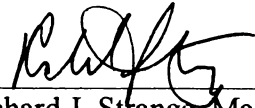


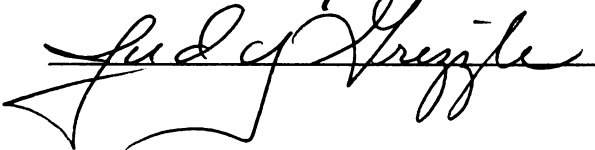
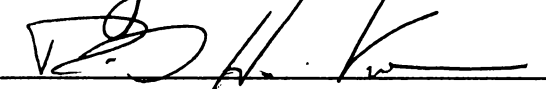
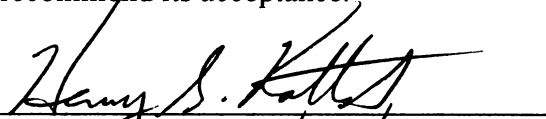
To the Graduate Council:

I am submitting herewith a dissertation written by Agnes M. Vanderpool entitled, "Using Salt-Enriched Diets to Reduce Stress-Induced Hypochloremia and Methemoglobinemia in Rainbow Trout". I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Animal Science.



Richard J. Strange, Major Professor

We have read this dissertation and
recommend its acceptance:



Acceptance for the Council:



Vice Provost and Dean of
Graduate Studies

USING SALT-ENRICHED DIETS TO REDUCE STRESS-INDUCED HYPOCHLOREMIA AND METHEMOGLOBINEMIA IN RAINOW TROUT

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Agnes M. Vanderpool
May 2002

Copyright © 2002 by Agnes M. Vanderpool
All rights reserved.

DEDICATION

This dissertation is dedicated to my grandmother, Mary Ward Maggard, who made sure that I developed a sense of adventure very early on, and to my parents Isaac and Kathryn Vanderpool for unfailing support and inspiration.

ACKNOWLEDGEMENTS

The completion of a scientific research project is never accomplished within a vacuum. There are many people who made this work possible through their support and assistance.

Dr. Richard Strange, Amanda Smith, Jingjuan Gong, Billy Goodrich, Randy Cooper, Rob Mottice, Ben Claxton and Jeff Highfill were highly instrumental in the care of the fish and conducting the experiments. Drs. Arnold Saxton and John Taylor provided invaluable assistance with statistical analyses. Mary Roberts provided laboratory training and assistance. Regina Brock provided clerical assistance. Dr. Larry Wilson provided advice and administrative assistance. My dissertation committee, chaired by Dr. Richard J. Strange and including Drs. Henry G. Kattesh, Judy Grizzle, Richard Heitman and Marshall Adams all guided the research and improved the quality of this manuscript many-fold.

Dwayne Oyer and the Tennessee Wildlife Resources Agency staff from Buffalo Springs Trout Hatchery provided the rainbow trout used for this research. Roger Long, Maintenance Manager for the JARTU facility helped me to keep the water flowing. Dr. Mark Campbell, facility manager for the JARTU labs provided invaluable technical and facility oversight assistance.

Finally, my parents Isaac and Kathryn Vanderpool, and my close friend and former husband Harley Spicer, provided a wealth of love and support throughout the years needed to see the project to completion.

ABSTRACT

Chronically stressful conditions in fish culture facilities can lead to outbreaks of disease and reductions in growth and performance. A secondary response to stress in fish is the sudden and significant loss of plasma chloride ions, a condition known as hypochloremia. This loss of plasma chloride has been implicated as a cause of mortality in severely stressed fish. The effect of increased dietary salt on reducing hypochloremia in rainbow trout subjected to a confinement challenge was investigated. Experimental diets included added NaCl or added CaCl₂.

Methemoglobinemia, an environmental disease of cultured fish caused by increases in nitrite concentrations in water can be reduced by the addition of salt to water. Increased dietary salt as either NaCl or CaCl₂ was investigated as an aid in reducing the development and severity of methemoglobinemia in trout exposed to elevated nitrite concentrations.

Trout were divided into five groups receiving a 3.6% NaCl diet, a 3.6% CaCl₂ diet, a 7.3% NaCl diet or a basal (no added salt) 0.7% NaCl diet. Experimental challenges were conducted during Days 72-76 of salt-feeding..

Trout subjected to confinement were sampled at Hours 0, 2, 4, 6, and 12, and at 24 and 72 hours of recovery for plasma chloride, plasma cortisol and glucose levels and percent blood hematocrits. Trout subjected to elevated nitrite were sampled at Hours 0, 2, 4, 6, and 8 for blood methemoglobin and plasma chloride concentrations.

Trout fed salt-enriched diets suffered less hypochloremia in the early hours of confinement. Trout fed the 7.3% NaCl diet had significantly higher recovery plasma chloride levels post-stress. Salt-enriched diets containing moderate levels of NaCl, may offer some benefit in recovering trout that have been exposed to stress.

Trout fed the 7.3% NaCl diet developed less hypochloremia in the early stages of nitrite exposure. Protective effects of this diet for hypochloremia were transient. Trout fed the 3.6% NaCl diet developed methemoglobinemia more slowly than trout fed the other diets in the early stages of nitrite exposure. Salt-enriched diets do not appear to offer long-term protection against the development and severity of methemoglobinemia in trout exposed to elevated nitrite.

TABLE OF CONTENTS

	PAGE
Chapter 1. Literature Review	1
Section 1 Stress in Aquaculture	1
1.1 The stress response in fish	1
1.2 Handling and confinement stress	5
1.3 Using dietary salt to reduce stress	10
1.4 Methemoglobinemia	13
1.5 Chloride cells and osmoregulation	14
1.6 Additional mechanisms of disease	22
1.7 Species differences in sensitivity to nitrite and methemoglobinemia	24
1.8 Individual differences in sensitivity to nitrite and methemoglobinemia	27
1.9 Research objectives	28
Chapter 2. Handling and Confinement Stress	29
Section 1. Introduction	29
Section 2. Materials and Methods	31
2.1 Overview	31
2.2 Confinement stress challenge	36
2.3 Assays	38
2.4 Experimental design	38
Section 3. Results	39
3.1 Growth	39
3.2 Confinement Results	40
3.3 Plasma cortisol levels	45
3.4 Plasma glucose levels	47
3.5 Hematocrit	49
Section 4. Discussion	50
4.1 Growth	50
4.2 Plasma chloride concentrations	51
4.3 Plasma cortisol concentrations	54
4.4 Plasma glucose concentrations	56
4.5 Hematocrit	58

Section 5.	Conclusions	59
Chapter 3.	Methemoglobinemia	61
Section 1.	Introduction	61
Section 2.	Methods	63
2.1	Overview	63
2.2	Nitrite challenge	64
2.3	Methemoglobin concentration Determination	66
2.4	Chloride cell counts	67
2.5	Plasma chloride concentrations	69
2.6	Experimental design and analysis	69
Section 3.	Results	70
3.1	Survival	70
3.2	Methemoglobin development	71
3.3	Influence of diet on methemoglobin development	71
3.4	Chloride cell development	74
3.5	Plasma chloride concentrations	77
Section 4.	Discussion	79
Section 5.	Conclusions	82
References		84
Appendices		95
Vita		164

LIST OF TABLES

Table	Page
2.1. Characterization for Basal (0.7% NaCl) Trout Diet	33-34
2.2. Day 0 Baseline Values for Rainbow Trout Used in A 133-Day Salt-Feeding Experiment	35
2.3. Average Percent Hematocrit Values for Salt-Fed Rainbow Trout Subjected to a 12-Hour Confinement Challenge	49
3.1. Day 0 Baseline Values for Rainbow Trout Used in A 133-Day Salt Feeding Experiment	70

LIST OF FIGURES

Figure		Page
1.1.	96-Hour LC ₅₀ Values for Rainbow Trout Exposed to NO ₂ -N	18
2.1.	Trout Rearing System, University of Tennessee	31
2.2.	Plastic Mesh Baskets Used to Confine Rainbow Trout During a 12-Hour Confinement Challenge	37
2.3.	Plasma Chloride Levels in Rainbow Trout Fed Salt-Enriched Diets	41
2.4.	Average Chloride Cell Numbers in Salt-Fed Rainbow Trout	42
2.5.	Average Plasma Chloride Concentrations in Rainbow Trout Confined for 12 Hours	43
2.6.	Average Plasma Cortisol Values in Rainbow Trout Confined for 12 Hours	46
2.7.	Average Plasma Glucose Values for Rainbow Trout Confined for 12 Hours	48
3.1.	Static Assay Tank Used for Nitrite Challenges For Rainbow Trout Fed Salt-Enriched Diets	65
3.2.	Chloride Cells on Lamellae and Interlamellar Spaces of Gills of Rainbow Trout Fed a Salt- Enriched Diet	68
3.3.	Pattern of Development of Methemoglobinemia In Trout Exposed to 18 mg/L N-NO ₂	72
3.4.	Methemoglobin Development by Diet and Hour of Exposure	73
3.5.	Average Chloride Cell Numbers in Salt-Fed Rainbow Trout	75
3.6.	Average Number of Chloride Cells by Diet	76
3.7.	Plasma Chloride Levels (meq/L) in Rainbow Trout Exposed to an 8-Hour Nitrite Challenge At 18 mg/L N-NO ₂	78

Chapter One

Literature Review

1. Stress in Aquaculture

1.1. *The stress response in fish*

The concept of stress and its deleterious effects on cultured fish has received widespread attention from fish culturists and researchers since the early 1940's. Early efforts to reduce mortalities during transport of fish led culturists to use ice to slow metabolism, provide aeration and to fast fish before shipment (Barton, 1997). Later studies focused on causes of delayed mortality including investigations of metabolic rate and blood lactic acid concentrations (Haskell, 1941; Black, 1956, 1957).

In 1950, Hans Selye introduced a theory for a general syndrome to describe responses of his test animals to a variety of stressful stimuli. Known as the General Adaptation Syndrome, this paradigm described an animal's response to a stressor in three stages. The first stage, termed the alarm reaction, is an animal's initial response to a stimulus and includes a disturbance in the physiological resting state of the animal. The second stage, called the stage of resistance, includes a period in which the animal tries to adjust to or compensate for the stressor to regain homeostasis. If an animal cannot adjust to or compensate for the stressor, it enters the third stage, the exhaustion phase. Entry into the exhaustion phase of the stress response can lead to development of a pathological state or to mortality. (Selye, 1950).

Subsequent research on the response to stressful stimuli by fish has led to a well-defined characterization of the endocrine and metabolic changes that occur when fish are confronted with stressful stimuli, hereafter called stressors. Although now known to be more complicated than the original General Adaptation Syndrome suggests, these responses in fish can still be described within the context of a general adaptive response to stress. Wendelaar-Bonga (1997) notes that the actions of a stressor on a fish are twofold: first, stressors produce effects that disturb homeostatic equilibrium. Second, stressors evoke a coordinated set of behavioral and physiological responses that are thought to be adaptive in helping the animal to cope with the perceived threat.

McDonald and coworkers (1997) examined salt disturbances, measured as changes in plasma Na^+ and Cl^- levels, in salmonids exposed to either chronic net confinement or continuous infusion of epinephrine. The rapid and immediate efflux of plasma electrolytes that was observed with the onset of stress was not sustained. The authors suggest that the adaptation of electrolyte loss to circulating epinephrine indicates that the initial severity of the stressor rather than the duration of the stressor determined the degree of osmotic disturbance. They conclude that reducing the initial stress response might be effective in reducing the overall detrimental effects of chronic stress in fish.

Specific mechanisms for the epinephrine-induced loss of plasma electrolytes is still not certain, but circumstantial evidence suggest increased blood pressure within the gills as a causative factor. Gonzalez and McDonald (1992) concluded that

ion efflux in response to epinephrine infusion or in response to specific stressors was larger than could be explained by functional surface area alone. They proposed that increased intra-lamellar pressure due to elevated blood pressure distorts and stretched paracellular tight junctions between gill cells and decreases the resistance to ion diffusion. Hence, paracellular permeability of the gills is increased and leakage of electrolytes occurs through paracellular channels.

Significant increases in circulating concentrations of epinephrine occur within seconds of the onset of an acute stressor and reach peak values within two minutes. Epinephrine release is followed by plasma cortisol and glucose responses, loss of plasma electrolytes, primarily Na^+ and Cl^- ions (McDonald and Milligan, 1992), increases in blood hematocrit and in cases where rigorous activity is involved as a stressor, declines in blood pH and increases in muscle concentration of lactic acid. The acute stress response is also characterized by a water influx across the gills, increased diuresis and increased renal electrolyte output (Wood and Randall, 1973; Adedire and Oduleye, 1984; McDonald and Rogano, 1986) and an increased urinary loss of electrolytes (Vermette and Perry, 1987) although this loss accounts for less than ten percent of branchial diffusion losses under resting conditions and even less under stress (McDonald, Walker and Wilkes, 1983; McDonald, 1983).

Related studies noted that corticosteroids released from interrenal tissues resulted in secondary metabolic changes such as increased plasma glucose (Leach and Taylor, 1982) and osmoregulatory disruption (Nikinmaa et al., 1983; Redding and Schreck, 1983). McDonald and Milligan (1997) noted that there is no evidence that

cortisol alone causes electrolyte or osmotic disturbances. However, the release of cortisol appears to be related and proportional to electrolyte loss, suggesting that overall ion loss may, in itself be stressful. Ion loss might therefore, lead to an amplification of the primary stress response and contribute to increases in cortisol and epinephrine.

By the early 1990's, the stress response of freshwater salmonids was well-characterized (Strange et al., 1978; Mazeaud and Mazeaud, 1981; Woodward and Strange, 1987; Randall and Perry 1992). Distinctions have been made among primary, secondary and tertiary responses to stress (Pickering, A.D., 1981). Primary responses involve releases of catecholamines from the brain and corticosteroids from the interrenal tissues. Secondary responses include the immediate actions and effects of these hormones in the blood and at the tissues and include increased cardiac output, increased oxygen uptake, mobilization of energy substrates and disruption of water and electrolyte balance. Tertiary responses occur at the level of the whole animal and include reduced growth, reproduction, immune response and less ability to tolerate additional stressors.

McDonald and Milligan provided a 1997 review of changes in the maximum amplitude of stress-induced primary and secondary physiological indices relative to resting levels. The majority of stress studies in fish to date are based on freshwater salmonids. The authors noted that an acute stressor may cause a 1000-fold increase in circulating epinephrine, a 200-fold increase in plasma cortisol, a three-fold increase in plasma glucose and up to a 40-fold increase in diffusive losses of Na^+ and Cl^- ions,

corresponding to a 35% loss of whole body NaCl. It is now generally accepted that losses of plasma electrolytes during exposure to acute stressors are caused by a catecholamine-mediated increased branchial efflux (Mazeaud and Mazeaud, 1981; McDonald and Milligan, 1997).

1.2. *Handling and Confinement Stress*

Handling and confinement of fish are necessary aquaculture practices that have long been recognized as stressful for the animals involved. The resultant stress response and its effects on fish health and performance has been the subject of multiple reviews (Mazeaud et al., 1977; Adams, 1990; Wedemeyer, Barton and McLeay, 1990 and Wedemeyer 1997; Barton and Iwama, 1991; and Wendelaar-Bonga, 1997).

Studies of handling and confinement in fish have focused on elevations of plasma cortisol and changes in blood glucose or electrolytes as indicative of the magnitude of the stress response. Wedemeyer in 1976 reported results of handling and crowding stress in Coho salmon. Blood glucose and chloride concentrations were measured in salmon held at densities of either six or twelve lb/ft³ water. Salmon reacted to both densities with significant hyperglycemia in the first four hours of exposure. This elevation of plasma glucose required eight days to return to resting levels. Wedemeyer also reported a mild hypochloremia in both groups of salmon but loss of plasma chloride concentrations did not correlate well with loading densities.

In 1977, Mazeaud and coworkers reviewed studies of handling and confinement in salmonid fishes. They reported that handling and confinement of fish caused rapid increases in circulating concentrations of the neurohormone epinephrine. There were no differences observed in this catecholamine response among the salmonid species examined, however, wide variations in the response of individual fish to stressful stimuli were reported. Immediately following this initial epinephrine response to a stressor, a rapid rise in plasma corticosteroids occurred followed by a pronounced rise in plasma glucose concentrations. The authors penned the terms, “primary” and “secondary” effects to describe the neuroendocrine versus the metabolic and osmotic disturbances observed in stressed fish respectively (Mazeaud et al., 1977).

Secondary effects of stress described by Mazeaud et al., (1977) included earlier work by Maetz and by Pic et al., in 1974, which suggested that circulating epinephrine was responsible for changes in gill permeability to water, leading to large increases in water diffusion into freshwater fish subjected to handling stress or epinephrine injection. This same review also reported work by Randall et al., (1972) which showed that stress induced by exercise caused losses of Na^+ ions across gills in rainbow trout and that osmoregulatory disturbances caused by handling were partially offset by holding fish in an isotonic saline environment (Hattingh et al., 1975; Miles et al., 1976).

In 1977, Strange and coworkers reported significant linear increases in plasma corticosteroid levels in Chinook salmon netted and confined in live cages for 24

hours. The authors concluded that plasma cortisol concentration was a sensitive indicator of the stress response in these fish. Mazeaud and colleagues (1977) confirmed the importance of activation of the hypothalamus-pituitary-interrenal axis in the stress response in their review of studies of stress in fish. Additional work by Strange and others in 1978 and Strange in 1980 showed that a variety of acute stressors caused significant elevations in plasma cortisol. Concurrent studies with salmonids showed that if an acute stressor is removed before compensation can occur, plasma cortisol levels often return to resting concentrations within 24 hours (Strange and Schreck, 1978; Strange et al., 1978; Barton et al., 1980; Specker and Schreck, 1980).

Adedire and Oduleye reported their work with tropical cichlids in 1984, showing that handling stress increased the water permeability of fish gills. The authors noted that increased circulating epinephrine levels led to an increase in gill lamellar perfusion rate and an increase in the number of perfused lamellae which, they proposed, affected branchial permeability. Epinephrine was shown to increase aortic blood pressure due to vasoconstriction, and to increase cardiac output in salmonids by Mazeaud and Mazeaud (1981). This increase in blood pressure and cardiac output was demonstrated to increase perfusion of gill lamellae and increase the number of lamellae perfused by Randall and Perry (1992).

Woodward and Strange (1987) reported increases in plasma glucose levels from 58 to 196 mg/dL in hatchery-reared rainbow trout subjected to a 12-hour net confinement. This response was even more severe in wild trout confined for 12

hours, with plasma glucose levels increasing from 55 to 284 mg/dL. Both wild and hatchery-reared trout required greater than 24 hours for plasma glucose concentrations to return to resting concentrations.

In this same study, Woodward and Strange observed significant losses of plasma chloride concentrations in wild trout. Mortalities in wild trout reached 12.5% during the stress event. No mortalities were observed in hatchery-reared trout which also showed significantly less plasma chloride loss in response to the same stressor.

McDonald and Milligan (1992) reported that net confinement of rainbow trout for eight hours resulted in immediate and substantial effects on Na^+ and Cl^- plasma ion concentrations with as much as a 40-fold increase in diffusional loss of electrolytes relative to diffusive ion loss in trout under non-stressed conditions. The authors noted a rapid adaptation of Na^+ and Cl^- efflux over the course of the stress event and suggested that the initial severity of the stressor determines the degree of osmotic disturbance.

Additional studies by Postlethwaite and McDonald (1995) addressed recovery from acute stress and showed that the return of body Na^+ and Cl^- concentrations to pre-stress levels occurred over a similar time course. The speed of recovery of ion concentrations was found to be dependent on the degree of the initial stress experienced, however recovery generally occurred within 24 to 48 hours post-stress. The mechanism of recovery of ionic balance was rapid with a progressive increase in Na^+ and Cl^- ion uptake by the gills at three-fold levels above controls within two

hours of the start of a four-hour confinement stressor. By the end of the stress period, the diffusive efflux of Na^+ and Cl^- ions had been reduced to near zero.

In a more recent study of confinement stress, Reubush and Heath (1997) examined the recovery of fed and fasted hybrid striped bass fingerlings following netting and restraint in air for five minutes. Fish were recovered in a range of salinities from five to 15 parts per thousand. Severity of the stress response was measured as whole body glucose, glycogen, lactic acid and blood osmolality. They report that blood osmolality was not greatly affected by salinity of recovery water but that all fish tested gained electrolytes.

Ruane and coworkers (1999) looked at differences between rainbow and brown trout in regulation of the hypothalamic-pituitary-interrenal axis during confinement. They utilized a 5.5 hour net confinement stressor and noted that confinement induced transient increases in plasma ACTH and cortisol levels in both species, with brown trout displaying a greater magnitude of response. Confinement caused increased hematocrit values and loss of plasma Na^+ and Cl^- concentrations in both species. Rainbow trout were reported to recover faster as plasma glucose concentrations and ionic balance disturbances persisted in the brown trout even after 46 hours post-stress. The authors noted that differences in the stress response of these two closely related species originate at the level of the pituitary gland and are observed throughout the secondary stress responses.

Mazik, Simco and Parker (1991) reported increased survival rates in both cold and warm water fish if NaCl was added in concentrations of 0.5 –1.0% to hauling

tanks. Recovery of fish was also improved by using NaCl-supplemented water after release from hauling tanks. An iso-osmotic NaCl concentration (0.9% NaCl in fresh water) has been shown to be more effective at reducing mortality in fish subjected to acute stressors than CaCl_2 added to increase water hardness, addition of anesthesia, use of cold water temperatures or hyperoxia (Nikinmaa et al., 1983; Redding and Schreck, 1983; Mazik and Simco, 1994).

1.3. *Using dietary salt to reduce stress*

Since 1969, the benefits of feeding salt-enriched diets for improving osmoregulatory capability in salmonids adapted to fresh water and transferred to sea water have been recognized. Juvenile salmonids raised in fresh water hatcheries and released into sea water were shown to suffer significantly fewer mortalities following transfer if fed salt-enriched diets prior to stocking (Zaug, and McLain, 1969; Basulto, 1976; Salmon and Eddy, 1990; Pelletier and Besner, 1992). Early studies of salt-enriched diets reported reduced growth and feed conversion in Coho and Atlantic salmon (Zaug, . and McLain, 1969; Basulto, 1976), however later studies with salt-enriched diets formulated to be isonitrogenous and isocaloric reported no adverse impacts on growth (Salman and Eddy, 1988; Zaug, et al., 1983; Gong, 1999).

Several studies have reported increased numbers of gill chloride cells (Salman and Eddy 1987; Gong, 1999) or increases in $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ in gill tissue following feeding with salt-enriched diets (Zaug and McLain, 1969; Zaug et al., 1983; Salman and Eddy 1987; Duston, 1992; Harms and Hinshaw, 1993, unpublished data). These observations are interesting in light of the fact that increases in gill chloride cell

number, Na^+ - K^+ -ATPase activity, and succinate dehydrogenase activity have all been shown to accompany adaptation to seawater by fresh water adapted salmonids (Sargent and Thomson, 1974; Giles and Vanstone, 1976; Boeuf et al., 1978; Saunders and Henderson, 1978; Folmar and Dickhoff, 1980; Philpott, 1980; Sardet, 1980 and Johnston and Cheverie, 1985). Changes in gill chloride cell number and activity concurrent with saltwater adaptation are attributed to the increased excretion of Na^+ and Cl^- ions across the gill epithelia (Bath and Eddy, 1979). Johnston and Cheverie (1985) demonstrated a significant negative correlation between increases in gill Na^+ - K^+ -ATPase activity and decreases in plasma Na^+ and Cl^- ion content. The similarities between changes in number and activity of gill chloride cells in fish fed salt-enriched diets compared to changes in gill chloride cells of fresh water-adapted salmonids transferred to seawater, led Langdon and Thorpe (1984) to suggest that chloride cells in salmonids fed salt-enriched diets may be functioning to excrete Na^+ and Cl^- ions as occurs in salt water adapted salmonids.

The ability of salt-enriched diets to increase plasma chloride concentrations is of interest in studies of stress in fish, due to the rapid and possibly fatal hypochloremia that has been shown to develop in salmonids exposed to acute stressors as previously discussed. Salman and Eddy (1988) reported significant increases in plasma chloride concentrations in rainbow trout 30 hours after feeding diets that were supplemented with 11.6% NaCl. Pelletier and Besner (1992) reported a short-term increase in plasma ion concentrations in brook trout fed diets supplemented with eight or 12% NaCl. This increase in plasma Na^+ and Cl^- levels

was detected between six to 12 hours after feeding but was undetectable if measured after a 24-hour fasting period, even in brook trout maintained on salt-enriched diets for a six-week period. However, upon osmotic challenge by transfer to seawater, brook trout fed the salt-enriched diets had markedly higher survival rates than trout fed control diets, and had significantly reduced plasma Na^+ and Cl^- concentrations reflecting a reduced influx of these ions relative to control (non-salt fed) fish.

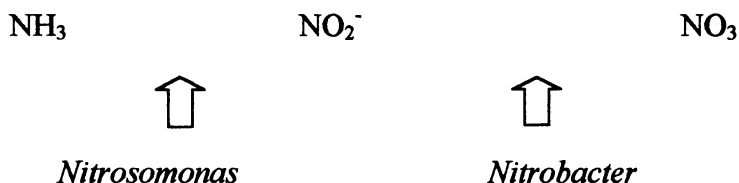
This body of research surrounding the effects of added salt in fish diets suggests that salt-enrichment may provide a means of reducing secondary responses to acute stress in fish, particularly the potentially fatal loss of plasma ions. The mechanism by which salt-enriched diets may offer protection from plasma electrolyte loss in fish under stress is not known, but several potential effects have been suggested including increases in the numbers and activity of gill chloride cells in fish fed salt-enriched diets, transient increases in the resting level of plasma chloride concentrations in salt-fed fish, and the sequestering of plasma electrolytes in tissues.

If salt-enriched diets can mitigate the additive effects of acute stressors in aquaculture, the use of these diets may lead to a decrease in the overall chronic stress load in fish. A decrease in the chronic stress response in cultured fish would translate into improved survival, performance, and decreased outbreaks of disease in aquaculture systems.

1.4. *Methemoglobinemia*

Methemoglobinemia, commonly called brown blood disease, is an environmental disease of fish. Methemoglobinemia develops when fish are exposed to elevated concentrations of nitrite (NO_2^-), a negatively charged ion, in the surrounding water. Nitrite is an intermediate product in the oxidation of ammonia, (NH_3) to the oxidized form, nitrate (NO_3). Ammonia is the primary waste product of fish, produced by the digestion of protein.

When ammonia is excreted into the water, it is oxidized by a variety of species of nitrifying bacteria, the best known of which are *Nitrosomonas* and *Nitrobacter*, that are ubiquitous to soil and water systems. These bacteria oxidize ammonia according to the following reaction:



The resulting nitrate ion, (NO_3) is relatively non-toxic to fish and other aquatic organisms. *Nitrobacter* are more sensitive to un-ionized ammonia than are *Nitrosomonas* (Lewis and Morris, 1986). Fish excrete un-ionized ammonia (NH_3) into the water. Under normal water quality conditions, NH_3 reacts quickly with water to form ionized, non-toxic ammonia NH_4^+ . This reaction is dependent upon pH, however. An increase of one pH unit will increase the concentration of un-ionized ammonia 10-fold (Piper et al, 1992). Therefore, changes in pH coupled with high

levels of ammonia excretion by fish can result in increased concentrations of unionized ammonia which differentially affect bacterial populations, specifically *Nitrobacter*, leading to rapid increases in nitrite concentrations in the water. In addition, mechanical failure of biofilters in recirculating aquaculture systems or treatment of fish diseases with antibiotics can result in both elevated ammonia and nitrite levels.

Methemoglobinemia develops when nitrite ions are actively taken up from the water and diffuse into the general circulation to contact the hemoglobin proteins. Once in the blood, the nitrite ions bind to hemoglobin molecules and cause the oxidation of divalent ferrous iron ions (Fe^{+2}) on the heme moieties of the hemoglobin to the ferric (Fe^{+3}) state. Oxidation to the ferric state causes a change in configuration of the hemoglobin molecule that makes it unable to reversibly bind oxygen. Hemoglobin that has been oxidized in this manner is termed methemoglobin.

Chloride cells in the gill epithelia of fish have been implicated in the concentration of nitrite and the development of methemoglobinemia. Chloride cells are normally involved in the uptake of negatively charged chloride ions (Cl^-) from the surrounding water.

1.5. Chloride Cells and Osmoregulation

Freshwater fish must maintain an internal concentration of solutes which is hyperosmotic relative to the dilute environment of the surrounding water. During normal osmoregulatory processes in fresh water, fish continually lose plasma

electrolytes and anions across the gill epithelia by passive diffusion into the water. Some solutes are also lost in the dilute urine produced to remove excess water that enters the fish by diffusion across gill membranes. To maintain the concentrations of monovalent ions needed for physiological functions, fresh water fish must have an active uptake mechanism to regain lost ions over and above the solutes consumed in food. As early as 1932, Keys and Wilmer noted the secretion of chloride from a specific type of cell in marine teleost gills. The term “chloride cell” was introduced by Copeland in 1948 to describe these specialized gill epithelial cells. Maetz followed later with a model for active ion exchange across fish gills (Maetz, 1964 and 1971). In the late 1990’s, the designation *alpha* or *beta* was applied to distinguish between chloride cells in marine versus fresh water fish species respectively (Moyle and Cech, 2000).

Additional support for this theory of methemoglobin development comes from numerous studies that have shown the protective effects of adding chloride ions to the culture water in preventing methemoglobin development and toxicity during nitrite exposure in fresh water fish (Perrone and Meade, 1977; Wedemeyer and Yasutake, 1978; Tomasso et al., 1979). Beta chloride cells are large, irregularly shaped cells located on gill filaments at the base of lamellae and in the interlamellar regions. Beta chloride cells may also be found on gill lamellae in fish that live in soft, ion-poor water. In fresh water species, gill chloride cells usually occur singly and contain numerous mitochondria and an extensive cytoplasmic microtubular system. Beta

chloride cells contain elevated concentrations of the enzyme $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ which is used to power the active ion exchange mechanisms (Karnaky, 1986).

In freshwater fish, beta chloride cells play an important role in the absorption of monovalent ions and maintenance of internal ionic homeostasis and electrical potential. A $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ system located on the basolateral membrane of beta chloride cells is involved in the active transport of three Na^+ ions out of the cell in exchange for two K^+ ions into the cell. This system maintains a high Na^+ concentration in the plasma and a low Na^+ ion concentration within the chloride cell cytoplasm. Active carriers on the apical surface of the cells are believed to exist for Cl^- ion uptake resulting in an elevated concentration of Cl^- ions inside the chloride cell. These carriers actively import Cl^- ions in exchange for the outward movement of HCO_3^- ions that are produced by the activity of carbonic anhydrase within the chloride cell. Maintenance of this concentration gradient for Na^+ and Cl^- ions establishes an electrochemical gradient which is negatively charged on the inside of the cell. The electronegative status inside the chloride cell assists in the passive inward diffusion of Na^+ ions from the surrounding water through sodium channels on the apical surface of the chloride cell. In addition to this electrochemical gradient, Na^+ ion influx is now thought to be further assisted by the active extrusion of H^+ ions via a transporter on the apical surface of the cell. The existence of a direct $\text{Na}^+ / \text{NH}_4^+$ or Na^+ / H^+ exchange mechanism is still debated (Lin and Randall, 1993, Karnaky, 1998).

Diffusive loss of Na^+ and Cl^- ions through paracellular channels is also documented (Moyle and Cech, 2000). Tight junctions between gill cells are blocked by Ca^{+2} ions to prevent excessive loss of Na^+ and Cl^- ions however, these junctions may be subject to greatly increased leakage of ions during the increase in blood and lamellar pressure which occurs during the primary stress response as discussed in Chapter 1. Paracellular efflux of HCO_3^- , OH^- , H^+ , and NH_4^+ have also been proposed but outflow of these ions may be constrained by the outflux of Na^+ and Cl^- ions (Moyle and Cech, 2000). The divalent cation calcium (Ca^{+2}) is also thought to be actively imported from the water through beta chloride cells (Verbosi et al., 1997; Patrick et al., 1997).

Thus, beta chloride cells are responsible for maintaining the osmotic and electrochemical balance of the gill tissues, for internal acid-base balance and blood pH, and for partial elimination of ammonia and metabolic carbon dioxide as HCO_3^- .

In 1980, Bath and Eddy found that chloride prevents methemoglobinemia and reduces the toxicity of nitrite exposure in channel catfish when the ratio of chloride ions to nitrite ions reached 16:1 in the water. Toxicity in catfish was found to occur above a $\text{NO}_2^-/\text{Cl}^-$ ratio of 0.25 which resulted in 30% methemoglobin in the blood (Schwedler and Tucker, 1983). Methemoglobin in channel catfish was found to be negligible in channel catfish when the $\text{NO}_2^-/\text{Cl}^-$ ratio was less than 0.06 (Tomasso et al., 1979). A 1986 review by Lewis and Morris examined reports of nitrite lethal concentration values from several studies of fresh water fish species. The review concludes that the relationship between the toxicity of NO_2^- concentration and the

concentration of Cl^- ions is a linear relationship. For example, 24 hour LC_{50} (lethal concentration) values for rainbow trout are 0.49 mg/L $\text{NO}_2^- \text{N}$ in water containing 0.35 mg/L chloride. This LC_{50} value increases to 28 mg/L $\text{NO}_2^- \text{N}$ in water containing 40 mg/L chloride. The 96 hour LC_{50} value for rainbow trout is 0.24 mg/L $\text{NO}_2^- \text{N}$ at 0.35 mg/L chloride, but increases to 11 mg/L $\text{NO}_2^- \text{N}$ in water having 40 mg/L chloride (Figure 1.1, Compiled from data reviewed by Lewis and Morris, 1986).

Additional reports of $\text{NO}_2^-/\text{Cl}^-$ ratios that have provided protection from methemoglobinemia for rainbow trout include ratios of 6:1 (Perrone and Meade, 1977) to 15:1 (Russo et al., 1981). The authors of these various studies conclude

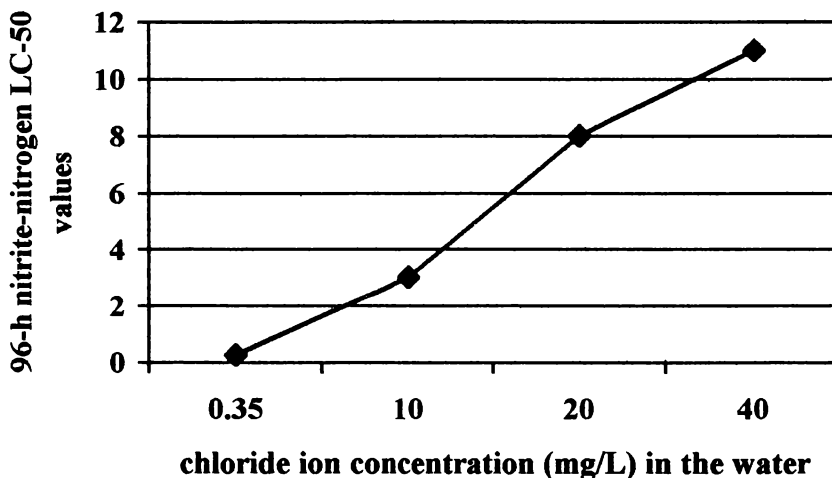


Figure 1.1: 96-Hour LC_{50} Values for Rainbow Trout Exposed to $\text{NO}_2^- \text{N}$

Changes in the 96-hour LC_{50} values for rainbow trout exposed to nitrite-nitrogen under increasing levels of chloride ion concentration. Figure was compiled from data reviewed by Lewis and Morris, Table 2, 1986 (SE = 0.0087, values are significantly different at $P \leq 0.01$).

that increasing the concentration of Cl^- ions in water helps to competitively lower NO_2^- uptake during exposure to elevated water nitrite concentrations.

As early as 1977, Crawford and Allen reported that addition of Ca^{+2} ions to culture water also provided protection from nitrite toxicity but did not reduce methemoglobin development in Chinook salmon. Wedemeyer and Yasutake in 1978 reported a more significant reduction in the toxicity of nitrite when calcium was present than when sodium was present. Their results with steelhead trout showed that the 96-hour LC_{50} was increased by a factor of 35 using 65 mg/L Cl^- added to the water as CaCl_2 as opposed to an increase in the LC_{50} of a factor of only 35 using 65 mg/L Cl^- added as NaCl. Unfortunately, the level of nitrite used in these studies was so high (30 mg/L) as to be likely to evoke other toxic reactions than might be observed at more realistic elevations of nitrite levels. Krous et al. in 1982 postulated that high calcium concentrations in the surrounding water might reduce the loss of Cl^- ions by diffusion through the gill epithelia by altering membrane permeability. Stemming the loss of chloride ions across the gills reduces the need for chloride uptake, leading to reduced nitrite uptake during periods of elevated nitrite exposure.

Bowser and coworkers in 1983 found that both NaCl and CaCl_2 provided equal protection against nitrite toxicity for channel catfish. Tomasso reported similar findings for channel catfish previously in 1980. Methemoglobin formation was not reported in either of these studies however. Lewis and Morris (1986) upon review of these earlier studies note that the various authors do not separate the effects of calcium from that of the associated anions added with the calcium. For this reason,

Lewis and Morris conclude that results on the benefits of calcium added to the surrounding water in preventing nitrite uptake, concentration and methemoglobinemia are still inconclusive.

Once taken up into the chloride cell, nitrite diffuses rapidly into the bloodstream. The binding of nitrite ions to hemoglobin is an autocatalytic process that is pH sensitive, with more alkaline pH levels slowing methemoglobin formation. The formation of methemoglobin allows hemoglobin molecules to act as a sink for nitrite ions, supporting a concentration gradient that sustains nitrite uptake and concentration in sensitive species.

The rate of plasma nitrite concentration increases slowly within the first four hours of exposure and then increases more rapidly from four to twelve hours of exposure. After four hours of exposure to 0.5 mmol/L $\text{NO}_2^- \text{N}$, the plasma nitrite concentration in rainbow trout exceeded the water nitrite concentration (Williams and Eddy, 1988). Blood methemoglobin levels in the trout also rose slowly within the first four hours of nitrite exposure, with an increasing rate of development over the next six hours. The authors suggest that an epinephrine-mediated stress response may be responsible for this pattern of plasma nitrite concentration and subsequent methemoglobin development.

Williams and Eddy in 1988 noted that during the first two hours of nitrite exposure, plasma chloride and sodium levels fell significantly in rainbow trout exposed to 0.5 mmol/L $\text{NO}_2^- \text{N}$ before plasma nitrite concentrations had begun to rise significantly. They suggested that this disturbance of plasma electrolyte

concentrations in the early stages of nitrite exposure might be due to an initial catecholamine release as part of a generalized stress response. In freshwater fish, increased levels of circulating catecholamines have been shown to stimulate increased passive ion loss (Bonga, 1997) as discussed in Section 1.1.

Concurrent with the loss of plasma electrolytes and Cl^- concentration, Williams and Eddy observed that the ratio of cation to anion loss remained equal during the first two hours of nitrite exposure. Following two hours of exposure, plasma nitrite levels began to rise. Compensation for plasma electrolyte and Cl^- loss occurred within four hours of nitrite exposure. Divalent cation concentrations of Ca^{+2} and Mg^{+2} ions remained unchanged throughout the 24-hour test. A significant loss of plasma K^+ was observed during the first four hours of exposure. This extracellular hypokalemia remained uncompensated after 24 hours of recovery. The trout erythrocytes initially swelled, then shrank by 30% between four and six hours of nitrite exposure, resulting in a decrease in hematocrit. After 24 hours of exposure, the trout displayed a wide spectrum of tolerance with some fish showing few signs of toxicity while other fish were moribund.

Jensen and coworkers in 1987 investigated changes in acid-base status and electrolyte concentrations in carp exposed to elevated nitrite levels. They noted a significant drop in plasma chloride and sodium levels and a rise in plasma potassium levels during the early hours of nitrite exposure with carp. Jensen postulated that the increase in plasma K^+ ion concentrations was due to a loss of potassium from skeletal muscle, because this reaction parallels that of fish that are heavily exercised.

However, he notes that the extracellular hyperkalemia under nitrite exposure occurs in the absence of extracellular acidosis that also occurs under exercise due to lactic acid production. Jensen suggests that the concurrent decrease in plasma Na^+ that occurs with nitrite-induced plasma hyperkalemia may be caused by the interference of nitrite ions with the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ enzyme system in chloride cells. In a later study Jensen (1990) investigated the effects of nitrite exposure on red blood cell function in vitro and in vivo in carp. He reported a significant loss of K^+ ions from carp red blood cells during shrinkage after exposure to nitrite. This loss of K^+ from red blood cells contributed significantly to a large plasma hyperkalemia during nitrite exposure.

1.6. *Additional Mechanisms of Disease*

Although the majority of research results on methemoglobinemia in fish support the contention that toxicity of the disease is due to tissue hypoxia, observations made by some researchers have led to suggestions for alternative mechanisms of toxicity. The observation by Holeton in 1971 that rainbow trout poisoned by exposure to carbon monoxide and inactivation of 90% of their blood hemoglobin were able to survive exposures of up to three hours at very low water temperatures (5°C) and low activity levels. In addition, Crawford and Allen in 1977 noted that inactive fish have low oxygen demands and may not be immediately threatened by severe methemoglobinemia.

In 1984, Arillo and colleagues reported tissue damage caused by hypoxia and acidification due to methemoglobinemia in the livers of rainbow trout. These fish were exposed to 0.45 mg/L NO_2^- N for 72 hours and displayed inhibition of liver lysosomal proteolytic activity and increased fragility of lysosomal membranes. Structural and biochemical damage in hepatocytes and in liver mitochondria was observed as well as decreased liver glycogen stores and decreased ATP levels. These authors conclude that liver hypoxia causes fatal liver dysfunction and leads to toxicity of methemoglobinemia.

Williams and Eddy in 1988 suggested that the energy demands of the NADH methemoglobin reductase may shorten the life of red blood cells. Increasing hemolysis of red blood cells in the spleen of sea bass exposed to 5.4 mmol/L NO_2^- N was reported by Scarano et al., 1984.

As noted previously, Williams and Eddy in 1988 reported a nitrite-induced extracellular hyperkalemia that translated into plasma hypokalemia after 24 hours of nitrite exposure. Similar results were reported by Jensen (1990), Stormer et al., (1996), and Knudsen and Jensen (1997). A significant loss of plasma K^+ resulting in plasma hypokalemia that is slowly compensated was suggested as a possible cause of cardiac failure in nitrite-sensitive species.

Several of these observations offer reasonable explanations for toxicity of methemoglobinemia rather than general hypoxia. However, the majority of these suggestions have yet to be well demonstrated as mechanisms of toxicity from nitrite exposure or methemoglobinemia. Additionally, several of these suggestions would

be more plausible for fish suffering from chronic nitrite exposure rather than a 24 to 48 hour exposure period which is more likely to occur in a fish culture system. Mortalities which occur during acute nitrite exposures and which are highly correlated to elevated plasma methemoglobin levels and plasma nitrite levels are more likely due to hypoxia. Research to further define the mechanism of toxicity for methemoglobinemia is needed.

1.7. Species Differences in Sensitivity to Nitrite and Methemoglobinemia

Normal methemoglobin levels in fish blood do not usually exceed 5% (Eddy and Williams, 1987). In addition to the $\text{NO}_2^-/\text{Cl}^-$ ionic ratio of the surrounding water as discussed above, the amount of methemoglobin that develops in fish blood with exposure to elevated nitrite also depends upon the species and the length of exposure time. The ability of the gills to concentrate nitrite in blood to many times the external water concentration appears to be species dependent. Important aquaculture species including salmonids, channel catfish and tilapia have rapid nitrite uptake and concentration. Other species including basses and sunfish show less uptake and concentration under similar exposure conditions. For instance, rainbow trout exposed to 10 mg/L NO_2^- -N concentrated seven times more nitrite than the water after 24 hours of exposure (Bath and Eddy, 1980). A 1986 study by Tomasso showed that bluegill exposed to 40 mg/L NO_2^- -N developed 15% methemoglobin while tilapia developed 70% methemoglobin. Channel catfish tested in the same study developed 82% methemoglobin after 24 hours, whereas goldfish developed only 62%

methemoglobin. Largemouth bass were found to exhibit a high tolerance for nitrite. Even with low chloride concentrations in the surrounding water, largemouth bass do not concentrate nitrite in the blood and chloride added to the water offered no additional protection from methemoglobin development in largemouth bass.

Species susceptibility to methemoglobinemia has been shown to be related to Cl^- uptake rates (Williams and Eddy, 1988). The ability of fresh water fish to transport ions and regulate acid-base status is strongly linked to the numbers and activity level of gill chloride cells as discussed above. Common carp are less sensitive to NO_2^- poisoning, but they have significantly fewer numbers of gill chloride cells than do rainbow trout, and anion uptake is slower than in trout (Williams and Eddy, 1986). This study also showed that carp hemoglobin has both a greater affinity for oxygen and for nitrite than does trout hemoglobin, however, the methemoglobin reductase enzyme system is more active in carp erythrocytes than in trout erythrocytes. In fish, methemoglobin is reduced to hemoglobin by the NADH methemoglobin reductase enzyme (Freeman et al., 1983; Scott and Harrington, 1985). NADH methemoglobin reductase is found in red blood cells.

Beta chloride cells are known to become hypertrophic and hyperplastic in trout exposed to chronic, sublethal levels of nitrite. Gaino and coworkers (1984) noted that chronic exposure to $32 \mu\text{mol/L NO}_2^- \text{N}$ for three days resulted in chloride cell hypertrophy. A later study by Sholtzke (1992) noted however, that this increase in beta chloride cell numbers plateaus at 14 days of exposure. Williams and Eddy (1986) suggested that the increase in beta chloride cell numbers during sublethal

nitrite exposure is probably due to decreased Cl^- ion uptake and reduced HCO_3^- excretion if the $\text{Cl}^-/\text{HCO}_3^-$ branchial exchange mechanism is disturbed by increased NO_2^- levels in the water and in the plasma. The increase in beta chloride cell numbers may indicate a compensatory response that allows nitrite-poisoned fish to maintain their plasma chloride levels despite the presumed competitive exclusion of chloride by nitrite.

A 1984 study by Gaino and colleagues supported earlier findings for beta chloride cell involvement in NO_2^- ion uptake by showing that increased nitrite levels cause an acceleration of Cl^- cell turnover producing hyperactivity in some cells which enhances degeneration. Greater numbers of chloride cells were observed to be in all stages of development including active, degenerating and completely degenerated stages in the gills of trout exposed to nitrite. Active chloride cells were also likely to be hypertrophic with nitrite exposure. Finally, plasma chloride concentrations remained constant in nitrite exposed trout regardless of the plasma nitrite concentration. This finding indicates an overall higher level of anion uptake in trout exposed to sublethal nitrite concentrations. If so, this mechanism would play a role in increased plasma nitrite concentrations in rainbow trout, a species proven to be sensitive to nitrite.

Species differences in tolerance to nitrite exposure and methemoglobinemia may be due to differences in methemoglobin reductase activity. A 1983 study by Freeman et al., examined the methemoglobin reductase system in 11 genera, 9 families and 13 species of fish. This study reported that 75% of fish examined (10 of

13 species) exhibited methemoglobin reductase activity. Three species were inconsistent in their concentrations of methemoglobin reductase, but the authors suggest that the reductase may be inducible if the substrate is present at an appropriate concentration to induce synthesis of the enzyme. No phylogenetic trend was observed for methemoglobin reductase activity among the genera tested.

1.8. *Individual differences in sensitivity to nitrite and methemoglobinemia*

Wide ranges of individual variation in methemoglobin development in fish of the same species were reported for the first time by Williams and Eddy in 1988. In this study, rainbow trout exposed to elevated nitrite from 0 to 12 hours with the most sensitive individuals developing lethal levels of blood methemoglobin within 12 hours of exposure, while more tolerant individuals retained sublethal levels of methemoglobin after 24 hours of exposure. Less sensitive species such as the common carp did not display as great an individual variation in methemoglobin development (Williams and Eddy, 1988).

The high level of individual variability in methemoglobin development in sensitive species has been reported in relatively few studies. In addition, standard errors for percent methemoglobin measurements are also rarely reported. Omissions of this type in the reporting of methemoglobinemia have made it very difficult to duplicate experimental results among various studies of nitrite toxicity in fish.

1.9. *Research objectives*

Our understanding of the causes of stress-induced hypochloremia coupled with findings from previous experiments with salt-enriched diets has lead us to see the potential for using salt supplementation in the diet to attempt to enhance stress resistance in cultured fish subjected to an acute confinement stressor. We believe that salt-enriched diets may provide some protection from disruption of osmoregulatory function and hypochloremia associated with the acute stress response. Results of these experiments are detailed in Chapter 2.

We also believe that salt-enriched diets may reduce the development of methemoglobinemia, an environmentally-induced disease that plagues intensive fish culture. Methemoglobinemia is caused by the active uptake of nitrite ions from the water by gill chloride cells when nitrite concentrations increase in production systems. Competitive uptake of nitrite ions to the exclusion of active chloride ion uptake has been shown to be the causative mechanism for this disease. Feeding of salt-enriched diets is expected to reduce the osmoregulatory disruption that occurs under acute exposure to increased nitrite concentrations in water. In this manner, salt-enriched diets may reduce the onset and/or severity of the disease. Results of these experiments are detailed in Chapter 3.

Remaining objectives include an examination of growth rates of rainbow trout fed salt-enriched diets for an extended (10 week) period, and an examination of changes in plasma chloride concentrations in these fish throughout the salt-feeding period. The results of those observations are reported in both Chapters 2 and 3.

Chapter 2

Handling and Confinement Stress

1. Introduction

Acute stress leading to mortality is rare in fish culture systems that are well-managed. Fish often survive acute stressors with transient primary stress responses (Wedemeyer, 1997). Chronically stressful conditions, however, can develop in intensive production systems where routine culture practices such as handling and confinement cause periodic acute stress responses. These responses may be additive over time affecting the metabolic or organismal level in fish resulting in reduced performance, impaired resistance to disease and impaired reproduction (Adams, 1990). Metabolic or organismal effects of chronic stress may therefore have serious consequences for aquaculture.

Acute stressors are known to cause secondary stress responses in freshwater fish including increased plasma glucose levels and disruptions of osmoregulatory and ionic balance due to a rapid loss of plasma electrolytes (primarily Na^+ and Cl^- ions) at the onset of the stress event (Woodward and Strange, 1987; McDonald and Milligan, 1992). Substantial losses of plasma electrolytes can contribute to fluid volume disturbances, cardiovascular collapse and failure of oxygen transport. A recent review of ionic and osmotic regulation in fish during stress suggests that the proximate cause for mortality in fish under acute stress is probably due to ion loss (McDonald and Milligan, 1997). Reducing ion loss during the initial stress response may be effective in reducing the long-term effects of chronic stressors.

Several actions to reduce the initial severity of acute stressors in cultured fish have been proposed. These include pre-stress fasting, use of anesthetics, increasing water hardness by addition of CaCO_3 or NaCl to water (Tomasso et al., 1980; Grizzle et al., 1993). Of these activities, the most commonly used in aquaculture is addition of NaCl to water. Theoretically, increasing the salinity of recovery water augments the recovery or prevents the loss of plasma electrolytes and helps to maintain osmotic homeostasis (Wedemeyer et al., 1990). Freshwater-adapted trout fed salt-enriched diets adapted more readily to the osmotic challenge of transfer to sea water and had greater numbers of chloride cells and increased $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity in gill epithelia as compared to trout fed normal diets (Zaugg et al., 1983; Salman and Eddy, 1987;). Salmonids maintained on salt-enriched diets were reported to have higher resting plasma chloride concentrations (Basulto 1976; Salman and Eddy, 1988). Given these increases in chloride cell numbers, gill $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity and plasma chloride concentrations, salt feeding may confer an advantage in maintaining ionic balance in fish subjected to acute stressors.

Few studies to date have investigated the value of salt-enriched diets in reducing the response to acute stressors in fish. Salt is an inexpensive and readily available substance that could be included in cultured fish rations as a routine diet to reduce mortality and improve performance of fish in the face of periodic acute stressors inherent to aquaculture. Our research objective was to determine the value of feeding diets enriched with chloride as NaCl (3.6 and 7.3% chloride as NaCl) and one diet enriched with chloride as CaCl_2 (3.6% chloride as CaCl_2) in reducing

primary and secondary stress responses in rainbow trout subjected to a handling and confinement stressor.

2. Methods and Materials

2.1. Overview

Rainbow trout (*Onchorynchus mykiss*) were obtained from the Tennessee Wildlife Resources Agency's Buffalo Springs Hatchery. 1000 trout were hand-graded for size at 21.6 cm +/- 1.3 cm, and transported to the aquaculture laboratory of the Johnson Animal Research and Teaching Unit (Figure 2.1).

Trout were equally divided and stocked into five, 862-L fiberglass tanks supplied with 23 L/min flowing dechlorinated water at 15.5° C. Dissolved oxygen

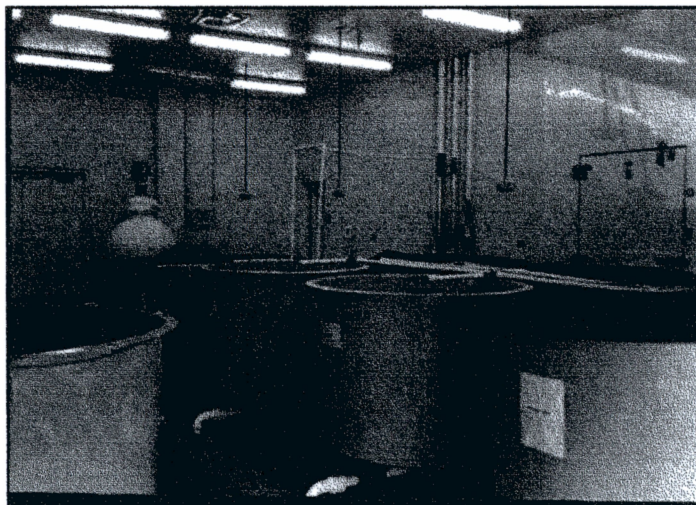


Fig. 2.1: Trout Rearing System, University of Tennessee

General rearing system at the University of Tennessee Johnson Animal Research and Teaching Unit (JARTU), used for culture of rainbow trout fed salt-enriched diets and a basal diet for confinement and methemoglobinemia experiments.

was maintained at 8.5 mg/L. Additional water quality parameters for the JARTU aquaculture laboratory are provided in Appendix A. Trout were acclimated for 15 days, during which they were fed a commercial trout diet (Ziegler, Gardners, PA) containing 38% protein. Trout were fed six days per week, twice per day at 1.5% of body weight (Piper et al., 1982).

Five experimental diets were formulated by Purina Mills Inc. and pelleted at the Purina TestDiet® laboratory (Richmond, IN). The experimental diets included a basal diet containing 0.7% chloride as NaCl, a high NaCl diet containing 7.3% added chloride as NaCl, a low NaCl diet containing 3.6% added chloride as NaCl, a high CaCl₂ diet containing 7.3% added chloride as CaCl₂ and a low CaCl₂ diet containing 3.6% added chloride as CaCl₂. The diets were identical with respect to basic ingredients except for added salt (Table 2.1). The high CaCl₂ diet was associated with increased mortalities during Days 2-4 of the experimental period. Two fish were lost on Day 2, two fish were lost on Day 3, and nine fish were lost on Day 4 relative to zero mortalities in the other tanks. Necropsy of mortalities showed blood in the stomach and intestines with hemorrhages in the wall of the body cavity. For this reason, feeding of the high CaCl₂ diet was discontinued.

Diets were formulated to be isocaloric and isonitrogenous to avoid protein dilution due to added salt. Using a completely randomized design, the basal and experimental diets were assigned to tanks containing trout with each tank receiving one diet over the course of the experiment. Prior to the initiation of salt feeding, trout

Table 2.1: Characterization for Basal (0.7% NaCl) Trout Diet

Basal Diet Characterization for the Purina TestDiet®

Neutral Detergent Fiber is approximately cellulose, hemicellulose and lignin. Acid Detergent Fiber is approximately cellulose and lignin. Physiological Fuel Value is the sum of decima fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4, 9, 4 kcal/g respectively.

Protein

Protein, % 48.3

Amino Acids

Arginine, % 2.86

Cystine, % 0.44

Glycine, % 3.44

Histidine, % 1.29

Isoleucine, % 2.06

Leucine, % 4.02

Lysine, % 3.64

Methionine, % 1.26

Phenylalanine, % 2.22

Tyrosine, % 1.39

Threonine, % 2.06

Tryptophan, % 0.52

Valine, % 2.94

Serine, % 2.05

Aspartic Acid, % 4.43

Glutamic Acid, % 6.67

Alanine, % 2.81

Proline, % 2.55

Taurine, % 0.15

Fats

Fat (either extract), % 10.1

Fat (acid hydrolysis), % 10.6

Cholesterol, ppm 1,370

Linoleic Acid, % 0.06

Linolenic Acid, % 0.12

Arachidonic Acid, % 0.14

Omega-3 Fatty Acids, % 2.04

Total Saturated Fatty Acids, % 3.68

Fiber

Fiber (max), % 14.1

Neutral Detergent Fiber, % 12.5

Acid Detergent Fiber, % 11.3

Nitrogen-Free Extract
(by difference), % 7.8

Carbohydrates

Starch, % 0.00

Glucose, % 0.00

Fructose, % 0.00

Sucrose, % 0.00

Sucrose, % 0.00

Energy

Total Digestible Nutrients, % 58.9

Gross Energy, kcal/gm 4.43

Physiological Fuel Value,
kcal/g 3.15

Metabolizable Energy,
kcal/g 1.63

Total Monounsaturated
Fatty Acids, % 2.34

Table 2.1. Continued**Minerals**

Ash, %	9.7
Calcium, %	2.33
Phosphorus, %	1.52
Phosphorus (non-phytate), %	1.49
Potassium, %	0.36
Magnesium, %	0.11
Sulfur, %	0.24
Sodium, %	0.94
Chlorine, %	1.49
Fluorine, ppm	61.0
Iron, ppm	574
Zinc, ppm	231
Manganese, ppm	99
Copper, ppm	16
Cobalt, ppm	0.55
Iodine, ppm	1.91
Chromium, ppm	1.66
Selenium, ppm	1.20

Vitamins

Carotene, ppm	0.0
Vitamin K (as menadione), ppm	5.5
Thiamin Hydrochloride, ppm	36
Riboflavin, ppm	36.0
Niacin, ppm	225
Pantothenic Acid, ppm	102
Choline Chloride, ppm	1,933
Folic Acid, ppm	6.7
Pyridoxine, ppm	28.77
Biotin, ppm	0.4
Vitamin B-12, mcg/kg	128
Vitamin A, IU/g	9
Vitamin D-3 (added), IU/g	2.4
Vitamin E, IU/kg	108
Ascorbic Acid, ppm	107.1

Calories Provided By

Protein	61.3%
Fat (ether extract)	26.8%
Carbohydrates	9.9%

Table 2.2: Day 0 Baseline Values for Rainbow Trout Used in a 133-day Salt Feeding Experiment

Baseline sample measures for length, weight and plasma chloride levels in rainbow trout taken prior to initiation of salt-feeding and following 14 days of acclimation. There were no statistical differences among the four tanks ($P \leq 0.05$).

<u>Tank</u>	<u>Length (cm)</u>	<u>Weight (kg)</u>	<u>Plasma Chloride (meq/L)</u>
1	22.4 $\pm$.46	.188 $\pm$.08	129.7 $\pm$ 1.80
2	21.7 $\pm$.27	.104 $\pm$.004	129.9 $\pm$ 1.75
3	22.2 $\pm$.34	.110 $\pm$.005	121.3 $\pm$ 2.36
5	22.6 $\pm$.32	.110 $\pm$.005	114.2 $\pm$ 1.53

from each tank were sampled on Day 0 and assessed for length, weight and plasma chloride levels to establish baseline data. Data were analyzed by ANOVA (SAS Version 8, 1999) to ensure that no tank effects existed for plasma chlorides (Table 2.2, Appendix D).

The total duration of the experiment was 133 days. Trout received experimental diets from Day 0 through Day 76 with stress challenges initiated on Day 76. Feeding of experimental diets was discontinued after the stress challenges and all trout were returned to the basal diet for an additional 57 days to assess persistence of effects of salt feeding following withdrawal of the salt-enriched diets.

Sampling for length and weight was performed on 10 fish and gill tissue samples were taken from five fish from each tank on Days 0, 13, 41, 76, 101, and 133. Ten trout were caught by net from each tank and euthanized by an overdose of tricaine methane sulfonate (100 mg/L). Fish were weighed, measured and exsanguinated into heparinized collection tubes by severing the caudal peduncle. The

blood was centrifuged for 5 minutes at 10,000 rpm. Plasma was removed and stored frozen at -10° C for later analysis of chloride. Following exsanguination, the heads of five fish randomly selected from the group of ten, were removed with gill arches intact and fixed in a 10% neutral buffered formalin solution for 48 hours. The central portion of the left gill arch from each fish was removed with surgical scissors and used as the standard reference point. Gill sections were dehydrated using alcohol, cleaned by xylene, infiltrated with paraffin and embedded in paraffin wax and were sectioned on a A/O A20 rotary microtome. The 5.0 µm-thick sections were stained with hematoxylin and eosin (H&E) stain (Trombetti et al. 1996, Gong, Jingjuan 1999). Slides were used for subsequent determination of chloride cell numbers.

2.2. *Confinement Stress Challenge*

Rainbow trout tested for confinement stress responses were reared under the general rearing conditions previously described. A 1264 L fiberglass tank was used to evaluate the trout stress response to confinement. The tank was equipped with four baskets made of black plastic mesh (Figure 2.2.). The baskets were secured to the sides of the tank with cable ties and placed to allow adequate water circulation between baskets. The confinement stress challenge was initiated on Day 76.

The tank was supplied with 23 L/min dechlorinated water at 16° C and a dissolved oxygen concentration of 8.0 mg/L was maintained throughout the challenge. Ten trout were removed from each tank and sampled immediately as the

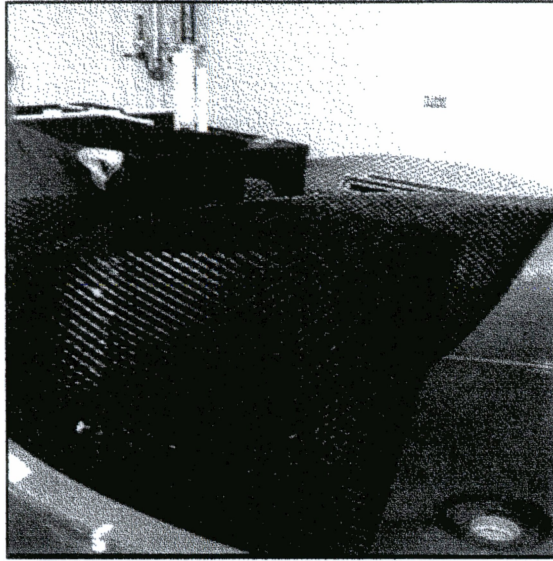


Fig. 2.2: Plastic Mesh Baskets Used to Confine Rainbow Trout During a 12-Hour Confinement Challenge

zero hour controls. Sixty trout from each tank were caught by net and placed into separate baskets in the challenge tank. The baskets were adjusted to a water depth that caused the fish to be intensely crowded so that the caudal fins of the fish were exposed to the air, but so that all fish heads remained underwater.

Ten trout were taken from each basket at hours 0, 2, 4, 6, and 12. Fish taken as samples were immediately euthanized by overdose with tricaine methane sulfonate (100 mg/L). Blood was collected from each fish and stored as previously described. Percent hematocrit was determined by centrifugation in an Adams Autocrit centrifuge (Clay-Adams Inc. NY) for 10 minutes and read on a microcapillary reader (Damon IEC Division).

Remaining trout from each basket were released into separate 852 L tanks with flow rates equivalent to the rearing conditions. Ten trout were taken from each

tank at 24 and 72 hours and sampled as described above to assess recovery from confinement.

2.3 *Assays*

Plasma cortisol levels were determined by radioimmunoassay (RIA) using Coat-A-Count® [I^{125}] competitive binding cortisol RIA kits (Diagnostic Products Corporation, CA). The Coat-A-Count® RIA for cortisol is designed for human plasma and contains standard curve calibrators that range from 0-500 ng/ml cortisol. This standard curve covers the range of cortisol concentrations documented for both wild and hatchery-reared rainbow trout subjected to confinement stress (Woodward and Strange, 1987). The Coat-A-Count® RIA was further characterized for rainbow trout plasma using a dilution assay for pooled plasma samples from “high” and “low” stress groups of trout. This characterization and the specific procedure for the Coat-A-Count® RIA are described in Appendix B.

Plasma glucose and chloride levels were determined using spectrophotometric enzyme assays (Sigma Chemical Co., MI).

2.4. *Experimental Design*

A completely randomized design with a 4x7 factorial for treatments of diet and time was used to evaluate stress responses, growth and chloride cell development in the rainbow trout fed salt-enriched diets as compared to a basal diet. Diet and time treatments were randomly assigned to individual fish. Two-way analysis of variance

was used to test for treatment and interaction differences. Least square means were compared by least significant difference, mean separation. Data were examined for equal variance and normality, and log transformations were used if necessary. All values were considered significant at $P \leq 0.05$. All average values are reported as mean $\pm$ standard error of the mean.

3. Results

3.1 Growth

The average length of rainbow trout increased from 22.2 cm $\pm$ 2 on Day 0 (stocking) to 28.8 cm $\pm$ 2 on Day 133. There were no differences in average lengths of trout among the experimental diets or in relation to the basal diet at any time tested (Appendix C). Changes in length averaged 1.3 cm per sampling period resulting in significant increases in average length at each sampling point.

Likewise, diet had no significant effect on weight gained by trout over the course of the experiment (Appendix C). Average weight of trout for all four diets increased significantly at each sampling point from .108 Kg $\pm$.002 at stocking to .290 Kg $\pm$.01 by Day 133. Changes in weight averaged .03 Kg per sampling period. Changes in length and weight were not significantly different among diets. The salt-enriched diets tested did not affect growth in rainbow trout over the course of this study.

Plasma chloride concentrations were measured at each sampling point to determine whether feeding with salt-enriched diets affected resting plasma chloride

levels. Average plasma chloride concentrations were not affected by diet (Figure 2.3).

Average plasma chloride measures across all four diets ranged from 115.9 mEq/l + 1.0 to 131.4 mEq/l + 1.5. Trout began receiving the various salt-enriched diets and the basal diet on Day 6 of the experimental period and by Day 13, trout fed the 3.6% CaCl₂ diet had plasma chloride values that were significantly higher than for trout fed the basal diet. This response was transient however, and disappeared by the Day 41 sample.

Plasma chloride values averaged across all four diets generally decreased over time from the Day 13 sample to the Day 76 sample when average plasma chloride values were at their lowest point. Plasma chloride measures increased again following the cessation of salt-feeding after Day 76 of the experiment.

Average numbers of gill chloride cells increase following initiation of salt feeding and decline with the cessation of salt feeding after Day 76 (Figure 2.4, Appendix E).

3.2. *Confinement Results*

Trout fed salt-enriched diets or a basal diet (no added salt) were tested for their response to confinement on Day 76 of a 133-day study.

During the confinement challenge, average plasma chloride levels in rainbow trout fed either salt-enriched diets or a basal (0.7% NaCl) diet did not differ at the Hour 0 baseline sample (Figure 2.5). Average plasma chloride levels for all trout

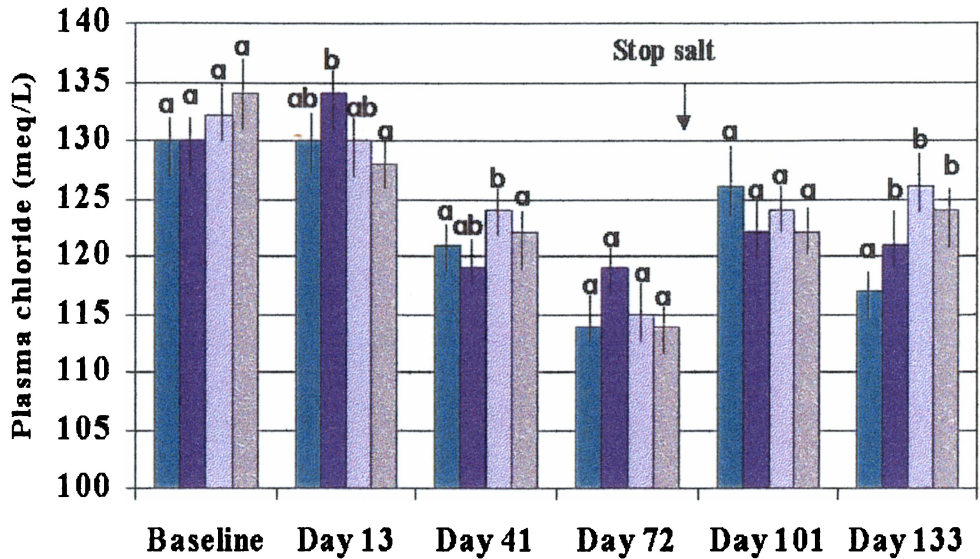


Figure 2.3: Plasma Chloride Levels in Rainbow Trout Fed Salt-Enriched Diets

Average plasma chloride concentrations in rainbow trout fed experimental salt-enriched diets or a basal diet for 76 days of a 133-day study. Green bars indicate plasma chloride concentrations in trout fed a 3.6% NaCl diet. Dark blue bars indicate plasma chloride concentrations in trout fed a 3.6% CaCl₂ diet. Light blue bars indicate plasma chloride concentrations for trout fed a 7.3% NaCl diet and gray bars indicate plasma chloride concentrations for trout fed a basal (0.7%) NaCl diet. Differences are significant at $P \leq 0.05$.

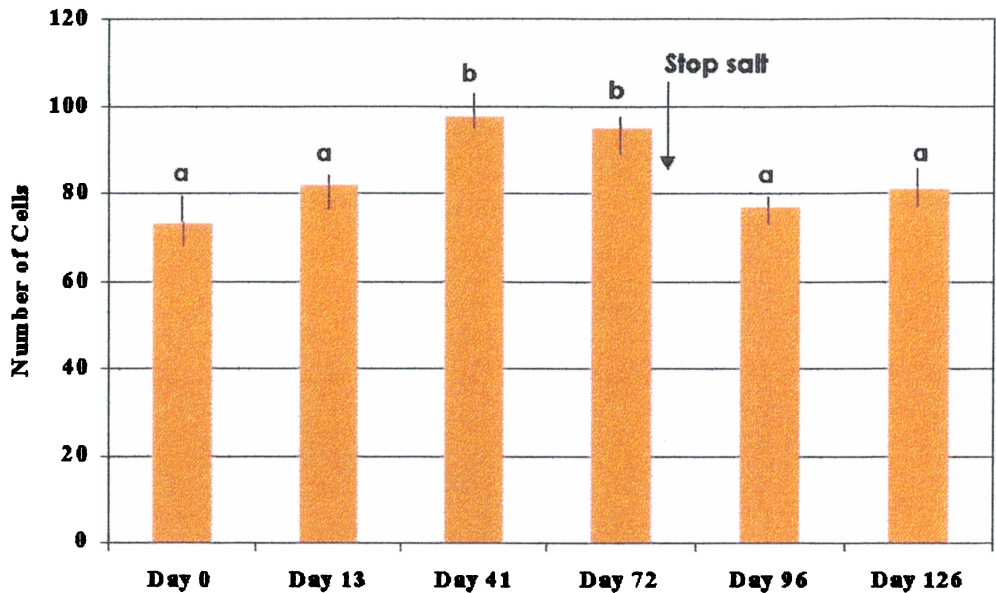


Fig. 2.4: Average Chloride Cell Numbers in Salt-Fed

Average gill chloride cell numbers from rainbow trout fed three salt-enriched diets from Day 0 to Day 76 of a 133-day study. Differences are significant at $P \leq 0.05$.

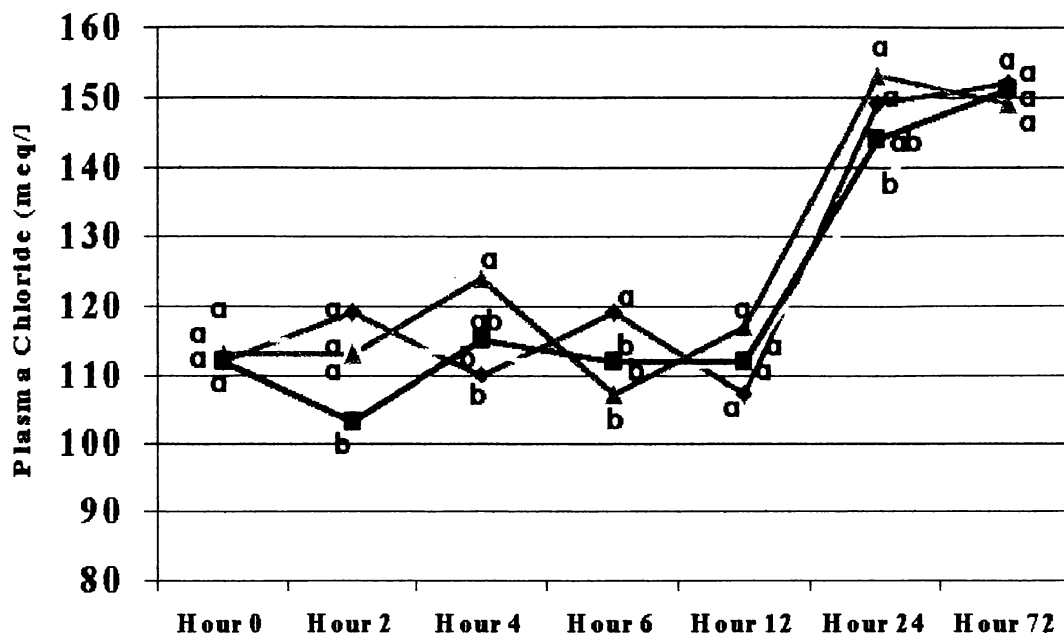


Figure 2.5: Average Plasma Chloride Concentrations in Rainbow Trout Confined for 12 Hours

Changes in average plasma chloride concentrations in rainbow trout fed three salt-enriched diets and a control basal diet during a 12-hour confinement challenge and a 72-hour recovery period. The red line indicates average plasma chloride levels in trout fed a 3.6% NaCl diet. The yellow line indicates average plasma chloride levels in trout fed a 3.6% CaCl₂ diet. The green line indicates average plasma chloride levels in trout fed a 7.3% NaCl diet and the blue line indicates average plasma chloride levels for trout fed a 0.7% NaCl basal diet. Differences are significant at $P \leq 0.05$.

combined did decrease slightly over the course of the experiment from $113.2 \text{ meq/L} \pm 0.93$ to $112.3 \text{ meq/L} \pm 2.04$ (Appendix F).

By Hour 2 of confinement, the average plasma chloride concentrations were significantly higher in trout fed all three of the salt-enriched diets compared to plasma chloride levels in trout fed the basal diet (Figure 2.5).

By Hour 4, these elevations in plasma chloride concentrations had decreased in all but trout fed the 7.3% NaCl diet. These trout still had plasma chloride levels significantly higher than those in trout fed the basal diet. After six hours of confinement, trout receiving the 3.6% NaCl diet had plasma chloride concentrations higher than those of trout fed the basal diet. By Hour 12 of confinement, there were no differences measured in plasma chloride levels among trout fed salt-enriched diets or fed the basal diet.

The confinement challenge was ended at 12 hours and trout were recovered for a 24-hour period. At Hours 24 and 72 of recovery, all trout sampled had significantly elevated plasma chloride concentrations relative to the Hour 0 baseline measures. Of the fish sampled at 24 hours, trout fed the 7.3% NaCl diet had plasma chloride levels that were significantly higher than those in trout fed the basal diet. At Hour 72, there were no differences among plasma chloride concentrations sampled, however plasma chloride levels in all trout remained significantly higher than baseline plasma chloride levels measured at Hour 0.

3.3. *Plasma cortisol levels*

Cortisol concentrations were significantly affected by diet, time of confinement and interaction of diet with time of exposure to confinement. The average plasma cortisol values over all four diets ranged from 7.0 ± 7.2 ng/ml to 130 ± 7.2 ng/ml during Hour 4 of confinement.

Average plasma cortisol concentrations sampled prior to the onset of the confinement challenge (Hour 0 values), were low, particularly the value for trout fed the 3.6% NaCl diet (Figure 2.6, Appendix G). During two hours of confinement, average plasma cortisol levels increased rapidly and significantly above the Hour 0 values in all trout sampled. Trout fed the 3.6% NaCl diet and the 3.6% CaCl₂ diet had average plasma cortisol concentrations significantly higher than the basal diet.

By Hour 4, plasma cortisol concentrations for all trout had once again increased significantly above the Hour 2 values. There were no differences at Hour 4, however, among trout fed different diets. Average plasma cortisol concentrations remained elevated at Hour 6 of confinement for trout fed the 3.6% CaCl₂ diet and the 7.3% NaCl diet but these cortisol values were not different from the basal diet. Plasma cortisol values in trout fed the 3.6% NaCl diet dropped sharply at the Hour 6 sample from a peak value of 132 ng/ml at Hour 4 to 45 ng/ml at Hour 6. Trout receiving the 3.6% NaCl diet were the only fish with significantly different average plasma cortisol values from the basal diet value at Hour 6. This drop in average plasma cortisol in trout fed the 3.6% NaCl diet rebounded by Hour 12 of confinement to 123 ng/ml which was a value significantly higher than that of the basal diet. The

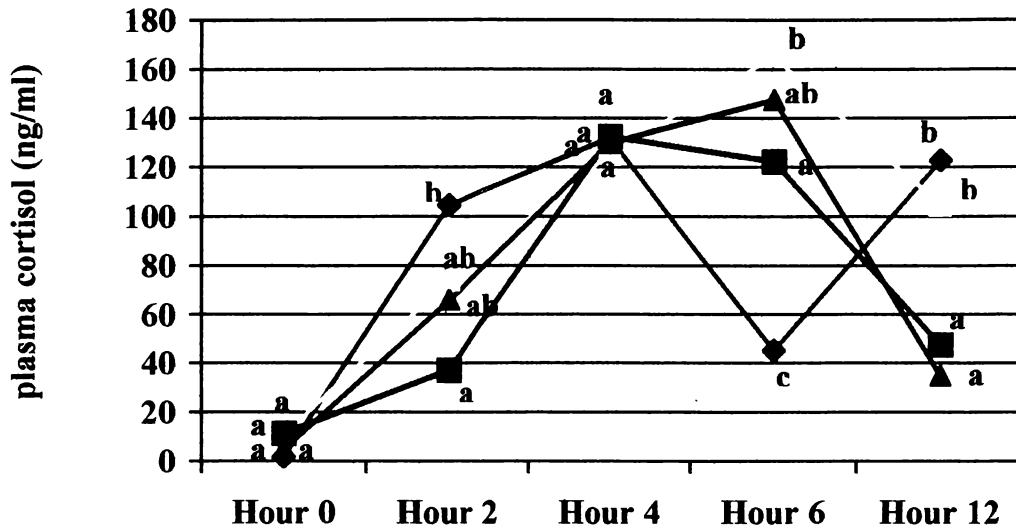


Figure 2.6: Average Plasma Cortisol Values in Rainbow Trout Confined for 12 Hours

Changes in mean plasma cortisol concentrations in rainbow trout fed salt-enriched and a control diet over the course of a 12-hour confinement stressor. The red line indicates cortisol concentrations in trout fed the 3.6% NaCl diet. The yellow line indicates cortisol concentrations in trout fed the 3.6% CaCl₂ diet. The green line indicates cortisol values in trout receiving the 7.3% NaCl diet and the blue line indicates plasma cortisol values for trout receiving the basal, 0.7% NaCl diet. Differences are significant at $P \leq 0.05$.

average plasma cortisol concentration for trout fed the 3.6% CaCl₂ diet was also significantly higher than the value for trout fed the basal diet at Hour 12.

3.4. *Plasma glucose levels*

Plasma glucose concentrations in rainbow trout exposed to a 12-hour confinement challenge ranged from an average level of 79 meq/L $\pm$ 2.2 at Hour 0 to an average high of 133 meq/L $\pm$ 5.7 at Hour 6 (Figure 2.7, Appendix H).

There were no differences in average plasma glucose levels among fish fed either experimental diets or the basal diet at the start of the experiment, Hour 0. Average plasma glucose levels rose for all trout between Hour 0 and Hour 2 of confinement (Figure 2.7).

By Hour 2 of confinement, trout fed the 3.6% CaCl₂ diet and those fed the 7.3% NaCl diet had average plasma glucose concentrations that were significantly higher than those for trout fed the basal diet; the trout on the 7.3% diet remained significantly higher at Hour 4. Average plasma glucose measures for trout fed the 3.6% NaCl diet were significantly higher than trout on the basal diet at Hour 4 of confinement. Trout receiving the 3.6% CaCl₂ diet had plasma glucose concentrations that rose sharply between Hour 4 and Hour 6 of confinement to peak at significantly higher glucose concentrations than those of trout fed the basal diet (Figure 2.7).

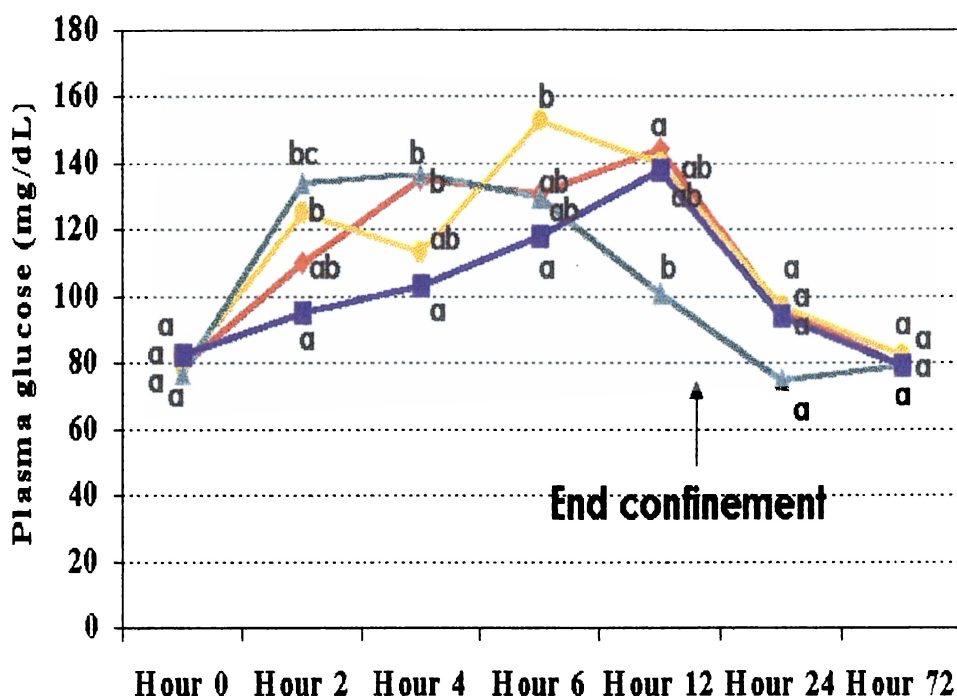


Figure 2.7: Average Plasma Glucose Values for Rainbow Trout Confined for 12 Hours

Changes in mean plasma glucose concentrations in rainbow trout fed three salt-enriched diets and a basal control diet and confined for 12 hours. The red line indicates mean plasma glucose levels for trout fed the 3.6% NaCl diet. The yellow line indicates mean plasma glucose levels for trout fed the 3.6% CaCl₂ diet. The green line indicates mean plasma glucose concentrations for trout receiving the 7.3% NaCl diet and the blue line indicates plasma glucose concentrations for trout fed the basal, 0.7% NaCl diet. Differences are significant at $P \leq 0.05$.

By Hour 12, all differences among diets with respect to plasma glucose levels had disappeared. Trout fed the 7.3% NaCl diet had an especially rapid drop in plasma glucose levels between Hour 6 and Hour 12 of confinement. Plasma glucose concentrations decreased during 24 hours of recovery following release from confinement to reach values that were not different from pre-stress values.

3.5. *Hematocrit*

Average percent hematocrit measures for rainbow trout fed either salt-enriched diets or a basal (0.7% NaCl) diet averaged 36% at the start of the twelve-hour confinement challenge (Appendix I). There were no differences in average percent hematocrits among trout receiving different diets at the Hour 0 baseline sample (Table 2.2).

Table 2.3: Average Percent Hematocrit Values for Salt-Fed Rainbow Trout Subjected to a 12-Hour Confinement Challenge

Confinement (Hours)	3.6% NaCl Diet	3.6% CaCl₂ Diet	7.3% NaCl Diet	Basal Diet
0	36 ± .02	36 ± .02	37 ± .02	36.5 ± .02
2	41 ± .02	39 ± .02	43 ± .02	40.5 ± .02
4	37 ± .02	39 ± .02	41 ± .02	40 ± .02
6	37 ± .01	38 ± .01	39 ± .01	38 ± .01
12	44 ± .01 ^{ab}	43 ± .01 ^{ab}	40 ± .01 ^b	45 ± .01 ^a
24	37 ± .02 ^a	43 ± .02 ^b	39 ± .02 ^{ab}	41 ± .02 ^{ab}
72	40 ± .02 ^b	41 ± .02 ^b	42 ± .02 ^b	34 ± .02 ^a

Differences are significant at $P \leq 0.05$.

Differences in percent blood hematocrit values among trout fed salt-enriched diets as compared to a basal diet were not apparent until the final sample at Hour 12 of confinement. At Hour 12, trout fed the 7.3% NaCl diet had significantly lower percent blood hematocrit levels than trout fed the basal diet.

There were no differences in percent hematocrit levels among trout fed different diets at 24 hours of recovery following confinement. At Hour 72 of recovery however, percent blood hematocrits were significantly higher in all groups of trout receiving salt-enriched diets relative to those values for trout fed the basal diet.

4. Discussion

4.1 *Growth*

Measures of length and weight were not significantly different among rainbow trout fed experimental salt-enriched diets or a basal diet without salt enrichment at any time tested. Although earlier studies of rainbow trout fed NaCl enriched diets indicated negative effects on growth and weight gain (Zaug and McClain, 1969; Basulto, 1976), these studies were conducted with diets that were not formulated to be isocaloric and isonitrogenous. Later studies using salt-enriched diets corrected for protein dilution due to added salt did not report evidence of reductions in performance due to diet (Salman and Eddy, 1988; Zaug et al., 1983; Gong, 1999). Trout grew consistently for all diets tested in our study including the control diet.

Therefore we concluded that feeding salt-enriched diets did not affect growth in rainbow trout over the course of this study.

4.2. *Plasma chloride concentrations*

Plasma chloride concentrations are known to decrease in stressed salmonids (Woodward and Strange, 1987; McDonald and Rogano, 1986; McDonald and Milligan, 1992). The average Hour 0 plasma chloride level observed in our study was $113.2 \text{ mEq/L} \pm 0.9$ across all four diets with no differences in average plasma chloride values among the diets. The average plasma chloride values at Hour 0 for trout used in this study were significantly lower than plasma chloride values reported by A.E. Smith (2001). Smith used rainbow trout fed diets identical to those used in this study and reported an average Hour 0 plasma chloride concentration across all four diets as $126.4 \text{ mEq/L} \pm 1.2$. Previous studies however, have reported similar values for average plasma chloride concentrations in unstressed trout as 113.7 meq/l (Basulto, 1976), 127 meq/l (Johnston and Cheverie, 1985), $126 \text{ meq/l} \pm 1.3 \text{ meq/l}$ (Avella et al.), and 103.4 ± 4.61 (Salman and Eddy, 1988). Concurrent measures of the primary stress response in our study, reported as plasma cortisol concentrations, do not indicate measurable levels of stress at Hour 0 in the trout used for this experiment.

Losses in plasma chloride concentrations are known to occur swiftly with the onset of acute stressors and the disruption of osmoregulatory processes. A significant loss of plasma chloride occurred during Hour 2 of confinement in trout fed the basal

diet. Trout receiving all three of the salt-enriched diets had significantly higher levels of plasma chloride at this time, but the plasma chloride levels were not different among the salt-enriched diets. Based on these observations, no distinction could be made concerning a specifically beneficial salt-enriched diet in reducing stress-induced hypochloremia in the early stages of the acute stress response. All three salt-enriched diets offered some apparent protection against hypochloremia as compared to the basal diet at two hours of confinement.

The manner in which plasma chloride concentrations rebounded in stressed trout at 24 hours following release was of note. All trout sampled at 24 and at 72 hours post-confinement had average plasma chloride concentrations that were significantly higher than plasma chlorides measured at the Hour 0 sample.

Measures of plasma chloride concentrations showed that trout fed salt-enriched diets had significantly less hypochloremia in the early stages of exposure to confinement than did trout fed the basal diet. Trout fed the 7.3% NaCl diet showed a higher recovery concentration of plasma chloride levels at 24 hour post-stress than did trout fed the other diets.

No significant elevations in plasma chloride levels were detected in trout fed salt-enriched diets as compared to a basal diet for samples taken on Days 41 and 72 of salt-feeding. The only significantly elevated plasma chloride concentration detected prior to the confinement challenge was on Day 13 for trout fed the 3.6% CaCl_2 diet. Salman and Eddy (1988) reported significant increases in plasma chloride concentrations within 30 hours post-feeding in trout fed a diet supplemented with

11.6% NaCl. Pelletier and Besner (1992) fed brook trout diets supplemented with eight or 12% NaCl. They measured a transient increase in plasma chloride that was undetectable after a 24-hour fasting period. Plasma chloride concentrations measured throughout the 133-day experimental period during our study were made on trout following a 12-hour fasting period. It is possible that we did not detect elevated levels of plasma chlorides in salt-fed trout because the response is only transient following feeding.

Several previous studies have reported increases in gill chloride cell numbers with salt-feeding (Salman and Eddy, 1987; Gong, 1999 and unpublished data from Harm and Hinshaw, 1993). Average numbers of gill chloride cells increased significantly in over baseline measures in rainbow trout fed salt-enriched diets in our study as well. Concurrent with the increase in gill chloride cells, plasma chloride concentrations were found to decline in trout from the onset of salt-feeding at Day 6 through the confinement challenge on Day 76. Plasma chloride values measured in remaining trout on Day 101 and again on Day 133 were found to increase. However, the changes in plasma chloride concentrations that we observed over time occurred in trout receiving the basal diet as well as the salt-enriched diets. Therefore we could not conclude that the salt-enriched diets tested had any effect on the changing plasma chloride concentrations over time.

4.3. *Plasma cortisol concentrations*

Plasma cortisol concentrations at Hour 0 for rainbow trout fed the 3.6% NaCl diet and the 7.3% NaCl diet were within the normal range of resting cortisol concentrations for salmonids (Pickering and Pottinger, 1985). Although trout receiving the basal diet and the 3.6% CaCl₂ had Hour 0 cortisol measures slightly above normal reported resting concentrations, (11.1 and 10.8 ± 1.1 ng/ml respectively), these values were not statistically different from the value of $4.6 \text{ ng/ml} \pm 1.4$ reported for the 7.3% NaCl diets.

Changes in plasma cortisol concentrations reflected a characteristic primary stress response to confinement as concentrations climbed rapidly and significantly over the first two hours to reach levels in all trout that have been reported to indicate stress (Barton and Schreck, 1987; Pickering and Pottinger, 1989; Ruane et al., 1999; A.E. Smith; 2001).

Trout fed the 3.6% NaCl diet had the most rapid increase in plasma cortisol levels within the first two hours of confinement. At Hour 2, these trout had plasma cortisol concentrations indicative of high levels of stress (Mazeaud et al., 1977; Strange et al., 1977; Woodward and Strange, 1987; Andersen et al., 1991). These observations suggest that a 3.6% NaCl diet provides no protection for the early cortisol response in rainbow trout subjected to an acute stressor.

Trout receiving the 7.3% NaCl diet had plasma cortisol concentrations that were not different from those of trout fed the basal diet at Hour 2. The 7.3% NaCl was the highest level of dietary chloride fed in our experiments. The 7.3% NaCl diet

apparently had no protective effect on the cortisol response in rainbow trout stressed by confinement for two hours because the average plasma cortisol concentrations of trout fed this diet were not less than those of trout fed the basal diet.

By Hour 4, trout fed all three salt-enriched diets performed the same in terms of plasma cortisol response as the trout fed the basal diet. No protection against elevated plasma cortisol levels was observed.

At Hour 6 of confinement, however, trout fed the 3.6% NaCl diet had average plasma cortisol levels significantly lower than those of trout fed the basal diet, the 3.6% CaCl₂ diet or the 7.3% NaCl diet. Given that plasma cortisol concentrations have been shown to increase continually with the duration of the stressor in rainbow trout up to 12 hours (Woodward and Strange, 1987), this result for trout fed the 3.6% NaCl diet was surprising. Raw data for these fish were examined for outliers, but seven out of ten trout sampled at Hour 6 from the 3.6% NaCl diet had average plasma cortisol values less than 50 ng/ml. These observations suggest that some protective effect against increases in plasma cortisol concentrations may be provided by the 3.6% NaCl diet after six hours of exposure to an acute stressor.

Overall, trout receiving the 7.3% NaCl diet had plasma cortisol concentrations which were generally lower than those in trout fed the 3.6% NaCl diet or the 3.6% CaCl₂ diet. However, the values for plasma cortisol in trout fed the 7.3% diet were not statistically different from trout fed the basal diet. Generally protective effects of salt-enriched diets on plasma cortisol responses in acutely stressed rainbow trout were not observed. With respect to measures of plasma cortisol, trout receiving a diet

supplemented with 7.3% NaCl had lower overall cortisol values than trout receiving the other salt-enriched diets tested, but these values were not significantly different from those for trout fed a basal diet.

4.4. *Plasma glucose concentrations*

Hour 0 measures of average plasma glucose concentrations in rainbow trout fed either salt-enriched diets or a basal diet were all similar ranging from 76.8 ± 4.3 mg/dL to 82.5 ± 4.3 mg/dL. These values were slightly higher than baseline plasma glucose levels previously reported (Wedemeyer, 1976; Woodward and Strange, 1987; Ruane et al., 1999). A significant increase was seen in average plasma glucose concentrations during the first two hours of confinement in trout fed all three experimental diets. This hyperglycemia was expected in light of the rapid primary stress response as indicated by changes in plasma cortisol concentrations in the first two hours of confinement.

The lack of a significantly elevated plasma glucose concentration until Hour 6 of confinement in trout fed the basal diet was surprising, given the significant increases in plasma cortisol levels which occurred in these fish at both Hour 2 and Hour 4 of confinement. Changes in plasma glucose concentrations are known to mirror changes in plasma cortisol with only a short lag time in stressed salmonids (Barton and Iwama, 1991).

Rainbow trout fed the CaCl₂ diet showed the maximum average level of hyperglycemia from all fish at Hour 6, whereas the other two salt-enriched diets had

plasma glucose concentrations which were not different from the basal diet.

Following Hour 6 of confinement, there were no further differences in average plasma cortisol concentrations among diets.

Interestingly, plasma glucose levels showed a sharp and significant decline in trout fed the 7.3% NaCl diet between six and 12 hours of confinement, even though these plasma concentrations were not significantly different from those of trout fed the basal diet. At 12 hours of confinement, these trout had average plasma glucose concentrations that were not different from the Hour 0 pre-stress levels. This decline continued through 24 hours of recovery following the release of confined trout. At the Hour 24 sample, trout fed the 7.3% NaCl diet had average plasma glucose measures that were low enough to be considered hypoglycemic (Woodward and Strange, 1987) but these values were not significantly different from values for trout fed the basal diet. Therefore, we cannot conclude that salt-enriched diets affect the attenuation of the glucose response in rainbow trout stressed by 12 hours of confinement in our experiment.

Trout receiving the 3.6% CaCl_2 diet had the highest overall stress responses seen for plasma cortisol and glucose. These results coupled with the acute mortalities of trout fed a 7.3% CaCl_2 diet in previous experiments in our laboratory lead us to conclude that diets containing elevated concentrations of chlorides as CaCl_2 may not be suitable for use as general diets for cultured fish.

4.5. Hematocrit

Percent blood hematocrit values averaged over all four diets did increase significantly at Hour 2 and at Hour 4 of confinement ranging from 36% to 40% $\pm$.01 respectively, although these values were not different among diets. Changes in percent hematocrit observed in confined trout were consistent with values reported recently by Ruane and coworkers (1999). The only difference in percent hematocrit values observed for trout subjected to a 12-hour confinement stressor occurred at the Hour 12 sample when trout fed the 7.3% NaCl diet had hematocrits significantly lower than those measured for trout fed the basal diet. These observations differed from those of A.E. Smith (2001) who observed significant and sustained increases in percent hematocrit values in rainbow trout fed a 3.6% CaCl_2 diet in response to an acid stress. She suggests that elevated plasma hematocrits in trout fed a 3.6% CaCl_2 diet may be caused by an overall increased stress response in calcium-fed fish due to the inability of the fish to ionoregulate excessive ingested Ca^{+2} ions. Excessive Ca^{+2} ions retained on cell membranes or within cell cytoplasm would increase intracellular osmolarity and cause increased influx or retention of water from the extracellular fluid, thus weakening cellular membranes. Movement of water out of the extracellular fluid and into cells would also decrease plasma fluid volume. This change might be reflected as increases in percent hematocrit levels (A.E. Smith, 2001).

There is no experimental evidence to date that increased ingested calcium results in increased retention of Ca^{+2} ions on cellular membranes or in the cytoplasm

in fish. Increases in circulating erythrocytes do occur in response to stress-induced release of catecholamines in fish as discussed previously. However significant changes in percent hematocrit values were not observed in our studies of trout confined for 12 hours. This difference could not be attributed to diet because percent hematocrit values did not differ among trout fed either salt-enriched or a basal diet. Our observations suggest that trout may respond differently to a confinement stress than to an acid stress.

5. Conclusions

Rainbow trout fed salt-enriched diets respond to acute confinement stress with classical primary and secondary stress responses measured as plasma cortisol, glucose, and chloride concentrations. Cortisol values for trout fed the 7.3% NaCl diet were not significantly lower than for trout fed the basal diet.

Measures of plasma chloride concentrations showed that trout fed salt-enriched diets had significantly less hypochloremia in the early stages of exposure to confinement than did trout fed the basal diet. Trout fed the 7.3% NaCl diet showed a higher recovery concentration of plasma chloride levels at 24 hour post-stress than did trout fed the other diets.

Trout receiving the 3.6% CaCl_2 diet had the highest overall stress responses seen for plasma cortisol and plasma glucose responses. These results coupled with the acute mortalities of trout fed a 7.3% CaCl_2 diet in previous experiments in our

laboratory lead us to conclude that diets containing elevated concentrations of chlorides as CaCl_2 are not suitable for use as general diets for cultured fish.

Although further research is needed to corroborate these results, we believe that diets enriched with chloride as NaCl at the 7.3% level may be beneficial in aiding the recovery of trout from episodes of acute stress and thus may reduce the more chronic detrimental effects of stress seen in cultured fish.

Chapter 3

Methemoglobinemia

1. Introduction

Methemoglobinemia is an environmental disease of fish caused by exposure to elevated nitrite (NO_2^-), concentrations in the water. Nitrite is a negatively charged ion produced during the oxidation of ammonia (NH_3 or NH_4^+) to its final oxidized form, nitrate, NO_3^- . Nitrite concentrations can increase in the water as ammonia, the nitrogenous waste product of fish, is oxidized after elimination. The oxidation of ammonia occurs due to the presence of nitrifying bacteria that are ubiquitous to soil and water systems.

Nitrite concentrations can become elevated in intensive fish culture systems where high feeding rates lead to increased ammonia excretion. Elevated nitrite levels have been reported from intensive recirculating aquaculture systems which rely on biofilters containing nitrifying bacteria to oxidize ammonia in the culture water prior to each pass of the water through the system (Liao and Mayo, 1974; Collins et al., 1975; Wedemeyer and Yasutake, 1978; Jewell and Cummings, 1990).

Methemoglobinemia is a well-documented disease in intensive catfish production ponds (Brown, 1981; Brown and Boyd, 1981; Schwedler and Tucker, 1983; Tomasso, 1994).

Methemoglobinemia is believed to occur in response to the concentration of nitrite in the blood of fish due to the competitive uptake of NO_2^- ions at the expense of chloride (Cl^-) ions (Meade and Perrone, 1980; Krous et al., 1982; Eddy et al., 1983;

Payan et al., 1984; Williams and Eddy, 1986). Chloride cells in the gill epithelia of fresh water fish contain active carrier sites for the uptake of Cl^- ions. Chloride is an important electrolyte for maintenance of osmotic balance and fluid volume in fish. The onset of methemoglobinemia is believed to be caused by the competitive inhibition of Cl^- ion uptake at active carrier sites on chloride cells by elevated concentrations of NO_2^- (Perrone and Meade, 1977; Bath, 1980; Bath and Eddy, 1980; Eddy et al., 1983). Once formed, methemoglobin cannot reversibly bind oxygen for transport to the tissues. Therefore, toxicity of methemoglobinemia is believed to result from tissue hypoxia caused by the reduced oxygen carrying capacity of the blood (Brown and McLeay, 1975).

Methemoglobinemia is a problematic disease for intensive aquaculture. The aquaculture industry spends an estimated \$ 2.5 million per year to add NaCl to the water of intensive culture systems to prevent outbreaks of this disease. The additional cost of labor and time to apply salt to large production systems as well as the environmental effects of adding salt to outdoor culture ponds at the recommended prophylactic concentrations are yet to be determined.

Rather than adding salt to the culture water, a more practical approach to mitigating the toxicity of methemoglobinemia in fish culture would be feeding salt-enriched diets. Because methemoglobinemia is believed to be caused by the uptake and concentration of NO_2^- ions through gill chloride cells in freshwater fish, salt-enriched diets might reduce the onset and severity of the disease in one of two ways. First, salt-enriched diets may increase the resting concentration of plasma chloride,

thereby reducing the rate of active chloride uptake and the activity of the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism in chloride cells. Previous studies by Salman and Eddy (1988), and Pelletier and Besner reported significant increases in plasma ion concentrations following feeding with salt-enriched diets.

Second, salt-enriched diets might increase plasma ion concentrations to levels that reverse the normal ion uptake activity of chloride cells causing active extrusion of Cl^- and Na^+ ions as occurs in chloride cells in salt-water adapted trout and other marine species (Langdon and Thorpe, 1984). If the chloride cells of freshwater rainbow trout could be made to excrete Cl^- ions, protection against the competitive uptake of NO_2^- ions would occur.

2. Methods

2.1. Overview

To examine the benefits of feeding salt-enriched diets in reducing methemoglobinemia, we used rainbow trout hand-graded for size at 21.6 ± 1.3 cm. Trout were stocked into 5, 862-L fiberglass tanks supplied with 23 L/min flowing water and maintained at 15.5 °C. Following a 14-day acclimation period, trout were fed one of four experimental diets for 73 days. The diets used were 3.6% chloride as NaCl, 3.6% chloride as CaCl_2 , 7.3% chloride as NaCl and a basal diet having 0.7% NaCl (Purina TestDiet® Laboratory, Richmond, IN). Rainbow trout were fed twice per day, six days per week at 1.5% of body weight (Piper et al., 1982). Following the acclimation period and prior to the start of salt-feeding, trout from each tank were

sampled for length, weight and plasma chloride levels, and numbers of gill chloride cells to establish baseline data for the experiment. On Days 72 and 73 of the experimental period, trout from each diet were exposed to 18 mg/L nitrite-nitrogen for eight hours and evaluated for development of percent blood methemoglobin to determine whether the salt-enriched diets would protect fish from the development or severity of methemoglobinemia. The total duration of the experiment was 133 days. Trout received experimental diets from Day 6 through Day 76 after which trout were returned to the basal diet to assess persistence of effects of salt feeding following withdrawal of the salt-enriched diets.

2.2. *Nitrite challenge*

To conduct the nitrite challenge, a static assay tank was prepared using a 1264-L circular fiberglass tank divided into two equal sections with a plastic mesh screen. The screen was secured with silicone so that fish could not move between the two sections. A water temperature of 15.5°C was maintained throughout the experiment. A dissolved oxygen concentration of 8.0 mg/L was maintained using two air diffuser stones (Figure 3.1).

Nitrite challenges were conducted over a period of two days, Day 72 and 73 of the study. On Day 72, 75.8g of NaNO₂ were dissolved in 1 L of water and added to the static assay tank to achieve a NO₂⁻ ion concentration of 18 mg/L. The nitrite concentration in the assay tank was monitored throughout the course of the experiment by enzymatic assay and values were read by spectrophotometer. Additional NaNO₂ was added as needed to maintain a constant nitrite concentration.

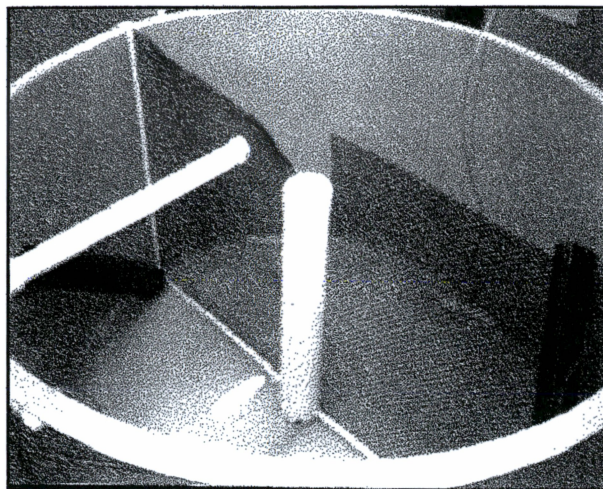


Fig. 3.1. Static Assay Tank Used for Nitrite Challenges for Rainbow Trout Fed Salt-Enriched Diets

To conduct the nitrite challenge, ten trout each from Tanks 1 and 3 were euthanized with overdose of tricaine methane sulfonate (100 mg/L) and exsanguinated by severing the caudal fin. These fish served as 0 Hour controls for methemoglobin levels for the 3.6% NaCl and the 7.3% NaCl diets. Forty trout from each of the two experimental diet tanks were immediately transferred to separate sides of the assay tank containing the elevated nitrite concentration. Ten trout were removed from each side of the assay tank at Hours 2, 4, 6, and 8 of nitrite exposure. Trout were euthanized, measured, weighed and exsanguinated. Trout from the experimental diet tanks receiving the 3.6% CaCl_2 and the basal 0.7% NaCl diets were subjected to the nitrite challenge using the same experimental methods and materials on the following day (Day 73).

2.3. *Methemoglobin concentration determination*

The percent blood methemoglobin level for each fish was measured on whole blood according to the methods of Evelyn and Malloy, 1938, as modified by Williams and Eddy, 1988 and Williams, M.T. (personal communication, 1999). 100 μ L of whole trout blood was added to a 16 x 125mm glass test tube containing a buffer solution of 5 ml of 10 mmol Tris base and .5% Triton X at pH 7.4. The blood was added to the buffer solution immediately upon exsanguination to lyse red blood cells and stop continued methemoglobin formation. The assay was performed in duplicate for each blood sample. The buffered blood was centrifuged for 7 minutes at 2000 rpm in an International Clinical centrifuge (International Equipment Co., MA) to remove cellular debris. Following centrifugation of whole trout blood in the buffered solution, the supernatant was stored under refrigeration for no more than six hours which preliminary experiments in our laboratory showed was the longest that methemoglobin levels were stable.

The supernatant was withdrawn and split evenly into two 16 x 125mm glass test tubes designated Tube 1 and Tube 2. Half of the supernatant from Tube 1 was transferred into 1.5 μ L plastic microcuvettes. The absorbancy of this sample was read spectrophotometrically at 630 nm. This reading measured the absorbancy of total hemoglobin in the whole trout blood and was listed as reading "A". A few milligrams of potassium cyanide were added to the remaining supernatant in Tube 1 to convert all methemoglobin present in the sample into cyanomethemoglobin. After 2 minutes, the supernatant in Tube 1 was transferred to a plastic microcuvette and a

reading was taken with the decrease in absorbancy noted as reading “B”. The difference between Readings A and B represented the proportion of hemoglobin that was present as methemoglobin in the sample. A few crystals of potassium ferricyanide were added to Tube 2 to convert all the hemoglobin present in the sample into ferrihemoglobin. Tube 2 was mixed by vortex mixer (Themodyne Type 16700 Maxi-Mix, Barstead Thermolyne Corp., MA) and allowed to react for 2 minutes. A reading on the change in this absorbancy was noted as reading “C”. Finally, a few crystals of potassium cyanide were added to the remaining supernatant in Tube 2 to convert the ferrihemoglobin to methemoglobin during a 2-minute reaction period. The absorbancy of total methemoglobin was then noted as “D”. The percent methemoglobin for each blood sample and duplicate was calculated as:

$$\% \text{ methemoglobin} = \frac{[\text{Reading A} - \text{Reading B}]}{[\text{Reading C} - \text{Reading D}]} \times 100$$

The duplicate values were averaged for each fish to give an average percent methemoglobin level per fish.

2.4. Chloride Cell Counts

Gill tissue samples were taken from five fish randomly selected from each tank on Days 0, 13, 41, 72-73, 101, and 133. Following exsanguination, heads of the fish were removed with gill arches intact and fixed in a 10% neutral buffered formalin solution for 48 hours. The central portion of the left gill arch from each fish was removed with surgical scissors and used as the standard reference. This portion

contained multiple pairs of gill filaments. Gill sections were dehydrated using alcohol, cleaned by xylene, infiltrated with paraffin and embedded in paraffin wax and were sectioned on a A/O A20 rotary microtome. The 5.0 μm -thick sections were stained with hematoxylin and eosin (H&E) stain (Trombetti et al. 1996, Gong, Jingjuan 1999). Slides were used for subsequent determination of chloride cell numbers (Appendix E).

Chloride cells were identified as large, darkly staining, irregularly shaped cells found at the base of the gill filaments and interlamellae regions of fish gill tissue (Figure 3.2). Chloride cells were counted for the first 25 pairs of lamellae from five gill filaments for each fish. The total number of chloride cells from each count was averaged to give an average number of chloride cells per fish.

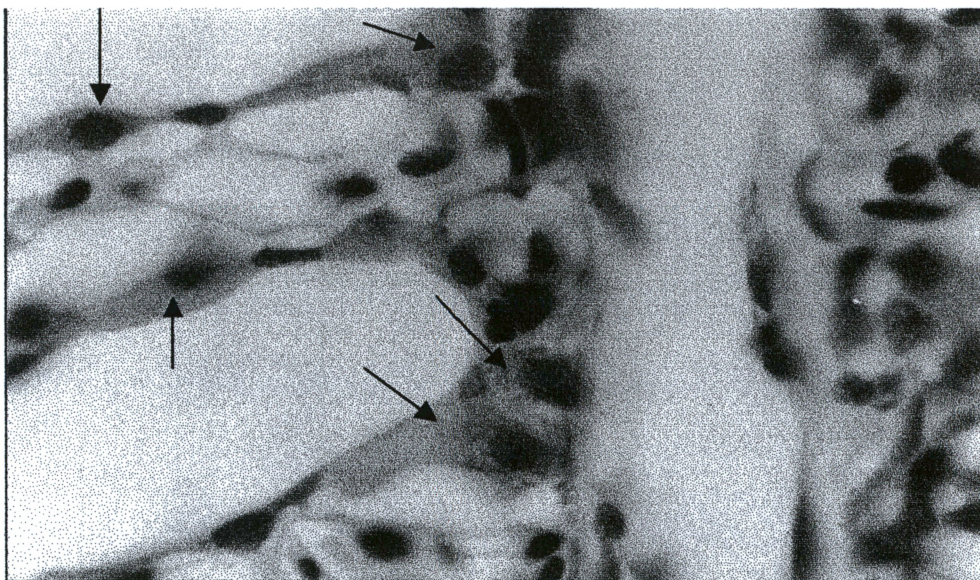


Figure 3.2. Chloride Cells on Lamellae and Interlamellar Spaces of Gills of Rainbow Trout Fed a Salt-Enriched Diet

2.5. *Plasma chloride concentration*

Plasma chloride concentrations were measured to determine whether feeding with salt-enriched diets affected resting plasma chloride levels. Sampling for plasma chloride was performed on 10 fish from each diet on Days 0, 13, 41, 72-73, 101, and 133. Ten trout were caught by net from each tank and euthanized by an overdose of tricaine methane sulfonate (100 mg/L). Fish were measured to the nearest .01 cm and weighed to the nearest .001 kg. Fish were exsanguinated into heparinized collection tubes by severing the caudal peduncle. The blood was centrifuged for 5 minutes at 10,000 rpm. Plasma was removed and stored frozen at -10° C for analysis of chloride. Plasma chloride concentrations were measured using a spectrophotometric enzymatic assay (Sigma Chemical Company, MI).

2.6. *Experimental design and analysis*

Prior to the initiation of salt feeding, trout from each tank were sampled on Day 0 and assessed for length, weight and plasma chloride levels to establish baseline data. Data were analyzed by ANOVA (SAS Version 8, 1999) to ensure that no tank effects existed for plasma chlorides (Table 3.1, Appendix D).

A completely randomized design, 4x5 factorial with treatments of diet and time was used to evaluate the response of salt-fed rainbow trout to an elevated nitrite concentration. A 4x6 factorial was used to evaluate changes in the numbers of gill chloride cells. All data were checked for normality and equal variance and percent

Table 3.1: Day 0 Baseline Values for Rainbow Trout Used in a 133-day Salt Feeding Experiment

Table 3.1. Baseline sample measures for length, weight and plasma chloride levels in rainbow trout taken prior to initiation of salt-feeding and following 14 days of acclimation. There were no significant differences at $P \leq 0.05$.

<u>Tank</u>	<u>Length (cm)</u>	<u>Weight (kg)</u>	<u>Plasma Chloride (meq/L)</u>
1	22.4 $\pm$.46	.188 $\pm$.08	129.7 $\pm$ 1.80
2	21.7 $\pm$.27	.104 $\pm$.004	129.9 $\pm$ 1.75
3	22.2 $\pm$.34	.110 $\pm$.005	121.3 $\pm$ 2.36
5	22.6 $\pm$.32	.110 $\pm$.005	114.2 $\pm$ 1.53

methemoglobin data were log transformed. Diet and time treatments were randomly assigned to individual fish. Two-way analysis of variance was used to test for treatment and interaction differences. Least square means were compared with least significant difference mean separation. All values were considered significant at the $P \leq 0.05$ level (SAS, Version 8, 1999).

3. Results

3.1. *Survival*

Overall survival of trout subjected to the nitrite challenge was high. No mortalities were reported from fish fed the 3.6% CaCl_2 diet or from fish fed the basal (0.7% NaCl) diet. Two mortalities each were recorded from fish fed the 3.6% NaCl diet and from fish fed the 7.3% NaCl diet. All four mortalities occurred between six and eight hours of nitrite exposure.

3.2. *Methemoglobin development*

Methemoglobin development in trout subjected to the nitrite challenge followed a characteristic pattern as described from previous studies. Average percent methemoglobin levels rose rapidly from four to eight hours of exposure (Figure 3.3). Average percent methemoglobin for all fish ranged from 9.3% at Hour 0 to 26.7% at Hour 8.

3.3. *Influence of Diet on Methemoglobin Development*

Average resting methemoglobin levels (Hour 0) for trout fed the 3.6% NaCl diet and the 7.3% NaCl diet were lower than for trout fed the basal diet prior to exposure to nitrite (Figure 3.4). Although normal resting levels of methemoglobin reported for salmonids by Eddy and Williams (1987) average less than 5%, high variability in sensitivity to nitrite among species and among individual fish within a species has been demonstrated (Williams and Eddy, 1988; Tomasso, 1986; Scott and Harrington, 1985; Freeman et al., 1983; Bath and Eddy, 1980). Nitrite concentrations tested enzymatically and read spectrophotometrically at Hour 0 from each of the experimental diet tanks prior to transfer of fish to the nitrite challenge tank did not indicate the presence of nitrite in the experimental diet tanks. Therefore, we attribute these differences in average methemoglobin blood levels at Hour 0 to the large individual variability among the fish with respect to resting levels of blood methemoglobin (Appendix J).

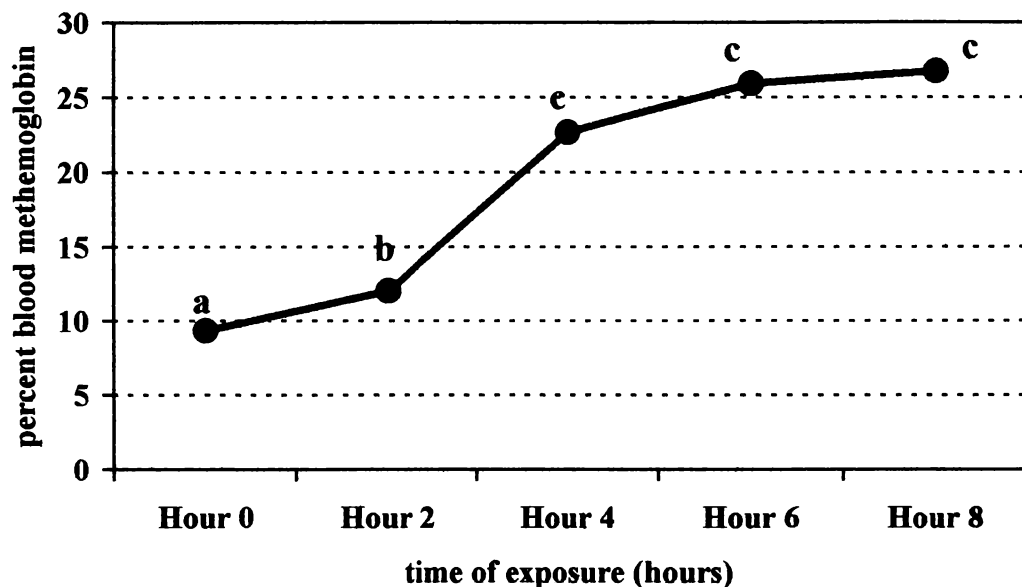


Fig. 3.3: Pattern of Development of Methemoglobinemia in Trout Exposed to 18 mg/L N-NO₂

Average across all treatments of percent blood methemoglobin levels by hour in rainbow trout exposed to 18 mg/L NO₂⁻N for an eight-hour period. Differences are significant at $P < 0.05$.

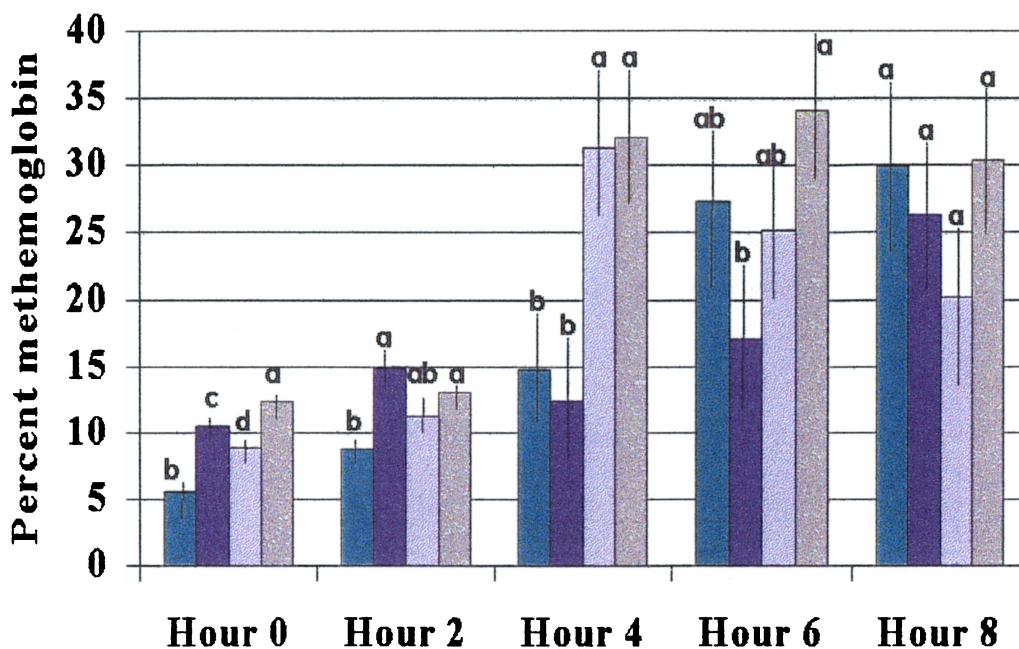


Fig. 3.4: Methemoglobin Development by Diet and Hour Of Exposure

Average percent blood methemoglobin levels in trout fed salt-enriched diets and exposed to 18 mg/L NO_2^- -N for an eight-hour period. Green bars indicate average percent methemoglobin in trout fed the 3.6% NaCl diet. Dark blue bars indicate average percent methemoglobin in trout fed the 3.6% CaCl_2 diet. Light blue bars indicate average percent methemoglobin in trout fed the 7.3% NaCl diet. Gray bars indicate average percent methemoglobin in trout fed the basal diet. Differences are significant at $P \leq 0.05$.

By Hour 4 of exposure, the onset of methemoglobin development in trout fed the 7.3% NaCl diet and the basal diet was evident. Trout fed the 7.3% NaCl diet had an average percent methemoglobin value of $31.3\% \pm 5.1$ (Figure 3.4). Trout fed the basal diet had an average percent methemoglobin value of $32.1\% \pm 4.6$. These values were significantly higher than those for trout receiving either the 3.6% NaCl diet or the 3.6% CaCl₂ diet.

Following longer exposure (Hour 6 and 8), there were no differences in average percent methemoglobin among fish from all four diets. Average blood methemoglobin levels remained elevated in all trout through eight hours of nitrite exposure.

Rainbow trout exposed to 18 mg/L nitrite showed extreme levels of individual variability in methemoglobin development. For instance, At Hour 4, percent methemoglobin levels in individual trout sampled from the basal diet group ranged from 14 to 56.7%. This range in individual results strongly affected standard errors for treatment groups, making it difficult to determine if differences in percent methemoglobin levels due to diet treatments did occur.

3.4. *Chloride cell development*

Trout were fed salt-enriched diets from Day 0 through Day 73 of the 133-day study. Prior to salt feeding, gill chloride cells averaged 73 cells per fish. Average chloride cells numbers for trout fed salt-enriched diets did increase significantly from Day 0 to Day 41 (Figure 3.5) however these increases were not significantly different from average gill chloride cell numbers in trout fed the basal diet (Figure 3.6) except

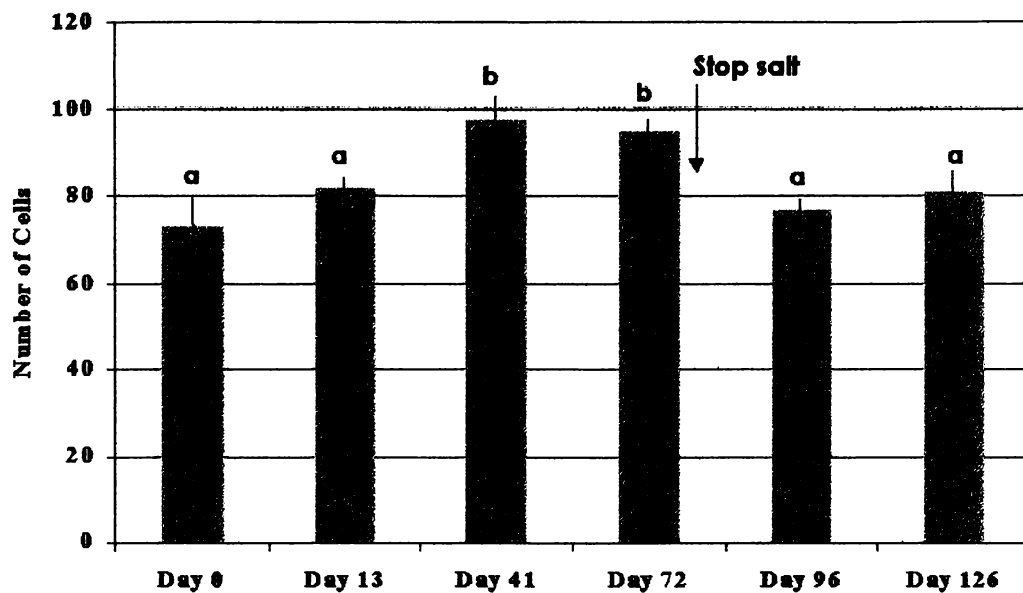


Fig. 3.5: Average Chloride Cell Numbers in Salt-Fed

Average gill chloride cell numbers from rainbow trout fed three salt-enriched diets from Day 0 to Day 72 of a 133-day study. Differences are significant at $P \leq 0.05$.

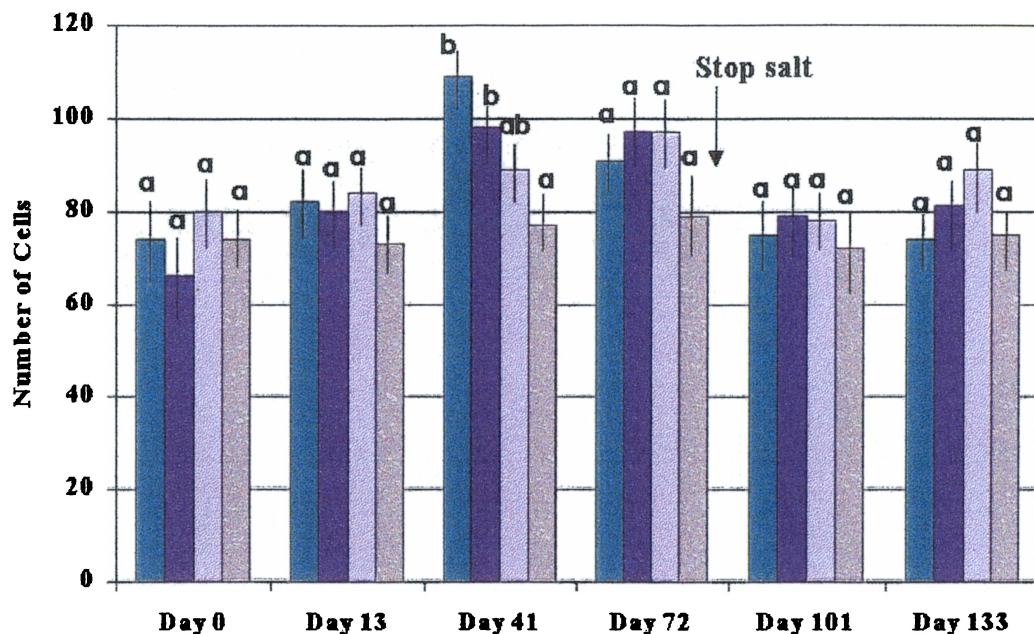


Figure 3.6: Average Number of Chloride Cells by Diet and Day of Sampling

Average number of gill chloride cells as sampled on Days 0, 13, 41, 72 101 and 133. Green bars indicate the average number of chloride cells in trout fed the 3.6% NaCl diet. Dark blue bars indicate the average number of chloride cells in trout fed the 3.6% CaCl₂ diet. Light blue bars indicate the average number of chloride cells in trout fed the 7.3% NaCl diet and gray bars indicate average chloride cell number in trout fed the basal diet. Gill chloride cells were counted from five fish from each of the four diets on each sampling day. Differences are significant at $P \leq 0.05$.

for one exception on Day 41 in trout fed the 3.6% NaCl diet. The average number of chloride cells remained significantly higher in trout receiving salt-enriched diets through Day 72 before dropping off to pre-salt diet levels following the cessation of salt-feeding on Day 73 (Figure 3.5). These changes were not different from average numbers of gill chloride cells in trout receiving the basal diet however. Feeding of salt-enriched diets was discontinued on Day 73 and all trout were returned to the basal diet of 0.7% NaCl. By Day 101, average gill chloride cell numbers had returned to pre-salt feeding levels, with an average of 77 cells per fish (Figure 3.5).

Mean separation of the changes in chloride cell numbers by individual diet showed that a significant increase in gill chloride cell numbers occurred after 41 days of salt-feeding (Figure 3.6) with the 3.6% NaCl diet. This effect was lost however as by Day 72 as there were no differences in gill chloride cell numbers for any trout fed salt-enriched diets as compared to the basal diet. Feeding of salt-enriched diets was stopped on Day 73 and all trout were returned to the basal diet. By Day 101, gill chloride cell numbers had returned to pre-salt diet levels but there were no differences among diets in gill chloride cell numbers (Figure 3.6).

3.5. *Plasma chloride concentrations*

Plasma chloride concentrations in trout fed salt-enriched diets or a basal diet and exposed to an eight-hour nitrite challenge were not affected by diet. Average plasma chloride concentrations for all trout ranged from 135.6 ± 1.7 mEq/L at Hour 0 to 127.1 ± 1.9 mEq/L at Hour 8 (Figure 3.7, Appendix K).

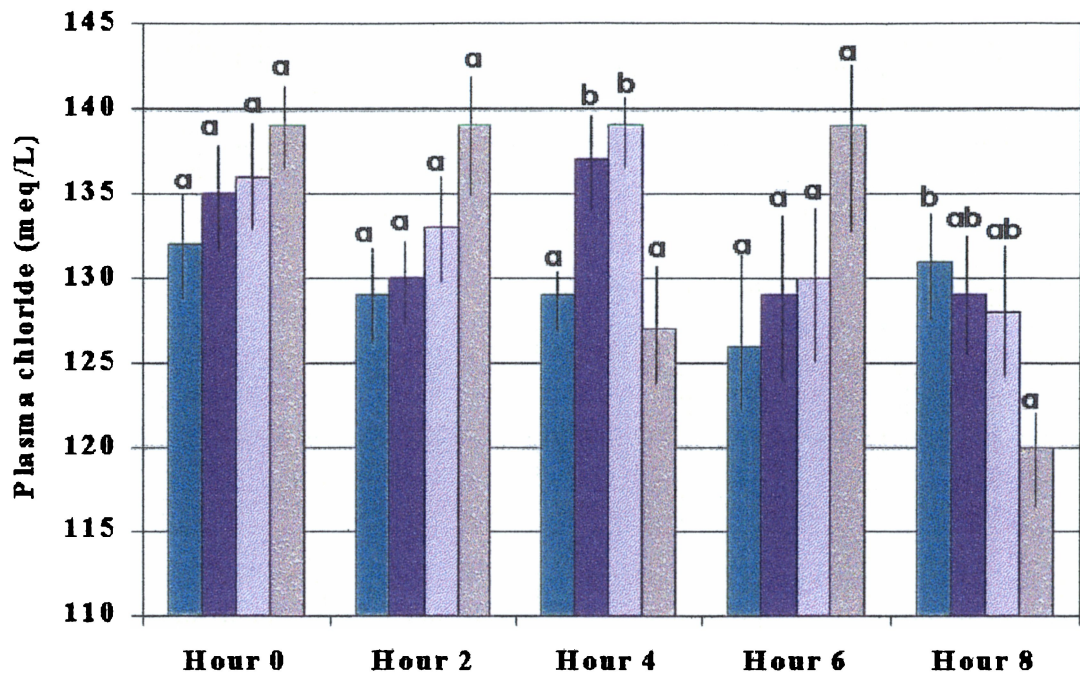


Figure 3.7: Plasma Chloride Levels in Rainbow Trout Exposed to an 8-Hour Nitrite Challenge

Mean plasma chloride concentrations (mEq/L) in trout fed salt-enriched diets or a basal diet and exposed to an 8-hour nitrite challenge at 18 mg/L nitrite nitrogen. Green bars indicate plasma chloride levels in trout fed a 3.6% NaCl diet. Dark blue bars indicate plasma chloride levels in trout fed a 3.6% CaCl₂ diet. Light blue bars indicate plasma chloride levels in trout fed a 7.3% NaCl diet. Gray bars indicate plasma chloride levels for trout fed a basal diet. Differences are significant at $P \leq 0.05$.

At Hour 4 of the nitrite challenge, plasma chloride concentrations were significantly higher in trout fed the 3.6% CaCl₂ diet and the 7.3% NaCl diet than in trout fed the basal diet (Figure 3.7). These effects were transient however, as there were no differences in plasma chloride concentrations among any of the diets by hour 6. At Hour 8, trout fed the 3.6% NaCl diet had significantly higher plasma chloride concentrations than trout fed the basal diet.

4. Discussion

Previously reported baseline values of percent methemoglobin for rainbow trout averaged less than 5% (Williams and Eddy, 1988). Baseline levels of percent methemoglobin in our experiment were higher (10.4 ± 0.6 in trout fed the 3.6% CaCl₂ diet and 12.4 ± 0.6 in trout fed the basal diet) in all but trout receiving the 3.6% NaCl diet. This effect could not be attributed to salt-enriched diets as the percent methemoglobin level was elevated in trout fed the basal diet as well. Lewis and Morris (1986) reported that percent methemoglobin values greater than 50% could be considered threatening to fish although salmonids are known to tolerate percent methemoglobin levels up to 70% for short periods. Based on these reported values, our Hour 0 values for percent methemoglobin, while higher than expected, are not indicative of the presence of methemoglobinemia.

A significant increase in percent blood methemoglobin was detected by Hour 4 of exposure to nitrite in rainbow trout fed the 7.3% NaCl diet and in trout fed the basal diet, with increases from 11.2 and 13% to 31.3 and 32.1% respectively. Diets

containing 3.6% chloride as either NaCl or CaCl₂ appeared to offer some protection from methemoglobin development in trout through Hour 4 of nitrite exposure. Of these two diets, the CaCl₂ enriched diet had the lowest level of percent methemoglobin ($12.4 \pm 4.8\%$).

Differences among diets with respect to percent methemoglobin levels were lost by Hour 6 of exposure to nitrite. Methemoglobin levels remained higher than baseline values in all fish throughout the remainder of the exposure period. Large standard errors associated with measures of percent methemoglobin from Hour 4 through Hour 8 demonstrate conclusively the need for large sample sizes in studies of methemoglobinemia in order to offset the individual variability in sensitivity. Many of our results suggest trends in methemoglobin development which might have provided meaningfully significant results had we used larger sample sizes during the nitrite challenges.

Previous research with freshwater-adapted juvenile trout and salmon fed salt-enriched diets has shown that salmonids have significantly reduced mortality and improved osmoregulatory ability when transferred to seawater (Zaug and McLain, 1969; Basulto, 1976; Salmon and Eddy, 1990; Pelletier and Besner, 1992). These observations suggest that salt-enriched diets assisted in the conversion of the function of freshwater chloride cells to that of salt water-adapted chloride cells which expel excess ions, including Cl⁻ ions, from the fish. Salt-feeding has been reported to increase the number of gill chloride cells in rainbow trout (Salman and Eddy, 1987; Gong, 1999) and to increase gill Na⁺-K⁺-ATPase, indicating greater chloride cell

activity (Zaug et al., 1983; Salman and Eddy, 1987). Our results showed that trout fed salt-enriched diets overall had a significant increase in average numbers of gill chloride cells during salt-feeding, but the increase in gill chloride cell numbers was not different from those in trout receiving the basal diet. Therefore we conclude that salt-enriched feeds had little effect on the average number of gill chloride cells (Figure 3.6). Our results for average gill chloride cell numbers show no evidence that the highest chloride level fed, 7.3% NaCl, caused significant increases in gill chloride cell numbers above those seen in trout fed the basal diet. Our results contrast with those of Gong (1999) who showed significant increases in gill chloride cell numbers in trout fed a medium level of NaCl rather than a high (12%) level of NaCl. No significant differences in numbers of gill chloride cells among any of the three specific diets could be detected due to the lack of adequate sample sizes. Likewise we found no evidence that salt-enriched diets affected gill chloride cell function as measured by changes in blood methemoglobin levels. If salt-enriched diets caused gill chloride cells to expel excess chloride ions, some protection against methemoglobin development might be expected during nitrite exposure, especially in trout fed the high 7.3% NaCl diet. Protection from methemoglobin development was observed at Hour 4 of nitrite exposure, but this occurred in trout receiving the medium level of NaCl enrichment, 3.6%, and in trout receiving the 3.6% CaCl_2 diet.

These results suggest that diets having moderate levels of chloride enrichment may be all that is necessary to provide some protective effects from the development of methemoglobin in the blood of rainbow trout.

Plasma chloride concentrations have been shown to decrease in salmonids exposed to elevated nitrite concentrations. Losses of plasma chloride concentrations occur within the first two hours of exposure to nitrite with compensation occurring by Hour 4 of nitrite exposure (Eddy et al., 1983; Jensen et al., 1987; Williams and Eddy, 1988). Significant declines in plasma chloride levels were not observed in trout fed salt-enriched diets until Hour 4 of our study, when trout fed the basal diet and the 3.6% NaCl diet had significantly reduced plasma chloride concentrations compared to trout fed the 3.6% CaCl diet and the 7.3% NaCl diet. Although these differences in plasma chloride concentrations among diets were transient, the observation suggests that trout fed salt-enriched diets, especially those with higher levels of NaCl, may have been somewhat protected from stress-induced hypochloremia during acute stress events.

By Hour 6 of nitrite exposure, trout fed the basal diet had compensated for the loss of plasma chloride concentrations as plasma chloride values had returned to pre-stress levels.

5. Conclusions

Salt-enriched diets containing moderate levels of chloride, particularly as NaCl, may offer some protection to rainbow trout exposed to elevated nitrite in the early stages of methemoglobin development (up to four hours). This effect is lost with prolonged exposure to nitrite however.

Salt-enriched diets, specifically the 3.6% CaCl_2 diet and the 7.3% NaCl diet, did show protective effects against the loss of plasma chloride concentrations at the time when methemoglobinemia was developing rapidly in nitrite-challenged trout. It is possible that salt-enriched diets provided an internal pool of electrolytes which are being sequestered somewhere other than in the plasma and can assist with boosting plasma electrolyte levels in trout under stress. The protective effects of the 7.3% NaCl diet on plasma chloride levels were transient however.

Based on these results, we conclude that salt-enriched diets do not offer long-term protection against the development and severity of methemoglobinemia in fish exposed to elevated nitrite concentrations. Diets enriched with moderate levels of NaCl may help to reduce methemoglobin development but further research using higher sample sizes is needed to offset individual variability in measures of percent methemoglobin to determine if such benefits truly exist. Some benefits in using salt-enriched diets to reduce the stress-induced loss of plasma chloride concentrations in nitrite-exposed fish appear to exist as well but the mechanism of this effect is yet to be understood.

REFERENCES

REFERENCES

- Adams, S.M., 1990. Biological indicators of stress in fish. Am. Fish. Soc. Symp. 8: 1-181.
- Adedire, C.O., Oduleye, S.P., 1984. Stress-induced water permeability changes in the tropical cichlid, *Oreochromis niloticus* (Trewavas). J. Fish. Biol. 25, 463-471.
- Andersen, D.E., Reid, S. E., Moon, T.W., Perry, S.F., 1991. Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Onchorynchus mykiss*). Can. J. Fish. Aquat. Sci. 48, 1811-1817.
- Arillo, A., Gaino, E., Margiocco, C., Mensi, P., Schenone, G., 1984. Biochemical and ultrastructural effects of nitrite in rainbow trout: liver hypoxia as the root of the acute toxicity mechanism. Env. Res. 34, 135-154.
- Barton, B.A., Peter, R.E., Paulencu, C.R., 1980. Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling, confinement, transport and stocking. Can. J. Fish. Aquat. Sci. 37, 805-811.
- Barton, B.A., Schreck, C.B., 1987. Metabolic cost of acute physical stress in juvenile steelhead. Trans. Am. Fish. Soc. 116, 257-263.
- Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the responses and effects of corticosteroids. Annu. Rev. Fish. Dis. 1, 3-26.
- Barton, B.A., 1997. Stress in finfish: past, present and future – a historical perspective. In: Iwama, G.K., Peicerking, A.D., Sumpter, J.P., and Schreck, C.B. (eds) Fish Stress and Health in Aquaculture, Society for Experimental Biology, Seminar Series 62, Cambridge University Press, Cambridge, New York, Melbourne. Pp. 1-34.
- Basulto, S. 1976. Induced saltwater tolerance in connection with inorganic salts in the feeding of Atlantic salmon (*Salmo salar* L.). Aquaculture. 8, 45-55.
- Bath, R.N. 1980. Some physiological effects of seawater and nitrite on the rainbow trout (*Salmo gairdneri*, Richardson). P.h.D. Thesis. University of Dundee. Dundee, Scotland. 206 Pp.

- Bath, R.N., Eddy, F.B., 1979. Salt and water balance in rainbow trout (*Salmo gairdneri*) rapidly transferred from fresh to sea water. J. Exp. Biol. 83, 193-202.
- Bath, R.N., Eddy, F.B., 1980. Transport of nitrite across fish gills. J. Exp. Zool. 214, 119-121.
- Black, E.C. 1956. Appearance of lactic acid in the blood of Kamloops and lake trout following transportation. Can. Fish Culturist. 18, 3-10.
- Black, E.C. 1957. Alterations in the blood level of lactic acid in certain salmonid fishes following muscular activity. I. Kamloops trout, *Salmo gairdneri*. J. Fish. Res. Bd. Can. 14, 807-814.
- Boeuf, G., Lassere, P., Harache, Y., 1978. Osmotic adaptation of *Onchorhynchus kisutch*. Walbaum. II. Plasma osmotic and ionic variations and gill $\text{Na}^+ - \text{K}^+$ ATPase activity of yearling coho salmon transferred to seawater. Aquaculture. 15, 35-52.
- Bonga, S.E.W., 1997. The stress response in fish. Physiol. Rev. 77, 591-635.
- Bowser, P.R., Falls, W.W., VanZandt, J., Collier, N. Phillips, J.D., 1983. Methemoglobinemia in channel catfish: methods of prevention. Prog. Fish. Cult. 45, 154-158.
- Brown, S.W. 1981. Water quality and off-flavor problems in commercial catfish culture in West Central Alabama. Master's thesis. Auburn University, Auburn, AL.
- Brown, D.A., McLeay, D.J., 1975. Effect of nitrite on methemoglobin and total hemoglobin of juvenile rainbow trout. Prog. Fish Cult. 37, 36-38.
- Brown, S.W., Boyd, C.E. 1981. Water quality in commercial catfish ponds (abstract). Procedures of the Catfish Farmers of America Res. Workshop. 3, 10-11.
- Collins, M.T., Garatzek, J.B., Shotts, E.B., Dawe, D.L., Campbell, L.M., Senn, D.E. 1975. Nitrification in an aquatic recirculating system. J. Fish. Res. Bd. Can. 32, 2025-2031.
- Crawford, R.E., Allen, G.H. 1977. Seawater inhibition of nitrite toxicity to Chinook salmon. Transactions. 106, 105-109.
- Duston, J., 1993. Effects of dietary betaine and sodium chloride on seawater adaptation in Atlantic salmon parr (*Salmo salar* L.). Comp. Biochem. Physiol. 105A, 673-677.

- Eddy, F.B., Kunzlick, P.A., Bath, R.N. 1983. Uptake and loss of nitrite from the blood of rainbow trout (*Salmo gairdneri*, Richardson) and Atlantic salmon (*Salmo salar* L.) in fresh water and in dilute seawater. J. Fish. Biol. 23, 105-116.
- Eddy, F.B., Williams, E.M., 1987. Nitrite and freshwater fish. Chem. Ecol. 3(1), 1-38.
- Evelyn, K.A., Malloy, H.T., 1938. Microdetermination of osyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 126, 655-662.
- Folmar, L.C., Dickhoff, W.W., 1980. The parr-smolt transformation (smoltification) and sea water adaptation in salmonids. A review of selected literature. Aquaculture. 21, 1-38.
- Freeman, L., Beiting, T.L., Huey, D.W. 1983. Methemoglobin reductase activity in phylogenetically diverse piscine species. Comp. Biochem. Physiol. 75B, 27-30.
- Gaino, E., Arillo, A., Mensi, P., 1984. Involvement of the gill chloride cells of trout under acute nitrite intoxication. Comp. Biochem. Physiol. 77A, 611-617.
- Giles, M.A., Vansone, W.E., 1976. Changes in ouabain-sensitive adenosine triphosphate activity in gills of coho salmon (*Onchorynchus kisutch*) during parr-smolt transformation. J. Fish. Res. Board Can. 31, 54-62.
- Gong, Jingjuan., 1999. Effects of dietary sodium chloride on chloride cell development and growth of rainbow trout. Master's thesis. University of Tennessee. Knoxville, Tennessee. 31 Pp.
- Gonzalez, R.J., McDonald, D.G., 1992. The relationship between oxygen consumption and ion loss in a freshwater fish. J. Exp. Biol. 163, 317-332.
- Grizzle, J.M., Cummins, K.A., Ashfield, C.J., 1993. Effects on environmental concentrations of calcium and sodium on the calcium flux in stressed 34-day-old striped bass. Can. J. Zool. 71, 1379-1384.
- Harms, C.A., Hinshaw, J.M., 1993. Effect of dietary sodium chloride on plasma electrolytes, acid-base status, and chloride cells in rainbow trout exposed to acute pH stress. Unpublished data. North Carolina State University, Raleigh, N.C.
- Haskell, D.C., 1941. An investigation on the use of oxygen in transporting trout. Transactions. 70, 149-160.

- Hattingh, J., Van Pletzen, A.J.J., 1974. The influence of capture and transportation on some blood parameters in freshwater fish. *Comp. Biochem. Physiol.* 49A, 607-609.
- Holeton, G.F., 1971. Oxygen uptake and transport by the rainbow trout during exposure to carbon monoxide. *J. Exp. Biol.* 54, 239-254.
- Huey, D.W., Beiting, T.L., 1982. A hemoglobin reductase system in channel catfish, *Ictalurus punctatus*. *Can. J. Zool.* 60, 1511-1513.
- Jewell, W.J., Cummings, R.J., 1990. Expanded bed treatment of complete recycle aquaculture systems. *Water Sci. Technol.* 22, 443-450.
- Jensen, F.B., Andersen, N.A., Heisler, N., 1987. Effects of nitrite exposure on blood respiratory properties, acid-base and electrolyte regulation in the carp (*Cyprinus carpio*). *J. Comp. Physiol. B.* 157, 533-541.
- Jensen, F.B. 1990. Nitrite and red cell function in carp: control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. *J. Exp. Biol.* 152, 149-166.
- Johnston, C.E., Cheverie, J.C., 1985. Comparative analysis of ionoregulation in rainbow trout (*salmo gairdneri*) of different sizes following rapid and slow salinity adaptation. *Can. J. Fish. Aquat. Sci.* 42, 1994-2003.
- Knudsen, P.K., Jensen, F.B. 1997. Recovery from nitrite-induced methaemoglobinemia and potassium balance disturbances in carp. *Fish Physiol. Biochem.* 16, 1-10.
- Krous, S.R., Blazer, V.S., Meade, T.L., 1982. Effects of acclimation time on nitrite movement across the gill epithelia of rainbow trout: the role of chloride cells. *Prog. Fish Cult.* 44, 126-130.
- Langdon, J.S., Thorpe, J.E., 1984. Response of the gill $\text{Na}^+ - \text{K}^+$ ATPase activity, succinic dehydrogenase activity and chloride cells to saltwater adaptation in Atlantic salmon, *Salmo salar* L., parr and smolt.
- Leach, G.J., Taylor, M.H., 1982. The effects of cortisol treatment and protein metabolism in *Fundulus heteroclitus*. *Gen. Comp. Endocrin.* 48, 76-83.
- Lewis, W.M., Morris, D.P., 1986. Toxicity of nitrite to fish: a review. *Transactions.* 115, 183-195.

Liao, P.B., Mayo, R.D., 1974. Intensified fish culture combining water reconditioning with pollution abatement. *Aquaculture*. 3, 61-85.

Maetz, J. 1971. Fish gills: mechanism of salt transfer in fresh water and sea water. *Philosophical Trans. Royal Society of London Series B Biological Sci.*

Mazeaud, M.M., Mazeaud, F., 1981. Adrenergic responses to stress in fish. In: *Stress and Fish*. Pickering, A.D. (ed.) Pp. 49-75.

Mazeaud, M.M., Mazeaud, F., Donaldson, E.M., 1977. Primary and secondary effects of stress in fish: some new data with a general review. *Trans. Am. Fish. Soc.* 196, 201-212.

Mazik, P.M., Simco, B.A., Parker, N.C., 1991. Influence of water hardness and salts on survival and physiological characteristics of striped bass during and after transport. *Trans. Am. Fish. Soc.* 120, 121-126.

Mazik, P.M., Simco, B.A., 1994. Effects of size, water hardness, salt levels and MS-222 on the survival and stress response in striped bass (*Morone saxatilis*). In: *High Performance Fish*. MacKinlay, D.D. (ed.) Pp. 425-430. *Am. Fish. Soc. Vancouver*.

McDonald, D.G., 1983. The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri* I. Branchial and renal net ion and H^+ fluxes. *J. Exp. Biol.* 102, 123-140.

McDonald, D.G., Robinson, J.G., 1993. Handling and confinement stress in the lake trout (*Salvelinus namaycush*) – effects of water hardness and genotype. *Trans. Am. Fish. Soc.* 122, 1146-1155.

McDonald, D.G., Milligan, C.L. 1992. Chemical properties of the blood. In: *Fish Physiology, Volume XIIB*, Hoar, W.S., Randall, D.J., Farrell, A.P. (eds.) Pp. 55-133. Academic Press, New York.

McDonald, D.G., Milligan, C.L. 1997. Ionic, osmotic and acid-base regulation in stress. In: *Iwama, G.K., Pickering, A.D., Sumpter, J.P., and Schreck, C.B. (eds.) Fish Stress and Health in Aquaculture, Society for Experimental Biology, Seminar Series 62*, Cambridge University Press, Cambridge, New York, Melbourne. Pp. 119-144.

McDonald, D.G., Milligan, C.L., 1992. Chemical properties of the blood. In: *Fish Physiology, Volume XIIB*. Hoar, W.S., Randall, D.J., Farrell, A.P. (eds.) Pp. 55-133. Academic Press, New York.

McDonald, D.G., Rogano, M.S., 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. *Physiol. Zool.* 59, 318-331.

- McDonald, D.G., Walker, R.L., Wilkes, P.R.H., 1983. The interaction of calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*, II. Branchial ionoregulatory mechanisms. *J. Exp. Biol.* 102, 141-155.
- Meade, T.L., Perrone, S.J. 1980. Effect of chloride ion concentration and pH on the transport of nitrite across the gill epithelia of Coho salmon. *Prog. Fish Cult.* 42(2), 71-72.
- Moyle, P.B., Cech, J.J. Jr. 2000. Hydromineral balance. In: *Fishes, an introduction to ichthyology*. Fourth ed. Prentice Hall, NJ. Pp. 79-96.
- Nikinmaa, M., Soivio, A., Nakari, T., Lindgren, S., 1983. Hauling stress in brown trout *Salmo trutta*. *J. Exp. Biol.* 63, 343-356.
- Payan, P., Girard, J.P., Mayer-Gostan, N., 1984. Branchial ion movements in teleosts: The roles of respiratory and chloride cells. In: Hoar, W.S., Randall, D.J. (eds) *Fish Physiology*, Vol XB, Academic Press, London, New York. Pp. 39-63.
- Pelletier, D., Besner, M. 1992. The effect of salty diets and gradual transfer to seawater on osmotic adaptation, gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ activation, and survival of brook charr, *Salvelinus fontinalis*, Mitchill. *J. Fish Biol.* 41, 791-803.
- Perrone, S.J., Meade, T.L., 1977. Protective effect of chloride on nitrite toxicity to Coho salmon (*Onchorynchus kisutch*). *J. Fish. Res. Bd. Can.* 34, 486-492.
- Philpott, C.W., 1980. Tubular system membranes of teleost chloride cells: osmotic response and transport sites. *Am. J. Physiol.* 238, 171-184.
- Pickering, A.D., 1981. *Stress and Fish*. (ed.) London: Academic Press.
- Randall, D.J., Perry, S.F., 1992. Catecholamines. In: *Fish Physiology*, Volume XIIB. Hoar, W.S., Randall, D.J., Farrell, A.P. (eds.) Pp. 255-300. Academic Press, New York.
- Pickering, A.D., Pottinger, T.G., 1985. Factors influencing blood cortisol levels of brown trout under intensive culture conditions. In: *Current Trends in Endocrinology*, Lofts, B., Holms, W.N. (eds.) Hong Kong: Hong Kong University. Pp. 1239-1242.
- Pickering, A.D., Pottinger, T.G., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish. Physiol. Biochem.* 7, 253-258.

- Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G., Leonard, J.R. (eds.) 1992. Nutrition and Feeding. In: Fish Hatchery Management. U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C.
- Postlethwaite, E.K., McDonald, D.G. 1995. Mechanisms of Na⁺ and Cl⁻ regulation in freshwater-adapted rainbow trout (*Onchorynchus mykiss*) during exercise and stress. J. Exp. Biol. 198, 295-304.
- Randall, D.J., Perry, S.F., 1992. Catecholamines. In: Fish Physiology. Hoar, W.S., Randall, D.J., Farrell, A.P. (eds.) San Diego, CA: Academic Press, Vol. XIIB. Pp. 255-300.
- Redding, J.M., Schreck, C.B., 1983. Influence of ambient salinity on osmoregulation and cortisol concentration in yearling coho salmon during stress. Trans. Am. Fish. Soc. 112, 800-807.
- Reubush, K.J., Heath, A.G., 1997. Effects of recovery water salinity on secondary stress responses of hybrid striped bass fingerlings. Prog. Fish. Cult. 59, 188-197.
- Ruane, N.M., Wendelaar Bonga, S.E., Balm, P.H., 1999. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. Gen. Comp. Endoc. 115, 210-219.
- Russo, R.C., Thurston, R.V., Emerson, K., 1981. Acute toxicity of nitrite to rainbow trout (*Salmo gairdneri*): Effects of pH, nitrite species and anion species. Can. J. Fish. Aquat. Sci. 38, 387-393.
- Russo, R.C., Thurston, R.V. 1991. Toxicity of ammonia, nitrite, and nitrate to fishes. In: Bruno, D.E., Tomasso, J.R. (eds). Aquaculture and Water Quality. World Aquaculture Society, Baton Rouge, LA. Pp. 58-59.
- Salman, N.A., Eddy, F.B. 1987. Response of chloride cell numbers and gill Na⁺-K⁺-ATPase activity of fresh water rainbow trout (*Salmo gairdneri*) to salt feeding. Aquaculture. 61, 41-48.
- Salman, N.A., Eddy, F.B., 1988. Effect of dietary sodium chloride on growth, food intake and conversion efficiency in rainbow trout (*Salmo gairdneri* Richardson) to salt feeding. Aquaculture. 70, 131-144.
- Salman, N.A., Eddy, F.B. 1990. Increased sea water adaptability of non-smolting rainbow trout by salt feeding. Aquaculture. 86, 259-270.
- Sardet, C., 1980. Freeze fracture of the gill epithelium of euryhaline teleost fish. Am. J. Physiol. 238, 207-212.

Sargent, J.R., Thomson, A.J., 1974. The nature and properties of the inducible sodium plus potassium ion dependent adenosine triphosphatase in gills of eels (*Anguilla anquilla*) adapted to fresh water and seawater. J. Biochem. 144, 69-75.

SAS Instiute Inc. 1999. *SAS/STAT Software Version 8*, SAS Institute, Inc., Cary, N.C.

Saunders, R.L., Henderson, E.B., 1978. Changes in gill ATPase activity and smolt status of Atlantic salmon (*Salmo salar*). J. Fish. Res. Bd Can. 35, 1542-1546.

Scarano, G., Saroglia, M.G. 1984. Hematological responses of sea bass *Dicentrarchus labrax* to sublethal nitrite exposures. Trans. Am. Fish. Soc. 113, 360-364.

Schotzke, N. 1992. Auswirkungen von nitrif auf die struktur der fischkieme. Diploma thesis. Univeristy of Innsbruck. Innsbruck, Austria.

Schwedler, T.E., Tucker, C.S. 1983. Empirical relationship between percent methemoglobin in channel catfish and dissolved nitrite and chloride in ponds. Transactions. 112, 117-119.

Scott, E.M., Harrington, J.P. 1985. Methemoglobin reductase activity in fish erythrocytes. J. Comp. Biochem. Physiol. 82B, 511-513.

Selye, H. 1950. Stress and the general adaptation syndrome. British Medical Journal. 1(4667), 1383-1392.

Smith, A.E. 2001. The effect of dietary salts on acid stress in rainbow trout (*Onchorynchus mykiss*). Master's thesis. University of Tennessee. Knoxville, Tennessee. 32 Pp.

Specker, J.L., Schreck, C.B., 1980. Stress responses to transportation and fitness for marine survival in coho salmon (*Onchorynchus kisutch*) smolts. Can. J. Fish. Aquat. Sci. 37, 765-769.

Stormer, J., Jensen, F.B., Rankin, J.C. 1996. Uptake of nitrite, nitrate, and bromide in rainbow trout, *Onchorynchus mykiss*: effects on ionic balance. Can. J. Fish. Aquat. Sci. 53, 1943-1950.

Strange, R.J., Schreck, C.B., Golden, J.T., 1977. Corticoid stress responses to handling and temperature in salmonids. Trans. Am. Fish. Soc. 106, 213-217.

- Strange, R.J., Schreck, C.B., 1978. Anesthetic and handling stress on survival and cortisol concentration in yearling Chinook salmon (*Onchorynchus tshawytscha*). J. Fish. Res. Bd. Can. 35, 345-349.
- Strange, R.J., Schreck, C.B., Ewing, R.D. 1978. Cortisol concentrations in confined juvenile Chinook salmon (*Onchorynchus tshawytscha*). Trans. Am. Fish. Soc. 107, 812-819.
- Strange, R.J. 1980. Acclimation temperature influences cortisol and glucose concentrations in stressed channel catfish. Trans. Am. Fish. Soc. 109, 298-303.
- Tomasso, J.R., Davis, K.B., Parker, N.C., 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass x striped bass) during netting and hauling. Proc. World. Mariculture. Soc. 11, 303-310.
- Tomasso, J.R., 1994. Toxicity of nitrogenous wastes to aquaculture animals. Rev. Fish. Sci. 2(4), 291-314.
- Tomasso, J.R., Simco, B.A., and Davis, K.B. 1979. Chloride inhibition of nitrite-induced methemoglobinemia in channel catfish (*Ictalurus punctatus*). J. Fish. Res. Bd. Can. 36, 1141-1144.
- Tomasso, J.R., Goudie, C.A., Simco, B.A., Davies, K.B. 1980. Effects of environmental pH and calcium on ammonia toxicity in channel catfish. Trans. Am. Fish. Soc. 109, 229-234.
- Tomasso, J.R., 1986. Comparative toxicity of nitrite to freshwater fishes. Aquat. Tox. 8, 129-137
- Trombetti, F. V., Ventrella, A., Pagliarani, R., Ballestrazzi, M., Galeotti, M., Trigari, G., Pirini, M., Borgatti, P.A. 1996. Response of rainbow trout gill, $\text{Na}^+ - \text{K}^+$ -ATPase, and chloride cells to T3 and NaCl administration. Fish Physiol. Biochem. 15(3), 265-274.
- Vermette, M.G. and Perry, S.F., 1987. The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*, III. Renal ionic fluxes. J. Exp. Biol. 128, 269-285.
- Wedemeyer, G.A., 1976. Physiological response of juvenile coho salmon (*Onchorynchus kisutch*) and rainbow trout (*Salmo gairdneri*) to handling and crowding stress in intensive fish culture. J. Fish. Res. Bd. Can. 33, 2699-2702.
- Wedemeyer, G.A., Yasutake, W.T., 1978. Prevention and treatment of nitrite toxicity in juvenile steelhead trout (*Salmo gairdneri*). J. Fish. Res. Bd. Can. 35, 822-827.

Wedemeyer, G.A., Barton, B.A., McLeay, D.J., 1990. Stress and acclimation. In: Methods for Fish Biology. Schreck, C.B., Moyle, P.B. (eds.) Bethesda, MD. Am. Fish. Soc. Pp. 451-489.

Wedemeyer, G.A., 1997. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama, G.K., Peicerking, A.D., Sumpter, J.P., and Schreck, C.B. (eds) Fish Stress and Health in Aquaculture, Society for Experimental Biology, Seminar Series 62, Cambridge University Press, Cambridge, New York, Melbourne. Pp. 35-71.

Wendelaar Bonga, S.E. 1997. The Stress Response in Fish. *Physiol. Rev.* 77, 591-625.

Williams, E.M., Eddy, F.B. 1986. Chloride uptake in freshwater teleosts and its relationship to nitrite uptake and toxicity. *J. Comp. Phys. B*, 156, 867-872.

Williams, E.M., Eddy, F.B., 1988. Anion transport, chloride cell number and nitrite-induced methemoglobinemia in rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) *Aquat. Tox.* 13, 29-42.

Wood, C.M., Randall, D.J., 1973. The influence of swimming activity on water balance in the rainbow trout (*Salmo gairdneri*). *J. Comp. Physiol.* 82, 257-276.

Woodward, C.C., Strange, R.J., 1987. Physiological stress responses in wild and hatchery-reared rainbow trout. *Trans. Am. Fish. Soc.* 116, 574-579.

Zaugg, W.S., McLain, L.R. 1969. Inorganic salt effects on growth, salt water adaption and gill ATPase of Pacific salmon. In: Neuhaus, O.W., Halver, J.E. (eds.). *Fish in Research*. Academic Press, New York, NY. Pp. 293-306.

Zaugg, W.S., Roley, D.D., Prentice, E.F., Gores, K.X., Waknitz, F.W. 1983. Increased seawater survival and contribution to the fishery of Chinook salmon (*Oncorhynchus tshawytscha*) by supplemental dietary salt. *Aquaculture*. 32, 183-188.

APPENDICES

Appendix A

Water Quality Report for JARTU

Aquaculture Lab, Spring 2000

Appendix A:

General Water Quality Parameters

Johnson Animal Research and Teaching Unit

Aquaculture Lab

Contaminant	Avg. Level Detected
Temperature	15.5°C
Chlorine	0.003 ppm
Dissolved Oxygen	8.5 ppm
Alkalinity	70 ppm
pH	7.39
Turbidity	0.13
Total Hardness	103
Calcium Hardness	78
Total Organic Carbon	1.4
Total Coliform	0%
Fecal Coliform and E. Coli	0%
Barium	34 ppb
Copper	7 ppb
Selenium	3 ppb
Fluoride	1 ppm
Nitrate (as Nitrogen)	1.3 ppm

Appendix B

Cortisol Coat-A-Count® Radioimmunoassay Procedure

Cortisol Coat-A-Count® Radioimmunoassay Procedure

1. Retrieve Coat-A-Count® kit from storage in refrigerator at 2-8° C.
2. Heat water bath to 37° C.
3. Line the inside of the hood with bench paper, taping it in place. Tape an empty plastic bag to the inside of the hood wall.
4. Put on glove, lab coat and radiation badge.
5. Place under the hood: vortex mixer, can of Radcon® spray, radiation marked tape, clean 100-ml beaker, repipet hand pipet with clean cartridge, foam decanting holders, plastic pan for decanting, paper towels, and radioactive cortisol from Coat-A-Count® kit. Contents of kit and plasma must be warmed to room temperature before beginning the assay.
6. Line lab bench with bench paper and place several test tube racks for small tubes on the bench.
7. The cortisol kit has packages of blue polypropylene 12 x 75 mm assay tubes. These tubes are coated with antibodies. Collect six plain (white) 12 x 75 mm plastic tubes from the lab stock. Plain white tubes are not coated with antibodies and are not included in the kit contents.

8. Label the six white tubes as follows:

- 1 TC
- 2 TC
- 3 TC
- 4 NSB
- 5 NSB
- 6 NSB

Place these tubes in order in a rack on the bench.

9. Now label the coated (blue) tubes with number only, but be sure to record the contents of each tube on a separate paper.

7	Calib A	16	Calib D	26	Ctrl	4
8	Calib A	17	Calib D	27	Ctrl	4
9	Calib A	18	Calib D	29	Ctrl	5
10	Calib B	19	Calib E	30	Ctrl	5
11	Calib B	20	Calib E	31	Ctrl	6
12	Calib B	21	Calib E	32	Ctrl	6
13	Calib C	23	Calib F	33	Pig-internal control	

14	Calib C	24	Calib F	34	Pig-internal control
15	Calib C	25	Calib F	35	High-stress trout
36	High-stress trout				
37	Low-stress trout				
38	Low-stress trout				
39	Unknown sample #1				
40	Unknown sample #1				

10. Continue the labeling process until all plasma samples have been accounted for in duplicate. Also number two tubes for Ctrl 4, 5, and 6 and two tubes for high and low stress trout plasma and place these at the end of the tubes as an internal control for drift within the assay. As the tubes are numbered, place them in order in the test tube racks.

11. For the moment, ignore Tubes #1-3, the total counts tubes. Into Tubes 4-6 and Tubes 7-9, pipet 25 μ l of the Calib A into the bottom of the tubes. Pipet 25 μ l of Calibrators B,C,D,E and F into the appropriate tubes. Finally, pipet 25 μ l of each unknown plasma sample into the appropriate tubes. Always pipet to the bottom of the tube.

12. Place the test tubes racks with all numbered tubes under the hood. Pour the radiolabelled cortisol solution into the beaker for easier access with the repipet. (This step MUST be done under the hood.) Using the repipet, add 1.0 ml of radioactive cortisol to each tube including Tubes 1-3, the TC tubes.

13. Vortex each tube.

14. Place the tubes into the water bath and incubate at 37° C for 45 minutes.

15. At the end of the incubation period, return the test tube racks to the hood and place the tubes into the foam decanting racks. BE CAREFUL- do not place the TC tubes #1-3 into the decanting rack. Tubes #1-3 are not decanted. Leave Tubes #1-3 in the test tube rack.

16. Flip the foam decanting racks upside down over the plastic pan and shake the foam rack thoroughly to remove excess fluid. All visible fluid should be removed from the tubes. Place the foam rack and tubes upside down on several sheets of paper towels under the hood and strike the tubes sharply with your hands to remove remaining drops of fluid. When tubes have been well-drained, place the tubes back into the test tube racks and take the racks to a gamma counter. Load the tubes into the gamma counter in numerical order.

17. As soon as the tubes are loaded, remove gloves and place in the plastic bag taped inside the hood. Wash hands with soap and water. Put on a new pair of gloves and carefully spray and wipe the hood area, water bath, vortex mixer and lab bench with the Radcon® spray. All paper towels, empty bottles, used repipet cartridges must be placed in the plastic bag in the hood. The beaker and plastic decanting pan must be emptied into a radiation sink and left to soak. The foam decanting racks should be placed into a plastic bag, taped closed with radiation tape and left under the hood.

18. Place all solid waste including paper towels and bench paper from the hood in the plastic bag taped in the hood. Remove and place gloves in this bag. Seal the bag with radiation tape and label according to date and isotope ^{125}I . This bag of solid waste goes into the disposal container for <90 day radiation.

19. Swipe 3-4 sites of possible contamination including lab bench, vortex mixer, water bath, and hood bench with small paper swipes and place these swipes into plain white 12 x 75 mm tubes and add these tubes to the gamma counter after the last of the samples.

20. When the counter is finished counting, remove all tubes and place them back in the test tube racks. Cover racks tightly with paraffin film. Place these racks under the hood for 24 hours in case a recount of certain tubes is required. Tubes can be recounted within a 24-hour period.

21. If no recounts are required, seal the tubes in a plastic bag with radiation-labelled tape. Discard the tubes into the solid waste container for radiation < 90 days. Remove all items from the soak sink, wash with soap and water. Place the test tube racks into the soak sink. Use gloves when handling and washing equipment. Complete all paperwork regarding usage and disposal of radioactive substances. Be sure to come back in 24 hours to wash the test tube racks.

Appendix C

Raw Data and Changes in Length and Weight of Rainbow Trout From 0 to 133 Days

Appendix C. Lengths and weights (means $\pm$ SE) for salt-fed rainbow trout from Day 0 to Day 133 ($P \leq 0.05$). Diet 1 represents values for trout fed a 3.6% NaCl diet. Diet 2 represents values for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent growth data for trout fed a 7.3% NaCl diet and a 0.7% (basal) diet respectively.

Diet	Day	Length (cm)	Weight (kg)
1	0	22.4 + 0.46	0.11 + 0.005
2	0	21.7 + 0.27	0.10 + 0.004
3	0	22.2 + 0.34	0.11 + 0.005
5	0	22.6 + 0.32	0.11 + 0.005
1	13	24.2 + 0.26	0.145 + 0.005 ^a
2	13	23.3 + 0.43	0.13 + 0.0085 ^{ab}
3	13	23.2 + 0.24	0.13 + 0.006 ^b
5	13	24.0 + 0.28	0.14 + 0.005 ^{ab}
1	41	25.5 + 0.30	0.17 + 0.006
2	41	25.0 + 0.21	0.16 + 0.005
3	41	25.0 + 0.33	0.16 + 0.006
4	41	25.0 + 0.25	0.17 + 0.007
1	72	25.2 + 0.40	0.17 + 0.008
2	72	25.7 + 0.47	0.18 + 0.01
3	72	26.0 + 0.20	0.20 + 0.007
5	72	25.85 + 0.56	0.19 + 0.01
1	101	27.3 + 0.24	0.24 + 0.008 ^{ab}
2	101	27.65 + 0.46	0.25 + 0.01 ^a
3	101	26.3 + 0.56	0.22 + 0.01 ^b
5	101	27.55 + 0.20	0.23 + 0.005 ^a
1	133	29.3 + 0.32	0.30 + 0.01 ^a
2	133	29.3 + 0.36	0.32 + 0.02 ^a
3	133	28.7 + 0.32	0.2755 + 0.009 ^{ab}
5	133	28.0 + 0.47	0.265 + 0.01 ^b

Appendix C Continued: Raw Data for Lengths and Weights of Rainbow Trout

<u>Diet</u>	<u>Fish</u>	<u>Day</u>	<u>Length (cm)</u>	<u>Weight (kg)</u>
1	1	0	20.5	0.09
1	2	0	23	0.115
1	3	0	21.5	0.1
1	4	0	21	0.085
1	5	0	20.7	0.09
1	6	0	23	0.11
1	7	0	24.5	0.125
1	8	0	22.7	0.115
1	9	0	24.6	0.14
1	10	0	22.1	0.1
2	1	0	21.5	0.105
2	2	0	21.5	0.09
2	3	0	22.4	0.115
2	4	0	22.6	0.12
2	5	0	21.6	0.1
2	6	0	21	0.095
2	7	0	23	0.13
2	8	0	20.2	0.09
2	9	0	22.3	0.095
2	10	0	21.2	0.1
3	1	0	23.4	0.145
3	2	0	21.3	0.1
3	3	0	21.5	0.1
3	4	0	23.9	0.13
3	5	0	20.9	0.105
3	6	0	23.1	0.115
3	7	0	21.1	0.095
3	8	0	23	0.12
3	9	0	22.1	0.1
3	10	0	21.7	0.095
5	1	0	23	0.11
5	2	0	23	0.13
5	3	0	24.3	0.13
5	4	0	23	0.11
5	5	0	21.7	0.095
5	6	0	22.3	0.105
5	7	0	21.6	0.11
5	8	0	22.3	0.1
5	9	0	21	0.08
5	10	0	23.7	0.135
1	1	14	24.7	0.155

1	2	14	24.2	0.145
1	3	14	24.7	0.16
1	4	14	23.9	0.145
1	5	14	24	0.155
1	6	14	23.5	0.125
1	7	14	25.8	0.165
1	8	14	24.6	0.15
1	9	14	23	0.125
1	10	14	23.4	0.13
2	1	14	23.3	0.125
2	2	14	21.7	0.105
2	3	14	23.7	0.13
2	4	14	23	0.125
2	5	14	24.8	0.16
2	6	14	22.1	0.125
2	7	14	22.4	0.11
2	8	14	22	0.1
2	9	14	25.6	0.185
2	10	14	24.8	0.155
3	1	14	24.5	0.159
3	2	14	23.6	0.165
3	3	14	22.8	0.13
3	4	14	23.8	0.125
3	5	14	23	0.14
3	6	14	22.4	0.11
3	7	14	23	0.13
3	8	14	23.5	0.13
3	9	14	21.9	0.11
3	10	14	23.6	0.135
5	2	14	23	0.115
5	3	14	22.9	0.12
5	4	14	22.7	0.135
5	5	14	25.5	0.155
5	6	14	24.4	0.155
5	7	14	24.5	0.14
5	8	14	24.8	0.16
5	9	14	24.3	0.135
5	10	14	24	0.135
1	1	41	24.5	0.165
1	2	41	27	0.195
1	3	41	24.5	0.16
1	4	41	26.1	0.145
1	5	41	25.7	0.16
1	6	41	26.5	0.17
1	7	41	26.2	0.195

1	8	41	24.2	0.135
1	9	41	24.8	0.165
1	10	41	25.4	0.185
2	1	41	25.2	0.17
2	2	41	25	0.165
2	3	41	24.3	0.155
2	4	41	24.5	0.155
2	5	41	25.1	0.16
2	6	41	25.9	0.16
2	7	41	24.8	0.15
2	8	41	24.5	0.17
2	9	41	26.3	0.19
2	10	41	24.4	0.135
3	1	41	25.9	0.175
3	2	41	22.9	0.13
3	3	41	23.5	0.13
3	4	41	25.6	0.175
3	5	41	26	0.195
3	6	41	25.6	0.16
3	7	41	24.9	0.175
3	8	41	24.6	0.155
3	9	41	25.7	0.165
3	10	41	25.4	0.17
5	1	41	25.4	0.175
5	2	41	25.3	0.18
5	3	41	25.1	0.165
5	4	41	24.6	0.14
5	5	41	24.6	0.165
5	6	41	24.1	0.145
5	7	41	25.6	0.18
5	8	41	26.5	0.21
5	9	41	25.2	0.16
5	10	41	23.7	0.14
1	1	72	23.4	0.175
1	2	72	25	0.19
1	3	72	25.5	0.2
1	4	72	26.4	0.23
1	5	72	24.1	0.17
1	6	72	26.8	0.2
1	7	72	24.6	0.2
1	8	72	27	0.22
1	9	72	23.8	0.15
1	10	72	24.5	0.155
2	1	72	24	0.15
2	2	72	25.8	0.19

2	3	72	24.5	0.18
2	4	72	27.6	0.25
2	5	72	26.2	0.22
2	6	72	26.2	0.215
2	7	72	22.9	0.12
2	8	72	25.6	0.225
2	9	72	27.5	0.21
2	10	72	26.4	0.215
3	1	72	26.1	0.195
3	2	72	27	0.23
3	3	72	25.6	0.18
3	4	72	25.4	0.21
3	5	72	25.6	0.195
3	6	72	26.1	0.205
3	7	72	25.5	0.19
3	8	72	25.4	0.165
3	9	72	26.9	0.23
3	10	72	26.8	0.22
5	1	72	25.3	0.195
5	2	72	24	0.165
5	3	72	26.3	0.17
5	4	72	26.9	0.2
5	5	72	28	0.235
5	6	72	22.9	0.13
5	7	72	28.6	0.24
5	8	72	24.8	0.17
5	9	72	26.5	0.2
5	10	72	25.2	0.195
1	1	101	27.8	0.265
1	2	101	27.7	0.28
1	4	101	26.7	0.215
1	5	101	28.2	0.245
1	6	101	27.5	0.245
1	7	101	25.5	0.195
1	8	101	27.6	0.25
1	9	101	27	0.205
1	10	101	27.8	0.23
2	1	101	27	0.225
2	2	101	29.1	0.27
2	3	101	27.7	0.265
2	4	101	27.5	0.24
2	5	101	27.8	0.235
2	6	101	28.5	0.26
2	7	101	27.4	0.245
2	8	101	24	0.18

2	9	101	28.5	0.28
2	10	101	29	0.31
3	1	101	27.3	0.23
3	2	101	23.5	0.17
3	3	101	27	0.235
3	4	101	27.5	0.245
3	5	101	27.7	0.25
3	6	101	25	0.155
3	7	101	27.5	0.24
3	8	101	27.5	0.27
3	9	101	23.1	0.16
3	10	101	27	0.245
5	1	101	27.5	0.25
5	2	101	28.5	0.255
5	3	101	27.8	0.25
5	4	101	27.8	0.245
5	5	101	27.3	0.225
5	6	101	28	0.21
5	7	101	28.3	0.24
5	8	101	26.7	0.215
5	9	101	27	0.215
5	10	101	26.6	0.225
1	1	133	28.1	0.28
1	2	133	29.5	0.29
1	3	133	31.3	0.36
1	4	133	28.8	0.31
1	5	133	29	0.255
1	6	133	28.4	0.27
1	7	133	28.3	0.26
1	8	133	29.4	0.32
1	9	133	30.5	0.315
1	10	133	29.4	0.325
2	1	133	27.1	0.225
2	2	133	29	0.275
2	3	133	30.3	0.36
2	4	133	28.6	0.31
2	5	133	28.2	0.275
2	6	133	29.5	0.305
2	7	133	29	0.33
2	8	133	29.8	0.355
2	9	133	30.3	0.375
2	10	133	31	0.395
3	1	133	28.5	0.295
3	2	133	31	0.335
3	3	133	29.1	0.275

3	4	133	28.8	0.28
3	5	133	28	0.255
3	6	133	28.6	0.27
3	7	133	29.3	0.295
3	8	133	28.3	0.275
3	9	133	28.8	0.24
3	10	133	27	0.235
5	1	133	25.6	0.185
5	2	133	29.1	0.31
5	3	133	27.3	0.265
5	4	133	30.3	0.335
5	5	133	28.1	0.255
5	6	133	28.2	0.285
5	7	133	27.1	0.24
5	8	133	27.1	0.23
5	9	133	26.8	0.24
5	10	133	30.1	0.305

Appendix D

Plasma Chloride Values for Salt-Fed Rainbow Trout From 0 to 133 Days

Appendix D: Plasma chloride concentrations for rainbow trout fed salt-enriched diets from Day 0 through Day 133. Diet 1 represents values for trout receiving a 3.6% NaCl diet. Diet 2 represents values for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent values for trout receiving a 7.3% NaCl diet and a 0.7% (basal) diet respectively.

<u>Diet</u>	<u>Fish</u>	<u>Day</u>	<u>Plasma Chloride (meq/L)</u>
1	1	0	126.3
1	2	0	134.5
1	3	0	140.3
1	4	0	126.5
1	5	0	126.2
1	6	0	123.6
1	7	0	124.0
1	8	0	126.9
1	9	0	133.2
1	10	0	135.2
2	1	0	129.8
2	2	0	130.3
2	3	0	126.2
2	4	0	127.1
2	5	0	132.5
2	6	0	131.8
2	7	0	126.7
2	8	0	138.7
2	9	0	127.4
2	10	0	126.9
3	1	0	130.0
3	2	0	135.4
3	3	0	110.6
3	4	0	134.7
3	5	0	151.2
3	6	0	139.4
3	7	0	118.2
3	8	0	135.6
3	9	0	116.2
3	10	0	152.7
5	1	0	128.7
5	2	0	125.6
5	3	0	132.3
5	4	0	150.7
5	5	0	121.4
5	6	0	131.1
5	7	0	136.0
5	8	0	123.8

5	9	0	144.7
5	10	0	143.4
1	1	13	131.8
1	2	13	132.5
1	3	13	128.7
1	4	13	137.6
1	5	13	135.1
1	6	13	134.9
1	7	13	123.6
1	8	13	124.7
1	9	13	120.5
1	10	13	129.4
2	1	13	133.8
2	2	13	136.3
2	3	13	132.0
2	4	13	138.9
2	5	13	131.8
2	6	13	132.2
2	7	13	139.2
2	8	13	133.4
2	9	13	136.5
2	10	13	122.0
3	2	13	130.5
3	3	13	116.4
3	4	13	136.1
3	5	13	126.0
3	6	13	126.3
3	7	13	131.2
3	8	13	139.6
3	9	13	146.3
3	10	13	127.4
5	1	13	122.35
5	2	13	133.1
5	3	13	132.5
5	4	13	125.3
5	5	13	116.4
5	6	13	128.7
5	7	13	130.2
5	8	13	136.01
5	9	13	127.6
5	10	13	127.1
1	1	41	137.2
1	2	41	113.5
1	3	41	125.6
1	4	41	124.2

1	5	41	115.6
1	6	41	122.0
1	7	41	124.2
1	8	41	122.0
1	9	41	110.9
1	10	41	118.4
2	1	41	124.2
2	2	41	110.9
2	3	41	124.35
2	4	41	115.5
2	5	41	118.2
2	6	41	117.6
2	7	41	127.1
2	8	41	123.8
2	9	41	112.4
2	10	41	120.0
3	1	41	130.2
3	2	41	130.5
3	3	41	119.8
3	4	41	116.0
3	5	41	127.6
3	6	41	119.6
3	7	41	125.4
3	8	41	131.1
3	9	41	127.1
3	10	41	114.7
5	1	41	124.7
5	2	41	114.55
5	3	41	125.4
5	4	41	121.6
5	5	41	117.8
5	6	41	130.9
5	7	41	112.55
5	8	41	122.35
5	9	41	120.2
5	10	41	126.0
1	1	72	117.6
1	2	72	113.19
1	3	72	117.4
1	4	72	116.4
1	5	72	118.0
1	6	72	109.8
1	7	72	118.7
1	8	72	105.1
1	9	72	117.3

1	10	72	108.2
2	1	72	123.3
2	2	72	113.1
2	3	72	112.4
2	4	72	115.1
2	5	72	124.0
2	6	72	127.2
2	7	72	123.1
2	8	72	118.2
2	9	72	118.7
2	10	72	119.3
3	1	72	114.0
3	2	72	117.6
3	3	72	109.5
3	4	72	117.6
3	5	72	111.5
3	6	72	117.3
3	7	72	118.2
3	8	72	110.2
3	9	72	122.2
3	10	72	117.45
5	1	72	108.2
5	2	72	128.7
5	3	72	106.6
5	4	72	98.6
5	5	72	112.2
5	6	72	108.2
5	7	72	129.8
5	8	72	120.0
5	9	72	115.5
5	10	72	117.3
1	1	101	140.0
1	2	101	127.25
1	3	101	142.0
1	4	101	116.5
1	5	101	120.4
1	6	101	126.3
1	7	101	123.4
1	8	101	124.5
1	9	101	130.2
1	10	101	105.1
2	1	101	126.0
2	2	101	121.8
2	3	101	125.3
2	4	101	114.2

2	5	101	116.2
2	6	101	120.4
2	7	101	122.0
2	8	101	115.1
2	9	101	126.7
2	10	101	133.4
3	1	101	128.3
3	2	101	131.2
3	3	101	126.5
3	4	101	124.0
3	5	101	119.3
3	6	101	109.3
3	7	101	129.2
3	8	101	123.8
3	9	101	121.6
3	10	101	122.5
5	1	101	119.6
5	2	101	124.7
5	3	101	134.15
5	4	101	98.4
5	5	101	124.2
5	6	101	119.1
5	7	101	129.1
5	8	101	119.6
5	9	101	124.0
5	10	101	126.2
1	1	133	119.3
1	2	133	119.6
1	3	133	112.9
1	4	133	115.3
1	5	133	107.8
1	6	133	123.3
1	7	133	110.6
1	8	133	135.2
1	9	133	108.03
1	10	133	122.9
2	1	133	111.6
2	2	133	120.4
2	3	133	130.0
2	4	133	110.7
2	5	133	121.1
2	6	133	115.1
2	7	133	141.6
2	8	133	117.8
2	9	133	116.7

2	10	133	120.7
3	1	133	121.4
3	2	133	119.3
3	3	133	119.6
3	4	133	121.6
3	5	133	128.7
3	6	133	128.7
3	7	133	132.3
3	8	133	127.1
3	9	133	127.3
3	10	133	133.1
5	1	133	128.9
5	2	133	121.6
5	3	133	114.4
5	4	133	116.4
5	5	133	128.2
5	6	133	128.2
5	7	133	115.6
5	8	133	136.3
5	9	133	130.5
5	10	133	125.3

Appendix E

Numbers of Gill Chloride Cells for Salt-Fed Rainbow Trout from Day 0 to Day 133

Appendix E: Number of gill chloride cells for rainbow trout fed salt-enriched diets for 76 days of a 133-day study. Diet 1 is the 3.6% NaCl diet. Diet 2 is the 3.6% CaCl₂ diet. Diet 3 is the 7.3% NaCl diet. Diet 5 is the 0.7% NaCl basal diet.

<u>Diet</u>	<u>Fish</u>	<u>Day</u>	<u>Number of cells</u>
1	1	0	45
1	3	0	94
1	4	0	96
1	5	0	65
1	10	0	77
2	1	0	48
2	5	0	74
2	6	0	51
2	8	0	67
2	9	0	113
3	2	0	94
3	3	0	62
3	5	0	61
3	8	0	110
3	9	0	97
5	1	0	124
5	2	0	103
5	3	0	65
5	6	0	42
5	8	0	81
1	1	13	79
1	5	13	98
1	6	13	62
1	8	13	86
1	10	13	85
2	2	13	118
2	3	13	61
2	4	13	82
2	6	13	90
2	7	13	116
3	2	13	89
3	3	13	53
3	4	13	115
			118

3	7	13	123
3	10	13	97
5	4	13	72
5	6	13	57
5	8	13	73
5	9	13	90
5.		13.	
1	1	41	122
1	4	41	99
1	7	41	83
1	9	41	74
1	10	41	96
2	1	41	129
2	6	41	73
2	7	41	65
2	9	41	61
2	10	41	93
3	5	41	80
3	6	41	63
3	7	41	80
3	9	41	75
3	10	41	62
5	1	41	60
5	3	41	87
5	6	41	108
5	7	41	92
5	9	41	78
1	3	72	73
1	4	72	103
1	7	72	77
1	9	72	81
1	10	72	53
2	2	72	84
2	4	72	79
2	5	72	46
2	7	72	96
2	9	72	43

3	4	72	99
3	5	72	107
3	6	72	73
3	8	72	97
3	10	72	70
5	1	72	75
5	2	72	142
5	3	72	77
5	5	72	57
5	8	72	100
1	1	101	71
1	2	101	38
1	4	101	66
1	6	101	94
1	10	101	66
2	1	101	129
2	2	101	56
2	3	101	79
2	4	101	83
2	8	101	68
3	4	101	103
3	7	101	59
3	8	101	92
3	9	101	64
3	10	101	74
5	2	101	110
5	4	101	68
5	6	101	66
5	7	101	80
5	9	101	76
1	3	133	112
1	4	133	73
1	6	133	56
1	8	133	64
1	9	133	53
2	2	133	107
2	3	133	90
2	5	133	66

2	7	133	62
2	9	133	80
3	1	133	88
3	3	133	78
3	4	133	98
3	5	133	94
3	9	133	89
5	1	133	98
5	5	133	82
5	7	133	108
5	8	133	95
5	10	133	73

Appendix F

Plasma Chloride Values for Salt-Fed Rainbow Trout During a 12-Hour Confinement Challenge and Recovered for 72 Hours

Appendix F: Plasma chloride values for salt-fed rainbow trout subjected to a 12-hour confinement challenge and recovered for 72 hours. Diet 1 represents plasma chloride values for trout fed a 3.6% NaCl diet. Diet 2 represents values for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent plasma chloride values for trout fed a 7.3% NaCl diet and a 0.7% NaCl (basal) diet respectively.

<u>Diet</u>	<u>Fish</u>	<u>Time</u>	<u>Plasma Chloride (meq/L)</u>
1	1	0	114.8
1	2	0	110.6
1	3	0	114.6
1	4	0	113.6
1	5	0	115.1
1	6	0	107.6
1	7	0	115.8
1	8	0	103.2
1	9	0	114.5
1	10	0	106.1
2	1	0	120.0
2	2	0	110.6
2	3	0	109.9
2	4	0	112.45
2	5	0	120.7
2	6	0	123.7
2	7	0	119.8
2	8	0	115.3
2	9	0	115.8
2	10	0	116.3
3	1	0	111.4
3	2	0	114.8
3	3	0	107.2
3	4	0	114.8
3	5	0	109.1
3	6	0	114.5
3	7	0	115.3
3	8	0	107.9
3	9	0	119.0
3	10	0	114.6
5	1	0	106.1
5	2	0	125.05
5	3	0	104.6
5	4	0	97.2
5	5	0	109.8
5	6	0	106.1

5	7	0	126.1
5	8	0	117.0
5	9	0	112.8
5	10	0	114.5
1	1	2	108.4
1	2	2	159.1
1	3	2	111.1
1	4	2	139.7
1	5	2	111.3
1	6	2	117.2
1	7	2	115.8
1	8	2	114.5
1	9	2	106.6
1	10	2	111.1
2	1	2	126.9
2	2	2	113.8
2	3	2	103.2
2	4	2	105.4
2	5	2	101.0
2	6	2	105.4
2	7	2	119.5
2	8	2	115.1
2	9	2	124.7
2	10	2	120.7
3	1	2	108.8
3	2	2	121.5
3	3	2	99.7
3	4	2	124.5
3	5	2	104.9
3	6	2	112.6
3	7	2	117.5
3	8	2	123.0
3	9	2	116.0
3	10	2	99.7
5	1	2	105.1
5	2	2	101.0
5	3	2	94.8
5	4	2	109.3
5	5	2	103.7
5	6	2	99.0
5	7	2	105.6
5	8	2	97.5
5	9	2	109.3
5	10	2	104.1
1	1	4	111.8

1	2	4	106.4
1	3	4	105.6
1	4	4	108.1
1	5	4	110.8
1	6	4	110.3
1	7	4	113.1
1	8	4	115.6
1	9	4	109.3
1	10	4	111.8
2	1	4	120.3
2	2	4	103.9
2	3	4	100.5
2	4	4	109.4
2	5	4	103.4
2	6	4	101.0
2	7	4	104.1
2	8	4	118.5
2	9	4	152.3
2	10	4	115.3
3	1	4	143.4
3	2	4	115.1
3	3	4	132.8
3	4	4	141.7
3	5	4	120.3
3	6	4	109.8
3	7	4	128.45
3	8	4	113.1
3	9	4	115.5
3	10	4	117.7
5	1	4	101.2
5	2	4	114.1
5	3	4	115.5
5	4	4	105.6
5	5	4	112.3
5	6	4	135.5
5	7	4	102.4
5	8	4	109.8
5	9	4	129.7
5	10	4	119.7
1	1	6	115.3
1	2	6	113.3
1	3	6	121.0
1	4	6	110.1
1	5	6	138.3
1	6	6	122.0

1	7	6	121.7
1	8	6	116.5
1	9	6	119.31
1	10	6	117.2
2	1	6	109.1
2	2	6	115.1
2	3	6	107.2
2	4	6	115.6
2	5	6	111.3
2	6	6	109.3
2	7	6	102.4
2	8	6	120.5
2	9	6	106.2
2	10	6	100.0
3	1	6	97.08
3	2	6	101.4
3	3	6	94.15
3	4	6	106.7
3	5	6	118.5
3	6	6	101.5
3	7	6	119.2
3	8	6	119.5
3	9	6	109.4
3	10	6	102.5
5	1	6	109.1
5	2	6	102.0
5	3	6	127.4
5	4	6	109.6
5	5	6	112.6
5	6	6	104.2
5	7	6	123.4
5	8	6	113.0
5	9	6	118.5
5	10	6	99.5
1	1	12	112.0
1	2	12	126.2
1	3	12	111.0
1	4	12	105.6
1	5	12	101.2
1	6	12	96.3
1	7	12	101.5
1	8	12	97.7
1	9	12	87.3
1	10	12	134.6
2	1	12	120.3

2	2	12	91.5
2	3	12	101.0
2	4	12	128.2
2	5	12	106.9
2	6	12	109.6
2	7	12	106.2
2	8	12	160.0
2	9	12	102.9
2	10	12	95.8
3	1	12	119.2
3	2	12	109.6
3	3	12	104.6
3	4	12	116.8
3	5	12	115.5
3	6	12	122.5
3	7	12	127.2
3	8	12	116.8
3	9	12	120.2
3	10	12	121.4
5	1	12	114.6
5	2	12	109.6
5	3	12	119.8
5	4	12	110.6
5	5	12	110.8
5	6	12	115.0
5	7	12	103.7
5	8	12	112.45
5	9	12	107.9
5	10	12	117.7
1	1	24	149.8
1	2	24	153.2
1	3	24	147.7
1	4	24	146.7
1	5	24	150.3
1	6	24	144.3
1	7	24	153.1
1	8	24	164.6
1	9	24	142.2
1	10	24	140.0
2	1	24	126.7
2	2	24	132.4
2	3	24	133.1
2	4	24	137.9
2	5	24	137.4
2	6	24	143.9

2	7	24	148.2
2	8	24	142.7
2	9	24	142.0
2	10	24	133.1
3	1	24	151.2
3	2	24	172.0
3	3	24	164.8
3	4	24	149.8
3	5	24	154.3
3	6	24	146.5
3	7	24	140.8
3	8	24	146.9
3	9	24	153.6
3	10	24	155.65
5	1	24	139.8
5	2	24	142.5
5	3	24	151.2
5	4	24	142.0
5	5	24	139.8
5	6	24	129.1
5	7	24	151.9
5	8	24	145.8
5	9	24	148.9
5	10	24	146.5
1	1	72	143.1
1	2	72	154.1
1	3	72	153.1
1	4	72	145.1
1	5	72	156.5
1	6	72	146.0
1	7	72	158.4
1	8	72	150.8
1	9	72	156.5
1	10	72	155.5
2	1	72	146.3
2	2	72	144.6
2	3	72	141.2
2	4	72	148.9
2	5	72	174.45
2	6	72	137.9
2	7	72	150.8
2	8	72	146.5
2	9	72	157.7
2	10	72	166.5
3	1	72	160.1

3	2	72	143.6
3	3	72	147.5
3	4	72	140.0
3	5	72	151.5
3	6	72	146.2
3	7	72	139.4
3	8	72	153.1
3	9	72	160.1
3	10	72	146.2
5	1	72	168.8
5	2	72	160.1
5	3	72	142.7
5	4	72	132.7
5	5	72	.
5	6	72	150.5
5	7	72	146.0
5	8	72	157.0
5	9	72	151.7

Appendix G

Plasma Cortisol Values for Salt-Fed Rainbow Trout During a 12-Hour Confinement Challenge

Appendix G: Plasma cortisol concentrations for salt-fed rainbow trout subjected to a 12-hour confinement stress.

<u>Diet</u>	<u>Fish</u>	<u>Day</u>	<u>Cortisol Concentration $\mu\text{g/L}$</u>
0.7% NaCl	1	0	10.75
0.7% NaCl	2	0	4.25
0.7% NaCl	3	0	19.85
0.7% NaCl	4	0	3.6
0.7% NaCl	5	0	5.7
0.7% NaCl	6	0	15.65
0.7% NaCl	7	0	4.9
0.7% NaCl	8	0	15.95
0.7% NaCl	9	0	3.7
0.7% NaCl	10	0	26.2
3.6% NaCl	1	0	2.25
3.6% NaCl	2	0	0.12
3.6% NaCl	3	0	0
3.6% NaCl	4	0	2.3
3.6% NaCl	5	0	4.7
3.6% NaCl	6	0	4.1
3.6% NaCl	7	0	1.9
3.6% NaCl	8	0	0
3.6% NaCl	9	0	0.465
3.6% NaCl	10	0	2.2
3.6% CaCl ₂	1	0	5.95
3.6% CaCl ₂	2	0	37.1
3.6% CaCl ₂	3	0	0.32
3.6% CaCl ₂	4	0	3.75
3.6% CaCl ₂	5	0	0
3.6% CaCl ₂	6	0	6.6
3.6% CaCl ₂	7	0	42.65
3.6% CaCl ₂	8	0	4.4
3.6% CaCl ₂	9	0	0
3.6% CaCl ₂	10	0	0
7.3% NaCl	1	0	3.95
7.3% NaCl	2	0	3
7.3% NaCl	3	0	0.095
7.3% NaCl	4	0	3.27
7.3% NaCl	5	0	4.85
7.3% NaCl	6	0	5.6

7.3% NaCl	7	0	3.7
7.3% NaCl	8	0	11.5
7.3% NaCl	9	0	5.55
7.3% NaCl	10	0	4.05
0.7% NaCl	1	2	47.65
0.7% NaCl	2	2	18.2
0.7% NaCl	3	2	17.65
0.7% NaCl	4	2	13.4
0.7% NaCl	5	2	60.3
0.7% NaCl	6	2	66.3
0.7% NaCl	7	2	20.95
0.7% NaCl	8	2	24.8
0.7% NaCl	9	2	14.65
0.7% NaCl	10	2	84.95
3.6% NaCl	1	2	83.2
3.6% NaCl	2	2	103.6
3.6% NaCl	3	2	193.75
3.6% NaCl	4	2	107.2
3.6% NaCl	5	2	88.9
3.6% NaCl	6	2	108.6
3.6% NaCl	7	2	91.3
3.6% NaCl	8	2	45.75
3.6% NaCl	9	2	38.45
3.6% NaCl	10	2	183.65
3.6% CaCl2	1	2	110.8
3.6% CaCl2	2	2	27.2
3.6% CaCl2	3	2	68.7
3.6% CaCl2	4	2	20.1
3.6% CaCl2	5	2	88.55
3.6% CaCl2	6	2	153.1
3.6% CaCl2	7	2	43.15
3.6% CaCl2	8	2	32.55
3.6% CaCl2	9	2	118.6
3.6% CaCl2	10	2	73.95
7.3% NaCl	1	2	28.05
7.3% NaCl	2	2	76.35
7.3% NaCl	3	2	51.65
7.3% NaCl	4	2	6.15
7.3% NaCl	5	2	120
7.3% NaCl	6	2	75.3
7.3% NaCl	7	2	88.5

7.3% NaCl	8	2	20.9
7.3% NaCl	9	2	126.1
7.3% NaCl	10	2	64.35
0.7% NaCl	1	4	203.55
0.7% NaCl	2	4	139.4
0.7% NaCl	3	4	69.35
0.7% NaCl	4	4	102.6
0.7% NaCl	5	4	131.95
0.7% NaCl	6	4	151.65
0.7% NaCl	7	4	209.65
0.7% NaCl	8	4	148.7
0.7% NaCl	9	4	51.55
0.7% NaCl	10	4	114.5
3.6% NaCl	1	4	130.6
3.6% NaCl	2	4	136.1
3.6% NaCl	3	4	246
3.6% NaCl	4	4	82.1
3.6% NaCl	5	4	130.15
3.6% NaCl	6	4	109.4
3.6% NaCl	7	4	121.95
3.6% NaCl	8	4	115.95
3.6% NaCl	9	4	142.55
3.6% NaCl	10	4	102.2
3.6% CaCl2	1	4	55.8
3.6% CaCl2	2	4	79.4
3.6% CaCl2	3	4	131.35
3.6% CaCl2	4	4	196.15
3.6% CaCl2	5	4	158.8
3.6% CaCl2	6	4	130.8
3.6% CaCl2	7	4	162.35
3.6% CaCl2	8	4	66.75
3.6% CaCl2	9	4	151.9
3.6% CaCl2	10	4	116.45
7.3% NaCl	1	4	150.9
7.3% NaCl	2	4	82.6
7.3% NaCl	3	4	104.6
7.3% NaCl	4	4	65.6
7.3% NaCl	5	4	132.2
7.3% NaCl	6	4	89.7
7.3% NaCl	7	4	230.3
7.3% NaCl	8	4	168

7.3% NaCl	9	4	124.6
7.3% NaCl	10	4	147.7
0.7% NaCl	1	6	160.7
0.7% NaCl	2	6	83.3
0.7% NaCl	3	6	55.9
0.7% NaCl	4	6	62.8
0.7% NaCl	5	6	75.05
0.7% NaCl	6	6	238.65
0.7% NaCl	7	6	220.25
0.7% NaCl	8	6	165.95
0.7% NaCl	9	6	93.65
0.7% NaCl	10	6	65.55
3.6% NaCl	1	6	43.3
3.6% NaCl	2	6	15.1
3.6% NaCl	3	6	22.85
3.6% NaCl	4	6	82.35
3.6% NaCl	5	6	51.25
3.6% NaCl	6	6	122.85
3.6% NaCl	7	6	48.6
3.6% NaCl	8	6	19.65
3.6% NaCl	9	6	36.7
3.6% NaCl	10	6	8.3
3.6% CaCl2	1	6	196.1
3.6% CaCl2	2	6	112.3
3.6% CaCl2	3	6	188.15
3.6% CaCl2	4	6	90.05
3.6% CaCl2	5	6	149.75
3.6% CaCl2	6	6	77.35
3.6% CaCl2	7	6	255.6
3.6% CaCl2	8	6	227.1
3.6% CaCl2	9	6	175.3
3.6% CaCl2	10	6	179.25
7.3% NaCl	1	6	143.1
7.3% NaCl	2	6	131
7.3% NaCl	3	6	165.2
7.3% NaCl	4	6	190.15
7.3% NaCl	5	6	101.25
7.3% NaCl	6	6	111.15
7.3% NaCl	7	6	57.3
7.3% NaCl	8	6	215.25
7.3% NaCl	9	6	281.4

7.3% NaCl	10	6	77.7
0.7% NaCl	1	12	36.15
0.7% NaCl	2	12	67.3
0.7% NaCl	3	12	71.9
0.7% NaCl	4	12	57.3
0.7% NaCl	5	12	54.8
0.7% NaCl	6	12	50.45
0.7% NaCl	7	12	60.2
0.7% NaCl	8	12	19.2
0.7% NaCl	9	12	27.8
0.7% NaCl	10	12	5.45
3.6% NaCl	1	12	221.4
3.6% NaCl	2	12	20.9
3.6% NaCl	3	12	127.6
3.6% NaCl	4	12	186.45
3.6% NaCl	5	12	121.6
3.6% NaCl	6	12	178.9
3.6% NaCl	7	12	239.3
3.6% NaCl	8	12	23.2
3.6% NaCl	9	12	102.35
3.6% NaCl	10	12	6.25
3.6% CaCl2	1	12	34.05
3.6% CaCl2	2	12	121.65
3.6% CaCl2	3	12	98
3.6% CaCl2	4	12	48
3.6% CaCl2	5	12	90.4
3.6% CaCl2	6	12	29.8
3.6% CaCl2	7	12	189.5
3.6% CaCl2	8	12	208
3.6% CaCl2	9	12	109.75
3.6% CaCl2	10	12	117.7
7.3% NaCl	1	12	35.15
7.3% NaCl	2	12	33.4
7.3% NaCl	3	12	19.35
7.3% NaCl	4	12	55.05
7.3% NaCl	5	12	26.7
7.3% NaCl	6	12	65.95
7.3% NaCl	7	12	49.8
7.3% NaCl	8	12	24.2
7.3% NaCl	9	12	30.5
7.3% NaCl	10	12	6.15

Appendix H

Plasma Glucose Values for Salt-Fed Rainbow Trout During a 12-Hour Confinement Challenge

Appendix H: Plasma glucose concentrations for salt-fed rainbow trout exposed to a 12-hour confinement challenge and then recovered for 72 hours. Diet 1 represents plasma glucose concentrations for trout fed a 3.6% NaCl diet. Diet 2 represents plasma glucose concentrations for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent plasma glucose values for trout fed a 7.3% NaCl diet and a 0.7% NaCl (basal) diet respectively.

<u>Diet</u>	<u>Fish</u>	<u>Time</u>	<u>Plasma Glucose (mg/dL)</u>
1	1	0	73.6
1	2	0	89.4
1	3	0	71.0
1	4	0	84.7
1	5	0	83.4
1	6	0	73.6
1	7	0	95.8
1	8	0	60.8
1	9	0	94.0
1	10	0	82.1
1	1	2	111.55
1	2	2	117.1
1	3	2	121.4
1	4	2	123.9
1	5	2	100.5
1	6	2	116.25
1	7	2	147.8
1	8	2	109.8
1	9	2	84.7
1	10	2	97.0
1	1	4	110.7
1	2	4	91.1
1	3	4	149.1
1	4	4	112.4
1	5	4	135.5
1	6	4	230.65
1	7	4	228.5
1	8	4	117.5
1	9	4	106.0
1	10	4	104.7
1	1	6	95.3
1	2	6	77.0
1	3	6	163.2
1	4	6	121.8
1	5	6	197.3

1	6	6	164.1
1	7	6	174.7
1	8	6	128.6
1	9	6	115.8
1	10	6	109.0
1	1	12	126.1
1	2	12	90.6
1	3	12	123.9
1	4	12	141.0
1	5	12	224.2
1	6	12	186.7
1	7	12	105.15
1	8	12	126.9
1	9	12	228.5
1	10	12	133.3
1	1	24	77.4
1	2	24	84.2
1	3	24	132.9
1	4	24	128.6
1	5	24	81.7
1	6	24	89.46
1	7	24	98.3
1	8	24	93.2
1	9	24	80.0
1	10	24	118.8
1	1	72	96.6
1	2	72	72.7
1	3	72	82.5
1	4	72	81.2
1	5	72	101.3
1	6	72	63.3
1	7	72	69.7
1	8	72	85.9
1	9	72	88.9
1	10	72	61.6
2	1	0	91.1
2	2	0	71.9
2	3	0	77.0
2	4	0	71.0
2	5	0	65.9
2	6	0	69.7
2	7	0	116.7
2	8	0	88.9
2	9	0	60.3

2	10	0	91.5
2	1	2	93.6
2	2	2	157.2
2	3	2	164.9
2	4	2	136.3
2	5	2	163.2
2	6	2	93.2
2	7	2	124.4
2	8	2	112.0
2	9	2	111.55
2	10	2	131.2
2	1	4	95.3
2	2	4	97.9
2	3	4	109.0
2	4	4	173.0
2	5	4	91.5
2	6	4	148.7
2	7	4	99.2
2	8	4	129.5
2	9	4	100.5
2	10	4	113.3
2	1	6	140.15
2	2	6	154.2
2	3	6	175.6
2	4	6	133.5
2	5	6	111.55
2	6	6	224.7
2	7	6	124.8
2	8	6	143.1
2	9	6	217.4
2	10	6	159.4
2	1	12	85.5
2	2	12	173.9
2	3	12	156.4
2	4	12	71.4
2	5	12	217.8
2	6	12	69.7
2	7	12	119.2
2	8	12	247.7
2	9	12	76.55
2	10	12	221.7
2	1	24	190.1
2	2	24	78.7
2	3	24	134.6

2	4	24	121.4
2	5	24	78.7
2	6	24	68.9
2	7	24	62.0
2	8	24	61.6
2	9	24	77.8
2	10	24	121.8
2	1	72	101.3
2	2	72	78.3
2	3	72	91.5
2	4	72	106.0
2	5	72	105.15
2	6	72	62.0
2	7	72	73.1
2	8	72	64.6
2	9	72	81.7
2	10	72	79.1
3	1	0	112.8
3	2	0	68.9
3	3	0	77.8
3	4	0	76.55
3	5	0	78.7
3	6	0	71.0
3	7	0	61.2
3	8	0	73.1
3	9	0	68.0
3	10	0	87.2
3	1	2	135.9
3	2	2	117.1
3	3	2	117.1
3	4	2	101.3
3	5	2	164.9
3	6	2	126.9
3	7	2	126.5
3	8	2	118.8
3	9	2	138.9
3	10	2	201.2
3	1	4	163.6
3	2	4	113.3
3	3	4	116.25
3	4	4	109.0
3	5	4	101.3
3	6	4	130.8
3	7	4	154.2

3	8	4	173.45
3	9	4	163.6
3	10	4	159.4
3	1	6	105.15
3	2	6	82.5
3	3	6	162.35
3	4	6	126.5
3	5	6	127.3
3	6	6	144.0
3	7	6	84.7
3	8	6	225.5
3	9	6	155.5
3	10	6	94.05
3	1	12	94.9
3	2	12	73.1
3	3	12	113.3
3	4	12	111.55
3	5	12	101.7
3	6	12	87.65
3	7	12	134.2
3	8	12	126.5
3	9	12	78.8
3	10	12	93.2
3	1	24	82.1
3	2	24	114.1
3	3	24	62.5
3	4	24	75.7
3	5	24	64.2
3	6	24	66.7
3	7	24	58.2
3	8	24	71.4
3	9	24	75.3
3	10	24	89.8
3	1	72	60.3
3	2	72	63.7
3	3	72	58.2
3	4	72	95.3
3	5	72	75.7
3	6	72	76.55
3	7	72	80.8
3	8	72	70.6
3	9	72	103.9
3	10	72	111.1
5	1	0	82.5

5	2	0	97.9
5	3	0	131.2
5	4	0	81.2
5	5	0	60.8
5	6	0	96.2
5	7	0	83.4
5	8	0	74.4
5	9	0	66.3
5	10	0	101.7
5	1	2	89.3
5	2	2	105.15
5	3	2	92.8
5	4	2	83.8
5	5	2	110.7
5	6	2	96.6
5	7	2	69.7
5	8	2	122.2
5	9	2	100.5
5	10	2	91.1
5	1	4	101.7
5	2	4	91.9
5	3	4	63.3
5	4	4	118.0
5	5	4	105.15
5	6	4	118.8
5	7	4	108.6
5	8	4	138.0
5	9	4	76.55
5	10	4	122.2
5	1	6	143.1
5	2	6	148.7
5	3	6	102.6
5	4	6	87.2
5	5	6	99.6
5	6	6	170.9
5	7	6	104.3
5	8	6	103.0
5	9	6	129.1
5	10	6	106.0
5	1	12	141.9
5	2	12	126.5
5	3	12	170.9
5	4	12	131.6
5	5	12	123.9

5	6	12	126.5
5	7	12	223.0
5	8	12	140.15
5	9	12	120.9
5	10	12	88.1
5	1	24	106.9
5	2	24	67.6
5	3	24	65.9
5	4	24	86.4
5	5	24	78.3
5	6	24	115.0
5	7	24	100.1
5	8	4	108.1
5	9	24	91.9
5	10	24	127.3
5	1	72	73.1
5	2	72	76.1
5	3	72	70.1
5	4	72	99.2
5	5	72	.
5	6	72	69.3
5	7	72	55.2
5	8	72	106.4
5	9	72	76.55
5	10	72	94.05

Appendix I

Percent Blood Hematocrit Levels for Salt-Fed Rainbow Trout Subjected to a 12-Hour Confinement Challenge

Appendix I: Percent blood hematocrit values for salt-fed rainbow trout subjected to a 12-hour confinement challenge and recovered for 72 hours. Diet 1 represents hematocrit values for trout fed a 3.6% NaCl diet. Diet 2 represents hematocrit values for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent hematocrit values for trout fed a 7.3% NaCl diet and a 0.7% NaCl (basal) diet respectively.

<u>Diet</u>	<u>Fish</u>	<u>Time</u>	<u>% Hematocrit</u>
1	1	0	0.41
1	2	0	0.4
1	3	0	0.35
1	4	0	0.35
1	5	0	0.33
1	6	0	0.32
1	7	0	0.34
1	8	0	0.37
1	9	0	0.3
1	10	0	0.42
2	1	0	.
2	2	0	0.39
2	3	0	0.43
2	4	0	0.37
2	5	0	0.35
2	6	0	0.31
2	7	0	.
2	8	0	0.35
2	9	0	0.39
2	10	0	0.31
3	1	0	0.34
3	2	0	0.37
3	3	0	0.39
3	4	0	0.5
3	5	0	0.4
3	6	0	0.29
3	7	0	0.34
3	8	0	0.34
3	9	0	0.34
3	10	0	0.37
5	1	0	0.39
5	2	0	0.23
5	3	0	0.44
5	4	0	0.4
5	5	0	0.39
5	6	0	0.2

5	7	0	0.37
5	8	0	0.43
5	9	0	0.39
5	10	0	0.41
1	1	2	0.35
1	2	2	0.41
1	3	2	0.45
1	4	2	0.38
1	5	2	0.44
1	6	2	0.36
1	7	2	0.35
1	8	2	0.42
1	9	2	0.46
1	10	2	0.46
2	1	2	0.34
2	2	2	0.4
2	3	2	0.18
2	4	2	0.44
2	5	2	0.46
2	6	2	0.34
2	7	2	0.44
2	8	2	0.4
2	9	2	0.51
2	10	2	0.39
3	1	2	0.42
3	2	2	0.42
3	3	2	0.4
3	4	2	0.47
3	5	2	0.48
3	6	2	0.41
3	7	2	0.39
3	8	2	0.4
3	9	2	0.46
3	10	2	0.46
5	1	2	0.45
5	2	2	0.35
5	3	2	0.44
5	4	2	0.42
5	5	2	0.34
5	6	2	0.43
5	7	2	0.4
5	8	2	0.36
5	9	2	0.39
5	10	2	0.47
1	1	4	0.32

1	2	4	0.36
1	3	4	0.42
1	4	4	0.37
1	5	4	0.35
1	6	4	0.32
1	7	4	0.35
1	8	4	0.41
1	9	4	0.43
1	10	4	0.39
2	1	4	0.44
2	2	4	0.29
2	3	4	0.47
2	4	4	0.45
2	5	4	0.41
2	6	4	0.3
2	7	4	0.32
2	8	4	0.42
2	9	4	0.41
2	10	4	0.41
3	1	4	0.36
3	2	4	0.4
3	3	4	0.41
3	4	4	0.37
3	5	4	0.56
3	6	4	0.37
3	7	4	0.45
3	8	4	0.38
3	9	4	0.39
3	10	4	0.42
5	1	4	0.37
5	2	4	0.38
5	3	4	0.42
5	4	4	0.41
5	5	4	0.4
5	6	4	0.45
5	7	4	0.38
5	8	4	.
5	9	4	0.43
5	10	4	0.4
1	1	6	0.34
1	2	6	0.36
1	3	6	0.38
1	4	6	0.38
1	5	6	0.42
1	6	6	0.4

1	7	6	0.32
1	8	6	0.34
1	9	6	0.39
1	10	6	0.39
2	1	6	0.37
2	2	6	0.44
2	3	6	0.39
2	4	6	0.25
2	5	6	0.42
2	6	6	0.31
2	7	6	0.39
2	8	6	0.4
2	9	6	0.36
2	10	6	0.45
3	1	6	0.36
3	2	6	0.44
3	3	6	0.44
3	4	6	0.42
3	5	6	0.37
3	6	6	0.39
3	7	6	0.39
3	8	6	0.38
3	9	6	0.38
3	10	6	0.36
5	1	6	.
5	2	6	0.46
5	3	6	0.44
5	4	6	0.37
5	5	6	0.39
5	6	6	0.33
5	7	6	0.36
5	8	6	0.37
5	9	6	0.35
5	10	6	0.38
1	1	12	0.4
1	2	12	0.4
1	3	12	0.51
1	4	12	0.45
1	5	12	0.5
1	6	12	0.42
1	7	12	0.45
1	8	12	0.43
1	9	12	0.36
1	10	12	.
2	1	12	0.41

2	2	12	0.42
2	3	12	0.46
2	4	12	0.44
2	5	12	0.43
2	6	12	0.44
2	7	12	0.39
2	8	12	0.45
2	9	12	0.45
2	10	12	0.44
3	1	12	0.4
3	2	12	0.4
3	3	12	0.37
3	4	12	0.36
3	5	12	0.43
3	6	12	0.37
3	7	12	0.31
3	8	12	0.49
3	9	12	0.49
3	10	12	0.37
5	1	12	0.43
5	2	12	0.43
5	3	12	0.45
5	4	12	0.41
5	5	12	0.52
5	6	12	0.42
5	7	12	0.47
5	8	12	0.51
5	9	12	0.42
5	10	12	0.46
1	1	24	0.34
1	2	24	0.33
1	3	24	0.29
1	4	24	0.39
1	5	24	0.44
1	6	24	0.34
1	7	24	0.34
1	8	24	0.33
1	9	24	0.42
1	10	24	0.44
2	1	24	0.46
2	2	24	0.41
2	3	24	0.44
2	4	24	0.42
2	5	24	0.5
2	6	24	0.34

2	7	24	0.3
2	8	24	0.5
2	9	24	0.44
2	10	24	0.45
3	1	24	0.34
3	2	24	0.34
3	3	24	0.4
3	4	24	0.42
3	5	24	0.45
3	6	24	0.39
3	7	24	0.41
3	8	24	0.3
3	9	24	0.41
3	10	24	0.43
5	1	24	0.44
5	2	24	0.4
5	3	24	0.37
5	4	24	0.41
5	5	24	0.43
5	6	24	0.4
5	7	24	0.47
5	8	24	0.36
5	9	24	0.33
5	10	24	0.49
1	1	72	0.39
1	2	72	0.4
1	3	72	0.37
1	4	72	0.49
1	5	72	0.43
1	6	72	0.42
1	7	72	0.32
1	8	72	0.39
1	9	72	0.46
1	10	72	0.34
2	1	72	0.43
2	2	72	0.42
2	3	72	0.43
2	4	72	0.4
2	5	72	0.44
2	6	72	0.35
2	7	72	0.41
2	8	72	0.41
2	9	72	0.36
2	10	72	.
3	1	72	0.46

3	2	72	0.41
3	3	72	0.41
3	4	72	0.39
3	5	72	0.41
3	6	72	0.49
3	7	72	0.45
3	8	72	0.37
3	9	72	0.44
3	10	72	0.4
5	1	72	0.47
5	2	72	0.41
5	3	72	0.38
5	4	72	0.23
5	5	72	0.29
5	6	72	0.3
5	7	72	0.23
5	8	72	0.38
5	9	72	0.26
5	10	72	0.44

Appendix J

Percent Blood Methemoglobin Values for Salt-Fed Rainbow Trout During an 8-Hour Nitrite Challenge

Appendix J: Percent blood methemoglobin levels in salt-fed rainbow trout subjected to an 8-hour nitrite challenge. Diet 1 represents methemoglobin values for trout fed a 3.6% NaCl diet. Diet 2 represents methemoglobin values for trout fed a 3.6% CaCl₂ diet. Diets 3 and 5 represent values for trout fed a 7.3% NaCl and a 0.7% (basal) diet respectively.

<u>Fish</u>	<u>Diet</u>	<u>Hour</u>	<u>% Methemoglobin</u>
1	1	0	3.8
3	1	0	4.2
4	1	0	4.8
5	1	0	5.8
6	1	0	5.1
7	1	0	2.3
8	1	0	6.9
9	1	0	8.4
10	1	0	8.3
1	1	2	8.1
2	1	2	7.2
3	1	2	6.2
4	1	2	7.5
5	1	2	11.5
6	1	2	9.4
7	1	2	8.7
8	1	2	9.7
9	1	2	9
10	1	2	10.2
1	1	4	7.3
2	1	4	8.7
3	1	4	9.8
4	1	4	21.6
5	1	4	26.5
6	1	4	16.9
7	1	4	11
8	1	4	11.6
9	1	4	14.5
10	1	4	18.7
1	1	6	34.1
2	1	6	32.9
3	1	6	8.9
4	1	6	11.3
5	1	6	25.6
6	1	6	9.9
7	1	6	9.2

8	1	6	71.8
9	1	6	36.7
10	1	6	32.6
1	1	8	10.9
2	1	8	30.8
3	1	8	8.4
4	1	8	20.9
5	1	8	43.4
6	1	8	20.6
7	1	8	44.9
8	1	8	59.7
9	1	8	
10	1	8	.
1	2	0	6.9
2	2	0	8.7
3	2	0	10.5
4	2	0	8.6
5	2	0	10.1
6	2	0	12.7
7	2	0	11.1
8	2	0	12.6
9	2	0	9.9
10	2	0	13.3
1	2	2	7.5
2	2	2	14.1
3	2	2	29.6
4	2	2	10.3
5	2	2	10.2
7	2	2	11.1
8	2	2	24.3
9	2	2	14.1
10	2	2	12.5
1	2	4	9.9
2	2	4	19.3
3	2	4	9.7
4	2	4	19.1
5	2	4	11.8
6	2	4	16.8
8	2	4	5.5
9	2	4	5.9
10	2	4	13.3
1	2	6	11.3
2	2	6	13.2
3	2	6	22.6

4	2	6	13.2
5	2	6	13.4
6	2	6	19.1
7	2	6	30.9
8	2	6	14.6
9	2	6	15.2
10	2	6	16.6
1	2	8	14.7
2	2	8	65.8
3	2	8	12.7
4	2	8	18.3
5	2	8	14.9
6	2	8	36.8
7	2	8	52
8	2	8	16.3
9	2	8	13.8
10	2	8	17.7
1	3	0	7.2
2	3	0	7.1
3	3	0	8.1
4	3	0	8.7
5	3	0	7.6
6	3	0	9
7	3	0	11.1
8	3	0	8.8
9	3	0	9.3
10	3	0	10.7
1	3	2	10.5
2	3	2	14.1
3	3	2	7.9
4	3	2	9.4
5	3	2	9.8
6	3	2	8.8
7	3	2	10.3
8	3	2	13.1
9	3	2	15.8
10	3	2	12.7
1	3	4	10.5
2	3	4	53.8
3	3	4	48.5
4	3	4	29.6
5	3	4	
6	3	4	.
7	3	4	69.2

8	3	4	12.3
9	3	4	12.6
10	3	4	14
1	3	6	13.3
2	3	6	20.7
3	3	6	5.5
4	3	6	39.8
5	3	6	13.9
6	3	6	54.7
7	3	6	45.5
8	3	6	33.1
9	3	6	13.9
10	3	6	10.9
1	3	8	30.2
2	3	8	15.2
3	3	8	15.8
4	3	8	13.1
5	3	8	12.3
6	3	8	15.4
7	3	8	13.7
8	3	8	46.1
9	3	8	.
10	3	8	.
1	5	0	9.8
2	5	0	9.9
3	5	0	12.3
4	5	0	13.2
5	5	0	10.6
6	5	0	11.5
7	5	0	13.9
8	5	0	13.9
9	5	0	14.2
10	5	0	14.8
1	5	2	10.9
2	5	2	12.5
3	5	2	11.6
4	5	2	13.1
5	5	2	12.2
6	5	2	10.8
7	5	2	12.4
8	5	2	16
9	5	2	14.7
10	5	2	16.1
1	5	4	41.9

2	5	4	49
3	5	4	14
4	5	4	32.5
5	5	4	56.7
6	5	4	54.2
7	5	4	14.2
8	5	4	24.2
9	5	4	14.1
10	5	4	20.7
1	5	6	42.4
2	5	6	16.9
3	5	6	21.2
4	5	6	21.2
5	5	6	21.7
6	5	6	100
7	5	6	22.7
8	5	6	28.6
9	5	6	24.5
10	5	6	41.5
1	5	8	32.9
2	5	8	23.2
3	5	8	23.4
4	5	8	23.6
5	5	8	13.8
6	5	8	53.9
7	5	8	56.3
8	5	8	19
9	5	8	36
10	5	8	21.7

Appendix K

Plasma Chloride Values for Salt-Fed Rainbow Trout During an 8-Hour Nitrite Challenge

Appendix K:

Plasma chloride values for rainbow trout fed salt-enriched diets and exposed to nitrite for 8 hours. Diet 1 represents the 3.6% NaCl diet. Diet 2 represents the 3.6% CaCl₂ diet and Diets 3 and 4 represent the 7.3% NaCl and the 0.7% (basal) diet respectively.

<u>Diet</u>	<u>Hour</u>	<u>Fish</u>	<u>Plasma Chloride (meq/L)</u>
1	0	1	133.3
1	0	2	123.8
1	0	3	130.95
1	0	4	121.7
1	0	5	118.2
1	0	6	130.5
1	0	7	115.2
1	0	8	153.8
1	0	9	140.4
1	0	10	150.5
1	2	1	139.8
1	2	2	120.6
1	2	3	122.3
1	2	4	124.1
1	2	5	120.6
1	2	6	129.0
1	2	7	156.6
1	2	8	120.0
1	2	9	130.1
1	2	10	126.9
1	4	1	122.5
1	4	2	135.0
1	4	3	120.6
1	4	4	118.5
1	4	5	117.2
1	4	6	130.5
1	4	7	127.1
1	4	8	139.6
1	4	9	144.95
1	4	10	131.8
1	6	1	.
1	6	2	110.9
1	6	3	143.9
1	6	4	126.2
1	6	5	148.8
1	6	6	116.3

1	6	7	150.5
1	6	8	124.1
1	6	9	109.2
1	6	10	101.4
1	8	1	133.3
1	8	2	122.3
1	8	3	139.6
1	8	4	159.6
1	8	5	130.1
1	8	6	133.1
1	8	7	109.6
1	8	8	121.5
1	8	9	.
1	8	10	.
2	0	1	131.8
2	0	2	135.9
2	0	3	138.3
2	0	4	115.4
2	0	5	132.9
2	0	6	141.3
2	0	7	134.8
2	0	8	142.4
2	0	9	139.1
2	0	10	139.6
2	2	1	140.9
2	2	2	113.9
2	2	3	124.9
2	2	4	128.8
2	2	5	140.2
2	2	6	132.2
2	2	7	106.6
2	2	8	150.3
2	2	9	134.6
2	2	10	124.5
2	4	1	134.6
2	4	2	135.9
2	4	3	127.7
2	4	4	141.3
2	4	5	136.1
2	4	6	147.5
2	4	7	145.2
2	4	8	137.2
2	4	9	142.4
2	4	10	126.0
2	6	1	134.2

2	6	2	121.7
2	6	3	128.15
2	6	4	117.2
2	6	5	149.3
2	6	6	137.8
2	6	7	132.9
2	6	8	126.4
2	6	9	113.1
2	6	10	126.9
2	8	1	123.6
2	8	2	120.6
2	8	3	142.15
2	8	4	116.7
2	8	5	144.5
2	8	6	124.7
2	8	7	114.1
2	8	8	136.7
2	8	9	128.4
2	8	10	136.3
3	0	1	137.2
3	0	2	134.8
3	0	3	132.7
3	0	4	134.0
3	0	5	149.7
3	0	6	110.3
3	0	7	149.0
3	0	8	127.5
3	0	9	141.3
3	0	10	146.0
3	2	1	113.3
3	2	2	136.55
3	2	3	138.7
3	2	4	138.3
3	2	5	128.15
3	2	6	126.2
3	2	7	141.7
3	2	8	138.1
3	2	9	134.8
3	2	10	138.9
3	4	1	133.5
3	4	2	132.2
3	4	3	132.7
3	4	4	153.3
3	4	5	144.5
3	4	6	129.4

3	4	7	130.1
3	4	8	132.5
3	4	9	158.1
3	4	10	142.8
3	6	1	133.5
3	6	2	118.2
3	6	3	136.55
3	6	4	132.5
3	6	5	130.1
3	6	6	122.7
3	6	7	122.3
3	6	8	113.7
3	6	9	142.4
3	6	10	152.9
3	8	1	140.2
3	8	2	113.1
3	8	3	134.8
3	8	4	113.9
3	8	5	131.6
3	8	6	134.8
3	8	7	129.9
3	8	8	124.5
3	8	9	126.2
3	8	10	130.1
5	0	1	141.1
5	0	2	143.4
5	0	3	135.3
5	0	4	115.9
5	0	5	145.6
5	0	6	137.8
5	0	7	142.4
5	0	8	148.2
5	0	9	140.6
5	0	10	142.8
5	2	1	146.0
5	2	2	131.4
5	2	3	147.8
5	2	4	.
5	2	5	.
5	2	6	133.3
5	2	7	151.8
5	2	8	137.8
5	2	9	129.9
5	2	10	133.5
5	4	1	126.2

5	4	2	127.1
5	4	3	135.7
5	4	4	121.7
5	4	5	127.9
5	4	6	129.4
5	4	7	122.5
5	4	8	.
5	4	9	.
5	4	10	.
5	6	1	122.1
5	6	2	111.1
5	6	3	148.0
5	6	4	146.7
5	6	5	151.2
5	6	6	111.8
5	6	7	181.1
5	6	8	.
5	6	9	121.7
5	6	10	155.5
5	8	1	104.0
5	8	2	110.0
5	8	3	118.2
5	8	4	119.5
5	8	5	135.7
5	8	6	117.8
5	8	7	111.6
5	8	8	130.9
5	8	9	116.5
5	8	10	139.8

VITA

Agnes Mary Vanderpool was born in Berea, Kentucky on February 2, 1965. She graduated as Valedictorian of Hazard High School in Hazard, Kentucky in May 1983. She entered Center College of Kentucky in Danville, Kentucky in August of 1983 and transferred to Florida State University in Tallahassee, Florida in August of 1986. She graduated from Florida State University in May 1989 with a B.S. degree in Biology. She then entered the graduate program for the U.S. Fish and Wildlife Service Cooperative Research and Education Unit at the University of Maine in Orono, Maine in August 1989. Agnes received her M.S. degree in Zoology with a concentration in Fisheries Biology from the University of Maine in 1992. In January 1992, Agnes accepted a position as the extension aquaculture specialist with the West Virginia University Extension Service in Morgantown, West Virginia, a position she held until May 1998. In 1998, Agnes entered the graduate program in Animal Sciences at the University of Tennessee, Knoxville, Tennessee to begin her doctoral program. While attending the University of Tennessee, she worked as a graduate research assistant in the Department of Forestry, Wildlife and Fisheries, and as a post-doctoral research associate. Agnes received her Doctor of Philosophy degree in Animal Science from the University of Tennessee in May 2002.