Effect of *Cryptobia salmositica*-induced anorexia on feeding behavior and immune response in juvenile rainbow trout *Oncorhynchus mykiss*

Adrian Chin, Fu Ci Guo, Nicholas J. Bernier, Patrick T. K. Woo*

Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

ABSTRACT: At 10°C, rainbow trout *Oncorhynchus mykiss* (n = 13 per group) infected with *Cryptobia salmositica* Katz, 1951 became anorexic at 3 wk post-infection (w.p.i.), with feed-intake decreasing significantly from 1.33 to 0.94 % body weight (b.w.). Anorexia was most severe at 4 w.p.i. (0.80 % b.w.), coinciding with peak parasitemia (9.2×10^6 parasites ml blood^{-1}) and anemia. At 8 w.p.i., fish had recovered their appetite although they still had contained detectable parasites (6.8×10^5 parasites ml^{-1}) and were anemic (pack cell volume, PCV, of 24.4 %). However at 5°C, anorexia occurred at 5 w.p.i. (0.81 % b.w.), and was most severe at 7 w.p.i. (0.40 % b.w.). At 8 w.p.i. (0.43 % b.w.), fish displayed high parasitemia (4.6×10^6 parasites ml^{-1}) and low PCV (10.8 %). Fish at 5°C had lower gastric evacuation (GE) rates (GE$_{48h}$) than 10°C fish, however there were no differences between infected and naïve fish at both temperatures. Before anorexia, there was no significant correlation between mean share of meal (MSM, a measure of how food was partitioned within a group) and coefficient of variation in feeding but this became significant during anorexia (p = 0.02 and p = 0.0002 at 10 and 5°C respectively). Significant correlations were detected between b.w. and MSM before onset of anorexia at 10°C (p = 0.005) and 5°C (p = 0.02); this was maintained at 10°C (p = 0.001) but not at 5°C (p = 0.98). Fish on an anorexic diet (0.93 % b.w.) responded well at 10°C to a live *C. salmositica* vaccine; this could partly be due to constant antigenic stimulation by the live vaccine.

KEY WORDS: Anorexia · *Cryptobia salmositica* · Temperature · Gastric evacuation · Feeding hierarchies · Immune response · X-radiography · Vaccine

INTRODUCTION

Cryptobia salmositica Katz, 1951, a protozoan hemoflagellate (Kinetoplastida) causes salmonid cryptobiosis which can result in morbidity and mortality. Clinical signs of disease are microcytic and hypochromic anemia, exophthalamia, abdominal distension with ascites, splenomegaly and hepatomegaly. Anorexia is also a consistent sign of the disease and immunodepression has been reported (see Woo 2001).

Anorexia, i.e. reduction in food consumption (Symons 1985), is a characteristic response of fishes to viral (Damsgard et al. 1998), bacterial (Neji & de la Noue 1988, Pirhonen et al. 2000) and parasitic (Thomas & Woo 1992, Dezfuli et al. 2003) diseases. It occurs in cryptobiosis, and its onset has been associated with the

acute phase of the disease, which is characterized by high parasitemia and severe anemia in rainbow trout (Li & Woo 1991, Thomas & Woo 1992). Conversely, a return of appetite has been associated with the establishment of an immune response against the pathogen that significantly reduces the parasitemia and anemia. Li & Woo (1991) showed anorexia to be beneficial, as it lowers plasma protein, thus reducing parasitemia and, consequently, the severity of the disease (Woo 1979).

Anorexia also occurs in fishes in response to environmental, social and physical stressors (Schreck et al. 1997, Bernier & Peter 2001a). Stress activates the hypothalamic–pituitary–interrenal (HPI) axis, resulting in the release of adrenocorticotropin hormone (ACTH) by the pituitary, the primary signal that initiates the synthesis and secretion of cortisol from interrenal cells

(Wendelaar Bonga 1997). This leads to elevated corti-
sol levels characteristic of a primary stress response
(Barton & Iwama 1991). The corticotropin-releasing
factor (CRF) and related neuropeptides are important
in regulating the HPI axis in the hypothalamus (Led-
eris et al. 1994), and appear to be involved in mediat-
ing the appetite-suppressing effects of stressors
(Bernier & Peter 2001b). In mammals, there is also
strong evidence that CRF-related peptides can regu-
late gastric evacuation and mediate stress-related
alterations in the motor functions of the gut. Although
Cryptobia salmositica-infected rainbow trout have
high circulating cortisol levels (Laidley et al. 1988) and
are characteristically anorexic, the impact of this para-
sitic infection on their gastric-evacuation rate has not
been documented.

Feeding hierarchies have been reported for
salmonids in both laboratory and field studies (Sloman
& Armstrong 2002). Size is an important factor (Sloman
& Armstrong 2002), and larger fish are more dominant
in terms of access to food and subsequent growth rate.
Much evidence in studies on mammals and fishes
implicates an increased susceptibility to disease con-
nected with a subordinate status (Peters et al. 1988,
Maule et al. 1989, Ebbersen et al. 1992, Padgett et al.
1998). This may be due to elevated cortisol levels in
subordinates, since cortisol is known to suppress the
immune response (Schreck 1996). However, there
have been virtually no studies on the impact of disease
on feeding hierarchies in fishes.

Humoral and cell-mediated immune responses are
important mechanisms against cryptobiosis (Ardelli &
Woo 2002, Mehta & Woo 2002). Live vaccine (attenu-
ated *Cryptobia salmositica*) protects rainbow trout (Li
& Woo 1995, Mehta & Woo 2002), chinook salmon
(Staines & Woo 1997), brook charr (Ardelli & Woo
1997), and Atlantic salmon (Ardelli & Woo 2002) from
this disease. Neutralizing, agglutinating, and comple-
ment-fixing antibodies have been detected in infected
and vaccinated rainbow trout (Jones & Woo 1987, Li &
Woo 1995, Mehta & Woo 2002). Cell-mediated immune
responses have been demonstrated, including
delayed-type hypersensitivity reactions (Thomas &
Woo 1990, Feng & Woo 1996), inhibition of macro-
phage migration (Thomas & Woo 1990), stimulation
and growth of peripheral leukocytes with concovalin A
(Ardelli & Woo 2002), and increased respiratory bursts
killing macrophages in the head kidney (Mehta & Woo
2002).

Infected rainbow trout show a significantly lower
humoral immune response to sheep red-blood cells
(sRBC) (Wehnert & Woo 1981, Thomas & Woo 1992)
and *Yersinia ruckeri* (Jones et al. 1986), and vaccina-
tion against yersiniosis has been unsuccessful in
acutely infected fish individuals (Jones et al. 1986). It
has been hypothesized that the immunodepression is
probably the result of antigenic competition, and
Thomas & Woo (1992) showed that anorexia con-
tributes to immunodepression.

The objectives of this present study were (1) to com-
pare the onset and severity of anorexia at 5 and 10°C
and its effect on gastric evacuation and feeding hierar-
chy in *Oncorhynchus mykiss*, and (2) to investigate the
effect of an anorexic diet on their humoral and cell-
mediated immune response to live *Cryptobia salmosit-
ica* vaccine.

MATERIALS AND METHODS

Source of *Cryptobia salmositica*. Pathogenic *C.
salmositica* (cloned Substrain T4) was initially isolated
from a leech vector on Vancouver Island (Woo 1978).
Pathogenicity has been maintained through serial pas-
sage in susceptible salmonids kept at 10°C, and the at-
tenuated *C. salmositica* (vaccine strain) is maintained at
10°C in minimum essential media (MEM) supplemented
with Hank's salt, L-glutamine, 25 mM 4-(2-hydroxy-
ethyl)-1-piperazineethanesulphonic (HEPES) buffer
and 20% fetal bovine serum (Gibco) (Woo & Li 1990).

Fish. Rainbow trout *Oncorhynchus mykiss* embryos
were purchased from Springs Trout Farm (Thames-
ford, Ontario) and raised in the laboratory. At 8 mo of
age, 114 fish (30 to 89 g) were divided into 7 groups (A
to G). There were no significant differences (p > 0.05)
in weight between groups. All fish in Groups A to D
(n = 13 per group) were fin-clipped for identification in
the gastric evacuation and feeding hierarchy study.
Fish in Groups A and C were infected with *Cryptobia
salmositica*, while fish in Groups B and D served as
naïve controls. Groups A and B were maintained at
10°C and Groups C and D at 5°C. Group E (n = 36) was
used to quantify parasitemia and PCV values during
the course of infection at 10°C. Groups F and G (n = 13
group⁻¹) were used to investigate the effect of anorexia
on immune response to the live vaccine at 10°C. Group
F was fed a quantity of food (anorexic diet) similar to
that eaten by Group A when the latter became
anorexic. Groups G and B were pair-fed. All groups
were maintained in individual 125 l tanks with aerated
recirculated well-water and an equatorial lighting
regime. Fish were acclimated to their treatment tanks
for 2 wk before the experiments began.

**Expt 1: Food consumption, gastric evacuation and
feeding hierarchy in juvenile rainbow trout infected
with pathogenic *Cryptobia salmositica* at 10 and 5°C.
Experimental design:** The fish were fed once a day
(between 09:00 and 11:00 h) with commercial dry trout
pellets (Martin Feed Mills, Elmira, Ontario) 42% pro-
tein, and the amount of food consumed was recorded.

Food pellets were distributed individually at different points in the tank and at different rates depending on the ingestion rates of the fish. Satiety was considered to be reached when 2 pellets had reached the bottom of the tank or feeding had continued for 10 min. Food consumed in a given week was expressed as a percentage of initial body weight (% b.w.). After 2 wk, fish in Groups A, C, and E were anesthetized with 2-phenoxylethanol and each fish was injected intraperitoneally (i.p.) with approximately 20 000 *Cryptobia salmositica* in 0.1 ml phosphate-buffered saline (PBS), while each fish in Groups B and D was injected i.p. with 0.1 ml of PBS. In Group E, 4 to 5 fish were killed weekly by a blow to the head, the caudal peducle was severed, and blood was collected with an heparinized syringe. Parasitemia was determined with a hemocytometer (Archer 1965), and PCV with a hematocrit centrifuge.

X-radiography — feed intake, gastric evacuation rate, and feeding hierarchy: Once a week, fish in Groups A to D were fed food that contained 5% ferrospheres (0.45 mm diameter; Draiswerke). The beads did not affect the palatability of the food, as there was no obvious change in food consumption. The fish were anesthetized 1 h after feeding with 2-phenoxyethanol, and x-rayed using an acu-ray HFJ series, universal portable X-ray (Sterne Equipment) at 50 kV, 1.05 mA s at 83 cm. The food intake of each fish was quantified by relating the number of beads found in its stomach to a standard curve ($r^2 = 0.99$, n = 16) (Jobling et al. 2000). Fish were again x-rayed 48 h later, and the remaining beads counted to estimate the amount of food cleared. Gastric evacuation (GE) of food was expressed as % beads cleared through the gastrointestinal tract after 48 h: $GE_{48h} = (beads_{0h} - beads_{48h})/beads_{0h}$. The 48 h period was chosen because (1) it maximized the time period between x-rays (minimizing stress without disrupting the feeding time), and (2) a preliminary time-course experiment had shown that 67% of juvenile rainbow trout maintained at 10°C (and fed once daily to satiation) had evacuated detectable beads at 48 h.

The hierarchy within Groups A to D was determined before and after the onset of anorexia in infected groups (A and C) by correlating the mean share of meal (MSM, a measure of how food was partitioned within the group) and coefficient of variation in food intake (CV) to determine the position of individual fish within the hierarchy of each group (McCarthy et al. 1992). MSM was calculated as: $MSMx$ = no. of beads ingested by fish x/total no. of beads ingested by group. CV, a measure of variability in food consumption from meal to meal by each individual fish was calculated by counting the number of beads ingested (x-radiographs) after feeding: |CV| = avg. food eaten (mass-specific)/SD (avg. food eaten).

Expt 2: Role of anorexic diet in immune response of juvenile rainbow trout to live *Cryptobia salmositica* vaccine. *Experimental design:* In the week when anorexia was first detected (as significant difference in food intake between infected and naïve groups) in infected fish (Group A) at 10°C (3 w.p.i.), the amount of food eaten in that week was averaged (0.94% b.w. d^{-1}) and this amount was then fed to Group F (pair-fed) for the duration of this experiment. Group F was fed this diet for 4 wk before vaccination. Groups G and B were pair-fed (Group B being the naïve control). The fish in Groups F and G were each vaccinated i.p. with 20 000 attenuated *C. salmositica* in 0.1 ml PBS. From Groups F, G, and B, 2 to 4 individuals were sacrificed weekly from 2 to 6 wk post-vaccination (w.p.v.) to determine cell-mediated and/or humoral immune response.

Cell-mediated immune response — macrophage isolation and respiratory-burst assay: Macrophages were isolated from the head kidney, and a respiratory-burst assay was performed (Secombes 1990). Briefly, fish were sacrificed with a blow to the head and bled dry via the caudal vein. The anterior kidney was dissected out aseptically, placed in 5 ml Leibovitz media (L-15) (Sigma) containing 2% fetal bovine serum (FBS, Gibco), heparin (10 U ml^{-1}, Fisher), and penicillin (100 µg ml^{-1})/streptomycin (100 U ml^{-1}) (P/S, Sigma) in a Petri dish. The head kidney was cut into fine pieces with scissors and the suspension was pipetted onto a 100 µm cell-sieve and pushed gently through and into a centrifuge tube. The resulting suspension was carefully layered onto a 34 to 51% Percoll (Amersham) density gradient, and centrifuged at $400 \times g$ for 40 min at 4°C. Cells were collected from the 34 to 51% interface, which was greatly enriched with macrophages. Cell viability was determined using 0.4% trypan blue (Sigma) in PBS, and the cell suspension was adjusted to 5×10^6 living cells ml^{-1} with L-15, 0.1% FBS and P/S. A volume (100 µl) of the cell suspension was placed into each well of a cell culture-grade 96-well microtiter plate (Corning) for 3 h to enable the cells to adhere and form a monolayer at 14°C. Adhered macrophages were then washed with L-15 and incubated overnight with L-15 supplemented with 5% FBS at 14°C. Macrophages from each fish were subjected to the following treatments in triplicate: 100 µl of phorbol myristate acetate (PMA) at 1 mg ml^{-1} (Sigma), *Cryptobia salmositica* lysate (antigen) at 5 µg ml^{-1} (Mehta & Woo 2002) and Hank's balanced salt solution (HBSS). Cells were incubated for 3 h at 14°C before addition of 100 µl nitroblue tetrazolium (NBT) at 1 mg ml^{-1} in HBSS for 2 h at 14°C. The supernatant was removed, and the cells were fixed in 100% methanol and allowed to air-dry. During incubation, activated macrophages produced O_2^-, which reacted with NBT to produce insoluble blue formazan. Formazan was dissolved using 80 µl 2 M

KOH and 93 µl dimethyl sulfoxide (DMSO, Sigma). The turquoise-blue solution was read on a multiscan spectrophotometer (Molecular Devices, MAXline microplate reader). The stimulation index (SI) was calculated as the ratio of triggered (with PMA or Antigen) to non-triggered (HBSS) optical-density (OD) readings.

Humoral immune response: enzyme-linked immunosorbent assay (ELISA): The method of Sitja-Bobbadilla & Woo (1994) was used with some modifications. Each well of 96-well flat-bottomed, high-binding polystyrene plates (Corning, USA) was coated with 50 µl of attenuated *Cryptobia salmositica* lysate (25 µg protein ml^{-1}), diluted in a carbonate-bicarbonate buffer (pH 9.6), and incubated overnight at 5°C. Unbound antigen was removed by washing 5 times with washing buffer (PBS + 0.05% Tween-20). A volume of 200 µl of blocking buffer (5% skim milk [w/v] in PBS) was added to each well and incubated at room temperature for 30 min followed by washing. Test plasma (50 µl) from sacrificed fish was added to each well in duplicate and incubated for 1 h at room temperature and then washed. Rabbit anti-salmon immunoglobulin (courtesy of K. Buchmann, Royal Veterinary & Agricultural College, Denmark), diluted 1:1000 in dilution buffer (PBS + 0.01% bovine serum albumin), was added to each well (50 µl), incubated for 1 h at room temperature, and then washed. This was followed by addition of 50 µl of peroxidase-conjugated goat anti-rabbit immunoglobulin (Sigma Aldrich) diluted 1:10 000 in dilution buffer, incubated for 1 h at

room temperature and then washed. The substrate *o*-phenyledamine (Sigma Aldrich) was added (50 µl) to each well and incubated for 15 min; the reaction was stopped with 50 µl of 3M HCl. The optical density in each well was read at 492 nm using a microplate reader (Molecular Devices).
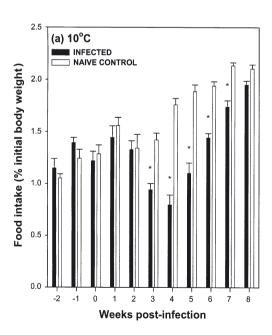
Statistical analysis. A 1-way ANOVA was used to compare between treatment groups each week. If variance was not equal among treatment groups, Kruskal-Wallis 1-way ANOVA on ranks was applied. Repeated-measures ANOVAs were used to compare changes in GE_{48h} as a function of treatment time. If a significant difference was found in the ANOVAs, Tukey's SD test or Dunn's test (ANOVA on ranks) were used for multiple pairwise comparison. Pearson's test was used to determine correlations between initial weight, MSM and CV. Significance was determined if $p < 0.05$.

RESULTS

Expt 1: Food consumption, gastric evacuation, and feeding hierarchy in infected juvenile rainbow trout at 10 and 5°C

Food consumption

At 10°C, there was no significant difference in food consumption between infected (Group A) and naïve (Group B) groups until 3 w.p.i., when Group A had a
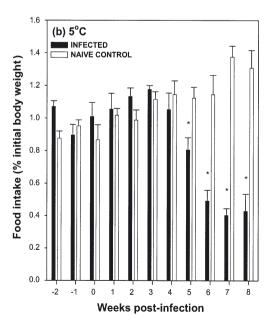

Fig. 1. *Oncorhynchus mykiss* infected with *Cryptobia salmositica.* Food intake (weekly mean ± SE; % of initial body weight) by juveniles (n = 13 group^{-1} dose of 20 000 parasites fish^{-1}) and naïve controls at (a) 10°C and (b) 5°C. *Significantly lower than naïve controls

significantly lower (p = 0.005) food consumption than Group B (0.94 and 1.42% b.w. respectively; Fig. 1a). Food consumption in Group A continued to be significantly lower until 7 w.p.i. Anorexia was most severe at 4 w.p.i. (0.80% b.w.), and at 8 w.p.i. there was no significant difference in food consumed between Groups A and B. During the anorexic period, all fish in Group A had ingested some food (data based on x-radiographs).

Fish at 5°C consistently consumed less food than fish at 10°C. Group C (infected) had significantly lower (p < 0.001) food consumption than Group D (0.81 and 1.14% b.w. respectively) at 5 w.p.i. until the end of the study at 8 w.p.i. (Fig. 1b). Complete absence of food was detected in 2 of 12 fish at 6 w.p.i., in 7 of 12 fish at 7 w.p.i., and in 5 of 12 fish at 8 w.p.i. Mean weekly food consumption was lowest at 7 w.p.i. (0.40% b.w.).

X-radiography confirmed that fish at 10°C became anorexic from 4 to 8 w.p.i., while fish at 5°C were anorexic from 5 w.p.i. until the end of the experiment.

Mortalities, parasitemia and PCV

At 10°C, parasites were detected in 5 of 5 fish (Group E) from 2 to 8 w.p.i. and were still detectable at 9 w.p.i. in 3 of 4 fish; 1 fish died at 5 w.p.i. in this group. Parasitemia increased sharply from 2 w.p.i., peaked at 4 w.p.i. (9.6×10^6 parasites ml^{-1} blood) and declined thereafter (Fig. 2). PCV decreased sharply from 33.3% at 3 w.p.i. to its lowest value of 18.0% at 5 w.p.i. (Fig. 2). At 9 w.p.i., fish displayed 24.4% PCV.

At 5°C, 2 fish died at 8 w.p.i. The dead fish displayed splenomegaly and contained large numbers of *Cryptobia salmositica* in the head kidney. The group was still anorexic and displayed high parasiteamia (4.2×10^6 parasites ml^{-1} blood), and low PCV (10.8%) at 9 w.p.i. (Fig. 2).

Gastric evacuation (GE)

The GE_{48h} in both infected and naïve groups decreased gradually at both temperatures. The difference between GE_{48h} at −1 and 8 w.p.i. was significant in Groups A, B, and D. In Group C there was a significant difference between GE_{48h} at 7 and 8 w.p.i., but some GE_{48h} values were missing, as 7 of 12 and 5 of 12 fish had not ingested food at these times respectively. Fish at 5°C consistently had a much lower GE_{48h} than fish at 10°C (Fig. 3). At 10°C, there was no significant difference between infected (A) and uninfected (B) groups during the course of the experiment. At 5°C, the infected group (Group C) had a significantly higher
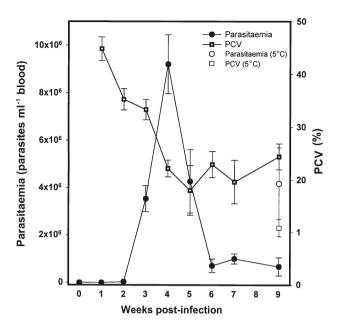

Fig. 2. *Oncorhynchus mykiss* infected with *Cryptobia salmositica*. Parasitemia and pack cell volume (PCV) values (mean ± SE) of juveniles (n = 5 per sample) infected with 20 000 *C. salmositica* over the course of infection at 10°C (solid symbols); open symbols: values at 5°C at 9 wk post-infection
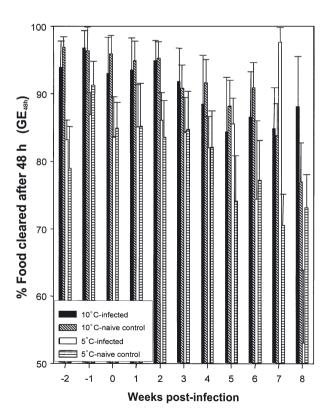

Fig. 3. *Oncorhynchus mykiss* infected with *Cryptobia salmositica*. Food cleared (mean percentage ± SE) by juveniles at 10 and 5°C after 48 h (n = 13 group^{-1})

GE$_{48h}$ (p < 0.05) than the uninfected group (D) at 7 w.p.i., but this could have been skewed, as 7 of 12 fish had not fed.

Feeding hierarchy

Relationships between MSM, b.w., and CV were examined using x-radiography before and after onset of anorexia. Infected fish at both temperatures had a higher, but not significant, CV compared to their naïve counterparts. There were significant positive correlations between MSM and b.w. before the onset of anorexia in all groups. This significant correlation was maintained during the anorexic period in Groups A and D but not in Groups B (p = 0.162) and C (p = 0.942) (Table 1). Group B displayed the least variation in b.w. within the group, and this would explain the lack of any obvious feeding hierarchy.

There were no significant correlations between MSM and CV before anorexia in Groups A, B, and C. The correlation in Group D was significant (p = 0.0126), but 1 fish had not ingested food in 5 of the 7 wk, resulting in an extremely high CV value (198.04) compared to the mean CV of the other 12 fish (35.12). Were the data from this fish removed from the analysis, then the correlation in Group D would not be significant (p = 0.104). During the anorexic period, neither of the uninfected groups (B and D) showed significant correlations, but both infected groups (A and C) did (p = 0.0168 and p = 0.0435 respectively). No significant correlations were found between b.w. and CV in any groups.

Expt 2: Role of anorexic diet in immune response of juvenile rainbow trout to live *Cryptobia salmositica* vaccine

Respiratory-burst assay

There were no significant differences in the stimulation index (SI) between macrophages from vaccinated and naïve fish, although the SIs of macrophages from vaccinated fish were generally higher (Fig. 4), and vaccinated fish also consistently yielded a greater number of macrophages. PMA consistently produced a higher SI than the *Cryptobia salmositica* antigen (ag) in both vaccinated and naïve fish (Fig. 4a). The SI produced by ag was often below 1 (Fig. 4b). In vaccinated fish, the SI with PMA was significantly higher than that with ag at 3 and 6 w.p.i. (p < 0.001 and p < 0.05 respectively). Vaccinated fish on both diets displayed the highest SI with PMA at 4 w.p.v. There were no significant differences in SI with PMA or ag between fish fed to satiation and fish fed an 'anorexic diet'.

ELISA

Antibodies against *Cryptobia salmositica* were detected at 2 w.p.v. Vaccinated fish had consistently higher antibody titers than uninfected controls. This difference was significant (p < 0.05) at 2, 4, and 5 w.p.v. However, there was no difference in antibody titer between fish fed to satiation and fish fed an anorexic diet.

Table 1. *Oncorhynchus mykiss.* Initial weight (g) and correlation analysis of mean share of meal (MSM) with initial body weight (b.w.), and with coefficient of variation (CV) in food intake before and after onset of anorexia in juveniles infected with *Cryptobia salmostica* and in naïve controls at 10 and 5°C. w.p.i.: weeks post-infection; *significant at p < 0.05

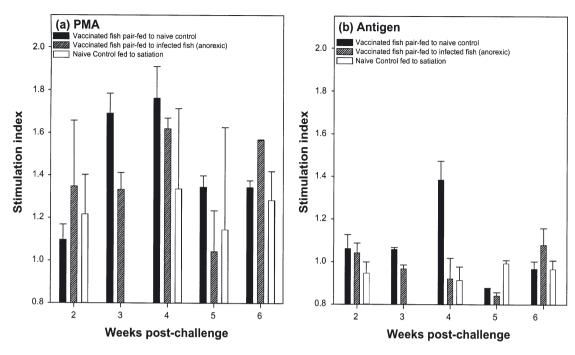| (Group) treatment	Initial wt (g) Mean ± SE	Range	CV (%) Mean ± SE	Correlation (p-value) of MSM with b.w.	CV
(A) Infected, 10°C (all)	60.1 ± 5.0	30.4–89.3	40.2 ± 3.1	0.0019*	0.0098*
before anorexia (–2 to 3 w.p.i.)			27.9 ± 3.0	0.0050*	0.37
after anorexia (4 to 8 w.p.i.)			50.1 ± 4.4	0.0013*	0.0168*
(B) Naïve, 10°C (all)	57.0 ± 3.3	38.4–72.7	36.9 ± 4.0	0.068	0.273
–2 to 3 w.p.i.			27.9 ± 3.0	0.0497*	0.279
4 to 8 w.p.i.			25.1 ± 2.5	0.162	0.886
(C) Infected, 5°C (all)	58.9 ± 4.3	35.8–85.7	57.6 ± 5.2	0.234	0.000655*
before anorexia (–2 to 5 w.p.i.)			30.7 ± 2.1	0.0338*	0.314
after anorexia (6 to 8 w.p.i.)			144.5 ± 35.8	0.942	0.0435*
(D) Naïve, 5°C (all)	57.1 ± 3.1	38.7–83.4	47.0 ± 9.2	0.0117*	0.00679*
–2 to 5 w.p.i.			47.6 ± 12.9	0.0177*	0.0126*[a]
6 to 8 w.p.i.			27.7 ± 6.3	0.0187*	0.165
[a]p = 0.104 after removal of outlier value					

Fig. 4. Stimulation index (mean ± SE) of macrophages stimulated with (a) PMA and (b) *Cryptobia salmositica* lysate from fish that were vaccinated with a live *C. salmositica* vaccine

DISCUSSION

Anorexia occurred in rainbow trout infected with *Cryptobia salmositica* at both 10 and 5°C and this was confirmed using x-radiography. The onset of anorexia coincided with a significant rise in parasitemia and a decrease in PCV at 10°C. Fish at 10°C resumed normal food intake at 8 w.p.i., while fish at 5°C were still severely anorexic and anemic, with high parasitemia, at 9 w.p.i., the termination of this experiment. Additionally, 2 fish died from cryptobiosis at this time and temperature. These results confirm earlier studies on the relationship between increasing parasitemia and the onset of anemia and anorexia (Li & Woo 1991, Thomas & Woo 1992). Lysis of erythrocytes occurs through (1) a metalloprotease secreated by the parasite (Zuo & Woo 1997, 2000), and/or (2) the formation of immune complexes on erythrocytes activating complement-mediated hemolysis (Thomas & Woo 1988, 1989). Anemia is most evident during the acute stage of the disease (high parasitemia) and it is at this time that fish are more susceptible to environmental hypoxia (Wehnert & Woo 1986), which is known to contribute to anorexia in fish (Kramer 1987, Jobling 1994). Acute disease incurs significantly higher bioenergetic costs (Beamish et al. 1996) and reduces the maximum scope aerobic activity (Kumaraguru et al. 1995).

Temperature affected the onset of anorexia, as fish kept at 10°C became anorexic at 3 w.p.i. while those at

5°C became anorexic at 5 w.p.i. Anorexia was much more severe at 5°C: the reduction in food intake was more pronounced and more than half the group (7 of 12) did not ingest food compared to fish at 10°C, all of which had some food in their stomachs. Further studies are necessary to elucidate the effect of temperature on anorexia during cryptobiosis.

Woo et al. (1983) also found a longer latent period in fish at 5°C. However, in contrast to this study, Woo et al. found that parasitemia in rainbow trout infected with *Cryptobia salmositoica* at 5°C was considerably lower than in fish infected at 10°C, even 70 d post-infection. Similarly, goldfish *Cassius auratus* infected with another pathogenic hemoflagellate, *Trypanasoma carrasi*, were more susceptible to infection at 20°C than at 10°C. At 5°C, the parasite multiplied less readily until it became acclimated to its new temperature; thereafter, the parasitemia pattern became similar to that of fish at 10°C. The susceptibility of fish to cryptobiosis at low temperatures should be further investigated.

Cryptobia salmosistica did not affect the GE$_{48h}$ in fish at either temperature. The 48 h period may have been too long, since different regions of the gastrointestinal tract have differential rates of evacuation (Tache et al. 2001). Olsson et al. (1999) found that stomach motility was greatly reduced in rainbow trout injected with the hormone cholecystokinin (CCK) which reduced gastric emptying of the stomach. In the present study, there

were large differences in GE$_{48h}$ between individuals in each group. Olsson et al. (1999) reported similar results, with the evacuation rate of some fish displaying a linear trend, and that of others an exponential shape. Stress may reduce appetite through the anorexigenic effects of the CRF and related peptides which also activate the HPI axis (Bernier & Peter 2001b). Although Laidley et al. (1988) found elevated cortisol levels in rainbow trout infected with *C. salmositica*; they were not significantly higher than in uninfected fish. These authors suggested that parasitemia did not activate the HPI axis. Recent studies in mammals (Tache et al. 2001) have shown that the delayed gastric evacuation induced by the CRF and related peptides is not related to activation of pituitary–adrenal hormones. The role of CRF-related peptides in mediating anorexia in *C. salmositica* infection or in the physiological regulation of gastric evacuation needs to be investigated.

MSM and CV can be used to assess social hierarchy in fish, with a negative correlation indicating a strong hierarchy (Jobling et al. 2000). McCarthy et al. (1992) found the correlation became stronger when food was given at lower rations and that larger fish were generally dominant (Sloman & Armstrong 2002). In the present study, there were significant correlations between initial weight and MSM in all groups before anorexia. This correlation was not present in the uninfected group at 10°C (Group B) or the infected group at 5°C (Group C). The lack of a significant correlation for Group B might be due to low variations in body size in this group. Group C was more anorexic than infected fish at 10°C (Group A), and the increased severity of disease in Group C may have contributed to the loss of size effects on hierarchy. There were no correlations between MSM and CV before the onset of anorexia. This was expected, as the fish were fed to satiation, thereby reducing competition. During the anorexic period, which represents the acute stage of the disease, there were significant negative correlations between MSM and CV in infected groups, but not in naïve groups. The significant correlation in infected fish suggests that the feeding hierarchy was strengthened by infection. This may also indicate variability in susceptibility to cryptobiosis, with dominant fish becoming more dominant (higher MSM and lower CV) and subordinate fish becoming more subordinate. This is consistent with the hypothesis that subordinate fish are more susceptible to disease (Peters et al. 1988, Maule et al. 1989). The correlation between MSM and b.w. was maintained at 10°C but not at 5°C. These results suggest that fish at 5°C are more severely affected by cryptobiosis, regardless of size or social status, while at 10°C the size hierarchy is maintained.

It has been hypothesized that, in parasitic infections, anorexia is beneficial to the host as it deprives parasites of nutrients, thereby affecting their survival and reproduction (Symons 1985, Li & Woo 1991, Kyriazakis et al. 1998). Nutritional modulation of *Cryptobia salmositica* infection has been demonstrated by Thomas & Woo (1990), and Li & Woo (1991) found higher parasitemia and lower PCV in fish fed a high-protein diet than in fish fed a low-protein diet. Plasma protein levels were significantly higher in fish fed a high-protein diet (52%) than in those fed a low-protein diet (37 and 22%), and this has been shown to increase parasite multiplication *in vitro* (Li & Woo 1991). Infected rainbow trout (on 37% protein diet) had a depressed humoral immune response to sRBCs (Wehnert & Woo 1981, Jones et al. 1986, Thomas & Woo 1992). Also, infected fish challenged with the bacterial pathogen *Yersinia ruckeri* suffered higher mortalities than naïve-controls, and infected fish had a significantly lower humoral immune response to the pathogen (Jones et al. 1986). Also, Thomas & Woo (1992) found that anorexia during cryptobiosis contributes to immunodepression, since naïve fish pair-fed with infected fish displayed a significantly lower antibody production in response to sRBCs. However, in the present study, anorexia did not affect the fish's cell-mediated or humoral immune response against live *C. salmositica* vaccine. This may have been the result of a combination of factors. For example, the continual antigenic stimulation provided by a live vaccine, compared to earlier studies which used sRBCs or a pathogen (*Y. ruckeri*) (Wehnert & Woo 1981, Jones et al. 1986, Thomas & Woo 1992). Also, the fish in the present study were fed a 42% protein diet compared to a 37% protein diet used in earlier studies. The higher protein diet would have increased plasma protein levels (Li & Woo 1991), and this increase might also have contributed to the lack of immunodepression in fish on an anorexic diet. Further studies are currently underway to test these hypotheses.

Respiratory-burst activity was higher in vaccinated fish than in naïve fish, but the difference was not statistically significant. Mehta & Woo (2002) found a significantly higher respiratory burst activity in fish infected or vaccinated with pathogenic and attenuated strains of *Cryptobia salmositica*. Additionally, *C. salmositica* was found to be a potent stimulator of respiratory-burst activity akin to PMA. The present study found that *C. salmositica* antigen consistently produced a lower SI (sometimes below 1), suggesting that the parasite may inhibit cell-mediated functions.

In conclusion, there was delayed onset of anorexia in fish kept at 5°C compared to fish kept at 10°C. At the lower temperature, anorexia was more prominent, PCV was lower, and feeding hierarchy was not main-

tained. There was no difference in gastric evacuation of food over 48 h, although the evaluation of gastric evacuation was not sensitive enough to detect regional differences in motility of the gastrointestinal tract (GIT). Cell-mediated and humoral immune responses to a live vaccine were not depressed in fish (on a 42% protein diet) fed the amount of food anorexic fish would eat. Cryptobiosis is ideal for studying changes in appetite-regulation, since *Cryptobia salmositica* infection induces anorexia in rainbow trout. Further work is needed to elucidate the mechanisms of anorexia and its relationship to immunodepression. This would include clarifying neuroendocrine pathways related to anorexia induced by infectious and non-infectious agents, and identifying regional differences in food evacuation in the GIT to better differentiate the effect of stress and/or disease on gut motility.

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