Effects of *Loma morhua* (Microsporidia) infection on the cardiorespiratory performance of Atlantic cod *Gadus morhua* (L).

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Abstract

The microsporidian *Loma morhua* infects Atlantic cod (*Gadus morhua*) in the wild and in culture and results in the formation of xenomas within the gill filaments, heart and spleen. Given the importance of the two former organs to metabolic capacity and thermal tolerance, the cardiorespiratory performance of cod with a naturally acquired infection of *Loma* was measured during an acute temperature increase (2 °C h^{-1}) from 10 °C to the fish's critical thermal maximum (CT_{Max}). In addition, oxygen consumption and swimming performance were measured during two successive critical swimming speed (U_{crit}) tests at 10 °C. While *Loma* infection had a negative impact on cod cardiac function at warm temperatures, and on metabolic capacity in both the CT_{Max} and U_{crit} tests (i.e. a reduction of 30–40%), it appears that the Atlantic cod can largely compensate for these *Loma*-induced cardiorespiratory limitations. For example, (i) CT_{Max} (21.0 ± 0.3 °C) and U_{crit} (~1.75 BL s^{-1}) were very comparable to those reported in previous studies using uninfected fish from the same founder population; and (ii) our data suggest that tissue oxygen extraction, and potentially the capacity for anaerobic metabolism, is enhanced in fish infected with this microsporidian.

Keywords: cardiac performance, critical thermal maximum, gill pathology, heart pathology, metabolism, oxygen consumption.

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Introduction

Microsporidial diseases pose significant challenges to the development of marine fish aquaculture, specifically in the North Atlantic (Murchelano, Despres-Patanjo & Ziskowski 1986; Bricknell, Bron & Bowden 2006; Kahn 2009), the North Pacific (Brown, Kent & Adamson 2010) and more recently the Red Sea (Abdel-Ghaffar *et al.* 2011). Of particular significance is the infection of gadoid fishes [e.g. Atlantic cod (*Gadus morhua*)] with the microsporidian *Loma morhua*; this species is recently identified separately from *Loma branchialis* (Brown *et al.* 2010). Microsporidian xenomas are characterized by their distinct morphology, with those produced by *Loma* sp. having a well-defined and characteristic thick granular amorphous wall and various developmental stages of the parasite (Lom & Dyková 2005). It is believed from data on related species (*Loma salmonae*) that the naïve host ingests spores that enter the host through the gut, that the sporoplasm is injected into a host cell that migrates to the heart and enters a merogony-like phase and finally that the parasite utilizes macrophage-mediated transport to the gill where endothelial and pillar cells hypertrophy to form xenomas (Sanchez, Speare & Markham 2000; Sanchez *et al.* 2001a; Rodriguez-Tovar *et al.* 2003). However, *L. morhua* xenomas regularly occur in the heart as well as in the gills and other organs (Murchelano *et al.* 1986). Infection with *Loma* leads to a reduced body condition, a decrease in energy stores (liver somatic index), and mild anaemia and leukaemia (Khan 2005). In salmonids, such as rainbow (*Oncorhynchus mykiss*) and brook

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trout (*Salvelinus fontinalis*) that are infected with *Loma salmonae*, decreases in specific growth rate are correlated with increases in routine and maximum metabolic rate, and reductions in routine but not maximum metabolic rate, respectively (Powell *et al.* 2005): the metabolic cost of the disease is largely attributed to changes in branchial O_2 permeability (Powell, Speare & Becker 2006).

The thermal biology of Atlantic cod has received considerable attention over the past decade (e.g. see Drinkwater 2005; Gollock *et al.* 2006; Perez-Casanova *et al.* 2008a,b; Hori *et al.* 2012). Although cod prefer cooler waters (8–15 °C; Pettersen & Steffansen 2003), local temperatures may fluctuate significantly on a seasonal and diurnal basis, and cod in the wild and in culture may not be able to escape acutely elevated water temperatures as high as 20 °C (Gollock *et al.* 2006; Righton *et al.* 2010). Given that it is widely recognized that limitations in metabolic scope and cardiac output are a key determinant of the upper thermal limits of fishes (Farrell 2002; Farrell *et al.* 2009; Gamperl, Swafford & Rodnick 2011; Keen & Gamperl 2012; Sandblom *et al.* 2014), that *Loma* infections impact fish metabolism (Powell *et al.* 2005) and that this parasite forms xenomas in the cod heart and gills, it might be expected that infection with this microsporidian constrains the upper temperature that infected cod can tolerate and/or reduces the capacity of individuals to perform other important physiologically (and metabolically) demanding functions such as swimming. Thus, we measured the following: (i) the cardiorespiratory physiology (oxygen consumption and cardiac performance) of adult, 10 °C acclimated, cod with varying severities of *L. morhua* infection when acutely exposed to increasing temperatures up to their critical thermal maximum (CT_{max}); and (ii) oxygen consumption in this same population of cod when they were subjected to two consecutive critical swimming speed (U_{crit}) tests.

Materials and methods

Experimental animals

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Institutional Animal Care committee of Memorial University of Newfoundland (protocol number 11–25 KG)

and the Norwegian Animal Welfare Authority (FDU). The Atlantic cod *G. morhua* L. used in this study (mean body mass ± SEM, 509.1 ± 12.3; total length 37.1 ± 0.2 cm; $N = 42$) were raised and maintained at the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, Memorial University of Newfoundland) in a 3000-L tank supplied with aerated sea water at 10–11 °C for at least 6 months prior to experimentation. The fish were fed daily with a commercial cod diet (EWOS) under a photoperiod of 8 hours light: 16 hours dark.

Critical thermal maximum experiment

Surgical procedures. Fish were individually netted and anaesthetized in sea water containing 100 mg L^{-1} MS-222 (tricaine methane sulphonate) until ventilatory movements ceased. The fish were then weighed and measured before being transferred to a surgical table where their gills were constantly irrigated with oxygenated, and chilled, sea water containing MS-222 (0.5 mg L^{-1}). To allow for the direct measurement of cardiac function (cardiac output *Q*; heart rate f_H; and stroke volume H_{sv}), a 2PS Transonic™ blood flow probe (Transonic Systems) was implanted around the ventral aorta using a method modified from Thorarensen, Gallaugher & Farrell (1996) and Gollock *et al.* (2006). In brief, the left operculum and underlying gills were elevated and secured in this position with umbilical tape, before a small 5–7 mm incision was made at the base of the junction between the second and third gill arches. The ventral aorta was then located and cleared of the surrounding connective tissue by careful dissection, and the flow probe was placed around the ventral aorta anterior to the intact pericardium. Finally, the cable from the flow probe was secured to the skin using 1-0 silk at positions immediately posterior to the operculum, just above the lateral line behind the left pectoral fin, and in between the first and second dorsal fin. A PE50 (Clay Adams Inc.) cannula, with a 45-degree bend approximately 2 cm from its tip, was also sutured at the upper edge of the opercular cavity for measurements of ventilation frequency. This cannula was then secured to the skin with 1-0 silk sutures using the same attachment points as for the flow probe cable.

Following surgery, the fish were transferred to 6.5-L tubular clear acrylic respirometers supplied with flush and recirculating pumps (EHEIM GmbH & Co. KG) and left to recover overnight (typically 20–24 h) with the pumps in the 'flush' mode. These respirometers were submerged in a 'water table' supplied with temperature-controlled (10 °C), and aerated, sea water from a large (~300 L) reservoir; the temperature in the reservoir controlled using a custom-designed heater/chiller (Technical Services, Memorial University of Newfoundland).

Experimental protocol. To begin the experiment, cardiac output Q, ventilation frequency (V_f) and oxygen consumption (MO_2) were measured at 10.0 °C. The fish were then challenged with an increase in temperature of 2 °C h^{-1} until loss of equilibrium [i.e. they reached their critical thermal maximum (CT_{Max})], with cardiorespiratory parameters and MO_2 measured at every 2 °C increase in temperature. This protocol was chosen to be consistent with previous studies that have assessed temperature-dependent cardiovascular limits and the relative contributions of stroke volume (H_{SV}) and heart rate (f_H) to increases in Q with temperature in various fishes (Gollock *et al.* 2006; Mendonca & Gamperl 2010; Gamperl *et al.* 2011; Keen & Gamperl 2012). After the fish lost equilibrium, water temperature was rapidly decreased to 10 °C, and the fish was removed from the respirometer and immediately killed in 10 °C sea water containing 0.3 g L^{-1} MS-222.

Data collection. Oxygen consumption measurements (in mg O_2 kg^{-1} h^{-1}) were made using a laptop computer running AutoResp® software (v2.1.0; Loligo Systems) that was interfaced with a fibre-optic oxygen meter (model OXY-4 mini) and associated with precalibrated dipping probe (PreSens) and Loligo's DAQ 4 and TEMP-4 modules. To make MO_2 measurements, the AutoResp® software switched between the 'flushing' and 'recirculating' EHIEM pumps for 8 min (making the respirometer a closed circuit), and began recording water oxygen levels after a 2-min wait period.

Cardiac output (Q, in volts) was recorded by connecting the flow probe leads to a Transonic T206 flow meter, whereas V_f (in min^{-1}) was measured by attaching the opercular cannula to a Gould Statham (Model P23-10) pressure transducer. This transducer was calibrated daily against a static water column. Signals from the Transonic flow meter and pressure transducers were amplified and filtered using a data acquisition system (MP100A-CE; BIOPAC Systems, Inc.) and universal interface module (UIM100C; BIOPAC Systems Inc.) connected to a laptop computer running AcqKnowledge® software (version 3.8.2; BIOPAC Systems, Inc.). As Transonic flow probes are temperature sensitive, and the characteristic of this relationship varies between probes, the temperature–flow relationship for each probe was measured at different flow rates (5–25 mL min^{-1}) over the temperature range used in the experiment using Transonic calibration tubing, a high precision peristaltic pump, a foam-lined chamber, and saline with a haematocrit of 15–20%. These relationships were then used to correct *in vivo* flow rates (Q) at various temperatures. Measurements of MO_2, Q, f_H and V_f were made for 10 min, just prior to the temperature being increased.

From the above data, the following parameters were calculated:

$$Cardiac\ stroke\ volume\ (H_{SV}, in\ mL) = Q/f_H$$

$$\begin{aligned}Arterio\text{-}Venous\ oxygen\ difference\ (C_aO_2 \\ - C_vO_2, in\ mg\ O_2\ mL^{-1}\ blood) \\ = MO_2/Q\end{aligned}$$

$$\begin{aligned}Perfusion\ conductance\ ratio\ (in\ mL\ blood \\ min^{-1}kg^{-1}mg\ O_2^{-1}) = Q/MO_2\end{aligned}$$

Swimming performance

To examine the effect of *Loma* sp. infection upon swimming performance, infected cod were initially placed in a custom-built (Technical Services, Memorial University of Newfoundland) 81-L Blazka-type swim tunnel filled with aerated sea water maintained at 10 °C, and with water velocity set to approximately 0.5 BL s^{-1}. This speed allowed the fish to maintain their orientation and position, without having to swim actively. At the end of a 24-h acclimation period, the swim tunnel was closed and oxygen consumption was measured over a 20-min period using a dipping probe that was inserted into the swim tunnel. This dipping probe was connected to a Fibox 3 oxygen meter (PreSens, Precision sensing GmbH) interfaced with a portable computer, and the output recorded using

Oxyview® software (LCDPST3-v2.01; PreSens). The water velocity was then increased in steps equivalent to 0.2 BL s^{-1}, with each step comprised of equivalent (10 min) flush and measurement periods. The water velocity was increased until the fish was unable to move away from the grid at the back of the swim chamber, at which point the velocity was reduced to 0.5 BL s^{-1} and the fish to allowed to recover. Oxygen consumption measurements were also made after 30 and 60 min of recovery at ~0.5 BL s^{-1}, and the fish was subsequently given a 2nd swim test (without measuring oxygen consumption) prior to being killed in 0.3 g L^{-1} of MS-222.

From these data, the following parameters were calculated:

$$\text{Critical swimming velocity, } (U_{\text{crit}}) = U_f + [U_i(t_f/t_i)]$$

where U_f is the last velocity at which the fish completed the entire period (20 min), U_i is the water velocity increment (0.2 BL s^{-1}), t_f is the time to fatigue and t_i is the duration of each velocity step.

$$\text{Gross cost of transport (GCOT, in mg kg}^{-1}\text{m}^{-1}) = (MO_{2i})/((U_i * L) * 36)$$

where MO_{2i} is the oxygen consumption at a given velocity (U_i) and L is the length of the fish.

Standard oxygen consumption ($MO_{2\text{standard}}$) was derived from a semi-log plot of swimming speed vs. log MO_2, and using the derived linear regression to extrapolate back to 0 BL s^{-1}. Routine oxygen consumption was that measured in fish that were resting quietly in the swim tunnel at 0.5 BL s^{-1}. Maximum oxygen consumption ($MO_{2\text{max}}$) was the highest oxygen consumption that each individual fish achieved, and metabolic scope was calculated by subtracting standard oxygen consumption from $MO_{2\text{max}}$.

Determination of the level of *Loma morhua* infection

After the fish were killed, the second gill arch on the left side was removed and fixed in 10% neutral buffered formalin for histological examination and confirmation of *Loma* sp. infection and the second gill arch on the right side was removed and placed in phosphate-buffered saline (PBS) for branchial xenoma quantification. The fish was then quickly dissected, and the whole heart was carefully removed to prevent damage to the atrium or bulbus arteriosus. After rinsing the heart in saline, it was blotted dry and weighed, the atrium and ventricle were weighed separately, and the ventricle measured for length (V_L), width (V_W) and height (V_H) as described by Powell, Nowak & Adams (2002) and Powell, Burke & Dahle (2011, 2012). The chambers of the heart were then fixed in 10% neutral buffered formalin for histological examination and xenoma quantification.

The filaments on the second gill arch on the right side were subsequently separated from the arch, and 40–80 individual filaments were then digitally photographed (at 10× magnification including an internal scale) and the number of visible xenomas recorded. The fixed ventricles were also digitally photographed, and the number of visible xenomas on the ventricle surface recorded.

Formalin-fixed gills and hearts were then embedded in paraffin wax and sectioned at 3–5 μm, stained with haematoxylin and eosin and examined using an Olympus BX51 microscope with an Olympus DP71 camera (Olympus Life and Material Sciences, Europa Gmbh) using Cell^B image software (Olympus Soft Imaging Solutions Gmbh).

Cardiac measurements and morphometrics

From measurements of the heart and its chambers, the following parameters were calculated:

Cardiac, Ventricular and Atrial somatic indices; CSI, VSI and ASI, respectively.
CSI = heart mass/fish mass; VSI = ventricular mass/fish mass; ASI = atrial mass/fish mass.
% contribution to heart mass (%V, %A): %V = ventricular mass/heart mass; %A = atrial mass/heart mass.
Ventricular shape ratios (L:W, L:H, H:W): L:W = V_L/V_W; L:H = V_L/V_H; H:W = V_H/V_W.

Statistical analyses

The data for MO_2, Q, f_H, H_{SV}, arterial-venous (AV) oxygen difference, perfusion conductance, V_f, and GCOT were all analysed using a one-way repeated-measures analysis of variance (RM ANOVA). Where the data were not normally
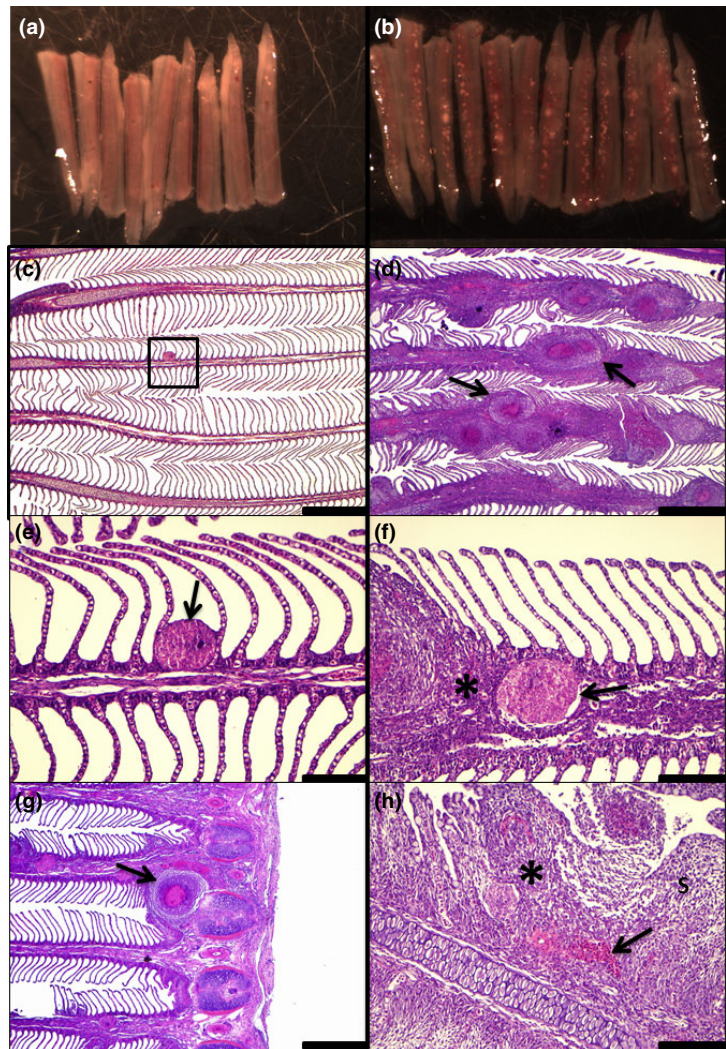
Figure 1 Images of gill filaments from Atlantic cod that were lightly (a) and heavily (b) infected with *Loma morhua*. The white 'spots' are xenomas. Histological sections of *Loma morhua* xenomas in the gills of lightly infected (c) and heavily infected (d) Atlantic cod (bar = 500 μm) showing limited (e) and extensive (f) filamental epithelial hyperplasia and mononuclear infiltration (*) (bar = 100 μm). Xenomas were also located in the gill arch at the base of the gill filaments (g, bar = 500 μm), where extensive inflammatory infiltration (*) and spongious hyperplasia (S) were associated with localized haemorrhage and xenoma disruption (h) (bar = 100 μm). Arrows indicate xenomas.

distributed, Freidman's RM ANOVAs on ranks were conducted. Individual differences were identified using either Student–Newman–Keuls or Tukey's post hoc analyses. The routine metabolic rate of cod scales allometrically with body mass with a slope of 0.8–0.85 (Post & Lee 1996; Killen *et al.* 2007). However, we report isometrically scaled metabolic rate data for comparison with previous studies on cardiorespiratory function in cod (Gollock *et al.* 2006; and Petersen & Gamperl 2010). Furthermore, the range of fish masses was small, and thus, the error in using isometric scaling was minimal. Pearson product–moment correlations were used to examine the relationship between the measured and derived parameters and the number of xenomas per filament or on the

surface of the heart. Comparisons between data collected in this study and those reported in Gollock *et al.* (2006) and Petersen & Gamperl (2010) were performed using unpaired *t*-tests. All analyses were performed using SigmaPlot 10.0 and SigmaStat 3.5 (Systat Software Inc.) graphical and statistical software. All values in the text, tables and figures are means + 1 standard error (SE).

Results

All of the fish used in this study were infected with *L. morhua* as determined by the presence of xenomas on the gills (Fig. 1a,b) and heart (Fig. 2a,b). However, the number, size and
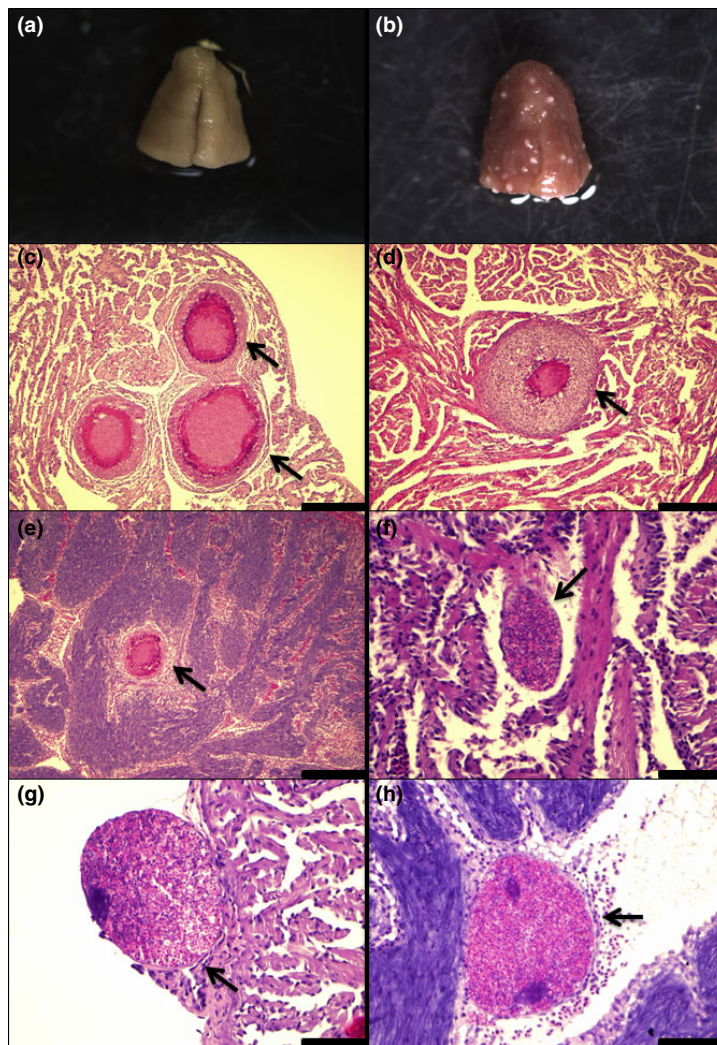
Figure 2 Image of lightly (a) and heavily infected (b) Atlantic cod ventricles showing white xenomas. Histological sections showing *Loma morhua* xenomas in the atrium (c), ventricle (d) and bulbus arteriosus (e) of the heart, with an accompanied fibrocytic and granulomatous inflammatory reaction (bar = 200 μm). Endocardial (f) and epicardial (g) and bulbus arteriosus endothelial (h) xenomas with limited inflammatory reaction (bar = 50 μm). Arrows indicate xenomas.

severity of the infection varied considerably between individuals, with some fish having few pathological signs of infection (Fig. 1c,e), whereas others showed extensive xenoma formation and branchial hyperplasia; the latter often associated with the filament tips and with fusion of gill lamellae (Fig. 1d,f,h). The level of infection in the gill ranged from 0.6 to 26.8 xenomas per filament (7.6 ± 1.1, median 5.5), whereas in the heart the number of xenomas ranged from 0 to 35 (5.9 ± 1.30, median 3.00) and they had an average density of 14.8 ± 3.3 xenomas g^{-1} ventricle (median 7.0; range of 0– 91.9) as determined by external examination. The xenomas in the filaments, both in the lamellae and at the base of the filaments on the gill arch, showed a characteristic amorphous external layer, contained numerous small spores, and were often associated with a fibrocytic or granulomatous tissue reaction (Fig. 1g). In the heart, xenomas were observed in the atrium, ventricle and bulbus arteriosus (Fig. 2c–e). Some of these were surrounded by an amorphous eosinophilic coat and a notable granulomatous reaction (Fig. 2c–e), whereas those associated with the endo- and epicardium generally showed no apparent inflammatory reaction (Fig. 2f–h). As a population, the density of branchial xenomas was not predictive of cardiac xenoma density (Pearson correlation coefficient = 0.130, *P* value = 0.417). The only significant correlation between morphological parameters and the number of xenomas or xenoma density was a negative correlation between condition factor (*K*) and cardiac xenoma density

Table 1 Pearson correlation coefficients and P values for the relationships between heart-somatic and morphological parameters, and (1) the mean number of branchial xenomas per filament and (2) the total number of visible cardiac xenomas per g ventricle mass in the Atlantic cod population used in the study

Parameter	Gill xenomas filament^{-1} Pearson	P value	Cardiac xenomas g^{-1} ventricle Pearson	P value
K	−0.081	0.615	−0.522	<0.001
THM	−0.137	0.393	−0.242	0.122
TVM	−0.023	0.888	−0.196	0.214
TAM	−0.107	0.504	−0.259	0.098
CSI	−0.101	0.530	0.085	0.591
VSI	0.062	0.698	0.171	0.278
ASI	−0.090	0.575	−0.098	0.538
%V	−0.097	0.548	−0.231	0.141
%A	−0.053	0.741	−0.167	0.290
V_L	0.105	0.512	0.0205	0.897
V_W	−0.088	0.584	0.018	0.908
V_H	−0.026	0.871	−0.0643	0.686
L:W	0.112	0.487	−0.026	0.870
L:H	0.119	0.459	0.057	0.719
H:W	−0.069	0.670	0.193	0.222

K represents condition index; THM, TVM and TAM are total heart, ventricle and atrial masses; CSI, VSI and ASI are cardio-, ventricular- and atrial-somatic indices; %V and %A are the relative proportion of cardiac mass contributed by ventricle and atrium, respectively; V_L, V_W and V_H are ventricular length, width and height, respectively; and L:W, L:H, H:W are ratios between the various measurements.

(Pearson correlation coefficient = −0.522, P value <0.001) (Table 1).

CT_{max} experiment

Oxygen consumption increased as temperature rose and reached a maximum value at 18 °C that was approximately twofold higher than measured at 10 °C (73.5 ± 3.5 mg O_2 kg^{-1} h^{-1}) (Freidman RM ANOVA on ranks X^2_5 = 102.8, P value <0.001) (Fig. 3a). Similarly, there were significant temperature-induced increases in cardiac output (Q, by 1.45-fold; from 21.5 ± 1.1 at 10 °C to 31.1 ± 2.4 mL kg^{-1} min^{-1} at 20 °C; Fig. 3b), heart rate (f_H, by 1.7-fold; from 46.8 ± 0.9 at 10 °C to 80.1 ± 2.3 beats min^{-1} at 20 °C; Fig. 3c) and the AV difference in blood oxygen content (by 1.45-fold; from 3.7 ± 0.30 at 10 °C to 5.4 ± 0.5 at 20 °C; Fig. 3f) [Freidman RM ANOVA on ranks (Q; X^2_5 = 38.8, P value <0.001: f_H; X^2_5 = 113.7, P < 0.001: C_aO_2-C_vO_2; X^2_5 = 33.1, P value <0.001)] (Fig. 3f). In contrast, cardiac stroke volume (H_{SV}; Fig. 3d) decreased slightly with temperature (by 14%; from 0.46 ± 0.03 at 10 °C to 0.40 ± 0.03 mL at

20 °C), and the perfusion convection requirement decreased significantly with temperature (by 30%; from 0.30 ± 0.02 at 10 °C to 0.21 ± 0.02 at 20 °C; Fig. 3g) (Freidman RM ANOVA on ranks H_{SV}; X^2_5 = 24.4, P < 0.001: MO_2/Q; X^2_5 = 31.3, P value < 0.001). Finally, ventilation frequency (V_f) increased significantly with increasing temperature (by 1.6-fold; from 40.2 ± 1.4 at 10 °C to 63.0 ± 1.7 ventilations min^{-1} at 20 °C; Fig. 3e) (RM ANOVA $F_{5,119}$ = 83.4, P value <0.001).

There was no significant correlation between the number of branchial xenomas and oxygen consumption at most temperatures. However, there was a strong negative correlation between these two parameters at 18 °C (Pearson correlation coefficient −0.58, P = 0.002) (Fig. 4a). Cardiac output was not significantly correlated with either branchial xenomas per filament or cardiac xenoma density at any of the temperatures (data not shown), and there were no significant correlations between heart rate and branchial xenomas per filament. Cardiac stroke volume and Q_{max} were also both negatively, but marginally (i.e. 0.01 > P < 0.05), correlated with cardiac xenoma density (H_{sv}; Pearson correlation coefficient = −0.36, P value = 0.093: Q; Pearson correlation coefficient = −0.37, P value = 0.093) (Fig. 4b,d). However, heart rate was positively correlated with cardiac xenoma density at all temperatures from 10 to 18 °C (Fig. 4c).

The mean critical thermal maximum (CT_{Max}) for this group of cod was 21.0 ± 0.3 °C. Upon reaching this upper temperature limit, all cardiorespiratory variables with the exception of H_{SV} (i.e. Q, f_H, and V_f) were substantially lower as compared with values recorded at 20 °C (Fig. 3b–e). There was a large degree of variation in the values of these parameters at CT_{max}, and thus, there were no statistically significant correlations between CT_{max} and either branchial xenomas (Pearson correlation coefficient = 0.017, P value = 0.94) or cardiac xenoma density at this temperature (Pearson correlation coefficient = 0.141, P value = 0.492) (Fig. 5).

Compared with the data presented by Gollock et al. (2006) for uninfected fish of the same stock origin, CT_{max} was only approximately 1 °C lower (21 vs. 22.2 °C; t_{33} = −6.11, P value = <0.001). This result was despite maximal MO_2 and Q (MO_2; t_{33} = −6.900, P value = <0.001: Q;
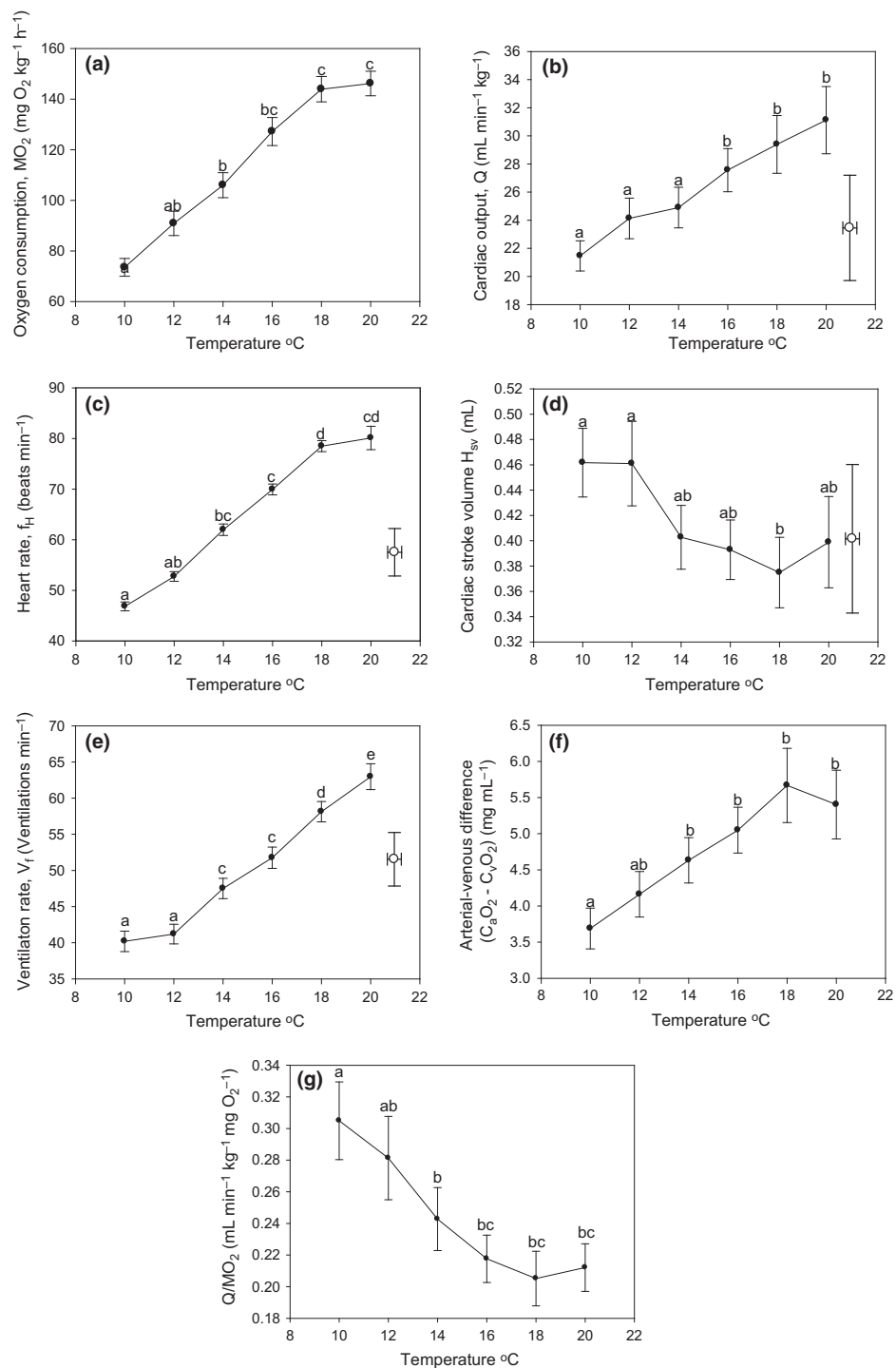
Figure 3 Oxygen consumption (a), cardiac output (b), heart rate (c), cardiac stroke volume (d), ventilation rate (e), arterial-venous blood O_2 content difference (f) and perfusion convection requirement (Q/MO_2) (g) of *Loma*-infected Atlantic cod acutely exposed to increasing temperature at 2 °C h^{-1}. Points (temperatures) without a letter in common are significantly different at $P < 0.05$. The open symbol represents values observed just after the fish reached their CT_{Max}. Values are means $\pm$ 1 SE.
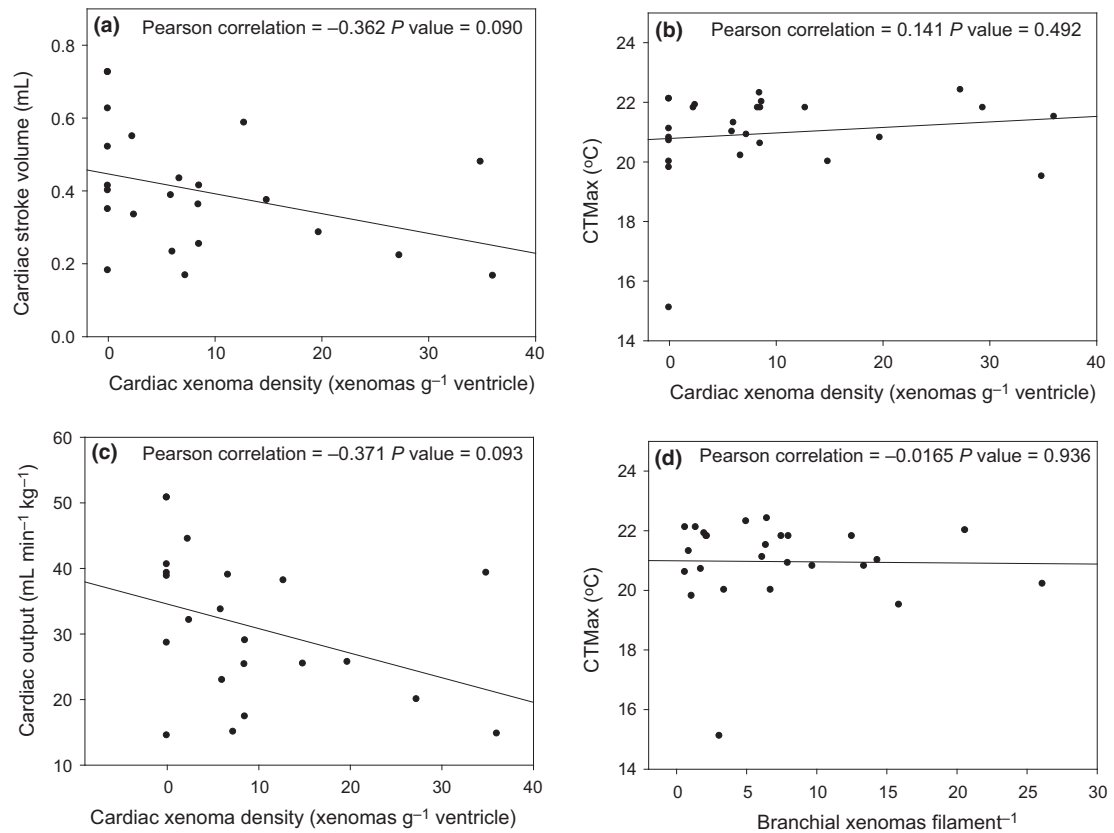
Figure 4 The relationship between (a) routine oxygen consumption rate at 18 °C and the mean number of branchial xenomas per gill filament; and between (b) stroke volume, (c) cardiac frequency at temperatures ranging from 10 to 18 °C and (d) cardiac output and cardiac xenoma density in Atlantic cod infected with *Loma morhua* and used in the CT_{Max} experiment.

$t_{33} = -4.86$, $P = <0.001$) being 30 and 40% lower, respectively, in this study. Resting ($t_{33} = -2.49$, P value = 0.018) and maximum H_{SV} ($t_{33} = -6.11$, P value <0.001) were also lower in the infected cod, and this was compensated for by an increase in resting heart rate (by 60%; $t_{33} = 5.75$, P value <0.001) but not maximum f_H ($t_{33} = 1.123$, P value = 0.270). Neither resting ($t_{33} = 1.123$, P value = 0.270) nor maximal ($t_{33} = 1.123$, P value = 0.270) perfusion conductance ratios (Q/MO_2) were statistically significant from those calculated from Gollock *et al.* (2006). However, Q/MO_2 was different between rest and maximal values for *Loma*-infected fish ($t_{50} = 3.182$, P value = 0.003) (Table 2). Similarly, while there were no significant differences between this study and Gollock *et al.* (2006) with regard to AV difference at rest ($t_{33} = -0.261$, P value = 0.796), the maximal value for AV difference for *Loma*-infected fish was higher than that derived from Gollock *et al.*

(2006) ($t_{33} = 1.827$, P value = 0.077) and the resting and maximal values for AV difference were only significantly different for *Loma*-infected fish ($t_{50} = -3.403$, P value = 0.001) (Table 2).

Swimming performance

Oxygen consumption increased significantly with water velocity and reached a maximum value (approximately twofold resting values) at 1.5 BL s^{-1} (RM ANOVA $F_{5,89} = 13.82$, P value <0.001). This swimming speed was approximately 0.2 BL s^{-1} below the cod's critical swimming velocity (1.71 BL s^{-1}; Fig. 6). After a 1-h recovery interval at 0.5 BL s^{-1}, oxygen consumption had, on average, only decreased to 77.1% of maximum. Nevertheless, the U_{crit} determined in the second U_{crit} trial was 1.76 BL s^{-1}. Gross cost of transport decreased with swimming velocity up to the critical swimming speed (U_{crit}) (Freidman RM ANOVA on ranks $X^2_6 = 68.981$, P value <0.001)
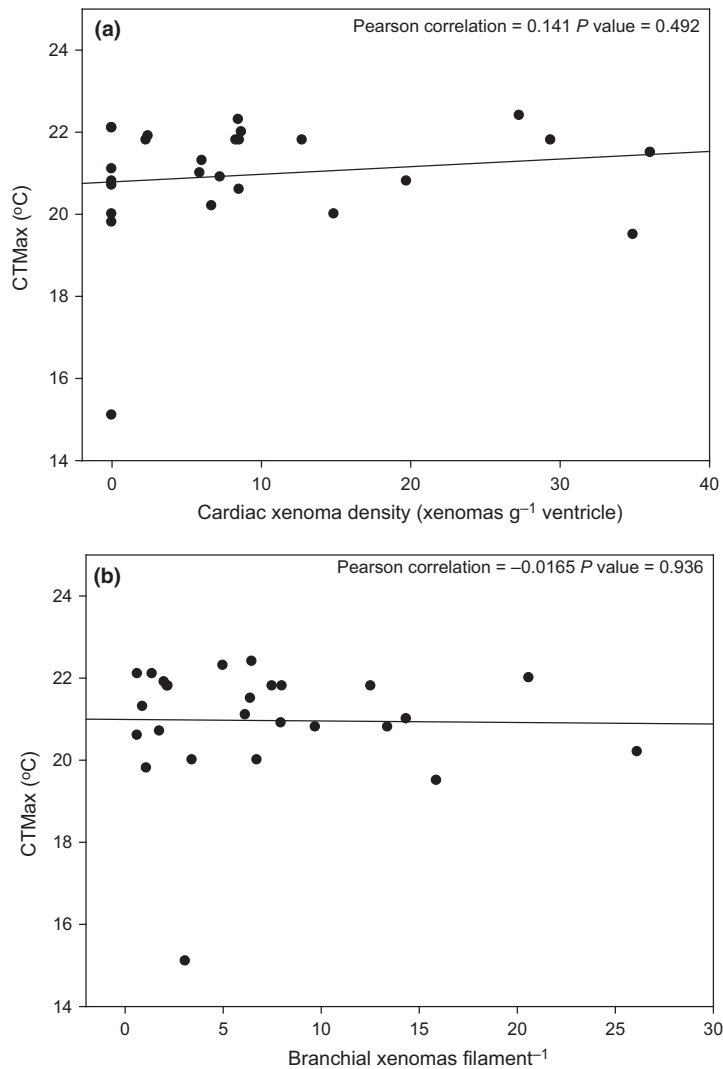
Figure 5 The relationship between CT_{Max} and cardiac xenoma density (a) and number of branchial xenomas per filament (b) in Atlantic cod infected with *Loma morhua*.

(Fig. 6). There were no significant correlations between, standard, resting (i.e. at 0.5 BL s^{-1}) or maximum oxygen consumption, U_{crit1} or U_{crit2}, and either gill xenomas per filament or cardiac xenoma density.

In comparison with data presented in Petersen & Gamperl (2010) for uninfected cod from the same founder population, maximal MO_2 (183.24 ± 10.31 as compared to 234.60 ± 21.20; $t_{22} = -2.444$, P value = 0.023) and metabolic scope (95.51 ± 11.97 as compared to 152.10 ± 20.70; $t_{19} = -2.463$, P value = 0.023) were significantly lower. In contrast, there were no significance differences between U_{crit} ($t_{22} = -0.227$, P value = 0.822), standard MO_2 ($t_{19} = 1.993$, P value = 0.061) or routine MO_2 ($t_{22} = 0.542$, P value = 0.593) between the two studies (Table 3).

Discussion

The Atlantic cod population used in this study was naturally infected with the microsporidian *L. morhua*. Nonetheless, the fish exhibited variable levels of infection, with all fish having branchial xenomas and cardiac xenomas as revealed through histological examination, but not external ventricular xenomas. The degree of host tissue inflammatory responses also varied greatly between individuals with some fish exhibiting xenomas with apparently little or no inflammatory reaction, as compared to others where extensive branchial hyperplasia or fibrocytic and granulomatous reactions were observed. Extensive hyperplastic and granulomatous reactions, and the associated infiltration of mononuclear cells, are characteristic of

Table 2 Comparison of oxygen consumption (MO$_2$, mg O$_2$ kg^{-1} h^{-1}), metabolic scope (the difference between resting and maximal MO$_2$), cardiac output (Q, mL min^{-1} kg^{-1}), the arterial-venous blood O$_2$ content difference, the perfusion conductance ratio (Q/MO$_2$), heart rate (f$_H$, beats min^{-1}) and stroke volume (H$_{SV}$, mL kg^{-1}) between the present study and those reported in Gollock *et al.* (2006). Both of these studies used adult cod reared from the same broodstock and a warming rate of 2 °C h^{-1}. Resting values are those recorded at 10 °C, whereas maximal values represent the highest values recorded prior to each fish reaching its critical thermal maximum (CT$_{Max}$) which are those observed just after the fish lost equilibrium

	Present study			Gollock *et al.* (2006)		
	Resting	Maximal	CT$_{Max}$ (21.0 ± 0.3* °C)	Resting	Maximal	CT$_{Max}$ (22.2 ± 0.2 °C)
MO$_2$	73.3 ± 3.5	146.2 ± 4.9*,#		82.2 ± 3.7	210.8 ± 7.2#	
Metabolic Scope	72.93 ± 4.26			128.6 ± 3.5		
Q	21.5 ± 1.1	31.1 ± 2.4*,#	23.4 ± 3.7	21.5 ± 0.8	52.6 ± 2.8#	28.4 ± 3.5
Q/MO$_2$	0.30 ± 0.02	0.21 ± 0.02#		0.26 ± 0.02	0.25 ± 0.02	
CaO$_2$-CvO$_2$	3.69 ± 0.28	5.67 ± 0.51#,§		3.82 ± 0.28	4.01 ± 0.51	
f$_H$	46.8 ± 0.9*	80.1 ± 2.3#	57.5 ± 4.7*	36.3 ± 1.7	71.8 ± 3.6#	37.4 ± 4.9
H$_{SV}$	0.46 ± 0.03*	0.40 ± 0.03*	0.40 ± 0.06	0.60 ± 0.04	0.76 ± 0.05	0.80 ± 0.08

Asterisks (*) indicate significant differences between the present study and that presented by or calculated from Gollock *et al.* (2006), whereas § represents differences at $P > 0.05 < 0.1$ and # represent significant differences between resting and maximal values within each study at $P < 0.05$. All data are means ± 1 SE.

Figure 6 Oxygen consumption (filled circles) and the gross cost of transport (open circles) of *Loma morhua*-infected Atlantic cod at different swimming velocities at 10 °C. The vertical line represents the mean critical swimming velocity (U$_{crit}$) for this group of fish. Points (swimming speeds) without a letter in common are significantly different ($P > 0.05$). Values are means ± 1 SE.
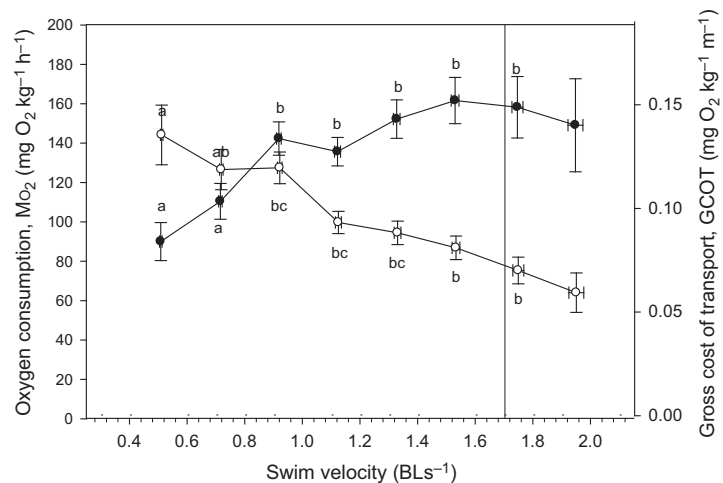
Table 3 Comparison of standard (MO$_{2standard}$), routine (MO$_{2routine}$) and maximum oxygen consumption (MO$_{2max}$), metabolic scope and critical swimming speed (U$_{crit}$) (BL s^{-1}) between the present study and the equivalent parameters measured by Petersen & Gamperl (2010) in cod from the same founder population.. Asterisks indicate significant differences between the two studies. Metabolic parameters are reported in mg O$_2$ kg^{-1} h^{-1}, and all values are means ± SE

	Present study	Petersen & Gamperl (2010)
MO$_{2standard}$	90.6 ± 9.3	64.3 ± 8.8
MO$_{2routine}$	90.0 ± 9.6[a]	82.5 ± 7.7[b]
MO$_{2max}$	183.2 ± 10.3*	234.6 ± 21.2
Metabolic Scope	96.5 ± 12.0*	152.1 ± 20.7
U$_{crit}$	1.71 ± 0.08	1.74 ± 0.06

[a]Based upon O$_2$ consumption measurements at 0.5 BL s^{-1}.
[b]Based upon O$_2$ consumption measurements at 0.25 BL s^{-1}.

Loma sp. infections where xenomas have ruptured and the spores released (Speare *et al.* 1998; Sanchez *et al.* 2000, 2001a,c; Sanchez, Speare & Markham 2001b; Lovy *et al.* 2004, 2006). The presence of spores and xenomas within the hyperplastic and granulomatous inflammatory tissue supports the suggestion of other authors (Rodriguez-Tovar *et al.* 2003) that autoinfection is characteristic with *L. morhua*.

Infection with *L. morhua* had an adverse effect on the fish, as indicated by the significant negative relationship between the number of cardiac xenomas and body condition. This response has been reported previously for Atlantic cod (Khan 2005) and fish infected with other parasites such as

Anguillicolla crassus in eels (*Anguilla rostrata*; Fenske, Secor & Wilberg 2010) and *Ichthyophonus hoferi* in Atlantic herring (*Clupea harengus*; Kramer-Schadt, Holst & Skagen 2010), and *Trichodina* sp., *Gyrodactylus* sp. and *Glugea stephani* in winter flounder (*Pleuronectes americanus*; Barker, Cone & Burt 2002; Khan 2004). However, care should be exercised when using condition index as the sole indicator of parasitism or fish health (Morton & Routledge 2006). With regard to *Loma* infections, it has been suggested that there is a metabolic cost related to osmoregulatory disturbances that occur at the time of rupture of the xenomas and the release or infective spores (Powell *et al.* 2006). However, it is difficult to consistently establish a relationship between condition factor and $MO_{2standard}$ or $MO_{2routine}$ post-infection [e.g. see present study vs. Petersen & Gamperl (2010); Powell *et al.* 2005], and thus, alternate explanations for the loss of body mass with parasite infection must be considered. One possibility is that *Loma* infection results in decreased appetite/food consumption. This would be consistent with what was observed by Khan (2005) for cod, and for rainbow trout (*Oncorhynchus mykiss*) infected with *Cryptobia salmositica* (Chin *et al.* 2004) and short-finned eel (*Anguilla australis*) infected with various internal parasites (Wang *et al.* 2006).

The CT_{Max} and U_{crit} values (21.0 °C and 1.71 BL s^{-1}) for *Loma*-infected Atlantic cod were slightly lower and higher, respectively, than those reported for non-parasitized fish from the same parental stock [22.2 °C, Gollock *et al.* 2006; 1.50 BL s^{-1}, Petersen & Gamperl (2010) (Tables 2 and 3)]. This was very surprising given that the fish used in the present study had lower maximal MO_2 values in the CT_{Max} and U_{crit} experiments (by 30–40%; Tables 2 and 3), that H_{SV} and Q were significantly compromised in *Loma*-infected fish (Fig. 5a and Table 2) and that a negative correlation was observed between branchial xenoma density and oxygen consumption at 18 °C (Fig. 4a). These results suggest that Atlantic cod can largely compensate for the negative effects of *Loma* infection on their cardiac function and metabolic capacity. With regard to the effects of *L. morhua* infection on H_{SV}, it is evident that our cod partially compensated by elevating resting heart rate (by ~30%; see Table 2). However, maximum f_H was not significantly greater in this study than reported in Gollock *et al.* (2006), and thus, maximum Q was still approximately 40% lower.

As there was no change in the ventricle's shape (i.e. its dimensions or their ratios; Table 1), as has been reported for a number of disease states including systemic hypertension (Powell *et al.* 2002) and haemolytic anaemia in Atlantic halibut *Hippoglossus hippoglossus* (Powell *et al.* 2012), it is unlikely that remodelling of the ventricle affected systolic function (i.e. ejection of blood from the heart). However, it is possible that the presence of xenomas in the myocardium resulted in a change in the elastic properties of the heart chambers and thus limited myocardial stretch and end-diastolic volume. Indeed, such an impact on myocardial properties has been suggested for other parasites that infect the heart such as *Stephanostomum tenue* (McGladdery *et al.* 1990) and *Kudoa thyrsites* (Kabata & Whitaker 1988).

With regard to how infected cod could reach CT_{Max} and U_{crit} values comparable to those of uninfected individuals despite a 40% decrease in maximum cardiac output, it appears that there are a number of possible explanations. First, based on the reported increase in AV O_2 difference (54%) and lower perfusion conductance ratio (30%) in *L. morhua*-infected cod when exposed to elevated temperature, and that $C_aO_2 - C_vO_2$ was higher in fish from this study vs. Gollock *et al.* (2006) under MO_{2max} conditions (by 42%; $P = 0.077$), it appears that tissues from cod infected with this parasite are more efficient at extracting O_2 from the blood. Interestingly, a very similar response is observed when cod (Petersen & Gamperl 2010) and rainbow trout (Moytka Norin & Gamperl unpubl.) are acclimated to chronic hypoxia (i.e. >8 weeks of exposure to waters with ~40% O_2 saturation). Acclimation to these conditions results in reduced (compromised) cardiac function, but values for Mo_{2max} and aerobic scope that are not different from normoxic-acclimated individuals when swum at 100% O_2 saturation or given a CT_{Max} test, respectively; that is, these fish can consume more oxygen per ml of blood pumped. Collectively, these data suggest that cod with *L. morhua* experience systemic hypoxaemia and that this results in tissue-level modifications that enhance the fish's MO_2 capacity despite reduced cardiac function. We did not look at tissue-level changes in this study. However, it is difficult to speculate about what alterations might have mediated the improvement in O_2 extraction. Hypoxia acclimation for prolonged periods does not lead to increased heart myoglobin levels (Driedzic,

Gesser & Johansen 1985; Hall *et al.* 2009), and the effect of hypoxic acclimation on muscle capillary density is variable (Johnston & Bernard 1982, 1984; Johnston, Bernard & Maloiy 1983; Sänger, Kim & Adam 1990). Further, we are not aware of any studies that have looked at the effects of this type of parasitic infection or chronic hypoxia on mitochondrial density, distribution or function, and chronic hypoxia does not lead to an increase in COX 1 mRNA expression in the cod heart or liver (Hall *et al.* 2009).

Second, the Atlantic cod has significant capacity for lactate production/anaerobic metabolism as shown in Nelson *et al.* (2002) and Dutil *et al.* (2007) and indicated by the fact that metabolic rate had only returned to 77% of MO_{2max} values by 1 h after U_{crit1}. Thus, it is probable that *L. morhua*-infected cod relied more on anaerobic metabolism during the CT_{Max} and U_{crit} challenges. Indeed, there are several lines of evidence that would support such a hypothesis. Nelson *et al.* (2002) demonstrated that some populations of Atlantic cod have been selected for, or conditioned to, support exercise performance through an enhancement of their use of anaerobic metabolism (i.e. there are intraspecific differences in anaerobic potential). Zhao *et al.* (2012) reported that hypoxia-acclimated juvenile qingbo (*Spinibarbus sinensis*) have significantly higher resting and post-exercise levels of plasma lactate than fish exposed to control (normoxic) conditions. Given that MO_{2max} was 30% lower in this study than measured in cod from Gollock *et al.* (2006) and Petersen & Gamperl (2010) despite similar values for CT_{Max} and U_{crit}, it is highly likely that anaerobic metabolism was used to make up the deficit in energy requirements during these two metabolically demanding challenges.

Finally, there was a significant negative correlation between cardiac xenoma density and condition factor, and studies have shown that food deprivation/starvation has an influence on the metabolism and swimming performance of Atlantic cod (Martinez *et al.* 2003, 2004; Lapointe, Guderley & Dutil 2006). These effects include a decrease in U_{crit}, an increase in MO_2 max and metabolic scope but increased the cost of transport, a reduction in the swimming speed at which burst-coast swimming begins and in the number of these movements utilized, and a diminished reliance on white muscle during swimming that results in less lactate production/accumulation.

However, it is unlikely that the condition of the fish used in this study contributed to the differences in metabolic capacity as compared to Gollock *et al.* (2006) and Petersen & Gamperl (2010). The average condition factor for fish used in this study was 0.99 ± 0.01, and the lowest condition factor recorded in this population was 0.77. This mean K value is higher than that of the fish used in Petersen & Gamperl (2010) (normoxic acclimated 0.94 ± 0.013; hypoxic acclimated 0.86 ± 0.03) and equivalent to, or greater than, that reported for the 'fed' cod (0.81–0.92) in the studies performed by Guderley and her colleagues (Martinez *et al.* 2003, 2004; Lapointe *et al.* 2006; Sylvestre *et al.* 2007).

Summary and perspectives

In this study, we showed that *L. morhua* infection of the heart and gills reduces the cardiac performance and maximum oxygen consumption of Atlantic cod, but does not diminish this species' upper thermal tolerance (CT_{Max}) or U_{crit}. These results are surprising given the critical importance of heart function and metabolic scope to these real-world challenges (Farrell 2009; Farrell *et al.* 2009; Petersen & Gamperl 2010; Gamperl *et al.* 2011). However, they provide another distinct example of the plasticity inherent in the fish's cardiorespiratory physiology, and how this allows this taxum to maintain function under conditions of prolonged oxygen limitation. With regard to the effect of *L. morhua* infection on cod cardiorespiratory function, there are several similarities with respect to how this species responds to long-term hypoxic acclimation (Petersen & Gamperl 2010). For example, under both conditions, there is a reduction in H_{SV} that is partially compensated for by an increase in f_H, and the fish improves oxygen extraction efficiency to make up for the deficit in cardiac function. The difference in the two studies, however, is the extent to which this increase in $CaO_2 - CvO_2$ can maintain the scope for oxygen consumption. In hypoxic-acclimated fish swum in 100% O_2 saturated water, it would be expected that the blood leaving the gills would be fully saturated and thus that there is sufficient blood oxygen carrying capacity which can be utilized (drawn down) to maintain MO_{2max} and metabolic scope. In contrast, *Loma* infection results in the formation of xenomas in the gills and the fusion of gill lamellae, and anaemia (Khan 2005), and likely

results in insufficient blood oxygen carrying capacity to allow improved oxygen extraction to meet metabolic demands. Thus, we suggest that this constant state of hypoxaemia promotes an increase in the capacity of *Loma*-infected cod to produce energy (ATP) through anaerobic metabolism and that it is the combination of improved oxygen extraction efficiency and enhanced capacity for lactate production that allows them to achieve comparable CT_{Max} and U_{crit} values as measured in uninfected fish. Clearly, this is a hypothesis that warrants further investigation, as are the mechanisms that result in reduced H_{SV} and enhanced oxygen extraction efficiency in both *L. morhua*-infected and hypoxic-acclimated cod (Petersen & Gamperl 2010) and rainbow trout (Moytka Norin & Gamperl, unpubl.).

Acknowledgements

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