Huress without stress: *Cryptobia* infection results in HPI axis dysfunction in rainbow trout

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Abstract

Despite clear physiological duress, rainbow trout (*Oncorhynchus mykiss*) infected with the pathogenic haemoflagellate *Cryptobia salmositica* do not appear to mount a cortisol stress response. Therefore, we hypothesized that the infection suppresses the stress response by inhibiting the key effectors of the hypothalamic–pituitary–interrenal (HPI) axis. To test this, we characterized the basal activity of the HPI axis and the cortisol response to air exposure in saline- and parasite-injected fish. All fish were sampled at 4 and 6 weeks post-injection (wpi). While both the treatment groups had resting plasma cortisol levels, the parasite-infected fish had lower levels of plasma ACTH than the control fish. Relative to the control fish, the infected fish had higher mRNA levels of brain pre-optic area corticotrophin-releasing factor (CRF) and pituitary CRF receptor type 1, no change in pituitary POMC-A1, -A2 and -B gene expression, higher and lower head kidney melanocortin 2 receptor mRNA levels at 4 and 6 wpi respectively and reduced gene expression of key proteins regulating interrenal steroidogenesis: StAR, cytochrome P450sc and 11β-hydroxylase. The parasite-infected fish also had a reduced plasma cortisol response to a 60-s air exposure stressor. Superfusion of the head kidney tissues of the parasite-infected fish led to significantly lower ACTH-stimulated cortisol release rates than that observed in the control fish. These novel findings show that infection of rainbow trout with *C. salmositica* results in complex changes in the transcriptional activity of both central and peripheral regulators of the HPI axis and in a reduction in the interrenal capacity to synthesize cortisol.

Introduction

Challenges that disturb the homoeostasis of an animal can be met by an activation of the stress response. A key component of this response in fish involves the stimulation of the hypothalamic–pituitary–interrenal (HPI) axis (Wendelaar Bonga 1997). Corticotrophin-releasing factor (CRF) from the pre-optic area (POA) is the principle hypothalamic regulator of the HPI axis (Bernier et al. 2009). CRF stimulates the secretion of the pro-opiomelanocortin (POMC)-derived peptide ACTH from the anterior pituitary via the CRF type 1 receptor (CRF-R1; Flik et al. 2006). In turn, ACTH binds to the melanocortin type 2 receptor (MC2R; Aluru & Vijayan 2008) of the interrenal cells in the head kidney and regulates the synthesis and secretion of cortisol, the primary stress hormone in teleosts (Barton 2002). The key rate-limiting steps for the production of cortisol...
include the transport of cholesterol across the inner mitochondrial membrane by StAR, the conversion of cholesterol to pregnenolone by the enzyme cytochrome P450 side-chain cleavage (P450scc) and the hydroxylation of 11-deoxycortisol to cortisol by 11β-hydroxylase (Mommsen et al. 1999). Given its importance in the maintenance of the dynamic steady state of the internal milieu in fish, several studies have characterized the impact of physical, environmental and social stressors on the principle effectors of the HPI axis (Huising et al. 2004, Bernier & Craig 2005, Fuzzen et al. 2010, Jeffrey et al. 2012). By contrast, much less is known about the regulation of the HPI axis in response to the challenges associated with diseases.

During immune challenges in mammals, activated macrophages produce pro-inflammatory cytokines such as tumour necrosis factor α (TNF) and interleukin-1β (IL1β (IL1B)) that activate the hypothalamic–pituitary–adrenal (HPA) axis (Turnbull & Rivier 1999). The resulting increase in circulating glucocorticoids affects numerous immune functions. Systemically, glucocorticoids primarily exert anti-inflammatory effects that protect the organism from the damaging effects of an over-stimulation of the immune system (Sapolsky et al. 2000). Similarly in fish, there is evidence that IL1B can stimulate the activity of the HPI axis (Holland et al. 2002, Metz et al. 2006) and that cortisol can inhibit the production of pro-inflammatory cytokines (Holland et al. 2003, Stolte et al. 2008, Castillo et al. 2009). Moreover, the elevated levels of plasma cortisol associated with either stressors or exogenous glucocorticoids are immunosuppressive and promote pathogen proliferation (Woo et al. 1987, Yada et al. 2002, Saeij et al. 2003a, Li et al. 2013). Overall, cortisol seems to play an important role in the regulation of the immune response in fish that can affect disease susceptibility and outcome (Verburg-van Kemenade et al. 2009).

Though cortisol is a key effector of the HPI axis during the immune response, its production during infection with protozoan parasites is equivocal. For example, rainbow trout infected with the pathogenic haemoflagellate Cryptobia salmositica (order Kinetoplastida, family Bodonidae) show no evidence of pituitary–interrenal axis activation either during disease onset or during disease progression (Laidley et al. 1988). Yet, C. salmositica-infected fish are characterized by pronounced abdominal distension with ascites, exophthalmia, anaemia, anorexia, suppressed growth and lethargy, i.e. clear signs of physiological duress (Woo 1979, 2003). Similarly, common carp (Cyprinus carpio) infected with Trypanosoma borreli (order Kinetoplastida, family Bodonidae), a close relative of C. salmositica, and human patients chronically infected with either Trypanosoma brucei or Trypanosoma cruzi (order Kinetoplastida, family Trypanosomatidae), the causative agents of African trypanosomiasis and Chagas disease respectively, are all characterized by cortisol levels that do not differ from those of the control subjects (Reinecke et al. 1994, Mazon et al. 2006, Pérez et al. 2011). In fact, both African trypanosomiasis and Chagas disease are characterized by dysfunctional adrenocortical and pituitary function (Reinecke et al. 1998, Corrêa-de-Santana et al. 2006, Pérez et al. 2011).

In this study, to determine how the HPI axis of rainbow trout is regulated during infection with C. salmositica, we characterized the basal activity of the HPI axis and the cortisol response to air exposure in saline- and parasite-injected fish. Using an in vitro superfusion set-up, we also determined the impact of C. salmositica infection on the cortisol synthesis capacity of the interrenals. Given the absence of any change in plasma cortisol levels despite evidence of severe illness (Laidley et al. 1988), we hypothesized that C. salmositica infection suppresses the stress response by inhibiting the key effectors of the HPI axis.

Materials and methods

Animals

Sexually immature rainbow trout of either sex were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada). Prior to experimental use, the fish were housed in the Hagen Aqualab at the University of Guelph (Guelph, ON, Canada) in 800 l tanks supplied with aerated well water at 12 ± 1°C, fed daily to satiation with commercial trout feed (4 PT Regular; Martin Mills, Elmira, ON, Canada) and kept under a 12 h light:12 h darkness photoperiod cycle. All procedures were approved by the local Animal Care Committee and conformed to the principles of the Canadian Council for Animal Care.

C. salmositica: preparation of inoculum

An ampoule of frozen C. salmositica (strain TP4; cryo-preserved at −90°C) was thawed and ~100 000 parasites were injected intraperitoneally into four rainbow trout (~150 g). At 4 weeks post-injection (wpi), once clinical signs of Cryptobia infection were detected, the fish were terminally anaesthetized using an overdose of tricaine methanesulphonate (MS-222) and parasites were collected from the blood via a caudal puncture using
Na₂EDTA-treated syringes and needles. The number of parasites was determined using a haemocytometer (Hausser Scientific, Horsham, PA, USA), and the blood was diluted with sterile PBS (pH 7.2) to a concentration of 100 000 parasites per 150 µl.

Experimental design

Experiment 1: effects of Cryptobia infection on the HPI axis in vivo A total of 80 fish (average weight ~140 g) were randomly assigned to one of eight 125-l tanks (n = 10 per tank) and allowed to acclimatize for 4 weeks. Following the acclimatization period, all fish were anaesthetized using buffered (NaHCO₃, 0.2 g/l) MS-222 (0.1 g/l; Syndel, Nanaimo, BC, Canada), and each fish in the four control tanks was inoculated intraperitoneally with 150 µl sterile PBS (control treatment); each fish in the remaining four tanks was injected with 100 000 parasites in 150 µl PBS (parasite treatment). At 4 and 6 wpi, all the fish from one tank in each treatment group were terminally anaesthetized using an overdose of MS-222 and sampled immediately to determine basal parameters. Blood was drawn via caudal puncture using a Na₂EDTA-treated syringe, and parasite load was determined using a haemocytometer (Mc2r); each fish in the four control tanks was injected with PBS and those from the other tanks were injected with C. salmositica as in Experiment 1. At 4 and 6 wpi, ten control and ten parasite-infected fish were terminally anaesthetized, and a blood sample was recovered to determine parasite load. To assess cortisol release in vitro, a 1 cm² area of the midline anterior head kidney was carefully dissected and immediately placed in 5 ml of ice-cold 0.015 M HEPES/Tris-buffered superfusion medium (128 mM NaCl, 2 mM KCl, 2 mM CaCl₂·2H₂O, 0.25% w/v glucose, 0.03% w/v BSA and 0.1 mM ascorbic acid, pH 7.4) for 30 min to flush residual endogenous cortisol from the dissection process. Individual samples (200–300 mg) were finely diced and placed on a fine-meshed filter in a superfusion chamber. The chamber was sealed and superfused using 13±0.5 °C carbogen (95% O₂ and 5% CO₂)-saturated medium at a flow-through rate of 40 µl/min via a multichannel peristaltic pump (Minipuls 3, Gilson, Inc., Middleton, WI, USA). During an initial equilibration period of 90 min, fractions were collected every 30 min until endogenous cortisol production reached a steady state. Following equilibration (t = 0 min), the head kidney preparations were superfused for 60 min with a medium containing 10⁻⁷ M human ACTH (hACTH, American Peptide Co., Sunnyvale, CA, USA) followed by superfusion with the medium alone for the remainder of the experiment. Fractions were collected every 20 min during peak cortisol production between 0 and 180 min and every 30 min thereafter until the end of the superfusion experiments. All fractions were immediately frozen at −20 °C and analysed for cortisol content using RIA. After superfusion, head kidney samples were removed from the chambers, sonicated (Vibracell, Sonics and Materials, Newtown, CT, USA) in 500 µl ddH₂O and analysed for protein content (Bio-Rad Protein Assay with BSA standards; Bio-Rad Laboratories). Cortisol release from each sample is expressed relative to the time 0 value and reported in pg/µg protein/min. The maximal cortisol release following the addition of ACTH, the time delay between the addition of ACTH and maximal cortisol release, and the total amount of cortisol released over the duration of the superfusion experiments were also calculated from each superfusion experiment.

Cortisol and ACTH analyses

Plasma and superfusion medium cortisol concentrations were measured in duplicate using RIA (Bernier et al. 2008). Briefly, 200 µl of standard, diluted plasma or superfusion medium were combined with
200 μl ³H-cortisol (5500–6000 c.p.m./tube, 70–100 Ci/mmol, PerkinElmer, Boston, MA, USA) and 200 μl of diluted rabbit anti-cortisol antibody (product code R4866, Clinical Endocrinology Laboratory, University of California Davis, CA, USA). The samples were incubated at 4°C for 16 h and then chilled on ice for 10 min prior to the addition of 200 μl of dextran-coated charcoal suspension in phosgel buffer (5.75 g dibasic sodium phosphate, 1.28 g monobasic sodium phosphate, 1 g gelatin and 0.1 g thimerosal per litre of dH₂O, pH 7.6). The samples were then vortexed and centrifuged at 1750g at 4°C for 12 min. The resultant supernatant was decanted directly into scintillation vials containing 5 ml of scintillation fluid and counted. Cortisol measurement in extracted plasma and medium were diluted to fall within the 20–80% range of the standard curve. A serial dilution of rainbow trout plasma gave a displacement curve that was parallel to the standard curve, and the lower detection of the assay was 15 pg/ml. All plasma cortisol samples were measured in a single assay with an intra-assay variability of 1% (n=4), while inter- and intra-assay variations of superfusion medium cortisol concentrations were 11.4% (n=6) and 1.9% (n=6) respectively.

Plasma ACTH levels were measured in duplicate using RIA. The reagents were provided, and the procedure was carried out as outlined in the ImmuChem Double Antibody hACTH kit (product code #07-106101; MP Biomedicals, Orangeburg, NY, USA). This assay has been validated previously for use in trout plasma (Craig et al. 2005). All samples were analysed in one assay that had a lower detection limit of 10 pg/ml and an intra-assay variation of 5.1% (n=8). Furthermore, a serial dilution of rainbow trout plasma gave a displacement curve parallel to the ACTH standard curve.

RNA extraction, first-strand cDNA synthesis and qRT-PCR
All tissues were homogenized and total RNA was extracted using TRIzol Reagent (Invitrogen). RNA pellets were re-dissolved in RNase-free water and quantified using TRIzol Reagent (Invitrogen). RNA pellets were quantified using TRIzol Reagent (Invitrogen). All tissues were homogenized and total RNA was extracted using TRIzol Reagent (Invitrogen). RNA pellets were re-dissolved in RNase-free water and quantified using TRIzol Reagent (Invitrogen). RNA pellets were then vortexed and centrifuged at 1750g at 4°C for 12 min. The resultant supernatant was decanted directly into scintillation vials containing 5 ml of scintillation fluid and counted. Cortisol measurement in extracted plasma and medium were diluted to fall within the 20–80% range of the standard curve. A serial dilution of rainbow trout plasma gave a displacement curve that was parallel to the standard curve, and the lower detection of the assay was 15 pg/ml. All plasma cortisol samples were measured in a single assay with an intra-assay variability of 1% (n=4), while inter- and intra-assay variations of superfusion medium cortisol concentrations were 11.4% (n=6) and 1.9% (n=6) respectively.

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DNA extraction, first-strand cDNA synthesis and qRT-PCR
All tissues were homogenized and total RNA was extracted using TRIzol Reagent (Invitrogen). RNA pellets were re-dissolved in RNase-free water and quantified using u.v. (A260) spectrophotometry. One microgram of total RNA was treated with DNase I and reverse transcribed to cDNA using Superscript II RNase H-reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Non-reverse-transcribed (no-RT controls) representatives from each tissue, treatment and sampling time were included during cDNA synthesis to monitor genomic contamination. Triplicates of each cDNA sample were amplified using an ABI StepOne Plus sequence detection system (Applied Biosystems). Each 15 μl reaction mixture contained 7.5 μl of Perfecta SYBR Green 2× PCR FastMix ROX (Quanta BioSciences, Gaithersburg, MD, USA), 3.75 μl of 5- to 15-fold diluted first-strand cDNA template or no-RT controls, and 1.875 μl of both forward and reverse primers (1.6 μM). Default cycling conditions were used: 5 min at 95°C followed by 40 cycles of 1 s at 95°C and 30 s at 60°C. This protocol was followed using a melting curve analysis to verify the specificity of the PCR products. 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). To account for differences in amplification efficiency, standard curves were constructed for each gene using known dilutions of cDNA. Input values were obtained by fitting the average threshold cycle (Ct) value to the antilog of the standard curve. To correct for minor variations in template input and transcriptional efficiency, the input values were normalized to the expression level of the housekeeping gene elongation factor 1α (ef1α). Initial pilot experiments revealed no changes in ef1α expression with parasite infection and thus ef1α was selected as a housekeeping gene for this study. Gene expression data are reported as fold change from the 4 wpi control treatment mRNA levels.

Statistical analyses
All results are presented as means ± s.e.m. For each variable in Experiment 1, a two-way ANOVA was carried out followed by a Holm–Sidak post hoc test to determine differences between the treatment groups and between the sampling times. In Experiment 2, a one-way repeated-measures ANOVA was carried out followed by a Bonferroni post hoc test to determine differences in cortisol release rate from the time 0 value within a given treatment. Differences in cortisol release rate, maximal cortisol release rate, response time to maximal cortisol release rate and total cortisol release between the treatments were determined by two-way ANOVA and by pairwise Holm–Sidak post hoc tests. Non-parametric data were log-transformed prior to analysis. All analyses were performed using SigmaStat 3.5 (SPSS, Inc.), and P<0.05 was considered statistically significant for all tests.

Results
In contrast to the control fish, the infected fish showed clear signs of disease at 4 and 6 wpi. The infected fish were anorexic (B N Madison, P T K Woo & N J Bernier 2013 unpublished observations) and lethargic and characterized by abdominal distension with ascites and...
exophthalmia. No parasites were detected in the control fish, while the parasitaemias in the infected fish were $2.41 \pm 0.75 \times 10^6$ and $1.79 \pm 0.62 \times 10^6$ parasites/ml of blood at 4 and 6 wpi respectively.

### Experiment 1: effects of Cryptobia infection on the HPI axis in vivo

Resting plasma cortisol levels were similar between the control and infected fish, and they ranged between 1.5 and 3 ng/ml (Fig. 1). Air exposure for 60 s significantly increased plasma cortisol levels in both treatments and sampling times. However, the average stress-induced increase in plasma cortisol levels over both sampling times was 31% lower in the infected fish than in the control fish.

In the pre-optic region of the brain, crf gene expression at both 4 and 6 wpi was fivefold higher in the infected fish than in the uninfected fish (Fig. 2A). Overall, pituitary crf-r1 transcript levels were higher in the infected fish than in the control fish, but the difference between the treatments was greater at 4 wpi than at 6 wpi (Fig. 2B). By contrast, the pituitary mRNA levels of pomc-a1, pomc-a2 and pomc-b did not differ between the treatments at either sampling time (Fig. 2C, D, and E), and basal plasma ACTH levels in the infected fish at both 4 and 6 wpi were 50% lower than those in the control fish (Fig. 2F).

Parasite infection was also associated with multiple changes in transcriptional activity in the interrenal cells of the head kidney. Relative to those in the control fish, while the mRNA levels of mc2r in the infected fish were increased by 2.8-fold at 4 wpi, they were decreased by nearly 70% at 6 wpi (Fig. 3A). Cryptobia infection also reduced the expression of head kidney star, p450scc and 11β-hydroxylase (Fig. 3B, C, and D). However, the reductions in transcript levels were only significant in the time-matched control treatment group at 6 wpi. Relative to those in the control treatment group, the mRNA levels of star, p450scc and 11β-hydroxylase in the infected fish at 6 wpi were reduced by 69, 64 and 75% respectively.

### Experiment 2: effects of Cryptobia infection on cortisol secretion in vitro

ACTH ($10^{-7}$ M) stimulated the rate of cortisol release from head kidney tissues in the control and infected fish at 4 and 6 wpi (Fig. 4A and B). However, the ACTH pulses elicited significantly larger increases in cortisol secretion rate in the control fish than in infected fish. Specifically, relative to the time-matched control fish, the infected fish at 4 and 6 wpi were characterized by 3.2-fold lower ACTH-stimulated maximal cortisol release rates (Fig. 4C). Also, a 40% longer response time to maximal cortisol release rate

### Table 1  Details of primers used for real-time PCR assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession no.</th>
<th>Efficiency (%)</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-Hydroxylase</td>
<td>AF179894.1</td>
<td>88.4</td>
<td>F: GCAGGAGGATCGTGAGAAGAC &lt;br&gt; R: GACGAAACTCACAACAGAGGATG&lt;br&gt;</td>
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<tr>
<td>crf</td>
<td>AF296672</td>
<td>90.8</td>
<td>F: ACAACGACTCAACTGGAAGATCTCG &lt;br&gt; R: AGGAAATTGAGCGCTGAGG&lt;br&gt;</td>
</tr>
<tr>
<td>crf-r1</td>
<td>AY533879.1</td>
<td>87.8</td>
<td>F: ACAGGCGCGCATGAGACA &lt;br&gt; R: CGTGGCCTGTTGAATCTG&lt;br&gt;</td>
</tr>
<tr>
<td>ef1α</td>
<td>AF498320</td>
<td>96.0</td>
<td>F: GAGGGTAGTATAGCTGAGAGAT &lt;br&gt; R: GGTCCGCTGGTTGAGAAG&lt;br&gt;</td>
</tr>
<tr>
<td>mc2r</td>
<td>NM_001124680.1</td>
<td>89.0</td>
<td>F: CGCTAGTACCATCCTCCA &lt;br&gt; R: GCCCTGGTTGCTATGAGT&lt;br&gt;</td>
</tr>
<tr>
<td>P450scc</td>
<td>S57305.1</td>
<td>96.9</td>
<td>F: GAGGGTAGGGAAGATCTGAGAGAT &lt;br&gt; R: GGTCCGCTGGTTGAGAAG&lt;br&gt;</td>
</tr>
<tr>
<td>pomc-a1</td>
<td>TC86162α</td>
<td>84.0</td>
<td>F: CTCGCTGCAAGACTCAACTCT &lt;br&gt; R: GAGTTGGTGGAGAGATGAGAGCT&lt;br&gt;</td>
</tr>
<tr>
<td>pomc-a2</td>
<td>TC89514α</td>
<td>87.4</td>
<td>F: CGAAGAACACTCTCTCTGAGAG &lt;br&gt; R: GAGGGTAGGGAAGATCTGAGAGAT&lt;br&gt;</td>
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<tr>
<td>pomc-b</td>
<td>X69809.1</td>
<td>89.7</td>
<td>F: GCCAGGCAAACCGCTAT &lt;br&gt; R: ATTGCAGTCATATGGCTTCATG&lt;br&gt;</td>
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<tr>
<td>star</td>
<td>NM_001124202.1</td>
<td>87.1</td>
<td>F: GAGGGTAGGGAAGATCTGAGAGAT &lt;br&gt; R: GGTCCGCTGGTTGAGAAG&lt;br&gt;</td>
</tr>
</tbody>
</table>

*EF1α, elongation factor 1α; F, forward; MC2R, melanocortin 2 receptor; R, reverse.

<sup>a</sup>Accession numbers from the rainbow trout gene index (www.tigr.org).
shown that pro-inflammatory cytokines such as IL1β, IL6 and TNFα increase CRF (CRH) gene expression in the hypothalamic paraventricular nucleus (PVN) in response to infection by pathogens (Turnbull & Rivier 1999). Similarly, the increase in brain crf mRNA levels and content in fish given a peripheral injection of the cytokine-inducing bacterial endotoxin lipopolysaccharide (Pepels & Balm 2004, Volkoff & Peter 2004) suggests that pro-inflammatory cytokines may mediate the increase in crf gene expression observed in this study. Although the production of pro-inflammatory cytokines peaks during the early stages of T. borreli infection in common carp, the expression of IL1β and TNFα is up-regulated in several immune organs up to at least 11 days post-infection (Fig. 4D) and 4.1-fold lower total cortisol release over the duration of the superfusion experiments (Fig. 4E) was observed in the infected fish.

**Discussion**

This study reports the first characterization of the effects of a parasitic infection in a teleost species on the transcriptional activity of the HPI axis and on its principle endocrine products, ACTH and cortisol. While our results confirm an earlier observation (Laidley et al. 1988) that infection of rainbow trout with the haemoflagellate C. salmositica does not affect baseline plasma cortisol levels, they also show that this disease has a significant impact on the HPI axis. Overall, C. salmositica infection of rainbow trout differentially affected the transcriptional activity of POA crf, pituitary crf-r1 and head kidney mc2r, star, p450sc and 11β-hydroxylase, key effectors of the HPI axis. C. salmositica infection also disrupted the stress response of rainbow trout, lowered basal plasma ACTH levels and reduced cortisol synthesis capacity.

Although a variety of stressors are associated with an increase in POA crf gene expression in fish (Bernier et al. 2009), to our knowledge, this is the first study to show that a parasitic infection can result in an increase in the transcription of this gene. Studies in mammals have
POA crf expression of *C. salmositica*-infected fish is most probably not responsible for the increase in pituitary *crf-r1* gene expression. Similarly, pro-inflammatory cytokines are not expected to mediate the increase in *crf-r1* gene expression, since previous studies have shown that treatment with either LPS or IL1B decreases pituitary *crf-r1* mRNA expression (Schmidt et al. 2003, De La Garza et al. 2005). Instead, since chronic intermittent hypoxia results in a sustained up-regulation of pituitary *crf-r1* mRNA expression in rats (Wang et al. 2004), the

(Engelsma et al. 2003, Saeij et al. 2003b). However, peak *C. salmositica* infection in rainbow trout (i.e. ~ 4 wpi) is associated with marked decreases in head kidney and spleen *il1b* mRNA expression (L E MacDonald, S L Alderman, S Kramer, P T K Woo & N J Bernier 2013 unpublished observations). Alternatively, the hypoxaemic conditions triggered by the ~ 50% reduction in haematocrit in *C. salmositica*-infected rainbow trout (Chin et al. 2004) may be responsible for the increase in POA *crf* gene expression. Previously, we have shown that hypoxia exposure can increase the gene expression of POA *crf* in rainbow trout (Bernier & Craig 2005).

At the pituitary level, peak *C. salmositica* infection was associated with a significant increase in *crf-r1* gene expression. Although increases in *crf-r1* gene expression are observed after the application of an acute stressor in mammals, chronic CRF stimulation of pituitary CRF-R1 leads to a down-regulation of *crf-r1* mRNA expression (Rabadan-Diehl et al. 1996, Kageyama & Suda 2009). Similarly, 24 h of chronic restraint stress in common carp concomitantly increases the gene expression of POA *crf* and reduces the gene expression of pituitary *crf-r1* (Huising et al. 2004). As such, the chronic increase in

**Figure 4** In vitro cortisol release rate from rainbow head kidney tissue in a superfusion set-up. Fish received an i.p. injection of either PBS (control treatment) or *Cryptobia salmositica* (parasite treatment) and head kidney tissues were sampled at 4 and 6 wpi. Following an equilibration period of 90 min, the head kidney tissues were stimulated with 10^{-7} M human ACTH for 60 min as indicated by the shaded portions. Asterisks indicate statistical differences with time 0 value within a treatment as determined by one-way repeated-measures ANOVA and by Bonferroni post hoc test. The time interval during which a difference was identified between the treatments is indicated by † symbol as determined by two-way ANOVA and by pairwise Holm–Sidak post hoc test. From the data presented in (A) and (B), the maximal cortisol release rate (C), the response time to maximal cortisol release rate (D) and the total cortisol release (E) in response to ACTH stimulation were also determined. Individual bars that do not share a common lowercase letter or treatments that do not share an uppercase letter are significantly different from each other as determined by two-way ANOVA and by pairwise Holm–Sidak post hoc test. The significance level for all the statistical tests was *P* < 0.05 (n = 10).
hypoxaemia that characterizes *C. salmositica* infection may be responsible for the increase in *crf-r1* transcription.

Despite increases in POA *crf* and pituitary *crf-r1* expression, *C. salmositica*-infected rainbow trout in this study were characterized by a lack of change in *pomc-a1*, *pomc-a2* and *pomc-b* mRNA levels and by a paradoxical decrease in plasma ACTH levels. Since CRF probably stimulates pituitary *pomc* gene transcription and ACTH secretion in fish as in mammals via CRF-R1 (Roberts *et al.* 1987, Flik *et al.* 2006), our results suggest that *Cryptobia* infection is associated with an activation of the HPI axis and a disruption in the signalling pathways of the corticotropes that mediate the stimulatory actions of CRF on ACTH release. Similarly, the blunted ACTH and cortisol responses to CRF treatment in patients infected with African trypanosomiasis suggest that a dysfunctional corticotropic response is associated with this parasitic disease (Reinecke *et al.* 1994).

The marked reduction in the capacity of the interrenal tissue of *C. salmositica*-infected fish to synthesize cortisol in response to ACTH treatment *in vitro* also implies that this disease leads to interrenal dysfunction. Likewise, the results of standard ACTH stimulation tests show that patients with African trypanosomiasis are characterized by adrenocortical insufficiency (Reinecke *et al.* 1994). Given the key role of STAR, P450SCC and 11β-hydroxylase in interrenal cell steroidogenesis (Mommsen *et al.* 1999, Fuzzen *et al.* 2010), the parasitaemia-induced reduction in the expression of these genes probably contributed to the observed reduction in cortisol synthesis capacity. However, despite having nearly identical cortisol responses to ACTH *in vitro*, the 4 and 6 wpi groups of fish infected with *C. salmositica* had a marked difference in head kidney *mc2r* gene expression. In a previous study, the up-regulation of *mc2r* expression in rainbow trout corresponded with an increase in interrenal tissue steroidogenic capacity and elevated cortisol production *in vitro* (Aluru & Vijayan 2008). Therefore, beyond a reduction in cortisol synthesis capacity, our results suggest that *Cryptobia* infection in rainbow trout also leads to a disruption in the signalling pathways that mediate the stimulatory actions of ACTH on interrenal steroidogenesis.

In accordance with an impairment of corticotropic and interrenal functions, we observed that *C. salmositica*-injected fish have a reduced cortisol response to a standardized 60-s air exposure stressor relative to saline-injected fish. While the magnitude of the parasitaemia-induced reduction in the stress response *in vivo* was relatively small in comparison with the marked suppression in *in vitro* cortisol synthesis capacity, the ACTH concentration used to stimulate maximal steroidogenesis in the superfusion experiments was approximately two orders of magnitude higher than the concentrations measured in the plasma of stressed rainbow trout (Doyon *et al.* 2006). By contrast, the lack of difference in baseline plasma cortisol levels between the treatments despite lower plasma ACTH levels in the parasite-infected fish suggest that non-ACTH corticotrophic signals may enhance the steroidogenic actions of ACTH in *C. salmositica*-infected rainbow trout (Bernier *et al.* 2009).

Parallel studies on the regulation of food intake and growth in *C. salmositica*-infected rainbow trout suggest that the cytokine leptin may be an important factor in the regulation of the HPI axis during parasitaemia. Leptin is a hypoxia-sensitive gene and its expression is stimulated by hypoxia-inducible factor 1 in response to an oxygen deficit in both mammals (Ambrosini *et al.* 2002) and fish (Chu *et al.* 2010, Bernier *et al.* 2012). In rainbow trout, *C. salmositica* infection is associated with a marked increase in liver leptin gene expression and plasma leptin concentrations (L E MacDonald, S L Alderman, S Kramer, P T K Woo & N J Bernier 2013 unpublished observations). Besides its important role in the homeostatic control of feeding and energy expenditure (Myers *et al.* 2008), leptin is involved in controlling the activity of the HPA (Roubos *et al.* 2012) and HPI (Gorissen *et al.* 2012) axes. In common carp, for example, leptin suppresses basal and CRF-induced ACTH secretion from superfused anterior pituitary glands and attenuates ACTH-stimulated cortisol production (Gorissen *et al.* 2012). In mammals, leptin inhibits the synthesis of corticosteroids by suppressing the adrenocortex expression of MC2R, StAR, P450scx, and other key steroidogenic enzymes (Kruse *et al.* 1998, Su *et al.* 2012). Moreover, although the actions of leptin on the synthesis and secretion of hypothalamic CRF and pituitary ACTH in mammals are still equivocal, leptin can increase PVN *crf* gene expression and blunt plasma ACTH responses to stress (Heiman *et al.* 1997, Malendowicz *et al.* 2007, Roubos *et al.* 2012). Therefore, while experiments are needed to identify the specific actions of leptin on the HPI axis of rainbow trout, given the above, it is conceivable that leptin is at least partly responsible for the blunted stress response and reduced cortisol synthesis capacity of *C. salmositica*-infected fish.

In addition to pro-inflammatory cytokines and leptin, pathophysiological changes associated with parasitaemia may contribute to the changes in HPI axis activity observed in this study. For example, in the head kidney of *C. salmositica*-infected rainbow trout and
T. borrelli-infected common carp, parasitaemia is characterized by a gradual proliferation of the interstitial haematopoietic tissue and an infiltration of extravascular parasites and phagocytes (Bunnajirakul et al. 2000, Bahmanrokh & Woo 2001). At peak parasitaemia, the extensive proliferation of lymphoid cells and infiltration of inflammatory cells lead to focal necrosis of the anterior kidney (Bunnajirakul et al. 2000, Bahmanrokh & Woo 2001). Moreover, during the later stages of T. borrelli infection in common carp, the activated phagocytes of the head kidney produce high levels of nitric oxide (NO; Wiegertjes & Forlenza 2010). While the role of NO in the regulation of the HPI axis has not been determined and the actions of NO on the HPA axis are complex, in mammals NO cannot inhibit adrenal steroidogenesis (Monau et al. 2010) and reduce the release of hypothalamic CRF and pituitary ACTH (Mancuso et al. 2010). Whether interrenal cell necrosis or NO production contributes to the dysfunction within the HPI axis in C. salmositica-infected rainbow remains to be determined.

This study reveals that the relative stress hyporesponsiveness of C. salmositica-infected rainbow trout results from complex alterations in the activity of the HPI axis. While the precise mechanisms responsible for this HPI axis dysfunction are not known, studies of haemoflagellate parasite-infected fish suggest that leptin, pro-inflammatory cytokines and NO production may be important factors. Whether the hyporesponsive HPI axis of C. salmositica-infected rainbow trout is adaptive or maladaptive remains to be determined. The fact that cortisol implants or stressors can increase parasitaemia and mortality in C. salmositica-infected rainbow trout and T. borrelli-infected common carp suggests that a hyporesponse HPI axis could be adaptive (Woo et al. 1987, Saeij et al. 2003a). By contrast, the anti-inflammatory properties of cortisol in fish (Saeij et al. 2003a, Stolte et al. 2008), the lack of a cortisol stress response in C. salmositica-infected rainbow trout could be harmful to the host and contribute to an increased morbidity (Verburg-van Kemenade et al. 2011). An intriguing possibility is that the sensitivity of head kidney phagocytes to the anti-inflammatory effects of cortisol may be mediated through an increase in the expression of glucocorticoid receptors in these cells (Stolte et al. 2008).

Overall, our results highlight the need for a better understanding of the contributions of the cortisol stress response to the pathogenesis of parasitic diseases in fish and of the complex interactions between the HPI axis and the immune system.

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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