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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, O_2-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 O_2-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

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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

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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*. 2006*a*). 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*. 2006*b*, Murashita *et al*. 2008, Rønnestad *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*. 2006*b*, Gorissen *et al*. 2009, Kobayashi *et al*. 2011), it decreases (Gorissen *et al*. 2009, Won *et al*. 2012) or increases (Kling *et al*. 2009, Rønnestad *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 Parabodonida, 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 O_2, 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 O_2-carrying capacity of rainbow trout and the relationship between O_2-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

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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 ($NaHCO_3$, 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 in a tank were terminally anesthetized at once with an overdose of 2-phenoxyethanol (2 ml/l, Sigma–Aldrich) and blood was obtained via caudal puncture using a K_2EDTA (0.5 M, pH 8.0)-treated syringe. The blood samples were used immediately to determine parasitemia, hematocrit, and hemoglobin concentration ($n=18$ per treatment). The remaining blood was centrifuged at 14 000 ***g*** for 3 min and the separated plasma was flash frozen in liquid nitrogen before storage at −80 °C for later analysis of plasma leptin-A1 ($n=12$ per treatment, i.e. six per tank). The liver was sampled to quantify the mRNA levels of *lep-a1*. At 4 wpi (peak anorexia), the brain was regionally dissected to isolate the hypothalamus according to the method of Bernier *et al.* (2008) and quantify the gene expression of neuropeptide Y (*npy*), agouti-related protein (*agrp*), pro-opiomelanocortin A2 (*pomc-a2*), and cocaine- and amphetamine-regulated transcript (*cart*). All tissues were immediately frozen in liquid nitrogen and stored at −80 °C for future analysis ($n=8$ per treatment, i.e. four per tank). Individual food intake was quantified from all fish using X-radiography (see 'Food intake quantification' section).

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 *il1β* 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 *il1β*. Individual food intake was quantified from all fish using X-radiography.

Experiment 3: effects of *Cryptobia* infection on O_2-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

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treatment), ii) an i.p. injection of an attenuated form of *C. salmositica* (vaccine TV4; 100 000 parasites in 150 µl PBS; vaccine treatment), or iii) an i.p. injection of the parasite *C. salmositica* (TP4; 100 000 parasites in 150 µl PBS; parasite treatment). The attenuated vaccine strain of *C. salmositica* 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 O_2-carrying capacity ($n=8$ per treatment). Individual food intake was quantified from all fish using X-radiography.

Experiment 4: validation of *Cryptobia* vaccine In order to confirm that the attenuated vaccine strain of *C. salmositica* 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 *C. salmositica* (vaccine TV4; 100 000 parasites in 150 µl PBS; vaccine treatment), or iii) an i.p. injection of the parasite *C. salmositica* (TP4; 100 000 parasites in 150 µl PBS; parasite treatment). The attenuated vaccine strain of *C. salmositica* used in this experiment was the same as used above in Experiment 3. PBS-, vaccine-, and parasite-injected fish were bled at 2 and 3 wpi to monitor parasitemia. At 5 wpi, both PBS- and vaccine-injected fish were challenged with an i.p. injection of the pathogenic strain (TP4; 100 000 parasites in 150 µl PBS). All pathogen-challenged fish were bled 2 and 4 weeks later to determine parasitemia.

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 (Hausser 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 O_2-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 O_2-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 O_2 electrode, and the change in O_2 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.

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Quantification of gene expression

Total RNA was extracted using TRIzol Reagent (Life Technologies) and the concentrations quantified using u.v. spectrophotometery 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 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 given sample was extrapolated using the linear regression of the target-specific standard curve. To correct for differences in template input and reverse transcriptase 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 ^{125}I-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

Gene	Accession no.	Efficiency (%)	Sequence (5′→3′)
agrp	CR376289	90.8	F: ACCAGCAGTCCTGTCTGGGTAA R: AGTAGCAGATGGAGCCGAACA
cart	CA380644	91.7	F: CCTCGACACAAGAAGTGTGAGAGA R: TGTAGTGCTCCAAGCAGTTGCT
ef1α	AF498320	95.5	F: GGGCAAGGGCTCTTTCAAGT R: CGCAATCAGCCTGAGAGGT
il1β	AJ278242	85.7	F: ATGGGAACCGAGTTCAAGGA R: AACACTATATGTTCTTCCACAGCACTCT
lep-a1	AB354909	95.1	F: GAGGGCATGGACCCATTTC R: GGCAGGCTTTCTATATGCTGATC
npy	AF203902	99.8	F: CGGTCAAACCCGAAAATCC R: TCTTCCCATACCTCTGCCTTGT
pomc-a2	TC89514[a]	87.4	F: CTGAAAACAACCTCCTGGAGTGT R: GAGAGGAGGGACAGAGGTAAGTAGAG

agrp, agouti-related protein; *cart*, cocaine- and amphetamine-regulated transcript; *ef1α*, elongation factor 1α; F, forward; *il1β*, interleukin 1β; *lep-a1*, leptin-A1; *npy*, neuropeptide Y; *pomc-a2*, pro-opiomelanocortin A2; R, reverse.
[a]Expressed sequence tag (EST) contiguous from the rainbow trout gene index (www.tigr.org).

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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 $tLep_{(110–123)}$ (Supplementary Fig. 1A, 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 charr (*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-A in Arctic charr (accession no. BAH83535) shares 71% sequence identity with $tLep_{(110–123)}$.

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 concentration, liver leptin gene 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 O_2-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 $\sim 11\times10^6$ parasites/ml at 3 wpi in *Cryptobia*-infected fish. Parasitemia declined rapidly thereafter and remained at $\sim 0.5\times10^6$ 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

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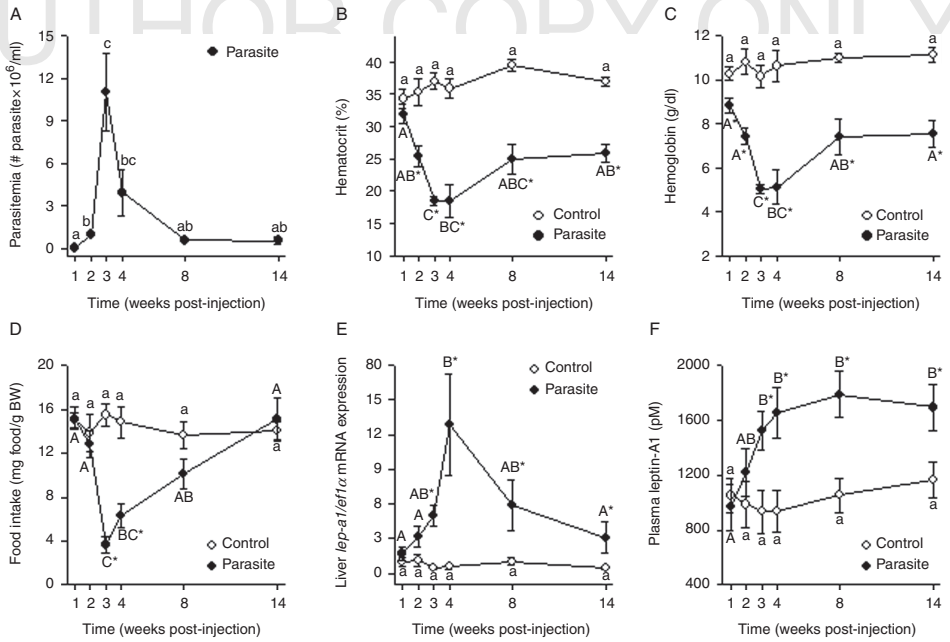

Figure 1

Effects of an i.p. injection of PBS (control treatment) or *Cryptobia salmositica* (100 000 parasites in PBS; parasite treatment) on (A) parasitemia ($n=18$), (B) hematocrit ($n=18$), (C) hemoglobin concentration ($n=18$), (D) food intake ($n=18$), (E) liver leptin-A1 (*lep-a1*) gene expression ($n=8$), and (F) plasma leptin-A1 levels ($n=12$). Parasites were not detected in the control treatment. The gene expression data are reported as the ratio of *lep-a1*:elongation factor 1α (*ef1*α) mRNA levels and expressed relative to the value of the control treatment. 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 concentration, liver *lep-a1* gene expression, and plasma leptin-A1 levels. Sampling times within the control treatment that do not share a common lowercase letter, or within the parasite treatment that do not share an uppercase letter, are significantly different from each other. *Difference between treatments at a given time. The significance level for all statistical tests was $P<0.05$. Values are mean ± S.E.M. BW, body weight.

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, *agrp*. Similarly, although *C. salmositica* infection resulted in a greater than twofold increase in *pomc-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*, *il1β*, 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 $4.53\pm1.14\times10^6$ 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 *il1β* 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

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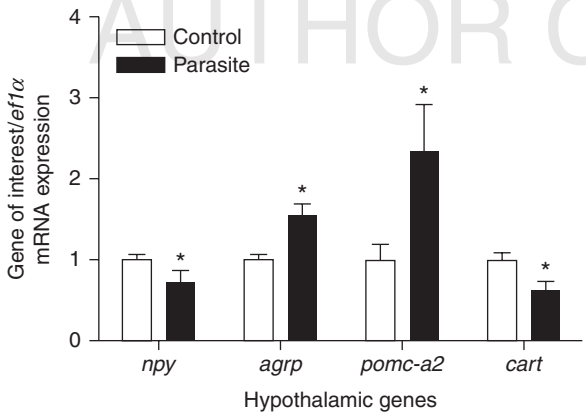

Figure 2
Effects of an i.p. injection of PBS (control treatment) or *Cryptobia salmositica* (100 000 parasites in PBS; parasite treatment) on the gene expression of hypothalamic neuropeptide Y (*npy*), agouti-related protein (*agrp*), pro-opiomelanocortin A2 (*pomc-a2*), and cocaine- and amphetamine-regulated 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 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, *C. salmositica* infection also resulted in a marked increase in *pomc-a2* 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 O_2-carrying capacity

At the time of acute disease, i.e. at 3 wpi, the parasitemia of parasite-injected 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 O_2-carrying capacity of the vaccine- and parasite-injected 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 ($R^2=0.61$, $P<0.0001$) between O_2-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 O_2-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, Bahmanrokh & Woo 2001). Herein, we show that the marked reduction in hemoglobin levels at peak parasitemia results in a

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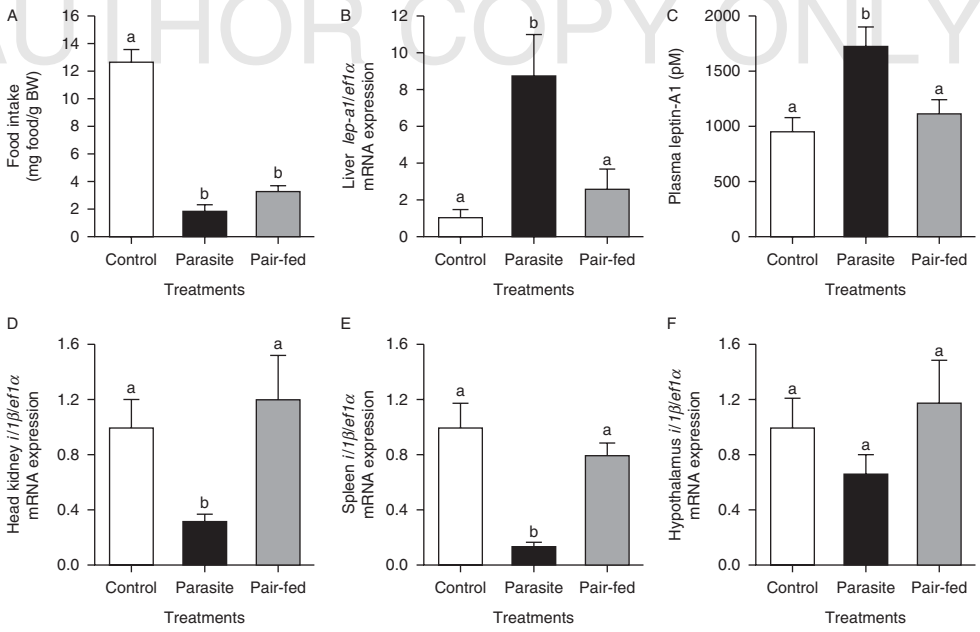

Figure 3

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 O_2-carrying capacity. Relative to parasite-injected fish, the smaller reduction in hemoglobin and O_2-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 O_2-carrying capacity.

The marked reduction in O_2-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 O_2 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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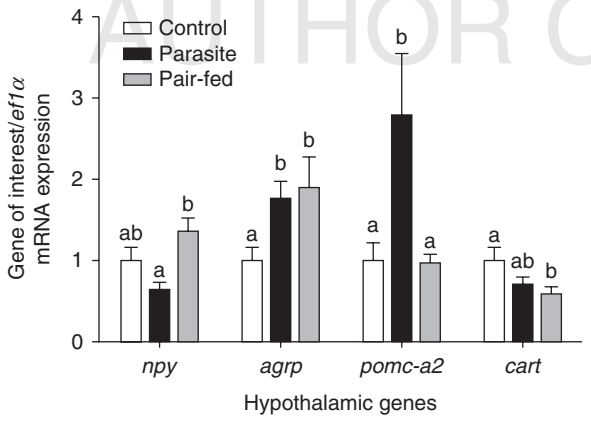

Figure 4

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 the gene expression of hypothalamic neuropeptide Y (*npy*), agouti-related protein (*agrp*), pro-opiomelanocortin A2 (*pomc-a2*), and cocaine- and amphetamine-regulated transcript (*cart*). 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. The expression data are reported as the ratio of the gene of interest:elongation factor 1α (*ef1*α) mRNA levels and expressed relative to the value of the control treatment. For a given gene, 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 $P<0.05$ ($n=8$). Values are mean+S.E.M.

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 (Huising *et al.* 2006*b*, 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 (Rønnestad *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),

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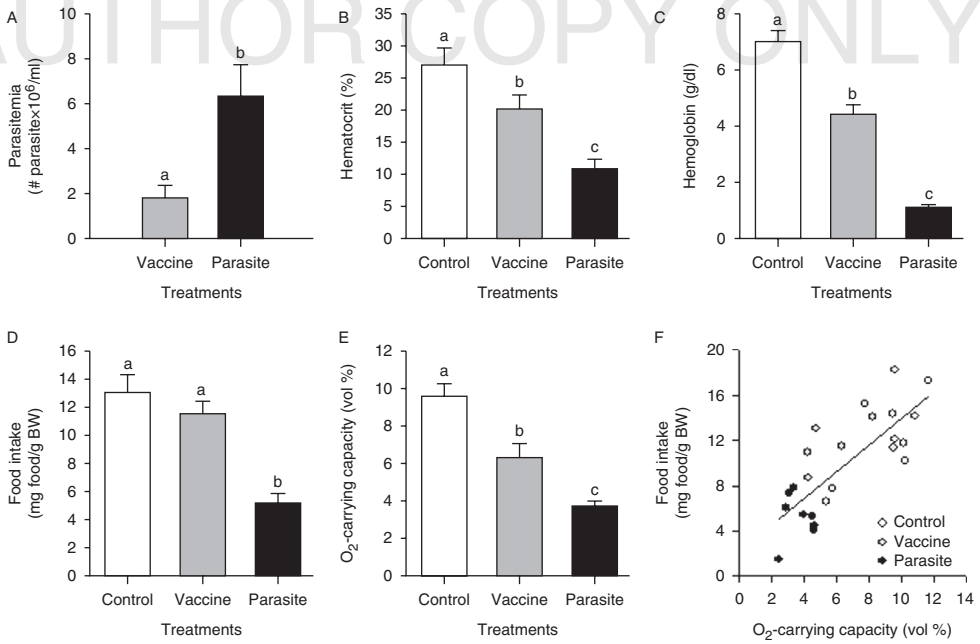

Figure 5

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 O_2-carrying capacity ($n=8$), and (F) the relationship between blood O_2-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 O_2-carrying capacity and food intake was analyzed using Pearson's product moment correlation test ($R^2=0.61$, $P<0.0001$). 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 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

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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 adrenocortex 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 hypoxemic 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 O_2 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.

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