ASSESSING THE PHYSIOLOGICAL IMPACT OF SEA LICE (*LEPEOPHTHEIRUS SALMONIS* KRØYER) ON ATLANTIC SALMON (*SALMO SALAR* L.)

by

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ABSTRACT

The objective of this thesis was to investigate the physiological changes that sea lice (*Lepeophtheirus salmonis*) cause in Atlantic salmon (*Salmo salar*). Sea lice are parasitic copepods that naturally infect salmonids in low numbers across the Northern Hemisphere with infrequent epizootics. The increase in the period of these epizootics in wild salmonids has mirrored the rise of the aquaculture industry in coastal areas. Therefore, it is important to answer basic physiological questions about parasite-host interactions such as sub-lethal lice levels required to affect performance, and the behavioural implications of increased lice infection.

Two preliminary experiments were performed using rainbow trout (*Oncorhynchus mykiss*) to refine the techniques used to measure fish physiological variables. The first was designed to determine which anaesthetic, between clove oil and MS-222, better minimised fish stress during surgery and recovery. Both clove oil and MS-222 reduced cortisol levels compared to non-anaesthetised controls within 10 minutes of blood sampling. However, fish exposed to MS-222 had elevated cortisol levels 1 hour after sampling. Based on this result, few other differences occurring between the two anaesthetics, and the fact that MS-222 is a human retinotoxicant, clove oil was used for all subsequent experiments. The second preliminary experiment was performed to determine proper recovery and interval times for critical swimming speed tests (U_{crit}), using hypoxia to stress fish in place of lice. Four hours of recovery following minor invasive surgery did not hinder the swimming performance of fish. Both 10 and 20 minute time intervals were found to be valid for measuring U_{crit}, blood and cardiac variables. During exercise, cardiac output ($\dot{Q}$) and heart rate (f_H) of normoxic fish increased (70 % and 25 % respectively) while those of hypoxic fish did not. A trend of a compensatory increase in stroke volume (V_S) with decreased f_H of hypoxic fish also was evident. Hypoxia reduced the U_{crit} of trout and lactate accumulation is not one of the physiological mechanisms. Blood

variables were measured in both studies, while cardiac variables and U_{crit} of fish also were measured in the latter.

The succeeding two experiments involved the artificial infection of Atlantic salmon with sea lice. In the first, the physiological impact of two sub-lethal levels of sea lice was measured on Atlantic salmon to determine when the health of fish is compromised. Fish with infection levels of 0.13 lice g^{-1} suffered a significant decrease (19 %) in U_{crit} and impaired osmoregulatory ability. Infection levels of 0.05 lice g^{-1} did not affect the swimming performance of salmon compared to controls. The second infection study was designed to determine if the observed behaviour of heavily infected wild salmonids in seawater (SW) returning early to native freshwater (FW) streams had a physiological basis. The U_{crit} of infected fish in SW decreased significantly (22 %) compared to controls, similar to the preceding study. However, salmon exposed to FW for 4 hours did not differ from controls with respect to U_{crit}. Evidence of the osmoregulatory ability of infected fish in SW being compromised did not occur. It is likely that energy use increased in the latter group to maintain osmotic balance. Blood and cardiac variables, and U_{crit} were measured in both studies.

The final study had an experimental and theoretical component. Lice with visibly ingested blood were first collected from infected fish, then their gut contents weighed. Juvenile trout then had repeated blood samples taken of two known quantities for one week and then had their U_{crit} measured. Gut contents and decline in performance due to blood loss were used to predict the possibility of blood loss due to sea lice infection decreasing the performance of their host. It was predicted that 5-10 % of the tissues consumed by lice is blood. At an infection level known to decreases U_{crit} (0.1 lice g^{-1}) lice would consume only 1 % of host blood, while 8 % loss is required to decrease U_{crit}. Significant reductions in U_{crit} purely due to blood loss would not occur until lethal infection levels (0.75+ lice g^{-1}) are reached. This series of studies has helped to answer some basic physiological questions about the interaction between sea lice and salmonids.

It is of great concern that sea lice can significantly affect salmonid physiology at infection levels lower than previously found on wild fish in areas of intensive aquaculture (0.5-2.1 lice g^{-1}). These findings support the implementation of continuous lice removal programs at all aquaculture sites to reduce infection levels in intensive aquaculture areas to those found in pristine areas (0.02 lice g^{-1}) that do not impair Atlantic salmon performance.

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COMMON MEASUREMENTS:

bl – body length

°C – degrees Celsius

m – metres cm – centimetres (10^{-2} metres) mm – millimetres (10^{-3} metres)

g – gram kg – (10^{3} grams) ng – nanograms (10^{-9} grams)

G – gravity

Hg – mercury

L – litres mL – millilitres (10^{-3} litres) µL – microlitres (10^{-6} litres)

min – minute s – seconds

mmol – millimoles ($6.023 * 10^{20}$ molecules of an element or compound; equal to the molecular weight in grams)

ppt. – parts per thousand

% – percentage

SE – standard error

GLOSSARY:

ATP – adenosine triphosphate

Blazka – swimming tunnel with two concentric tubes and a propeller that forces water at a known velocity through the inner tube towards an end cap where it is redirected to the outer tube and back to the propeller

Clove oil – active ingredient eugenol

Doppler – pulsed Doppler ultrasonic flow probe

f_H – heart rate (beats min^{-1})

FW – freshwater SW – seawater

MS-222 – tricaine methanesulfonate

P_{O2} – partial pressure of oxygen

$\dot{Q}$ – cardiac output (mL min^{-1} g^{-1} fish) $\dot{Q}_{max}$ – cardiac output at U_{crit}

Q_{10} – magnitude of change to a measured variable due to a 10 °C change in temperature

U_{crit} – critical swimming speed, maximum prolonged swimming ability of fish

V_S – stroke volume (mL $beat^{-1}$ g^{-1} fish)

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CHAPTER 1: GENERAL INTRODUCTION

SIGNIFICANCE OF SEA LICE

Atlantic salmon (*Salmo salar* L.) have a complex life cycle that involves moving from freshwater streams as smolts, after a residency of 2-5 years, into seawater in order to feed for 1-3 years (Scott & Crossman, 1973). During their return to the their native stream these fish stop feeding and undergo several morphological and physiological changes in order to journey upriver to the spawning grounds. The swimming ability of Atlantic salmon is, therefore, very important to its survival and fitness. Due to the finite energy reserves of salmon upon return to freshwater, any energy loss due to anthropomorphic or natural stressors may impair fish from reaching spawning grounds or decrease their spawning success.

Sea lice (*Lepeophtheirus salmonis*) are parasitic copepods that have long been known as a natural low-level pathogen of salmonids (Wilson, 1905; Scott & Scott, 1913). The life cycle of the sea louse is comprised of five phases and ten stages (Johnson & Albright, 1991; Schram, 1993). These include two free-swimming nauplius larval stages, one copepodid stage that infects the host fish, four chalimus larval stages attached to the host by a frontal filament, and two preadult stages followed by the adult that are free-moving on the host and sexually dimorphic. Lice feed on the skin, mucus, and blood of fish, causing local oedema and haemorrhaging of skin tissues (Kabata, 1974; Brandal *et al.*, 1976; Wootten *et al.*, 1982). High infections of lice (0.75-1.0 lice g^{-1} pre-adults; ie. 375-500 lice on a 500 g fish) can severely affect the osmotic balance of host fish, and lead to mortality (Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997). This parasite not only infects different salmonid species under aquaculture conditions, but also has been reported to infect wild species off the coasts of Canada (Templeman, 1967), Ireland (Boxshall, 1974), Japan (Nagasawa & Takami, 1993), Norway (Finstad *et al.*, 1994), and the United Kingdom (MacLean *et al.*, 1990).

Currently there is a debate as to whether lice from aquaculture facilities are increasing mortality of wild fish or if these infections are a secondary attribute of a more complex problem,

with little evidence to support either hypothesis (Dawson *et al.*, 1999). While epizootics of lice have been reported in wild fish for over 60 years (White, 1940), they typically are infrequent. The increase in the period of these epizootics in wild salmonids has mirrored the rise of the aquaculture industry in coastal areas over the past 15 years (Bjørn *et al.*, 2001; Bjørn & Finstad, 2002). Lice infections in aquaculture and wild fish within near-shore waters are suggested to be consistently higher than on fish found offshore or upstream. However, the paucity of information on the prevalence and intensity of infection among farmed salmon reported by Pike (1989) remains true today, making a comparison between farmed and wild lice levels very difficult. Jawarski & Holm (1992) found cultured Atlantic salmon *(Salmo salar)* to be heavily infected with lice (2 % of body covered), but did not include infection numbers or a means to calculate them. The two thousand lice found on a single farmed salmon by Brandal & Egidus (1977) likely is rare, but Jacobsen & Gaard (1997) did report 299 lice found on a single first year salmon (187 *L. salmonis*, 112 *Caligus elongatus*). This fish was surmised to have either escaped from an aquaculture facility or recently travelled from an inshore area with high level of infection. Sharp *et al.* (1994) also reported wild sea trout with an average and maximum infections of 63.9 and 216 lice respectably. Wild fish caught close to shore also are reported to have high levels of lice infection. Estuarine Arctic charr (*Salvelinus alpinus*) caught in traps by Finstad *et al.* (1995) had a mean intensity of 150-200+ lice per fish (0.5-0.65+ lice g^{-1}). Sea trout (*Salmo trutta*) released by Birkeland & Jakobsen (1997) into estuarine waters with nearby fish farms also became infected with extremely high levels of lice copepodids (mean intensity of 241; 8.3 lice g^{-1}). Jacobsen & Gaard (1997) found wild Atlantic salmon near Norway also were infected with lice, but at much lower levels than reared salmon. They found longline-caught salmon had a lice prevalence of 99.2% and a mean intensity of 29.8. These fish were not in close proximity to shore-bound aquaculture facilities. Lower intensity numbers ranging from 1 to 25, also have been reported for wild Atlantic salmon, sea trout, and all Pacific salmon with some lice

prevalence well under 100% (Templeman, 1967; Boxshall, 1974; Wootten *et al.*, 1982; Nagasawa, 1987).

Levels of infection among wild fish may be highest inshore because of their proximity to large aquaculture facilities where sea lice generally are found in high concentration. Anadromous salmonids actually can be infected more than once as they leave the rivers to feed in the ocean and again travel upstream to overwinter or spawn. Infection during upstream travel is not thought to be as detrimental to the fish because the levels of lice have been reported to decrease (White, 1940; Birkeland & Jacobsen, 1997) due to their intolerance to low salinity (Hahnenkamp & Fyhn, 1985). There are conflicting reports as to the severity of louse infection once in fresh water, however. MacLean *et al.* (1990) found the majority of lice infecting Atlantic salmon were lost within 48 hours, and the few remaining attached lasted a maximum of only six days. However, Finstad *et al.* (1995) found the level of infection on Arctic charr can remain near 60 lice per fish (0.2 lice g^{-1}) after one week of exposure to fresh water. It is unknown whether this level of infection may be detrimental to salmon migrating upriver to spawn or overwinter. Along with imposing physiological limitations on already energy-taxed fish, sub-lethal lice infection may cause chronic stress. Elevated cortisol levels due to chronic stress can cause immunosuppression, leading to an increased susceptibility to secondary infection (Ellis, 1981; Wootten *et al.*, 1982; Pickering & Pottinger, 1989; McKinnon, 1998).

Currently the perceived problem is both cultured and wild salmon are being parasitised by sea lice at increasing levels. Lice may be able to continuously re-infect both populations of fish and persist to feed as the fish migrate into fresh water to overwinter or spawn. Therefore, it is important to attempt to answer basic physiological questions about sub-lethal parasite-host interactions, such as the number of lice required to affect fish performance and the behavioural implications of increased lice numbers.

MEASURING THE PHYSIOLOGICAL IMPACT OF LICE

In order to study the physiological changes caused by sea lice, blood and cardiovascular variables of fish were measured along with their maximum prolonged swimming ability. These physiological variables typically are measured by drawing blood samples, attaching a Doppler flow probe to the ventral aorta, and performing critical swimming speed tests (U_{crit}), respectively. The Doppler flow probe forms a cuff at its distal end that is attached surgically through a small incision in the lateral wall of the isthmus, and anchored to the skin with sutures. The Doppler system measures the total amount of blood passing through the ventral aorta directly from the heart. This measurement is performed using an electrically-stimulated crystal to project an ultrasound beam of known frequency through the aorta wall, recording the sound wave reflected back to the crystal, and calculating the proportional decrease in wavelength (Doppler shift) caused by moving erythrocytes (Abel & McCutchen, 1979). The recorded cardiac output ($\dot{Q}$) is the product of heart rate (f_H) and stroke volume (V_S). The U_{crit} method is a measure of the maximum prolonged, or aerobic, swimming capacity of fish, and is recognised as an integrated measure of fish physiological performance (Brett, 1964; Beamish, 1978; Nelson, 1989; Randall & Brauner, 1991; Plaut, 2001). However, the methods used for $\dot{Q}$ and U_{crit} in past studies have been highly variable. Therefore, the best methods for testing the effects of an external parasite on its host had to be determined. Rainbow trout (*Oncorhynchus mykiss*) were used as a model salmonid system in two preliminary studies (Chapters 2 & 3) owing to their similar physiological response to sea lice as Atlantic salmon (Fast *et al.*, 2002) and the availability of that species.

The first problem was related to surgery because of the need to attach a Doppler cuff to the ventral aorta of each fish in order to measure the cardiac variables ($\dot{Q}$, f_H, and V_S). The loss of sensation during surgery is important for immobilisation of fish and reduction of stress (Brown, 1993; Burka *et al.*, 1997). Anaesthetics are used to depress the central and peripheral nervous systems (Summerfelt & Smith, 1990). Several chemical anaesthetics have been used to

sedate fish including MS-222 [3-aminobenzoic acidethyl ester methanesulfanate], Benzocaine hydrochloride [*p*-aminobenzoic acid ethyl ester], Metomidate [1-(1-phenylethyl)-*H*-imidazole-5-carboxylic acid methyl ester], and Etomidate [1-(1-phenylethyl)-*H*-imidazole-5-carboxylic acid ethyl ester], among others (see Iwama & Ackerman, 1994). While MS-222 is one of the most commonly used fish anaesthetics (Marking & Meyer, 1985), a study by Iwama *et al.* (1989) suggested it might act as a stressor under certain circumstances. Meanwhile, several studies have shown clove oil (active ingredient eugenol) to be a cheap and effective new alternative anaesthetic to MS-222 for use in fisheries science (Endo *et al.*, 1972; Hikasa *et al.*, 1986; Soto & Burhanuddin, 1995; Anderson *et al.*, 1997; Munday & Wilson, 1997; Peake, 1998; Waterstrat, 1999; Taylor & Roberts, 1999). However, while the physiological response of fish to MS-222 has been studied fairly extensively, the ability of clove oil to suppress the normal stress response of salmonids (rainbow trout used in Chapter 2) in the same manner is not known. Therefore a comparative study was performed between clove oil and MS-222 to measure the physiological response of fish to each anaesthetic.

The second issue was the testing of existing U_{crit} methods during measurement of a second stressor. Hypoxia was used in place of lice to cause combined stress effects, with U_{crit}, while measuring cardiac variables of rainbow trout (Chapter 3). Exposure of trout to low oxygen levels was performed because several studies have examined the effects of hypoxia using either U_{crit} or cardiac output, but not the two methods in conjunction. Very few studies have attempted to measure the effects of a stressor on cardiovascular variables during swim testing. Gallaugher *et al.* (1995) and Gamperl *et al.* (1995) used anaemia and hypoxia respectively, although the latter exercised trout only to 1.0 body lengths per second (bl s^{-1}), and did not approach U_{crit}. However, knowledge of the effects of hypoxia on cardiac variables and U_{crit} individually is quite extensive. Low levels of oxygen are known to physically challenge fish by increasing their lactate levels and respiration rates (Holeton & Randall, 1967; Farrell *et al.*, 1998). In salmon,

heart function also changes from a high heart rate, low stroke volume to a low heart rate, high stroke volume due to vagus stimulation from blood acidosis (Holeton & Randall, 1967; Randall, 1970; Wood & Shelton, 1980; Gamperl *et al.*, 1994a). These low oxygen changes have been shown by Jones (1971) to significantly lower rainbow trout U_{crit} when exposed to half air-saturation (68-78 mmHg). Farrell *et al.* (1998) found the critical swimming speed of sockeye salmon was not affected by moderate hypoxia (100-106 mmHg), but repeat swimming performance was lowered significantly. The sockeye swim speeds likely were not affected because the reduced oxygen level still was too high to affect maximum power output (Farrell *et al.*, 1989).

Testing of combined stressors also was necessary to determine the proper time interval and recovery period that could be used within the time constraints imposed by lice that continually moult through life stages and become reduced in number. A major concern in the lice infection studies was reduction of the overall experiment time in order to minimise differences in lice growth, while still obtaining cardiac variable results with low variation. The debate over the proper time interval for critical swimming studies using water current speed increments was mentioned by Jones (1971) and has continued in the literature up to recent times. The time interval used varies from as low as two minutes (Anderson *et al.*, 1997), to the often used 10-20 minute range (Davis *et al.*, 1963; Dahlberg *et al.*, 1968; Farrell *et al.*, 1998), upwards to the original 60 minute time intervals set by Brett (1964). Davis et al. (1963) found doubling the time interval from 10 to 20 minutes did not significantly lower critical swim speeds. Likewise, fish tested by Jones (1971) at 20 and 40 minute time intervals at a speed increment of 9 cm s^{-1} showed no significant differences in critical swim speed. The amounts of time fish have been allowed to recover from anaesthesia and surgery in the swimming chamber before performing the U_{crit} test also has been highly variable. Recovery time used in other studies varies from 1-2 hours (Keen & Farrell, 1994; Anderson *et al.*, 1997; Korsmeyer *et al.*, 1997) to

12 hours (Thorarensen *et al.*, 1996; Farrell *et al.*, 1998), up to 24+ hours (Kiceniuk & Jones, 1977; Gallaugher *et al.*, 1995) after exposure or surgery. Jones (1971), however, reported no significant differences in critical swim speed when testing fish after 2 hour and 20 hour swim tube acclimation times holding fish at a speed of 2-4 cm s^{-1}. Results from this study helped determine the length of time increments and recovery times to be used for the reliable measurement of cardiac output in fish experiencing multiple stressors. The preliminary experiments (Chapters 2 & 3) were crucial to the success of the subsequent sea lice infection studies (Chapters 4-6).

Experiments involving artificial infection of Atlantic salmon with sea lice were performed to answer basic physiological questions about the impact lice have on their host. The first lice experiment involved determining the effects of two sub-lethal levels of sea lice on salmon blood and cardiac physiology during maximum prolonged swimming (Chapter 4). Swim performance testing had never been performed on salmonids infected with lice. MacKinnon (1997) reported a pronounced increase in louse pathology once the chalimus IV moults to the preadult stage. Therefore, my working hypothesis was a significant reduction in swimming performance would occur at a given level of infection with adult lice. To this end, the numbers and life stages of lice were counted before U_{crit} testing according to the criteria set out by Johnson & Albright (1991) and Schramm (1993), using the same method as Bjørn & Finstad (1998). While the effects of lethal lice levels had been reported, the infection level required to initially compromise the osmoregulatory capacity, cardiac output and swimming ability of Atlantic salmon was not known. There have been conflicting reports about what sub-lethal level of lice infection begins to affect salmon. Jacobsen & Gaard (1997) commented an average of 30 predominantly adult lice seemed not to affect first or second year Atlantic salmon (38-85 cm fork length), although no testing was performed. However, an intensity as low as 10 lice on mature fish has been reported to cause weight loss due to reduction in feeding (MacKinnon, 1997).

Other studies have defined the lethal infection level as starting between 0.75 lice g^{-1} (Grimes & Jakobsen, 1996; 30 pre-adult lice on 40 g Atlantic salmon post smolts) and 1.0 lice g^{-1} (Bjørn & Finstad, 1997; 90 pre-adult and adult lice on 90 g sea trout post smolts). This level of lice infection chronically elevates cortisol levels and eventually causes osmoregulatory breakdown. Based on these parameters, two different sub-lethal infection levels were chosen along with uninfected control fish to help answer the question of what level of louse infection is detrimental to the health and performance of salmonids.

The second lice experiment was performed because heavily infected wild salmonids have been observed returning early to native streams during the past ten years (Tully *et al.*, 1993a, 1993b; Birkeland, 1996; Birkeland & Jakobsen, 1997; Tully *et al.*, 1999; Bjørn *et al.*, 2001). Whether physiological stress relief of fish to lice infection was the cause of this behaviour is unknown. Sea lice have been shown to affect the osmotic balance of salmonids in seawater (Grimes & Jakobsen, 1996; Birkeland & Jakobsen, 1997). The initial drop off rates for salmon lice are high once the fish reach a river (McLean *et al.* 1990), but a large proportion can continue to infect migrating fish through the transition into freshwater for upwards of 2 weeks (Finstad et al. 1995). Many species of salmon travel long distances inland to spawn or overwinter. This migration is taxing physiologically, due to the initial change in salinity, physical obstacles, and high water velocities encountered, and would only be compounded by lice infection. Therefore, it is important to determine what effect lice have on the swimming performance of salmon in changing salinity conditions. Fish were infected with sub-lethal lice levels known to compromise salmon performance (determined in Chapter 4) and subjected to changes in salinity (from 33 to 0 ppt.) prior to swim testing (Chapter 5). This design helped to determine if the osmotic balance of infected fish returning to FW is restored and if this restoration benefits swimming performance.

The involvement of sea lice was theoretical to a large degree in the final study that dealt with the question of host blood loss due to parasitic feeding (Chapter 6). Sea lice are grazer-type feeders (Kabata, 1974), but their feeding rates and the percentage of haemorrhaged blood ingested along with mucus and skin tissues from their host are unknown. Approximately 30 % of adult lice (90% gravid females) do feed on blood (Brandal *et al.*, 1976) and lice with visibly ingested blood were collected from infected fish and their gut contents weighed. Repeated blood sampling is known to increase cortisol and decrease haematocrit levels in fish (Iwama *et al.*, 1989; Gallaugher & Farrell, 1998), similar to the effects of high sea lice infection (Bjørn and Finstad 1997). Therefore, repeated caudal punctures were used to simulate blood feeding by lice prior to swimming performance testing. Gut contents of lice and decline in the swimming performance of fish due to blood loss were used in a predictive model to determine whether blood loss due to sea lice infection can affect host physiology.

The following chapters in this thesis represent the first attempt to determine the physiological responses of fish to combined stressors involving sea lice at sub-lethal levels of infection. The methodological studies (Chapters 2 & 3) combined with those involving artificial infection with sea lice (Chapters 4-6) serve to answer specific questions about the physiological impact of lice on Atlantic salmon. Aspects of blood and cardiorespiratory physiology, as well as the maximum prolonged swimming performance of fish were measured because they are good integrated indicators of physiological disturbance.

*Chapter 2: The Ability of Clove Oil and MS-222 to Minimise Handling Stress in Rainbow Trout (*Oncorhynchus mykiss* Walbaum)

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INTRODUCTION

Anaesthetics are used primarily to immobilise fish by depressing their central and peripheral nervous systems (Summerfelt & Smith, 1990). Loss of sensation is important for the reduction of stress during several fisheries research procedures such as handling, transport, blood sampling and surgery (Brown 1993; Burka *et al.*, 1997). Aquaculture facilities routinely monitor fish health using a number of techniques including blood sampling. Physiological studies examining the chemical properties of the blood utilise one of two approaches to blood sampling: acute sampling of anaesthetised fish via caudal or cardiac puncture; and chronic sampling via an indwelling catheter (McDonald & Milligan, 1992). Acute sampling procedures are useful in obtaining resting or baseline levels of blood variables, but can potentially alter blood chemistry more than chronic procedures especially when repeated samples are drawn. In general, the use of an anaesthetic for blood sampling requires a lethal dose that has been shown not to affect resting blood (Strange & Shreck, 1978; Barton *et al.*, 1986). The main drawback of this procedure is the animal must be sacrificed. I used a technique whereby blood was sampled rapidly without anaesthetic. This provided us with an initial, undisturbed sample and also induced a handling stress that was monitored by subsequent sampling.

I tested the ability of tricane methanesulfonate (MS-222) and eugenol (clove oil) to potentially suppress normal stress response by comparing blood variables following sampling via caudal puncture. MS-222 is one of the most commonly used anaesthetic for fish and is the only one registered in North America (Marking & Meyer, 1985). The effects of MS-222 during anaesthesia have been investigated on a variety of blood chemistry variables including glucose and lactate (Sovio *et al.*, 1977), haematocrit (Reinitz & Rix, 1977), cortisol (Strange & Schreck, 1978; Barton & Peter, 1982; Iwama *et al.*, 1989), amino acids (Morales *et al.*, 1990), lipids (Harrington *et al.*,1991), cholesterol (Wedemeyer, 1970), electrolytes (Houston *et al.*, 1971), and gases (Iwama *et al.*, 1989). Some results in the study by Iwama *et al.* (1989) suggest the

administration of MS-222 may act as a stressor under certain circumstances. This information is critical for fisheries researchers who utilise MS-222 in experiments, particularly those investigating stress related parameters, where such effects may potentially alter the results of the research.

A growing number of studies have demonstrated the efficacy of clove oil and suggested it as an effective alternative anaesthetic to MS-222 for use in fisheries science (Endo *et al.*, 1972; Hikasa *et al.*, 1986; Soto & Burhanuddin, 1995; Anderson *et al.*, 1997; Munday & Wilson, 1997; Peake, 1998; Waterstrat, 1999; Taylor & Roberts, 1999). Few studies, however, have addressed the physiological impacts of clove oil compared with MS-222 (Prince & Powell, 2000). Anderson *et al.* (1997) demonstrated that clove oil, like MS-222, had no post-exposure effect on swimming performance of juvenile or adult rainbow trout (*Oncorhynchus mykiss*). In addition they found that clove oil induced anaesthesia more rapidly than MS-222, but it also caused an extended recovery period compared with MS-222. Their findings have been confirmed in juvenile rainbow trout (Keene *et al.*, 1998) and chinook salmon (*Oncorhynchus tshawytscha*) (Cho & Heath, 2000).

In this study I compare the physiological effects of equal concentrations of clove oil and MS-222 in stressed pre-spawning adult rainbow trout. I acquired samples from fish under both basal physiological conditions as well as from fish stressed by blood sampling using caudal puncture in two separate experiments. The objectives were as follows: (1) compare physiological effects associated with exposure to clove oil and MS-222 following handling stress; and (2) examine the ability of each anaesthetic to minimise the handling stress response associated with blood sampling.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

All fish were obtained from a 50:50 sex ratio population at the Rainbow Springs Trout Farm, Thamesford, ON. The fish were held in well water (11.5 ^{o}C ± 0.5 SE) for at least 2 weeks prior to experimentation at the Waterloo Biotelemetry Institute, University of Waterloo. Fish were fed a pellet trout food (Martin Mills Incorporated, Elmira, ON) *ad libitum*. Feed was withheld for 48 hours prior to, and during, the experimental period to ensure the fish were in a post-absorptive state before induction of anaesthesia.

For the first experiment forty-eight rainbow trout were held in a 6500 L tank. Fish were divided into separate anaesthetic treatments and measured following initial rapid blood sampling (clove oil: length = 29.5 ± 2.8 cm (mean ± SE); weight = 288.8 ± 9.8 g, MS-222: length = 30.3 ± 2.6 cm; weight = 314.5 ± 10.2 g). Samples were collected at six time intervals following exposure to each anaesthetic treatment including: observation of full anaesthesia, observation of total behavioural recovery, one hour, four hours, 24 hours, and 48 hours post anaesthetic exposure. Eight fish were sampled per time interval.

In the second experiment fifty-four rainbow trout held in a 4000 L tank. Fish were sampled rapidly and divided into three treatment groups (clove oil: length = 20.0 ± 2.7 cm; weight = 91.1 ± 3.0 g, MS-222: length = 20.3 ± 2.4 cm; weight = 92.7 ± 2.8 g, Non-anaesthetised Controls: length = 20.3 ± 2.4 cm SE; weight = 93.8 ± 2.9 g). Following exposure to each anaesthetic treatment, samples were collected at three of the six sampling times used in the first experiment, e.g., observation of total behavioural recovery (control fish sampled at similar time to anaesthetised fish), one hour, and four hours post anaesthesia exposure. Six fish were sampled per time interval.

SAMPLING PROCEDURES

Experiment 1

To induce initial stress, fish were netted quickly and gently, removed from the holding tank, and held on a foam pad to prevent vigorous struggling. A 1 mL sample of blood was taken by caudal puncture using a heparinized syringe similar to Houston (1990). This initial sample acted as an initial stressor for each fish. The process took less than 30s once a fish was removed from the tank. Each fish then was exposed to either a 20 L anaesthetic bath of 60 mg L^{-1} clove oil (6.6 x 10^{-5} mols) (88 % min. eugenol; Hilltech Canada Inc., Vankleek Hill, Ontario), or 60 mg L^{-1} MS-222 (6.0 x 10^{-5} mols) (Sigma, St. Louis, MO) buffered with 120 mg L^{-1} $NaHCO_3$ (as per Burka *et al.*, 1997) until full anaesthesia (Induction 3) as defined by McFarland (1959) was reached. Due to its incomplete solubility in water at temperatures below 15 °C clove oil first was dissolved in ethanol at a ratio of 1:10 (clove oil: ethanol). The maximum ethanol concentration in this study (540 mg L^{-1}) should have no effect on fish physiology because the 96 hour effective concentration (EC_{50}) for 80 g rainbow trout is 12 000-16 000 mg L^{-1} (Mayer & Ellersieck, 1986).

Once anaesthetised, each fish was marked with a spaghetti tag inserted into its dorsal musculature to identify its specific treatment and time group. Fish were placed into a 20 L recovery bath of aerated water, free of anaesthetic and time to full recovery was measured. Full recovery, as defined by McFarland (1959), occurred when the fish experienced total behavioural recovery and swimming behaviour had returned to normal. Induction and recovery times were judged visually and measured with a stopwatch to the nearest second. The eight fish in each time group were transferred into one recovery tank (200 L) and held until their second sampling time, except for those in the full anaesthesia and full recovery time groups which were sampled individually directly from the 20 L anaesthetic or recovery bath. The final blood sample was taken using the same method as the first.

After each blood sample was taken, the syringe was placed immediately on ice. Blood samples were analysed for glucose, lactate and haematocrit using a Stat Profile 9 Plus Analyzer (NOVA Biomedical, Waltham, MA, USA). Remaining blood was centrifuged at 5000 x G for 5 min to collect plasma. The plasma was stored at –20 °C for later analysis of cortisol. Plasma cortisol levels were quantified using a commercial radioimmunoassay kit (ICN Biomedicals Inc., Irvine, CA) from duplicate 20 μL samples.

Experiment 2

The second experiment focused on four critical time points: initial (individually for each time point), observation of recovery, one hour and 4 hours post anaesthetic exposure. The procedures for the second experiment were the same as the first experiment except as follows. A non-anaesthetised control group was sampled at all time intervals, fish were identified with pectoral fin clips instead of spaghetti tags, and the final blood sample was taken after quickly placing all six fish within a treatment simultaneously into a 20 L lethal bath (200 mg L^{-1}) of MS-222 as per Prince & Powell (2000).

STATISTICS

The data for both studies was analysed using two-way analysis of variance (ANOVA) and Tukey's multiple comparison post-hoc tests. Length and weight of the fish were used as covariates, each blood variable was a dependent variable, and the treatments and sample times were the independent variables All data was transformed after analysis, for graphical presentation in Fig. 2.1, by subtracting the value of the initial basal sample from the final sample.

RESULTS

EXPERIMENT 1

Change in all blood variables from resting values to anaesthesia-exposed are presented in Fig. 2.1, while absolute values are referenced below. Changes in plasma cortisol and

haematocrit did not differ between clove oil and MS-222-exposed fish. In contrast, the increase in plasma glucose from resting levels at Induction 3 was significantly greater in clove oil-exposed (2.3 ± 0.4 mmol L^{-1}) versus MS-222 (0.4 ± 0.2 mmol L^{-1}) exposed fish ($P < 0.001$). Likewise, at full recovery the rise in plasma lactate from rest was significantly greater for clove oil (4.6 ± 0.2 mmol L^{-1}) than for MS-222 (3.3 ± 0.3 mmol L^{-1}) exposed fish ($P = 0.005$).

Plasma cortisol levels significantly increased from initial resting levels and peaked at one hour following anaesthetic exposure for clove oil (160.4 ± 20.8 ng L^{-1}) and MS-222 (178.1 ± 18.1 ng L^{-1}) ($P < 0.01$). Plasma cortisol levels remained elevated up to 48 hours post-sampling. Whole blood glucose levels peaked at one hour for MS-222 (7.0 ± 0.7 mmol L^{-1}) and at 4 hours for clove oil (7.8 ± 0.8 mmol L^{-1}) ($P < 0.01$). Both anaesthetics induced elevated glucose levels up to 48 hours after initial blood sampling. Whole blood lactate plasma levels peaked one hour after exposure for clove oil (6.8 ± 0.1 mmol L^{-1}) and MS-222 (6.5 ± 0.6 mmol L^{-1}), but were elevated from Induction 3 until 4 hours post sampling ($P < 0.01$). Haematocrit levels peaked at Induction 3 for clove oil (33.4 ± 1.1 %) and MS-222 (33.6 ± 0.7 %), and decreased significantly ($P < 0.01$) from basal levels at 24 hours in both clove oil and MS-222-exposed fish, respectively (22.0 ± 1.4 mmol L^{-1}; 19.0 ± 0.9 mmol L^{-1}) ($P < 0.01$).

Overall, fish entered Induction 3 significantly faster when exposed to clove oil compared to MS-222 (Table 2.1). In contrast, fish recovered from anaesthesia significantly faster with MS-222 compared to clove oil. Fish length and weight did not influence the reaction to either anaesthetic with respect to changes in blood physiology or recovery times.

EXPERIMENT 2

Three critical time points were examined following blood sampling and exposure to clove oil, MS-222, or no anaesthetic (control). Absolute values are referenced below, while calculated differences between final and initial values are used in Fig. 2.1. After initial blood sampling

resting plasma cortisol levels were significantly lower in both clove oil (30.4 ± 15.1ng L^{-1}increase; $P = 0.03$) and MS-222 (12.2 ± 3.3 ng L^{-1} increase; $P = 0.02$) anaesthetic treatments compared to non-anaesthetised controls (124.9 ± 38.1 ng L^{-1}). The increase in whole blood lactate was significantly lower one hour after exposure to clove oil (3.3 ± 0.6 mmol L^{-1}) compared with control fish (6.6 ± 0.6 mmol L^{-1}); ($P < 0.01$) or fish exposed to MS-222 (5.6 ± 0.4 mmol L^{-1}; $P = 0.03$). Haematocrit levels were significantly higher than basal levels at recovery in clove oil (2.8 ± 1.1 % increase) and MS-222 treated fish (1.3 ± 1.4 % increase) compared to control fish (4.2 ±1.2 % decrease) ($P < 0.01$). One hour after exposure, the haematocrit of fish exposed to MS-222 was not different from basal levels (0.0 ± 1.4 %), but was significantly higher than in fish exposed to clove oil (3.2 ± 1.0 % decrease) and controls (6.3 ± 1.7 % decrease) ($P < 0.01$).

Plasma cortisol levels peaked in the control group at recovery (212.8 ± 29.9 ng L^{-1}) and at one hour for fish exposed to MS-222 (115.4 ± 11.1 ng L^{-1}). Fish anaesthetised with clove oil showed no significant changes in plasma cortisol from resting values. Blood glucose was significantly higher than resting levels by one hour and peaked at 4 hours for all three treatments (clove oil 11.6 ± 1.4 mmol L^{-1}; MS-222 11.7 ± 0.6 mmol L^{-1}; control 11.0 ± 0.7 mmol L^{-1}) ($P < 0.01$). Blood lactate levels of MS-222-exposed and control fish were significantly higher at recovery, and peaked in all three groups at one hour (clove oil 6.4 ± 0.9 mmol L^{-1}; MS-222 7.7 ± 0.4 mmol L^{-1}; control 9.6 ± 0.6 mmol L^{-1}) ($P < 0.05$). Haematocrit levels were significantly lower by recovery in control fish (19.8 ± 2.1 %) and remained depressed through to 4 hours ($P < 0.05$). At one hour haematocrit levels from fish exposed to clove oil (27.0 ± 1.2 %) had fallen significantly below basal levels while fish in the MS-222 group (19.0 ± 1.5 %) had a similar depression at by 4 hours.

DISCUSSION

PHYSIOLOGICAL EFFECTS

Overall, fish exposed to MS-222 and clove oil had similar responses following initial blood sampling with few exceptions. These findings are similar to those of Cho & Heath (2000) who observed no physiological differences in chinook salmon anaesthetised with different concentrations of clove oil (20 mg L^{-1}) and MS-222 (50 mg L^{-1}). The present results also agree with Anderson *et al.* (1997) who found no differences in swimming performance of rainbow trout exposed to clove oil or MS-222.

While in most cases clove oil and MS-222 elicited similar responses there were several important differences. Extended recovery times associated with clove oil (Table 1) may have led to the significantly higher blood lactate levels at recovery compared with MS-222–exposed fish (Fig. 1). However, beyond one hour no lactate differences occurred. Similarly, Black & Connor (1964) observed increased plasma lactate levels in rainbow trout during anaesthesia with MS-222. The significant increase in blood glucose following clove oil exposure may be caused by a release of catecholamines if fish perceive the anaesthetic that, unlike MS-222, has a scent and alters the appearance of water. This release would mobilise energy stores from the liver, however I did not observe a corresponding cortisol response and the effect disappeared upon recovery.

More important were the physiological differences between fish exposed to an anaesthetic and the control fish observed in the second experiment. These differences were evident at recovery when fish exposed to either anaesthetic exhibited baseline cortisol and haematocrit levels compared to those of control fish. Plasma cortisol levels measured in anaesthetised fish suggest both clove oil and MS-222 are effective as short term stress reducers. Normally, cortisol peaks by one hour post-stress (Sumpter *et al.*, 1986), but thus was not the case in the control group that peaked at recovery. However, at one hour following exposure cortisol

levels of fish exposed to MS-222 rose significantly while clove oil-exposed fish remained unchanged from initial levels. This result supports the possibility that administration of MS-222 can act as a stressor under certain circumstances (Iwama *et al.*, 1989).

The increase in haematocrit levels following anaesthetic exposure may be due to a combination of erythrocyte swelling (Iwama *et al.,* 1989) and low initial blood loss because of slowed heart rate. Swelling can occur when oxygen levels are reduced because of low gill irrigation, and has been reported for rainbow trout during exposure to MS-222 (Lowe-Jinde & Niimi, 1983). Neither the suppression of cortisol nor the maintenance of haematocrit were evident after recovery. Haematocrit of anaesthetised fish likely fell to control levels once gill irrigation increased due to a combination of minor blood loss and erythrocytes returning to normal size.

Anaesthetics are used routinely to reduce stress in fish during blood sampling (Strange & Schreck, 1978; Barton & Peter, 1981), but prolonged exposure can induce stress as well (Strange & Schreck, 1978; Iwama *et al.*, 1989). While previous studies have demonstrated clove oil is as effective as MS-222 prior to a stress such as blood sampling (Keene *et al.,* 1998), this is the first study to show it also can be effective when used following sampling.

Both clove oil and MS-222 reduced cortisol levels within 10 minutes after blood sampling compared to non-anaesthetised controls. Similar results have been shown for MS-222 (Strange & Schreck, 1978) and etomidate (Limsuwan *et al.*, 1983), but this is the first study to demonstrate clove oil has this same effect. The above studies found the increase in cortisol levels of handled, non-anaesthetised control chinook salmon and channel catfish (*Ictaluras punctatus*) respectively, did not occur in fish anaesthetised prior to handling.

CLOVE OIL AS AN ALTERNATIVE

Induction times were significantly shorter for fish exposed to 60 mg L^{-1} clove oil compared to 60 mg L^{-1} MS-222 while recovery times were substantially longer. This finding is in agreement with previous studies and appears to be consistent for a number of salmonid species over a wide range of sizes (Anderson *et al.*, 1997; Keene *et al.*, 1998; Cho & Heath, 2000). The reduced induction time with clove oil may be related to its high lipid solubility (Keene *et al.*, 1998). The more rapid recovery times for MS-222 may be related to the initial stimulatory effect it has upon heart rate (Randall, 1962) in contrast to clove oil which has an inhibitor effect (Hikasa *et al.*, 1986). It is clear clove oil is a suitable alternative from an efficacy point of view. The distinct differences in induction and recovery times between clove oil and MS-222 observed here and in other studies do not seem to affect fish physiological variables in a consistent manner. However, the fact that fish exposed to MS-222 had significantly elevated cortisol levels at one hour suggests the anaesthetic may not be as effective at reducing stress as clove oil.

Perhaps the largest drawback of using MS-222 as an anaesthetic is the fact it is a known retinotoxicant to humans, requiring protective clothing to avoid contact during use (Bernstein *et al.*, 1997). Officially, clove oil is not sanctioned for use as a fish anaesthetic in the USA (FDA 2002). However, it has been listed as 'generally recognised as safe' (GRAS) in humans at levels not exceeding 1500 mg L^{-1} (Nagababu & Lakshamaiah, 1992; FDA, 2002) because it is organic and its main ingredient, eugenol, is non-toxic. The potential applications of clove oil as a stress reducer in place of MS-222 are numerous. Clove oil may reduce mortality in the live wells of boats, used for catch and release fishing, by lowering stress from capture and crowding, and minimising oxygen and waste product uptake, all of which are significant problems (Gustaveson *et al.*, 1991). Other potential uses include exposure to clove oil following blood sampling for research purposes. When fish are captured for non-lethal sampling, they can be sampled immediately for blood to obtain close to resting levels, then anaesthetised, allowed to recover and released.

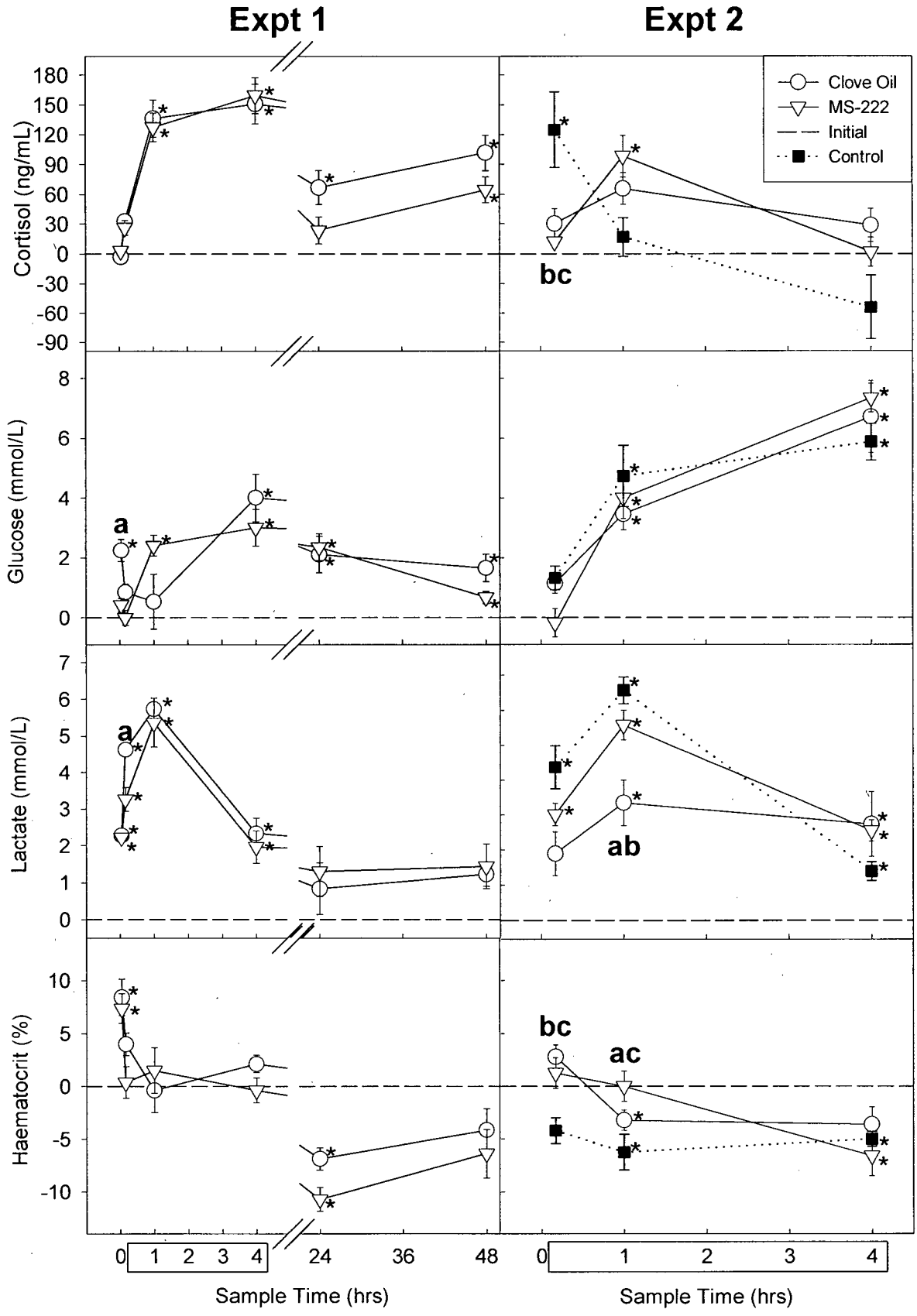
There are few differences between the effects of the anaesthetics clove oil and MS-222 on the physiological response of fish to stress. Both anaesthetics maintain haematocrit levels initially at recovery, possibly due to lowered heart rate and erythrocyte swelling. However, clove oil is more effective at reducing the short-term stress response induced by handling and blood sampling. Clove oil should be considered an effective alternative fish anaesthetic to MS-222 because it has similar physiological effects and can be used to reduce stress in situations requiring immediate release of fish. Another benefit is the fact clove oil is approximately ten times less expensive than MS-222, based upon cost per kg basis (Keene *et al.* 1998).

Table 2.1: Induction and recovery times of rainbow trout anaesthetised with clove oil or MS-222 at equal concentrations (60 mg L^{-1}). Induction 3 occurred at a total loss of equilibrium. Results are expressed as mean ± SE and include data from separate experiments 1 and 2.

Expt.	Anaesthetic	Sample Number	Time to Induction 3 (s)	P-value*	Time to Recovery (s)	P-value*
1	Clove Oil	8	126.3 ± 4.6	0.002	538.6 ± 27.1	< 0.001
	MS-222	8	148.8 ± 5.5		311.5 ± 27.4	
2	Clove Oil	6	68.6 ± 4.9	< 0.001	660.6 ± 36.4	< 0.001
	MS-222	6	103.1 ± 7.3		368.7 ± 38.2	

*P-values smaller than 0.05 indicate significant differences between anaesthetic treatments.

Figure 2.1: Changes in plasma cortisol, whole blood glucose, lactate and haematocrit levels of rainbow trout subjected to initial blood sampling via caudal puncture then anaesthetised with clove oil (60 mg L^{-1}) and MS-222 (60 mg L^{-1}) in two experiments. Post-initial sample times included Induction 3, Recovery, 1h, 4 h, 24 h, and 48 hr. Dashed lines represent initial control values set to zero. An asterisk (*) indicates significant difference ($P < 0.05$) from resting levels at each time point. Letters indicate a significant difference between clove oil and MS-222 treatments (a); control and clove oil treatments (b); and control and MS-222 treatments (c). Results are expressed as mean differences (final - initial sample) ± SE; N = 8 Experiment 1; N = 6 Experiment 2.


Expt 1
Expt 2
Clove Oil
MS-222
Initial
Control
Cortisol (ng/mL)
Glucose (mmol/L)
Lactate (mmol/L)
Haematocrit (%)
a
bc
a
ab
bc
ac
Sample Time (hrs)
Sample Time (hrs)

*Chapter 3: Cardiovascular changes in trout stressed by hypoxia and maximum prolonged swimming

*A version of this chapter has been submitted for publication. Wagner, G.N. & McKinley, R.S. (2003). Cardiovascular changes in trout stressed by hypoxia and maximum prolonged swimming. *Comparative Biochemistry and Physiology.*

INTRODUCTION

Fish in the natural environment are subjected to a combination of stressors that can affect their general physiological state. These stressors include changes in abiotic factors (ie. oxygen, salinity and temperature) as well as biotic pressures (ie. predation and food acquisition). Most of these individual stressors have been used under laboratory conditions to examine their impact on fish oxygen consumption (ie. energy use and allocation). However, insight into how interacting physiological systems partition energy can be gained by evaluating the effects of combined stressors.

A recognised integrated measure of fish physiological performance is critical swimming speed (U_{crit}) (Brett, 1964; Beamish, 1978; Nelson, 1989; Randall & Brauner, 1991; Plaut, 2001), a measure of the maximum prolonged, or aerobic, swimming capacity of fish. Physiological processes such as cardiac output ($\dot{Q}$), gas transfer, osmotic balance and muscle contractility affect U_{crit}. Cardiac output itself combines several of these processes and is the product of heart rate (f_H) and stroke volume (V_S).

Hypoxia and aerobic exercise often have opposite effects on heart rate, so it is unknown what will occur to the cardiac output of intact fish exposed to both simultaneously. At rest, hypoxia causes acidosis and a subsequent decrease in blood oxygen carrying capacity, leading to an increase in fish respiration rate (Holeton & Randall, 1967; Farrell *et al.*, 1998). In resting salmonids this change causes normally high f_H to decrease and V_S to increase (Holeton & Randall, 1967; Randall, 1970; Wood & Shelton, 1980; Gamperl *et al.*, 1994a). Half air-saturation (68-78 mmHg) has been shown by Jones (1971) to significantly lower the U_{crit} of rainbow trout (*Oncorhynchus mykiss*). Even moderate hypoxia (100-106 mmHg) has been found to significantly lower repeated swimming performance of sockeye salmon (*Oncorhynchus nerka*) (Farrell *et al.*, 1998). Indirect measurements of $\dot{Q}$ during exercise have indicated a 3- to 5-fold increase occurs primarily due to increases in V_S (Stevens & Randall, 1967; Kiceniuk & Jones,

1977; Randall & Daxboeck, 1982). Farrell *et al.* (1994) found artificially exercised perfused trout hearts exposed to hypoxic conditions (70, 45, 25 mm Hg) had reduced maximum cardiac output. However, they noted the difficulty in exactly reproducing the maximum mechanical heart performance of intact trout. Thorarensen *et al.* (1996) directly measured $\dot{Q}$ during critical swim testing of rainbow trout and found $\dot{Q}$ increased until 97.3 % of U_{crit} was reached. This increase in $\dot{Q}$ was due to a 38 % increase in f_H and a 25 % increase in V_S, which differ greatly from earlier calculated results. Gamperl *et al.* (1995) found heart rates of hypoxic trout did not increase similar to increased cardiac output when exercised at 1.0 bl s^{-1}. However, these fish were not tested at U_{crit} that may produce different results due to increased heart performance.

The ideal time interval for critical swimming studies using water current speed increments has been debated in the literature. Time intervals have varied from as low as 2 minutes (Anderson *et al.*, 1997) up to 60 minutes originally set by Brett (1964) for sockeye salmon and validated for largemouth bass by Farlinger & Beamish (1977). However, many studies have used time intervals in the 10-20 minute range (Davis *et al.*, 1963; Dahlberg *et al.*, 1968; Jones, 1971; Farrell & Steffensen, 1987; Farrell *et al.*, 1998). Davis *et al.* (1963) found doubling the time interval from 10 to 20 minutes did not significantly lower critical swim speeds. Likewise, trout tested by Jones (1971) at 20 and 40 minute time intervals at a speed increment of 9 cm s^{-1} showed no significant differences in critical swim speed. The purpose of the present study was to 1) assess the impact of different swim testing methods on rainbow trout maximum prolonged swimming capacity, 2) determine whether a trade off between cardiac variables occurs at U_{crit} under hypoxic conditions, and 3) assess the underlying physiological mechanisms.

MATERIALS AND METHODS

A total of 12 fish were used in a preliminary experiment to determine if 4 hours of fish recovery before critical swim testing and after surgical attachment of a Doppler cuff, was enough

time to produce repeatable results. Surgical methods were identical to the main experiment and ten minute time intervals were used. The following information was derived from this test:

a) No detectable differences in the maximum swimming speed were found with recoveries of 4 or 24 hours.

b) Surgical attachment of Doppler cuffs did not affect U_{crit} after 4 hours recovery.

EXPERIMENTAL ANIMALS

Thirty-two prespawning rainbow trout (37.3 ± 0.4 cm; 660.2 ± 22.6 g) were obtained from a 50:50 sex ratio population at the Rainbow Springs Trout Hatchery, Thamesford, Ontario. Fish were held in a round 2500 L tank supplied with aerated well water (8.9 ± 0.2 SE °C) at the Waterloo Biotelemetry Institute, University of Waterloo. Fish were fed five-point floating trout food (Martin Mills Incorporated, Elmira, ON) and allowed to acclimatise to laboratory conditions for at least two weeks before surgery. Food was withheld for 24 hours prior to each surgery to induce a post-absorptive state.

EXPERIMENTAL DESIGN

Four treatments contained 8 fish each: 1) 10 minute time intervals at P_{O2} = 146.1 ± 2.6 mmHg; 2) 10 minute intervals at 73.9 ± 1.7 mmHg; 3) 20 minute intervals at P_{O2} = 147.2 ± 3.6 mmHg; 4) 20 minute intervals at 74.2 ± 0.6 mmHg. Critical swimming speed and cardiac output of two fish from a single treatment were tested each day, on a rotating treatment basis over 12 days.

Individual fish were netted from one of the holding tanks, anaesthetised in a 60 ppm clove oil bath (active ingredient eugenol, 88 % min.; Hilltech Canada Inc., Vankleek Hill, ON, Canada) and placed on a foam surgery table. The gills were irrigated with seawater containing a maintenance anaesthetic dose of 30 ppm clove oil. A small incision (~5 mm) was made on the right side of the fish, posterior to the last gill arch directly above the ventral aorta to isolate and

fit it with a Doppler cuff (545C–4 Directional Pulsed Doppler Flowmeter, Bioengineering, University of Iowa, USA). The trailing wire of the cuff was anchored to the skin using 3-0 silk suture (Johnson and Johnson Medical Products, Peterborough, ON, Canada). After surgery, each fish was placed individually in a 130 L Blazka-type swim chamber with a modified net plug to prevent the fish from drafting in the turbulent area near the cap. The fish was allowed to acclimatise for four hours at a water velocity of 0.2 m s^{-1} (approximately 0.5 bl s^{-1}). Swimming at this low speed is referred to as 'resting' in the present study. For the hypoxic treatment groups, oxygen partial pressure was reduced in the header tank of the swimming chamber (110 L) during the latter 2 hours of the recovery period. Oxygen was reduced by 1/6 oxygen partial pressure every 15 minutes (final level ≈ 75 mmHg) by bubbling nitrogen through the water column. Fish swam at the final low oxygen level for the last 30 minutes of the recovery period as well as the entire U_{crit} test.

Based on a narrow range of speed increments (0.05-0.15 m s^{-1}) used in past studies (Jones, 1971; Kiceniuk & Jones, 1977; Keen & Farrell, 1994; Gallaugher *et al.*, 1995; Thorarensen *et al.*, 1996; Anderson *et al.*, 1997; Korsmeyer *et al.*, 1997; Farrell *et al.*, 1998) the intermediate increment of 0.1 m s^{-1} (approximately 0.25 bl s^{-1}) was used for U_{crit} testing beginning at 0.4 m s^{-1}. Fish were deemed fatigued when they could no longer continue swimming against the current and rested for more than 30 s on the downstream grate. Critical swimming speed (U_{crit}) was calculated using the formula of Brett (1964):

$$U_{crit} = V + (t\Delta t^{-1})\,\Delta v\,, \quad \text{units} = \text{m s}^{-1}$$

where V is the highest velocity maintained for the prescribed incremental period (m s^{-1}), t is the elapsed time at the final velocity, Δt is the time increment in minutes, and Δv is the velocity increment (0.1 m/s). Cardiac output ($\dot{Q}$), heart rate (f_H) and stroke volume (V_S) were recorded throughout the trial. Fish were allowed to acclimate to each new speed increment (10

or 20 min.) to approach a physiological steady state and the final two minutes of data were analysed.

BLOOD SAMPLING

After swim testing fish were removed from the swim tunnel and euthanised in a water bath containing 200 ppm clove oil. A blood sample was taken by caudal puncture within 30 seconds of submersion. Cortisol (ICN Biomedicals Inc. radioimmunoassay), glucose (Medisense Glucose Sensor: Precision Q.W.I.D.), haematocrit (6500-200 Ames Microspin Haematocrit Centrifuge), chloride and lactate (Sigma Diagnostics 461-3; 735-10) levels were measured. Calibration of the Doppler cuffs included removing the head of each fish and infusing (Harvard Apparatus PHD 2000) beef blood through the ventral aorta. Acute hypoxia does not affect resting blood variables, such as lactate and haematocrit (Farrell *et al.*, 1998), so they were assumed to be similar for all treatment groups prior to swim testing.

STATISTICS

Swimming performance results of the preliminary experiment were compared using repeated measures ANOVA. Main experiment blood and U_{crit} data were analysed using one-way ANOVA and Tukey's multiple comparison method. In all cases the length and weight of each fish were tested for covariation and included if significant. The cardiac variables ($\dot{Q}$, f_H, and V_S) were analysed using two-way ANOVA with repeated measures. When interaction occurred, all simple effects were individually tested for significance.

RESULTS

Swimming performance of fish in the 10 minute hypoxic treatment (1.9 ± 0.1 bl s^{-1}) was significantly lower (24 % reduction) than normoxic fish at 10 minute intervals (2.5 ± 0.1 bl s^{-1}) (Fig. 3.1). The same was true in the 20 minute treatment where hypoxic fish had significantly lower (21 % reduction) U_{crit} values (1.9 ± 0.1 bl s^{-1}) than normoxic fish (2.4 ± 0.01 bl s^{-1}).

The only blood variable to change significantly during the hypoxia study was lactate that followed a similar pattern to U_{crit} (Fig. 3.2). Hypoxic fish at both 10 minute (3.7 ± 0.6 mmol L^{-1}) and 20 minute (3.2 ± 0.3 mmol L^{-1}) time intervals had significantly lower ($P < 0.05$) lactate levels than fish in either normoxic treatments (6.7 ± 0.4 mmol L^{-1} and 6.1 ± 0.8 mmol L^{-1} respectively). Averages for the blood variables that did not differ significantly between treatments after U_{crit}, were as follows: calcium (1.4 ± 0.0 mmol L^{-1}), chloride (110.3 ± 1.9 mmol L^{-1}), cortisol (163.4 ± 34.5 ng L^{-1}), glucose (8.0 ± 0.7 mmol L^{-1}), haematocrit (37.3 ± 1.8 %), potassium (3.2 ± 0.2 mmol L^{-1}), and sodium (146.0 ± 1.7 mmol L^{-1}).

All cardiac variables are reported in Table 3.1. Resting values for all three variables did not vary between treatments. Both $\dot{Q}$ and f_H increased significantly from resting levels at U_{crit} in normoxic fish at 10 minutes (64 % and 25 % increase respectively) and 20 minutes (75 % and 26 % increase respectively), but not in hypoxic fish. At U_{crit}, hypoxic fish in the 10 minute treatment had significantly lower f_H (24 % slower) than normoxic fish with the same time interval. Stroke volume did not vary significantly from rest to U_{crit}, or between treatments. The relative contributions of f_H and V_S to the increase in cardiac output at U_{crit} (Fig. 3.3) was equal (~50 %) in both normoxic treatments where $\dot{Q}$ increased significantly. This similar proportion occurred despite the fact the increase in V_S was not statistically significant. The contributions of f_H and V_S were highly variable in the two hypoxic treatments where $\dot{Q}$ did not change significantly from rest.

DISCUSSION

METHODOLOGICAL TESTING

In previously published studies, the amount of time fish have been allowed to recover from anaesthesia and surgery prior to swim testing has been highly variable. Recovery time used in other studies varies from a few hours (Keen & Farrell, 1994; Anderson *et al.*, 1997;

Korsmeyer *et al.*, 1997), 12 hours (Thorarensen *et al.*, 1996; Farrell *et al.*, 1998), up to 24+ hours (Kiceniuk & Jones, 1977; Gallaugher *et al.*, 1995) after exposure or surgery. Jones (1971), however, reported no significant differences in trout critical swimming speeds after recovering in an exercise tunnel for 2 or 20 hours with a water current of 2-4 cm s^{-1}. Similarly, in our preliminary experiment I found minor invasive Doppler surgery did not hinder trout swimming ability after a four hour recovery period.

No differences occurred between 10 and 20 minute time intervals when comparing blood variables or critical swim speeds. These results confirm previously reported findings that U_{crit} values are not affected within the 10-40 minute interval range (Davis *et al.*, 1963; Jones, 1971). Fish in the 10 minute interval treatments were in the same zone of steady performance (ie. low variability due to acclimation) as fish in the 20 minute intervals, similar to the findings of Kiceniuk (1976). Some differences in cardiac output did occur, however (Table 3.1). In the 10 minute interval treatments the maximum f_H of normoxic fish was 24 % faster than hypoxic fish. Therefore, the lower f_H expected for fish exposed to hypoxia that was not seen at rest did occur during swim testing. A trend towards higher heart rates also was seen for fish in the 20 min. normoxic treatment (18 % higher than hypoxic fish), but was not significant (P = 0.125). Another trend was seen for the V_S of hypoxic fish at U_{crit} that were 8 % larger than normoxic fish in the 10 minute intervals and 34 % larger in the 10 minute intervals. Although these differences in V_S were not significant, they still compensated for the significantly lower f_H of hypoxic fish resulting in all four treatments having similar $\dot{Q}$ at U_{crit}. The trend for exercising hypoxic fish in both 10 and 20 minute time intervals was a lower f_H and higher V_S compared to normoxic fish, although the level to which these variables changed differed somewhat in magnitude. These results indicate the use of either time interval is valid for testing trout aerobic swimming performance. It is interesting to note that Kiceniuk (1976) found cardiac variables of swimming trout did not reach a steady state until after approximately 30 minutes. A lack of steady state

might explain the different magnitudes of change in the cardiac variables. While valid for measuring major cardiovascular changes, as evidenced in this and other studies (Kolok & Farrell, 1994; Gamperl *et al.*, 1994a; Korsmeyer *et al.*, 1997), intervals above 30 minutes may improve the measurement of more subtle changes.

HYPOXIA & EXERCISE

The 21-24 % reduction in blood lactate levels of hypoxic fish compared to normoxic fish (Fig. 3.2) mirrored their similarly reduced swimming performance (Fig. 3.1). The fact lactate did not accumulate in the systems of hypoxic fish at U_{crit} supports findings described in Richards *et al.* (2002a) suggesting that fatigue is not lactate-dependent. Blood lactate levels increase during anaerobic muscle activity (when pyruvate is broken down to produce ATP with lactate as an end-product) and by metabolic inertia (pyruvate production exceeding oxidation because of imbalance between the transformation and catalytic rates of glycogen phosphorylase and pyruvate dehydrogenase) during aerobic activity. During prolonged swimming trials, anaerobic energy is required only at the beginning of each new time interval and after 80 % of the fish's critical speed has been reached (Webb, 1971; Richards *et al.*, 2002a). Therefore, lower lactate production as a metabolic end-product may be due to hypoxic fish swimming fewer intervals even though their $\dot{Q}_{max}$ was not significantly different from normoxic fish.

While hypoxia can cause an increase in trout haematocrit, the normal increase associated with aerobic swimming (see review by Gallaugher & Farrell, 1998) was not exacerbated. The lack of differences in glucose and cortisol results shows all fish were stressed during exercise regardless of the oxygen level. Also, the fact ionic balance in the blood did not differ between hypoxic and normoxic fish suggests the ability of the latter group to osmoregulate was similar during exercise.

The average increases in $\dot{Q}$ (70 %), f_H (25 %), and V_S (26 %, but not significant) for normoxic fish are similar to those found for exercised rainbow trout by Thorarensen *et al.*

(1996). Heart rates of normoxic fish were significantly higher than hypoxic fish only at U_{crit} (Table 1), as the typical inhibiting effect of hypoxia on f_H was not seen in resting fish. However, hypoxia likely counteracted the normal increase of f_H with exercise as found by Gamperl *et al.* (1995) because f_H did not increase significantly as in normoxic fish (Table 3.1). The subsequent contributions of f_H and V_s to maximum $\dot{Q}$ in the hypoxic treatments were highly variable (Fig. 3.3). This increased variability lead to a lack of increase in $\dot{Q}$ (Table 3.1) and is a good indicator that hypoxia affected the cardiovascular system. Also, $\dot{Q}$ did not differ between treatments even though hypoxic fish swam at a significantly reduced U_{crit}. The fact that this measure of energy use was similar with decreased swimming also indicates a direct effect of hypoxia on the physiology of fish rather than their behaviour. The mechanism for a direct physiological effect is not yet fully understood. The observed trend of raised V_s combined with decreased f_H may allow increased O_2 uptake at the gills and supply to the heart by lengthening blood residence time within the heart and gills (Farrell, 1984; Farrell *et al.*, 1989) and helped facilitate coronary oxygen delivery (Gamperl *et al.*, 1995). When fish near U_{crit} this process may override the normal increase in f_H that Gamperl *et al.* (1994a) reported for hypoxic trout swimming at slower speeds. Measurements of coronary blood flow and oxygen extraction, not performed in the present study, may elucidate the lack of an increase in $\dot{Q}$ at U_{crit} compared to resting levels in hypoxic fish. If both these variables increased in hypoxic fish, a significant increase in $\dot{Q}$ may not be required.

Similar to the present study, Jones (1971) reported no significant differences in trout U_{crit} between 2 and 20 hours of recovery and several other studies have used 1-2 hour recovery times (Keen & Farrell, 1994; Anderson *et al.*, 1997; Korsmeyer *et al.*, 1997). However, there is some evidence that shorter recovery times may increase cardiac variability. Kiceniuk (1976) found cannulated trout had higher average heart rates than uncannulated fish after 18-24 hours recovery in a swim tunnel. Gamperl *et al.* (1994b) also found cortisol levels of cannulated trout confined

within isolation boxes remained above pre-confinement levels up to 6-8 days. A similar recovery period of 8-10 days was required for heart rates of cod to stabilise after cardiac output surgery (Webber *et al.*, 1998). These findings may help explain the fact hypoxic fish did not exhibit typically low resting heart rates, but does not necessarily indicate fish were not fully recovered within the swimming tunnel after 4 hours. While an original resting steady state may not have occurred, maximum cardiac and swimming performance levels achieved by the fish indicated they were fully recovered from surgery.

Methodological tests were very informative for the design of the present study and future experiments involving U_{crit}. Four hours of recovery following minor invasive surgery did not hinder the cardiac or swimming performance of fish. The use of 10 and 20 minute time intervals are valid for swim testing and blood variable measurements. Direct measurement of trout $\dot{Q}$ at U_{crit} during exposure to low levels of oxygen has provided insight into the effects of combined stessors on fish physiology. During exercise, $\dot{Q}$ and f_H of normoxic fish increased while those of hypoxic fish did not. A trend of a compensatory increase in V_S of hypoxic fish during exercise also was evident. Hypoxia reduced trout U_{crit} but lactate accumulation is not one of the physiological mechanisms. More studies that examine the effects of stressors on exercising fish are needed in order to determine the mechanisms involved in energy partitioning between the interacting physiological systems.

Table 3.1: Comparison of the mean cardiac output ($\dot{Q}$), heart rate (f_H) and stroke volume (V_S) at rest and U_{crit} for rainbow trout exposed to high (~150 mmHg) and low (~75 mmHg) oxygen using 10 and 20 minute time intervals. An asterisk (*) indicates a significant increase ($P < 0.05$) for the variable from rest to U_{crit}. Dissimilar letters within a row indicate statistical difference between treatments at rest or U_{crit}.

Cardiac Variable	Rest				U_{crit}			
	High O_2 10 min.	Low O_2 10 min.	High O_2 20 min.	Low O_2 20 min.	High O_2 10 min.	Low O_2 10 min.	High O_2 20 min.	Low O_2 20 min.
$\dot{Q}$ (mL kg^{-1} min^{-1})	29.5 ± 3.5a	29.9 ± 3.4a	23.7 ± 3.7a	36.9 ± 7.7a	48.4 ± 7.6a*	38.8 ± 5.3a	41.6 ± 7.1a*	45.7 ± 9.3a
f_H (beats^{-1})	62.2 ± 5.1a	59.7 ± 5.2a	61.2 ± 4.9a	56.6 ± 5.6a	77.6 ± 3.4a*	62.5 ± 5.4b	77.3 ± 2.1a*	65.1 ± 2.2ab
V_S (mL kg^{-1})	0.51 ± 0.06a	0.52 ± 0.07a	0.41 ± 0.08a	0.71 ± 0.15a	0.63 ± 0.11a	0.67 ± 0.11a	0.53 ± 0.09a	0.71 ± 0.12a

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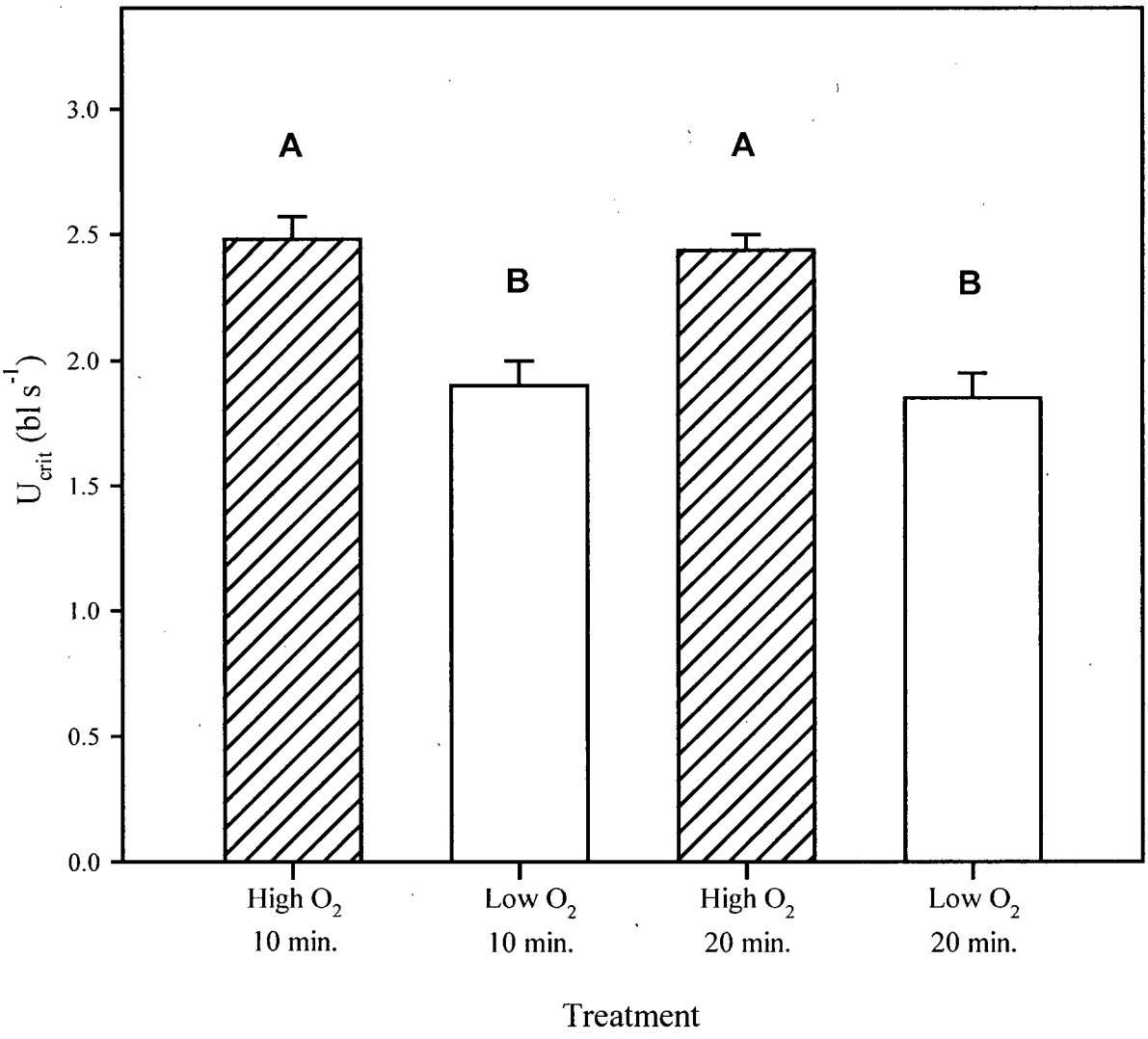

Figure 3.1: Critical swimming speeds (U_{crit}) of rainbow trout exposed to high (~150 mmHg) and low (~75 mmHg) environmental oxygen, using 10 and 20 minute time intervals (mean ± SE, n = 8). Dissimilar letters indicate statistical difference between treatments at $P < 0.05$ significance.

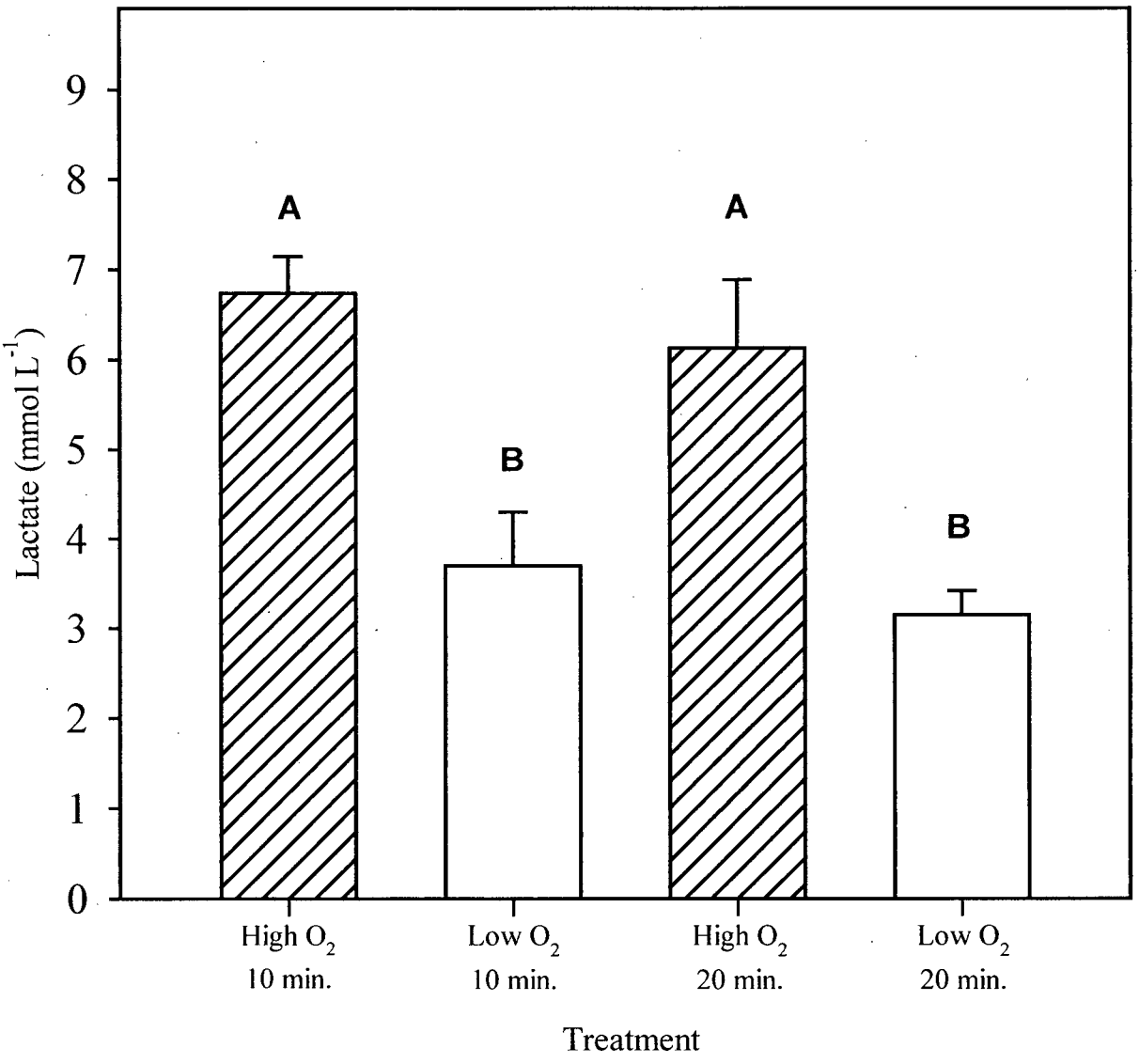

Figure 3.2: Blood lactate of rainbow trout after critical swim testing at high (~150 mmHg) and low (~75 mmHg) oxygen, and using 10 and 20 minute time intervals (mean ± SE, n = 8). All fish were terminally sampled using an overdose (200 ppm) of clove oil. Dissimilar letters indicate statistical differences between treatments at $P < 0.05$ significance.

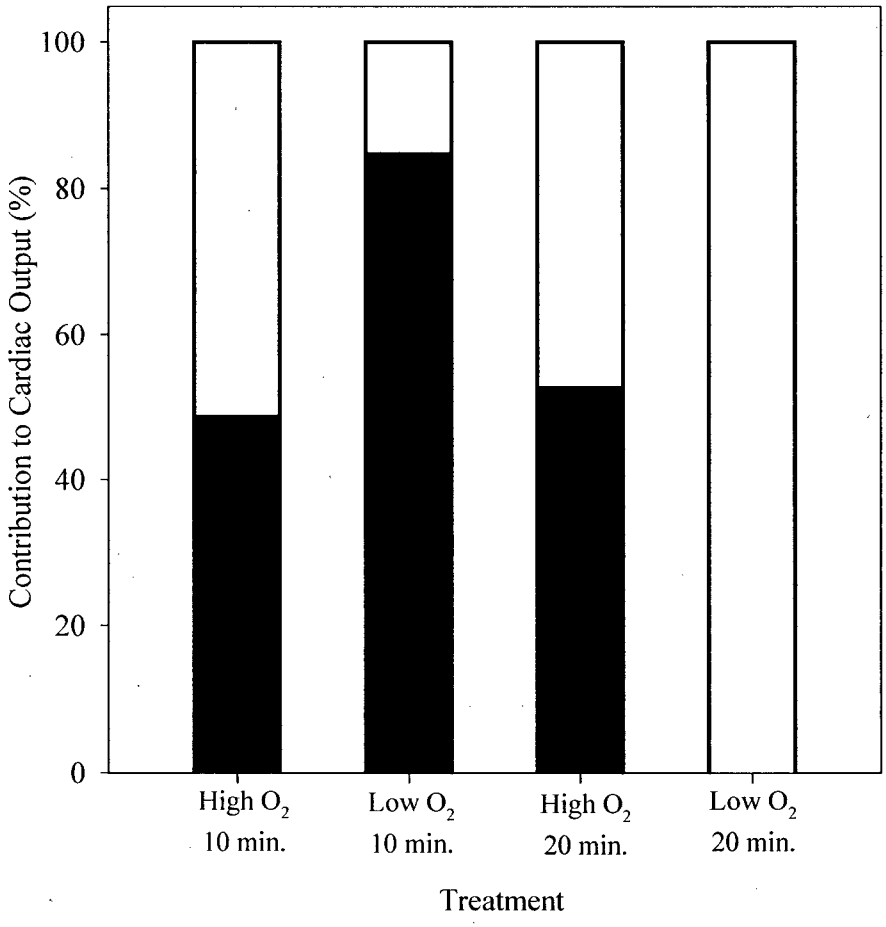

Figure 3.3: Average contribution of heart rate (white bars) and stroke volume (black bars) to the change in cardiac output from resting values for critically swim tested rainbow trout.

*Chapter 4: Physiological Impact of Sea Lice on Swimming Performance of Atlantic Salmon

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INTRODUCTION

The swimming ability of Atlantic salmon (*Salmo salar* L.) is very important to their survival and fitness. Salmon cease feeding upon their return to native streams and, therefore, have a finite energy reserve available to complete their ascent and spawn. Loss of this energy due to anthropomorphic or natural stressors causes re-allocation of energy reserves and a decrease in overall fitness (Brett & Groves, 1979). This deleterious combination may impair fish from reaching spawning grounds or decrease spawning success.

One natural stressor is the salmon louse (*Lepeophtheirus salmonis*), a parasitic copepod known to infect salmonids across the entire Northern Hemisphere. Lice infection levels in wild salmon populations are a concern because they may be increasing along regular migratory routes where intensive aquaculture is present (Bjørn *et al.*, 2001; Bjørn & Finstad, 2002). Sea lice can affect the osmotic balance of fish possibly leading to death (Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997), but their effects on host cardiac and swimming performance have not been reported. These physiological measures are important because exercise is an integrated indicator of deleterious changes in gas exchange and muscle performance (Nelson, 1989; Randall & Brauner, 1991) while cardiac output can indicate component changes within these systems. Blood variables also are useful measures of physiological disturbance. Cortisol and glucose are primary indicators of chronic stress; chloride and haematocrit for osmoregulatory ability; and lactate for muscle activity during exercise.

Problems with osmoregulatory maintenance due to damage by lice may force energy to be reallocated to this system, as well as cause later problems with performance. Both changes would affect migration and spawning. The purpose of the present study was to determine the level of salmon lice required to compromise the osmoregulatory capacity, cardiac output and swimming ability of Atlantic salmon.

MATERIALS AND METHODS

Sixty pre-spawning Atlantic salmon (38.2 ± 4.3 SE cm, 609.3 ± 201.8 g; stock #L04/98 Imsa River) of a 50:50 sex distribution were reared and held at the Norwegian Institute of Nature Management (NINA) Research Station, Ims, Norway (55 °N; 15 °E). Four 2300 L round tanks containing seawater (33.5 ± 1.2 ppt; 9.5 ± 0.2 °C) held 15 fish each under flow-through conditions. Fish were fed micro 30 sinking fish food (48% protein, 24% fat, 8% ash; EWOS AS, Bergen, Norway) once daily to satiation. After two weeks acclimatisation, fish were infected with sea lice copepodids. Infection was performed according to Bjørn & Finstad (1998) with 5600, 2500 and 1000 copepodids, respectively, to attain levels of approximately 150, 65 and 25 lice per fish based on 65 % infection success and 65 % lice survival. The latter two treatments were combined because numbers of lice did not differ significantly. Control fish were treated the same as infected fish without exposure to lice. Feeding was stopped at least 24 hours prior to swim testing to induce a post-absorptive state. Water temperature during the swim testing period was 10.4 ± 0.2 °C.

SWIM TRIALS

Eight fish from each treatment were tested in swimming performance trials once adult male lice stages appeared (identified according to Johnson & Albright, 1991), approximately 4.5 weeks after infection. Individual fish were netted from one of the holding tanks, anaesthetised in a 60 ppm clove oil bath (active ingredient eugenol, 88 % min.; Hilltech Canada Inc., Vankleek Hill, ON, Canada) and placed on a foam surgery table. The gills were irrigated with seawater containing a maintenance anaesthetic dose of 30 ppm clove oil. A small incision (~5 mm) was made on the right side of the fish, posterior to the last gill arch directly above the ventral aorta to isolate and fit it with a Doppler cuff (545C–4 Directional Pulsed Doppler Flowmeter, Bioengineering, University of Iowa, USA). The trailing wire of the cuff was anchored to the skin using 3-0 silk suture (Johnson and Johnson Medical Products, Peterborough, ON, Canada).

After surgery, all sea lice present on the fish were counted and the stages identified. Each fish was placed individually in a 130 L Blazka-type swim chamber with a modified net plug to prevent the fish from drafting in the turbulent area near the cap. The fish was allowed to acclimatise for four hours at a water velocity of 0.2 m s^{-1}.

Water velocity was increased from the initial speed of 0.2 m s^{-1} to 0.4 m s^{-1} to begin the swim trial with incremental speed increases of 0.1 m s^{-1} every 10 minutes. Fish were deemed fatigued when they could no longer continue swimming against the current and rested for more than 30 s on the downstream grate. Critical swimming speed (U_{crit}) was calculated using the formula of Brett (1964):

$$U_{crit} = V + (t\Delta t^{-1})\,\Delta v, \quad \text{units} = \text{m s}^{-1}$$

where V is the highest velocity maintained for the prescribed incremental period (m s^{-1}), t is the elapsed time at the final velocity, Δt is the time increment in minutes, and Δv is the velocity increment (0.1 m/s). Cardiac output ($\dot{Q}$), heart rate (f_H) and stroke volume (V_S) were recorded throughout the trial. Fish were allowed to acclimate to each new speed increment to approach a physiological steady state and the final two minutes of data were analysed. Two fish from one treatment were tested each day on a rotational basis between the four treatments over a period of 16 days.

BLOOD SAMPLING

After swim testing fish were removed from the swim tunnel and euthanised in a water bath containing 200 ppm clove oil. A blood sample was taken by caudal puncture within 30 seconds of submersion. Cortisol (ICN Biomedicals Inc. radioimmunoassay), glucose (Medisense Glucose Sensor: Precision Q.W.I.D.), haematocrit (6500-200 Ames Microspin Haematocrit Centrifuge), chloride and lactate (Sigma Diagnostics 461-3; 735-10) levels were measured. Calibration of the Doppler cuffs included removing the head of each fish and infusing (Harvard

Apparatus PHD 2000) beef blood through the ventral aorta. Resting blood samples were taken from the remaining fish, in each treatment tank, at the end of the experiment.

STATISTICS

Data from blood analysis and U_{crit} were analysed by one-way ANOVA and Tukey's multiple comparison method. In all cases length and mass of each fish was tested for covariation and included if significant. Time post-infection also was tested as a covariate to determine if female lice development into adult stages over the 16 days of swim testing accounted for any variation in the results. The cardiac variables ($\dot{Q}$, f_H, and V_S) were analysed using two-way ANOVA. When interaction occurred, all simple effects individually were tested for significance.

RESULTS

Adult male lice stages first appeared at 4.5 weeks and swim testing started 3 days later. Final infection levels were (0.13 ± 0.02 lice g^{-1}; ave: 81.3 ± 6.7 lice $fish^{-1}$; range: 43-149 lice $fish^{-1}$) and (0.02 ± 0.00 lice g^{-1}; ave: 11.1 ± 2.1 lice $fish^{-1}$; range: 5-21 lice $fish^{-1}$) when swim trials began. During the first three days of swim testing adult male (95.5 %) and second pre-adult female (84.7 %) predominated respective lice stages; the remainder were composed of second and first pre-adults respectively. During the final three days adult males (100%) remained high and adult females (93.0 %) predominated respective lice stages. Lice maturation during the swim testing period did not account for significant variation in any of the performance or blood results.

BLOOD VARIABLES

Resting and post-exercise levels of the blood variables are presented in Fig. 4.1. Chloride levels of fish with higher infection (184.4 ± 11.3 mmol L^{-1}) were significantly higher than lower infection (142.0 ± 3.7 mmol L^{-1}) or control fish (159.5 ± 3.5 mmol L^{-1}) after swim testing. Post-

exercise levels of chloride also increased significantly from resting values (112.1 ± 5.2 mmol L^{-1}) in the higher lice treatment. Resting levels of blood glucose were significantly higher in the higher lice (4.5 ± 0.8 mmol L^{-1}) and lower lice (3.5 ± 0.8 mmol L^{-1}) treatments compared with control fish (2.7 ± 0.7 mmol L^{-1}). After exercise glucose increased significantly from resting levels only in fish with lower infection (5.0 ± 1.0 mmol L^{-1}) and controls (5.1 ± 0.9 mmol L^{-1}). Cortisol levels of fish with lower infection increased after exercise (242.2 ± 12.2 ng mL^{-1} from 67.0 ± 7.7 ng mL^{-1}) while haematocrit levels decreased (34.2 ± 1.9 % from 41.4 ± 2.8 %). The mean cortisol level of resting control fish (4.6 ± 0.7 ng mL^{-1}) was 11-14 times lower than infected fish (higher infection: 51.1 ± 20.2 ng mL^{-1}; lower infection: 67.0 ± 16.0 ng mL^{-1}) and an order of magnitude lower than exercised controls (187.4 ± 80.2 ng mL^{-1}).

PERFORMANCE

The U_{crit} of fish with higher infection (2.1 ± 0.1 bl s^{-1}) was significantly lower than fish with lower infection levels (2.5 ± 0.1 bl s^{-1}) and controls (2.6 ± 0.1 bl s^{-1}) (Table 4.1). Resting values for all three cardiac variables did not vary between treatments (Fig. 4.2). Both $\dot{Q}$ and f_H increased significantly at U_{crit} from resting levels in fish with lower infection and controls, but not in the higher infection treatment. Also, the higher-infected fish had significantly lower f_H (9 % slower) than controls. Stroke volume did not vary significantly from rest to U_{crit}, or between treatments at either state.

DISCUSSION

Pre-adult sea lice infection levels of 0.75-1.0 lice g^{-1} increase cortisol levels and cause osmoregulatory breakdown in Atlantic salmon and sea trout smolts (Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997). This physiological stress also can cause immunosuppression leading to an increased susceptibility to secondary infection (Ellis, 1981; Pickering & Pottinger, 1989). These physiological impairments can lead directly to increased mortality (Wootten *et al.*, 1982;

Bjørn & Finstad, 1998), but sub-lethal infections also may affect the health and survival of wild salmon. The level of lice infection that causes osmoregulatory breakdown is within the same range of 0.5-2.1 lice g^{-1} (50-200 lice per fish average) found on wild sea trout and Arctic charr in an area of intensive aquaculture in Norway (Bjørn *et al.*, 2001). These infections on wild fish predominately were copepodids and chalimi larvae, but indicate that levels of the more pathogenic mobile stages would be high as well. Lice numbers used in the lower treatment of the present study are similar to those found by Bjørn *et al.* (2001) on wild fish in areas distant from salmon farms. Fish in these pristine areas averaged less than ten lice of all developmental stages. At a relative infection intensity of 0.13 lice g^{-1} (43-149 lice per fish average), the level of infection in our higher treatment was much lower than previously shown to affect salmonids, but significant physiological changes still occurred. The fact wild fish near areas of aquaculture may have infection levels much higher than this indicates possible future problems with these populations with respect to their health and fitness.

The present study is the first to demonstrate that sea lice can reduce significantly the swimming performance of Atlantic salmon. Fish with an infection level of 0.13 lice g^{-1} suffered a 19 % reduction in performance over control fish (Table 4.1). The fact salmon with 0.02 lice g^{-1} swam as well as control fish indicates a low abundance of lice does not affect the overall health of fish. Therefore, wild salmon that average fewer than 0.05 lice g^{-1} in pristine areas (Bjørn *et al.*, 2001; Bjørn *et al.*, 2002) should be as healthy as fish without lice.

One component change in the physiology of fish with higher lice infection was a 47 % increase in blood chloride levels during exercise (Fig. 4.1). Resting chloride levels did not show a typical chronic stress response (Schreck, 1990; Thomas, 1990; Wendelaar Bongo, 1997), but increased significantly higher than lower-lice fish and controls during exercise, 184.4 ± 11.3 mmol L^{-1}. This level of chloride is well above the normal resting (120-140 mmol l^{-1}) and exercise ranges (140-160 mmol L^{-1}) for Atlantic salmon in seawater. Grimes & Jakobsen (1996)

found chloride levels of resting Atlantic salmon increased above 180 mmol L^{-1} after 25 days of infection with sea lice. Fish in their study were much smaller (40 g) than fish in the present study and had much higher infection levels (39-132 lice $fish^{-1}$). The fact chloride increased only with exercise in the present study indicates resting fish with sub-lethal infection levels are able to compensate for osmoregulatory problems induced by feeding lice. During exercise fish with higher infection levels were unable to cope with the combination of stressors and ions entering from seawater. The resulting hyperchloremia likely caused acidosis that can affect oxygen carrying capacity and muscle performance. Similar to resting chloride levels, haematocrit levels of the higher infected fish did not differ between treatments and were similar to uninfected Atlantic salmon (Bjørn & Finstad, 1997), indicating resting fish were able to osmoregulate normally. Both shrinking of erythrocytes due to increased plasma ions and blood loss due to lice feeding on blood (Brandal *et al.*, 1976) can lead to a decrease in haematocrit (Iwama *et al.*, 1989; Grimes & Jakobsen, 1996). However, neither possibility seems to explain why the post-exercise haematocrit of fish fell significantly after exercise only in the lower infection treatment. High resting levels of glucose in infected fish likely reflect added stress from the presence of lice (Bjørn & Finstad, 1997). The secondary stress response of mobilising energy reserves from the liver also was evident after exercise for lower lice and control groups, but glucose levels of fish with higher infection already may have become asymptotic. The trend of higher cortisol levels in infected-fish also suggested they were chronically stressed compared to control fish, similar to infected sea trout (Bjørn & Finstad, 1997). This would confirm our glucose and chloride findings because elevated cortisol in seawater causes secondary increases in chloride and glucose due to increased gill permeability to ions and glucogenesis in the liver (Schreck, 1990; Thomas, 1990; Wendelaar Bonga, 1997). However, most increases in cortisol were not significant possibly because of the large variation within infected fish. Lactate levels also were highly variable, but no trends were evident indicating similar muscle activity between treatments.

Another physiological component change of higher-infected fish was a 9 % reduction in f_H at maximum performance (Fig. 4.2). Normally, $\dot{Q}$ increases directly with increased exercise due to increases in f_H and V_S (Stevens & Randall, 1967; Kiceniuk & Jones, 1977; Thorarensen *et al.*, 1996). Additional stressors may change $\dot{Q}$ during exercise, or affect f_H or V_S, usually in a compensatory fashion (Gallaugher *et al.*, 1995; Brodeur *et al.*, 1999). For fish with higher infection, a lower f_H at U_{crit} resulted in $\dot{Q}$ not increasing from resting levels. Cardiac output did not differ between groups, but fish with higher lice infection swam at a significantly reduced critical swim speed. This indicated another stressor, in this case lice infection, directly affected the physiology of fish rather than their behaviour. The mechanism for this direct physiological effect is not yet fully understood. Fish with higher lice may lose energy to the increasingly compromised osmoregulation, while also subjected to a lower oxygen carrying capacity of the blood due to acidosis caused by hyperchloremia. A negative oxygen feedback loop may then occur during exercise if the heart cannot supply itself with enough oxygen (see Farrell, 1983; Farrell *et al.*, 1989). The subsequent stress-induced hypoxia of the heart would cause the f_H to slow and V_S to increases similar to when subjected to hypoxia (Holeton & Randall, 1967; Randall, 1970; Wood & Shelton, 1980; Gamperl *et al.*, 1994a). In the present experiment, V_S of fish with higher infection did not increase significantly compared to the other treatments, but did contribute to the overall increase in $\dot{Q}$. Sub-lethal infection by sea lice can thus compromise the osmoregulatory ability and cardiac performance of Atlantic salmon during exercise. These detrimental physiological changes led to a subsequent reduction in swimming performance. This intensity of infection probably would not lead directly to mortality because resting fish can osmoregulate and function normally. However the chronic system stress and decline in performance may be detrimental to the survival of wild salmon. Infected fish may not be able to forage or escape predation because of reduced swimming ability. This level of lice infection was much lower than previously reported to be detrimental so these findings raise concern for the

health and fitness of wild salmon in areas of high lice abundance. These conclusions apply to salmonids such as sea trout and Arctic charr due to similar lice pathogenicty on these species (Bjørn & Finstad, 1998). Other salmonids such as coho and chinook salmon are more resistant to infection (Johnson & Albright, 1992) and may be less susceptible to heavy lice burdens.

Table 4.1: The effect of sea lice infection on the critical swimming speeds (U_{crit}) of Atlantic salmon. An asterisk indicates the treatment is significantly lower at $P < 0.05$.

Level of Infection (lice g^{-1})	N	Mass (g)	Fork Length (cm)	U_{crit} (bl s^{-1})
0	7	535.7 ± 48.1	37.0 ± 1.1	2.6 ± 0.1
0.02	12	656.5 ± 71.1	38.5 ± 1.6	2.5 ± 0.1
0.13	7	633.3 ± 87.1	39.0 ± 1.8	2.1 ± 0.1*

Figure 4.1: Blood plasma levels of chloride (A), glucose (B), lactate (C), haematocrit (D) and cortisol (E) taken at rest (triangles) and immediately after swim testing (circles) from Atlantic salmon infected with two levels of sea lice. An asterisk indicates significant differences at $P < 0.05$ between resting and post trial values. Dissimilar letters above symbols indicate significant difference ($P < 0.05$) between treatments.

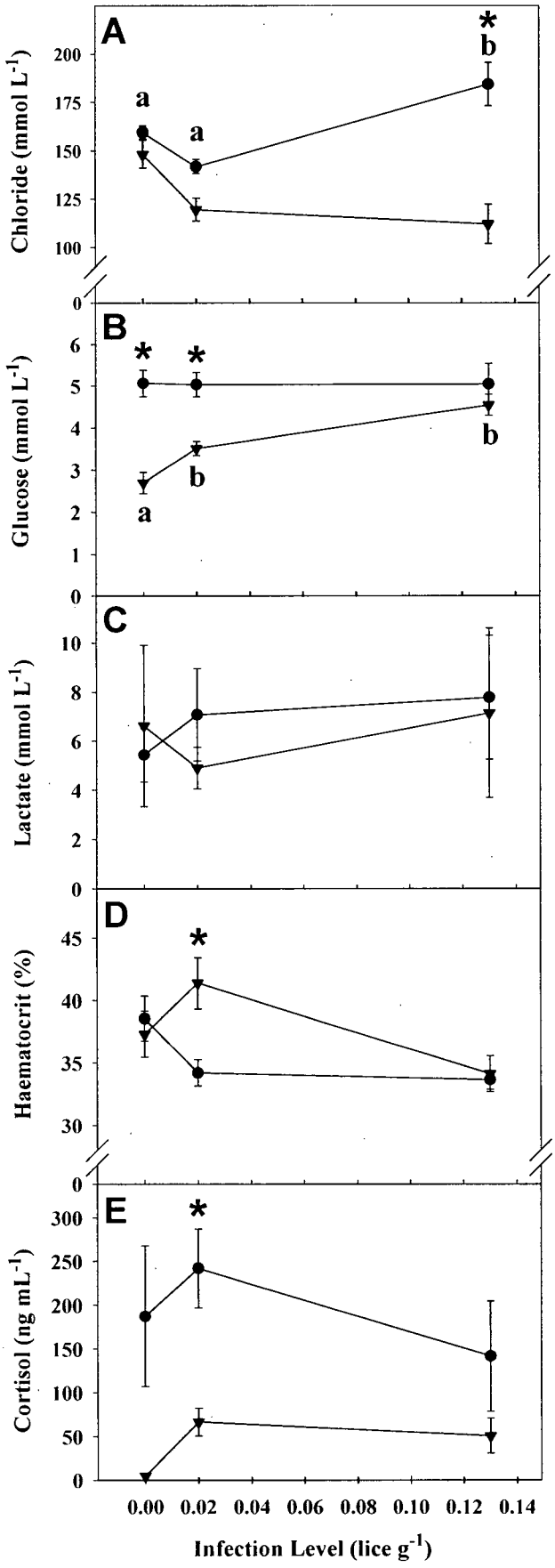
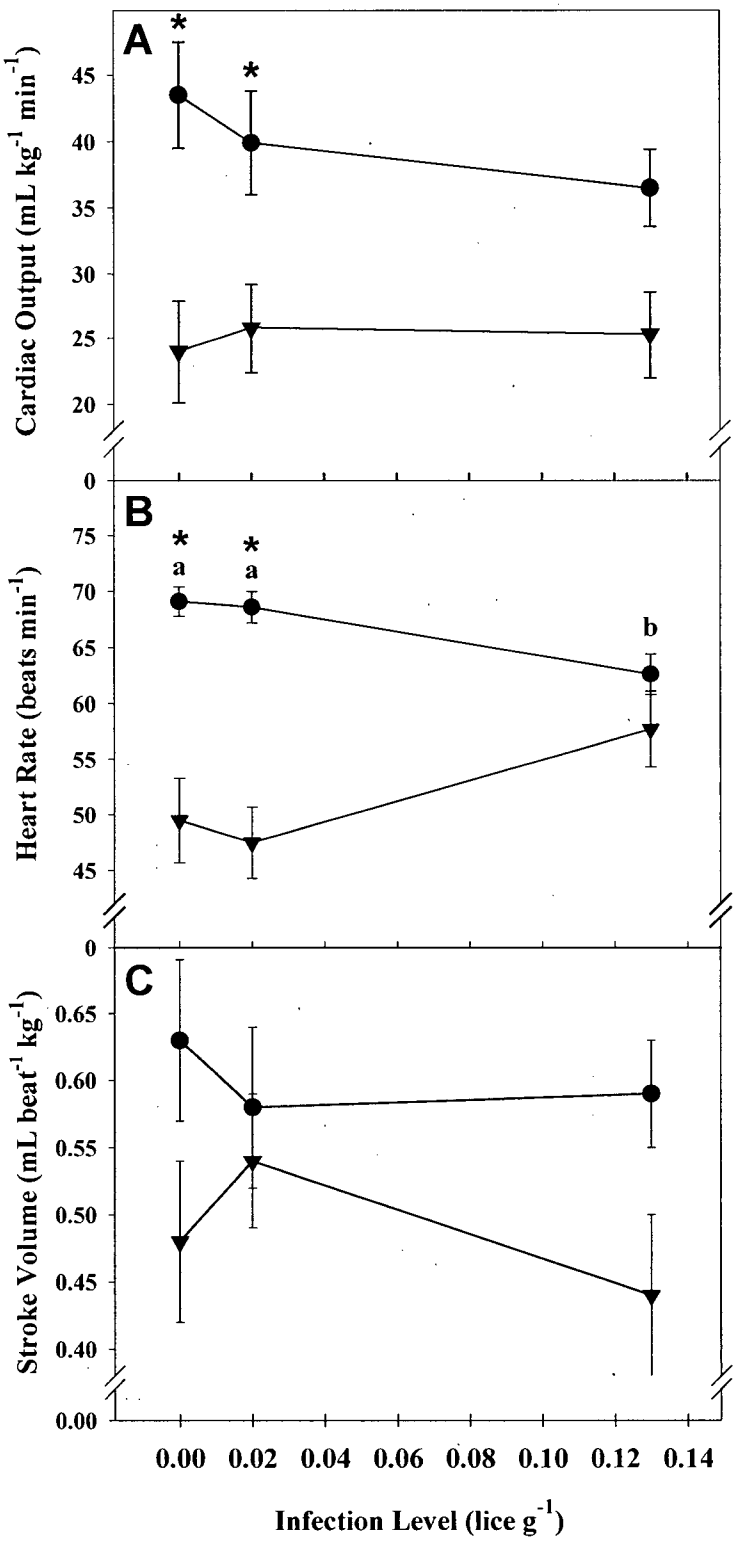

A
Chloride (mmol L-1)
200
175
150
125
100
0
a
a
*
b
B
Glucose (mmol L-1)
6
5
4
3
2
1
0
*
*
a
b
b
C
Lactate (mmol L-1)
10
8
6
4
2
0
D
Haematocrit (%)
45
40
35
30
0
*
E
Cortisol (ng mL-1)
300
250
200
150
100
50
0
*
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Infection Level (lice g-1)

Figure 4.2: Comparison of cardiac output (A), heart rate (B) and stroke volume (C) at rest (triangles) and immediately after swim testing (circles) for Atlantic salmon infected with two levels of sea lice. An asterisk indicates a significant increase ($P < 0.05$) for the variable from rest to U_{crit}. Dissimilar letters above symbols indicate significant difference ($P < 0.05$) between treatments.


A
B
C
Cardiac Output (mL kg-1 min-1)
Heart Rate (beats min-1)
Stroke Volume (mL beat-1 kg-1)
Infection Level (lice g-1)
*
a
b
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14

*Chapter 5: Short-term Freshwater Exposure Benefits Sea Lice-Infected Atlantic Salmon

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INTRODUCTION

Anadromous salmonid post-smolts are able to live in either seawater (SW) or freshwater (FW) because of several physiological adaptations. Osmotic balance is maintained in SW by active drinking, secretion of sodium and chloride by chloride cells in the gills, and secretion of divalent ions by the intestine and kidney (see reviews by Wendelaar Bonga, 1997 and Høgåsen, 1998). However, external stressors can disrupt this carefully maintained balance, causing ion influx and water loss. Infection levels of sea lice (*Lepeophtheirus salmonis* Krøyer) between 0.75-1.0 lice g^{-1} have been shown to disrupt the osmotic balance of salmonids and lead to morbidity (Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997; McKinnon, 1998; Finstad *et al.*, 2000; Bjørn *et al.*, 2001). Systemic stress also can occur at sub-lethal infection levels leading to osmoregulatory, skin, and gill epithelial changes (0.05 lice g^{-1}, Nolan *et al.*, 1999) and a reduction in swimming performance (0.13 lice g^{-1}, Chapter 4).

During the past ten years, sea lice-infected sea trout (*Salmo trutta*) (Tully *et al.*, 1993a, 1993b; Birkeland, 1996; Birkeland & Jakobsen, 1997; Tully *et al.*, 1999; Bjørn *et al.*, 2001) and Atlantic salmon (Jon Backer, Norwegian Institute of Nature Research Station, Ims, Norway, pers. comm.) post smolts have been observed returning early to FW. The current theory based on these observations is infected fish return to FW to restore osmotic balance and remove sea lice (Bjørn *et al.*, 2001; Bjørn, 2002) that cannot osmoregulate in hyposaline conditions (Hahnenkamp & Fyhn, 1985). Two problems this theory does not address are the high numbers of lice able to survive in fresh water for 1-2 weeks (Finstad *et al.*, 1995) and the physiological stress caused by salinity transfer (Evans, 1984; Wendelaar Bonga, 1997). Prolonged lice survival may extend the tenure of fish in FW and lead to long-term health problems such as weight loss (Birkeland, 1996). Salinity transfer can compromise swimming performance (Brauner *et al.* 1992, 1994) and possibly compound the stress from lice infection. The purpose of this study was to determine if the physiological performance of salmon infected with sub-

lethal levels of lice is improved or compromised further by short-term transfer to freshwater. The physiological performance of infected and non-infected Atlantic salmon (*Salmo salar* L.) was determined by measuring critical swimming speeds (U_{crit}), cardiovascular performance (cardiac output, $\dot{Q}$; heart rate, f_H; stroke volume, V_S) and blood variables.

MATERIALS AND METHODS

Sixty post-smolt Atlantic salmon (38.0 ± 0.4 SE cm; 652.8 ± 20.3 g) of a 50:50 sex distribution were reared and held at the Norwegian Institute of Nature Research (NINA) Research Station, Ims, Norway (55 °N; 15 °E). Two 2300 L round tanks containing seawater (33.5 ± 1.2 ppt; 9.5 ± 0.2 °C) held thirty fish each under flow-through conditions. Fish were fed micro 30 sinking fish food (48% protein, 24% fat, 8% ash; EWOS AS, Bergen, Norway) once daily to satiation. Fish in one tank were infected with sea lice copepodids while the control tank was sham infected. Infection was performed according to Bjørn & Finstad (1998) with 5500 copepodids to attain levels of approximately 75 pre-adult and adult lice per fish, based on 65 % infection success and 65 % lice survival. Feeding was stopped at least 24 hours prior to swim testing to induce a post absorptive state.

Critical swimming performance trials were conducted between June and August of 2002. Swim testing of infected fish started once adult male lice stages appeared (identified according to Johnson & Albright, 1991), 10 days after the end of control fish swimming trials. Treatments each contained nine fish and included: (SW-Infected) lice-infected fish swim tested in seawater; (FW-Infected) lice-infected fish changed to freshwater 4 hours prior to swim testing; (SW) non-infected fish swim tested in seawater; (FW) non-infected fish changed to freshwater 4 hours prior to swim testing. Surgical attachment of Doppler cuffs, lice identification, swimming performance testing, and blood sampling were performed as described in Chapter 4 except as follows. One uninfected fish was swim tested each day for 18 days on a rotational basis between

the two salinity treatments in order to minimise differences between treatments. Following the maturation of infective sea lice, fish in the infected treatments were tested in the same manner as above. Fish were allowed to recover in the swim tunnel for 8 hours prior to swim testing. FW-treated fish slowly were changed to FW over the final 4 hours of the recovery period. Resting blood samples were taken from the remaining fish in each treatment tank at the end of the experiment by lethally anaesthetising the entire tank using 200 ppm clove oil and rapidly sampling all fish.

Temperature of the FW was controlled using a SW flow through cooler unit. Using this method, FW and SW temperatures were maintained within 0.5 °C. Temperatures of SW and FW increased prior to critical swimming trials of infected fish (12.8 °C) and were significantly higher ($P < 0.05$) than initial trials for uninfected control fish (9.2 °C) (Table 5.1). This temperature increase had a large positive affect on U_{crit}, $\dot{Q}$ and f_H and inverse affect on V_S, and was used as a covariate in statistical analyses. Therefore, the Q_{10} relationships determined for rainbow trout by Keen & Farrell (1994) were used to estimate corrected values for the affected variables (Q_{10} U_{crit} = 1.35; Q_{10} $\dot{Q}$ = 1.26; Q_{10} f_H = 1.52) and have been included in Table 1 and 2 as a reference.

Data from blood analysis and U_{crit} were analysed by three-way ANOVA and Tukey's multiple comparison method. In all cases fish length and mass, and water temperature were tested for covariation and included if significant. Time post-infection also was tested as a covariate to determine if female lice development into adult stages over the 18 days of swim testing accounted for any variation in the results. Cardiac variables ($\dot{Q}$, f_H and V_S) were analysed using two-way ANOVA. When interaction occurred, all simple effects were tested individually for significance.

RESULTS

Adult male lice stages first appeared at 5 weeks and swim testing started 3 days later. Final infection levels were (0.08 ± 0.01 lice g^{-1}; ave: 46.0 ± 4.4 lice $fish^{-1}$, range: 25–93 lice $fish^{-1}$) when swim trials began. During the first four days of swim testing adult males (55 %) predominated with second pre-adult females (19 %) and adult females (26 %) composing the rest of the lice stages present. Lice stages during the rest of the swimming trials were composed solely of adult males (57 %) and adult females (43 %). Lice maturation during the swim testing period did not affect the performance or blood results.

BLOOD VARIABLES

Calcium and glucose were affected by increased temperatures in the second half of the experiment and were adjusted statistically by using temperature as a co-variate in analysis. Resting and post-exercise levels of the blood variables are presented in Fig. 5.1. Chloride levels of control and infected fish in SW (159.5 ± 4.1 mmol L^{-1}; 159.9 ± 3.9 mmol L^{-1}) were significantly higher than similar groups in FW (144.5 ± 2.5 mmol L^{-1}; 142.3 ± 1.4 mmol L^{-1}) after swim testing. After exercise, potassium decreased from resting levels by 34 % only in uninfected fish in both SW and FW. Post-exercise levels of lactate increased from rest by 44.1 % in uninfected control fish in SW. Infected fish in SW had significantly lower levels of lactate after exercise than the other three treatments. Cortisol, glucose and haematocrit levels increased significantly from resting levels in all treatments after exercise regardless of salinity or infection. Post-exercise glucose levels increased by approximately 43 % and haematocrit levels increased by 12 %.

PERFORMANCE

Infected salmon swam significantly slower in SW (2.14 ± 0.08 bl s^{-1}; Q_{10}-corrected = 1.88 bl s^{-1}) than in FW (2.81 ± 0.08; Q_{10}-corrected = 2.51 bl s^{-1}) and slower than non-infected control fish in either SW or FW (Table 5.1). Resting $\dot{Q}$ of infected fish in FW was significantly

higher than the other three treatments (Table 5.2). Subsequently, $\dot{Q}$ of these fish did not increase from resting levels during exercise to the same degree ($P < 0.10$ versus $P < 0.05$) as the other treatments. Control fish in FW (72.6 ± 1.8 beats min^{-1}) and SW-infected fish (70.7 ± 3.08 beats min^{-1}; Q_{10}-corrected = 58.5 beats min^{-1}) had the highest and lowest respective maximum f_H. Control fish Vs were significantly lower than infected fish at U_{crit}. Resting Vs of FW-infected fish (0.84 ± 0.17 mL kg^{-1}) was significantly higher than fish in the other treatments. Maximum Vs of fish in FW did not vary significantly from resting values.

DISCUSSION

The present study shows that critical swimming speed of sea lice-infected Atlantic salmon in SW increases to uninfected levels after short-term exposure to FW. The infection intensity of 0.08 ± 0.01 lice g^{-1} was commensurate with sub-lethal levels (0.13 ± 0.02 lice g^{-1}) shown in Chapter 4 to affect the swimming performance of post-smolt Atlantic salmon in SW. A similar 22 % decline in U_{crit} occurred in the present study for infected fish in SW compared to uninfected control fish (Table 5.1). Brauner *et al.* (1992, 1994) found transfer to SW followed by FW did not affect the swimming performances of FW acclimatised coho (*Oncorhynchus kisutch*) parr and smolts. Although these results are similar to the present study, the fact coho were not adults and were held at each salinity change for 24 hours prior to testing make a comparison tenuous. Also, unlike coho salmon that slowly acclimatise to salinity decreases in estuaries over days and weeks, Norwegian Atlantic salmon can enter home river systems abruptly (Thorstad *et al.*, 1998). This naturally short estuary residence time may explain why control fish were able to acclimate to FW quickly and suffer no decrease in performance.

Blood physiology was altered by salinity change and to a lesser extent by lice infection. Unlike results presented in Chapter 4, chloride levels of infected fish in SW did not increase during exercise (Fig. 5.1) and were within normal exercise limits for Atlantic salmon (Wendelaar

Bonga, 1997). This result is important because the link between sub-lethal lice infection and decreased swimming performance initially was speculated to be owing to osmoregulatory imbalance, leading to hyperchloremia. However, swimming performance of infected fish in SW decreased with no significant change in chloride or potassium ions. This lack of change in blood variables indicates infected fish were able to maintain osmoregulatory balance during exercise and eliminates the possibility of direct ion effects on oxygen carrying capacity and muscle performance. A possible explanation for the decrease in U_{crit} despite normal chloride levels is the use of excess energy by SW-infected fish due to increased energy costs in maintaining osmotic balance. Elevated cortisol and glucose levels in resting infected fish can indicate chronic stress and mobilised energy reserves (Ruane *et al.*, 2000; Chapter 3), but neither differed significantly in resting fish. Blood glucose after exercise is expected to be similar for fish swimming to the same level because aerobically exercising fish primarily use red muscle that utilises fatty acids as metabolic substrate (Richards *et al.*, 2002b). However, glucose levels in SW-infected fish that had lower U_{crit} were not lower than other fish. Post-exercise lactate levels were significantly lower for infected fish in SW. This decrease may be due to the fact anaerobic white muscle is used only at the beginning of each new time interval and after 80 % of the fish's critical speed has been reached (Webb, 1971; Richards *et al.*, 2002a). Therefore, infected fish in SW that swam fewer intervals may have produced less lactate as a metabolic end-product. Similar lactate results have been found for rainbow trout (*Oncorhynchus mykiss*) with reduced U_{crit} in FW due to hypoxia (Chapter 2). An increase in oxygen consumption would show SW-infected fish had increased metabolic rates, but oxygen was not directly measured in this study. Infected fish in SW had $\dot{Q}_{max}$ similar to other fish, but oxygen use still could have been higher due to increased arterial-venous O_2 differential (Kiceniuk & Jones, 1977; Wendelaar Bonga, 1997). Oxygen consumption in Atlantic salmon eventually decreases after transfer to FW (Maxime *et al.*, 1990) so any increase would be due solely to lice infection. The fact potassium

levels decreased after exercise only in uninfected fish indicates lice infection caused some stress to the osmoregulatory system. Lack of major osmotic changes may be due to the fact lice levels in the present study (0.08 lice g^{-1}) were lower than those in Chapter 4 (0.13 lice g^{-1}). A threshold for lice-induced loss of osmotic balance may exist near lice concentrations of 0.1 lice g^{-1}.

Salinity change affected several blood variables (Fig. 5.1). Increased chloride levels after exercise in SW is typical of the osmoregulatory compromise that occurs in the gills. As surface area increases and tension decreases in order to increase oxygen uptake, permeability of the gills also increases (Schreck, 1990; Thomas, 1990; Wendelaar Bonga, 1997) allowing passive ion entry and water loss. Maxime *et al.* (1990) also found transfer of Atlantic salmon from SW to FW caused chloride levels to decrease after 6 hours. Short-term exposure of fish to FW did not affect potassium levels, as in the present study. Haematocrit levels did decrease significantly with exercise in all treatments. Stressed fish in SW can have decreased haematocrit due to shrinking of erythrocytes because of plasma ion increase (Iwama *et al.*, 1989). However, why haematocrit decreased in FW fish as well is not known, especially when erythrocytes typically are released from the spleen during exercise (Pearson & Stevens, 1991; Gallaugher *et al.*, 1992, 1998).

Changes in cardiac variables indicate both FW exposure and lice-infection cause systemic stress. Resting $\dot{Q}$ of uninfected fish, and infected fish in SW (Table 5.2) were similar to values reported by Brodeur *et al.* (2001) for swimming rainbow trout while fish in the FW-infected treatment that had significantly higher $\dot{Q}$ due to increased Vs. Elevated resting Vs indicates the combined stresses of lice infection and salinity change acted on the heart. Stressors can alter $\dot{Q}$ during exercise or affect f_H or Vs, usually in a compensatory fashion (Gallaugher *et al.*, 1995; Brodeur *et al.*, 1999). Hypoxia similarly increases Vs (Holeton & Randall, 1967; Randall, 1970; Wood & Shelton, 1980; Gamperl *et al.*, 1994a), but a subsequent decrease in resting f_H did not occur in the present study. Increased resting $\dot{Q}$ did not translate into a

significantly higher $\dot{Q}$ at maximum performance. All fish had similar $\dot{Q}_{max}$ that increased significantly from rest except FW-infected fish. The latter group did show the same trend of increased $\dot{Q}$ ($P < 0.1$), however, high resting values decreased their cardiac scope. Similar $\dot{Q}$ at rest and U_{crit} indicates fish had delivered arterial oxygen to the tissues at similar rates regardless of lice infection and salinity. Transition to FW also affected the Vs of fish. Unlike fish remaining in SW, Vs of fish in FW did not significantly change from resting values during exercise. Therefore, the 30 % increase in $\dot{Q}$ of control and infected fish in FW was due almost solely to increased heart rate. Normally, $\dot{Q}$ increases in a direct relationship with exercise due to increases in f_H and Vs (Stevens & Randall, 1967; Kiceniuk & Jones, 1977; Thorarensen *et al.*, 1996). Transfer to FW instead reduced this normal increase in Vs of both control and infected fish so that values did not change significantly from rest. Although f_H of infected fish in SW showed a trend of increase with exercise ($P < 0.1$) similar to the other three groups, comparatively f_H were lower at U_{crit}.

Sea lice have a similar physiological effect on Atlantic salmon and sea trout (Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997). Dawson *et al.* (1997) reported sea lice survival was higher on sea trout than Atlantic salmon, but no differences in lice numbers occurred at 3-5 weeks infection. During this period lice moulted from mainly commensal chalimus into pathogenic adult stages (Grimnes & Jakobsen, 1996; Bowers *et al.*, 2000) and numbers still were at physiologically detrimental levels (~ 0.1 lice g^{-1}). Due to similar lice survival and impact, heavily infected sea trout likely return early to FW for the same physiological reasons as Atlantic salmon.

The fact swimming performance of infected fish improved upon transfer to FW confirms a link between the physiological effects of lice and the early return of heavily infected salmonids to FW. The lice infection intensity was well below the range of 0.5-2.1 lice g^{-1} found on wild sea trout and Arctic charr (*Salvelinus alpinus*) in an intensive aquaculture area of Norway (Bjørn *et*

al., 2001). If lice populations are high in these areas, behaviour of wild salmonids may be altered due to the physiological impact of the lice. Although early return to FW allows recovery from lice infection, fish still can have negative long-term health effects from extended FW residence. These effects include minor osmoregulatory disturbances, weight loss and increased mortality (Birkeland, 1996; Bjørn *et al.*, 2001).

The present study demonstrates short-term FW exposure counteracts the decline in Atlantic salmon swimming performance caused by lice infection in SW. Changes in cardiac and blood variables indicate salinity change and sub-lethal lice infection cause systemic stress, but within 4 hours of FW exposure, swimming ability of Atlantic salmon is not compromised. Osmotic balance of infected fish was not disrupted in SW, but energy use may have increased in the osmoregulatory and cardiovascular systems. The fact infected Atlantic salmon suffer no major physiological penalties passing from SW to FW while benefiting from improved performance and lice removal supports a physiological cause for the return to FW of heavily infected salmonids.

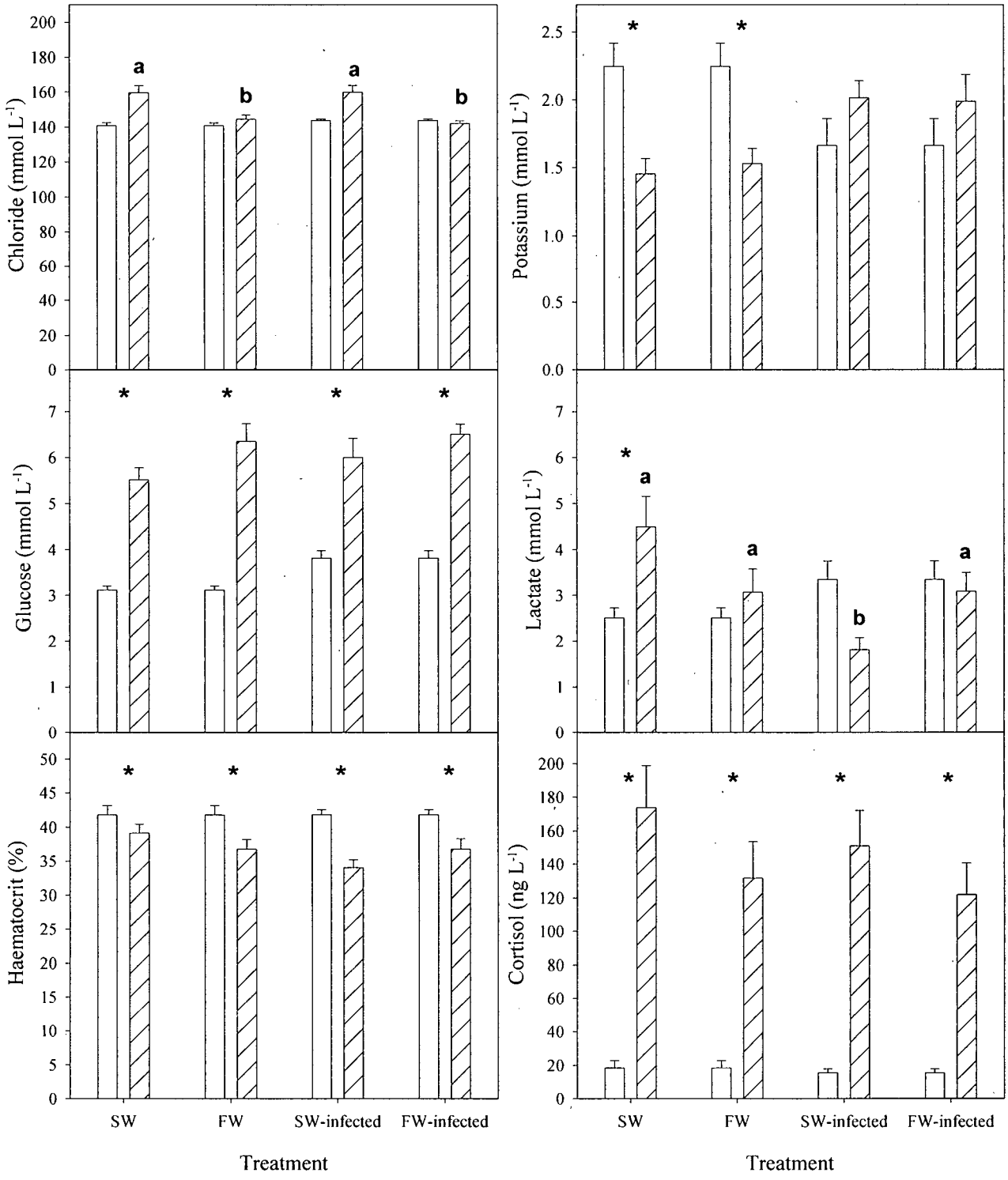
Table 5.1: The effect of salinity change on the critical swimming speeds (U_{crit}) of control and sea lice-infected (0.08 lice g^{-1}) Atlantic salmon. Seawater (SW) acclimatised fish were maintained in SW or changed to freshwater (FW) prior to swimming trial. Dissimilar letters indicate statistical between treatments at $P < 0.05$ significance. Temperature was used as a covariate; numbers in parentheses indicate Q_{10}-corrected values.

Treatment	N	Mass (g)	Fork Length (cm)	Temperature (°C)	U_{crit} (bl s^{-1})
SW	8	646.75 ± 50.07	37.34 ± 0.89	9.13 ± 0.18 [a]	2.42 ± 0.04 [a]
FW	9	673.78 ± 42.06	37.73 ± 0.72	9.22 ± 0.15 [a]	2.61 ± 0.08 [a]
SW-infected	9	656.78 ± 41.58	38.81 ± 1.00	12.89 ± 0.34 [b]	2.14 ± 0.08 [b] (1.88)
FW-infected	9	633.33 ± 35.14	38.13 ± 0.87	12.67 ± 0.26 [b]	2.81 ± 0.08 [a] (2.51)

Table 5.2: The effect of salinity change on the cardiac output ($\dot{Q}$), heart rate (fh) and stroke volumes (Vs) of control and sea lice-infected (0.08 lice g^{-1}) Atlantic salmon during critical swimming performance (U_{crit}) tests. Seawater (SW) acclimatised fish were maintained in SW or changed to freshwater (FW) in the swimming chamber 4 hours prior to exercise. Asterisk (*) indicates a significant increase ($P < 0.05$) for the variable from rest to U_{crit}. Cross (†) indicates an increase ($P < 0.1$) for the variable from rest to U_{crit}. Dissimilar letters indicate the heart variables are significantly different at rest or U_{crit}. Temperature was used as a covariate; numbers in parentheses indicate Q_{10}-corrected values and calculated Vs.

Treatment	Water Temperature (°C)	$\dot{Q}$ (mL kg^{-1} min^{-1})		fH (beats min^{-1})		Vs (mL $beat^{-1}$ kg^{-1})	
		Rest	U_{crit}	Rest	U_{crit}	Rest	U_{crit}
SW	9.13 ± 0.18 [a]	35.8 ± 3.8 [a]	60.7 ± 2.8 [a*]	54.6 ± 1.6 [a]	62.7 ± 1.6 [a*]	0.66 ± 0.08 [a]	0.96 ± 0.05 [a*]
FW	9.22 ± 0.15 [a]	44.0 ± 4.7 [a]	69.4 ± 5.5 [a*]	58.1 ± 2.9 [a]	72.6 ± 1.8 [b*]	0.79 ± 0.11 [a]	0.95 ± 0.09 [a]
SW-infected	12.89 ± 0.34 [b]	30.1 ± 3.5 [a] (27.4)	64.8 ± 3.2 [a*] (59.0)	64.9 ± 2.2 [a] (53.7)	70.7 ± 3.0 [c†] (58.5)	0.47 ± 0.04 [a] (0.51)	0.93 ± 0.06 [b*] (1.01)
FW-infected	12.67 ± 0.26 [b]	54.4 ± 8.3 [b] (49.8)	71.2 ± 7.0 [a†] (65.4)	70.4 ± 5.4 [a] (59.0)	79.5 ± 4.0 [a*] (66.6)	0.84 ± 0.17 [b] (0.84)	0.94 ± 0.12 [b] (0.98)

Figure 5.1: Blood plasma levels of chloride (A), potassium (B), glucose (C), lactate (D), haematocrit (E) and cortisol (F) taken at rest (clear bars) and immediately after critical swim testing (hatched bars) from control and sea lice-infected (0.08 lice g^{-1}) Atlantic salmon. An asterisk indicates significant differences at $P < 0.05$ between resting and post trial values. Dissimilar letters above bars indicate significant difference ($P < 0.05$) between treatments. Temperature was used as a covariate.


Chloride (mmol L-1)
Potassium (mmol L-1)
Glucose (mmol L-1)
Lactate (mmol L-1)
Haematocrit (%)
Cortisol (ng L-1)
a
b
*
SW
FW
SW-infected
FW-infected
Treatment

*Chapter 6: Anaemia and Salmonid Swimming Performance: The Effects of Sub-lethal Sea Lice Infection

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INTRODUCTION

A known cause of blood loss in nature is the feeding of sea lice (*Lepeophtheirus salmonis*) that penetrate the skin of fish (Brandal *et al.*, 1976; Wootten *et al.*, 1982). In small numbers this loss may be insignificant, but sea lice can occur in very high numbers (100+ lice $fish^{-1}$; 3.0+ lice g^{-1}) in both laboratory studies and wild populations (Grimnes & Jakobsen, 1996; Bjørn *et al.*, 2001). High infection levels (0.75-1.0+ lice g^{-1}) lead to salmon morbidity, likely due to osmotic imbalance and compromised immune response (Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997). Sub-lethal lice infections (0.1 lice g^{-1}) cause a significant decrease in the critical swimming performance (U_{crit}) of Atlantic salmon (*Salmo salar* L.) (Chapter 4). Blood loss due to ingestion and leakage from wounds (Grimnes & Jakobsen, 1996; Dawson *et al.*, 1999) has been reported, but anaemia has not been considered as a main stressor even though it is known to decrease swimming performance of salmonids (Jones, 1971; Gallaugher *et al.* 1995).

In the present study, blood parasitism was simulated by taking caudal puncture blood samples from juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum) over a 5 day period. Repeated blood sampling causes stress in fish (Gallaugher & Farrell, 1998; Chapter 2). Single samples above 10% total blood volume can lead to anaemia and repeated sampling below 1 % is recommended for mammals and birds that average slightly higher total blood volumes than fish (Morton *et al.*, 1993; Stoskopf, 1993). Haematocrit did not change in infected salmon at the sub-lethal infection levels used in Chapter 4, so anaemia was not responsible for decreased U_{crit}. However, it is important to determine the point at which infected fish may become anaemic due to blood loss from lice feeding as this may occur prior to known lethal levels of infection. This information is important to increase basic knowledge of how lice affect the physiology of their host. Anaemia is known to occur at haematocrit values < 22 % (Gallaugher *et al.* 1995) so blood

sampling results were used in conjunction with the development of a sea lice blood-feeding model in order to predict the point at which blood ingestion will cause anaemia in infected fish.

MATERIALS AND METHODS

EXPERIMENTAL FISH

Juvenile rainbow trout were obtained from a 50:50 sex ratio population at the Rainbow Springs Trout Hatchery, Thamesford, Ontario. Fish were placed in recirculated well water (8.1 ± 0.2 °C; mean ± SEM) held in a 1500 L tank. Standard three-point sinking trout food was fed to fish held in this tank for approximately two months. Twenty-four fish were placed into groups of six in four 80 L opaque tubs. Each tub had an individual source of flow-through well water (P_{O2} = 146.6 ± 3.1 mmHg) and aeration, and was covered with a plastic grate. Fish were acclimatised for two weeks prior to initial blood sampling. Food was withheld for 24 hours prior to swim testing in order to induce a post-absorptive state.

REPEATED BLOOD SAMPLING

Fish total blood volume is approximately 5 % of body mass (Gallaugher & Farrell, 1998); fish in the present study that weighed on average 150 g, contained approximately 7.5 ml of blood. Percentage of total blood volume removed was calculated for the 5 day period over which the samples were taken. Each group of six fish was assigned one of four treatments: 1) 0.5 ml blood samples (25.9 ± 0.7 cm; 184.7 ± 15.8 g) (3.2 % of total blood volume day^{-1}); 2) 1.0 ml blood samples (24.4 ± 0.7 cm; 149.9 ± 12.7 g) (8.0 % of total blood volume day^{-1}); 3) sham blood sampling (23.1 ± 0.9 cm; 131.6 ± 16.6 g); 4) no anaesthesia controls (22.2 ± 0.6 cm; 122.5 ± 13.3 g). Following acclimatisation, the first three treatments were sampled every two days on a rotational basis for three total samples. Fish in an individual treatment were anaesthetised as a group in 60 ppm clove oil (active ingredient eugenol, 88 % min.: Hilltech

Canada Inc., Vankleek Hill, Ontario). Blood samples were taken immediately after knockout while sham treatment fish were treated in the same fashion without drawing blood. Control fish were not anaesthetised or sampled until after swim testing.

SWIM TESTING

Critical swimming speed testing (U_{crit}) spanned four days with all fish from one treatment being tested each day because of the initial one day stagger in sampling. Individual fish were anaesthetised using 60 ppm clove oil to reduce handling stress, then placed individually into a Blazka-type swim chamber with a modified net plug to prevent drafting in the turbulent area near the cap. Two identical swim chambers were used for all swim testing that was performed using a protocol similar to that described in Chapter 4, with a 4 hour recovery time, 0.1 m s^{-1} speed increments, and 10 minute time intervals. A final blood sample was taken immediately after swim testing, by euthanasia of each fish using a 200 ppm clove oil bath.

EXPERIMENTAL SEA LICE

Sea lice were collected from Atlantic salmon during two separate physiological experiments in 2001 and 2002 at the Norwegian Institute of Nature Management (NINA) Research Station, Ims, Norway (55 ^{o}N; 15 ^{o}E). Adult lice with blood visible in their digestive tracts were patted dry on absorbent lint-free tissue (wet mass), or dried under incandescent light for 1 hour (dry mass) before being weighed. Gut contents were excised from each louse using fine forceps and a scalpel (no. 11 blade). Incisions were made through the cephalothorax and abdomen along one side of the gut, the contents of which were then removed and weighed. Lice sexes were present in a 50:50 ratio and approximately 45 % of female and 10% of male lice had blood visible in their digestive tracts.

STATISTICS

All data from blood analysis and critical swimming speeds were analysed using one-way ANOVA and Tukey's multiple comparison method. The mass of each fish was used as a

covariate when analysing all results except those for repeated blood sampling. Fish were not individually weighed during repeated blood sampling in order to increase sampling speed. Linear regression analysis was performed on gut and whole body masses. For repeated sampling the dependant variables included individual blood parameters and U_{crit}. All treatments were independent variables grouped by sample time.

RESULTS

Given that the same amount of blood was taken from fish with variable mass, the exact amount of blood relative to the body mass also varied. Blood loss was used as a covariate in all analysis including U_{crit}. The average amount of blood removed from fish was approximately 3.2 % of total blood volume (2.81 ± 0.23 ml kg^{-1} $sample^{-1}$) in the 0.5 ml treatment and 8.0 % of total blood volume (6.89 ± 0.51 ml kg^{-1} $sample^{-1}$) in the 1.0 ml treatment. The difference in relative amounts of blood removed was significant ($P < 0.05$) at a ratio of 1:2.4.

Of the blood parameters measured prior to swim testing, only chloride, haematocrit and cortisol changed significantly between treatments or with time (Fig. 6.1). On day 1, Initial chloride levels of the 1.0 ml treatment (113.8 ± 1.4 mmol l^{-1}) were significantly lower than the 0.5 ml treatment (122.9 ± 1.3 mmol l^{-1}). Haematocrit levels in the 1.0 ml treatment (17.2 ± 2.2 %) fell significantly below those of the 0.5 ml treatment (24.7 ± 1.6 %) on day 3. A similar trend occurred on day 5 with a decrease in the 1.0 ml treatment (16.2 ± 2.1 %) compared to the 0.5 ml treatment (21.8 ± 2.2 %), but not at the same level of significance ($P = 0.095$). Although cortisol levels were elevated in the 0.5 ml treatment on day 3 (50.9 ± 20.8 ng ml^{-1}) and day 5 (48.7 ± 19.0 ng ml^{-1}) compared to the 1.0 ml treatment (day 3: 7.1 ± 2.2 ng ml^{-1}; day 5: 8.1 ± 4.4 ng ml^{-1}), the variance between individual fish was high (day 3 range: 5.7 to 140.1 ng ml^{-1}; day 5 range: 4.3 to 112.8 ng ml^{-1}) and significant differences did not occur.

The only statistically significant changes ($P < 0.05$) with time occurred for chloride levels (Fig. 6.1). In the 0.5 ml treatment, chloride was significantly higher on day 5 (124.2 ± 1.0 mmol l^{-1}) than day 3 (119.9 ± 0.7 mmol l^{-1}), but unchanged from the first sample day. Chloride levels remained significantly high in fish of the 1.0 ml treatment after day 3 (day 1: 113.9 ± 1.4 mmol l^{-1}; day 3: 120.6 ± 1.0 mmol l^{-1}; day 5: 121.1 ± 1.1 mmol l^{-1}). Some interesting trends in haematocrit and cortisol occurred as well, although not at the same level of significance ($0.05 > P < 0.10$). In the 1.0 ml treatment, the haematocrit of fish sampled on day 5 (16.2 ± 2.1 %) fell below those sampled on the first sample day (23.2 ± 1.9 %) ($P = 0.076$). Haematocrit levels did not vary significantly in the 0.5 ml treatment. Although the cortisol levels of fish in the 0.5 ml treatment were highly variable, an increasing trend ($P = 0.085$) was seen by day 3 (day 1: 3.1 ± 0.41 ng ml^{-1}; day 3: 50.9 ± 20.7 ng ml^{-1}; day 5: 48.7 ± 19.0 ng ml^{-1}). No such trend occurred in the 1.0 ml treatment.

Immediately after swim testing, only blood chloride and haematocrit levels changed significantly between the four treatments (Fig. 6.2). Chloride levels of blood sampled fish (0.5 ml: 123.2 ± 1.7 mmol l^{-1}; 1.0 ml: 122.2 ± 1.9 mmol l^{-1}) were significantly higher than those of control fish (113.1 ± 1.0 mmol l^{-1}), but not significantly different from the sham treated fish (115.9 ± 1.4 mmol l^{-1}). Haematocrit levels of both sampled treatments (0.5 ml: 24.5 ± 1.9 %; 1.0 ml: 16.7 ± 3.4 %) were significantly lower than sham treated (33.3 ± 1.7 %) and control (37.8 ± 2.5 %) trout.

Changes in the U_{crit} of repeatedly sampled fish also occurred (Fig. 6.3). Critical swim testing showed fish that lost the most blood had significantly reduced U_{crit} (3.8 ± 0.1 bl s^{-1}) compared to sham (4.8 ± 0.2 bl s^{-1}) and control fish (4.7 ± 0.3 bl s^{-1}). The U_{crit} of fish that had the least amount of blood sampled (4.0 ± 0.1 bl s^{-1}) did not vary significantly from any of the treatments.

Sea lice body mass was significantly correlated with mass of their gut contents (Fig. 6.4). Although the regression value for dry masses ($r^2 = 0.85$, N = 24) was higher than for wet masses ($r^2 = 0.65$, N = 24) both were significant at $P < 0.05$. Regression lines were similar (dry mass slope of 0.28 versus wet mass slope of 0.29), so wet masses were used in calculations because they are more practical to measure in lab and field situations. The regression equation from the wet mass correlation:

$$y_1 = \text{gut mass} = 0.29 \times (\text{louse body mass}) - 0.0004 \quad (1)$$

was used to develop a model to predict feeding rates of sea lice for different concentrations of blood (Fig. 6.5). Data for infection level (lice g^{-1}) and blood ingestion rate (% total blood volume day^{-1}) were developed as follows:

$$y_2 = \text{infection level} = \text{no. lice/ fish mass} \quad (2)$$

$$y_3 = \text{clearance rate} = y_1 \times 24 \text{ hours} \quad (3)$$

$$y_4 = \text{blood ingestion} = [\text{no. lice} \times y_3 \times K^1 \times K^2] / \text{fish blood volume} \quad (4)$$

where K^1 = % of lice with ingested blood $\approx$ 30 % (Brandal *et al.*, 1976; G.N. Wagner, unpublished observations) and K^2 = % of ingested tissue consisting of blood (50, 25, 10 and 5 %). Average gut mass (y_1) was calculated using the average wet mass of lice (0.0161 g) measured in the present study. The clearance rate (y_3) was estimated to be the average gut volume every hour (Huskin *et al.*, 2000). The fish blood volume used was 5 % body mass (Gallaugher & Farrell, 1998).

DISCUSSION

Although Morton *et al.* (1993) recommend taking repeated blood samples below 1 % of total blood volume, 5 days of 3.2 % serial samples did not significantly decrease fish haematocrit. Gallaugher *et al.* (1992) found similar serial removal of 3.5-5 % of blood did not decrease haematocrit in rainbow trout. However, haematocrit of fish in the lower blood loss treatment was reduced significantly after exercise, possibly because splenic erythrocyte stores normally released during exercise (Pearson & Stevens, 1991; Gallaugher *et al.*, 1992, 1998) had already been depleted. During exercise in freshwater, fluid can shift into the plasma compartment causing hemodilution (Gallaugher *et al.*, 1992; Wendelaar Bonga, 1997) and fish in the lower blood loss treatment were unable to compensate (34 % decrease to 25 % haematocrit). The U_{crit} of these fish subsequently was reduced and not significantly different from control fish or fish in the 1.0 ml treatment. Blood loss of 8.0 % total blood volume did decrease haematocrit over the 5 day sample period, as well as post-exercise. Similar serial blood losses of 6-12 % have been reported to decrease haematocrit in rainbow trout and Atlantic cod (*Gadus morhua*) (Gallaugher *et al.*, 1992; Gallaugher & Farrell, 1998) leading to progressive anaemia. Gallaugher *et al.* (1995) found a 50 % reduction in haematocrit over 48 hours was correlated with a significant decrease in rainbow trout U_{crit} and increased resting cardiac output. A similar 54 % haematocrit reduction to only 16.7 % significantly decreased U_{crit} in the present study. These haematocrit results are similar to those of Gallaugher *et al.* (1995) and serve to corroborate their proposed 22 % haematocrit transition point to anaemia. It is important to estimate the point at which anaemia can occur in sea lice-infected fish. If this point is below known lethal infection levels, anaemia may help precipitate the morbidity of host fish.

Along with causing anaemia, 8% blood loss also led to a significant increase in blood osmolarity during U_{crit}. Chloride may have increased due to fluids shifting from interstitial spaces to the plasma (Gallaugher *et al.*, 1992; Gallaugher & Farrell, 1998). Despite significant

change, chloride levels did remain within normal limits for rainbow trout (85-130 mmol l^{-1}; Wedemeyer *et al.*, 1990). However, these changes could be further exacerbated when combined with the osmotic challenges posed by skin lesions and increased energy demand from lice feeding (Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997; Chapter 4). Chloride levels have been shown to decrease in Atlantic salmon when transferred between seawater and freshwater without affecting haematocrit or U_{crit} (Chapter 5). This lack of change suggests environmental salinity alone does not play a significant role in post-smolt salmonid swimming physiology, unless significant perturbations occur such as lice infection.

Sampling stress was not a factor in the present study. Cortisol levels of repeatedly sampled fish were surprisingly low, especially in the 1.0 ml treatment, possibly due to the sampling method. Cortisol did increase in all fish with exercise as expected, because of increased physiological stress (Wendelaar Bonga, 1997).

Intake rates of suspension-feeding copepods have been examined fairly thoroughly either by calculation or direct measurement (Peters & Downing, 1984; Huskin *et al.*, 2000; Champalbert & Pagano, 2002). These copepods can process 8-38 ml of water per day, acquiring up to 60 % of their total body carbon. No such feeding studies exist for parasitic copepods that feed on host body tissues including blood. Blood-feeding terrestrial insects can ingest bloodmeals of 110 % their body mass (Emmanuelle-Machado *et al.*, 2002), but do so within a matter of seconds. Sea lice, however, are grazers (Kabata, 1974) and do not fit the food ingestion models of filter-feeding copepods or haematophagous insects. Regardless of the mode used by parasites to acquire bloodmeals, intake rates are dependant on digestion and the egestion of food from their digestive tracts. While haematophagous insects require 20+ hours to digest a single meal (Feldman *et al.*, 1990), individual copepodids can average egestion rates of 1.6 mL $hour^{-1}$ (Huskin *et al.*, 2000). In the present study, the clearance rate of lice was conservatively estimated to be the average gut volume (0.004 mL) every hour based on their continuous

browsing type of feeding (Kabata, 1974) and the much higher rates of filter feeding copepods (0.3-1.6 mL hr-1)(Peters & Downing, 1984; Huskin *et al.*, 2000). Also, an assumption was made that all blood loss in infected fish was due to consumption by lice, although leakage of blood components from severe skin lesions may occur (Wootten *et al.* 1982; Grimnes & Jakobsen, 1996; Dawson *et al.*, 1999). Finally, gut content mass (g) was converted to ml based on a 1:1 density ratio with water. While this exact ratio is improbable, most fish tissues have the same approximate density as water so differences in calculated feeding rates should be negligible.

The strong correlation between lice body size and gut content mass ($r^2 = 0.85$) allowed the use of the regression equation from the relationship to help calculate feeding rates for different lice infection levels. The percentage of tissues consumed consisting of blood was not estimated above 50 % for two main reasons. The results of Brandal *et al.* (1976) indicate blood in the digestive tract of lice is diluted compared to host blood. Also, at a blood feeding rate above 50 % the model predicts consumption would far exceed amounts known to cause morbidity in rats (McGuill & Rowan, 1989) for louse concentrations that do not cause morbidity in fish (0.4-0.7 lice g^{-1}). The decline in swimming performance predicted to occur at 50 % blood consumption, due to 8 % blood loss day^{-1}, matches declines reported by in Chapter 4 for louse infection levels of 0.1 lice g^{-1}. However, haematocrit was not reduced at that infection level. Also, at infection levels above 1.2 lice g^{-1} feeding rates at 50 % blood consumption would exceed the total amount of blood in a fish on a daily basis.

Lice averaging a consumption of 15-25 % blood tissue component are predicted to consume only 0.8-1.7 % of host blood at lower infection levels (0.1 lice g^{-1}) known to decrease the U_{crit} of Atlantic salmon (Chapter 4). This value is near the 1 % level recommended for serial blood sampling of laboratory animals (Morton *et al.*, 1993) and well below 8 % blood loss that affects swimming performance. However, at a sub-lethal infection level of 0.5 lice g^{-1} lice

feeding at this rate could begin to cause anaemia due to the consumption of 6-12 % up to 14-30 % total blood volume of fish day^{-1}. This range encompasses values of continuous blood loss known to decrease salmonid swimming performance (Gallaugher *et al.*, 1995; present study) and single blood sample loss that causes haemorrhagic shock and mortality in rats (McGuill & Rowan, 1989; Morton *et al.*, 1993). Therefore, this anaemic condition would compound the osmotic and energy problems of fish during exercise (Chapter 4) at this sub-lethal infection level. This compounding effect most likely would help lead to morbidity with further increases in lice concentration. Significant 30-40% reductions in haematocrit were observed in fish with pre-adult lice levels of 0.75-1.0+ lice g^{-1} experiencing high mortality rates 30 days post-infection (Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997).

The present study confirms that blood loss of 8 % total blood volume causes a decrease in rainbow trout U_{crit}. Total blood volume losses of 3.2 % can reduce erythrocyte stores, but do not affect swimming performance. Based on a predictive feeding rate model, it is probable that 15-25 % of the tissue consumed by sea lice is blood. This consumption of blood at higher sub-lethal infection levels (0.5+ lice g^{-1}) may cause anaemia and a further decrease in swimming performance. Anaemia would only compound the osmotic balance problems due to infection and likely precipitate the morbidity seen at lethal lice levels above 0.75 lice g^{-1}.

Figure 6.1: Blood parameters of rainbow trout repeatedly sampled over a five day period (mean ± SE, n = 6). Open bars represent 0.5 ml serial blood samples; shaded bars represent 1.0 ml serial blood samples. An asterisk (*) indicates significance ($P < 0.05$) between individual treatments. A cross (†) indicates a trend ($P < 0.10$) between individual treatments. Dissimilar letters above bars indicate differences within treatments at $P < 0.05$ significance except for trends in haematocrit and cortisol ($P < 0.10$).

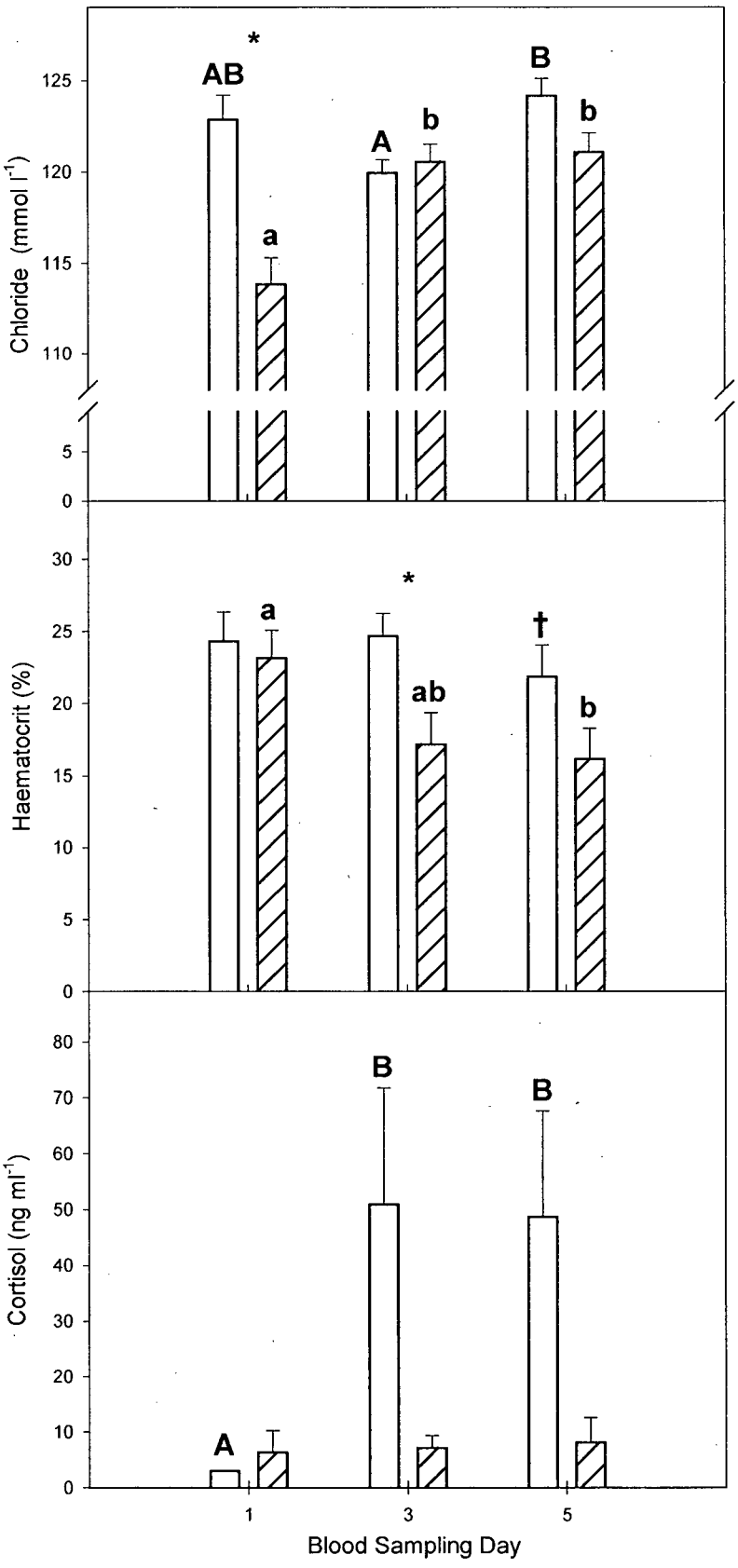
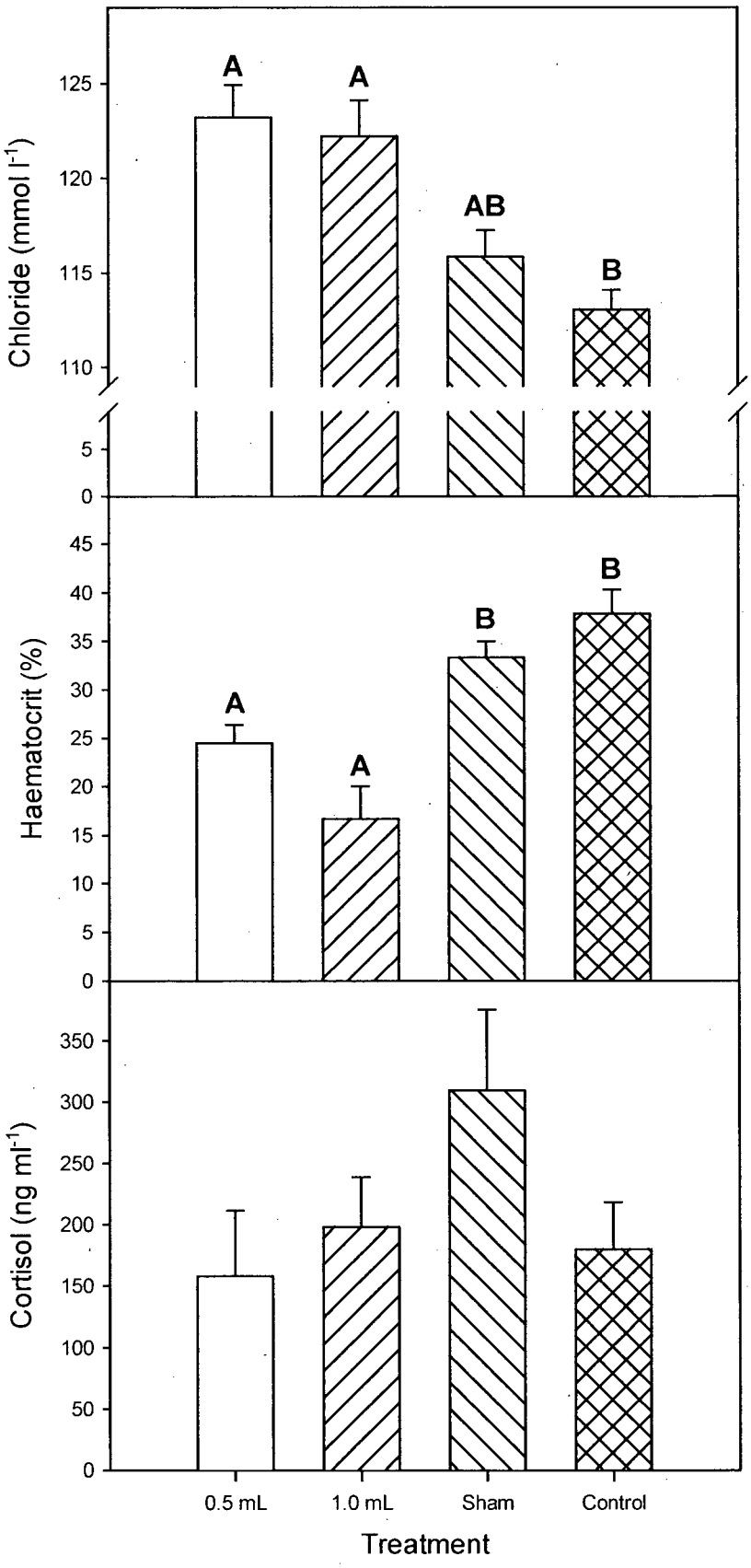

Chloride (mmol l-1)
125
120
115
110
5
0
AB
*
a
A
b
B
b
Haematocrit (%)
30
25
20
15
10
5
0
a
*
ab
†
b
Cortisol (ng ml-1)
80
70
60
50
40
30
20
10
0
A
B
B
1
3
5
Blood Sampling Day

Figure 6.2: Blood parameters of repeatedly blood sampled 0.5 ml (open) and 1.0 ml (forward-hatched), sham (backward-hatched) and control (cross-hatched) rainbow trout after critical swim testing (mean ± SE, n = 6). Dissimilar letters above bars indicate differences among treatments ($P < 0.05$).


Chloride (mmol l-1)
125
120
115
110
5
0
A
A
AB
B
Haematocrit (%)
45
40
35
30
25
20
15
10
5
0
A
A
B
B
Cortisol (ng ml-1)
350
300
250
200
150
100
50
0
0.5 mL
1.0 mL
Sham
Control
Treatment

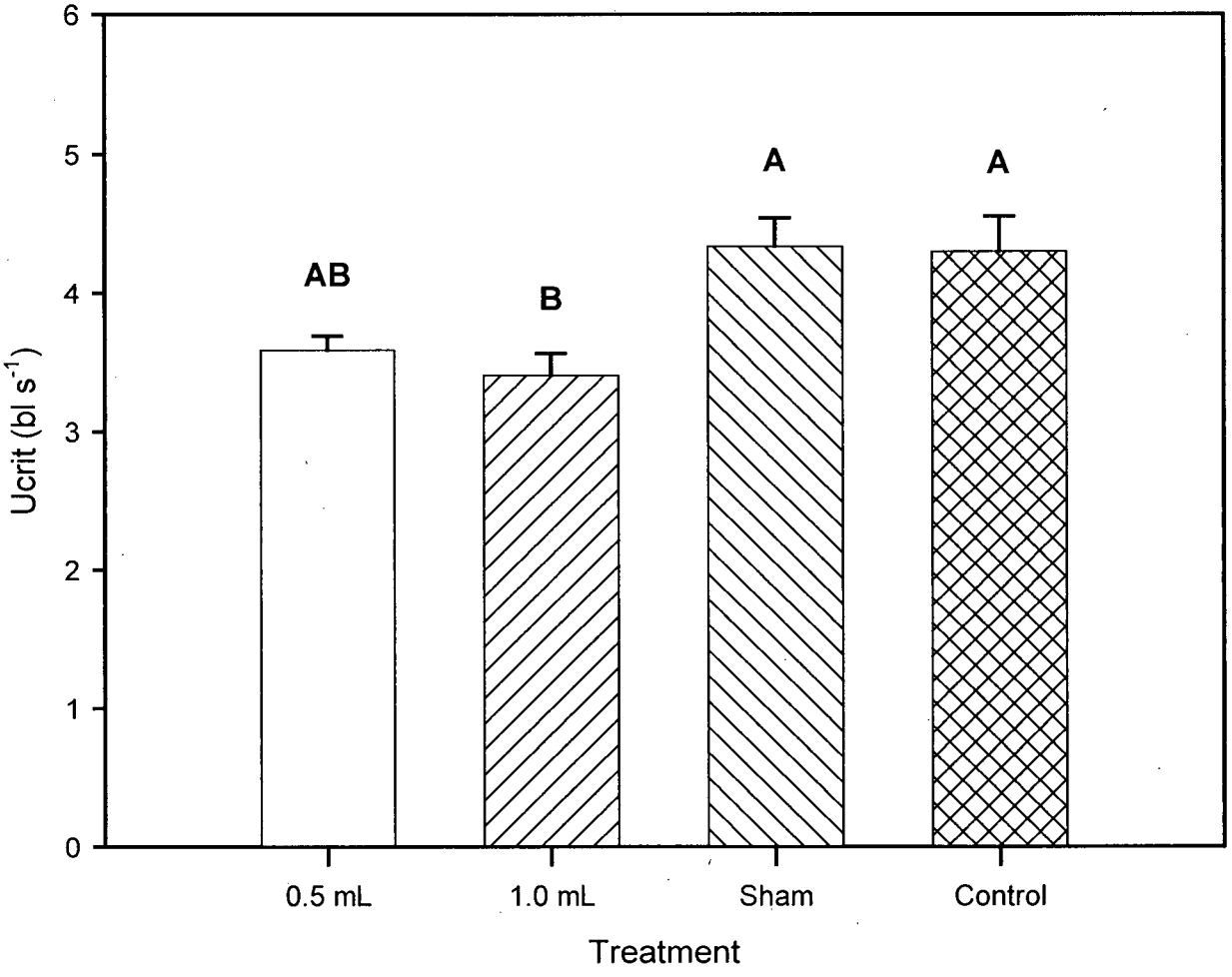


Figure 6.3: Critical swimming speeds (U_{crit}, in body lengths per second) of repeatedly blood sampled 0.5 ml (open) and 1.0 ml (forward-hatched), sham (backward-hatched) and control (cross-hatched) rainbow trout (mean ± SE, n = 6). Dissimilar letters above bars indicate differences among treatments ($P < 0.05$), with body mass as a covariate.

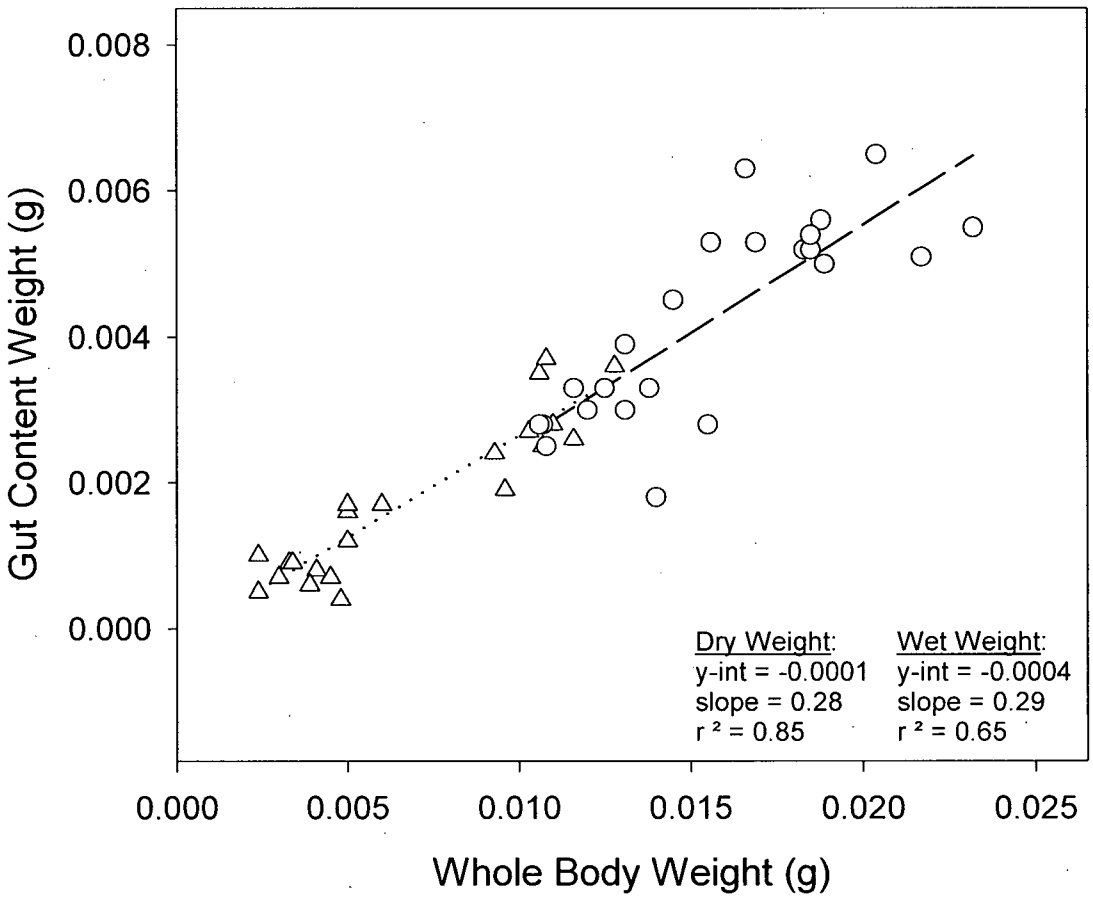


Figure 6.4: Relationship between body mass and mass of gut contents for sea lice with blood visible in digestive tracts. Dry (triangles) and wet (circles) masses were measured in 2001 and 2002. Regression lines are represented for dry (dotted) and wet (dashed) mass correlation.

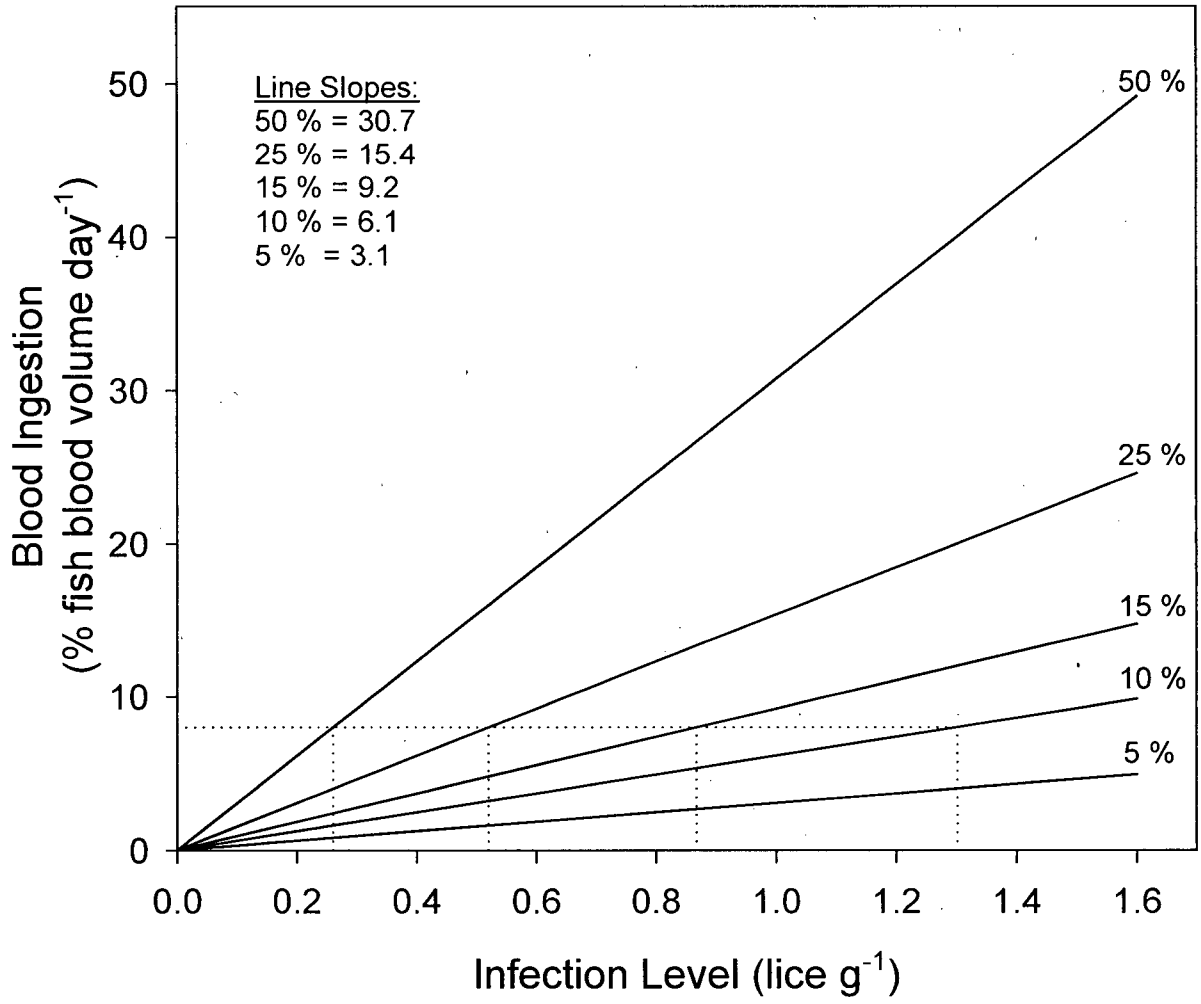


Figure 6.5: Predicted feeding rates of sea lice for given infection levels. Each line represents the feeding rate based on a given percentage of tissues consumed that consisted of blood. Dotted drop-lines indicate lice infection levels above which the amount of blood ingested would affect swimming performance based on results of the present study.

CHAPTER 7: GENERAL DISCUSSION

ADVANCES IN EXPERIMENTAL METHODS

The first two experimental chapters of this thesis (Chapters 2 & 3) are comprised of studies that empirically tested the materials and methods to later be used in studies involving the artificial infection of Atlantic salmon with sea lice. Several advances were made in these preliminary studies towards determining the best methods for testing the null hypothesis that sub-lethal infections of sea lice do not affect the physiology of Atlantic salmon. While these experiments did not involve actual lice infection, the techniques that were tested and refined allowed for subsequent accurate measurement of fish physiological variables. This improvement was necessary because the lice infection levels used were sub-lethal in nature and, therefore, detection of physiological changes was likely to be more abstruse.

Similar to other recent studies (Keene *et al.*, 1998; Cho & Heath, 2000), clove oil was found to be an effective anaesthetic and comparable to MS-222 with regard to the physiological response of fish (Chapter 2). However, clove oil also was shown to have other benefits including being more effective at short-term (1 hour) stress reduction, lower in cost (factor of 10), and non-toxic. Clove oil was a more effective short-term stress reducer because fish cortisol levels increased one hour following exposure to MS-222. Meanwhile cortisol levels of clove oil-exposed fish did not change from initial resting levels. Iwama *et al.* (1989) also suggested MS-222 may act as a stressor to fish, which seems to be corroborated in this study. While anaesthetics are used routinely to reduce stress in fish during blood sampling (Strange & Schreck, 1978; Barton & Peter, 1981) this is the first study to demonstrate that clove oil also is effective at reducing stress when used following sampling.

The benefits of clove oil anaesthetic use do not extend solely to physiological studies involving surgery. Suggestions for other researchers and the aquaculture industry include using low levels of clove oil during handling (eg. blood sampling, antibiotic injection, tank transfer)

and transport to minimise the stress levels of fish. Due to the organic nature of clove oil, it also can be used to suppress the nervous system of fish after sport fishing angling, as it will simply biodegrade. During short-term retention in live-wells, the anaesthetic would reduce stress and waste products, thereby decreasing mortality of the fish once released (Gustaveson *et al.*, 1991). The main question remaining about the efficacy of clove oil compared to MS-222 has to do with its faster induction and slower recover times. However, the amount of time brain receptors are blocked by the binding of either anaesthetic does not seem to significantly affect fish physiological variables.

Methodological test results corroborate those of previous studies that showed shorter recovery times (Keen & Farrell, 1994; Anderson *et al.*, 1997; Korsmeyer *et al.*, 1997) and time intervals (Davis *et al.*, 1963; Jones, 1971; Farrell *et al.*, 1998) do not significantly affect fish swimming performance. The use of 4 hours of recovery and 10 minute time intervals were found to be valid testing methods for measuring U_{crit} and blood and cardiac variables for studies involving combined stressors (Chapter 3). These methods were successfully used in subsequent studies (Chapters 4-6), producing substantial results. It was important to empirically test these different methods before their use in subsequent studies because, although they has been used in previous studies, in some cases their validity for accurate physiological measurement had not been tested. Similar empirical testing of common fish surgery practises were performed by Wagner *et al.* (2000) because the efficacy of methods used for over 50 years seldom were determined prior to use.

While the cardiac results ($\dot{Q}$, f_H, and V_S) were more variable than in studies involving long term recoveries of several days (Gamperl *et al.*, 1994a; Webber *et al.*, 1998) and 30+ minute time intervals (Gallaugher *et al.*, 1995), similar resting and maximum levels occurred in most cases. As well, fish swimming performance and blood variables were comparable to the above studies involving long-term recovery. The cardiac variables of fish constantly are

readjusted to attempt to maintain balanced oxygen delivery to the tissues and remove metabolic waste products at all level of activity. Despite the fact cardiovascular variables may require somewhere between 18-24 hours (Kiceniuk, 1976) and several days after surgery to return to absolute basal levels, it is implausible for most researchers to wait that length of time between treatments because of time and equipment limitations. Thus, certain concessions have to be made with respect to the statistical resolving power of cardiorespiratory measurements while still maintaining the ability to make viable conclusions from the data obtained. This certainly is one area of methodological research that remains to be clarified.

An advance in the understanding the causal factors of fatigue during prolonged swimming was made in Chapter 3. While the definitive answer for what causes exhaustion and the subsequent cessation of swimming still remains unclear, one possible culprit has been eliminated. Blood lactate levels were shown not to be linked to fatigue, as fish exposed to hypoxia had significantly slower U_{crit} values than normoxic fish as well as significantly lower lactate levels. This result along with those of Richards *et al.* (2002a) suggest that energy reallocation and direct stressor effects are the most likely causal mechanisms for fatigue in fish. In the case of hypoxia and exercise being the combined stressors, low oxygen may have had direct effects on muscle performance such as decreased oxygen availability for cell respiration and blood acidosis causing disruption of cell membrane activity. As well, acidosis caused indirect effects on the heart through vagus nerve stimulation due to reduced oxygen carrying capacity of the blood (Holeton & Randall, 1967; Randall, 1970; Wood & Shelton, 1980; Gamperl *et al.*, 1994a). Specifically, hypoxic stress decreased U_{crit} similar to past studies (Jones, 1971; Farrell *et al.*, 1998), and affected the cardiovascular system. The observed trends of raised $\dot{V}s$ and decreased f_H in exercising hypoxic fish corroborate the theory that blood residence time within the heart and gills increases to maximise O_2 uptake (Farrell, 1984; Farrell *et al.*, 1989) and increase coronary oxygen delivery in some teleosts (Gamperl *et al.*, 1995). These findings

provided a comparative benchmark for the studies involving sea lice because they were the first measurements of stressor effects in combination with maximum prolonged swim testing. It is important for future studies to further examine the effects of stressors on exercising fish in order to determine the mechanisms involved in energy partitioning between interacting physiological systems.

PHYSIOLOGICAL IMPACT OF LICE

Due to the complex life history of Atlantic salmon that involves large-scale ocean migrations and difficult upstream travel to spawn, the swimming performance of this fish is extremely important to its survival and reproductive fitness. The ability of salmon to traverse potentially high water flows and obstacles upon their return to native streams is impeded by their cessation of feeding. It is because of this finite energy reserve that returning salmon are vulnerable to stressors that can reduce energy stores and affect their ability to reach the spawning grounds and successfully spawn. It is therefore imperative that salmon maximise their energy reserves, in the form of fat reserves, during their ocean feeding time. The results of the studies performed in this thesis indicate that energy conservation in the presence of increased numbers of external parasites such as sea lice may not be possible. Using the tested and refined materials and methods of the preliminary studies, several questions have been answered about the effects of sub-lethal levels of sea lice on their host. Studies involving artificial infection with varying levels of sea lice were performed along with exposure to seawater (SW) and freshwater (FW) and repeated blood loss (Chapters 4-6). The null hypothesis that sub-lethal infections with sea lice do not affect the physiology of Atlantic salmon was rejected because of significant declines in U_{crit} and changes to blood and cardiac variables. These changes could lead to an increased depletion of energy stores in salmon in SW before their return to FW, with potential deleterious consequences to swimming ability and spawning success.

Sea lice were found to have significant effects on the physiology of their host at sub-lethal levels much lower than infection levels that lead to morbidity (0.75-1.0 lice g^{-1}; Wootten *et al.*, 1982; Grimnes & Jakobsen, 1996; Bjørn & Finstad, 1997). Infection levels of approximately 0.1 lice g^{-1} altered the cardiac performance of Atlantic salmon during exercise and led to 19-22 % reductions in U_{crit} (Chapter 4 & 5). While the osmotic balance of infected fish was not disrupted consistently, energy use may have increased in the osmoregulatory and cardiovascular systems. Evidence of energy increase included the significant reduction in U_{crit} and changes to f_H and V_S, while $\dot{Q}_{max}$ did not differ significantly. This theory of energy loss also was supported by the fact short-term exposure to FW improved the U_{crit} of infected fish back to control levels (Chapter 5). Infected fish exposed to FW also had increased heart rates at U_{crit} compared to those of infected fish (~ 0.1 lice g^{-1}) in SW (Chapter 4 & 5) and hypoxic fish (Chapter 3). Beyond the alleviation of osmotic imbalance that results in reduced energy use, fish also benefit from the fact that lice cannot osmoregulate in FW and eventually detach. These physiological benefits help to explain the behaviour of heavily infected wild fish that return early to native streams. Fish infected with lice, at levels far below those found to be lethal, can be subject to chronic system stress and a decline in prolonged swimming performance that may be detrimental to their survival. Reduced swimming performance can negatively impact the ability of infected fish to forage or escape predators, and reduce energy reserves needed for FW migration and gamete development. Based on a predictive lice-feeding model, it is possible that these physiological changes are due in part to blood loss at higher sub-lethal levels (Chapter 6). From 15-25 % of the host tissues sea lice feed on was predicted to be blood. At sub-lethal concentrations of lice, this level of consumption would not significantly affect the swimming performance of fish. However, blood loss at lice infections nearing lethal levels (0.5+ lice g^{-1}) can contribute significantly to host morbidity. The accrued results of the sea lice infection studies indicate a serious need to curb the increase in lice epizootics observed during the past two

decades because of the serious impact that sub-lethal infection levels can have on the physiology of their hosts.

PLAN OF ACTION

The fact sea lice can significantly affect the physiology of salmonids at levels 5-20 times lower than have been found on wild fish in areas of intensive aquaculture (0.5-2.1 lice g^{-1}; Bjørn *et al.*, 2001) is of great concern. Although these numbers were obtained in a single year and the intensity of infections at local aquaculture sites were not recorded, the implementation of lice removal programs at all aquaculture sites would be a proactive approach. If lice elimination is conducted, infection levels in intensive aquaculture areas could be reduced to levels similar to those being found in pristine areas (0.02 lice g^{-1}; Bjørn *et al.*, 2001; Bjørn & Finstad, 2002) that do not impair Atlantic salmon (Chapter 4). The types of treatments vary from topical bath application at reduced water volume (Wootten *et al.*, 1982; Grave *et al.*, 1991) to oral application as a component of fish diet (Stone *et al.*, 1999, 2000; Ramstad *et al.*, 2002).

Two related problems exist with current lice reduction programs. The first problem is the lack of knowledge about the percentage of lice actually killed during treatment. Knowing this percentage is important because lice simply may detach and become free living for up to 30 days or more (Bron *et al.*, 1993; Ritchie, 1997) until a new host fish is found. The most likely new hosts are wild fish travelling in the immediate area of sea cages while reattachment to fish in the cages also is possible. Oral treatments seem to have more promise than bath treatments because of continued lice reduction for 20+ days, and the ability to remove chalimus stages as well as adults. The second problem with current removal programs is their proper implementation by aquaculture facilities typically is not enforced consistently by a governing body. Without consistent use of either treatment, new lice populations quickly can become re-established. The findings of this dissertation warrant that these two problems be addressed. Until such time, the

physiology and behaviour of known susceptible species (ie. Atlantic salmon, Arctic charr, sea trout) in areas of intensive aquaculture might continue to be negatively impacted, with possible serious repercussions to populations already in distress.

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