goldfish (Volkoff & Peter 2004). However, peak anorexia in Cryptobia-infected rainbow trout was associated with a marked reduction in IL1β mRNA expression in the periphery and with no change in hypothalamic IL1β mRNA levels. Also, while common carp (Cyprinus carpio) infected with a related hemoflagellate parasite, Trypanoplasma borreli (order Parabodonida, class Kinetoplastea), are characterized by an upregulation of IL1β and Tnfα gene expression in the head kidney, liver, and spleen, the response generally peaks within 2 days of infection (Engelsma et al. 2003, Saeij et al. 2003). Although these preliminary results suggest that IL1β does not contribute to the regulation of food intake during peak C. salmositica infection in rainbow trout, an alternative explanation for the observed reduction in head kidney and spleen IL1β gene expression is a redistribution and/or depletion of leukocyte populations from these hematopoietic tissues in response to infection. Moreover, multiple cytokines are known to have anorexigenic properties in mammals (Buchanan & Johnson 2007). As such, we suggest that future studies are needed to directly assess the role of inflammatory cytokines in the regulation of food intake during infection in fish, as well as their potential contribution to the regulation of leptin secretion.

The role of leptin during C. salmositica infection in rainbow trout may not be limited to the regulation of food intake. For example, the inhibitory effects of leptin on pituitary adrenocorticotropin hormone secretion and interrenal cell cortisol production in fish (Gorissen et al. 2012), and its ability to suppress the expression of key adrenocortisol steroidogenic enzymes in mammals (Kruse et al. 1998, Su et al. 2012) likely contribute to the blunted stress response and reduced cortisol synthesis capacity of C. salmositica-infected rainbow trout (Madison et al. 2013). Similarly, given the important roles of leptin in the regulation of hematopoiesis (Bennett et al. 1996), angiogenesis (Anagnostoulis et al. 2008), and the immune system (Carlton et al. 2012, Mariano et al. 2013), a promising avenue for future research in C. salmositica- and T. borreli-infected fish may be to explore the contribution of leptin to the regeneration of hematopoietic tissues during the chronic stage of infection and its effects on the innate and acquired immune responses that characterize these diseases (Woo & Ardelli 2014). Recent studies in fish have shown that leptin can function as a hyperglycemic factor (Baltzegar et al. 2014) and stimulate metabolic rate (Dalman et al. 2013). Therefore, given the considerable bioenergetic cost of C. salmositica infection (Beamish et al. 1996, Woo 2003), leptin may also play an important role in regulating energy expenditure or promoting catabolic processes during the sustained phase of infection. Finally, a pleiotropic role for leptin during hypoxic conditions in fish is suggested by the stimulatory effects of chronic hypoxia exposure on the expression of lepr in several peripheral tissues (Wong et al. 2007, Cao et al. 2011, Bernier et al. 2012).

Though a clinical sign of many diseases is a loss of appetite, very little is known about the specific mechanisms that mediate anorexia in diseased fish. In support of our hypothesis, our findings implicate leptin, in concert with other orexigenic (e.g. NPY) and anorexigenic (e.g. α-MSH and CRF) neuropeptides, as a potential mediator of Cryptobia-induced anorexia. As previously observed in hypoxic common carp (Bernier et al. 2012), our results also suggest that hepatic lep gene expression in fish is more sensitive to deficits in O2 availability than in nutrient availability. Overall, as many fish pathogens bring about disease through the production of factors that are highly hemolytic or that agglutinate erythrocytes (Bernier 2010, Woo & Bruno 2011), we suggest that leptin may mediate anorexia in a variety of fish diseases.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0615.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research was supported by grants to P T K W and N J B from Natural Sciences and Engineering Research Council of Canada (NSERC).

Acknowledgements
The authors thank Barry Madison, Erin Bristow, Sylvana Miller, and Fu Ci Guo for their excellent technical assistance and Dr Dominique Bureau for his help in the development of the labeled feed used for food intake quantification. Dr M M Vijayan is gratefully acknowledged for providing us with recombinant Arctic char leptin-A. The authors are also grateful to Drs James Ballantyne and Pat Wright for access to equipment and to Bob Frank and Matt Cornish for technical support in the Hagen Aqualab.

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Received in final form 31 March 2014
Accepted Preprint published online 16 April 2014