THE PHYSIOLOGICAL STRESS RESPONSE OF SALMONIDS:

CONSIDERATIONS FOR FIELD PROCEDURES AND ENVIRONMENTAL MONITORING

By

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Abstract

Investigating the physiological state of wild salmonids is challenging on many levels. The sensitive nature of an integrated physiological stress response directs how biological data is collected in the field and, consequently, how the results are interpreted. This thesis is comprised of two main components. The first component encompasses laboratory-based studies addressing the potential confounding effects of: 1) anaesthesia with either tricaine methanesulphonate (tricaine) or clove oil (eugenol) prior to blood/tissue sampling, and 2) capture by electroshocking, on the immediate and short-term responses of plasma/serum cortisol and glucose concentrations, haematocrit, plasma/serum lysozyme activity, and total leucocyte abundance in juvenile chinook salmon (*Oncorhynchus tshawytscha*). The second component involves a field-based exploration of the *in situ* physiological status, using the same five physiological traits, of wild bull trout (*Salvelinus confluentus*) in the Torpy River watershed, B.C., in relation to selected habitat attributes (stream gradient, discharge rate, and riparian canopy-closure).

Anaesthetization and electroshocking did not significantly alter values for the five physiological traits provided that post-capture blood sampling occurred immediately. Tricaine and clove oil immobilization produced similar effects on the physiological stress response of juvenile chinook salmon. Clove oil (eugenol) shows promise as a viable and safe alternative to tricaine for aquacultural purposes and in laboratory- and field-based research.

Electroshocking is an acute stressor from which juvenile chinook salmon can recover physiologically (usually within 12-24 h). Handling without shocking, however, significantly reduced serum lysozyme activity for up to 2 wks post-stress. Radiographs indicated that while

some degree of spinal abnormality exists naturally in domestic chinook juveniles, individuals exposed to a single brief shock incur significantly more spinal deformities.

Some of the variation in the stress physiology and non-specific immune function of wild bull trout in the Torpy River system were explained by the combined effects of stream gradient, discharge rate, and riparian canopy-closure. The physiological measurements of wild bull trout generally did not differ from those reported in the literature for other salmonid species. The "background" effects of these habitat features on the physiology of wild salmonids must be considered when interpreting field-collected data.

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GENERAL INTRODUCTION

The Integrated Stress Response

The close association between organism and environment renders fish highly sensitive to changes in their aquatic habitat. A fish can respond to such changes behaviourally through avoidance or physiologically through biological adjustment processes. It is thought that a psychological component also exists (Schreck 1981; Barton 1997). If avoidance is impossible or unlikely, then the fish pays a physiological price for continued exposure to an altered or sub-optimal environment.

External stimuli that can induce an altered physiological state in an organism are called stressors. Stressors can be of biological, chemical, or physical origin. A stress response is a dynamic process of adaptation to a stressor or stressors. A physiological stress response is often indicative of an organism's capacity to deal with stressors, depending on the nature of the stressor. Four levels of biological organization are affected by stressors and form the basis of the integrated stress response (Wedemeyer *et al.* 1984; Wedemeyer 1996; Wendelaar Bonga 1997). The stress response is often species-specific as well as being variable among populations of the same species (Schreck 1981). There can be a cumulative effect from repeated or multiple stressors (Barton *et al.* 1986; Mesa 1994).

The primary (1°) response is a neuro-endocrine response through the hypothalamus and pituitary gland near the brain, and the interrenal tissues located within the head kidney, collectively known as the hypothalamic-pituitary-interrenal (HPI) axis. The hypothalamus detects changes in the external environment (i.e. stressors) and relays this message to the pituitary gland which initiates a hormonal response by secreting adrenocorticotropic hormone

(ACTH), among others. This, in turn, stimulates the interrenal cells to release corticosteroids (e.g. cortisol) into the circulatory system. The modifying effects of cortisol at target organs (gills, intestines, and liver) reflect its role in hydromineral balance and energy mobilization (Wendelaar Bonga 1997). Cortisol has also been implicated in reproductive function, growth, and immunocompetence (Barton and Iwama 1991; Balm 1997; Pankhurst and Van Der Kraak 1997). Significant increases in blood cortisol levels occur naturally in two life-phases of salmonids: during smoltification and spawning. Smoltification is the process whereby changes in behaviour, morphology, and physiology prepare young salmon for life in seawater. Cortisol concentrations also increase during final maturation and spawning stages (Barton and Iwama 1991). These life events are considered to be stressful and have been demonstrated to be associated with immunosuppression (Maule *et al.* 1987; Maule *et al.* 1993).

The secondary (2°) response is manifested by altered blood chemistry and changes in histology. The progression of physiological responses from the cellular (primary) level to the onset of responses at the tissue (secondary) level reflects the initial adaptive nature of a stress response. Some of these changes are through direct and indirect effects of the primary hormonal response. Examples of such characteristics are hyperglycemia (increased blood glucose levels), faster blood-clotting, and greater gill perfusion. Should exposure to the stressor(s) continue (i.e. chronic stress), the secondary response usually becomes maladaptive (e.g. immunosuppressive).

Changes at the whole-animal level are indicative of the tertiary (3°) response and often reflect the maladaptive consequences of stress. A fish may exhibit reduced overall vigour, lower disease resistance, reduced growth, impaired maturation, poor reproductive success, and altered behaviour, all of which can result in lower survival rates (Wendelaar Bonga 1997).

The quaternary (4°) response involves adverse effects that extend to and become apparent at the population-level such as lower recruitment of fish stocks. If prolonged, these changes may eventually affect the ecosystem through disturbed trophic interrelationships (Wedemeyer 1996).

Effects of Stress on Immune Function

An organism's capacity to raise an effective immune response against various stressors and pathogens has become an increasingly important area of research. The adverse effects of stress on fish immunocompetence are well-established (Maule *et al.* 1989; Pickering and Pottinger 1989; Ainsworth *et al.* 1991; Pickering 1993). Pickering (1993) discussed several mechanisms by which chronically elevated cortisol levels could reduce the effectiveness of specific and non-specific immune function. The number and performance of circulating leucocytes (white blood cells, wbcs) can change substantially in response to both acute and chronic stressors (Ellsaesser and Clem 1986; Ainsworth *et al.* 1991; Pulsford *et al.* 1994).

Stress can also compromise non-specific (innate) immunity. The non-specific immune system is important at all life-stages, from the embryonic development of fish through to adulthood. Lysozyme, an important component of the non-specific immune system, is responsive to various stressors (Möck and Peters 1990; Demers and Bayne 1997; Rotllant *et al.* 1997). Lysozyme (also called muramidase) is a hydrolytic enzyme that can be found in hen egg whites, tears, mucous and colostrum. In fish, it is can be found in eggs (Yousif *et al.* 1991) and in very young fish which rely heavily upon their non-specific immune system until their specific immune system matures (Manning *et al.* 1982; Takemura 1996). It is one of several hydrolytic enzymes released from neutrophils and macrophages. Lysozyme helps to compromise the

integrity of bacterial cell walls by hydrolysing the β1-4-glycosidic bonds between *N*-acetyl muramic acid (NAM) and *N*-acetyl-glucosamine (NAG) subunits which form an integral component of bacterial cell walls. Lysozyme is particularly effective in destroying Gram positive cells since NAM and NAG are exposed on the cell exterior. Lysozyme relies on other components of the non-specific immune system (i.e. complement) to gain access to the NAM and NAG between the double-walled membrane of Gram negative bacteria. The lysozyme of fish, however, appear to be quite effective against some Gram negative pathogens (Grinde 1989).

Monitoring Fish Physiology in the Field: Problems and Considerations

Tremendous advancements in the field of fish stress physiology have been, and continue to be, achieved through controlled laboratory-based research. Such studies not only contribute to growing knowledge of the physiological impacts of specific stressors on different fish species, but also serve to validate and improve the assays required to make accurate and precise measurements.

Investigating the physiological stress of any organism in field-based studies is challenging on many levels. First, the establishment of environmental controls is impeded by dynamic interactions among various habitat features. Such ecological relationships make it difficult to isolate or identify potential environmental stressors. Wild salmonids are subject to environmental variations on a daily and seasonal basis, thus confounding the interpretation of physiological data (Adams 1990).

Second, the establishment of physiological controls requires a knowledge of the physiological baseline range for wild fish. In light of the first point mentioned above, this may

be problematic since habitats vary from system to system and, hence, the physiological makeup of local fish populations may also vary (i.e. intraspecies variation). The physiological values of wild and hatchery-reared fish are often compared since those of domestic fish have been studied extensively and are well documented. But hatchery-reared and wild salmonids can differ greatly in their physiological response to acute stressors and this difference has been attributed to dissimilar rearing environments (Woodward and Strange 1987; Salonius and Iwama 1993).

Third, is it possible to differentiate between physiological responses due to normal environmental/seasonal fluctuations and those due to sublethal environmental disturbances? The emphasis of using animals as environmental biomarkers has largely been limited to toxicants or pollution (Peakall 1992). Since natural habitat fluctuations cannot be avoided, it is important to examine their effects on the physiology of fish and, subsequently, to take these effects into consideration when interpreting results.

Investigating adverse environmental effects in fish populations in the field has been primarily limited to the quaternary (population level) stress response by monitoring disease incidence, changes in stock recruitment or returning number of spawning adults, or altered growth rate. The main disadvantage of monitoring fish populations at the quaternary level is that by the time adverse effects are apparent, the damage has already been done. Preventative (proactive) detection strategies are long overdue and mitigation is often ineffective because of delayed (reactive) management action. Early detection is ideal and may be achieved through the tracking of physiological attributes at the primary and secondary levels rather than at later stages when damage may be extensive and/or irreparable.

This thesis is generally divided into two main parts. Chapters 1 and 2 address the potential confounding effects associated with experimental procedures (anaesthetization and

capture by electroshocking) performed in the field to collect biological samples. Through the incorporation of the research protocols outlined in the previous two chapters, Chapter 3 examines the extent to which selected habitat features, not normally considered as stressors, contribute to the physiological variation in wild fish. Analysis and interpretation of field-collected data may be erroneous if these effects are ignored.

CHAPTER 1.

Comparison of Tricaine (MS222) and Clove Oil Anaesthesia Effects on the Stress Physiology and Immune Function of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*)

1.1 ABSTRACT

The physiological stress response and immune function of juvenile chinook salmon (*Oncorhynchus tshawytscha*) anaesthetized with either tricaine methanesulphonate (50 mg/L) or clove oil (20 ppm) were compared using unanaesthetized fish as controls. The feasibility of replacing tricaine with clove oil as an alternative fish anaesthetic, particularly in fish stress research, is also explored. Haematocrit, serum cortisol, serum glucose, serum lysozyme activity, and differential leucocyte counts were determined from blood samples collected prior to, during, and upon recovery from anaesthesia, and at specified intervals up to 72 h post-recovery. Differences between the two anaesthetic groups were not significant for any trait measured during anaesthetic induction and after recovery. Serum lysozyme activity of control fish, however, was significantly lowered for 72 h post-stress. Clove oil may be a cost-effective and environmentally safe alternative to tricaine in aquacultural operations and for laboratory- and field-based research.

1.2 INTRODUCTION

Chemical and non-chemical means to immobilize fish help to minimize the full impacts of handling procedures on their physiology. Physiological studies require the minimization of possible confounding factors, including the anaesthetization that is necessary before performing specific surgical or physiological procedures. Although anaesthesia benefits the fish by reducing the impact of a greater, more severe, stressor(s), it is also inherently stressful (Soivio *et al.* 1977; Strange and Schreck 1978; Barton and Peter 1982; Laidley and Leatherland 1988; Iwama *et al.* 1989). This is of critical importance in the field of fish stress physiology and related research that examine stressors other than anaesthesia. The issues of physiological changes due to anaesthesia and whether these changes influence subsequent biological sampling must be addressed. This study investigated the post-recovery (72 h) physiological stress response of juvenile chinook salmon briefly immobilized in either tricaine or clove oil solutions.

A general anaesthetic is an external agent that produces a reversible loss of sensation (i.e. anaesthesia) by paralyzing sensory nerve endings or nerve fibres and is accompanied by a loss of consciousness. Anaesthetics depress the central nervous system (CNS) by inhibiting the generation or transmission of nerve impulses. This can be achieved by preventing the influx of Na^+ ions into the nerve cell thereby stopping the initiation of a nerve impulse (i.e. prevent nerve cell depolarization). Or conversely, the outflux of Na^+ ions from a nerve cell can be blocked to maintain a depolarized state which would prevent additional impulses from being transmitted (i.e. prevent repolarization). A neural block can also occur by preventing the synthesis of neurotransmitters or their transmission across synaptic gaps.

Certain chemicals can be used to relieve pain or tension (i.e. analgesics, muscle relaxants) or to induce a state of calm/stupor (i.e. narcotics, sedatives, tranquilizers) in an organism.

Tranquilizers, for example, result in a calmed state but not in analgesia (Summerfelt and Smith 1990). Consequently, these substances are not administered as true anaesthetics since the treated animals are often capable of responding given sufficient stimulation or provocation (Giovanoni and Warren 1983).

Types of Anaesthetics

A fish is usually immobilized, either chemically or non-chemically, to facilitate safer fish handling. The choice of anaesthetic depends on several considerations: (1) availability, (2) cost effectiveness, (3) ease of use, (4) nature of the study, and (5) safety to the user. Chemical fish anaesthetics fall into three main categories depending on route of administration or physical state: inhalation, parenteral injection, and gas. Non-chemical means of fish immobilization are hypothermia (cold shock) and electroshock (galvanonarcosis). For more extensive listing and greater detail of specific anaesthetics, refer to Ross and Ross (1984) and Iwama and Ackerman (1994). Table 1.1 summarizes anaesthetic types and includes some advantages and disadvantages as they pertain to field research or aquacultural operations.

For field sampling purposes, anaesthesia induced by either parenteral injection or by gas administration are not practical. Parenteral anaesthetics are strictly for laboratory-based research and are not approved for use in food fish or released wild fish. Injecting anaesthetics into individual fish is also time-consuming and labour intensive. Although effective, carbon dioxide produces shallow anaesthesia at concentrations that are difficult to control and maintain (Ross and Ross 1984), and the gas cylinders are unwieldy and costly for field purposes. However, carbon dioxide is advantageous in that no chemical residue remains in tissues.

During anaesthetic induction, a fish undergoes several stages (Table 1.2). The point at which a fish is considered fully anaesthetized and ready for handling or surgery occurs when

Table 1.1. Descriptions of different types of fish anaesthetics (Ross and Ross 1984). Advantages and disadvantages are given as they relate to efficacy in the field or in aquaculture.

Class	Type	Method / Route	Examples	Advantages	Disadvantages
CHEMICAL	Inhalation	Chemical in solution and ventilated over gills. Drug enters circulatory system directly at gills.	tricaine (MS222) benzocaine quinaldine 2-phenoxyethanol metomidate	• most common method • easy to use • easy to transport	• only tricaine is approved for use in food fish and released wild fish (requires a minimum 21 d withdrawal period in both cases)
	Parenteral injection	Injection into the internal body cavity (intraperitoneal), into the circulatory system (intravascular), or into a muscle mass (intramuscular).	sodium thiopentone ketamine-HCl xylazine-HCl	• long-lasting	• long induction times • strictly for laboratory-based research • inefficient for large number of fish (i.e. aquaculture)
	Gas	Bubbling a soluble gas into the water.	carbon dioxide (CO_2)	• no residual traces in tissues	• shallow anaesthesia • difficulty to control and maintain effective levels • unwieldy equipment
NON-CHEMICAL	Hypothermia	Reduced metabolism (i.e. lower O_2 use) by exposure to lower water temperatures.	Ice slurries or ice blocks in water	• most effective in fish acclimated to $\geq$ 10°C.	• light anaesthesia only applicable for transport, but not surgery or invasive tissue sampling
	Electroshock	Induces galvanonarcosis with low intensity electroshock.	---	• instant effect	• user-risk • potential for extensive physiological effects and physical injuries in fish

Table 1.2. Stages of anaesthesia induction and recovery (from Summerfelt and Smith 1990).

Stage of anaesthesia	Descriptor	Behaviour exhibited
0	Normal	Reactive to external stimuli; opercular rate and muscle tone normal.
1	Light sedation	Slight loss of reactivity to external visual and tactile stimuli; opercular rate slightly decreased; equilibrium normal.
2	Deep sedation	Total loss of reactivity to external stimuli except strong pressure; slight decrease in opercular rate; equilibrium normal.
3	Partial loss of equilibrium	Partial loss of muscle tone; swimming erratic; increased opercular rate; reactive only to strong tactile and vibrational stimuli.
4	Total loss of equilibrium	Total loss of muscle tone and equilibrium; slow but regular opercular rate; loss of spinal reflexes.
5	Loss of reflex reactivity	Total loss of reactivity; opercular movements slow and irregular; heart rate very slow; loss of all reflexes.
6	Medullary collapse	Opercular movements cease; cardiac arrest usually follows quickly.
Stage of recovery		Behaviour exhibited
1		Reappearance of opercular movement.
2		Partial recovery of equilibrium with partial recovery of swimming motion.
3		Total recovery of equilibrium.
4		Reappearance of avoidance swimming motion and reaction in response to external stimuli, but still behavioural response is stolid.
5		Total behavioural recovery. Normal swimming.

there is a total loss of equilibrium, breathing rate is slow but steady, and there is no reactivity to external stimuli (described by stage 5 in Table 1.2). Should exposure to anaesthetics continue, the fish will eventually experience medullary collapse with death following shortly thereafter. In practice, chemical immobilization of fish is rarely taken to this final stage (stage 6). Even if opercular movements cease, there is a short time interval during which recovery is still possible. Recovery is unlikely, however, once cardiac arrest occurs. The stages of recovery (Table 1.2) are essentially the reverse of the induction process. It is important to note that, in practice, the assessment of full recovery is often based upon the restoration of behavioural (i.e. regained equilibrium), rather than physiological, characteristics. This gives a degree of latitude in the interpretation of "recovery." From a stress physiology perspective, a return to a physiological state similar to that before exposure to the biological, chemical, or physical stressor(s) signifies complete recovery.

Literature on the effects of different anaesthetics, particularly tricaine methanesulphonate, on fish physiology is extensive (Soivio *et al.* 1977; Strange and Schreck 1978; Barton and Peter 1982; Iwama *et al.* 1989). Tricaine methanesulphonate (3-aminobenzoic acid ethyl ester methanesulphonate) is a highly soluble white crystalline powder (1.0 g tricaine/0.8 mL H_2O, 20°C). Tricaine is its generic name. When dissolved in water, it greatly reduces the pH of the water due to the presence of methanesulphonic acid. A tricaine anaesthetic bath solution is thus commonly buffered, usually with sodium bicarbonate ($NaHCO_3$). Tricaine acts directly on the sensory centres of the CNS (Summerfelt and Smith 1990). Although quite potent and effective in low concentrations, tricaine is expensive and is a suspected carcinogen, although not a mutagen (Yoshimura *et al.* 1981, cited in Summerfelt and Smith 1990).

Currently, MS222™ and Finquel™ (two formulations of tricaine methanesulphonate) are the only anaesthetics recognized by the U.S. Food and Drug Administration for safe use in fish

destined for human consumption (Kelsch and Shields 1996), provided that a 21 d withdrawal period elapses before the fish are harvested. A 21 d holding period is also recommended for wild fish anaesthetized in the field with tricaine prior to release back into the water (Ross and Ross 1984; Kelsch and Shields 1996). In Canada, the Bureau of Veterinary Drugs recommends that fish treated with tricaine should ideally be held for 5 days prior to release as long as the water temperature is ≥10°C (S. St-Hilaire[1], pers. comm.). The higher temperature allows for faster metabolic removal of the drug from the fish. At water temperatures <10°C, a 21 d withdrawal period is then advised. Unless the cost and feasibility of holding facilities in the field can be overcome, a mandatory withdrawal period is a clear logistic disadvantage.

Clove oil is a relatively recent addition to the array of chemicals available for fish immobilization. Clove oil has received favourable reviews as an alternative fish anaesthetic for a variety of fish species (Hikasa *et al.* 1986; Soto and Burhanuddin 1995; Anderson *et al.* 1997; Munday and Wilson 1997; Keene *et al.* 1998) as well as for crustaceans (Gardner 1997). Eugenol (4-allyl-2-methoxyphenol), the active component of clove oil, is obtained from the buds, leaves, and stems of the *Eugenia caryophyllus* plant. Clove oil has several advantages over tricaine in fisheries research, assessment studies, and aquacultural applications. It is an easily and inexpensively obtained organic distillate that is used as a food additive and possesses anti-fungal and anti-bacterial properties. Because clove oil is organic, no withdrawal period is currently required for fish intended for human consumption. In addition, it is does not pose a chemical health hazard to the user. The main disadvantage of clove oil is its photosensitivity leading to gradually reduced effectiveness over time. This is easily prevented by storing the

[1] D.V.M., Department of Fisheries and Oceans, Pacific Biological Station, Hammond Bay Road, Nanaimo, BC, V9R 5K6.

clove oil in an opaque container. Clove oil achieves its anaesthetic properties by inhibiting prostaglandin H (PGH) synthase, an enzyme required for the synthesis of the neurotransmitter prostaglandin H (Dewhirst and Goodson 1974; Thompson and Eling 1989; Pongprayoon *et al.* 1991). Hence, inter-neuron impulse conduction is impeded.

Three other studies examined clove oil (eugenol) anaesthetization in salmonids (Endo *et al.* 1972; Anderson *et al.* 1997; Keene *et al.* 1998). Endo *et al.* (1972) studied the efficacy of clove oil anaesthesia at various dose levels in rainbow trout (*Oncorhynchus mykiss*) as did Keene *et al.* (1998) who additionally examined lethal concentrations (LC_{50}) of clove oil for rainbow trout. Anderson *et al.* (1997) investigated the effects of clove oil immobilization on the swimming performance of rainbow trout. Iwama *et al.* (1989) obtained physiological (stress) measurements from cannulated rainbow trout anaesthetized in five different chemicals, but clove oil was not one of the drugs studied and post-recovery stress effects were not examined. Therefore, studies designed to provide insight into the effects of clove oil (or eugenol) anaesthetization on fish stress physiology are required.

This experiment was conducted to fulfill three goals. First, it addresses the issue of physiological changes during anaesthesia. The best stage at which to handle a fish or collect tissue/blood samples is when the fish has lost equilibrium and does not respond to external stimuli (see stage 5 induction, Table 1.2). Ideally, this stage of anaesthesia is reached within 5 min (often within 2-3 min) of initial exposure to the anaesthetic. It is possible that the length of this time interval is sufficient for certain physiological variables (e.g. blood hormone levels) to change significantly from "pre-anaesthesia" values. Are biological samples collected at Stage 5 anaesthesia reflecting true "pre-stress" measurements? or, are they confounded by anaesthetic effects?

Second, if exposure to an anaesthetic is stressful, how long do post-recovery effects persist? The impact of multiple stressors is known to be cumulative (Mesa 1994; Barton and Peter 1982), and repeated stressors elicit a stress response of successively greater magnitude (e.g. cortisol response). Physiological values determined at various time intervals after exposure to an experimental stressor may be further compounded by any residual effects of the anaesthetic.

Third, although the physiological impacts of tricaine in fish (particularly salmonids) are well-documented, its use in the field poses several problems. The main drawback is that fish anaesthetized with tricaine must be held for a minimum of 5 d prior to release. This is not an option if holding tanks in the field are unavailable. In addition, used tricaine solutions cannot be discharged into natural water systems. Replacing tricaine with clove oil would permit immediate release of fish back into the water. Clove oil would be a cost-effective alternative since it is potent at very low concentrations (approximately 10-40 ppm) in both fresh- and seawater. Therefore, in addition to investigating the stress effects of immobilization with the most commonly used fish anaesthetic (tricaine), this study also explores the possibility of replacing tricaine with clove oil for physiology research and its practicality for use in the field.

1.3 MATERIALS AND METHODS

Approximately 500 juvenile (age 1 year) chinook salmon (*O. tshawytscha*) were obtained from Yellow Island Aquaculture Ltd. (Y.I.A.L.) located on Quadra Island, British Columbia. Mean body weight and fork length were 40.18 ± 0.56 g and 15.6 ± 0.1 cm, respectively. The juveniles were transferred from on-site seawater netpens to a freshwater holding trough (3 000 L) and allowed to acclimate for 2.5 weeks at 7°C (October - November 1997). Fish were fed daily during the acclimation period and were fasted for 24 h prior to the start of the study.

1.3.1 Preparation of Anaesthetics, Treatment, and Sampling

Two aerated 80 L anaesthetic baths were prepared using freshwater (7°C). The first bath contained a buffered tricaine solution (50 mg/L, 1:1 ratio of tricaine to sodium bicarbonate, $NaHCO_3$) while the second bath held a solution of clove oil (20 ppm). A preliminary trial with juvenile chinook salmon of similar size established that a clove oil concentration of 20 ppm induces stage 5 anaesthesia (Table 1.2) in the same time interval as the prepared tricaine solution (within 2 min). A solution of 20 µL clove oil/L H_2O yields a final concentration of about 20 ppm as clove oil density is 1.04 g/mL (similar to water density). Because essence of clove oil is insoluble at water temperatures below 15°C, the clove oil was first dissolved in 95% ethanol (1:10 ratio of clove oil to ethanol) before adding to freshwater (Anderson *et al.* 1997). Keene *et al.* (1998) stated that the 96 h effective concentration (EC_{50}) of ethanol for 80 g rainbow trout was 12 000 – 16 000 ppm. Thus, the level of ethanol used to dissolve the clove oil in this study is not likely to have affected the results.

One hundred and twenty control fish were netted from the holding trough and carefully divided among 8 tanks (n=15 fish/tank) without being anaesthetized. Two treatment groups of fifty fish each were netted from the trough and gently transferred to the prepared anaesthetic baths, one group in each type of anaesthetic. When all treatment fish reached stage 5 anaesthesia (within 2 min), they were carefully transferred to freshwater tanks (140 L) for recovery (n=5 fish/tank). Since groups of fish were kept separate according to treatment and intended sampling time, each tank was sampled only once, thus eliminating the effects of multiple sampling from the same tank.

Treatment fish were sampled at 10 time intervals: prior to anaesthetic exposure, stage 5 anaesthesia, stage 5 recovery, and 1, 2, 6, 12, 24, 48, and 72 h post-recovery (n=5 fish/sampling). Unanaesthetized control fish were sampled at 8 time intervals (n=15 fish/sampling). Control fish

were not sampled at stage 5 anaesthesia and stage 5 recovery since they were not exposed to anaesthetics except for euthanization before blood sampling.

Prior to blood sampling by caudal severance, fish were euthanized in a lethal solution of tricaine (200 mg/L buffered with $NaHCO_3$). While anaesthetization with lower tricaine concentrations (25-50 mg/L) can elicit a cortisol response (Strange and Schreck 1978), exposure to levels ≥100 mg/L does not produce immediate changes in serum cortisol levels (Strange and Schreck 1978; Barton *et al.* 1986; Laidley and Leatherland 1988) which could confound experimental results. Body weights, fork lengths, and haematocrits were measured and blood smears were made for differential leucocyte counts. The remaining blood was allowed to clot at about 4°C and centrifuged to collect serum. The serum was stored at -80°C for later analysis of cortisol and glucose concentrations, and lysozyme activity.

1.3.2 Haematocrit Measurement

Heparinized haematocrit (hct) capillary tubes were filled with blood and sealed at one end with a putty sealant (Critoseal™) and centrifuged in a haematocrit centrifuge (IEC Clinical Centrifuge). Manual calipers were used to measure the packed red cell volume. Haematocrit (hct) values were expressed as the percent ratio of the length of the rbc column to that of the total sample column in the hct tubes.

1.3.3 Serum Cortisol Determination

Serum cortisol was determined with iodine-125 (I^{125}) radioimmunoassay (RIA) kits (Diasorin, Stillwater, MN) modified as in Heath *et al.* (1993). Serum samples (10 μL) with I^{125}-labelled reagents in antibody-coated tubes were incubated in an air incubator (VWR Scientific mini hybridization oven, Model 2700) for 45 min at 37°C. Radioisotopic activity (counts per

minute, cpm) was determined by a gamma counter (IsoData, Series 500). The cpm were then converted to cortisol concentration (μg/mL) using CurveExpert software (v.1.2). A standard curve was obtained using known standard concentrations of 0, 10, 30, 100, 250, and 600 ng/mL cortisol.

1.3.4 Serum Glucose Determination

Serum glucose was measured with an enzyme-based colourimetric diagnostic kit (modified Trinder method, procedure no. 315; Sigma Chemical Company, St. Louis, MO; see Heath *et al.* 1993). This time-dependent assay is based on two enzyme reactions:

1) glucose + H_2O + O_2 $\xrightarrow{\text{(glucose oxidase)}}$ gluconic acid + H_2O_2

2) H_2O_2 + 4-aminoantipyrine + ρ-hydroxybenzene sulphonate $\xrightarrow{\text{(peroxidase)}}$ quinoneimine dye + H_2O

The intensity of the resulting pink colour, measured with a UV-Vis light spectrophotometer (Perkin-Elmer UV/Vis Spectrometer, Lambda 2S) is directly proportional to glucose level.

1.3.5 Serum Lysozyme Activity Measurement

Serum lysozyme activity was determined by the lysoplate method developed by Osserman and Lawlor (1966) with modifications (Lie *et al.* 1986; Grinde *et al.* 1988) using 0.06 M sodium phosphate buffer adjusted to pH 6.24 using 1.0 N sodium hydroxide, NaOH. Saline agar media plates with a known concentration of *Micrococcus lysodeikticus* (M-3770; Sigma Chemical Company, St. Louis, MO) were made (0.6 g *M. lysodeikticus* and 1.169 g NaCl per 1.0

L buffer). Twenty-five millilitres of this media were auto-pipetted into sterile petri plates (100 mm diameter). Serum samples (15 uL) were pipetted into 8 wells which had been punched around the perimeter of the media plates with a 3 mm diameter cork borer attached to a water vacuum apparatus. Three additional wells were punched in the centre for the external standard, hen egg white lysozyme (HEWL) (L-3770, Sigma Chemical Company, St. Louis, MO). A quality control turbidimetric assay (EC 3.2.1.17, Sigma Chemical Company, St. Louis, MO) was performed to determine enzymatic activity levels of HEWL standards. HEWL standards were made up in concentrations ranging from 100 to 20 000 μg/mL. Under the specified conditions (i.e. temperature, pH, substrate concentration), one unit (1.0 U) of enzyme activity is equal to the amount of enzyme that yields a change in spectrophotometric absorbance by 0.001 per minute (Grinde *et al.* 1988).

Lysoplates containing standards and serum samples were placed in a moist chamber (an insulated lidded foam box with wet paper towels on the bottom) and incubated overnight for 17 h at approximately 21°C. After incubation, the diameters of the resulting clearance zones were delineated with a fine-tip marker and measured with manual calipers in 3 different directions. The mean diameter was obtained and included in the statistical analysis. Lysozyme activity is proportional to the log of the clearance zone diameter according to the following equation, using linear regression analysis (where A and B were empirically calculated from known standard concentrations):

$$Y = A + B * \log (X)$$

where Y = diameter of clearance zone (mm)
A = constant
B = slope
X = lysozyme activity (U/mL)

1.3.6 Differential Leucocyte Counts

Blood smears were air-dried and fixed in methanol for 5 min. The slides were stained in a combination Giemsa-Wright staining solution (Starplex, Toronto, ON) for 1-1.5 minutes, rinsed with deionized water, and air-dried before being examined under immersion oil microscopy. Cells that touched the outer boundaries of the microscopic field were not included in the count. Ten randomly selected microscopic fields (minimum = 50 rbcs; maximum = 200 rbcs) were examined per slide. In each field, the number of rbcs was tallied and leucocytes (lymphocytes, neutrophils, and thrombocytes) were identified (differentiated) and counted (Ellis 1977; Yasutake and Wales 1983; Houston 1990).

1.3.7 Statistical Analysis

Total leucocyte populations typically do not exceed 2-3% of whole blood. Percentage, or proportional, data tend to deviate from normality when values are <30% or >70% (Zar 1984). Therefore, arcsine square root transformation (Zar 1984) was performed on differential leucocyte counts (each leucocyte type expressed as a percentage of total leucocytes) and on total leucocyte abundance (expressed as a percentage of total red blood cell, rbc, counts) for each fish. A one-way ANOVA using treatment as a factor was performed at each sampling time to test for differences among the three groups (control, tricaine, and clove oil). Where *F*-values were significant, a Bonferroni post-hoc test was used to determine which groups differed (Sokal and Rohlf 1981; SYSTAT® 7.0: Statistics 1997). A one-way ANOVA with a Bonferroni post-hoc test was also used to detect within-group differences among sampling times for each physiological trait.

1.4 RESULTS

All treatment fish reached stage 5 anaesthesia within 2 min. One fish in the tricaine group died approximately 2 h after being immobilized and placed in freshwater. No mortalities occurred among controls and fish anaesthetized with clove oil.

1.4.1 Anaesthetic Effects During Induction

Table 1.3 shows the physiological measurements obtained for the control, tricaine, and clove oil groups at the pre-anaesthesia, stage 5 induction, and recovery sampling times (no data for controls at stage 5 and recovery). Pre-anaesthetic physiological values were not significantly different among the three groups ($P>0.05$). There were no significant differences between tricaine and clove oil fish at stage 5 induction for serum cortisol, serum glucose, and total leucocyte counts. Differences existed between the two anaesthetic groups with tricaine-treated fish having higher values for haematocrit ($P<0.02$) and serum lysozyme activity ($P<0.04$) at stage 5 induction. However, stage 5 induction values for all physiological traits for each treatment group, including haematocrit and serum lysozyme activity, did not differ from their respective pre-anaesthetic values.

1.4.2 Short-term Post-recovery Physiological Responses

Haematocrit (Figure 1.1-A)

All groups exhibited a general decline in hct throughout the experiment. By 72 h post-recovery, hct for all groups were significantly lower than their respective pre-stress values (control $P<0.04$; tricaine $P<0.001$; clove oil $P<0.01$). Hct for tricaine fish were lower than

Table 1.3. Physiological values (mean ± 1 SE) of juvenile chinook salmon under three treatments: "Control" (no anaesthetic), tricaine-anaesthetized "T", and clove oil-anaesthetized "CL". Values are given for pre-anaesthetic exposure, stage 5 induction, and upon recovery. Stage 5 induction and recovery values for control fish are not given as fish were not briefly immobilized in anaesthetic. A "*f*" indicates significant difference between the tricaine and clove oil treatment groups.

	Group	Pre-anaesthesia	Stage 5 induction	Recovery
Haematocrit	Control	59.0 ± 2.6	—	—
(%)	T	59.2 ± 6.3	65.2 ± 2.2 *f*	55.8 ± 0.4
	CL	58.5 ± 2.2	57.2 ± 1.3	51.6 ± 3.4
Serum cortisol	Control	38.27 ± 12.57	—	—
(ng/mL)	T	33.84 ± 13.74	60.58 ± 12.76	151.90 ± 24.67
	CL	40.32 ± 5.87	103.31 ± 28.88	161.60 ± 8.98
Serum glucose	Control	55.11 ± 3.89	—	—
(mg/dL)	T	75.59 ± 4.59	61.32 ± 1.52	72.21 ± 1.28
	CL	63.97 ± 4.46	82.65 ± 11.72	68.97 ± 3.70
Serum lysozyme	Control	21 139.0 ± 3 503.5	—	—
activity	T	12 139.9 ± 3 195.8	18 490.6 ± 2 965.7 *f*	15 296.6 ± 3 745.0
(units/mL)	CL	12 014.3 ± 2 158.4	10 372.8 ± 1 314.4	17 707.6 ± 2 409.8
Total leucocyte	Control	0.22 ± 0.06	—	—
abundance	T	0.43 ± 0.15	0.35 ± 0.10	0.73 ± 0.21
(% total RBC)	CL	0.37 ± 0.17	0.51 ± 0.24	0.61 ± 0.21
Lymphocyte	Control	19.67 ± 9.19	—	—
abundance	T	30.00 ± 10.22	31.24 ± 7.96	44.41 ± 13.38
(% total WBC)	CL	5.00 ± 5.00	18.00 ± 11.14	21.18 ± 12.94
Neutrophil	Control	25.44 ± 9.20	—	—
abundance	T	17.33 ± 7.99	6.86 ± 4.30	14.82 ± 5.41
(% total WBC)	CL	10.00 ± 10.00	15.67 ± 8.69	23.33 ± 19.44
Thrombocyte	Control	28.22 ± 10.14	—	—
abundance	T	32.67 ± 14.24	41.91 ± 11.62	40.77 ± 17.86
(% total WBC)	CL	65.00 ± 18.71	46.33 ± 17.52	55.49 ± 18.81

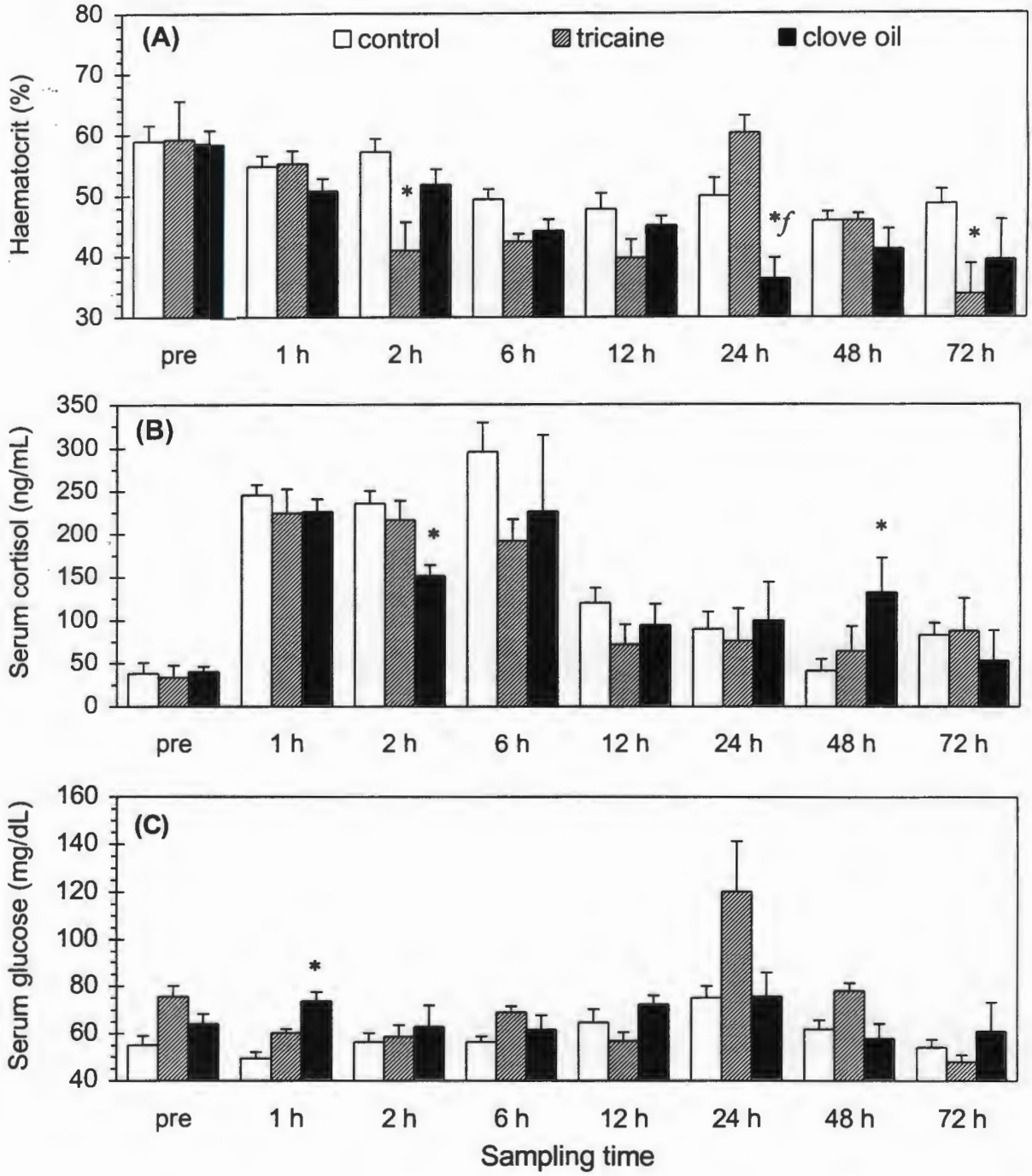


Figure 1.1. Mean post-recovery physiological measurements (± 1 SE) of juvenile chinook salmon that were handled without anaesthesia (control), anaesthetized with tricaine, or anaesthetized with clove oil: **(A)** haematocrit, **(B)** serum cortisol, and **(C)** serum glucose. A "*" indicates significant difference between treatment and control groups, while "*f*" indicates significant difference between treatment groups at the 95% confidence level.

control values at 2 h ($P<0.005$) and 72 h ($P<0.04$). Hct of clove oil fish were lower than control values at 24 h ($P<0.04$). Differences between treatment groups were detected only at 24 h post-recovery ($P<0.004$).

Serum cortisol (Figure 1.1-B)

Control and tricaine fish displayed significant increases in serum cortisol concentrations from their respective pre-stress levels at 1, 2, and 6 h post-recovery (at each sampling time: control $P<<0.001$; tricaine $P<0.01$). Cortisol levels then declined to values similar to pre-stress levels. Despite an elevation in serum cortisol levels for clove oil fish, this increase was not statistically significant ($0.05<P<0.06$). No differences were found between the two anaesthetic groups throughout the study. Cortisol concentrations of clove oil fish were lower than those of controls at 2 h ($P<0.02$), but higher than control values at 48 h ($P<0.04$).

Serum glucose (Figure 1.1-C)

Serum glucose levels of control fish were higher than their pre-stress values at 24 h ($P<0.02$) whereas neither anaesthetic groups experienced significant deviations from their respective pre-stress glucose levels throughout the experiment. No differences were found among groups except at 1 h when the clove oil group displayed higher values than the controls ($P<0.001$).

Serum lysozyme activity (Figure 1.2-A)

The control fish exhibited a dramatic reduction (by two-thirds) in serum lysozyme activity by 1 h post-handling and remained significantly depressed at 20-30% of pre-handling levels for the rest of the study ($P<<0.001$ at all sampling times). Conversely, neither of the

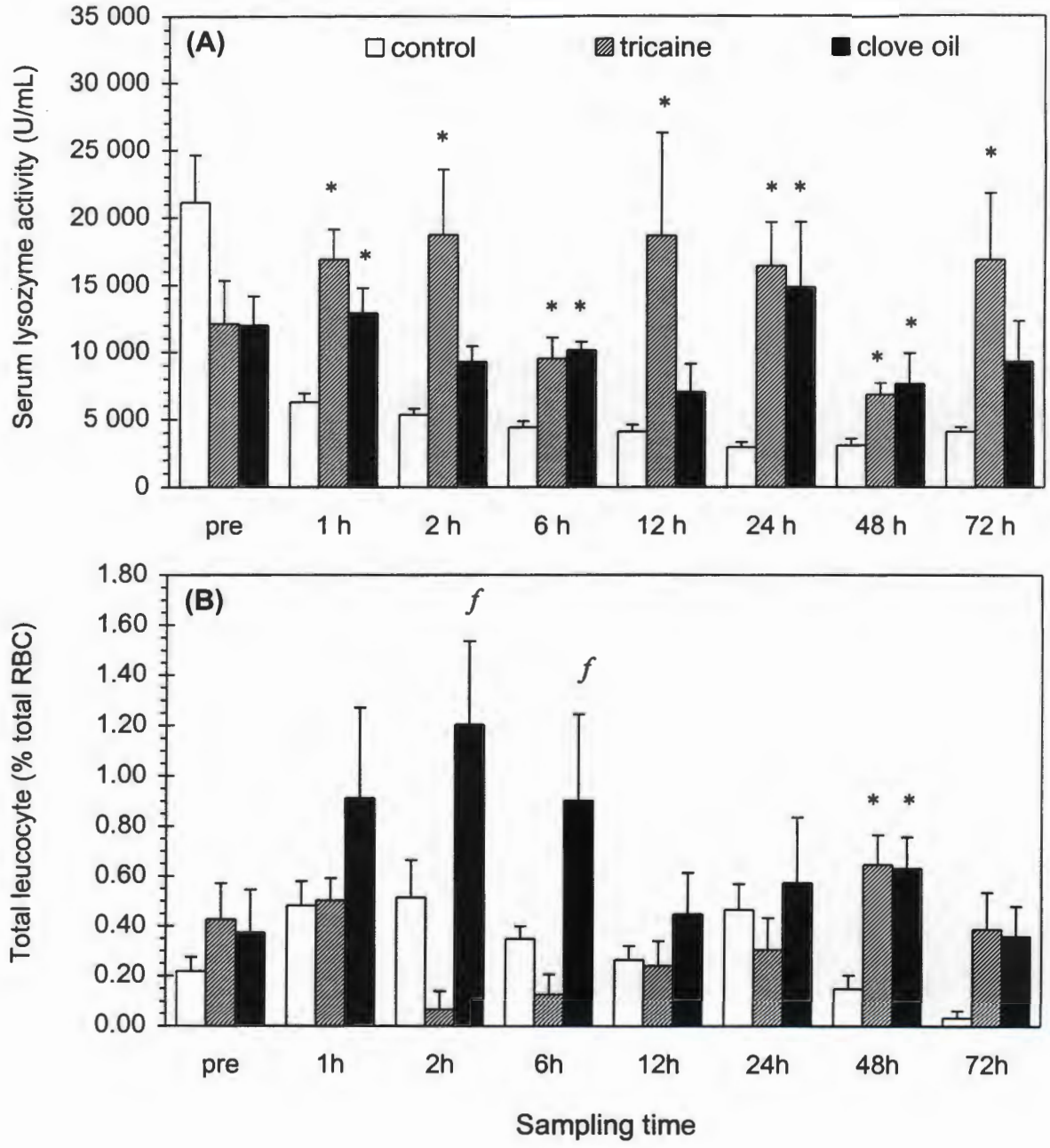


Figure 1.2. Mean post-recovery immunological measurements (± 1 SE) of juvenile chinook salmon that were handled without anaesthesia (control), anaesthetized in tricaine, or anaesthetized in clove oil: **(A)** serum lysozyme activity and **(B)** total leucocyte abundance. A "*" indicates significant difference between treatment and control groups, while "*f*" indicates significant difference between treatment groups at the 95% confidence level.

treatment groups displayed a similar decrease in lysozyme activity levels and, in fact, did not exhibit significant changes throughout the study. Lysozyme activities of tricaine fish remained higher than those of control fish at all post-recovery sampling times. Lysozyme activity levels of clove oil fish were also consistently and significantly higher than control levels except at 2, 12, and 72 h post-recovery. No differences were detected between treatment groups at any post-recovery sampling time.

Differential leucocyte counts (Figure 1.2-B)

Transformed values of differential leucocyte ratios and total leucocyte ratios were analysed. None of the three groups (control, tricaine, and clove oil) experienced significant changes in total leucocyte abundance relative to their pre-stress values throughout the study. Similarly, changes in the abundance of lymphocytes, neutrophils, and thrombocytes within each experimental group were not evident.

Control fish exhibited a slight leucocytosis (an increase in the number of circulating leucocytes) by 1 h and again at 24 h with a slight leucopenia (reduced number of circulating leucocytes) by 72 h. These changes, however, were not significant. Tricaine-anaesthetized fish also experienced a slight leucopenia by 2 h with a gradual increase in leucocyte abundance until about 48 h. These changes were also not significant. Clove oil-treated fish experienced a leucocytosis over the first 2 h, after which leucocyte abundance gradually declined. Again, these changes in total leucocyte abundance were not significant from pre-anaesthesia values.

Total leucocyte levels of clove oil fish were higher than those of tricaine fish at 2 h and 6 h post-recovery ($P<0.004$ at both sampling times). This difference may be attributed to the relatively higher (but not significant) proportion of thrombocytes in clove oil fish at 2 h and 6 h, as well as a relatively greater (again, not significant) proportion of lymphocytes at 2 h. The

tricaine and clove oil group had higher total leucocyte abundance than the control group at 48 h post-recovery ($P<0.002$ for both treatment groups) attributed to relatively more thrombocytes than the controls.

1.5 DISCUSSION

Clove oil immobilizes fish effectively at very low doses. In this study, juvenile chinook salmon reached stage 5 anaesthesia within 2 min at a concentration of 20 ppm at 7°C. The concentration and induction time are comparable to those of other species of salmonids of similar size at similar water temperatures (Endo *et al.* 1972; Anderson *et al.* 1997; Keene *et al.* 1998). Anaesthetic efficacy is temperature-dependent (Endo *et al.* 1972; Hikasa *et al.* 1986), but this feature was not examined in this thesis. It is clear, however, that even in water temperatures ranging from 7 - 14°C, salmonids are effectively anaesthetized with very low concentrations of clove oil.

A practical concern is the influence of anaesthetics on fish physiology during anaesthesia when blood/tissue sampling normally occurs. Physiological values could change significantly during induction due to anaesthetic effects alone. This is especially important for physiological traits that change rapidly (e.g. cortisol). Although differences existed between the tricaine and clove oil groups at stage 5 anaesthesia for haematocrit and serum lysozyme activity, neither were different from their respective pre-stress values. Anaesthetization in either tricaine or clove oil does not appear to alter haematocrit, serum cortisol, serum glucose, serum lysozyme activity, total leucocyte abundance and differential leucocyte counts significantly during the time interval from pre-exposure to stage 5 induction.

Laidley and Leatherland (1988) reported that increases in plasma cortisol levels can occur within 4-6 min of a disturbance and that these elevations were not significant until about 12-14 min post-stress. In this study, the interval of 4-6 min appears to be sufficient for anaesthetizing and sampling fish since there were no significant changes in serum cortisol levels for tricaine and clove oil groups from pre-anaesthesia to stage 5 induction. Since initiation of the serum cortisol response is the quickest, the cortisol response will be the limiting factor in the design and timing of physiological studies such as this one. Thus, either tricaine or clove oil would be suitable for anaesthetizing fish in stress physiology research in which tissue and/or blood samples must be obtained.

Various anaesthetics can have different impacts on the physiology of fish (Iwama *et al.* 1989). Overall, tricaine and clove oil had similar post-recovery effects on haematocrit, serum cortisol and glucose levels, serum lysozyme activity, and leucocyte abundance. While it is understood that chemical immobilization of fish helps to minimize the effects of handling procedures, the results presented here indicate that the degree of CNS depression achieved in this study with the tricaine or clove oil levels used does not necessarily mitigate certain physiological responses, particularly serum cortisol levels.

All three groups in this study exhibited a gradual decline in hct for 72 h. This is consistent with Soivio *et al.* (1977) who demonstrated that post-recovery hct values for rainbow trout anaesthetized in a buffered tricaine solution (100 mg/L) declined to levels below pre-anaesthesia values for up to 7 d. Such a decline in hct may reflect a "rebound" effect after the acute increase in oxygen demand associated with deep anaesthesia. Tricaine is commonly believed to induce hypoxia (Brown 1993), but this is probably true for any anaesthetic which diminishes ventilation rate as induction progresses (Iwama *et al.* 1989). Upon recovery from anaesthesia, hct may decline to compensate for the transient increase in oxygen requirements.

Strange and Schreck (1978) found that yearling chinook salmon remained capable of eliciting a significant cortisol response after a continuous 3 h exposure to different tricaine concentrations (up to 50 mg/L). Iwama *et al.* (1989) found that anaesthesia in general produced a downward trend in plasma cortisol levels during anaesthetic induction. However, Iwama *et al.* (1989) collected blood samples when fish attained the deepest inductive state (cessation of opercular movements) and the tricaine concentration used was higher (100 mg/L). Fish that reach such deep anaesthesia have been exposed to the chemical for an extended period of time and a tricaine concentration of 100 mg /L does not elevate plasma cortisol levels with (Strange and Schreck 1978).

Significant hyperglycemia (increased blood glucose concentrations) can be evident within 16-32 min post-stress (Laidley and Leatherland 1988). In contrast, none of the groups in this study exhibited hyperglycemia relative to pre-stress values regardless of treatment. Only the tricaine group displayed a transient increase in serum glucose levels at 24 h. It is interesting to note that although serum cortisol levels rose significantly for control and tricaine fish, neither group exhibited the hyperglycemia that is commonly believed to be associated with higher cortisol concentrations. Similar results were demonstrated by Andersen *et al.* (1991) who challenged the traditional glucose-immobilizing role of cortisol.

The considerable and extended decrease in serum lysozyme activity for handled control fish in this experiment was unexpected. Demers and Bayne (1997) found that lysozyme concentrations increased after a 30 s aerial exposure, although they also stated that a number of their experimental fish did not respond similarly. On the other hand, Möck and Peters (1990) reported significant reductions in plasma lysozyme activity for up to 24 h post-stress in rainbow trout that had been handled for 30 min, and a 55.8 – 79.8% reduction in lysozyme activity in rainbow trout subjected to transport. The fact that serum lysozyme activities of the tricaine and

clove oil groups in this study did not change significantly throughout the experiment may indicate that anaesthetic exposure immediately after a brief stressor can successfully mitigate its effects, both during anaesthesia and following recovery. Because the nature of this study did not require the brief anaesthetization of control fish, it is not known how prompt the lysozyme response is after handling stress; however, it is clear that values decreased profoundly within 1 h post-stress. Demers and Bayne (1997) observed that plasma lysozyme activities in rainbow trout can increase 10 min after a brief aerial stressor.

Anaesthetization with either clove oil or tricaine may help to alleviate the stress effects of handling on circulating leucocytes. Lymphocytopenia (a decrease in lymphocyte numbers) with an associated neutrophilia (an increase in neutrophil numbers) is a common response after an acute stressor (McLeay 1975; Pulsford *et al.* 1994). A similar response, however, was not observed in this study probably because anaesthetization is simply not an acute disturbance *per se* but rather an agent to mitigate the effects of other acute stressors.

Tricaine and clove oil are highly effective in immobilizing fish without producing significant changes in blood characteristics during anaesthesia. This is particularly important when the determination of pre-anaesthesia physiological status is necessary.

The potential for lingering anaesthetic effects and their influence on subsequent repeated sampling must be recognized. Both anaesthetics yield similar short-term post-recovery responses. Given these results, there appears to be no physiological repercussions in choosing clove oil over tricaine to immobilize juvenile chinook salmon, and likely other salmonids. Clove oil is highly effective at anaesthetizing juvenile chinook salmon at very low concentrations (20 ppm) and does not cause anaesthesia-induced mortality. This study provides further evidence to support clove oil (eugenol) as a viable and safe alternative to tricaine as a fish anaesthetic in aquacultural and fisheries applications.

CHAPTER 2.

Electroshocking Effects on the Stress and Immune Responses of Juvenile Chinook Salmon (*O. tshawytscha*)

2.1 ABSTRACT

Juvenile chinook salmon (*O. tshawytscha*) were subjected to a single brief electroshock in two separate experiments (in 1996 and 1997) to determine immediate and extended post-shock physiological stress and immune responses. In 1996, radiographs of unshocked and shocked fish were examined for spinal column abnormalities. Haematocrit, serum cortisol, serum glucose, serum lysozyme activity, and total leucocyte counts were monitored over a 72 h period (1996) and a 3 wk period (1997). Although domesticated chinook salmon appeared to have a natural occurrence of spinal abnormalities (vertebral compressions), shocked fish had a significantly higher incidence of vertebral compressions and misalignments. Haematocrit declined for up to 12 h post-shock. Electroshocking elicited a significant increase in serum cortisol and glucose from which fish typically recovered within 12-24 h. Electroshocking did not affect serum lysozyme activity, but handling without shocking immediately and significantly reduced lysozyme activity for up to 2 wks. Total leucocyte abundance was not affected, but numbers of neutrophils and thrombocytes in shocked fish were significantly higher than in unshocked controls at 2 wks and 3 wks, respectively. Estimates from blood samples collected immediately (within 2-3 min) after shocking indicate that electrofishing can be used to determine the pre-capture physiological status of fish.

2.2 INTRODUCTION

A primary goal in research investigating the stress physiology of fish is to minimize extraneous confounding trauma sustained by the organism due to experimental protocols. In Chapter 1, the stress effects of anaesthesia were investigated. However, in the field, wild fish must be caught before they can be anaesthetized. Capture stress can influence many physiological variables. The choice of fish capture method depends on the nature of the study, the planned biological measurements, and the characteristics of the study site (e.g. small stream versus deep lake). It is ideal to catch fish efficiently and harmlessly while simultaneously minimizing the level of stress they incur. In cases where the death of a fish is undesirable (e.g. radiotelemetry, mark-recapture studies), the researcher must return the fish to the water without having significantly compromised its survival during the process of capture and handling.

Many live-capture methods are based upon entrapment or entanglement. These include baited pot/parlour traps (e.g. minnow traps) or seine and trammel nets. The main advantage of these capture tools is that trapped fish remain physically unharmed until the researcher collects the fish. In stress physiology research, the main disadvantage is that captured fish generally cannot be sampled immediately. Because traps and nets are typically left for a considerable period of time (often up to 24 h) and because the stress response resulting from confinement or crowding is initiated rapidly (Barton *et al.* 1980; Woodward and Strange 1987; Rotllant and Tort 1997), estimations of pre-capture physiological status are not possible. The phenomenon of "ghost fishing", in which fish continue to be caught in neglected or lost traps and nets, is another significant drawback of these types of fish collection methods and can result in considerable ecological damage. It is evident, therefore, that when immediate blood/tissue sampling is required in the field, the fish collection method chosen must be both rapid and harmless.

Fish can be immobilized (i.e. anaesthetized) by an electric current at low intensities (see Chapter 1). Curry and Kynard (1978) induced galvanonarcosis in yearling rainbow trout, *O. mykiss* (standard length 50 - 87 mm), with a head-to-tail voltage gradient of 2.1 – 4.9 V using continuous direct current. However, fish are rarely anaesthetized in this manner mainly because of the potential hazards to the user and its impracticality under field conditions. At higher output levels, electricity can result in a stronger form of immobilization. This technology has greater application in the field as a means to capture, rather than to anaesthetize, fish. Electrofishing, or electroshocking, is a highly effective method of collecting fish in small streams (backpack electrofishing) and in larger rivers or lakes (boat electrofishing). The use of electrofishing has become increasingly common in fisheries research and population assessment studies. An electric current passed through the water can temporarily stun and immobilize fish (induce galvanonarcosis) for capture. Once the electric current is stopped, the fish can regain voluntary movement almost immediately. It is for these reasons of convenience and efficiency that electrofishing is often chosen over other fish collection methods for stream surveys, mark-recapture studies, and population estimation studies. It is for these same reasons that electroshocking is potentially problematic because fish without visually apparent injuries are assumed to be unharmed (Sharber and Carothers 1988; McMichael 1993; Hollender and Carline 1994; Dalbey *et al.* 1996; Kocovsky *et al.* 1997). As was the case for anaesthesia, recovery from electroshocking is judged by the re-establishment of equilibrium and not necessarily by the return of physiological variables to "pre-stress" values.

Internal injuries such as hemorrhages and spinal damage have been associated with electroshocking (Sharber and Carothers 1988; McMichael 1993; Sharber *et al.* 1994; Dalbey *et al.* 1996). McMichael (1993) and Sharber *et al.* (1994) reported that higher pulse frequencies of direct current (DC), rather than higher voltages, resulted in a greater incidence of spinal injuries

and hemorrhages. The severity of injury is also related to the type of current used. Alternating current (AC), continuous direct current (cDC), and pulsed direct current (pDC) have all been shown to be effective in catching fish. The use of AC, however, has long been recognized to be the most damaging (Hauck 1949), while continuous DC is considered to be the least damaging because the lack of a pulsing current reduces muscle contraction intensity and frequency (Reynolds 1996). Intermediate to these two extremes is pulsed DC which allows for "forced swimming" in affected fish. Forced (involuntary) swimming is a phenomenon in which a fish's body flexes during the electrical "on" times and relaxes during the "off" times. The resulting swimming action induces galvanotaxis (directional attraction towards the anode) which facilitates capture.

Numerous studies have described how electroshocking influences growth (Dwyer and White 1995; Dalbey *et al.* 1996; Dwyer and White 1997), reproductive capacity of adults (Maxfield *et al.* 1971; Marriott 1973), egg development and survival (Dwyer *et al.* 1993; Dwyer and Erdahl 1995; Tipping and Gilhuly 1996; Muth and Ruppert 1996), and behaviour (Curry and Kynard 1978; Mesa and Schreck 1989). More recently, electroshocking effects on the physiological stress response have also been investigated (Woodward and Strange 1987; Mesa and Schreck 1989; Barton and Grosh 1996; Barton and Dwyer 1996). There is also evidence that a brief electroshock influences specific immune function of fish by altering the abundance of circulating leucocytes. Schreck *et al.* (1976) found that rainbow trout exposed to a brief electroshock (230 volts; 2.3 amperes) exhibited an immediate but transient increase in the number of circulating thrombocytes, while granulocytes (e.g. neutrophils) may decline in response to an electroshock (Barton and Grosh 1996). It is thought that the effects of electroshocking are similar to those of recovery from hypoxia (Schreck *et al.* 1976), possibly due to oxygen debt resulting from severe muscular activity or tetanus. To my knowledge,

electroshocking impacts on non-specific immune function in fish have not yet been explored. Changes in any of these traits imply specific disturbances which can compromise the fitness of fish in the wild.

Since the physiological status of wild salmonids in relation to various habitat attributes is addressed in this thesis (see Chapter 3), the possibility that the capture method itself acts as a confounding stressor had to be assessed (Bouck 1984). Backpack electroshocking was chosen for the field study (Chapter 3) because it produces an instant, non-lethal immobilization effect on fish. Examination of the physiological stress response necessitates immediate post-capture tissue/blood sampling. This is especially true for rapid-response traits such as serum/plasma cortisol (see Chapter 1). The use of traps or nets likely would have resulted in variable physiological confounding effects that may not accurately reflect pre-capture status.

The experiments discussed in this chapter were designed to examine the physiological and physical effects of a brief electroshock in juvenile chinook salmon. Specific issues addressed include the timing and magnitude of physiological changes, the severity of any electroshocking-induced spinal abnormalities, and the occurrence of "recovery", where recovery is defined as the return of physiological values to pre-shock levels. Domestic juvenile chinook salmon were used as a model species in place of wild-caught salmonids (i.e. bull trout) because the removal and terminal sampling of wild bull trout from the Torpy River study site were prohibited (Ministry of Environment, Lands and Parks, MoELP). Body size (fork lengths) of the chinook salmon used in these studies and those of the bull trout captured in the Torpy River watershed were comparable. The results of these experiments may indicate whether the physiological values measured from wild bull trout were valid estimates of pre-capture physiological condition, and not artefacts of capture stress. The prospect of post-capture survival upon release is also assessed.

2.3 MATERIALS AND METHODS

2.3.1 Field Experiments and Sampling

Electroshocking studies were conducted in 1996 (8-12 Nov) and 1997 (27 Nov-18 Dec) at Yellow Island Aquaculture Ltd. (Y.I.A.L.) on Quadra Island, British Columbia.

1996 Experiments

In 1996, two replicate trials (trials I and II) were performed on juvenile chinook salmon (mean body weight and fork length: 78.79 $\pm$ 1.65 g and 18.8 $\pm$ 0.1 cm, respectively). One month prior to the start of the experiments, the fish for trials I and II were transferred from seawater netpens and housed separately in freshwater in two different holding tanks (3 000 L). In each trial, the fish were exposed to a 10 s electroshock (200 V, 80 Hz) with a backpack electroshocker (Smith-Root, Model 12-A) capable of programmable output waveforms (POW) and fitted with an aluminum ring anode (11 inch diameter). The specific settings on the electroshocker ("M7") used in this experiment provides a "wide-to-narrow varying width" pulse waveform (Smith-Root, Inc. 1994). At this setting, the pulses sweep from an initial pulse width of 8 ms to 0.4 ms in a 2 s time-frame at a frequency of 80 Hz, and maintains a 0.4 ms pulse width at the same frequency until the electrical power is shut off. Ambient water conductivity was 97.1 microSiemens/cm (μS/cm) measured at 7.9°C. Immediately after shocking, fish were gently transferred to tanks (tank volume = 140 L; n=10 fish/tank). Blood sampling procedures followed those described in Chapter 1 (Section 1.3.1). Pre-shock fish (n=10) were sampled for blood by caudal severance. Electroshocked fish were similarly sampled at 0 h (immediately after shocking), 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 h post-shock (n=10/sampling).

To determine the physical effects of electroshocking on the spinal column, unshocked and shocked fish (n=24/group) were euthanized in a solution of tricaine methanesulphonate (200 mg/L). Spinal radiographs (left-lateral and dorso-ventral aspects) of these 48 fish were taken on mammogram film (4 fish/film) at the Campbell River and District General Hospital, Campbell River, British Columbia.

1997 Experiments

In 1997, a second electroshocking study was conducted on juvenile chinook salmon (mean body weight and fork length: 40.41 $\pm$ 0.45 g and 15.5 $\pm$ 0.1 cm, respectively) with the inclusion of unshocked controls throughout the extended 3-week sampling period. Fish were transferred from seawater to freshwater 2.5 wks prior to the start of the experiments and housed in a single holding tank (3 000 L). Unshocked control fish were gently transferred to 11 tanks (tank volume = 140 L; n=20 fish/tank). The remaining fish were exposed to a 10 s electroshock at the same settings used in 1996 and immediately transferred to 140 L tanks. Since each tank represented a single sampling time, no tank was sampled twice. Ambient water conductivity was 108.1 μS/cm at 7.4°C. Pre-shock controls (n=15) were sampled via caudal severance. Shocked and unshocked fish were sampled at the same time intervals, staggered 30 min apart (n=15/sampling), at 0, 1, 2, 4, 6, 12, 24, 48, 72 h, and 1, 2, and 3 weeks post-shock. Radiographs were not taken in 1997.

In the 1996 and 1997 studies, haematocrits were measured, blood smears made, and serum collected and stored at -80°C until serum cortisol and glucose concentrations, and serum lysozyme activity could be determined.

Blood smears made in 1996 did not stain well. Under immersion oil microscopy, red blood cells (rbcs) and leucocytes appeared to have lysed, leaving only exposed nuclei. An

attempt to restain blood smears was unsuccessful. The high proportion of slides with lysed cells (>90% of individual blood smears) was deemed sufficient to exclude differential leucocyte analysis from the 1996 database. It is possible that the glass slides were too cold (the study took place in November in an unheated facility) and blood could not dry fast enough (J. W. Heath[2], pers. comm.). While a blood smear normally takes 30-60 s to dry by air, during the 1996 studies blood smears took about 5-10 min to air dry. In 1997, care was taken to keep drying blood smears slightly warm. Differential leucocyte counts were determined from the 1997 stained blood smears.

In the 1997 study, the inflow tubing supplying water for the tank containing the unshocked fish to be sampled at 1 wk became clogged with debris. Since no mortalities occurred in this tank, it was inferred that the lack of freshwater inflow was short-term (e.g. a few hours).

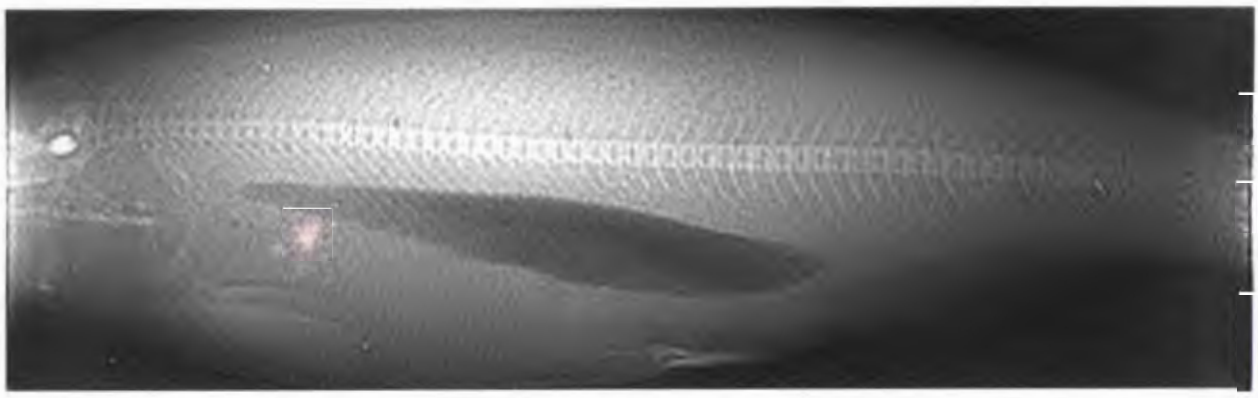
2.3.2 Laboratory Assays

The determination of haematocrit, serum cortisol, serum glucose, serum lysozyme activity, and differential leucocyte counts are described in Chapter1 (Sections 1.3.2 to 1.3.6).

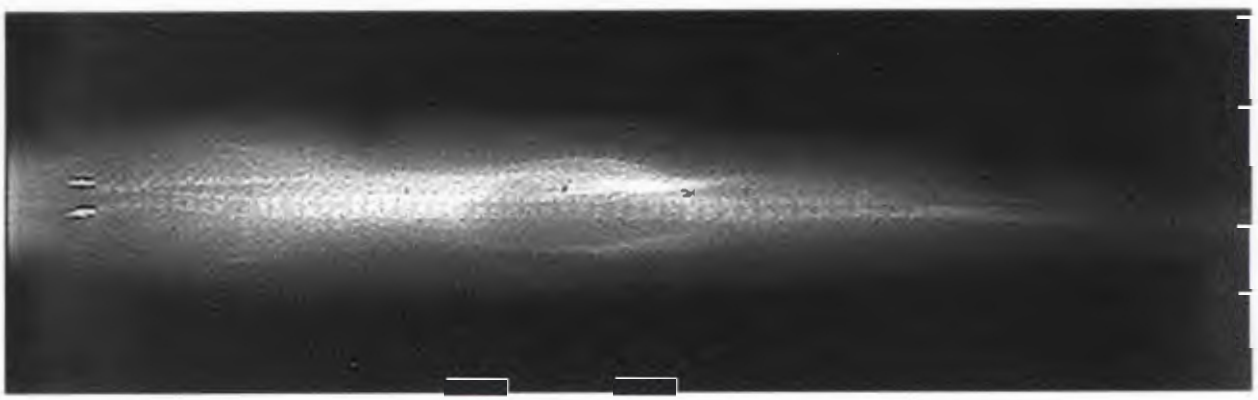
2.3.3 Radiograph Examination

Radiographs were placed on a portable light table and examined under a table-mounted magnifier. The left-lateral (LAT) and dorso-ventral (DV) aspects of spinal columns for each fish were inspected (refer to Figures 2.1-A and 2.1-B). Examining both aspects for all 48 fish constituted one radiograph session. Within a single radiograph session, the total number of

[2] J. W. Heath, Research Director, Yellow Island Aquaculture Ltd., 1681 Brook Crescent, Campbell River, BC, V9W 6K9.



(A)



(B)

Figure 2.1. Radiographs of entire spinal column of unshocked juvenile chinook salmon: (A) left-lateral (LAT) and (B) dorso-ventral (DV) aspects. The fish is facing to the left in both figures.

vertebrae were counted for each fish and were numbered in an anterior-posterior direction. The first distinct vertebra located behind the head was counted as number 1. The type of spinal abnormality (spinal lesion), if any, was noted and its location identified as the vertebra(e) number(s) involved. Spinal lesions were classified as misalignments, compressions, or obvious fractures. The condition of the spinal column was ranked according to a rating system with values ranging from 0 to 3 (see Table 2.1; from Reynolds 1996). This rating system is intended to help standardize injury identification (i.e. yields ranked data) and does not necessarily establish a relationship to actual fish health (Reynolds 1996). A compression was documented as a reduction of intervertebral spacing compared with those of neighbouring vertebral gaps (Figure 2.2-A). Some spinal columns had a "wavy" appearance. Compressions in such situations were identified as those that occur at the apex of each curve (Figure 2.2-B). Serial compressions can also occur along straight portions of the spinal column in which no intervertebral gap is visible between a number of consecutive vertebrae (Figure 2.2-C). Vertebral misalignments were identified as vertebrae with a discernable shift from alignment compared to adjacent vertebrae (Figure 2.2-D). Dorso-ventrally oriented vertebral shifts can be detected from LAT x-rays, but not as easily from DV x-rays. Likewise, lateral shifts in vertebrae are discernable only from a DV aspect, but not from a LAT aspect. Spinal fractures include crushed vertebrae or complete separation of a number of vertebrae (Figure 2.3).

Three preliminary examination sessions were performed to become familiar with interpretation methods and identification of spinal abnormalities. The radiographs were then assessed an additional three times to collect data for final analysis. The ranking for each fish (for each aspect) was taken to be the two matching ranks out of the three sessions. A final overall rank was assigned to each fish by noting its worst injury regardless of radiograph aspect. For

Table 2.1. Rating system for evaluating the severity of electrofishing-induced spinal injuries in fish (from Reynolds 1996).

Rank	Injury type and description
0	No spinal damage apparent.
1	Compression (distortion) of vertebrae only.
2	Misalignment of vertebrae, including compression.
3	Fracture of $\geq$ 1 vertebrae or complete separation of $\geq$ 2 vertebrae.

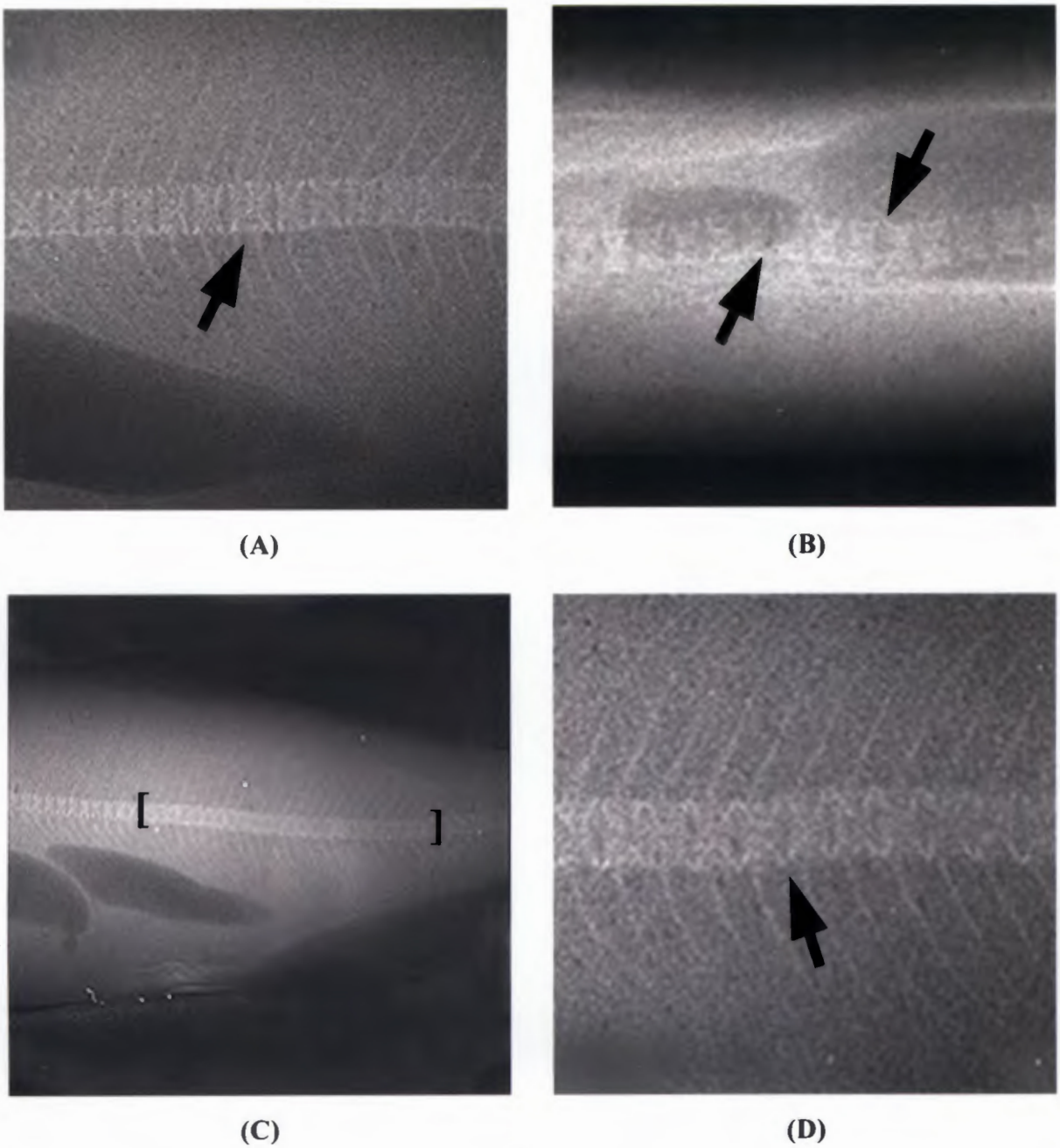


Figure 2.2. Radiographs showing various spinal abnormalities (lesions) in juvenile chinook salmon subjected to a 10 s electroshock of pDC (200 V; 80 Hz). Injury description and radiograph view is given: **(A)** compression between two vertebrae (LAT), **(B)** apical compressions (DV), **(C)** serial compressions (LAT, within brackets), and **(D)** vertebral shift (LAT).

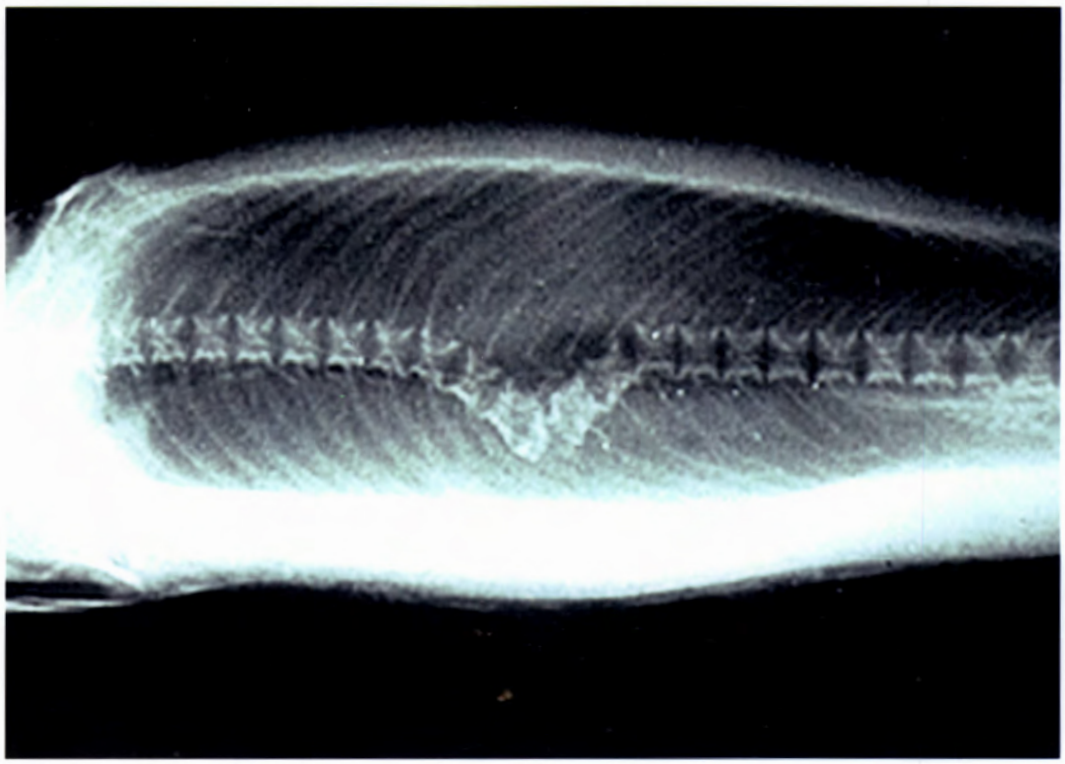


Figure 2.3. Dorso-ventral radiograph of an electroshock-induced spinal fracture in a rainbow trout (printed with permission by N. Sharber).

example, if examination of a lateral x-ray showed no injury for one fish (rank of 0) but its corresponding DV aspect indicated vertebral misalignments (rank of 2), the fish would be given a final overall rank of its worst injury (misalignment = rank of 2). There are two potential sources of error with radiograph interpretation: 1) error with different people examining the same information and 2) error with a single person over time. To minimize biased interpretations between radiograph sessions, one person assessed all radiographs. As well, the identification number of each x-ray was masked with a "dummy" label for each session and were mixed and remasked for subsequent sessions.

2.3.4 Statistical Analysis

The ratios of total leucocyte numbers to erythrocyte numbers and of leucocyte type to total leucocyte number were arcsine square-root transformed (Zar 1984) before analysing the data. To test for between-group differences in the 1996 (trial I versus trial II) and 1997 (unshocked versus shocked) experimental groups, a one-way ANOVA was performed at each sampling time. A one-way ANOVA with Bonferroni adjustment was performed to detect within-group differences across all sampling times for each physiological trait (Sokal and Rohlf 1981; SYSTAT® 7.0: Statistics 1997) for the 1996 and 1997 datasets.

The 1996 radiograph data were analysed by a two-way Pearson chi-square test. Sparse data in a sufficient number of cells (i.e. frequency was <5) yielded results in which little confidence could be placed (SYSTAT® 7.0: Statistics 1997). To overcome this problem, all spinal lesions ranked as 1 were combined with those ranked as 2. This compares normal and abnormal spinal conditions regardless of the degree of abnormality (i.e. rank 1 and rank 2 spinal lesions were considered equally deviant from normal spinal condition). Comparisons were made

between control and shocked fish according to radiograph aspect (LAT versus DV). Final injury ranks were also compared between the control and shocked groups.

2.4 RESULTS

2.4.1 Results of the 1996 Electroshocking Study

Haematocrit 1996 (Figure 2.4-A)

All but one capillary tube broke for the 72 h sampling group in trial I (n=1). Haematocrit values in both trials declined throughout the study. No differences were found between trials I and II except at 6 h post-shock ($P<<0.001$). In trial I, post-shock haematocrit values were lower than control (C) levels at 6 h ($P<0.001$) and 12 h ($P<<0.001$). Haematocrits of fish in trial II did not change significantly from their respective pre-shock values for the duration of the study.

Serum cortisol 1996 (Figure 2.4-B)

Serum cortisol levels for both trials were significantly elevated from pre-shock levels by 0.5 to 1 h post-shock. Trial II cortisol concentrations were higher than those of trial I at 0 h ($P<0.01$), 1 h ($P<0.004$), and 24 h ($P<0.02$). This relationship was reversed at 48 h when trial I values were higher ($P<0.04$). In trial I, cortisol concentrations were higher than pre-shock levels at 0.5 h and 4 h ($P<0.04$ at both sampling times) and at 6 h ($P<0.02$). By 12 h, levels returned to and remained at values similar to pre-shock concentrations. Trial II cortisol levels were higher than its control values at 1 h ($P<0.02$) and remained elevated (but not significantly so) until 12 h.

Serum glucose 1996 (Figure 2.4-C)

Serum glucose levels of trial I were significantly higher than those of trial II for the first

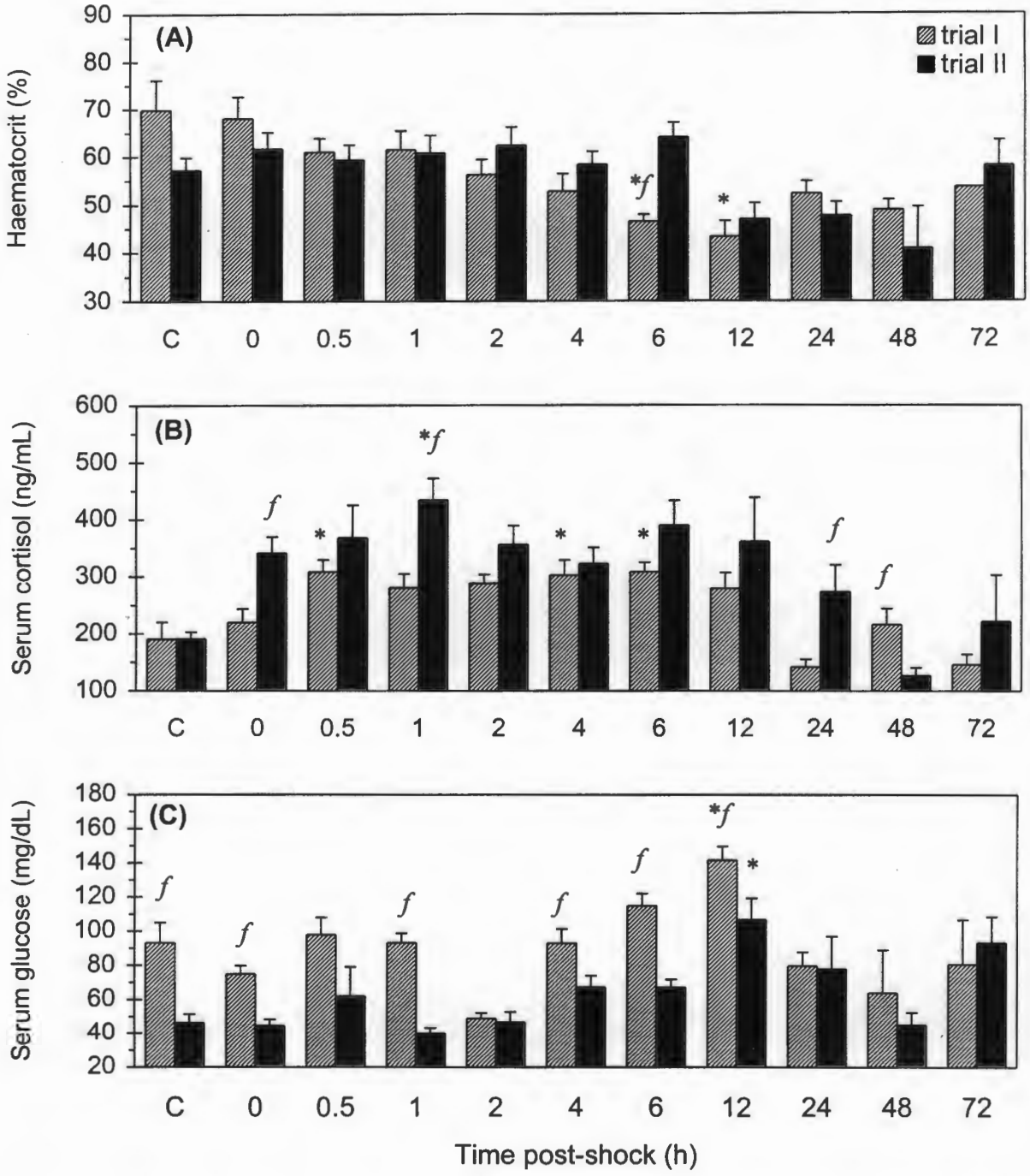


Figure 2.4. Mean physiological values (± 1 SE) of juvenile chinook salmon subjected to a 10 s electroshock of pDC (200 V; 80 Hz) in two replicate trials in 1996: **(A)** haematocrit, **(B)** serum cortisol, and **(C)** serum glucose. A "*" indicates significant difference from pre-shock controls values and "*f*" indicates significant difference between both trials at the 95% significance level.

12 h after electroshocking, except at 0.5 h and 2 h when there were no differences. No further differences were found between the trials after 12 h. In both trials, glucose concentrations were significantly higher than their respective pre-shock values only at 12 h (trial I, $P<0.04$; trial II, $P<0.004$), after which glucose levels returned to values similar to controls.

Serum lysozyme activity 1996 (Figure 2.5)

Serum lysozyme activity of trial II fish were consistently and significantly higher than those of trial I fish at all sampling times. Therefore, the results are presented as proportional changes relative to their respective pre-shock levels. In both trials, lysozyme activity did not differ from their respective pre-shock values throughout the study, except at 48 h post-shock for trial II when lysozyme activity levels were significantly lower than pre-shock values ($P<0.04$).

Radiographs 1996 (Table 2.2)

Table 2.2 presents the observed frequencies of fish exhibiting varying levels of spinal conditions. No fish displayed injuries ranking 3 (fractures or separation of vertebrae; see Table 2.1). All vertebral compressions were detected from LAT x-rays while none were detected from DV x-rays. A greater number of vertebral shifts were identified from DV radiographs than from LAT radiographs.

Control fish had a significantly higher frequency of normal spinal columns while shocked fish exhibited a higher frequency of abnormal spinal columns in LAT radiographs ($P<0.002$; $\chi^2=10.24$; df = 1). This pattern was also found from DV radiographs ($P<0.02$; $\chi^2=6.00$; df = 1). Electroshocking had a significant impact on spinal condition ($P<0.04$; $\chi^2=4.55$; df = 1). Shocked fish had a higher proportion of abnormal spinal columns than did control fish.

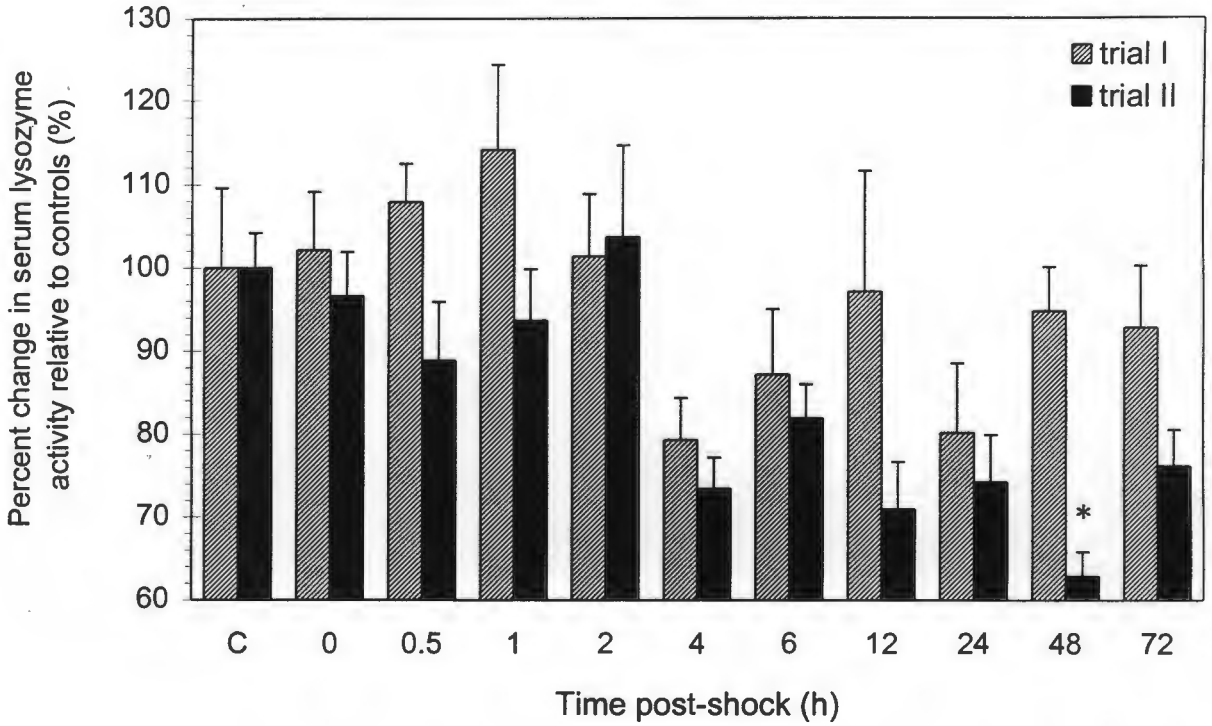


Figure 2.5. Percent change in serum lysozyme activity relative to pre-shock control values (control levels are 100%) in juvenile chinook salmon subjected to a 10 s electroshock of pDC (200 V; 80 Hz) in replicate trials in 1996. A "*" indicates significant difference from control values at the 95% confidence level.

Table 2.2. Observed frequencies of spinal conditions in unshocked and shocked juvenile chinook salmon (n=24 per group) ranging from normal uninjured spinal columns (rank 0) to misalignments and compressions (rank 2). Shocked fish were exposed to a 10 s electroshock of pDC (200 V; 80 Hz). The data is presented according to radiograph aspect and the overall spinal condition. Frequencies in the shaded sections were combined for statistical analysis (normal versus abnormal vertebrae).

	NORMAL	ABNORMAL		Statistical results (Pearson chi-square)
	Rank 0	Rank 1	Rank 2	
Lateral aspect:				
control	19	2	3	$P<0.002$ $\chi^2 = 10.24$ df = 1
shock	8	12	4	
Dorso-ventral aspect:				
control	12	0	12	$P<0.02$ $\chi^2 = 6.00$ df = 1
shock	4	0	20	
Final ranking:				
control	8	2	14	$P<0.04$ $\chi^2 = 4.55$ df = 1
shock	2	1	21	

2.4.2 Results of the 1997 Electroshocking Study

Haematocrit 1997 (Figure 2.6-A)

Control and shocked fish exhibited a decline in hct for the first 12 h after electroshocking. No differences were found between control and shocked groups except at 1 wk ($P<0.01$) when control fish had higher hct values. The control group displayed hct levels that were lower than their respective pre-stress (0 h) values at 12 h ($P<0.04$), 48 h ($P<0.004$), 2 wks ($P<0.004$), and 3 wks ($P<0.02$). Conversely, the shocked group showed no significant changes in hct, although values had diminished during the experiment.

Serum cortisol 1997 (Figure 2.6-B)

In control and shocked groups, serum cortisol concentrations had greatly increased by 1 h post-stress and remained significantly elevated for about 6 h. Cortisol levels of control fish returned to pre-stress levels by 12 h. Cortisol levels of shocked fish remained elevated and significantly higher than those of control fish (but not significantly higher than its respective 0 h values) until 24 h post-shock. Control fish displayed a transient increase in cortisol at 1 wk while shocked fish showed a similar transient increase at 2 wks.

Serum glucose 1997 (Figure 2.6-C)

Shocked fish showed significant elevations in serum glucose levels within the first 12 h compared to control fish. These increased glucose concentrations were higher than those of control fish at 1 h, 2 h, 6 h, and 12 h ($P<<0.001$ at these sampling times). At 24 h and 1 wk, control fish had higher glucose levels than those of shocked fish (24 h, $P<0.01$; 1 wk, $P<<0.001$). Glucose levels among control groups did not differ throughout the study except at 1 wk when

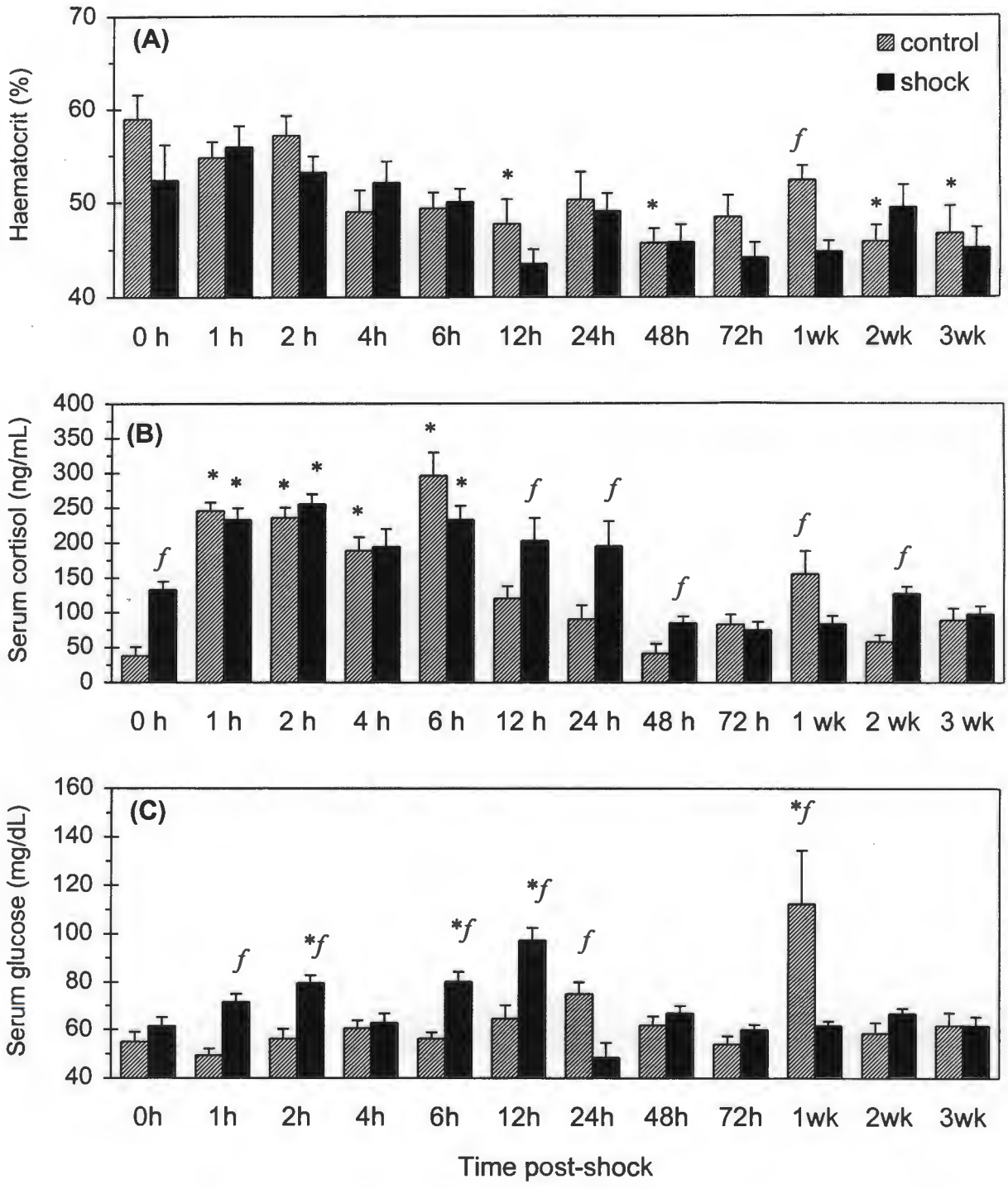


Figure 2.6. Mean physiological values (± 1 SE) of unshocked and shocked juvenile chinook salmon subjected to a 10 S electroshock of pDC (200 V; 80 Hz) in the 1997 study: **(A)** haematocrit, **(B)** serum cortisol, and **(C)** serum glucose. A "*" indicates significant difference from time 0 h and "*f*" indicates significant difference between unshocked and shocked groups at the 95% confidence level.

the fish displayed a transient increase in glucose that was significantly higher than their respective pre-stress values ($P<<0.001$). By 2 wks, control glucose levels had returned to pre-shock levels. Shocked fish exhibited glucose concentrations higher than their respective pre-shock values at 2 h ($P<0.02$), 6 h ($P<0.02$), and 12 h ($P<<0.001$). Glucose levels of the shocked group returned to and stayed at values similar to pre-shock levels for the remainder of the study.

Serum lysozyme activity 1997 (Figure 2.7-A)

The serum lysozyme activity of control and shocked fish were similar at the start (0 h) and end (3 wks) of the study. At all other sampling times, control fish sustained a severe depression in lysozyme activity that was significantly lower than that of shocked fish ($P<<0.001$ at all of these sampling times). Fish exposed to electroshocking did not experience significant changes in serum lysozyme activity throughout the study.

Differential leucocyte counts 1997 (Figure 2.7-B)

Total leucocyte abundance for unshocked and shocked fish declined for the first 48 h post-stress. Shocked fish exhibited higher total leucocyte numbers than those of control fish at 0 h ($P<0.04$), 48 h ($P<0.04$), 72 h ($P<0.05$), 2 wks ($P<0.004$), and 3 wks ($P<0.02$). Neither group displayed significant changes in total leucocyte abundance from their respective 0 h values throughout the study. Within-group differences were not found in the control group for abundance of lymphocytes, neutrophils, and thrombocytes. There were no significant changes in lymphocyte abundance within the shocked group, but neutrophil and thrombocyte numbers were higher than time 0 h values at 2 wks ($P<<0.001$) and 3 wks ($P<0.02$), respectively.

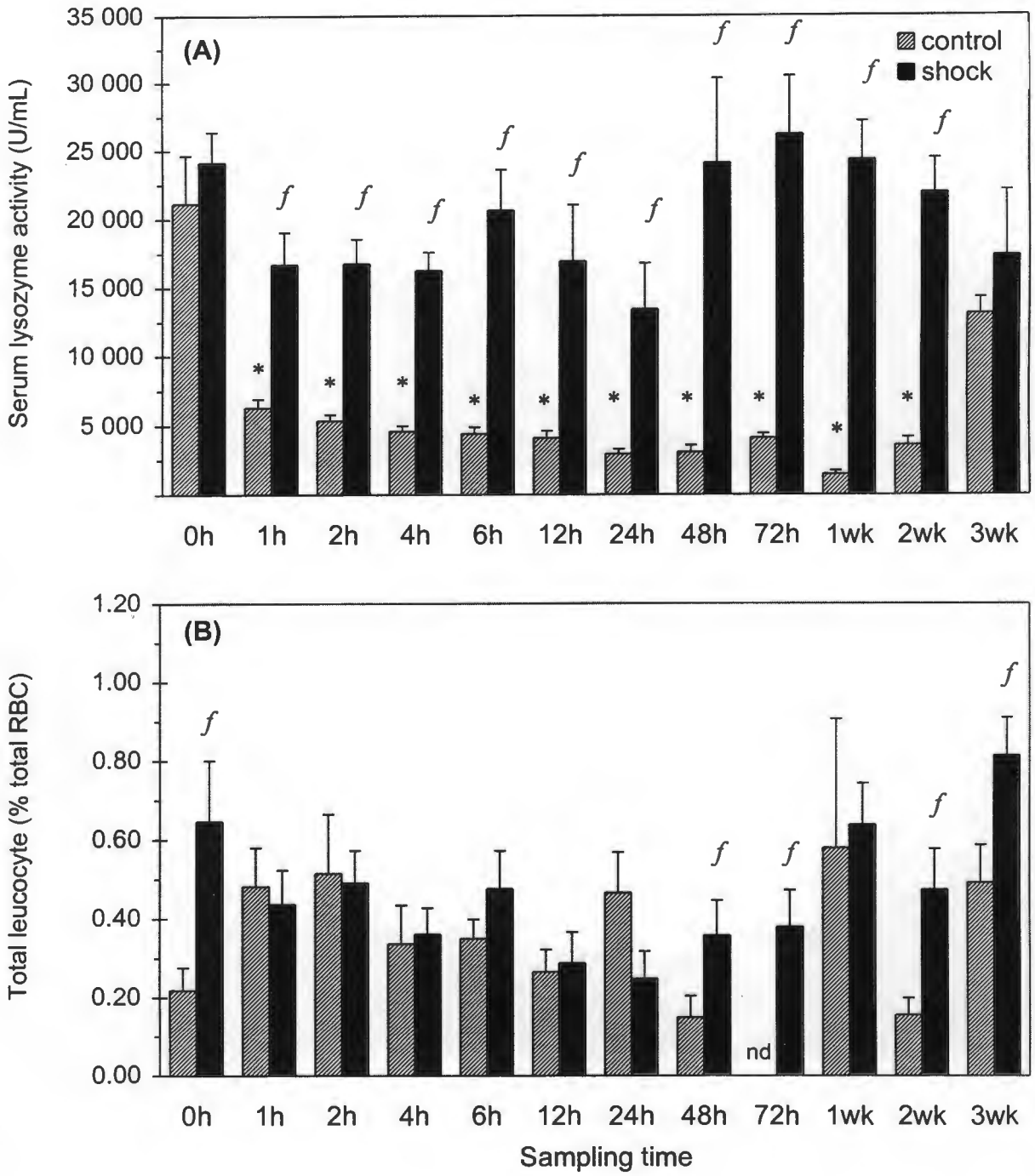


Figure 2.7. Mean immunological values (± 1 SE) of unshocked and shocked juvenile chinook salmon subjected to a 10 s electroshock of pDC in 1997: **(A)** serum lysozyme activity and **(B)** total leucocyte abundance. A "*" indicates significant difference from 0 time values and "*f*" indicates significant difference between unshocked and shocked groups at the 95% confidence level. No data was obtained for controls at 72 h ("nd").

2.5 DISCUSSION

A brief electroshock is sufficient to elicit an array of physiological responses in juvenile chinook salmon. Some responses were pronounced but short-lived, or gradual but more extended, while others did not appear to be significantly altered. Of greater concern is the higher incidence of spinal lesions in fish exposed to a brief shock. Electroshock-induced abnormalities were not externally obvious, but have the potential to influence the overall fitness of the fish.

The higher abundance of total leucocytes in shocked fish at time 0 h is likely attributed to a greater proportion of thrombocytes immediately after electroshocking. Schreck *et al.* (1976) reported similar findings for electroshocked rainbow trout. In contrast, Barton and Grosh (1996) found that granulocytes (neutrophils and thrombocytes) declined within the first 15 min after shocking. The slight increase in circulating neutrophils and thrombocytes from 48 h to 3 wks post-shock may account for the higher total leucocyte numbers in shocked fish than in control fish. Schreck *et al.* (1976) and Barton and Grosh (1996) monitored leucocyte abundance for only 3 h post-shock and so are not comparable to the results presented here. Although lymphopenia (a reduction in lymphocyte numbers) is a typical response to an acute stressor (McLeay 1975), such a response was not seen in this study

The response of blood lysozyme activity to an acute stressor depends on the nature of the stressor (Möck and Peters 1990; Røed *et al.* 1993). Bouck *et al.* (1978) reported that four different capture methods (angling, seining, electroshocking, and direct netting) had no significant effect on the activities of four plasma enzymes (lysozyme was not one of them) within 2 min of capture. Similar findings were obtained in this study for lysozyme activity in response to electroshocking. Demers and Bayne (1997) found that plasma lysozyme increased significantly within 10 min of an acute aerial stressor. The results presented in this chapter

indicate that handling alone can produce a severe drop in serum lysozyme activity. Thus, an increase in lysozyme activity does not necessarily indicate a stressed state in fish and that the type of stressor must also be considered.

The results presented here support other published research showing that a brief electroshock is sufficient to affect the spinal columns of fish. In contrast to the unshocked control fish, the shocked fish in this study had a greater incidence of vertebral misalignments and compressions. Although misalignments (i.e. shifts) are generally considered to be more harmful than compressions, the direction/orientation of vertebral shifts can have significant influence on the severity of the injury or the degree of incapacitation (J. Speyers[3], pers. comm.). The higher number of vertebral misalignments identified from DV radiographs suggests that lateral (side-to-side) shifts in vertebrae are more likely to occur than dorso-ventral shifts. Lateral vertebral shifts are less likely to break and are often not as severe as dorso-ventral shifts. The spinal columns of animals such as fish, birds, and reptiles tend to be more flexible than those of mammals. Animals with more flexible spinal columns can sustain a relatively greater degree of lateral vertebral shifts and still function normally. In contrast, dorso-ventral shifts of vertebrae are more conducive to breakage and are functionally more debilitating because the nerves may be pinched. Such injuries are more likely to be permanent and possibly result in paralysis. Even if the injury were to heal, significant pressure would then be permanently exerted on the nerve-rich spinal cord located within the bony spinal column. Given these results, it is recommended that both LAT and DV radiographs of the same fish be viewed in order to ascertain the severity and type of any electroshock-induced injury.

[3] D.V.M., All Mobile Veterinary Hospital, 2380 Ospika Boulevard, Prince George, BC, V2N 3N5.

In this study, radiographs were taken of a group of unshocked fish and a separate group of electroshocked fish. It may have been better to take pre- and post-shock radiographs of the same group of fish. This would have provided a direct comparison of "before-and-after" spinal conditions of the same organism. However, this was not logistically feasible. The radiograph results presented in this chapter represents further evidence that a greater proportion of spinal lesions occur in shocked fish than in unshocked fish, although there appears to be a natural occurrence of spinal abnormalities in unshocked domesticated fish. Such abnormalities can arise naturally through injuries incurred under intensive aquacultural practices or temperature fluctuations during embryonic development (Seymour 1959).

The transient increase in hct, serum cortisol, and serum glucose in control fish (in 1997) at 1 wk post-stress may reflect the temporary interruption in freshwater inflow into the tank. Reduced dissolved oxygen content of the water was likely not severe enough to cause mortalities due to asphyxiation, but may have been sufficiently hypoxic to cause a slight increase in oxygen demand. A rise in haematocrit can occur in two ways in order to accommodate a higher oxygen requirement: (1) polycythemia (increased number of circulating rbcs) by the release of additional rbcs from the spleen, or (2) rbc swelling due to haemoconcentration (Perry and McDonald 1993). The transient rise in serum cortisol and serum glucose are probably due to the hypoxia-induced increase in circulating catecholamines (Wendelaar Bonga 1997). Catecholamines (mainly adrenaline) rapidly promote the conversion of liver glycogen into glucose and initiate the elevation in serum cortisol through activation of the HPI axis (Wendelaar Bonga 1993).

Differences between the two replicate trials in 1996 were mainly evident for serum glucose concentration and, in particular, serum lysozyme activity. Although the absolute values for trials I and II were significantly different for serum glucose (part of the study) and serum lysozyme activity (throughout the entire study), it is important to note that the relative response

profiles were similar. The reason for the difference in serum lysozyme activity between the two 1996 trials is unknown. Lysoplates were made in advance using the same batch of phosphate buffer; hence, the pH and solute molarity of the buffer were the same. Aliquots of the HEWL standards were also made in advance and frozen until time of use. Reconstituted HEWL can keep for several months in the frozen state (Sigma Chemical Company, technical representative, pers. comm.). The quality of HEWL standards could not have degraded within the 1-2 days that it took to perform the lysoplate assay for the 1996 samples. Since the fish used in trials I and II were held in separate tanks (3 000 L), it is possible there were tank effects. But potential tank effects were not reflected by differences in the pre-shock values of other variables (hct and serum cortisol).

In 1997, the higher serum cortisol levels in shocked fish at time 0 h may be a result of some initial chasing before being exposed to an electroshock. Unfortunately, this was unavoidable as all experimental fish were held in a common holding tank (3 000 L volume) unlike 1996 when 2 holding tanks were used. Unshocked fish had to be removed first and divided among experimental tanks. The remaining fish were then exposed to a brief electroshock, but it is likely that they were chased somewhat beforehand. The shocked fish, therefore, may have been initiating a stress response (i.e. cortisol response) at the time of shocking. The 1996 results for serum cortisol, however, supports the fact that immediate post-shock blood sampling does not yield significantly increased cortisol values. This is in agreement with Maule and Mesa (1994) who also reported that immediate post-shocking sampling is sufficient to avoid artefactual increases in plasma cortisol.

This chapter has addressed three main concerns regarding the use of electroshocking as a fish collection tool. First, it must be recognized that fish can incur some degree of spinal

abnormality as a result of electroshocking. Some spinal injuries can heal, but the risk of chronic debilitating harm remains.

Second, capture by electroshocking is an acute stressor from which fish can recover physiologically. The timing of recovery, defined here as the return of physiological values to "pre-stress" levels, can vary among different biological traits. In this study, recovery seemed to occur within a day (24 h) for some blood characteristics (i.e. serum cortisol and glucose, leucocyte abundance) while others remained altered for an extended time period (i.e. haematocrit). Still others did not appear to be significantly affected at all by a brief electroshock (i.e. serum lysozyme activity). Handling alone (dipnetting and gentle transfer to a different tank) resulted in a rapid and severe drop in post-stress lysozyme activity for up to 2 wks. Clearly, collecting fish in the wild with an electroshocker will initiate a physiological stress response.

Third, in order to have confidence in measuring pre-capture physiological status of a fish, immediate blood sampling is imperative (within 2-3 min). The results presented here demonstrate that certain blood traits do not change significantly as long as post-shock sampling is rapid. Backpack electroshocking can be used safely to ensure pre-capture status of a fish.

CHAPTER 3.

Influence of Stream and Riparian Habitat Attributes on the Physiology and Immunocompetence of Bull Trout (*Salvelinus confluentus*) in the Torpy River Watershed

3.1 ABSTRACT

Examining the stress physiology of wild salmonids *in situ* is problematic for several reasons: 1) interrelationships among biotic and abiotic habitat features hinder the isolation or identification of potential environmental stressors and impedes the establishment of controls, 2) the stress response of wild fish is relatively unknown and may be quite variable, and 3) it is difficult to differentiate between the effects of normal environmental fluctuations and those of sublethal disturbance. Habitat features contributing to physiological variation are those that can also confound detection of the effects of suspected environmental disturbances. The goal of this study was to determine the extent to which habitat features influence the variation in physiological measurements of wild bull trout in the Torpy River watershed. Bull trout (n=109) were captured by electroshocking and non-destructively sampled for blood in 13 of the 31 surveyed streams. The influence of habitat attributes (canopy-closure, stream gradient, and discharge rate) on haematocrit, plasma glucose and cortisol concentrations, plasma lysozyme activity, total leucocyte counts, and fish abundance were examined using simple and multiple linear regression analysis. Leucocyte abundance did not appear to be significantly influenced by these habitat attributes. Variation in plasma cortisol and glucose levels and haematocrit (15-20%), plasma lysozyme activity (>60%), and fish abundance (about 40%) could be accounted for by the combined effects of canopy-closure, stream gradient, and discharge rate. The physiological status of wild bull trout is strongly influenced by their habitat and care should be taken when interpreting results.

3.2 INTRODUCTION

Physiological Measurements of Wild Fish: Problems and Considerations

Monitoring changes in the physiological and immunological status of wild fish *in situ* is complex. Three main areas of concern contribute to this difficulty. First, physiological and environmental controls are difficult to establish in the field (Depledge 1989; Adams 1990; Peakall 1992). This problem stems from the dynamic interactions among biotic and abiotic habitat features which make it difficult to isolate or identify potential environmental stressors and nearly impossible to establish an environmental (site) control. Wild salmonids are subject to diel and seasonal environmental fluctuations which can also confound physiological measurements. The issue of other potentially interfering factors such as the methods commonly performed to obtain haematological values were addressed in Chapters 1 (anaesthesia) and 2 (capture stress by electroshocking). These are unavoidable aspects of experimental procedures used in field research that require the physical capture and handling of fish to obtain biological information.

Second, it is uncertain as to what constitutes a "normal" physiological range for wild fish. How would baseline values be established? A range of "resting" physiological values have been reported for domesticated salmonids (Wedemeyer *et al.* 1990). Domestic and wild salmonids, however, can differ greatly in their physiological response to acute stressors (Casillas and Smith 1977; Woodward and Strange 1987). The stress response of fish of the same genetic stock can also differ if they were raised under different rearing environments (Salonius and Iwama 1993). Published literature abounds with information on the physiological stress response of domesticated fish species to specific stressors common to aquaculture such as handling, tagging, grading, and transport (Barton *et al.* 1980; Ellsaesser and Clem 1986; Barton and Iwama 1991; Sharpe *et al.* 1998). Studies investigating more environmentally applicable factors (e.g. toxicant

or pollution effects, disease transmission) usually do so under controlled laboratory settings (McLeay 1975).

Third, is it possible to differentiate between physiological responses due to normal environmental/seasonal fluctuations and those due to sublethal environmental disturbances? The emphasis of using animals as environmental biomarkers has largely been limited to toxicant or pollution effects (Peakall 1992). Since natural habitat variation is unavoidable, it is important to examine its effects on the physiology of fish and, subsequently, to take these effects into consideration when interpreting comparative results. This final point forms the crux of the problem in environmental monitoring.

Depledge (1989) proposes using physiological and behavioural changes as early warning signals for significant marine pollution. He outlines 3 categories of condition/impairment levels:

1) homeostasis (normal processes)
2) compensation (energy-consuming processes)
3) non-compensation (failure of physiological and behavioural processes)

Interestingly, these stages closely parallel those of a stress response which is initially adaptive (energy-consuming processes of compensation) in a disturbed organism, but becomes increasingly maladaptive, and ultimately fatal (non-compensation), in extremely stressful situations.

Depledge (1989) makes a distinction between changes in physiology/behaviour (i.e. impairment) and the consequences of such changes (i.e. disability). An organism that must compensate for a disturbance is obligated to divert energy from other important processes such as growth and reproduction. A portion of an organism's compensatory energy reserve may mask small deviations from "normal" biological ranges (some impairment) with very little disability incurred. Advanced impairment (maladaptive aspect of stress response) can lead to severe

disability that may become evident at the population level, and potentially at the ecosystem level. Hence, an environmental monitoring strategy should attempt to forestall detrimental effects by detecting the early changes that predispose an organism to severe impairment and eventual disability. An effective program, however, will also examine the degree to which inherent habitat features contribute to normal physiological variation.

Bull Trout and Their Habitat

Bull trout, or bull charr (*Salvelinus confluentus* Suckley) are endemic to North America ranging from northern California (41°N) to the Yukon River drainage basin (62°N) and from western Alberta and Montana (114°W) to northwestern British Columbia (133°W) (Haas and McPhail 1991). It is believed that bull trout are now locally extirpated in northern California (Haas and McPhail 1991). Until 1978, bull trout were taxonomically confused with their coastal congener, the Dolly Varden (*S. malma*). They are morphologically similar, but are phylogenetically distinct (Cavender 1978; Grewe *et al.* 1990). There is evidence, however, that Dolly Varden and bull trout hybridize where sympatric populations exist (Baxter *et al.* 1997).

Bull trout are a coldwater-adapted, highly piscivorous fish species with narrow habitat requirements. Their abundance increases with increasing latitude (Haas and McPhail 1991) as well as with increasing elevation (Donald and Alger 1993). They prefer higher gradient headwater streams where maximum summer water temperatures generally do not exceed 18°C and are typically below 12°C (Fraley and Shepard 1989; Goetz 1997). Bull trout have been known to occur in stream gradients as high as 30% (Cannings and Ptolemy 1998).

Because bull trout have specific habitat requirements for rearing and spawning, as well as a general sensitivity to disturbances at all life-stages, they are considered excellent indicators of environmental disturbances (Fraley and Shepard 1989; Cannings and Ptolemy 1998). As a

result, bull trout have been blue-listed in British Columbia. A blue-listed species is not currently threatened, but it is at higher risk due to current or anticipated declining population numbers, restricted movement, or increasing loss of suitable habitat (Cannings and Ptolemy 1998).

Study Area and Local Bull Trout Populations

The Torpy River watershed (Figure 3.1), located approximately 100 km east of Prince George, British Columbia, is part of the Upper Fraser River drainage system in the sub-boreal interior ecoregion (McPhail and Carveth 1993; Demarchi 1995, cited in Cannings and Ptolemy 1998). The Torpy watershed was chosen as the study site because of increased harvesting activities to utilize wood killed by the Hemlock looper (*Lambdina fiscellaria lugubrosa* Hulst). This insect reduces timber quality through extensive foliage destruction resulting in tree mortality.

The Upper and Lower Torpy Rivers drain an area of roughly 700 km^2 (7 000 ha). Most of the perennial streams that are tributary (primarily first and second order streams) to the Torpy River mainstem are small with channel widths typically ranging from 1 – 3 m (the widest is about 6 m), and shallow enough for wading during the summer. Streams in the Upper Torpy tend to have high gradients (averaging 10-12%) and low water conductivities (above 30 µS/cm, but below 200 µS/cm), while Lower Torpy streams have low gradients (below 10%, but usually 4-5%) and high water conductivities (above 100 µS/cm, but below 300 µS/cm).

Adult bull trout from the Lower Torpy have been reported to migrate up to 360 km into the Fraser River within a 6 month period (C. Harris[4], pers. comm.). It is not certain whether bull

[4] Consultant, AquaFore Consulting Ltd., 3815 Willow Road East, Prince George, BC, V2N 5Z3.

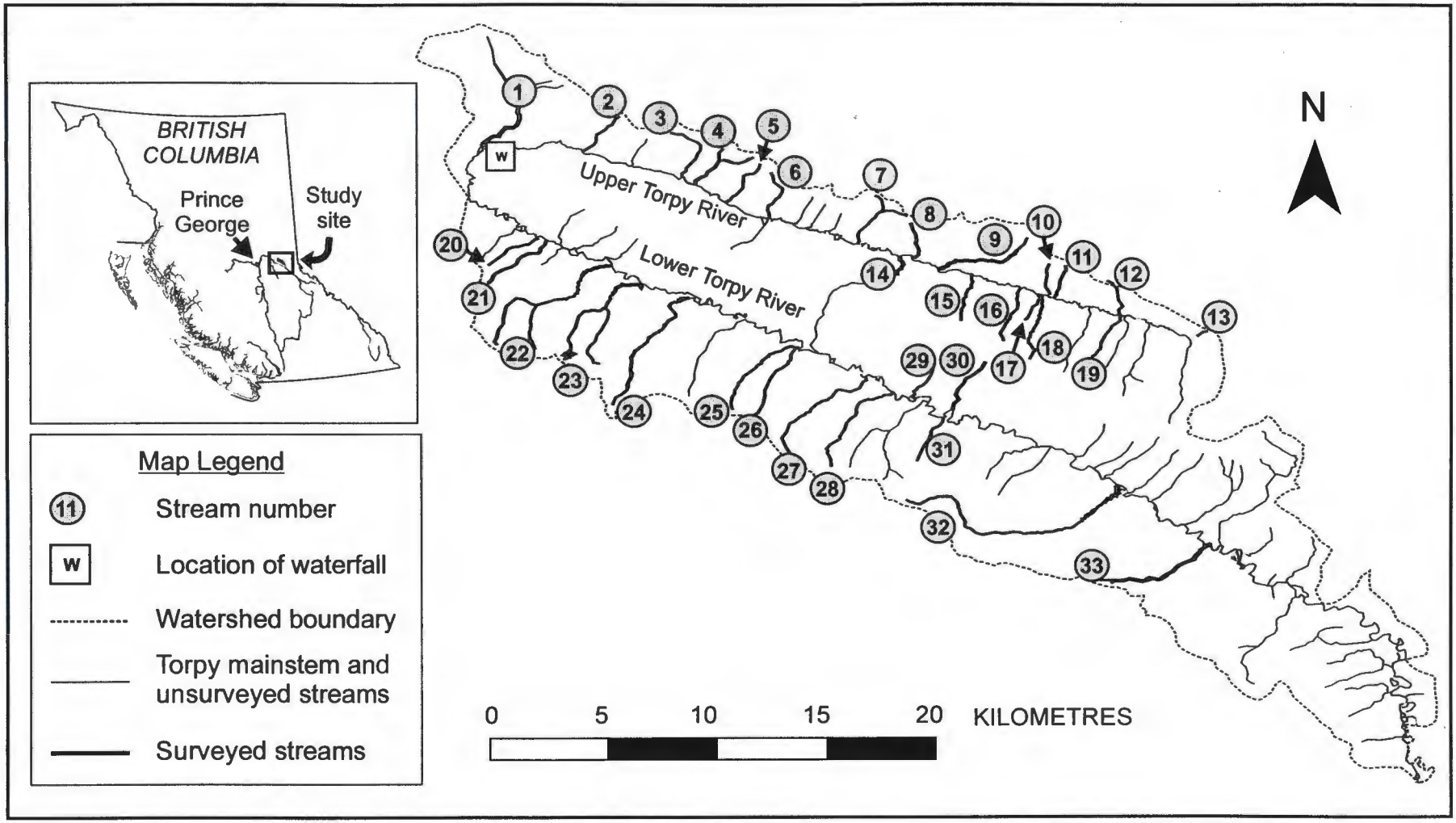


Figure 3.1. Map of the Torpy River watershed with watershed boundaries shown. Stream identification numbers of surveyed streams (bold lines) are given.

trout in the Upper Torpy represent a migratory or resident population. A large waterfall located near the hairpin turn between the Upper and Lower Torpy Rivers (Figure 3.1) is considered to be a significant barrier to fish movement to or from the Upper Torpy (D. Cadden[5], pers. comm.). Thus, it is speculated that bull trout in the Upper Torpy are resident fish (i.e. non-migratory), but this has not been confirmed.

Research Objectives

The principal goal of this study was to assess the extent to which adjacent forest cover (canopy-closure) and selected stream features in the Torpy River watershed influence physiological traits of wild bull trout at the primary (cellular) and secondary (tissue) levels of biological organization (see General Introduction). The impacts of environmental disturbances are commonly monitored at the late tertiary (whole animal) or quaternary (population/ecological) levels at which point the damage is extensive and/or irreparable. This chapter is not intended to ascertain whether bull trout in the Torpy watershed are stressed *per se*, but rather to determine whether certain habitat features contribute to normal physiological variation that may confound the interpretation of biomonitoring data. Emphasis is on the importance of taking the effects of inherent habitat features into consideration when analysing field-collected data.

3.3 MATERIALS AND METHODS

3.3.1 Stream Surveys, Fish Capture, and Sampling

Fieldwork was conducted in 1996 (31 July – 24 September) and 1997 (26 July - 22

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August). Habitat variables of 34 streams were measured in reaches that were within, and representative of, the sampled (electroshocked) stream sections. Stream gradient was measured with a clinometer over a distance ranging from 20-30 m depending on visibility due to riparian vegetation and stream sinuosity. Mean water velocity was determined by the float method (Gore 1996) where the travel time of a floating stick for a specified distance was measured in triplicate with a stopwatch. This value was multiplied by a correction factor of 0.85 to account for streambed roughness (Gore 1996). Cross-sectional area of the stream was calculated by multiplying the wetted channel width and mean depth. Mean depth was calculated from depth measurements taken along a transect (the same line of measurement for wetted width) at 10 cm intervals. Discharge rate (m^3/s) was obtained by calculating the product of water velocity (m/s) and the cross-sectional area (m^2) of the wetted stream. Ambient water conductivity and temperature were measured in triplicate with a portable, dual-readout water conductivity meter (CheckMate 90, Corning Inc.).

All fish were captured by 2-pass backpack electroshocking (Smith-Root, Model 12-A) using voltages ranging from 200-300 V at a frequency of 80 Hz (pulses per second). All electroshocking occurred during the day from early morning to late afternoon (about 0800 to 1800 h) with a 2-person crew. Stream sections were electroshocked in 100 m segments at a time using barrier nets to delineate the upstream and downstream boundaries and to prevent fish movement into and out of each section.

Upon capture, fish were immediately netted into an anaesthetic bath solution of tricaine methanesulphonate (50-60 mg/L buffered 1:1 with sodium bicarbonate). Fish were anaesthetized within 2 min and blood sampling was completed within an additional 1-2 min. Blood samples were collected ventrally from the dorsal artery with a tuberculin (1 mL) syringe fitted with a 25 gauge needle. Both were pre-coated with ethylenediamine tetraacetic acid (EDTA) as this

anticoagulant does not interfere with molecular genetic analyses of blood. Fork length was measured and the adipose fin was clipped. A drop of blood (about 5 μL) was stored in 95% ethanol in a small microcentrifuge tube for genetic identification of bull trout species. Another drop was used for blood smears. The syringe containing the remaining blood was covered with crushed ice until haematocrit (hct) values could be read and the plasma obtained. Captured fish missing an adipose fin were assumed to have been sampled previously and were released back into the stream. Fish captured in stream #27 (see Figure 3.1) were very small (mean fork length was 7.5 cm). It is difficult to collect sufficient blood sample volumes non-destructively from very small fish. As they were not sampled for blood, no physiological data was obtained from these fish. Furthermore, the fish collection permit for this study prohibited the terminal sampling of bull trout in the Torpy watershed (MoELP). They were visually identified as bull trout (McPhail and Carveth 1993) and included in the fish abundance and presence-absence data analysis. Thirty-eight rainbow trout (*Oncorhynchus mykiss*) were captured in the Lower Torpy, but blood sample collection was possible from only 28 rainbow trout. Rainbow trout numbers were insufficient for statistical analysis and so are excluded from the final results and discussion sections in this chapter.

Since the diagnostic assays required only a small amount of whole blood or plasma and no tissue samples, non-destructive blood sampling was performed as the death of the animal was unnecessary. In salmonids, total blood volume is approximately 3-5% of total body weight. Removing 30-40% of this volume does not compromise the long-term survival of fish in the laboratory (G. Iwama[6], pers. comm.). A minimum of 30 μL of plasma was required to perform

[6] Professor, Faculty of Agricultural Sciences, University of British Columbia, Suite 248-2357 Main Mall, Vancouver, BC, V6T 1Z4.

all laboratory assays without replication (60 μL for replicate assays). Since preliminary sampling of some bull trout in the Torpy watershed indicated that hct values ranged from 30 to 70%, about 100 μL of whole blood was needed from each fish. This volume is below that deemed safe to be removed from fish. Genetic identification of bull trout followed the protocols described by Baxter *et al.* (1997) and was modified by using *HaeIII* as the restriction enzyme.

3.3.2 Laboratory Analysis

The determination of haematocrit, plasma cortisol, plasma glucose, plasma lysozyme activity, and differential leucocyte counts are described in Chapter 1 (see Sections 1.3.2 to 1.3.6).

3.3.3 Extraction of Canopy-Closure Data Using A Geographic Information System (GIS)

Clinnick (1985, cited in Barling and Moore 1994) stated that a possible method in defining the area necessary in order to protect a water system is to delineate the catchment area contributing to runoff into the system. Such areas are separated from similar adjacent catchment basins by ridges (Stanford 1996). This method was chosen for this study because it would identify riparian and upland areas that likely contribute to stream variation. The identification of individual catchment areas, or "streamsheds", was achieved through the use of a geographic information system (GIS) using the ARC/INFO® method.

In brief, a digital vector database containing information for forest cover, topographical elevation lines (contour interval = 20 m), streams, and roads for the Torpy River region was obtained from Northwood Pulp and Timber Ltd. in Prince George, British Columbia. Thirty-one surveyed streams were identified and their respective streamsheds manually digitized in ArcEdit (ARC/INFO®, Environmental Systems Research Institute 1993) using contour elevation data. Three streamsheds (stream #s 1, 13, and 34) were not digitized because digital forest cover data

for the entire streamshed were not available. Hence, these streams were excluded from analysis. No bull trout were captured in these three streams. Canopy-closure information was extracted from datafiles associated with forest cover "patches" found within streamshed boundaries. Because forest cover data within each streamshed consisted of a mosaic of patches, or polygons (Figure 3.2), a single weighted canopy-closure index value (CCWT) was calculated for each stream, using the following equation, to account for the different levels of canopy-closure and area of each polygon:

$$CCWT = \frac{[(A_1 * cc_1) + (A_2 * cc_2) + \ldots + (A_x * cc_x)]}{A_T}$$

where CCWT = weighted canopy-closure value
A_x = area of individual forest cover polygons within a streamshed
cc_x = canopy-closure value for individual forest cover polygons within a streamshed
A_T = total area of streamshed

More specifically, vector data for nine maptiles encompassing the Torpy region were used. Streams that were sampled in the field were identified by generating a route system using the logging road network and calculating the distance to each stream (the distance along the route system to the node representing the point where the road crosses the stream) as pre-determined in the field using the odometer of the field vehicle and TRIM (Terrain Resource Inventory Mapping) maps. Forest cover polygons contained within the boundaries of each streamshed were clipped using the IDENTITY command in ARC/INFO®. The resulting streamshed canopy-closure information was extracted and unloaded according to attribute (streamshed identification number and associated forest cover polygons, polygon area, percentage canopy-closure).

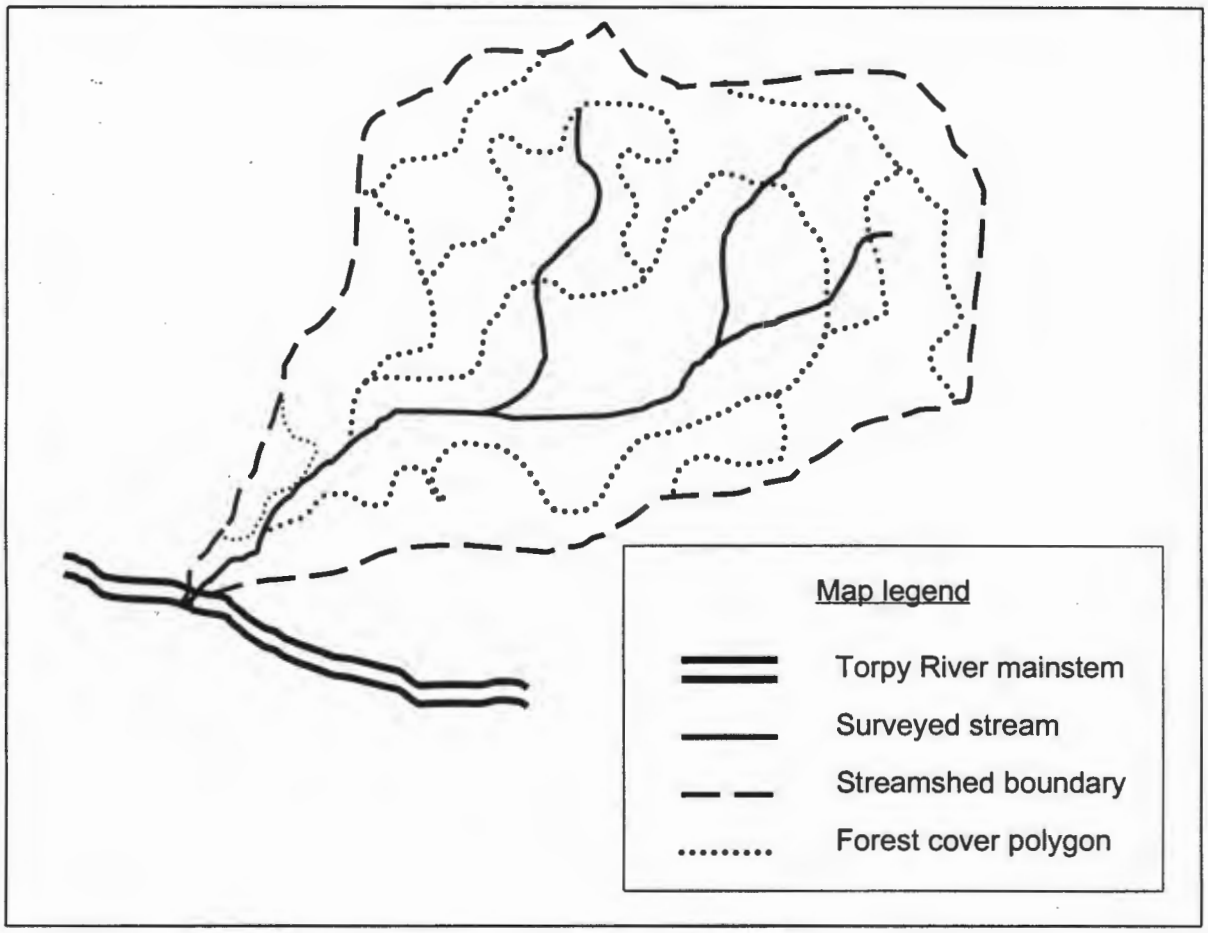

Figure 3.2. Example of forest cover polygons delineated within individual digitized streamshed boundaries.

3.3.4 Statistical Analysis

Weighted canopy-closure index (CCWT), discharge rate, stream gradient, wet channel width, and mean channel depth were tested for collinearity. Significant collinear relationships existed among discharge rate, wet channel width, and mean channel depth ($P<<0.001$ for any pair combinations among the 3 variables). Wet channel width and mean channel depth, therefore, were excluded from simple and multiple regression models. Simple linear regression analysis was performed on individual physiological measurements using CCWT, stream gradient, and discharge rate as independent variables. Multivariate regression analysis was performed on individual physiological measurements, fish abundance, and presence-absence data via the General Linear Model (Sokal and Rohlf 1981; SYSTAT® 7.0: Statistics 1997) using models incorporating the same habitat features. A sequential Bonferroni adjustment of alpha, α, (Rice 1989) was performed to correct for the numerous multiple regression tests.

3.4 RESULTS

3.4.1 Stream Surveys and Fish Sampling

Genetic analysis of blood samples confirmed that the sampled fish were bull trout. Thirty-one streams tributary to the Torpy River mainstem were sampled. Of the 31 streams surveyed, bull trout were present in 13 streams (11 in the Upper Torpy and 2 in the Lower Torpy). All bull trout survived the process of capture, anaesthesia, and blood sampling. Stream measurements and electrofishing results are shown in Table 3.1.

Table 3.1. Measurements of habitat features for the 31 analysed streams in the Torpy River watershed. Stream identification numbers correspond to those provided in Figure 3.1.

Stream ID:	Fish abundance (/ 100 m)	CCWT [a] (%)	Gradient (%)	Discharge (m^3/s)	Depth (m)	Wet channel width (m)	Conductivity (µS/cm)	Water temp. (°C)
UPPER TORPY								
2	0	36.6	15.0	0.22	0.13	2.10	103.8	4.8
3	5.7	21.3	16.0	0.71	0.30	3.39	36.1	5.3
4	3.6	30.2	15.0	0.18	0.13	3.18	41.1	5.4
5	4.4	17.8	17.0	0.22	0.11	2.32	57.2	5.5
6	2.9	16.6	15.0	0.11	0.12	1.73	78.0	5.9
7	2.6	23.1	12.5	0.29	0.15	2.95	64.6	5.4
8	0	37.1	11.0	0.04	0.18	0.66	144.3	5.2
9	1.1	44.0	2.0	0.26	0.11	3.53	30.6	16.1
10	0	43.1	4.0	0.01	0.04	0.40	82.8	7.8
11	0	43.5	19.0	0.08	0.08	1.73	44.9	9.5
12	0	47.9	8.0	0.03	0.07	1.25	nd [b]	nd
14	4.1	43.0	15.0	0.13	0.10	3.07	165.6	5.3
15	3.3	22.1	7.0	0.22	0.16	1.67	88.4	10.8
16	2.5	23.4	10.0	0.26	0.13	1.84	132.4	9.7
17	0	45.5	7.0	0.01	0.05	0.60	199.9	8.9
18	3.2	19.1	6.0	0.26	0.15	2.13	67.9	9.5
19	6.7	35.5	7.5	0.11	0.11	3.11	58.9	8.6
LOWER TORPY								
20	0	23.4	5.0	0.05	0.09	1.11	224.0	8.1
21	0	27.7	4.0	0.24	0.18	1.70	249.0	8.7
22	0	23.4	1.0	0.95	0.23	5.34	238.3	5.7
23	0	26.2	5.0	0.40	0.15	3.89	190.3	5.4
24	0	19.5	8.5	0.61	0.22	6.23	239.3	5.2
25	0	17.9	9.0	0.07	0.17	3.96	236.0	4.8
26	0	32.9	5.0	0.47	0.08	2.52	236.7	8.2
27	4.0	21.7	2.0	0.26	0.15	3.45	218.0	11.4
28	0	21.4	4.0	0.40	0.17	3.95	170.0	10.4
29	0	59.3	5.0	0.14	0.08	3.16	43.1	11.2
30	0	48.6	5.0	<0.01	0.03	0.55	109.7	11.6
31	0	19.3	5.0	0.66	0.21	3.90	192.0	12.6
32	1.6	41.6	4.5	1.28	0.33	5.24	293.0	5.3
33	0	46.0	4.5	0.26	0.12	3.05	227.0	11.9

[a] CCWT = weighted canopy-closure.

[b] nd = no data.

3.4.2 Physiological Measurements and Fish Abundance

Mean physiological values of wild bull trout in the Torpy River watershed are presented in Table 3.2. These values may serve as guidelines for other coldwater salmonids in the wild. Generally, the mean glucose level was well within the approximate normal range reported for other salmonids (approx. 41-151 mg/dL, Wedemeyer *et al.* 1990) while mean hct and cortisol values were slightly above the reported normal ranges (hct: 24-52%, cortisol: 0-40 ng/mL, Wedemeyer *et al.* 1990). Mean plasma lysozyme activity of the bull trout were comparable to values of control/"unstressed" salmonids described in other studies (approx. 15-60 U/mL, Sakai 1983; Möck and Peters 1990). Mean total leucocyte abundance was somewhat below the reported normal range (approx. 0.8-1.8 % of total rbcs, Wedemeyer *et al.* 1990).

The results of the simple linear regression analysis are presented in Table 3.3. Plasma cortisol, total leucocyte abundance, and fish presence-absence are excluded from the table as the simple linear regression tests were not significant for these three dependent variables. The results of the multiple regression analysis on physiological measurements and fish abundance are presented in Table 3.4. Total leucocyte abundance is excluded from the table as multiple regression tests were also not significant for this dependent variable.

Plasma lysozyme activity

Weighted canopy-closure and gradient strongly influenced plasma lysozyme activity (Table 3.3) in simple linear regression analysis. All multiple regression models were significant for plasma lysozyme activity (Table 3.4). The greatest variation in lysozyme activity was explained by the combined effects of CCWT, gradient, and discharge rate.

Table 3.2. Mean physiological values (±1 SE) of bull trout sampled in individual streams. Fish numbers are shown in parentheses.

	Mean Physiological Measurements					
Stream ID:	Fork length (cm)	Hct (%)	Cortisol (ng/mL)	Glucose (mg/dL)	Lysozyme activity (U/mL)	Total WBC (% rbc)
3	18.8 ± 0.7 (16)	52.6 ± 3.2 (16)	73.95 ± 9.81 (16)	70.53 ± 6.67 (16)	12.91 ± 0.38 (15)	0.71 ± 0.19 (16)
4	21.2 ± 1.0 (8)	72.5 ± 2.8 (8)	73.06 ± 20.73 (8)	69.72 ± 7.36 (8)	12.05 ± 0.52 (8)	0.22 ± 0.05 (8)
5	13.0 ± 1.2 (15)	43.3 ± 2.8 (10)	86.24 ± 13.57 (11)	83.58 ± 16.09 (11)	13.15 ± 0.72 (11)	0.40 ± 0.13 (7)
6	11.7 ± 1.0 (2)	38.6 (1)	27.18 (1)	82.34 (1)	8.46 (1)	0.24 ± 0.05 (2)
7	20.0 ± 0.5 (6)	71.2 ± 1.5 (6)	23.17 ± 8.04 (6)	73.27 ± 3.62 (6)	14.05 ± 1.13 (6)	0.36 ± 0.12 (6)
9	15.3 ± 2.4 (3)	28.9 ± 0.3 (2)	29.2 ± 4.00 (2)	65.07 ± 0.37 (2)	20.37 ± 0.02 (2)	0.24 ± 0.12 (2)
14	14.0 ± 0.4 (18)	54.6 ± 2.5 (15)	45.05 ± 5.97 (17)	52.98 ± 4.86 (17)	10.61 ± 0.47 (17)	0.45 ± 0.10 (17)
15	19.2 ± 2.8 (4)	32.7 ± 12.1 (3)	14.99 ± 9.72 (3)	82.60 ± 0.88 (3)	19.91 ± 0.91 (3)	0.45 ± 0.23 (3)
16	15.8 ± 1.5 (8)	31.5 ± 2.2 (3)	44.77 ± 11.53 (3)	83.82 ± 8.52 (3)	20.01 ± 1.53 (3)	0.25 ± 0.18 (3)
18	19.7 ± 0.8 (8)	41.2 ± 4.5 (8)	30.71 ± 11.54 (8)	91.36 ± 8.32 (8)	22.28 ± 0.73 (8)	1.45 ± 0.64 (8)
19	19.1 ± 0.3 (3)	nd [a]	16.10 (1)	62.87 ± 1.84 (2)	22.43 ± 0.31 (2)	0.80 ± 0.10 (2)
27	7.5 (1)	nd	nd	nd	nd	nd
32	14.3 ± 2.0 (12)	49.4 ± 3.2 (7)	78.41 ± 17.71 (10)	99.45 ± 3.52 (10)	11.41 ± 0.95 (11)	0.11 ± 0.04 (12)
Grand mean:		51.4 ± 1.7	57.35 ± 4.70	74.81 ± 3.13	13.99 ± 0.48	0.50 ± 0.08
Minimum:		9.3	2.78	30.07	7.33	0 [b]
Maximum:		85.5	194.31	190.45	25.99	4.84

[a] nd =no data.

[b] Does not necessarily indicate absence of leucocytes but rather that none were found during blood smear examination.

Table 3.3. Results of simple linear regression analysis of individual physiological measurements of bull trout captured in the Torpy River watershed.

	Dependent Variables			
Independent variables:	Hct	Glucose	Lysozyme	Fish abundance
Weighted canopy-closure (ccwt)	ns [a]	ns	$P<0.003$ $r^2 = 0.14$ slope = -15.82	ns
Gradient	$P<0.02$ $r^2 = 0.10$ slope = 1.07	$P<0.02$ $r^2 = 0.09$ slope = -1.82	$P<<0.0001$ $r^2 = 0.22$ slope = -0.43	$P<0.02$ $r^2 = 0.32$ slope = 0.19
Discharge rate	ns	$P<0.04$ $r^2 = 0.08$ slope = 22.61	ns	ns

[a] ns = not significant.

Table 3.4. Results of multiple linear regression analysis of physiological traits and fish abundance using weighted canopy-closure (CCWT), stream gradient, and discharge rate as independent variables. Four models were run for each dependent variable. Probabilities (dots[a]) and regression coefficients (in parentheses) are provided for each factor within a model. Total *P*-values and coefficients of determination (r^2) for each model are given at the far right. Dashes indicate which independent factor was not included in the model. Models showing the combined effects of all three habitat factors are highlighted in bold text.

		Independent Variables							
Dependent variable:		CCWT		Gradient		Discharge		TOTAL MODEL	
Plasma	(1)	••••	(-23.09)	••••	(-0.57)	—		*P*<<0.001	$r^2 = 0.49$
lysozyme	(2)	—		••••	(-0.64)	••••	(-5.84)	*P*<<0.001	$r^2 = 0.43$
activity	(3)	••	(-14.77)	—		ns [b]		*P*<0.004	$r^2 = 0.16$
	(4)	••••	**(-22.00)**	••••	**(-0.75)**	••••	**(-5.47)**	***P*<<0.001**	**$r^2 = 0.67$**
Plasma	(1)	•	(-79.96)	•••	(-2.28)	—		*P*<0.003	$r^2 = 0.16$
glucose	(2)	—		ns		ns		ns	
	(3)	ns		—		••	(25.05)	*P*<0.01	$r^2 = 0.13$
	(4)	•	**(-81.90)**	•	**(-1.76)**	**ns**		***P*<0.002**	**$r^2 = 0.20$**
Plasma	(1)	ns		ns		—		ns	
cortisol	(2)	—		••	(3.30)	•••	(46.95)	*P*<0.002	$r^2 = 0.17$
	(3)	ns		—		ns		ns	
	(4)	**ns**		••	**(3.25)**	•••	**(47.05)**	***P*<0.005**	**$r^2 = 0.17$**
Hct	(1)	ns		•••	(1.22)	—		*P*<0.01	$r^2 = 0.15$
	(2)	—		••	(1.16)	ns		*P*<0.03	$r^2 = 0.11$
	(3)	ns		—		ns		ns	
	(4)	**ns**		•••	**(1.31)**	**ns**		***P*<0.02**	**$r^2 = 0.16$**
Fish	(1)	ns		•••	(0.14)	—		*P*<0.02	$r^2 = 0.38$
abundance	(2)	—		••	(-0.81)	ns		*P*<0.03	$r^2 = 0.37$
	(3)	ns		—		ns		ns	
	(4)	**ns**		*	**(0.15)**	**ns**		***P*<0.04**	**$r^2 = 0.41$**

[a] • < 0.05; •• <0.01; ••• <0.005; •••• <0.001

[b] ns = not significant.

Plasma glucose

In simple linear regression, gradient and discharge rate significantly influenced plasma glucose concentrations (Table 3.3). It is surprising, therefore, that the 2-factor model combining gradient and discharge was not significant. All other models were significant for glucose concentrations (Table 3.4). The greatest amount of variation in glucose levels was also explained by the 3-factor model with CCWT, gradient, and discharge rate.

Plasma cortisol

No habitat feature significantly influenced plasma cortisol levels in simple linear regression analysis. Multiple regression models (2- and 3-factors) incorporating gradient and discharge rate, however, were significant (Table 3.4).

Haematocrit

Stream gradient was the only factor that strongly influenced hct in simple linear regression. All multifactor models incorporating gradient were significant (Table 3.4). The combined effects of CCWT and discharge rate were not significant in explaining the variation in hct.

Fish abundance

Variation in fish abundance due to habitat features followed the same pattern as hct. Gradient alone was a significant factor (Table 3.3) as were multifactor models including gradient (Table 3.4). The combined effect of CCWT and discharge did not significantly contribute to variation in fish abundance.

3.5 DISCUSSION

It is evident that the physiology of bull trout in the Torpy River watershed is influenced by specific habitat features. Canopy-closure, stream gradient, and discharge rate contributed to variation in haematocrit, plasma glucose and cortisol levels, and plasma lysozyme activity, but not in total leucocyte abundance. These three habitat traits also influenced bull trout abundance. Whether the physiological status of the bull trout in this study reflects a stressed state *per se* or an acclimatized ("normal") state to their surroundings is not self-evident. It is apparent, however, that existing riparian and upland forest cover and stream (geomorphic) traits account for a part of the observed physiological variation. Neglecting to take these "background" effects into consideration could lead to erroneous interpretation of field-collected data.

Ambient temperature is a major driving force of biochemical processes, particularly in poikilotherms (Hazel 1993). Fish in the wild, however, are acclimatized to natural fluctuations in water temperature and, thus, may not exhibit a physiological response typical of an acute thermal stress (Hazel 1993). It was not possible to incorporate temperature as an environmental factor, however, as the measurements obtained in the field represent a single point in time and were not monitored over an extended period.

Based on the simple linear regression tests, each habitat feature contributed, to some extent, to the variation in at least 1 of 4 dependent factors (hct, glucose concentration, lysozyme activity, and fish abundance), but not in cortisol levels or leucocyte numbers. Variation in plasma cortisol concentrations, however, could be accounted for by multiple linear regression analysis. The fact that a portion of the variation in all dependent factors (except for total leucocyte numbers) was significantly attributed to the combined effects of canopy-closure, stream gradient, and discharge rate emphasizes the importance of examining a number of

habitat/environmental traits (Bozek and Hubert 1992; Watson and Hillman 1997). It also underscores the fact that multiple habitat factors not normally considered as stressors do influence the physiology of fish. Lanka *et al.* (1987) demonstrated that geomorphic and stream habitat variables had significant combined influences on the abundance of brown trout (*Salmo trutta*), rainbow trout (*O. mykiss*), brook trout (*Salvelinus fontinalis*), and cutthroat trout (*O. clarki*) in small streams.

It is generally recognized that the environmental/rearing history (i.e. acclimation temperature, nutritional status, size, development stage) and genetic makeup of a fish can influence the nature of its physiological stress response (Adams 1990; Salonius and Iwama 1993). These "external" factors may also alter an organism's capacity to respond to additional stressors, acute or chronic, in an adaptive manner (Schreck 1981; Adams 1990; Barton and Iwama 1991). In short, how much of a fish's compensatory reserve remains if a portion of that reserve is used to deal with environmental fluctuations?

Biotic and abiotic habitat attributes are not the only factors that can influence variation in fish physiology. Although not investigated specifically in this study, seasonal and diel effects have an impact on physiological characteristics. Pickering and Pottinger (1983) found that plasma cortisol in brown trout exhibited a pronounced diel rhythm with peak levels occurring during darkness (approximately 2000 – 0800 h) and lower, relatively stable, concentrations occurring during the day from roughly 0800 – 1700 h. The bull trout sampled in this study were captured during the day from about 0800 – 1800 h which encompasses the period of lower cortisol levels described by Pickering and Pottinger (1983). Thus, the cortisol values obtained from the bull trout likely represent lower-limit levels and not an elevated value that may be misinterpreted as a stressed state.

Pickering and Pottinger (1983) also found that brown trout displayed seasonal peak plasma cortisol levels in the spring and early summer. A supporting study by Maule *et al.* (1993) demonstrated that coho salmon (*O. kisutch*) displayed a similar seasonal pattern in plasma cortisol concentrations (peaks in March-April and June-July). Again, the bull trout in this study generally were sampled outside of these periods (late July – August/September).

There also appears to be a seasonal pattern for lysozyme activity. Muona and Soivio (1992) found that the plasma lysozyme activity of domestic Atlantic salmon (*Salmo salar* L.) were generally higher during the winter months and declined to lower-limit levels in late spring. It is not known, however, whether lysozyme activities began to increase again at the onset of autumn or winter. It is likely that the plasma lysozyme activities measured in the Torpy River bull trout reflect non-peak values.

Adams (1990) cautions against relying on a single physiological variable as an "all-purpose" bioindicator, stating that a single variable or a number of variables measured at the same level of biological organization cannot adequately describe complex population- or ecosystem-level conditions. Because of its implication in many functions (e.g. reproductive maturation, smoltification, immune function), an increased cortisol concentration is often the basis by which an organism is judged to be stressed. But adverse impacts are not reflected by elevated cortisol levels alone. In fact, blood cortisol responses can vary depending on the nature of the stressor. A significant short-term peak in cortisol levels is generally associated with an acute, rather than chronic, stress response (Adams 1990; Barton and Iwama 1991). For example, plasma/serum cortisol levels may increase from "pre-stress" values of 0-70 ng/mL to values well over 100-200 ng/mL in response to an acute stressor (Barton *et al.* 1986; Pickering and Pottinger 1989; Barton and Iwama 1991; Mesa 1994). Cortisol levels in chronically stressed fish, however, may be within normal physiological range, although slightly raised, but still at much

lower levels than those usually associated with an acute response (Pickering and Pottinger 1989; Wendelaar Bonga 1997). It is also possible for fish to exhibit an apparent lack of cortisol response despite exposure to lethal concentrations of toxicants/pollutants. Barton and Iwama (1991), however, warn that this does not necessarily indicate a lack of stress response, but that chemicals such as cadmium or endrin appear to suppress the typical cortisol response. Mean cortisol levels observed in the Torpy River bull trout were not vastly different from those reported for other charrs and non-charr salmonids, and were much lower than values given for acutely stressed fish (refer to Barton and Iwama 1991).

Because the integrated stress response and immunocompetence are closely linked (Maule *et al.* 1989; Pickering 1993; Wendelaar Bonga 1997), lysozyme has recently been investigated in several studies as a component of a physiological stress response (Möck and Peters 1990; Rotllant *et al.* 1997; Demers and Bayne 1997). It is unclear from the literature whether high or low levels of plasma/serum lysozyme benefit fish. Fevolden *et al.* (1993) examined rainbow trout that naturally produced low or high lysozyme levels, indicating a genetic component to lysozyme activity. Thus, a high lysozyme activity could be an indicator of a natural "high-producer" rather than a stressed individual. Fevolden *et al.* (1993, 1994) also proposed that high lysozyme activities do not necessarily translate into greater disease resistance and that, in fact, significantly increased levels of lysozyme were more indicative of a stressed state than an enhanced immune response. If this is the case, then the negative relationship between canopy-closure and plasma lysozyme activity in bull trout in the Torpy watershed might be interpreted as implying that salmonids found in streams with low levels of canopy-closure are more stressed rather than having an immunological advantage. This line of reasoning is not entirely tenable as lysozyme activity can also decrease in response to various stressors (Möck and Peters 1990; Hajji *et al.* 1990, cited in Wedemeyer 1997). As was evident in the results of Chapter 2, brief handling

alone was sufficient to reduce serum lysozyme activity significantly and sustain these lowered levels for over 2 wks. Nevertheless, it is clear that a component of the non-specific immune system (i.e. lysozyme activity) is responsive to canopy-closure levels within a stream's catchment basin. This study and those of others (Grinde *et al.* 1988) demonstrate that fish are sensitive to their surroundings and that this sensitivity can be reflected by variations in lysozyme activity.

The question is thus raised of whether physiological traits that are insensitive to environmental fluctuations but sensitive to specific xenobiotic or anthropogenic/chronic disturbances would serve better as bioindicators (Bouck 1984). Which physiological parameter fits this description? At the primary (hormonal) and secondary (cellular/tissue) levels of organization, this may not be possible. Adams (1990) states that the primary and secondary stress responses are sensitive, but rapid, relatively short-term, and quite generalized. Responses that are slower are usually those of the tertiary and quaternary levels, but detectable effects at these response levels are often associated with damage that may be irreversible. If fisheries/resource management decisions were based solely on monitoring blood cortisol concentration or lysozyme activity, a number of problems arise. First, a single physiological variable is a poor indicator of complex ecosystem interrelationships. Such a restricted approach disregards not only the organism, but also the ecosystem in which it lives, as a functioning whole. Second, since adequate environmental/physiological controls are nearly impossible to establish in the field, what is the basis of comparison to conclude that the fish are stressed? And third, the response profile of certain physiological variables is dependent on the nature of the stressors(s); thus, high or low values do not necessarily mean stressed and unstressed states, respectively.

Haematocrit and blood glucose levels may be more representative of normal fluctuations due to feeding activity or the energy expended in order to maintain stream position in the faster flowing waters of high gradient or high discharge streams. A rapid and pronounced increase in glucose concentrations, due to the action of catecholamines (adrenaline in salmonids), is a commonly reported acute stress response with post-stress glucose levels generally reaching 150-300 mg/dL, or greater (Barton *et al.* 1986; Woodward and Strange 1987). Higher gradient usually implies higher water velocities and greater discharge rate (Hunter 1991) and so one would expect fish to expend more energy under such conditions. The significant positive relationship of discharge rate on plasma glucose levels supports this hypothesis. The significant negative relationship between gradient and glucose levels, however, as well as the apparent lack of collinearity between gradient and discharge rate, initially seem counter-intuitive and do not appear to support the energy-expenditure hypothesis. The Torpy watershed, in particular the Upper Torpy, consists of small headwater streams. Despite the high gradients characteristic of such streams, they tend to have step-pool profiles and have more pools for a given length of stream reach than do low gradient streams (Bisson and Montgomery 1996). As a result, the effects of high stream gradient may be countered by the presence of numerous plunge pools. Since water velocities are reduced in small plunge pools (Hunter 1991), where most of the bull trout were captured, then the discharge rate might also be lowered despite high stream gradients. This could have resulted in plasma glucose measurements that were lower than expected (i.e. within normal range). Although water flow rate through plunge pools was reduced, it may have been sufficient to claim some level of energetic cost from the bull trout in these small headwater streams. The significant positive effect of gradient on hct suggests that the energy requirements may also influence oxygen demand.

The issue of fish being exposed to slight electroshocking, within the "fright" or "awareness" zone of influence near the electroshocker anode, before actual capture is a possibility that should be addressed. Are the observed fish numbers an underestimation due to fish escaping shocking and capture? Is it possible that fish shocked from a distance, that once captured and sampled, have already initiated a hormonal stress response? These are two aspects of electroshocking that were not addressed in Chapter 2, but have direct bearing in this study where wild bull trout may have been exposed to shocking some distance from where the research crew was located. This effect was probably negligible for two reasons. First, to address the issue of observed fish abundances, relatively low fish numbers in the sampled streams may be attributed to two factors: 1) bull trout abundance in streams may have been underestimated if fish were escaping shocking and capture, or 2) bull abundance in small streams is naturally low, particularly in small shallow streams such as those found in the Torpy watershed. Like many stream-dwelling salmonids, bull trout are territorial and prefer to keep out of sight from neighbouring conspecifics. Visual isolation may be more difficult to achieve in small (first and second order) streams thereby forcing neighbouring fish to be spaced further apart. Thus, relative fish abundance is normally low and may not necessarily reflect fish escape. Second, to address the issue of confounded cortisol levels, fish that were inadvertently shocked from a distance would probably initiate a rapid stress (hormonal) response. These fish would exhibit relatively high plasma cortisol levels. In all surveyed streams, the vast majority of sampled fish were caught during the first (upstream) pass of electroshocking. If fish were shocked from a distance prior to capture and sampling, their plasma cortisol values (rapid-response hormonal trait) likely would have reflected this with a trend of increasing cortisol levels as sequential fish sampling progressed in an upstream direction. This was not the case, however, and simple linear regression of fish sampling order within a stream on cortisol concentrations was not significant.

The results presented in this chapter are the first to document the physiological and immunological status of wild bull trout *in situ*. Although the grand means did not differ greatly from the approximate ranges given by Wedemeyer *et al.* (1990), the range observed in this study highlight the considerable variation in the physiological traits studied. It is of interest to note that the lowest plasma cortisol level obtained in this study is on par with that of unstressed domesticated salmon. Pankhurst and Sharples (1992, cited in Wendelaar Bonga 1997) reported the lowest cortisol values to date (<8 ng/mL) in wild fish (snapper, *Pagrus auratus*) captured and sampled underwater. One may be confident that capturing wild fish by electroshocking and anaesthetizing them before collecting blood samples do not appear to result in artefactual haematological measurements.

To my knowledge, this thesis is also the first to describe physiological variation in wild salmonids in relation to habitat/stream variables commonly used to define/predict fish abundance and distribution (Kozel *et al.* 1989. Bozek and Hubert 1992; Rieman and McIntyre 1995; Watson and Hillman 1997). Although the majority of published literature focuses on the effects of stream features on fish abundance, the results presented here support the hypothesis that inherent stream traits such as those examined in this study can also influence the physiology of fish. While this study is not an attempt to determine whether bull trout in the Torpy River system are stressed, it identifies certain habitat attributes inherent to the stream on three different levels that exert significant influence on bull trout physiology: 1) those which cannot be modified (e.g. gradient), 2) those determined by season and climate (e.g. discharge rate), and 3) those that can be modified either through environmental stochastic events or anthropogenically (e.g. forest cover, and to a certain extent discharge rate). Of the three habitat features investigated in this study, forest cover (canopy-closure) offers the greatest degree of flexibility for resource management purposes.

In view of the results of this study, it is important to remember that multiple habitat features contribute to normal physiological variation in wild salmonids. No single habitat factor or physiological trait can adequately describe the state of an environment or the health/vigour of a fish population. Just as the integrated stress response occurs at different levels of biological organization and the interactions among these levels, effective biomonitoring programs and research projects should adopt a similarly comprehensive approach when investigating the physiological condition of wild fish populations.

4.0 CONCLUDING REMARKS AND RECOMMENDATIONS

Fish are susceptible to many biological, chemical, and physical stressors. Their capacity to cope with such disturbances is often reflected by their integrated physiological stress response. Some stressors, such as anaesthetization and capture, are those that cannot be avoided under normal experimental procedures. Still others may not be considered stressful *per se* (i.e. inherent habitat traits such as stream gradient or stream size), yet they represent factors that influence the physiology of fish in the wild. Modification of other habitat factors (i.e. canopy-closure) through either natural events or human activities have the potential to contribute to a suboptimal environment. The influence of these features on the physiology of wild fish must be addressed.

In Chapter 1, the effects of anaesthetization on the stress physiology of chinook salmon were examined. The comparison between tricaine and clove oil anaesthetization served a second purpose of investigating a more environmental- and user-"friendly" means of immobilizing fish in the laboratory and, more importantly, in the field where time constraints and the absence of holding facilities do not favour holding tricaine-anaesthetized fish for the recommended withdrawal period.

As a capture tool, the backpack electroshocker is acutely stressful to fish. In this study (Chapter 2), the incidence of spinal abnormalities was found to be significantly greater in shocked fish. This indicates that electroshocking can induce spinal abnormalities that are not externally obvious. Lateral vertebral shifts occurred with a greater frequency in the spinal columns of shocked fish. Although lateral shifts in vertebrae are relatively less debilitating than dorso-ventral shifts, particularly in organisms with more flexible spinal columns (i.e. fish, reptiles, and birds), it does not provide sufficient justification to continue electroshocking without due care. It is impractical to advocate the discontinuation of electroshocking despite the growing evidence showing that even a single brief electroshock can induce spinal abnormalities

in salmonids. Backpack electroshocking is indeed efficient and maximizes catch-per-unit-effort. But long-term repercussions from multiple capture within a field season and over a number of subsequent seasons must be considered.

Adams (1990) stated that many physiological traits have limited value as environmental stress indicators because it is difficult to differentiate between capture/handling effects and environmental disturbance effects. One of the goals of this study was to obtain an *in situ* "snapshot" of the physiological status of wild bull trout. The results presented in the preceding three chapters indicate that this objective is possible with the field protocols chosen. Capture by backpack electroshocking in small streams with immediate anaesthetization of fish does not alter physiological measurements significantly from pre-capture values provided that the time interval from capture to sampling is minimized (within 2-3 min). Fish are capable of recovering physiologically from an acute stressor within a relatively short time period. It must be recognized, however, that while certain physiological traits return to pre-stress values fairly quickly, others may remain disturbed for an extended interval (i.e. reduced serum lysozyme activity in response to handling). This thesis presents field procedures that can be adopted to optimize data collection in the field without damaging the environment (i.e. water contamination with inorganic anaesthetic chemicals) or significant harm to the fish (i.e. non-destructive blood sampling).

The concept of biomonitoring implies a temporal aspect which was not possible in this study. Effective monitoring programs are performed on a regular basis and at a frequency that allows for early detection of potentially adverse changes. The work presented in Chapter 3 does not attempt to ascertain whether the bull trout in the Torpy watershed are stressed, but it does provide some insight into the influence of external factors on data interpretation. Of the three habitat attributes investigated in this study, canopy-closure represents a factor that can be modified under resource management operations. This thesis provides some evidence that a

range of canopy-closure values can directly or indirectly influence the physiology of wild salmonids and that it is no longer a simple matter of one extreme (i.e. clearcut) or the other (uncut).

Based on the results provided in this thesis, the first two recommendations are suggested in future research designs with the goal of examining the *in situ* physiological condition of wild fish populations in small water systems:

1) The field procedures (anaesthesia and capture by electroshocking) described in this thesis and employed in order to examine the stress response/status of wild salmonids is limited by sampling design. Ideally, fish should be captured and sampled one at a time rather than caught en masse and then sampled. This is logistically possible for a minimum 2-person crew, but it is also time-consuming. Wider streams and/or marshes would probably require a relatively larger crew. Therefore, one should balance the costs of labour with time constraints specific to a given project, particularly in larger watersheds or in larger, more complex aquatic systems.

2) Backpack electroshocking is the only fish capture method that allows for immediate fish collection and sampling in the field. It is vital, however, to remember that fish can sustain extensive sublethal spinal lesions/injuries that may ultimately reduce their overall fitness.

From an environmental monitoring perspective, the following suggestions are made to emphasize the need to recognize system (site)-specific characteristics, their impacts on the physiology of local fish populations, and the direction of future research for the health of wild salmonids:

3) The results presented in this thesis may be specific to the bull trout in the Torpy River watershed. Results may not be similar for salmonids in other aquatic systems. It is important to keep in mind the differences among different salmonid species in terms of habitat and thermal preferences, behavioural characteristics, and site-specific traits, as well as how these traits can also influence the results in comparative studies.

4) Given the potential for future comparative studies, it is important to investigate a suite of physiological traits that reflect more than one level of biological organization, or reflect more than one aspect of physiological impact (e.g. reproduction, growth, health, nutritional status).

5) Biomonitoring requires tracking environmental and biological changes, if any, over time. This thesis was constricted to single point estimations of physiological and habitat features. Thus, the physiological values reported in this project cannot accurately assess the current health/stress condition of wild bull trout. It would be desirable for similar future studies to collect data at regular intervals for a longer period of time. This would provide a more concrete framework within which to interpret fluctuations or significant changes in physiological measurements in relation to habitat changes as being benign, potentially damaging, or detrimental.

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