EFFECTS OF FEEDING AND TEMPERATURE ON ACID-EXPOSED TROUT

EFFECTS OF FEEDING AND TEMPERATURE ON ACID-EXPOSED JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) DURING A GLOBAL WARMING SCENARIO

By

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TITLE: Effects Of Feeding And Temperature on Acid-Exposed Juvenile Rainbow Trout (*Oncorhynchus mykiss* Walbaum) During A Global Warming Scenario

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Abstract

Juvenile rainbow trout were chronically exposed to acidified softwater, alone and in combination with a slight temperature increase in order to understand the possible effects of global warming and environmental acidification in freshwater fish. The second goal was to determine the role of diet in the response to acid stress and elevated temperatures. In the first two exposures, a simulated global warming scenario (+2°C) was applied for 90 days in winter (8-12°C), in the presence and absence of sublethal acidity (pH 5.2). In the first trial, fish were fed to satiation twice daily, while in the second trial, fish were fed only 1% of their wet body weight every four days (~0.25% daily). A slight increase in temperature caused a marked increase in oxygen consumption, nitrogenous waste excretion and growth, although there did not appear to be any specific pH effects.

During the Satiation Exposure, fish exposed to low pH especially at slightly elevated temperatures had increased appetites compared to non-acid exposed fish. This increased appetite suggested that NaCl losses brought about by low pH exposure, stimulated appetite in some way, thereby alleviating any ionoregulatory disturbances. During the Limited Ration Exposure, ionoregulatory disturbances occurred during low pH exposure, with more dramatic effects in fish at slightly elevated temperatures. Trout maintained on a limited diet had a higher mortality rate, lower plasma and whole body Na^+ and Cl^- concentrations, and elevated cortisol levels compared with fish fed to satiation. Thus, it became clear that fish could use food to compensate for the stresses of increased temperature and low pH.

The third exposure was conducted to determine whether food simply provided the necessary fuel to meet the increased cost of living in a low pH environment, or whether

food directly provided the dietary salts necessary to replace branchial ion loss. Diets were formulated at two levels of energy (regular: 16.31MJ/kg or low: 9.77MJ/kg) and two levels of NaCl (regular: 263 mmols/kg or low: 43 mmols/kg) using a factorial design (2x2=4 treatments). In addition, a fifth group of fish were not fed during the exposure. All five groups of fish were challenged with pH 4.0 to induce a rapid ionoregulatory disturbance and then held at pH 5.2 for the next 28 days. During this month, fish were fed 0.6% of their body weight of one of the four diets. Fish fed the low salt diets incurred typical long-term ionoregulatory disturbances with decreased whole body Na+, K+ and Cl- concentrations. These effects were not seen in fish fed regular salt diets, regardless of energy content, showing that it is the salt content of the food rather than the energy content which is critical in protecting against the deleterious effects of low environmental pH. Interestingly, fish fed the regular energy/low salt diet had high cortisol levels and increased mortality while fish fed the regular salt diets, low energy/low salt diets and starved fish did not have a high rate of mortality. These results may have been due to differences in metabolic rate and therefore oxygen consumption (M_{O2}). Starved fish had the lowest M_{O2}. Fish fed the regular energy diet had increased post-prandial M_{O2} due to the specific dynamic action evoked in fish by ingestion of protein-rich food. An increase in oxygen consumption may have caused an increase in branchial ion loss, thereby exacerbating the ionoregulatory deficit associated with chronic acid exposure. This is detrimental when dietary salts are unavailable to replace branchial losses. Overall, the salt content of food may play an important role in ameliorating the deleterious effects of chronic low pH, while the energy content of food may complicate the response.

Acknowledgments

Someone once said that this section is the only part of your thesis that comes from your heart. When I look back over the last two years, I find that most of this work has come from the support and guidance provided by the people around me, making this section even more sentimental. I would like to thank my committee, Chris Wood, Pat Chow-Fraser and Mike O'Donnell for excellent ideas and scientific guidance and Colin Nurse for stepping in at the last moment . I would especially like to convey my appreciation to my supervisor Chris Wood, who went to great lengths to help me meet my goals for this thesis (4:30 am is not really that early!).

Two years ago, after my first "exposure" to B112, I thought there was no way I would ever figure out the reverse osmosis unit, the plumbing, the feeding cycles, the zillions of assays and machines. However, the "Global Warmers" (inference to the project only!) proved to be incredible. Ian Morgan was an excellent mentor and friend, getting me through those late night alarms and sampling periods with plenty of laughs and stories. Tyler Linton, "my pardner in fish" who was always there not only to catch me literally but to encourage me during those days of smelly fish deaths and writers block. Jacqui Dockray for her words of wisdom on acid surges and fish kills helped me keep perspective on the project and increased my appreciation for all the work she put in for "Exposure 2".

There are so many people to thank for all their help especially in the Wood and McDonald labs. I would especially like to thank Sara Croke, a brave soul willing to work with me doing radioactive fluxes. I remember fondly those late night laughs and the ultimate horror when we realized when the 36 hours sampling period would be! Thank you to Derek Alsop and Nathan Webb who were always willing to help sample fish, build systems, and listen to my stories. I would also like to thank my summer buddies, Pam

Wright and Danielle McDonald who were always willing to lend a hand or take a break, and share some ice cream, remembering there are no calories when you share. Jim McGeer and Nic Bury who somehow managed to find time to read and edit my papers providing excellent feedback and ideas, making thesis writing much more enjoyable.

I would like to thank my family, especially my mother, Cynthia D'Cruz for listening patiently to all my laboratory ups and downs and offering words of encouragement at all hours of the night. I would like to acknowledge my two best friends for their continued support, love and slave labour, David Fortier and Kevin Macdonald. I know there are no words to express my appreciation for everything you did (though I am sure there may be enough beer). Thank you especially to Kevin for always reminding me of what was important in life, showing me the humour in all disasters and motivating me to keep going. I could not have done it without you.

Thesis Format

This thesis is organized into three Chapters and one Appendix. The first Chapter is a general overview of the study and pertinent background information. The second and third Chapters have been written as manuscripts for future submission to peer reviewed scientific journals. Abstracts have been included for Chapter 2 and 3. Concluding Remarks summarize the results of each Chapter, and an Appendix describes a low pH challenge experiment that highlights some of the controversy around whether acclimation to low pH occurs. Literature cited follows Chapters 2, 3 and the Appendix, while the general references include references from Chapter 1 and the Concluding Remarks.

Chapter 1: General Introduction.

Chapter 2:

Title: Physiological Effects of Sublethal Acid Exposure in Juvenile Rainbow Trout on a Limited or Unlimited Ration during A Simulated Global Warming Scenario.

Authors: L.M.D'Cruz, J.J.Dockray, I.J. Morgan and C.M.Wood.

Comments: The Limited Ration Exposure was performed exclusively by L.M.D under the guidance of I.J.M, and supervision of C.M.W. The unlimited ration exposure was conducted by J.J.D under the supervision of C.M.W. whereas all analyses for both exposures were performed by L.M.D.

Chapter 3:

Title: The Influence of Dietary Salt and Energy on the Response to Low pH in Juvenile Rainbow Trout

Authors: L.M.D'Cruz and C.M.Wood

Comments: Data were generated exclusively by L.M.D with the supervision of C.M.W.

Appendix 1: This Appendix may be submitted to a peer-reviewed journal as a note.

Authors: L.M.D'Cruz, I.J.Morgan and C.M.Wood.

Comments: Flux experiments were conducted by both L.M.D and I.J.M. Data were analyzed by L.M.D under the guidance of I.J.M, and supervision of C.M.W.

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Chapter 1

General Introduction

Acid Rain

Over the last 140 years, acidic precipitation has caused a decrease in pH of 0.5 to 1.5 units in softwater lakes in North America and Europe (Kemp 1994). The deleterious effect of acid rain was recognized as early as the mid 1920's, but it was not until the late 1970's that governments sponsored large scale studies of the problem (Schindler 1988). The consequences of this acidification have now been chronicled in numerous reports (e.g. Harvey and Lee 1982; Kelso et al.1986; and Schindler 1988). Although sulphur dioxide (SO_2), nitrogen oxides (NO_x) and other organic compound emissions are much lower than in the early 1970's levels, they are still twice as high as in the early 1900's (Irving 1991). Decreased emissions have also corresponded to less rapid changes in lake pH, but due to decreased buffering capacity as a result of former emissions, these lakes are still very susceptible (Schindler 1988). In Canada, 7.6% of the surface area is comprised of freshwater lakes of which, 38% are susceptible to acid atmospheric deposition because of their low buffering capacity (Kelso et al. 1990). The majority of these hydrogen ions (36 - 77%) may come from snowpack melt (Kelso et al. 1986).

Physiological Effects of Low pH in Fish

The ecological impact of freshwater acidification on fish has been studied extensively (e.g. Beamish 1972; Beamish et al. 1975; Leivestad et al. 1976; Kelso and Gunn 1984; Lacroix et al. 1985; Lacroix and Townsend 1987; Kelso et al. 1990). Rainbow trout (Oncorhynchus mykiss) are quite sensitive to low pH (Grande 1978) and mechanisms for the deleterious effects accompanying low pH exposure have been well documented in trout (Fromm 1980; Milligan and Wood 1982; Wood and Macdonald 1982;

Wood 1989; Butler et al. 1992). The presence of high external [H^+] at the gills causes a rapid loss of ions due to both a decrease in active ion uptake and enhanced diffusive loss (Leivestad et al. 1976; Wood 1989; Reid 1995). This disturbance in whole body ion regulation results in a fluid shift from the extracellular fluid into the intracellular fluid causing an increase in hematocrit and plasma protein concentration. Thus, blood viscosity is augmented, increasing arterial blood pressure leading to circulatory failure (Milligan and Wood 1982). Softwater intensifies ion loss as low water [Ca^{2+}] weakens apical tight junctions, thereby increasing paracellular ion loss of Na^+, K^+ and Cl^- (Marshall 1985). During chronic sublethal exposure to acidity, there is a partial inhibition of influx and enhanced efflux, but within a couple of days, efflux decreases considerably resulting in a stabilization of electrolyte balance (McDonald 1983; McDonald et al. 1983; Booth et al. 1988). However, recovery to control plasma ion concentrations does not occur (Audet et al. 1988). Morphological changes to fish gills due to low pH have not been consistently reported (Leino and McCormick 1984; Lacroix et al. 1990; Laurent and Perry 1991; Audet and Wood 1993; Balm and Pottinger 1993).

Global Warming

Climate is a consequence of the way the atmosphere redistributes heat energy absorbed by the earth from the sun. Recent measurements of the composition of atmospheric gases, trapped in ice cores, reveals that a major departure from past patterns is emerging. Carbon dioxide concentration is 20% higher than over the past 220 000 years, methane concentrations have doubled, while nitrous oxide levels have increased by about 8% (IPCC 1995). These increases have been attributed to anthropogenic emissions. If these gases influence climate then increased concentrations would lead to more heat being trapped, leading to higher atmospheric temperatures and an enhanced greenhouse effect. Using General Circulation Models, air temperatures are predicted to increase by 1.3 to

4.5°C over the next 50 to 100 years, with a rise of 1.8°C by the year 2030 (Hansen et al. 1988; Mohnen and Wang 1992).

Global Warming and Fish

Increasing seasonal and annual water temperatures in North America have been reported in response to global climate warming (Hengeveld, 1990). As fish are poikilothermic, their body temperatures and metabolic rates are set by the surrounding environment, so the effects of global warming are likely to be substantial (Schindler et al. 1990). A slight increase in temperature has potentially harmful effects for fish, even mortality, when water temperature approach the upper thermal limits of certain cold water species such as brook and lake trout; in addition, decreased viable thermal habitats results in density-dependent stress (Matthews and Zimmerman 1990; Magnuson et al. 1990; Meisner 1990; Keleher and Rahel 1996). A study in the Experimental Lakes Area of Ontario has shown that air temperatures have increased by 2°C in the last 20 years, resulting in increased evaporation rates, decreased precipitation and decreased available cold water habitats for stenothermal fish species (Schindler et al. 1990).

In general, within a normal range of temperatures for a fish species, increased temperature results in increased metabolic rate, whereas above the normal range increased temperature results in a decreased metabolic rate (Jobling 1997). A recent study from our laboratory, examining a slight increase of temperature over the natural thermal regime supported this conclusion (Reid et al. 1995). When temperatures reached their upper peak during summer, there was a 20% reduction in growth, appetite, gross conversion efficiency and protein turnover in rainbow trout subjected to the "global warming scenario". However, at earlier lower temperatures during this study, these rates had been stimulated by the slight increase in temperature.

A subsequent question that arises is what are the physiological consequences of a small temperature elevation above the normal range for a freshwater fish during winter?

The effects of temperature upon the rates at which physiological processes proceed is described by the term Q_{10} (Jobling 1994). Q_{10} is calculated as follows:

$$Q_{10} = (V_2/V_1)^{10[T(2) - T(1)]},$$

where V_1 and V_2 are the rates of the physiological process measured at temperatures T(1) and T(2), respectively. Thus, as rates of physiological processes decrease outside the upper thermal range, but increase within normal range, especially at lower temperatures, the Q_{10} for freshwater fish would be quite high during winter temperatures (Jobling 1994). As winter water temperatures may be affected to a great extent by global warming (Mohen and Wang 1992), it is essential to examine the effects of a winter warming scenario on the physiology of freshwater fish.

Physiological Effects of Low pH and Temperature

Clearly, freshwater fish in many parts of North America and Europe may face the impact of acidification and global warming in combination. However, only a few studies have been conducted to examine the combined effects of temperature and acidic water. One such study was conducted by Kwain (1975) based on incremental changes in temperature and the subsequent effects of pH on embryonic development of rainbow trout. In general, low pH caused more adverse effects at higher temperatures. However, this study was based on different environmental questions, and was not focused on the global warming issue.

The Global Warming Project

In the past decade, it has become apparent that research is needed to properly assess the combined impact of global warming and the effects of acidic precipitation on freshwater fish. Hence, the NSERC Strategic "Global Warming Project"
was developed in our laboratory. One objective of the study was to obtain data that quantified the bioenergetic, physiological and toxicological responses of rainbow trout to chronic small elevations in water temperature over the annual cycle, in the presence and

absence of sublethal levels of low pH in softwater. The study was designed to be as similar to the natural world as possible. For example, most organisms are chronically exposed to pollutants and thus long-term studies were used. In addition, exposures used waters that followed the natural fluctuating thermal regime of inshore Lake Ontario and photoperiod that followed the season. A conservative estimate of the global warming scenario, +2°C and an environmentally realistic low pH of 5.2 were used.

The first long-term exposure was conducted in the summer of 1993 (Reid et al. 1995; Dockray et al. 1996). Fish were fed to satiation and the slight temperature elevation increased the cost of living as near incipient lethal temperature were reached (Dockray et al. 1996). Growth, gross conversion efficiency and protein turnover were reduced. However, pH 5.2 did not increase the cost of living beyond the effects of +2°C alone, and the "typical" ionoregulatory disturbances reported in earlier low pH studies were not seen.

In the second long-term exposure, fish were fed a restricted ration of approximately 1% of their wet body weight per day (Dockray 1995). Again, fish exposed to +2°C had reduced growth rates, while trout chronically exposed to pH 5.2 alone, did not suffer from ionoregulatory disturbances.

Low pH and Feeding

As mentioned, Dockray (1995) and Dockray et al. (1996) found that fish chronically exposed to low pH did not exhibit any of the typical ionoregulatory disturbances. In fact, fish fed to satiation and exposed to pH 5.2 showed a higher gross energy intake and weight gain, and more efficient energy conversion, similar to the findings of Wilson et al. (1994). The fish fed a limited ration of 1% and exposed to pH 5.2 again had better growth than non-acid exposed fish. This led the authors to propose that fish were eating their way *out of trouble.* It also shifted this aspect of the "Global Warming Project" to focus more on the role of nutrition during chronic exposure to low pH.

Little is known about nutritional effects on the responses of fish to environmental toxicants (Lanno et al. 1989). While numerous studies have been conducted to examine the effects of low pH on freshwater fish, the great majority have not considered the possible role of either dietary quantity or quality. Many studies have either not stated or defined the feeding regime, or else have used the commonplace expression "ad libitum" feeding without reporting how satiety was measured.

However, studies such as those of Dockray (1995) and Dockray et al. (1996) during low pH exposure, Salman and Eddy (1987) and Smith et al. (1995) during circumneutral pH exposure indicate that feeding may critically influence ion balance. Further indirect evidence is available as to the importance of feeding during low pH exposure. Balm and Pottinger (1993) reported no mortality or prolonged change in plasma Cl^-, hematocrit or plasma protein levels when trout were fed 2% of their body weight during a two week, pH 4.0 exposure. This pH is close to the 4 day LC50 in unfed rainbow trout (Graham and Wood 1981). Sadler and Lynam (1986) reported no ionoregulatory disturbance occurred in yearling brown trout that were exposed to low pH conditions (4.4-5.2) and fed 2% of their body weight per day. Conversely, when feeding was restricted, ionoregulatory disturbances were reported such as in the Audet et al. (1988) study (pH 4.8) when fish were fed to satiation once a week; and the aforementioned Sadler and Lynam (1986) study (pH 4.4-5.2), in which fish were starved. Similarly, Cunningham and Shuter (1986) noted decreased pH tolerance of smallmouth bass during starvation. In fish fed a ration just sufficient to maintain weight but not grow, typical ionoregulatory disturbances were reported during exposure to pH= 4.0 - 5.2 (Booth et al. 1988; Butler et al. 1992). Together, these data suggest that feeding has a strong influence on the response of freshwater fish to low pH.

A strong possibility for the beneficial role of food during low pH exposure may be as a dietary source of replacement salts. Dietary salts may play a larger role in maintaining

ion balance when net branchial ion balance is disturbed by acid stress (Smith et al. 1989 and 1995). There is nearly 100% absorption of NaCl by the gut (Smith et al. 1989) and mechanisms of intestinal absorption by fish appear similar to those of mammals, mainly by active transport of Na^+ via Na^+/K^+ATPase followed by passive movement of Cl^- (Fange and Grove 1979). In a pristine environment, freshwater fish are capable of controlling optimum blood Na^+ levels and when deviations occur the appropriate regulatory mechanisms are affected, such as increasing branchial efflux when high salt diets are ingested (Smith et al. 1995) or increasing chloride cell production in softwater to increase uptake (Laurent 1984; Laurent and Perry 1991).

The other possibility is that food provides the necessary fuel to initiate physiological processes (i.e. increased chloride cells, improved transport mechanisms) to maintain homeostasis during low pH exposure. The cost of ion-osmoregulation has been estimated at up to 20% of the metabolic rate of rainbow trout in freshwater (Rao 1968). Previous studies have shown that low pH exposure increases energy expenditure (Butler et al. 1992; Hargis 1976; Waiwood and Beamish 1978). Dockray et al. (1996) found that chronic exposure to low pH seemed to stimulate appetite, suggesting a higher cost of living. Thus, the importance of food may be to provide the fuel necessary to maintain ion-osmoregulation in an adverse condition such as low pH.

Effects of Low pH and Increased Temperatures on Food Intake during Winter: A Hypothetical Approach

Although, feeding rates of fish in the wild are difficult to estimate (Elliott 1972; Boisclair and Marchand 1993), it is generally accepted that fish eat less in the winter than in the summer (Elliott 1972; Boisclair and Marchand 1993, Smith et al. 1989, Smith and Griffith 1994). In the wild, rainbow trout feed on invertebrates, particularly choronomids, during winter (Smith and Griffith 1994). Low pH has been shown to decrease the number of invertebrates (Schindler 1988), thus reducing the food supply of freshwater fish. In a

study by Hogg and Williams (1996) in which a stream just outside of Toronto, was artificially "globally warmed" by 2-3.5°C, total invertebrate densities were shown to be decreased, particularly Chironomida. In addition, as a slight increase in temperature should increase metabolic rate during winter (Jobling 1997) and low pH has been shown to increase energy expenditure (Hargis 1976; Waiwood et al. 1992; Butler et al. 1992), a reduction in food supply may be especially detrimental to freshwater fish in these circumstances.

The Present Study

As mentioned, in the "Global Warming Project" two summer exposures were conducted earlier to determine the effects of low pH and a slight temperature elevation, alone and in combination, on rainbow trout (Dockray 1995). This thesis followed up this work with two more long-term winter studies. The second purpose of these exposures was to determine if dietary ration influenced the response of rainbow trout to these environmental stresses. If diet did have a significant influence, then the next step was to determine the mechanism involved.

Experimental Design

Experimental design and sampling methods employed were similar to those of Dockray (1995) with the additional measurement of plasma cortisol. Four treatment groups were used: control temperature and pH 5.2 (0/5.2), control temperature and ambient pH (0/6.1), +2°C above control temperature and ambient pH (2/6.1), and +2°C above control temperature and pH 5.2 (2/5.2). These studies were conducted in synthetic softwater using natural fluctuating water temperatures.

In the first two exposures (90 days each), the experimental set up is shown in Figure 1- 1. The system has been described in detail by Dockray et al. (1996). In brief, Hamilton City tap water was passed through a reverse osmosis unit where it was softened and deionized. The product water was then pumped to a main head tank where hardwater

was added back to attain [Ca^{2+}] and [Na^{+}] of ~25 and 100 μmol/ L, respectively. This synthetic softwater (pH 6.1) was then gravity fed into two sub-head tanks. The gravity-feed to one of these two sub-head tanks passed through a heat exchanger where 2°C was added to the control temperature. The water from each of these two tanks was further split into two more sub-head tanks, resulting in the four treatment head tanks. H_2SO_4 (0.2 N) was metered into one of the control and one of the +2°C temperature head tanks. The water from each treatment head tank flowed into duplicate 205 L polypropylene tanks.

In the third exposure (30 days), the above exposure system was modified (Figure 1- 2). Hamilton tapwater was still passed through the reverse osmosis unit and pumped to a head tank where hardwater was added back to attain [Ca^{2+}] and [Na^{+}] of ~25 and 75 μmol/ L, respectively. This synthetic hardwater was gravity-fed to a second head tank into which H_2SO_4 (0.2 N) was added. pH was maintained at pH 4.0 for 12 hours and then held at pH 5.2 for the rest of the experiment. This experiment examined the effects of low pH alone; there was no temperature elevation component.

Physiological Measurements

By measuring various physiological parameters, a more substantial assessment of the cost of living can be made. During the current studies, in-tank oxygen consumption was used as an indirect measure of metabolic rate. As all aerobic processes require ATP and oxygen is used in the production of ATP, it is a good measure of the integrative cost of living. Growth measurements in conjunction with feeding amount provided a relative measure of the gross food conversion and an indirect measure of energy expenditure, i.e. if fish consumed more food but did not grow, it may be inferred that the cost of homeostasis was higher.

Plasma ions, protein and hematocrit, and whole body ions are all parameters influenced by low pH exposure; and they provide an index of the level of acid stress (Giles et al. 1984; Reid 1995). Plasma cortisol was also measured, because it is released in

response to stressors and its functional significance in physiological processes is documented (reviewed by Barton and Iwama 1991). Cortisol is released mainly from interrenal cells upon stimulation by adrenocorticotrophic hormone. In general, the magnitude and duration of the cortisol response reflects the severity and duration of the stressor. Proximate analysis of protein, lipids and carbohydrates also provides information about how fish are utilizing their energy stores, with a decrease suggesting that energy costs are beyond that supplied by food.

Chapter 2

Chapter 2 describes the results of feeding two rations, satiation and limited, on the physiological and metabolic response of juvenile rainbow trout during chronic exposure (90 days) to low pH (5.2) at relatively low water temperature (i.e. winter months), alone and in combination with a slight temperature increase (+2°C). When trout were fed to satiation approximately ~1 to 3 % of their body weight/day (Satiation Exposure), +2°C caused a marked increase in appetite, nitrogenous waste excretion and oxygen consumption. This increase in oxygen consumption was attributed to the apparent specific dynamic action of feeding, which is the extra oxygen consumed to support food assimilation, interconversion and deamination (Beamish 1974a; LeGrow and Beamish 1986; Brown and Cameron 1991). Satiated fish exposed to pH 5.2 showed no ionoregulatory disturbances or mobilization of cortisol. Moreover, these acid exposed fish had increased appetites, resulting in increased growth. In comparison, during the Limited Ration Exposure (1% every 4 days ~0.25%/day), fish did not grow and showed typical ionoregulatory responses to acid stress with lower whole body [Na^+] and [Cl^-], increased cortisol production and greater mortality. Detrimental effects were greater at +2°C. The +2°C thermal regime had no effect on either oxygen consumption or nitrogenous waste excretion in fish fed a limited ration suggesting that the specific dynamic action of feeding is more temperature dependent than basal metabolism. Overall, +2°C and pH 5.2

increased the cost of living as determined by increased feeding in satiated fed fish and greater mortalites in fish maintained on a limited ration.

These findings also showed that fish given sufficient food can eat their way *out of trouble*. Hence, the third exposure (Chapter 3) was carried out to determine if food compensated for any increased energy expenditures and/or provided dietary salts to aid in maintaining ion balance during low pH exposure.

Chapter 3

Chapter 3 describes the results of feeding four different diets and starvation on the response of juvenile rainbow trout to pH 5.2 after an acute exposure to pH 4.0. Four diets were designed: regular energy/low salt, low energy/low salt, regular energy/regular salt and low energy/regular salt. Fish were fed 0.6% of their body weight per day of one of the four diets. Irrespective of the energy content of the diet, fish fed the low salt diets suffered from typical ionoregulatory disturbances with lower whole body Na^+, Cl^- and K^+ concentrations. Thus, dietary salts served to replace branchial ion losses during acid exposure.

Fish fed the regular salt diets, the low energy/low salt diets and starved fish had a low rate of mortality. However, fish fed the regular energy/low salt diets not only suffered from ion imbalance, but had increased cortisol levels and an increased rate of mortality. Evidence from measuring oxygen consumption suggests that the specific dynamic action of eating following an energy rich meal may correspond to a loss of ions. Hence, fish fed the regular energy/ low salt diets were further compromised in an acidified environment because there were unable to replace increased branchial ion losses with dietary salts.

Appendix 1

A brief appendix describes the results of a low pH challenge. Rainbow trout were challenged with pH 4.0 after being maintained on a limited ration and exposed to low pH

and a slight temperature elevation for 90 days (described in Chapter 2). Branchial Na^+ and Cl^- movements were monitored during the 24 hour flux using radioisotopes $^{22}Na^+$ and $^{36}Cl^-$. Results from this experiment can be interpreted in a number of ways showing some of the controversy that exists when deciding if fish acclimate to low pH. Using death as the most non-disputable marker of stress, fish previously exposed to low pH and an elevated temperature do not acclimate to low pH.

The information contained within this thesis will hopefully increase our understanding of the response of rainbow trout to a marginalized environment, providing insight into the future of Canada's freshwater resources. The data also provide information that will aid in planning studies for future low pH experiments and provide some partial explanations for the differing results in the literature.

Figure 1-1

The experimental system used to expose juvenile rainbow trout to increased temperatures and sublethal low pH in the Satiation and Limited Ration Exposure of Chapter 2. Synthetic softwater filled the head tank and hardwater was titrated back to yield a final concentration of Ca^{2+} and Na^{+} ~25 and 100 μmol/ L, respectively. Water from the main head tank was then subdivided: two treatments were heated +2°C above control and the other half of the water remained at the control temperature. H_2SO_4 was added to produce two treatments of pH 5.2. Thus there were four treatments: control temperature/pH 5.2 (0/5.2), control temperature/control pH (0/6.1), control temperature +2°C/control pH (2/6.1) and control temperature +2°C/pH 5.2 (2/5.2).

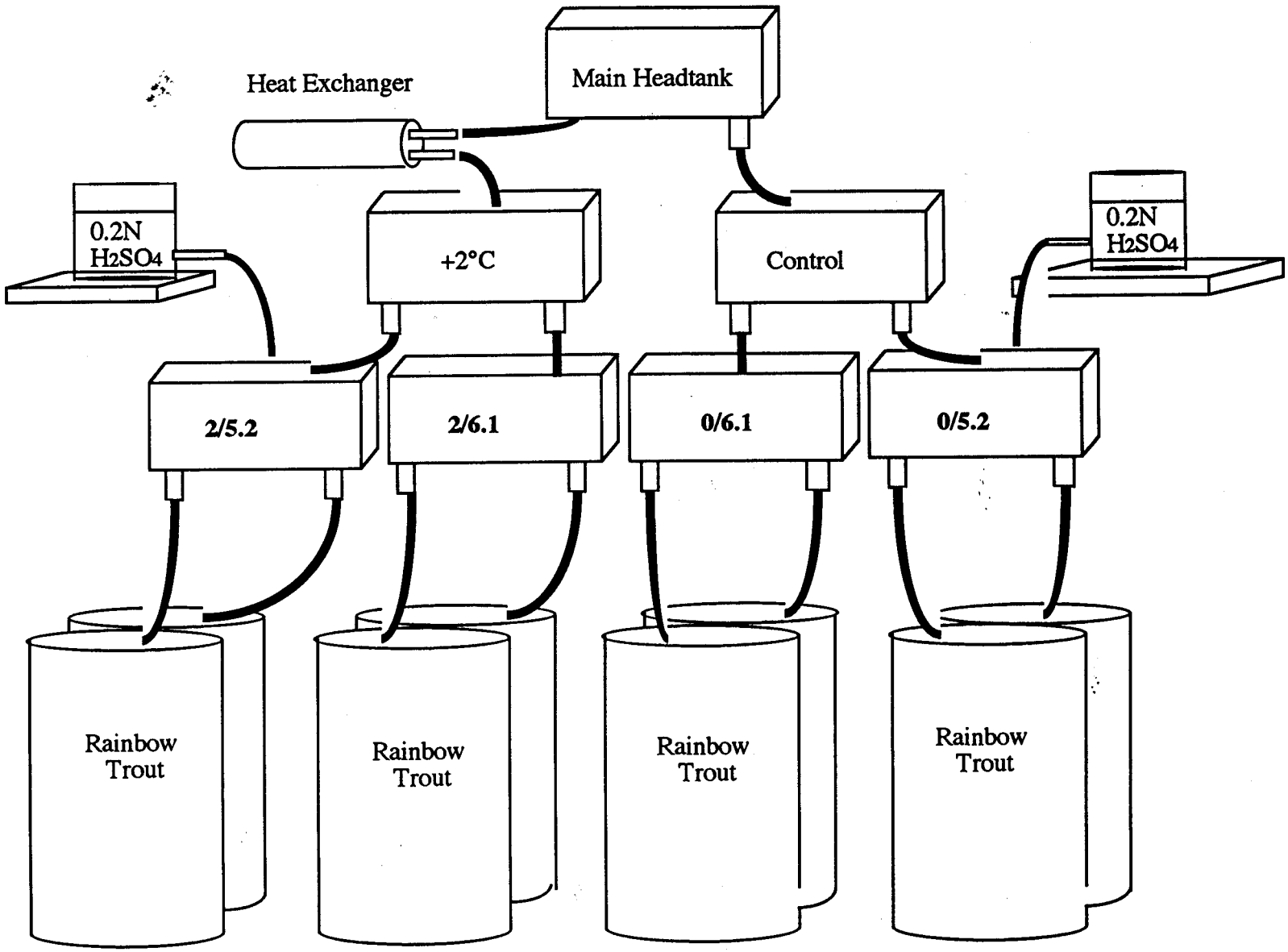
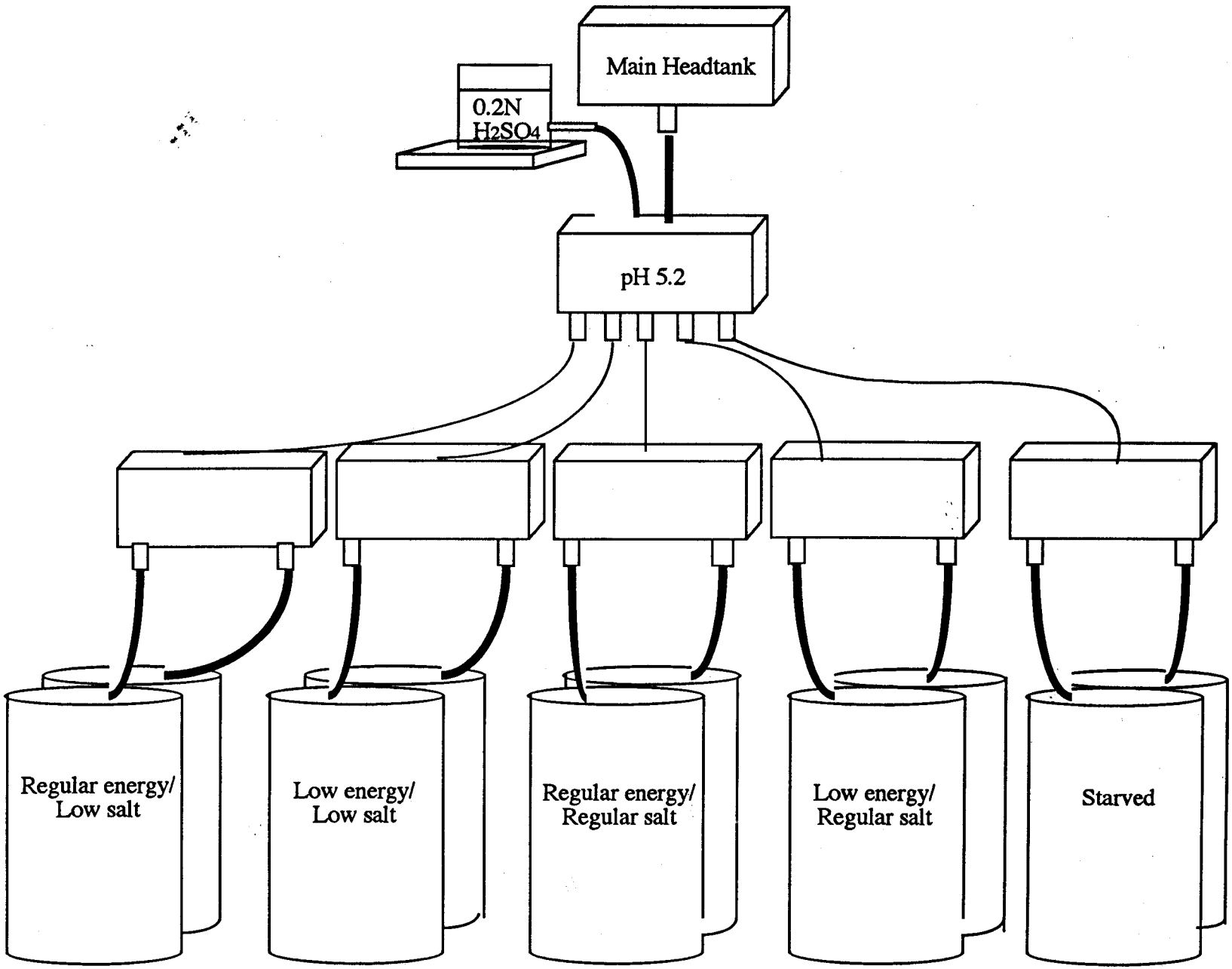

Heat Exchanger
Main Headtank
0.2N
H2SO4
+2°C
Control
0.2N
H2SO4
2/5.2
2/6.1
0/6.1
0/5.2
Rainbow
Trout
Rainbow
Trout
Rainbow
Trout
Rainbow
Trout

Figure 1-2

The experimental system used to expose juvenile rainbow trout to sublethal low pH in Chapter 3. Synthetic softwater filled the head tank and hardwater was titrated back to yield a final concentration of Ca^{2+} and Na^{+} ~25 and 100 μmol/ L, respectively. H_2SO_4 was added to the main head tank and then the water were subdivided into five head tanks. During this exposure, fish were either starved or fed with one of four diets: regular energy/low salt, low energy/low salt, regular energy/regular salt, or low energy/regular salt.


Main Headtank
0.2N
H_2SO_4
pH 5.2
Regular energy/
Low salt
Low energy/
Low salt
Regular energy/
Regular salt
Low energy/
Regular salt
Starved

Chapter 2

Abstract

Rainbow trout were exposed to a simulated global warming scenario (+2°C), in the presence and absence of sublethal acidity (pH 5.2) in synthetic softwater for 90 days during winter (8-12°C). Effects of feeding on the metabolic response to this marginalized environment were investigated. Fish were either fed to satiation, ~1 to 3 % of their wet body weight daily, or fed 1% once every four days . When trout were fed to satiation, +2°C caused a marked increase in appetite, nitrogenous waste excretion and oxygen consumption. Surprisingly, satiation fed fish exposed to pH 5.2 showed no ionoregulatory disturbances. Moreover, these acid exposed fish had increased appetites, resulting in increased growth. In comparison, fish maintained on a limited ration did not grow and showed typical ionoregulatory responses to acid stress with lower whole body [Na^+] and [Cl^-] and greater mortality. Detrimental effects were greater at +2°C. The +2°C thermal regime had no effect on either oxygen consumption or nitrogenous waste excretion in fish fed a limited ration. Overall, +2°C and pH 5.2 increased the cost of living as determined by increased feeding in satiation fed fish and greater mortalites in fish maintained on a limited ration. Most importantly, these findings suggest that fish given sufficient food can compensate for any increased energy expenditure and difficulties in maintaining ion balance associated with low pH exposure.

Introduction

Effects of low pH on the physiology of freshwater fish, particularly rainbow trout (Oncorhynchus mykiss) have been documented extensively (reviewed by Wood 1989; Reid 1995), but research continues because large numbers of softwater lakes in North America and Europe are still affected by acidic precipitation from sulphur dioxide and nitrogen oxide emissions (Kelso et al. 1990; Kemp 1994). Field studies have shown that low pH increases fish mortality, decreases species diversity, and reduces reproductive success and rates of growth (Beamish 1974b; Beamish et al. 1975; Scheider et al. 1979; Lacroix and Townsend 1987).

Understanding of the mechanisms of toxicity associated with acid exposure in the environment is best achieved through laboratory experiments designed to simulate field conditions. Acute exposure to environmentally representative pHs (4.0-5.8) results in a disturbance to ionoregulation due to inhibition of active salt uptake because of competition between H^+ and Na^+ and stimulated diffusive ion losses (McDonald and Wood 1981; Wood 1989). Disruption of branchial ion transport probably accounts for the decrease in whole body ions seen in fish after chronic exposure to low pH (Neville 1985; Lacroix 1985; Audet et al. 1988; Booth et al. 1988). However, in recent studies by Wilson et al. (1994a and b) and Dockray et al.(1996) where fish were fed to satiation during exposure to pH 5.2 for extended periods, no decreases in whole body ion concentrations were reported. These results suggest that the uptake of dietary salts may compensate for branchial ion losses incurred during low pH exposure. Further support for this hypothesis comes from Sadler and Lynam's work (1986) on brown trout, Salmo trutta. They demonstrated decreased growth, decreased mineral content and lower plasma chloride, muscle water, potassium and sodium content in acid-exposed starved fish, but no significant changes in these parameters in acid-exposed fed fish. Previous work has also

revealed that the susceptibility of largemouth bass to low pH may change depending on the amount fed (Kwain et al. 1984; Leino and McCormick 1992). These results highlight the need to examine the role of nutrition in the response of rainbow trout to low pH.

In addition to the acid rain problem, the Intergovernmental Panel on Climate Change (IPCC 1995) estimated an increase in global temperature of 1.8°C by the year 2030, with larger temperature changes occurring in winter months (Mohnen and Wang 1992). This global warming may significantly impact fish as they are poikilothermic, especially if food availability is also affected. Dockray et al. (1996) and Reid et al. (1997) investigated the cost of living for rainbow trout in waters of low pH and slightly elevated temperatures (+2°C) during summer when temperatures reached near incipient lethal levels (26.2°C; Elliott 1982). Parameters such as oxygen consumption, nitrogenous waste excretion, protein synthesis rates, appetite, growth, and partitioning of food energy were used as indicators of metabolic cost whilst indicators such as whole body and plasma ions were measured to determine physiological effects. Overall, exposure to low pH resulted in increased energy intake and weight gain, and better conversion efficiency, while the addition of +2°C reduced gross energy intake and increased fecal energy losses. This study was unique as it followed the natural, fluctuating thermal profile for inshore Lake Ontario during the summer of 1993 , creating a more ecologically relevant study (Reid et al. 1995). However, winter months are of particular interest in the study of environmental acidity in Ontario, because of the build-up of acidic precipitation which then melts, causing between 36 to 77% of the annual acid export from watersheds to lakes (Semkin and Jeffries 1988; Kelso et al. 1990).

The purpose of this study was to examine the effects of two rations (satiation and limited ration) on the physiological and metabolic response of rainbow trout during chronic exposure (90 days) to low pH (5.2) at relatively low water temperatures (8 to

12°C). The second, equally important purpose was to determine the effects of a slight temperature increase (+2°C) on the cost of living. Methods similar to Dockray et al. (1996) were employed with the additional measurement of plasma cortisol, to monitor the stress response of fish to this warmer, slightly acidified environment.

Materials and Methods

Two 90 day exposures, both in synthetic softwater were conducted from January to April, during the winters of 1994 and 1996. The 1994 experiment will be referred to as the Satiation Exposure, and the 1996 experiment will be referred to as the Limited Ration Exposure, in reference to the different feeding regimes. Methods used were similar to those described by Dockray et al (1996) and are summarized below with any deviations from this method noted.

Pre-exposure Holding

Juvenile rainbow trout (Oncorhynchus mykiss; 7 - 15 g) were obtained from local hatcheries. Upon arrival, fish were held in two 400L polypropylene tanks that were supplied with Hamilton dechlorinated tap water (Ca^{2+} =0.98 ± 0.11mM, Na^{+}=0.56 ± 0.03mM, pH 7.6-8.0) and fed dry trout pellets (Zeigler, Salmon Starter #3) at a ration 1% of their wet body weight daily. This food was used for both subsequent experiments. After three weeks, fish were acclimated to synthetic softwater by slowly increasing the amount of softwater to the tank and decreasing the amount of tapwater. After experimental water chemistry conditions were achieved (Ca^{2+} =0.025 ± 0.002mM, Na^{+}= 0.076 ± 0.007 mM, pH 6.1-6.2), fish were held for another 3 weeks before the exposures were started. Softwater was obtained by passing Hamilton tapwater, which closely follows the thermal fluctuations of inshore Lake Ontario, through a reverse osmosis unit (Anderson Water Conditioning Equipment, Dundas, ON). Photoperiod mimicked the natural photoperiod throughout acclimation and exposures.

Exposure System and Experimental Design

The synthetic softwater flow (~pH 6.1) was subdivided into two head tanks, with half of the water being fed by gravity through a heat exchanger that raised the temperature by 2°C. Water was further subdivided, with H_2SO_4 (0.2N) titrated to the control and +2°C water resulting in four treatments: control temperature/sublethal pH 5.2 (0/ 5.2), control temperature/control pH 6.1(0/6.1), control temperature plus 2°C/control pH 6.1 (2/6.1) and control temperature plus 2°C/sublethal pH5.2 (2/5.2). Two 205 L polypropylene tanks per treatment received water at an average rate of 0.8 L/min. Sublethal pH 5.2 was maintained by pH statting; pH in the treatment tanks was continually monitored by a Leeds and Northrup Meridian II® Combination industrial electrode which controlled a Cole Parmer Instrument Co. solenoid valve (CP#01367-70), that opened and closed to deliver H_2SO_4 to the head tank. Low pH and the +2°C temperature elevation were monitored via a Ladder Logic and Texas PLX Model TI315 programmable controller that was connected to an alarm and a Safe House Model 49-433A automatic message dialer system (see Dockray et al. 1996 for a more detailed description). Vigorous aeration maintained P_{O2} above 120 torr and prevented P_{CO2} build up. Temperature and pH were measured daily with independent probes, and $[Na^+]$ and $[Ca^{2+}]$ were monitored weekly using atomic absorption spectroscopy (Varian AA-1275).

In the Satiation Exposure, approximately 142 fish were placed in each tank, thus 284 fish were exposed to each of the four treatments. In the Limited Ration Exposure, 85 fish were placed in each tank; as a result 170 fish were exposed to each of the four treatments.

Feeding Regimes

In the Satiation Exposure fish were fed by hand to satiation twice daily (8:30 and 16:30) as described by Wilson et al. (1994) and appetite was monitored by weighing bags

of the Zeigler diet, pre and post feeding. Faecal matter was removed daily and tanks were cleaned weekly. During the Limited Ration Exposure, fish were fed 1% of their wet body weight once every four days (~0.25% daily ration). Pellets were spread over the water surface to allow all fish access to food. This regime was chosen in order to minimize formation of a social hierarchy (McCarthy et al. 1993). Gross conversion efficiencies were calculated as the wet weight of food eaten divided by the wet weight gained over the 90 day exposure.

Physiological Measurements

Sampling Protocol

During the Satiation Exposure, sampling and physiological measurements (oxygen consumption, nitrogenous waste excretion, blood and plasma composition, proximate composition, and whole body ion analysis) were conducted over 3 - four day periods, ending on days 0, 75 and 90, respectively. In the Limited Ration Exposure, fish sampling and physiological measurements were conducted over 4 - three day periods ending on days 0, 30, 60 and 90, respectively. Oxygen consumption and nitrogenous waste excretion measurements were made on days 0, 30, 60, and 90 for both exposures.

Growth

Whole tank biomass was determined weekly in the Satiation Exposure and monthly in the Limited Ration Exposure. Fish were quickly netted en masse into a 10L bucket that was lined with a plastic sieve and filled with water of the appropriate pH and temperature. The bucket and contents were weighed on a GSE 450 Scale Systems balance (Michigan, USA) and then the sieve and fish were lifted free of the bucket and the fish were replaced in the appropriate tank. The bucket, sieve, and water were then reweighed. The difference in weight was the whole tank biomass.

Additionally, as there is more inter-individual variability in growth when food intake is restricted (McCarthy et al. 1993), individual fish (n=24 per treatment) were followed throughout the Limited Ration Exposure. The number of marked fish declined, to as low as n = 8, over the 90 days due to mortality and loss of identifiable marking in some treatments. Before the exposure began, fish were chosen using a random number table, lightly anesthetized with MS222 which was neutralized with $NaHCO_3$, and then marked with a unique identifier using a Pan-jet needleless injector (Wright Dental Group, Scotland). Weight and fork length were measured.

Condition factor was calculated using the weights and lengths of the individual fish sampled at each time period, using the equation:

$$CF = 100 \times (\text{body weight (g)})/(\text{total length (cm)})^3.$$

Specific growth rate (SGR) was estimated using the equation:

$$SGR = (\ln wt_{90} - \ln wt_0)/ 90 * 100\%$$

in which 90 represents the number of exposure days, and wt_{90}= weight at day 90 and wt_0= weight at day 0. Average weights of the fish sacrificed on days 0 and 90 were used in this calculation for the Satiation Exposure. In the Limited Ration Exposure, the SGR calculation employed the individual weights of marked fish followed throughout the 90 days.

Metabolic Rates

All oxygen consumption and nitrogenous waste excretion data were corrected for fish size differences using the weight exponent 0.824, determined for rainbow trout by Cho (1992).

i) Oxygen Consumption

In the Satiation Exposure, oxygen consumption (M_{O2})was determined using closed system respirometry every second hour over a 10 h period from 7:00 and 17:00

hours. Fecal matter was siphoned from the tanks. Each tank was then sealed with an air-tight, transparent lid and water within the tank was recirculated using a pump (Little Giant, 1EUAA-MD). Water samples were taken every 20 minutes for an hour and then water flow and aeration were resumed for one hour. P_{O2} was measured using a thermostatted Radiometer/Copenhagen E5046 oxygen electrode and Cameron Instrument Co. oxygen meter. The rate of oxygen depletion was determined for each hour using oxygen solubility coefficients from Boutilier et al. (1984), factored by time, volume and total fish weight to yield the rate of M_{O2}. Mean M_{O2} values were graphed against time and the curve produced was integrated, for each treatment, to give an overall mean M_{O2} value for that 10 hour period. During the Limited Ration exposure, oxygen consumption and nitrogenous waste excretion were determined simultaneously over 48 hours using the same methods. The measurements made in the first 24 hours were of fish that had not been fed for three days, while measurements in the second 24 hour period started one hour after fish had finished eating. These two different measurement regimes were used in these two exposures, to best capture the different daily patterns of M_{O2} due to feeding regimes

ii) Nitrogenous Waste Excretion

In the Satiation Exposure, in-tank nitrogenous waste excretion (M_N) was measured over 24 hours, using six 1 hour periods. Water flow to the tank was discontinued and samples were taken at the beginning and end of the one hour periods. Flow to the tank was then resumed during the following three hours. Water samples were frozen at -20°C for later analysis of ammonia-N by the salicylate-hypochlorite assay (Verdouw et al. 1978) and urea-N by the diacetyl monoxime method after a five fold concentration of the water sample by lyophilization (Lauff and Wood 1996). Nitrogenous waste production was determined by the difference between N concentration at the beginning and end of the flux period and was factored by time, volume and total fish weight. Data over the 24 hour

sampling period were graphed against time, and the area under the curve was determined for each treatment to give a 24 hour nitrogenous waste production rate. In the Limited Ration Exposure, similar methods were used to determine M_N. Due to the smaller biomass, six 3 hour sample periods were measured over the 48 hour period. Again, this encompassed the two different stages in the feeding regime.

Fractional protein utilization (FPU) was determined for each treatment at each sampling period. FPU is an index of the fraction of MO_2 that is supported by protein metabolism i.e. the degree that the fish depends on protein as a fuel source. FPU is calculated by dividing the nitrogen quotient (NQ = the ratio of the moles of nitrogen produced to moles of oxygen consumed) by the theoretical maximum NQ in which protein supports all aerobic metabolism. This theoretical maximum for fish has been determined by Kutty (1972) to be 0.27.

Whole Body and Blood Sampling

In the Satiation Exposure, 20 fish were randomly chosen from each treatment tank on each sampling day. Ten fish were rapidly killed by a blow to the head, blotted dry, and weight and length were measured. Blood was collected into ammonium heparinized capillary tubes after caudal severance . Hematocrit was determined following centrifugation at 10 000g, and plasma protein was measured using a hand-held refractometer (American Optical). Remaining plasma was frozen at -70°C for subsequent analysis of plasma [Na^+], [Cl^-], and cortisol. The other ten fish were killed with a lethal dose of MS222. These fish were immediately freeze-clamped using aluminum tongs that were chilled in liquid nitrogen and then stored at -70°C.

During the Limited Ration Exposure, ten fish, at each sampling period were rapidly killed by a blow to the head; plasma was taken and then the bodies were freeze clamped as previously described. Fish with unique identifiers were not sacrificed prior to

day 90. To ensure that the marked fish were representative of fish in the tank, all fish were handled during the measuring of the marked fish.

Sample Processing

Whole bodies were ground frozen using an IKA (M10/M20) grinding mill cooled to -70°C with dry ice and methanol. Approximately 2g of this ground tissue was oven dried at 80°C until a constant weight was achieved, to determine water content. Remaining frozen tissue was lyophilized (Labconco Lyph-Lock 6) and stored desiccated at -20°C for proximate analysis. Whole body protein, lipids and carbohydrates were quantified on the freeze dried tissue. Protein was measured by the Lowry assay as modified by Miller (1959), lipids by the chloroform/methanol extraction method (Folch et al. 1957), and standard enzymatic analyses (Bergmeyer 1985) were employed to measure glucose, glycogen and lactate. The sum of the latter three was taken as an estimate of carbohydrates. Whole body ions were determined from lyophilized tissue which was digested in 8% perchloric acid at a 9:1 ratio. Whole body $[Na^+]_{wb}$ $[Ca^{2+}]_{wb}$ and $[K^+]_{wb}$ and plasma $[Na^+]_p$ were then determined by atomic absorption spectroscopy and $[Cl^-]_{wb}$ and $[Cl^-]_p$ were determined using coulometric titration (Radiometer CMT10). Cortisol was measured using a I^{125} Radioimmunoassay (ICN Biomedicals).

Statistical Analysis

Values are given as the mean ± standard error (SEM) (n= number of fish). There were no significant differences between replicate tanks and thus data were combined in all analyses. Mean values were compared using one-way ANOVA (SAS JMP Version 2.0.5). When the F-value indicated significance, a Tukey-Kramer comparison of all pairs was used to determine treatment differences within a sampling period. Note that statistical comparisons were not performed for the metabolic rate measurements (oxygen consumption and nitrogenous waste excretion) because only whole tank measurements

were made for each treatment. In these cases, the reported SEM's were for repeated measurements of the same tank. To determine significant differences between the Satiation and Limited Ration exposures, an unpaired Student's T test was used to compare the day 90 data of the same treatment groups. A Chi-Square Test for Distribution was used to determine if there were any significant differences in mortality amongst treatments. The level of significance chosen was $p<0.05$.

Results

Temperature Profile and pH

Average low pH regime was 5.14 ± 0.03 for the Satiation Exposure and 5.27 ± 0.02 for the Limited Ration Exposure, while the control pH was 6.1 ± 0.03 for both exposures. As the exposures deliberately used water that followed the natural thermal fluctuations of inshore Lake Ontario, and these varied in the two years, unavoidably, the temperatures regimes were slightly different. In the Limited Ration Exposure, temperatures were about 2°C higher than in the Satiation Exposure. However, temperature profiles in the two exposures were similar with temperatures remaining relatively constant from day 0 to day 75, and then increasing steadily until day 90 (Figure 2-1).

Mortalities

During the Satiation Exposure less than 10% of fish died from treatment effects (data not shown). However, in the Limited Ration Exposure, significant treatment related mortality was seen. Most mortality occurred in the acid exposed fish between days 50 to 90; cumulative mortality was 34 % in the 0/5.2 treatment and 39% in the 2/5.2 treatment. These values were significantly greater than for the fish at circumneutral pH (Figure 2-2).

Appetite, Feeding, Growth and Condition Factor

Satiation Exposure

In this exposure, fish in the +2°C treatments consumed 1.5% to 3% of their body weight in food daily, while fish at the control temperatures consumed 1% to 2% of their wet body weight daily. Acid exposed fish had a slightly increased appetite compared with their respective controls. For example, the 2/5.2 group consumed 29.7 g of food/fish by the end of the exposure, while the 2/6.1 group consumed only 23.2 g/fish (Figure 2-3). Increased food consumption was accompanied by increased growth in all treatment groups (Table 2-1). Specific growth rate (SGR) was greatest in the fish exposed to 2/5.2 at 2.07%/day (Table 2-1). Fish exposed to +2°C gained significantly more weight than fish at control temperatures (Figure 2-3) and utilized their food more efficiently (Table 2-2). Throughout the 90 days, gross conversion efficiency was slightly higher in the +2°C treatments and at this elevated temperature, acid exposed fish utilized their food slightly more efficiently than non-acid exposed fish. This same pattern was not repeated in fish at control temperatures. By day 90, the 2/5.2 fish had the highest weight which was significantly different from those of the control temperature treatments, but not statistically different from the 2/6.1 treatment (Figure 2-3).

Limited Ration Exposure

In this exposure, when fish were fed only ~0.25% of their body weight per day, fish at control temperatures were fed ~85% less food than fish in the same treatments during the Satiation Exposure, while fish at +2°C received ~95% less food than their respective treatments in the Satiation Exposure (Figure 2-3). Fish fed the limited ration had significantly lower condition factor (CF) and SGR's compared to the Satiation Exposure, independent of treatment. Fish maintained on this limited ration had relatively constant

weights (Figure 2-3) and no significant differences in SGR (Table 2-1). Fish were in a catabolic state from days 0-30 and 60-90 (Table 2-2).

Plasma

Satiation Exposure

Plasma $[Na^+]_p$ and $[Cl^-]_p$ showed only a few treatment related changes (Table 2-3). On day 75, the 0/5.2 fish had a higher $[Na^+]_p$ compared to the 0/6.1 treatment and by day 90, 2/5.2 fish had a lower $[Na^+]_p$ than the 0/5.2 treatment, though this was not statistically different from the other two treatments. $[Cl^-]_p$ was significantly higher in the 0/5.2 treatment compared to the pH 6.1 treatment. Hematocrit fluctuated, with transient differences at day 75. By day 90, hematocrit in the 2/6.1 treatment was higher than in the control temperature treatments, though this temperature difference did not prevail in the 2/5.2 group which was no different from any of other 3 treatments. Plasma protein showed no changes over the exposure in any treatments (Table 2-3). Cortisol, as determined by a radioimmunoassay was high in the day 0 sampling but this may have been an artifact of sampling such small fish (Figure 2-4). By day 75, values were between ~4-7 ng/mL and increased by day 90 to ~8-11ng/mL.

Limited Ration Exposure

$[Na^+]_p$ and $[Cl^-]_p$ showed a number of transitory differences, but one trend that continued throughout the exposure was the significantly lower plasma ion concentrations in the 2/5.2 treatment (e.g.$[Na^+]_p$ ~108mM and $[Cl^-]_p$~106mM at day 90) (see Table 2-3). Hematocrit and plasma protein showed a number of transitory differences (Table 2-3) and on day 90, the 2/5.2 group had elevated levels compared to the other treatments. Cortisol levels at day 30 had increased from day 0 and then surged at day 60, correlating with the increased rate of mortality. By day 90, cortisol levels had decreased from day 60 values but were still elevated with the highest level in the 2/5.2 treatment (Figure 2-4).

Satiation and Limited Ration Exposure

When comparing treatment groups between the two ration regimes on Day 90 (Table 2-3), fish maintained on the limited diet at pH 6.1 had a higher $[Na^+]_p$ than fish in the Satiation Exposure at pH 6.1. On the other hand, the 2/5.2 limited ration fish had lower ion levels than their satiation fed counterparts. However, there were no significant differences between the 0/5.2 treatment groups for $[Na^+]_p$ and $[Cl^-]_p$ based on feeding regime. This trend did not hold for $[Cl^-]_p$ for fish at pH 6.1. Satiated fish in the 2/6.1treatment had a higher chloride level than fish in the Limited Ration Exposure, but this difference was not seen at control temperatures. Hematocrit was significantly higher in the Satiation Exposure except between the 2/5.2 groups, in which values for the Limited Ration Exposure were higher than the Satiation Exposure. Plasma protein was significantly higher, approximately two fold, in all satiation fed fish compared to the Limited Ration Exposure while cortisol levels were much higher, approximately six fold, in the Limited Ration Exposure (Figure 2-4).

Whole Body Ions and Whole body Analysis

Satiation Exposure

Whole body ions: $[Na^+]_{wb}$, $[Cl^-]_{wb}$, and $[K^+]_{wb}$ did not show any variation amongst treatments except for modest differences in $[Cl^-]_{wb}$ on Day 75 (see Figure 2-5). Water content decreased from ~77% to 73% over the 90 day exposure in all treatments (data not shown). Although protein increased slightly, the main compensating change was due to lipids which increased from ~5% to 7-9% (Figure 2-6). There were no treatment effects on water content, protein or lipids during the exposure. The only difference among treatments was for stored carbohydrates (glucose, lactate and glycogen) (Table 2-4) which were higher in the 2/5.2 treatment.

Limited Ration Exposure

$[Na^+]_{wb}$ and $[Cl]_{wb}$ decreased over the 90 days, most rapidly in the 2/5.2 group, and by day 90 both acid exposed groups showed lower levels of $[Na^+]_{wb}$ and $[Cl^-]_{wb}$. $[K^+]_{wb}$ was lowest in the 0/5.2 treatment from day 60 onwards (Figure 2-5). Water content increased over the 90 days from ~75% to ~79 - 80% in all treatments (data not shown). This increase was accounted for by a slight decrease in protein and a larger decrease in lipids. Protein showed a transient difference among treatments (Figure 2-6) but by day 90 there were no differences due to treatment. Lipids decreased by ~2-4% in all treatments over the 90 days except in the 0/5.2 group. There were no differences in stored carbohydrates among treatments on day 90.

Satiation and Limited Ration Exposure

By Day 90, $[Na^+]_{wb}$ and $[Cl^-]_{wb}$ were higher in the non-acid exposed fish of the Limited Ration Exposure compared to the satiated fed fish. Acid exposed fish, regardless of feeding regime, showed no feeding related differences in either $[Na^+]_{wb}$ or $[Cl^-]_{wb}$, but $[K^+]_{wb}$ was lower in fish fed a limited ration. Water content was significantly lower in the satiation fed fish compared to fish fed a limited ration. Protein was higher in all the satiation fed fish except for the 0/6.1 treatment, where there were no differences in protein content between the exposures. Lipids were higher in the satiation fed fish except for the 0/5.2 group.

Metabolic Rates

While statistical comparisons could not be performed (see Methods), a number of clear trends were apparent.

Satiation Exposure

At each sample period, fish exposed to the elevated temperature (2/5.2 and 2/6.1) showed an increased oxygen consumption (M_{O2}) compared with the fish at control

temperatures (Figure 2-7). By day 90, when the control temperature had increased to about 10°C, the difference between the +2°C and control temperature groups was not as marked. Acid exposure had no effect on M_{O2}.

Urea excretion accounted for ~11% and ammonia excretion for ~89% of the total nitrogenous waste, regardless of temperature or pH. Nitrogenous waste excretion rate (M_N) was higher throughout the exposure in the +2°C groups. Again, acid exposure had no effect on M_N (Figure 2-7). FPU increased from day 0 until day 60 in all treatments; by day 90 use had decreased in all treatments (Figure 2-7). The 2/5.2 treatment tended to use protein more than the other three treatments.

Limited Ration Exposure

M_{O2} for 24 hours prior to and post feeding were not dramatically different (~2.4 ± 0.2 μmol/g/hr compared to ~2.6 ± 0.2 μmol/g/hr), so the values were averaged together for Figure 2-7, There were no treatment effects on M_{O2} (Figure 2-7).

Urea excretion accounted for about 21% of the total nitrogenous waste excretion and ammonia excretion for ~79%. Neither temperature nor low pH caused any consistent variation in M_N. However, M_N showed a marked difference between unfed and fed states being ~33% higher in the 24 hours after feeding. In addition, feeding resulted in increased protein utilization, while utilization decreased ~30-50% after three days of not being fed. However, for the ease of comparison with the Satiation Exposure, pre and post values again were averaged together for both M_N and FPU (Figure 2-7). A higher FPU in the acid exposed fish after feeding (specific data not shown) indicates that these fish were using more protein than the non-acid exposed fish, throughout the 90 days (Figure 2-7).

Satiation and Limited Ration Exposure

M_{O2} and M_N was much lower for all groups, regardless of thermal regime or acid exposure in the Limited Ration Exposure as compared with the satiation fed fish. In

addition, acid exposed fish had a higher FPU suggesting increased reliance on protein for fuel.

Discussion

Temperature and food consumption are the two major influences on bioenergetics in freshwater fish (Jobling 1994). The present study examined how differences in the amount of food consumed influenced the physiological and metabolic response of rainbow trout to low pH, in conjunction with a slight increase in temperature (+2°C), equivalent to the rise expected for a global warming scenario (IPCC 1995). These experiments deliberately used a natural water source and therefore fluctuations in temperature occurred due to natural variation both within and between the two exposures. However, in the two separate years, fish were exposed to a similar thermal pattern: relatively constant temperatures from day 0 to 75, with a more rapid increase from days 75 to 90. Therefore comparisons made at day 90 were between similarly treated fish.

Low pH effects

Dockray et al. (1996) proposed that the ability to compensate for ionoregulatory disturbances due to low pH may depend on the amount of food consumed by fish. Results from these experiments clearly confirm that given sufficient food, fish can compensate for any increased energy expenditures and/or ionoregulatory disturbances. Fish on a limited ration, especially at slightly elevated temperatures, generally showed typical ionoregulatory disturbances (McDonald and Wood 1981; Fugelli and Vislie 1982; Lacroix 1985; Brown et al. 1984; Audet et al. 41988; Wood 1989), with lower levels of $[Na^+]_{wb}$, $[Cl^-]_{wb}$, and $[K^+]_{wb}$ as in the present study. Analysis of the plasma showed similar results, with lower $[Na^+]_p$, $[Cl^-]_p$ and increased hematocrit in acid exposed fish, especially, at +2°C compared to the control temperatures. In contrast, when fish were fed to satiation (given approximately ~1 to 3% of their body weight/day) and exposed to low pH, no whole body

ionoregulatory disturbances were found by day 90, though $[Na^+]_p$ values had declined in the 2/5.2 treatment (see temperature and feeding effects). Present results are consistent with the work of Sadler and Lynam (1986) who found greater ionoregulatory disturbances in starved fish at low pH.

Interestingly, these exposures also supported work by Smith et al. (1995) who found that fish could regulate transbranchial Na+ efflux depending on dietary salt consumption in non acid waters. In their work fish who consumed higher amounts of NaCl had increased Na^+ efflux. Thus, in the Satiation Exposure, the non-acid exposed fish probably had increased Na+ efflux, explaining why their whole body Na+ levels were lower than non-acid exposed fish maintained on the limited ration.

Better growth, as demonstrated by a higher SGR and higher CF was seen in the fish fed to satiation in the 2/ 5.2 treatment, compared to the non-acid exposed fish at this temperature. However, at control temperatures, there was no stimulatory effect of pH on either growth or M_{O2}. These results are contradictory to other investigators who found that sublethal low pH increased M_{O2}, (Hargis 1976; Waiwood and Beamish 1978; Butler et al. 1992), subsequently leading to reduction in growth. However, in two previous studies, when a similar satiation regime was employed, increased growth was reported in acid exposed fish (Wilson et al. 1994a; Dockray et al. 1996). A possible explanation for such discrepancies may be compensation for branchial ion loss by dietary means. Recent work by Smith et al. (1995) showed that fish vary branchial Na^+ influx and efflux depending on dietary salt intake. In acid exposed fish, appetite may have been stimulated by a Na^+ deficit (Salman and Eddy 1987), thereby, replacing branchial ion loss and whole body reserves, with a secondary effect of increased growth. In the +2°C fish, M_{O2} was not increased with increased feeding in the acid-exposed fish, and food was converted more efficiently, however, the acid-exposed and non-acid exposed fish had a similar level

of growth (Table 2-2). These results are different from juvenile Salmo salar which had depressed appetites when exposed to low pH (Waiwood et al. 1992).

Whole body composition of fish fed to satiation and exposed to low pH showed no treatment related differences with regards to lipids and protein components. However, carbohydrates stores were higher in 2/5.2 treatment by day 90 . In the Limited Ration Exposure, there were only slight changes in weights in the four treatments, in contrast to the large weight gain in the satiation fed fish. Whole body lipids decreased and water content increased, except in the 0/5.2 treatment, suggesting that fish were in a state of catabolism, using their lipid stores for energy. In the 0/5.2 treatment, lipid stores were 7% in comparison to ~2-4 % in the other three treatments. As water content, protein and stored carbohydrates were similar to the other treatments, the inherent difference in this treatment may be due to significantly decreased amounts of inorganic content (ash). The feeding cycle employed in the Limited Ration Exposure (1% every four days) may have helped fish conserve whole body protein (Kaushik and Gomes 1988).

Temperature and Feeding Effects

Routine M_{O2} was used as an integrative measure of the cost of energy expenditure in these exposures. Routine M_{O2} includes not only basal metabolism but also activity related metabolism, and may be influenced by temperature, food intake and energy requirements for the biochemical and mechanical aspects of feeding and digestion (specific dynamic action (SDA; Cho and Kaushik 1990). In the present study, food intake was found to greatly influence M_{O2}. M_{O2} was ~50% less in fish fed 0.25%/day, which approximated a maintenance ration (a level of dietary intake at which animals neither gain nor lost weight; Kaushik and Gomes 1988) compared to fish fed to satiation. M_{O2} differences between feeding regimes were probably due to both increased SDA and increased activity in the fish fed to satiation, i.e. a higher metabolic rate caused by

increased food intake (Beamish 1974a; Brett and Groves 1979; Cho and Kaushik 1990; Alsop and Wood 1997).

Temperature also impacted greatly on metabolic rate in satiated fish but had no effect on fish fed a limited ration. This may be because the latter fish tended towards a state of starvation (Brett and Groves 1979; Jobling 1994) and thus, regardless of temperature maintained a very low metabolic rate. However in satiated fish a slight temperature increase caused a ~25 to 35% increase in M_{O2}. This increase in M_{O2} was probably due to increased SDA and a higher metabolic rate due to the increased temperature (Brown 1946; Brett 1971). These results suggest there is a greater temperature dependence of SDA than there is for basal metabolism. Work by Soofiani and Hawkins (1982) indicated a similar situation in juvenile cod, in which increasing temperatures more dramatically influenced SDA than basal metabolism.

Satiation feeding resulted in relatively high growth rates for all treatments, with large accumulation of lipid stores. Distinct differences in growth rates between +2°C and control temperatures occurred mainly due to increased appetite. As well, fish in the +2°C treatments were closer to their optimal temperature for growth (15 and 20°C; Cho and Kaushik 1990). Fish maintained on the limited diet lost weight gradually. The weight loss between days 0-30 was probably due to "adaptation" to the new eating regime (Brett et al. 1969; Kaushik and Gomes 1988). Between, days 30-60 there was improved food conversion efficiencies, however, the +2°C did not influence this conversion. From days 60-90, gross food conversion efficiency was again quite low, especially in the acid exposed treatment. This period encompassed the most dramatic increase of water temperatures and the highest rate of mortality, suggesting that during this period, dietary energy was being utilized in maintaining ionoregulation rather than maintaining body weight. When fish were fed a limited ration and held in a more constant environment, i.e.

not adapting to the feeding regime or changing temperatures, food conversion efficiencies (Table 2-2) were higher than in fish fed to satiation, similar to results reported by Paloheimo and Dickie (1966). Jobling (1980), Miglavs and Jobling (1989) and Quinton and Blake (1990) have demonstrated that starvation or restricted feeding leads to reduced lipid content and a replacement of this lipid with water in fish tissues as seen in the present results.

The slight increase in water temperatures from approximately day 75 to 90 seemed to have been stressful for both satiation fed and limited ration fish exposed to the 2/5.2 treatments, though effects were less dramatic in the former exposure. In the Limited Ration Exposure, fish in the 2/5.2 treatment had greater mortality and ionoregulatory disturbances. Effects in the satiated fed 2/5.2 treatment included increased ionoregulatory disturbance with lowered $[Na^+]_p$ and $[Cl^-]_p$. These effects were not mimicked in 0/5.2 treatment, suggesting that this increase in temperature was more stressful for the 2/5.2 exposed fish. A possible explanation may be that because satiated fish in the 2/5.2 treatment already had elevated metabolic rates, increasing water temperatures resulted in additional ion loss. This phenomena has been coined the "osmorespiratory compromise" in which increased MO_2 corresponds with increased movement of all permeable electrolytes across the gills (Randall et al. 1972; Gonzalez and McDonald 1992). As the fish in the 0/5.2 treatment had a lower metabolic rate, consequently, they were able to meet the increasing energy demands brought about by the rising water temperatures.

Nitrogenous waste excretion followed similar trends to M_{O2} with rates being much higher in satiation fed fish. Increased feeding corresponded to increased nitrogen losses associated with the assimilation and deamination of protein (Jobling 1981). In addition, satiated fish used protein as fuel to a greater extent, as determined by a higher FPU value (Figure 2-7). Nevertheless endogenous protein stores were still increased because of the

higher levels of plasma protein compared to the fish maintained on a limited ration. Though protein utilization decreased from day 60 in the satiation fed fish, it was higher in acid exposed fish compared to control pH fish. In the Limited Ration Exposure, prior to feeding, the nitrogenous waste excretion profile suggests M_N was of an endogenous sources, and the increase after feeding was due to exogenous nitrogen sources (Jobling 1981). By day 90, the decreased levels of whole body protein indicate that these fish were utilizing protein energy stores (Jobling 1981).

Indicators of Stress

The generalized stress response, characterized by activation of the pituitary interrenal axis, results in release of cortisol into circulation (Donaldson 1981). Elevated plasma cortisol levels have been reported in a number of longer term low pH studies (Brown et al. 1984,1986; Brown et al. 1989; Audet and Wood 1993) while in shorter term exposures to low pH, basal cortisol levels were recovered (Goss and Wood 1988) or did not increase (Balm and Pottinger 1993). In the present study, cortisol levels were elevated in the treatments experiencing greater ionoregulatory disturbances, but also seemed greatly affected by ration level. Indeed, all treatments in the Limited Ration Exposure had elevated levels compared with the satiation fed fish. However, Anderson et al. (1991) found that cortisol was not elevated in starved fish, suggesting there are distinct differences in the stress response depending on nutritional status. For example, in this study when fish were fed ~1 to 3 % of their body weight daily, the interrenal axis was not activated while fish fed 1% of their body weight every four days showed a marked elevation in cortisol. Thus, either no food or an abundance of food prevents activation of the interrenal axis.

Increased mortality during the Limited Ration Exposure also indicates that the fish were stressed. This mortality is probably not size related as Toneys and Coble (1980)

reported that there were no differences in mortality depending on fish size in a over-winter studies (40 g in Satiation Exposure versus 10g in Limited Ration Exposure). Mortality in the Limited Ration Exposure was probably due to impaired ionoregulation which resulted in a shift of fluid from the extracellular compartment to the intracellular compartment thereby resulting in decreased circulatory efficiency (Milligan and Wood 1982).

The decreasing levels of protein, and generally decreased levels of lipids in limited ration fish, suggests that at this thermal regime (8-12°C), feeding restriction is itself stressful. Further support for this is provided by the satiated fish with their high endogenous energy stores that did not change regardless of thermal or pH regime.

Summary

When fish were fed to satiation at temperatures between 8 and 12°C, they did not suffer from ionoregulatory disturbances or mortalites when exposed to low pH and increased temperature. In fact, metabolic rate, growth and appetite were increased dramatically in the +2°C fish. Acid exposure at this temperature stimulated appetite, possibly because of a need to replace ions lost through branchial efflux with those from dietary salts, acid exposure also improved food conversion efficiency. However, when +2°C fish were faced with the need to acclimate to slightly higher temperatures due to seasonal changes, ion loss increased, though this may have been transitory. We speculate that given enough time and food, fish would have replaced this ionic loss.

When fish were maintained on a limited ration, acid exposure resulted in ionoregulatory disturbances and hematological changes resulting in a higher mortality rate. These data support the hierarchy of energy allocation, such that fish with lower energy intakes and growth rates have proportionately greater responses to stresses (Rice 1990). A slight elevation in temperature did not increase metabolic rate or growth and tended to be detrimental, especially for the acid exposed fish. These fish were not able to compensate

for the increased energy expenditure and salts needed to maintain homeostasis during this thermal regime. The Limited Ration Exposure is probably more ecologically relevant as food deprivation is a more common occurrence in winter (Smith et al. 1989; Smith and Griffith 1994). Thus, a slight increase in temperature will be detrimental to the fish population living in a marginalized environment.

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Table 2-1: Condition Factor (CF), (after 90 days of exposure) and Specific Growth Rate (SGR) (%/day) over 90 days in each treatment. SGR's for the Satiation Exposure were calculated using the average weights for thirty fish at day 0 and sixty fish per treatment at day 90 and therefore could not be evaluated statistically. SGR's for the Limited Ration exposure were calculated using the individually marked fish (n=8-24) that were monitored throughout the 90 day exposure. No significant differences for SGRs occurred within the Limited Ration Exposure. There were only significant differences ($p<0.05$) for CF in the Satiation Exposure, and these are indicated by treatment groups that do not share a letter.

	Satiation Exposure				**Limited Ration Exposure**			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**	**0/5.2**	**0/6.1**	**2/6.1**	**2/5.2**
CF	1.05 ± 0.01^{a}	1.05 ± 0.01^{a}	1.08 ± 0.01^{ab}	1.12 ± 0.01^{b}	0.91 ± 0.09	0.87 ± 0.02	0.79 ± 0.02	0.77 ± 0.02
SGR (%/day)	1.25	1.27	1.78	2.07	0.02 ± 0.1	0.03 ± 0.1	-0.22 ± 0.1	-0.17 ± 0.1

Table 2-2: Food conversion efficiencies (wet weight gained by fish: wet weight of food eaten) calculated for each 30 day period. Measurements were determined by calculating the amount of food consumed per tank and the change in whole tank biomass for that period.

a) Satiation Exposure

Time Period	**Treatment**			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**
0-30	0.56	0.56	0.82	1.07
30-60	0.97	1.13	1.34	1.39
60-90	0.72	0.98	1.11	1.18

b) Limited Ration Exposure

Time Period	**Treatment**			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**
0-30	-0.61	-0.42	-0.23	-0.46
30-60	1.21	1.48	0.55	1.21
60-90	-1.26	-0.25	-0.77	-1.95

Table 2-3: Measured plasma $[Na^+]_p$, plasma $[Cl^-]_p$,hematocrit (%) and plasma protein (g/100ml) for each sampling period. Mean values ± SEM (n=10-20) are shown. Treatments that share a letter are not significantly different from one another other ($p<0.05$).

a) Satiation Exposure

	Day 75				Day 90			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**	**0/5.2**	**0/6.1**	**2/6.1**	**2/5.2**
$[Na^+]_p$ (mM)	131.2^a	124.8^b	128.7^{ab}	131.9^{ab}	131.3^a	125.0^{ab}	124.8^{ab}	117.8^b
	± 1.6	± 1.0	±2.0	±2.2	± 1.8	±1.4	±.9	± 1.4
$[Cl^-]_p$ (mM)	122.4	124.5	124.3	127.7	128.2^a	122.9^b	123.6^b	125.1^{ab}
	± 1.9	± 1.5	± 1.0	± 1.8	± 1.4	±1.8	± 0.6	± 1.1
Hematocrit	34.7^{ab}	30.7^a	37.8^{bc}	40.7^c	36.2^a	39.5^a	45.7^b	42.0^{ab}
(%)	± 1.0	± 1.5	± 1.3	± 1.9	± 0.8	± 2.3	± 1.7	± 1.4
Protein	5.4	5.3	5.6	5.5	5.78	5.36	5.59	6.2
(g/100ml)	± 0.2	± 0.2	± 0.2	± 0.4	± 0.2	± 0.1	± 0.2	± 0.4

Table 2-3 continued

b) Limited Ration Exposure

	Day 30				Day 60				Day 90			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**	**0/5.2**	**0/6.1**	**2/6.1**	**2/5.2**	**0/5.2**	**0/6.1**	**2/6.1**	**2/5.2**
$[Na^+]_p$ (mM)	129.2^b	133.9^a	135.2^a	124.0^b	125.2^a	26.23^a	124.9^a	116.1^b	124.9^a	129.3^a	130.5^a	108.3^b
	± 2.5	± 1.4	± 1.9	± 3.6	± 2.7	± 2.4	± 1.4	± 2.2	± 3.8	± 0.9	± 1.5	± 4.0
$[Cl^-]_p$ (mM)	123.6^b	127.9^a	126.4^a	118.6^b	120.7^a	129.9^a	124.0^a	105.5^b	125.0^a	119.4^a	125.6^a	106.8^b
	± 2.6	± 1.3	± 4.1	± 4.2	± 1.0	± 1.9	± 2.2	± 4.4	± 1.3	± 2.5	± 1.4	± 3.9
Hematocrit	40.5^a	37.1^{ab}	37.0^{ab}	33.2^b	30.0^{ab}	29.0^b	33.7^a	39.7^c	33.2^a	30.0^{ab}	31.6^a	36.2^b
(%)	± 2.7	±1.7	±1.3	±1.3	±1.0	±1.3	±0.9	±1.2	±1.2	±1.0	±1.1	±1.2
Protein	2.7	2.9	2.9	2.5	3.0^a	2.8^a	2.6^a	3.8^b	2.2^a	2.9^b	2.4^{ab}	3.0^b
(g/100ml)	±0.1	±0.2	±0.1	±0.2	±0.2	±0.2a	±0.1a	±0.2b	±0.2	±0.1	±0.2	±0.2

Table 2-4: Stored carbohydrates (the sum of glucose, lactate, and glycogen) at Day 0 and 90, expressed as a percentage of whole body composition. Significant differences were seen only in the Satiation Exposure and are indicated by treatments that do not share a letter ($p<0.05$).

a) Satiation Exposure

	Day 0	**Day 90**			
		0/5.2	**0/6.1**	**2/6.1**	**2/5.2**
carbohydrates	0.39	0.39^{a}	0.30^{ab}	0.29^{b}	0.35^{ab}
±SEM	0.03	0.03	0.03	0.03	0.03
n	4	10	9	9	6

b) Limited Ration Exposure

	Day 0	**Day 90**			
		0/5.2	**0/6.1**	**2/6.1**	**2/5.2**
Carbohydrates	0.30	0.20	0.29	0.24	0.25
±SEM	0.02	0.03	0.04	0.04	0.03
n	6	8	6	6	8

Figure 2-1

Water temperatures experienced by juvenile rainbow trout over the two 90 day exposure periods. Control temperatures gradually increased from 8°C to 12°C, with a more rapid increase in water temperatures between days 75 and 90. The solid lines represent the Satiation Exposure thermal regime (January to April, 1994) and the broken lines represent the Limited Ration thermal regime (January to April, 1996).

Figure 2-1

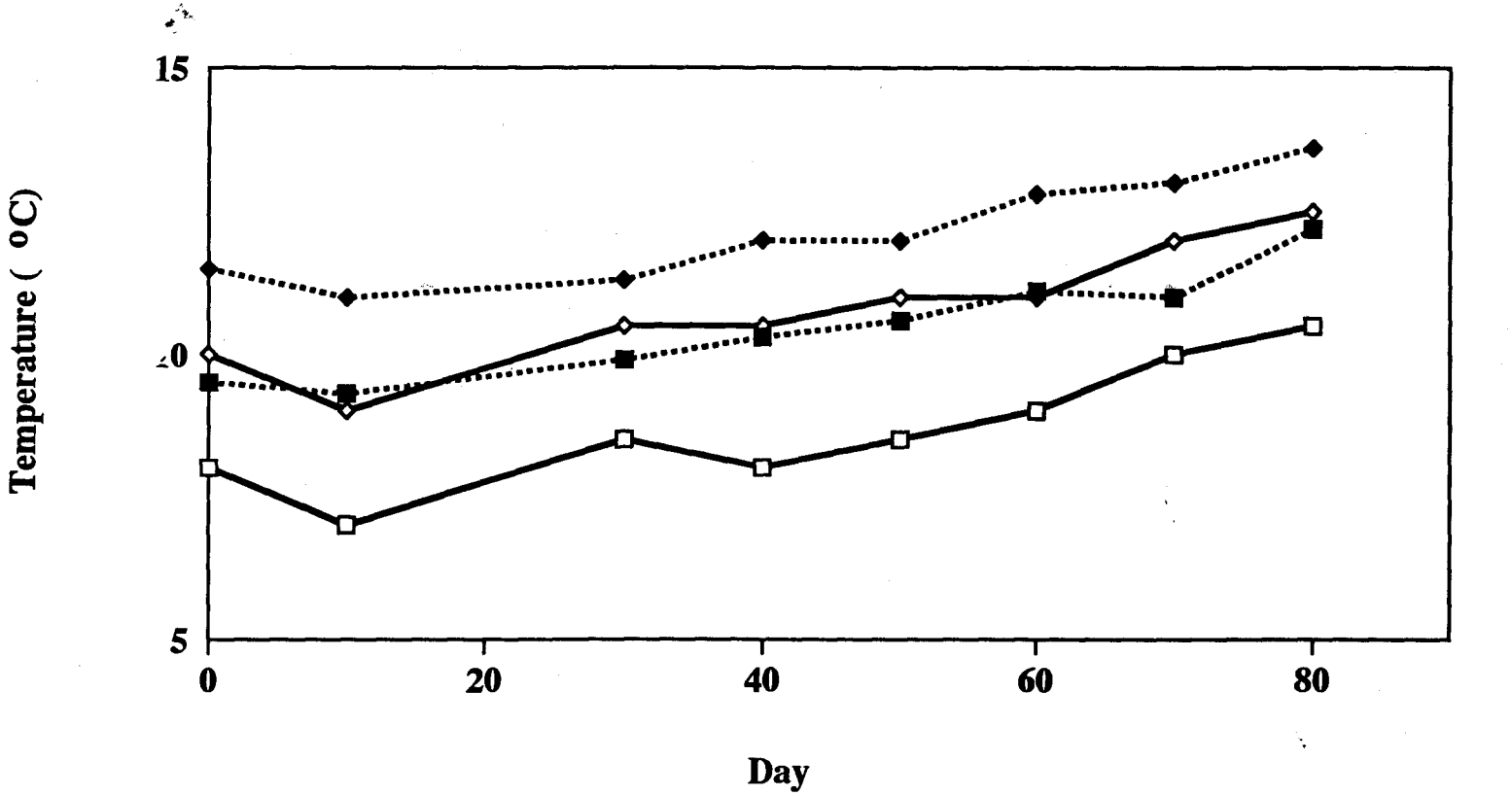

Temperature (°C)
15
10
5
0
20
40
60
80
Day
Limited Diet: Control Temperature
Satiation: Control Temperature
Limited Diet: +2 °C
Satiation: +2°C

Figure 2-2

Cumulative mortality for the Limited Ration Exposure over the 90 days. Based on a Chi-Square Analysis the acid exposed fish have a significantly higher mortality, denoted by an *.

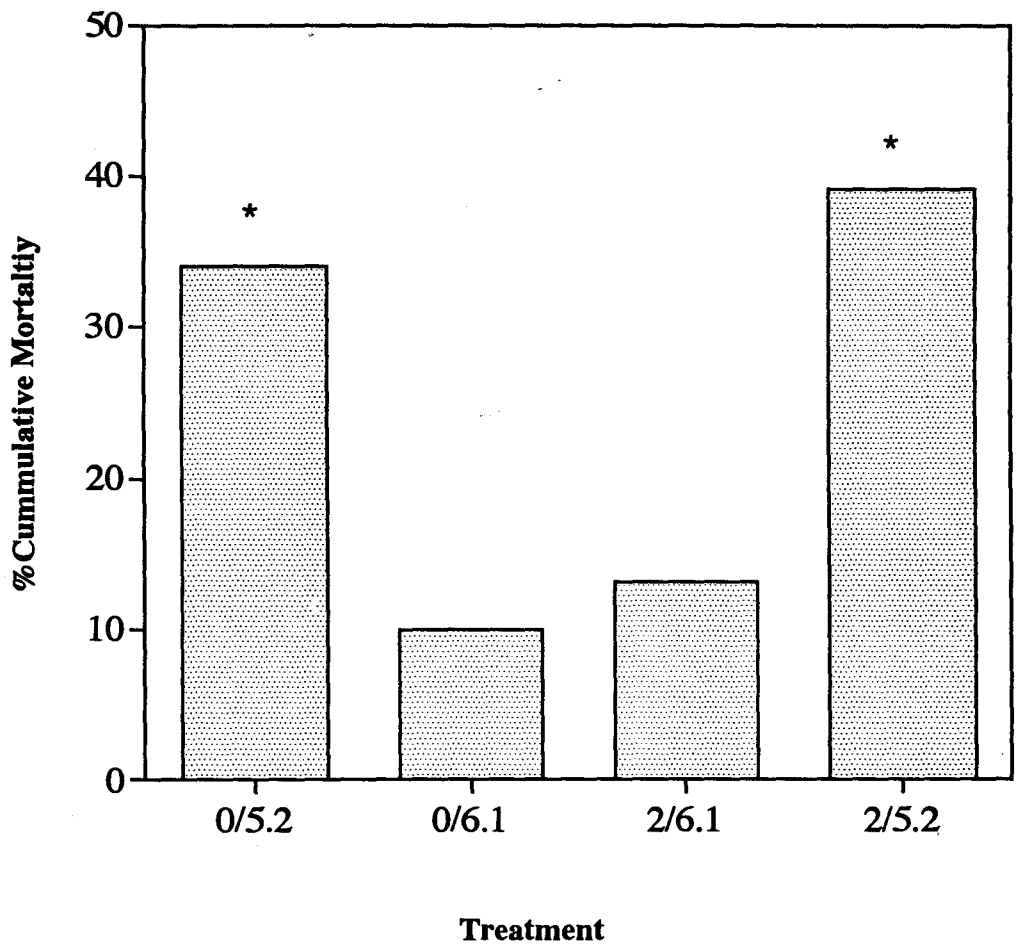

50
40
30
20
10
0
% Cummulative Mortaltiy
*
*
0/5.2
0/6.1
2/6.1
2/5.2
Treatment

Figure 2-3

Cumulative food intake, expressed on a wet weight basis (g/fish) in (A) Satiation Exposure and (B) Limited Ration Exposure. Absolute growth expressed as wet body mass (g), in (C) Satiation Exposure and (D) Limited Ration Exposure. Cumulative food intake "appetite" was calculated by the amount of food eaten per tank divided by the number of fish present; statistical comparisons are based on 180 daily appetite measurements. In the Satiation Exposure (A), fish in the +2°C thermal regimes consumed more food than fish at control temperatures. In comparison, in the Limited Ration Exposure, fish consumed 80% - 95% less than the fish fed to satiation. In the Satiation Exposure, (n=40 except in the 2/5.2 treatment where n=20), the fish in the +2°C treatments grew the most. Growth in the Limited Ration Exposure (n=20) was minimal. Within exposures, significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.

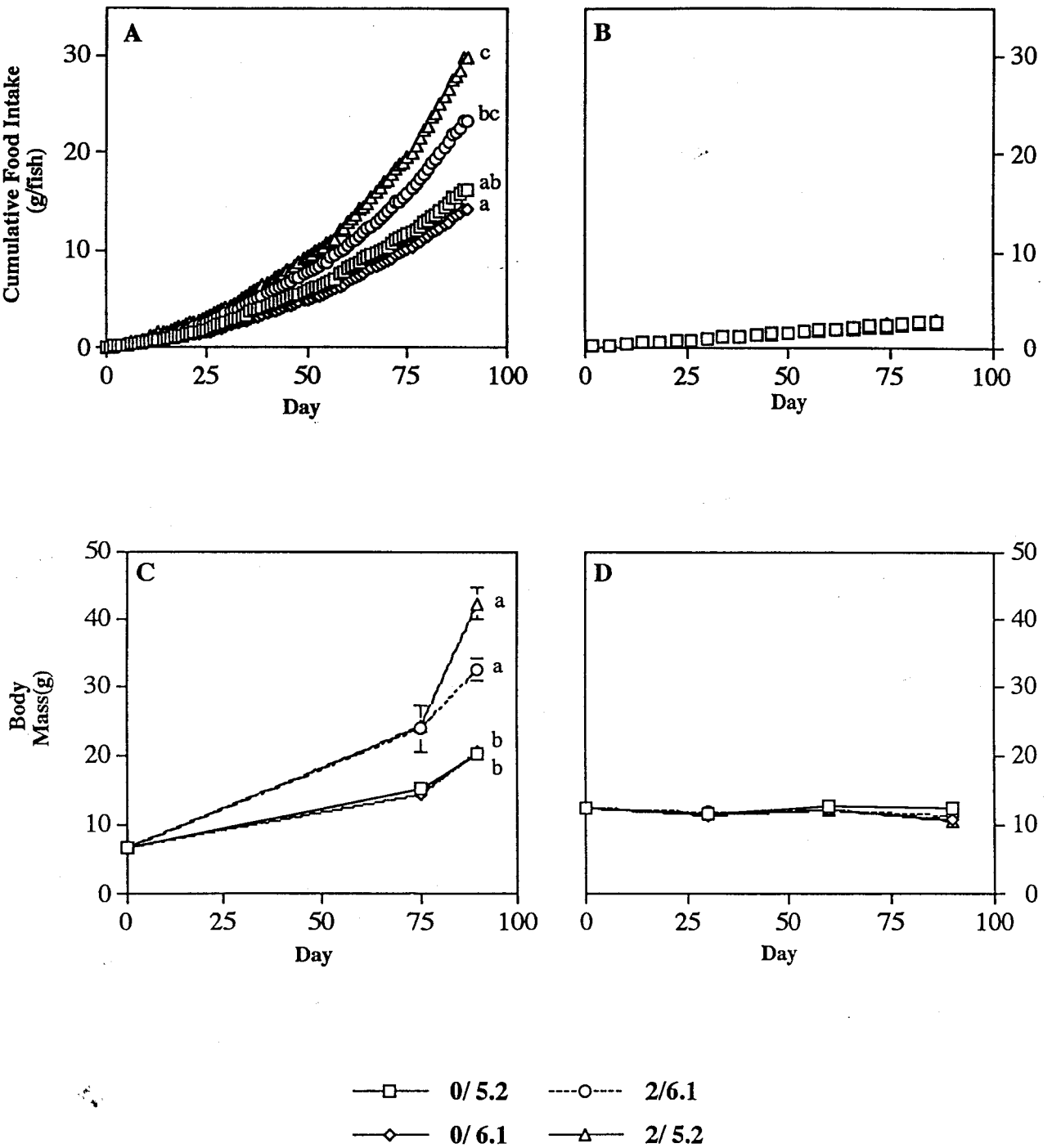

A
B
C
D
Cumulative Food Intake (g/fish)
Body Mass(g)
Day
c
bc
ab
a
a
a
b
b
0/ 5.2
2/6.1
0/ 6.1
2/ 5.2

Figure 2-4

Plasma cortisol (ng/mL) in the (A) Satiation Exposure and (B) Limited Ration Exposure. The black solid bar represents values are Day 0. The lightly speckled and heavily speckled outside bars represent the acid exposed fish, while the broken cross hatched and the full cross hatched represent the fish at control pH (6.1). The two bars on the left represent fish at control temperatures while the two bars on the right represent fish at +2°C above control temperatures. Values are given as means ± SEM (n=12 to 20) except in the Satiation Exposure 2/5.2 treatment where (n=10). Significant differences ($p<0.05$) were only seen in the Limited Ration Exposure on Day 30 and 90, and are indicated between means that do not share a common letter.

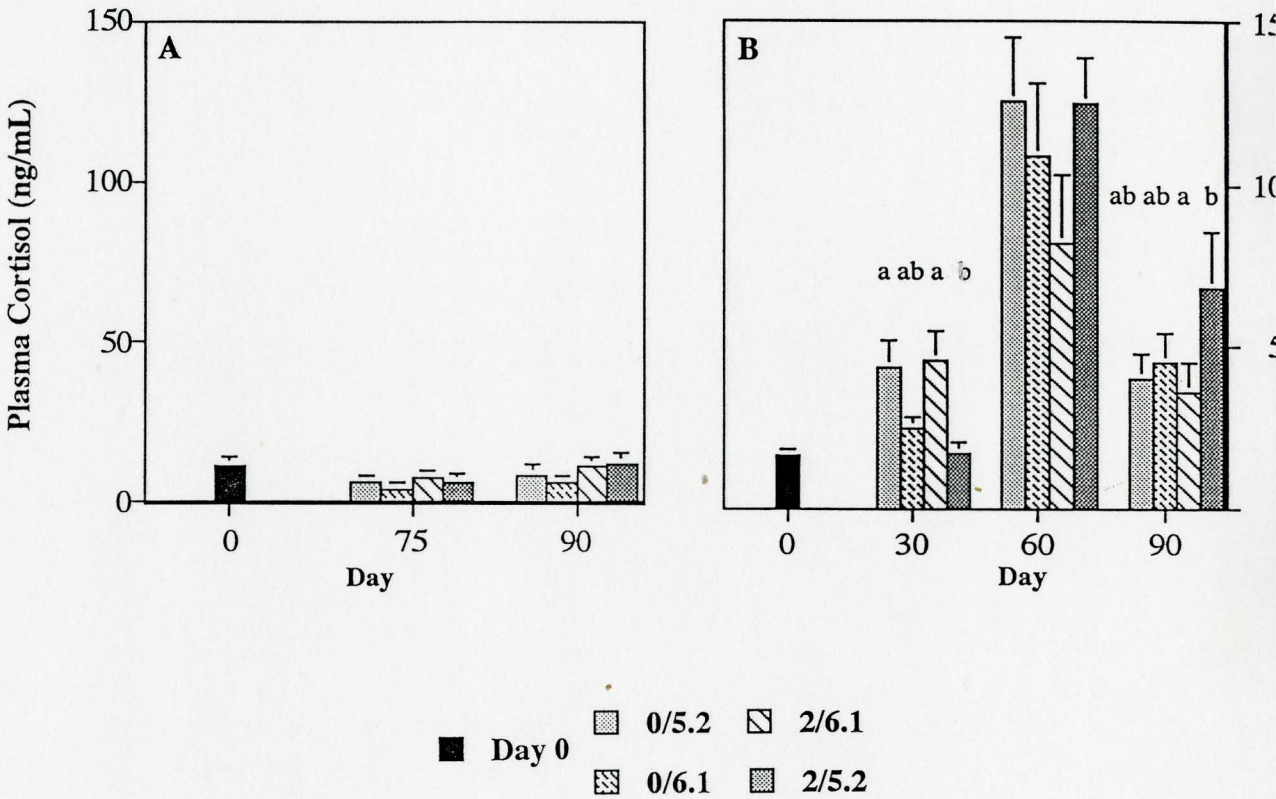

Plasma Cortisol (ng/mL)
150
100
50
0
A
0
75
90
Day
B
a ab a b
ab ab a b
0
30
60
90
Day
Day 0
0/5.2
2/6.1
0/6.1
2/5.2

Figure 2-5

Whole body ions (mmol/kg). (A - C) Satiation Exposure $[Na^+]_{wb}$, $[Cl^-]_{wb}$ and $[K^+]_{wb}$ and (D - F) Limited Ration Exposure $[Na^+]_{wb}$, $[Cl^-]_{wb}$, and $[K^+]_{wb}$. Other details are in the legend of Figure 2-4. Values are given as means $\pm$ SEM; n= 6-12 in the Satiation Exposure and n= 12-18 in the Limited Ration Exposure. Significant differences ($p<0.05$) are indicated by treatment means that do not share a common letter. If there are no letters present for the sampling period, there are no significant differences between means.

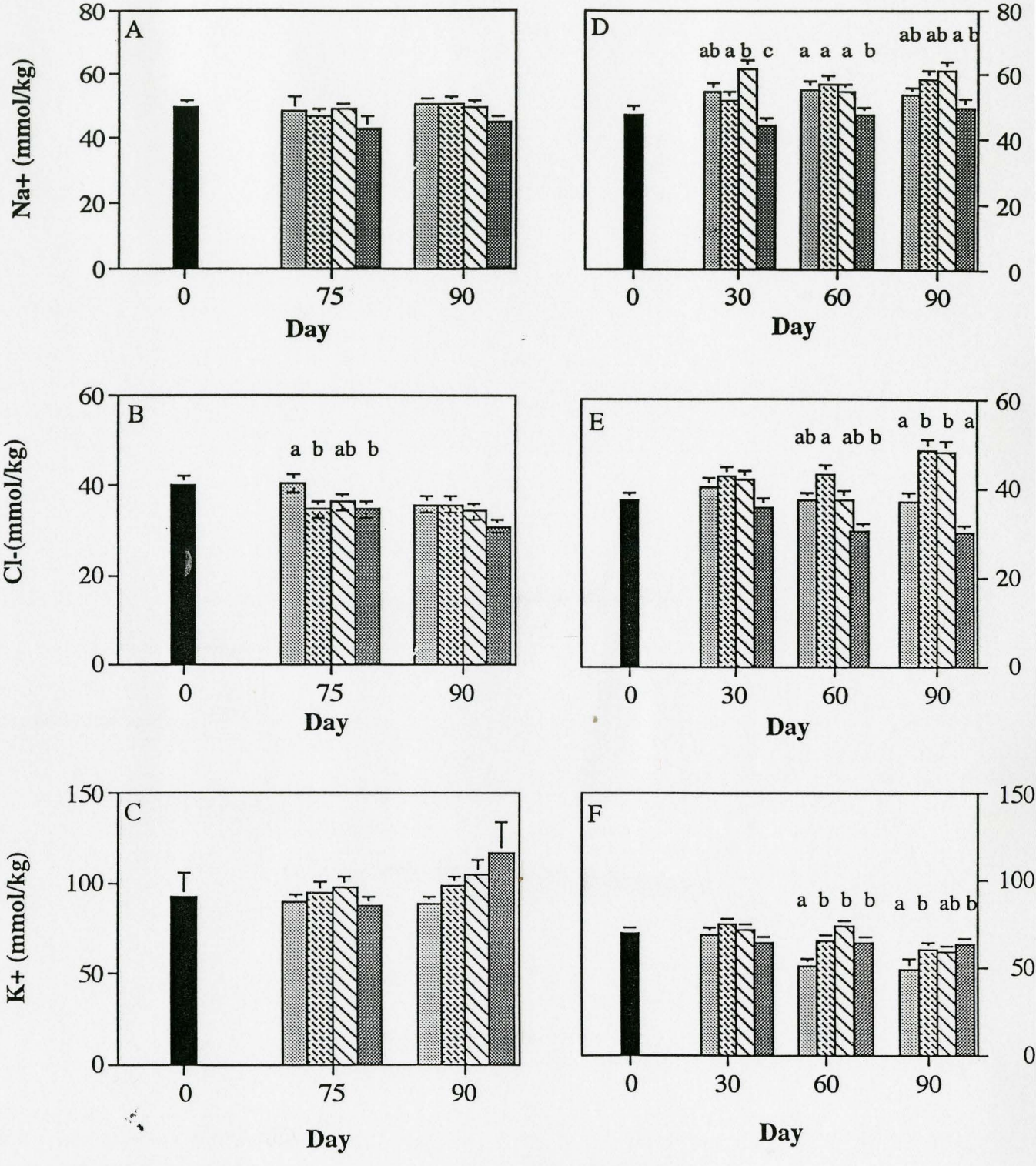

A
B
C
D
E
F
Na+ (mmol/kg)
Cl-(mmol/kg)
K+ (mmol/kg)
Day
0
75
90
30
60
a b ab b
ab a b c
a a a b
ab ab a b
a b b a
ab a ab b
a b b b
a b ab b
Day 0
0/5.2
2/6.1
0/6.1
2/5.2

Figure 2-6

Whole body protein (%) in the (A) Satiation Exposure, (n=12 except in the 2/5.2 treatment where n=6) and (B) Limited Ration Exposure (n=20). Whole body lipids (%) in the (C) Satiation Exposure (n=12 except in the 2/5.2 treatment where n=6) and (D) Limited Ration Exposure (n=20). Other details are in the legend of Figure 2-4. Values are given as means $\pm$ SEM. Significant differences ($p<0.05$) are indicated between means that do not share a common letter. If there are no letters present for the sampling period, there are no significant differences between means.

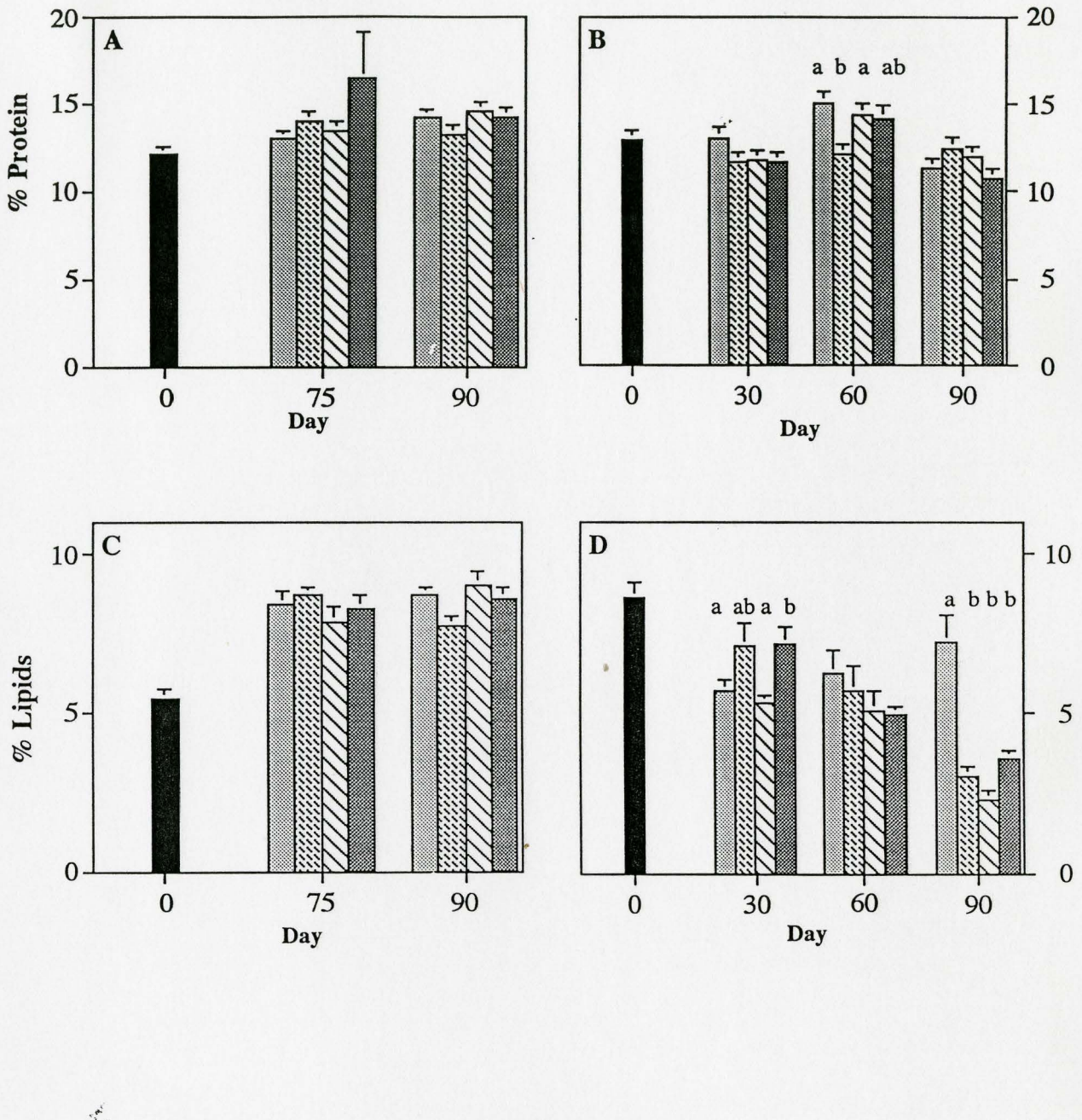
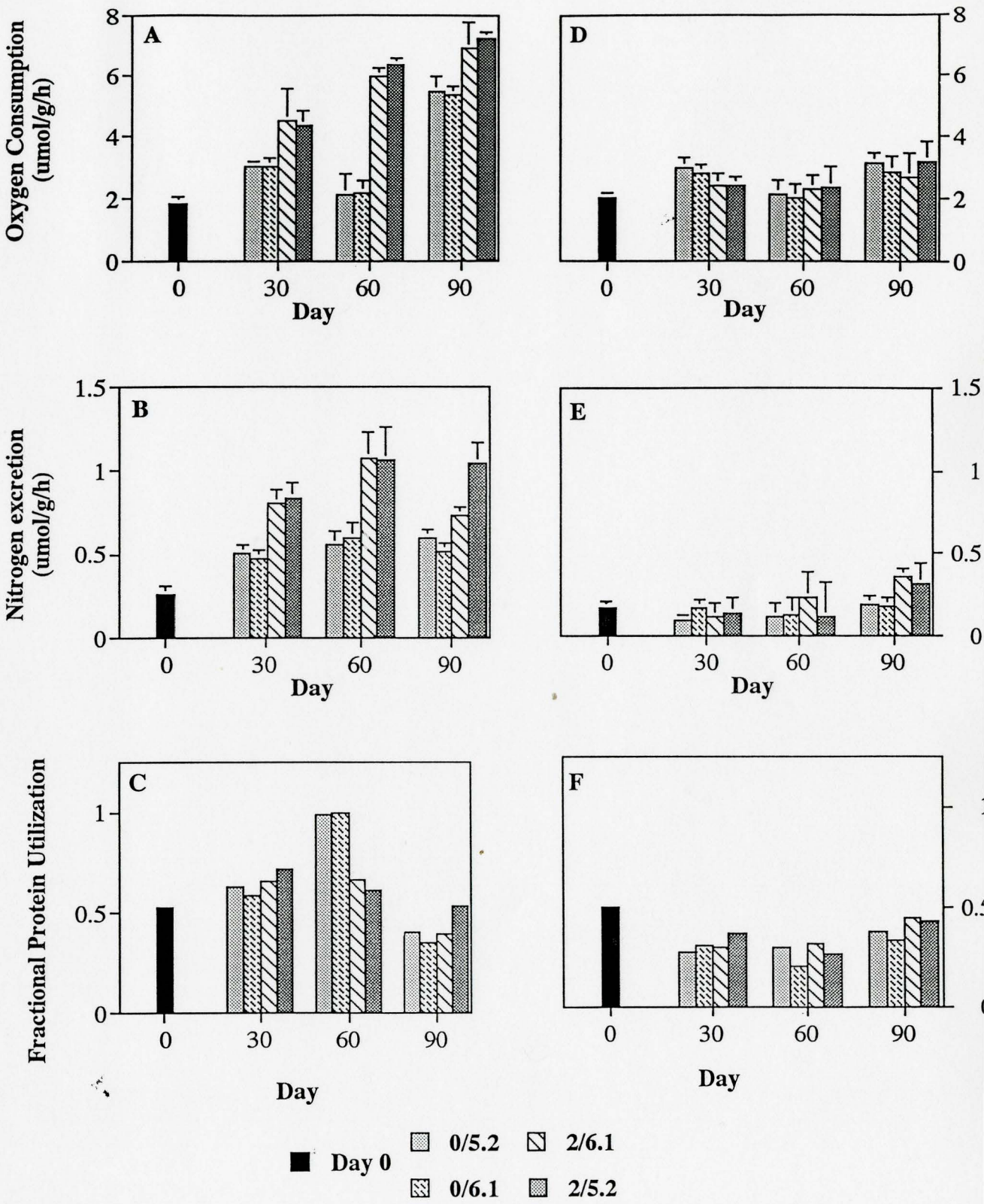

A
B
C
D
% Protein
% Lipids
Day
0
75
90
30
60
a b a ab
a ab a b
a b b b
Day 0
0/5.2
2/6.1
0/6.1
2/5.2

Figure 2-7

Metabolic rates for the Satiation and Limited Ration Exposures. (A-C) Satiation Exposure: routine oxygen consumption rates (μmol/g/hr), routine nitrogenous excretion (total ammonia + urea excretion rates (μmol/g/hr)) and fractional protein utilization, (D-F) Limited Ration Exposure: routine oxygen consumption (μmol/g/hr), routine nitrogen excretion (total ammonia + urea rates (μmol/g/hr)) and fractional protein utilization. Data for in-tank oxygen consumption and nitrogen excretion were corrected for size differences using the weight exponent determined by Cho (1992). Rates for the Satiation Exposure were determined for the total number of fish in one duplicate tank, over an 10 hr period during the day, including feeding periods. Rates for the Limited Ration Exposure were determined for the total number of fish in each tank and replicate treatments were averaged together, oxygen consumption was measured over a 48 hr period including feeding periods. Error bars represent measurement standard error only, and thus no statistical comparisons can be carried out. Other details are in the legend of Figure 2-4. FPU was determined by: moles N produced/moles O2 consumed/0.27. The denominator is the theoretical maximum nitrogen quotient as determined by Kutty (1972).


Oxygen Consumption (umol/g/h)
A
D
Nitrogen excretion (umol/g/h)
B
E
Fractional Protein Utilization
C
F
Day
0
30
60
90
Day 0
0/5.2
2/6.1
0/6.1
2/5.2

Chapter 3

Abstract

After an initial 12 hour pH 4.0 challenge, juvenile rainbow trout were exposed to acidified softwater (pH 5.2) for 28 days, to elucidate the importance of diet in their response to acid stress. Diets were formulated using a factorial design (2x2=4 treatments) at two levels of energy (regular: 16.3 MJ/kg or low: 9.8 MJ/kg) and two levels of NaCl (regular: ~263 mmol/kg or low: ~64 mmol/g) . In addition, a fifth group of fish were not fed during the exposure. Following the initial acid challenge, typical ionoregulatory disturbances were seen; however, after 28 days fish fed the regular salt diets had recovered ionic homeostasis, regardless of the energy content of the diet. Fish maintained on the regular energy/low salt diet exhibited the most deleterious effects, including elevated cortisol levels and a 4.1%/day mortality rate. Fish fed the low energy/low salt diet, regular salt diets, and starved fish were not as adversely affected by the acid stress. Evidence from measuring oxygen consumption suggests that following an energy rich meal, fish show an elevated metabolic rate which may correspond to a loss of ions. This mechanism may involve the osmorespiratory compromise, the term used to describe the conflict between gas exchange and ion regulation in the gills of freshwater fish. Thus, fish fed the regular energy/low salt diets were further compromised in an acidified environment because they were unable to replace increased branchial ion losses with dietary salts. These results showed that it is the salt content of the food rather than the energy content which is critical in protecting against the deleterious effects of low pH.

Introduction

Freshwater fish constantly lose ions to the external medium by diffusion across the gills and body surface and by excretion in the faeces and urine. Nevertheless, ionic balance is maintained by active ion uptake at the gills (Evans, 1993) and through the diet (Cowey and Sargent, 1979). However, when fish are faced with a low pH environment, additional ionoregulatory stress occurs. Na^+ influx at the gills is depressed (H^+ competing with Na^+ for transport sites and/or access to channels) and there is enhanced paracellular ion loss due to increased diffusive capacity, leading to greater whole body Na^+, K^+ and Cl^- loss (McDonald and Wood 1981; Fugelli and Vislie 1982; Wood 1989; Reid 1995). This net ion loss through the gills suggests that dietary salts may become much more important in maintaining homeostasis during acid stress (Smith et al. 1995). For example, when fish were starved or fed a very limited diet during exposure to low pH, typical ionoregulatory disturbances were seen (Fromm 1980, Neville 1985; Audet et al. 1988; Booth et al. 1988; Butler et al. 1992, Chapter 2). Conversely, when fish were fed and exposed to low pH, ionoregulatory disturbances were much reduced or did not occur (Kwain 1984; Menendez 1976; Sadler and Lynam 1986; Wilson et al. 1994; Dockray et al. 1996, Chapter 2). If dietary factors are important in reducing ion loss there are two possibilities: 1) food provides the necessary fuel to meet increased costs of living in a low pH environment, or 2) dietary salt replaces branchial ion loss.

Previous studies have shown that a low pH environment may cause growth impairment in trout (Menendez 1976; Cleveland et al. 1986). Some studies related this decreased growth to reduced food consumption (Brown et al. 1984; Lacroix and Townsend 1987; Tam et al. 1988),while others related it to increased energy expenditure (Butler et al. 1992; Hargis 1976; Waiwood and Beamish 1978; Waiwood et al. 1992). Wilson et al. (1994), Dockray et al. (1996) and Chapter 2, found that chronic exposure to

low pH seemed to stimulate appetite, suggesting a higher cost of living (Reid et al. 1996, Reid et al. 1997). Sadler and Lynam (1986) argued that food compensated for the increased energy expenditure needed to combat ionoregulatory imbalance caused by moderately low levels of pH. On the other hand, evidence supporting dietary replacement of branchial losses comes from Smith et al. (1989, 1995) who found that dietary ions may play a critical role in maintaining whole body ion homeostasis at circumneutral pH. Salman and Eddy (1987) reported that appetite may be stimulated by decreased whole body levels of Na^+ and this may explain the increased food intake by acid exposed fish reported by Dockray et al. (1996) and in Chapter 2. Thus, the present study was carried out to further elucidate whether it is the energy in the diet or NaCl in the diet that aids fish in maintaining ionoregulatory balance when chronically exposed to an acidic environment.

Materials and Methods

Pre-exposure Holding

Approximately, eight hundred juvenile rainbow trout (10 - 12g), were purchased from Humber Valley Springs Farm, Orangeville, Ontario and held in two 600L polypropylene tanks that were supplied with dechlorinated Hamilton tap water (Ca^{2+} =1.0 $\pm$0.1 mMol, Na^+=0.60$\pm$0.03 mMol, pH 7.6 -7.8, temperature 18°C). After two weeks, acclimation to softwater was started, by progressively increasing the flow of softwater and decreasing the flow of hardwater to the tank over a one week period. Deionized water was produced through reverse osmosis (Anderson Water Conditioning Equipment, Dundas, ON) and then small amounts of tap water were titrated back to yield water with 0.057 $\pm$ 0.010 mMol Ca^{2+} and 0.047 $\pm$ .007 mMol Na^+. During acclimation, fish were fed Zeigler Salmon Starter #3 daily, at a ration of 1% of their wet body weight (see Table 3-1 for dietary composition). Photoperiod mimicked the seasonal conditions during the

acclimation and exposure (September to November, 1996). Fish were held in softwater for another three weeks.

Experimental Design and Exposure System

The 800 fish were divided among ten 205L polypropylene tanks and held for the last 7 days of the three week softwater acclimation period, before the acid challenge was started on October 23, 1996. Low pH was attained by titrating H_2SO_4 (0.2N) into the synthetic softwater. This water was then divided into five head tanks, each feeding two replicate tanks. This low pH was maintained by pH statting, with a Leeds and Northrup Meridian II® Combination industrial electrode that continually monitored pH and controlled the opening and closing of a Cole Parmer Instrument Co. solenoid valve (CP#01367-70) delivering H_2SO_4 to the headtank. At the start of the experiment, in-tank pH was lowered over 3 hours to pH 5.0, fish were then held at this pH for 24 hours, following which pH was lowered to pH 4.0 for another 12 hours. The latter pH was slightly more severe than the 4 day LC 50 (Graham and Wood 1981) and is documented to cause severe ionoregulatory disturbance in rainbow trout (Wood 1989). After this challenge, pH was raised over 4 hours and held at 5.27 ± 0.02 for the next 28 days. Chronic exposure to pH 5.2 does not cause mortality when trout are fed to satiation (Dockray et al. 1996). Vigorous aeration maintained P_{O2} and prevented P_{CO2} build up. Starting on day 1 of the 28 day period, fish were fed one of four diets: regular energy/low salt, low energy/low salt, regular energy/regular salt, low energy/regular salt (Table 3-1). Each diet was fed to two tanks of fish, yielding 5 replicate treatment tanks; in addition, two tanks of fish were not fed throughout the 28 days of acid exposure.

Water temperatures decreased steadily from 18°C to 13.5°C during the 28 day exposure, reflecting the normal seasonal pattern for this autumn period. Temperature and

pH for each tank were measured daily and [Na^+] and [Ca^{2+}] were monitored weekly using atomic absorption spectroscopy (Varian AA-1275).

Diet and Feeding Regimes

Diets were formulated in the Department of Nutritional Science at the University of Guelph. Diets were developed to contain all essential nutrients, but differing amounts of energy, by manipulation of the digestible protein and lipid content. Proteins and lipids are generally considered to be the main dietary fuels for trout as carbohydrates are poorly utilized (Cowley and Sargent 1979, Cho and Kaushik, 1990). The values used to determine the energy content of food were 23.6 MJ/kg, and 39.5 MJ/kg for protein and lipid, respectively (Braefield and Llewellyn 1982). Based on the percentage of protein and lipids (Table 3-1), the regular energy diets had approximately 16.3 MJ/kg of food while the low energy diets had 9.8 MJ/kg of food. Thus, the low energy diets in this exposure were 40% lower in energy than the regular energy diets. Cellulose, a poor metabolic fuel, was used to make up the differences in the low energy diets (Cho and Kaushik, 1990).

Na^+ and Cl^- contents were about 80% lower than regular salt diets, a difference achieved by decreasing the amount added in the mineral premix. All diets were steam-pelleted and crumbled to sizes readily consumed by the fish. Fish were fed a daily ration of 0.6% of their wet body weight, except on sampling days when they were not fed. Following the sampling day, fish were fed 1.2% of their body weight to compensate for this missed feeding. All food was consumed. Feeding amount was determined from the whole tank biomass. This was determined after each sampling day except for day 0 using the bulk weighing technique described in Chapter 2.

Physiological Measurements

Sampling Protocol

On day -1 and day 0, ten fish were sampled, one fish from each tank. Six fish were sampled from each tank on day 11 and 20, and ten fish from each tank were sampled on days 5 and 28. Fish were rapidly killed by a blow to the head, blotted dry, and weight and fork length were measured. Blood was collected by caudal severance into ammonium heparinized capillary tubes. Hematocrit was determined by centrifugation at 10 000 x g; plasma from this tube was removed for measurement of plasma protein using a hand-held refractometer (American Optical). The remaining plasma was frozen at -70°C for subsequent analysis of plasma Na^+ and cortisol. Plasma was not available for cortisol analysis on day 20. Fish were freeze-clamped using aluminum tongs that were chilled in liquid nitrogen and then the fish were stored at -20°C for later analysis.

Following this whole body sampling, whole tank biomass was measured. In addition, fish were individually measured to determine changes in growth. In order to determine a mean starting fish size, prior to acid exposure twenty fish from each tank were randomly chosen, lightly anesthetized with MS222 that had been buffered with $NaHCO_3$, and weight and fork length were determined. On days 11 and 28, all fish in each tank were anesthetized with MS222 and the same parameters were measured.

Metabolic Rates

Whole tank oxygen consumption was determined on day 15 over 4 one hour periods prior to feeding and 3 one hour periods after feeding. Duplicate tanks for each treatment were measured. Tanks were sealed with air-tight, transparent lids and the water within each tank was recirculated using a pump (Little Giant, 1EUAA-MD). For the 4 hour prefeeding period, water samples were taken every 60 minutes and P_{O2} was measured by injecting water samples into a thermostatted Radiometer/Copenhagen E5046

electrode connected to a Cameron Instrument Co. oxygen meter. Fish were then fed with their respective diets and 0.5 hours later the same method was resumed to measure oxygen consumption post-prandially for a further 3 hours. Measurement of oxygen consumption was stopped if P_{O2} decreased below 100 torr. The rate of oxygen depletion was determined for each hour using oxygen solubility coefficients from Boutilier et al. (1984), factored by time, volume and total fish weight to yield the rate of M_{O2}. Data for each hour were averaged, and factored by time, volume and total fish weight, the latter being corrected for size differences using the weight exponent 0.824 that was determined by Cho (1992) for rainbow trout.

Sample Processing

Plasma $[Na^+]$ was determined by atomic absorption spectroscopy. Cortisol was measured using a I^{125} radioimmunoassay (ICN Biomedicals). Frozen whole bodies were ground using an IKA (M10/M20) grinding mill cooled to -70°C with a dry ice and methanol mixture. About 2g of this ground tissue was oven dried to a constant weight to determine water content. Remaining tissue was lyophilized (Labconco Lyph-Lock 6) and stored at -20°C for proximate body analysis. Whole body ions were determined from lyophilized tissue which was digested in 8% perchloric acid at a 9:1 ratio. Whole body $[Na^+]_{wb}$, $[Ca^{2+}]_{wb}$ and $[K^+]_{wb}$ were measured using atomic absorption spectroscopy and $[Cl^-]_{wb}$ was determined by a colorimetric method (Zall et al. 1956). Proximate analysis was measured only for fish sampled on days -1, 0 and 28. Whole body protein and lipids were quantified using the Lowry assay as modified by Miller (1959) and chloroform/methanol method extraction (Folch et al. 1957), respectively. Standard enzymatic analyses (Bergmeyer 1985) were employed to determine glucose, glycogen and lactate; the sum of the three was used as a measure of total carbohydrates.

Gross food efficiencies were determined by dividing the amount the fish grew by the amount of food consumed during the 28 days, and condition factors for each fish were determined as the quotient of the weight (g)/fork length $(cm)^3$ x 100. Mortalites were calculated for intervals between sampling periods and expressed as % per day.

Statistical Analysis

Values are given as the mean ± standard error (SEM), except in the case of oxygen consumption where the mean and the range of replicate measurements are reported. There were no significant differences between replicate tanks and therefore data were combined in all analyses. Mean values were compared using one-way ANOVA (SAS JMP version 5.0); when the F-value indicated significance, the Tukey-Kramer comparison of all pairs was used to determine treatment differences within a sampling period and to compare mortality rate over the 28 days. To determine if fish recovered from the initial acid stress, a one way ANOVA was used to compare means of whole body ions (Na^+, Cl^- and K^+) and plasma Na+, if the F-value indicated significance, the Dunnett's test was then used to compare the means to the values at day -1. An unpaired Students' t-test was used to compare values on day -1 and day 0, and also to compare proximate composition values between day -1 and day 28. A 95% confidence level was chosen.

Results

Mortality

Mortalites were relatively high for all treatments between days 0-11 (shortly after the pH=4.0 challenge). They decreased between days 11-20, and then increased again between days 20 and 28 (Figure 3-1). The highest mortality rate throughout the exposure was experienced by fish fed the regular energy/low salt diet, with an mean of 4.13% fish deaths/day, which was significantly different from the other four treatments. Mortality rate was lowest for starved fish with a rate of 0.46 %/day while the rest of the treatments

had rates varying from 0.75 to 1.35%/day. The difference in mortality rate amongst these four treatments was not significant.

Growth and Food Conversion Efficiency

By day 11 differences in weight gain were already noted, and by day 28, fish fed the regular energy/regular salt diet were significantly larger than either fish fed the low energy/regular salt diet or starved fish, both of which had lost weight (Figure 3-2). The weight of trout fed the regular energy/low salt diet was not significantly different. The low energy/regular salt fed fish were larger than the starved fish yet smaller than the fish fed the regular energy/regular salt diet.

Initial condition factor (CF) was 0.95$\pm$ 0.01, and by day 11 this had not changed for any of the treatments. However, by day 28, CFs for fish fed the low salt diets were significantly greater: regular energy/low salt =1.20$\pm$0.04 and low energy/low salt = 1.21$\pm$ 0.02 respectively. Note, however, that the high mortality rate in the fish fed the regular energy/low salt diet may have skewed the data . On the other hand, fish fed low energy/regular salt diets and starved fish had lower CF than at day 0, 0.90 $\pm$ 0.02 and 0.87 $\pm$ 0.01, respectively. CF values for fish fed the regular energy/regular salt diet were intermediate at 1.11 $\pm$ 0.03.

Gross food conversion efficiencies were calculated (Table 3-2). The regular energy/regular salt diet was far better utilized than the three other diets. The low energy/low salt diet produced the poorest conversion efficiency (- 0.28). The fish resorted to catabolism of internal stores during this exposure.

Metabolic Rate

As only replicate measures on two tanks per treatment were made, these data could not be evaluated statistically. Nevertheless, clear trends were apparent. Prior to feeding, fish fed the low salt diets had lower M_{O2} values than fish fed the regular salt diets (Figure

3-3). Post-prandially, the similarities between similar salt levels ended and changes in M_{O2} corresponded with the energy content of the food. Fish fed the regular energy diets experienced an increase in M_{O2} (~32-47% increase post- prandially) compared to the low energy diets in which M_{O2} did not change. Starved fish had the lowest M_{O2} (~1.34-1.41mmol O_2/g/hr) compared to any of the other treatments.

Plasma Analyses

Cortisol levels rose significantly following the severe acid challenge, then decreased in all five treatments until day 28 when the regular energy fish had higher levels, especially the fish fed a regular energy/low salt diet. Cortisol levels remained relatively low in the fish fed the low energy diets and starved fish. Increasing cortisol levels corresponded with decreasing water temperatures, decreasing fish number, and length of acid exposure (Figure 3-4).

$[Na^+]_p$ showed a significant decrease following the initial acid challenge of pH 4.0 (Figure 3-5). After 28 days of exposure to pH 5.2, $[Na^+]_p$ was lower in fish fed low salt diets, especially the regular energy/low salt diet while $[Na^+]_p$ was higher in the fish fed the regular salt diets and starved fish. Thus, fish fed the regular salt diets recovered from the acid stress, while fish fed the low salt diets had significantly lower $[Na^+]_p$ than at day -1. Table 3-3 shows the plasma protein and hematocrit values over time. There were no significant differences among treatments for hematocrit though it did increase by day 28 from the pre-acid challenge values measured on day -1. Plasma protein changed during the exposure, with lower levels in starved fish (2.8 g/100 mL) compared to fish fed the regular energy/low salt diets (5.8 g/100mL). The other three groups exhibited intermediate levels of plasma protein at this time.

Whole Body Ions and Proximate Composition

$[Na^+]_{wb}$ and $[Cl^-]_{wb}$ did not change significantly immediately after the acid challenge, in contrast to $[K^+]_{wb}$ and $[Na^+]_p$. By day 11 of the exposure, lower levels for all three electrolytes were seen in fish fed low salt diets (Figure 3-6). $[Na^+]_{wb}$ was again lower in the low salt diets on day 28, especially in the fish fed the regular energy/low salt diets, while the regular salt fed fish and the starved fish had similar $[Na^+]_{wb}$ values to the day -1 values. $[K+]_{wb}$ also showed the same trend in these treatments on day 11 but not on day 28 when $[K+]_{wb}$ actually increased in the low salt diet groups. $[Cl^-]_{wb}$ exhibited generally similar trends to $[Na+]_{wb}$ with lower levels in the low salt diets. $[Ca^{2+}]_{wb}$ showed no treatment effects during the exposure (data not shown). The overall mean for $[Ca^{2+}]_{wb}$ was 305.3 $\pm$ 4.6 mmol/kg (n=236).

Whole body protein did not change in any of the treatments over the 28 days, with levels staying between ~8.6% and ~10% (Figure 3-7). However, lipids and carbohydrates both showed variations among the treatments. On day 28, lipid levels were lower in the low energy diets and starved fish (~6.4-7%) compared with fish fed the regular energy diets (~8.7-8.9%), however, only the low energy/regular salt fish had a lower lipid content than fish at day -1. Water content was inversely proportional to lipid levels, thus starved fish had the highest percentage of water and the regular energy/regular salt fish had the lowest. Stored carbohydrates (glucose, glycogen and lactate) which made up a much smaller percentage of the proximate composition (0.2 to 0.6%) showed no definite pattern with higher levels in fish fed the low energy/low salt diet and the regular energy/regular salt diets.

Discussion

Fish fed regular salt diets did not experience any electrolyte imbalance during chronic acid exposure regardless of the energy content of the food; clearly answering the

question about whether it is the energy or salt in food that is responsible for preventing ionoregulatory disturbances. This work supports the hypothesis put forth in previous studies by Dockray et al. (1996) and in Chapter 2. Fish can replace branchial ion losses during chronic acid exposure with dietary salt, a prime example of how the quality and quantity of a diet can modify the toxicity of a pollutant (Lanno et al. 1989; Cho and Kaushik 1990). In addition, this study shows that on a fixed ration, a balanced ratio of energy and salt are needed to protect against the deleterious effects of acid exposure.

Dietary Salts and Acid Exposure

As Smith et al. (1989) pointed out, very little research has been directed at accounting for the role that dietary ions may play in ionoregulation. The present experiment clearly indicates that dietary NaCl aids in maintaining whole body ion homeostasis when net branchial Na^+ uptake is impaired due to low pH exposure. Fish fed regular salt diets recovered from the acute acid stress even in the face of a continuing sublethal stress.

Calculations based on food ion concentrations (Table 3-1), feeding ration (0.6% body weight per day) and whole body ion pools (Figure 3-6) found that fish on low salt diets consumed about 0.6 to 1.09% (258 to 468 umols Na+/kg) of their Na^+ pool per day, and this was insufficient to maintain Na^+ levels. Fish on regular salt diets consumed about 3.4 to 3.94% (1462 to 1694 umol/kg) of their Na^+ pool per day which seemed to correct for any ionoregulatory imbalances. This is substantially lower than the 10% per day that fish were taking in when they were fed to satiation (Dockray et al. 1996). Based on Na^+ net flux measurements of fish chronically exposed to pH 5.2 (Appendix), fish maintained on a limited dietary regime of commercial fish food (Zeigler Diet, Table 3-1) lose from 28.36 umol/kg/hr to 71.46 umol/kg/hr of Na^+. Thus, the fish were losing approximately 1200 umol/kg/day (using a net loss of 50 umol Na^+/kg/hr) per day which was substantially

higher than dietary Na^+ intake for the low salt diets, explaining the decrease in whole body Na^+. In comparison, fish fed the regular salt diets were taking in a small excess of Na^+. This extra was probably not stored (Figure 3-6) but released across the gills (Smith et al. 1995). These findings and calculations regarding Na^+ indicate show that dietary salts replace branchial ion losses during acid stress.

Death probably ensued in the fish fed the regular energy/low salt diets (4.13%mortality/day) due to hematological changes secondary to the loss of plasma ions which results in fluid shifts from the extracellular fluid volume (ECF) to the intracellular fluid volume. Ion loss during acid exposure leads to contraction of the ECF volume, increased haematocrit, and catecholamine induced discharge of stored red blood cells leading to increased blood viscosity and peripheral resistance and ultimately circulatory failure (Milligan and Wood 1982).

Benefits of dietary salt may be two fold, replacing branchial ion loss and stimulating branchial uptake. High salt diets have been shown to increase chloride cell number and Na^+/K^+ ATPase resulting in enhanced ionic uptake (Salman and Eddy 1987). In addition, low dietary salt intake has been reported to increase food conversion efficiencies but not affect growth (Salman and Eddy 1988). In the present exposure, fish fed low salt diets had better food conversion efficiencies than the low energy/regular salt fed fish which were in a catabolic state.

Dietary Energy Sources (Protein and Lipids) and Acid Exposure

Metabolic rate, as measured by oxygen consumption (M_{O2}), is a composite measure of the energy expenditures of living, including parameters such as ionoregulation, swimming and the cost of feeding and digesting food (Cho, 1990). In this exposure, when fish were fed regular energy diets, there was a marked increased in oxygen consumption following feeding, ~32-47%, while fish maintained on low energy diets

showed no peak in M_{O2}, post-prandially. This increase due to feeding is attributed to specific dynamic action (SDA) . SDA is the term used to describe the energy expended by fish during digestion, absorption, interconversion and resynthesis of substrates for retention in tissues, and formation and excretion of metabolic wastes (Beamish 1974, Jobling 1981; Cho and Kaushik 1992). The main biochemical basis for SDA appears to be the energy expended for interconversion and synthesis (Brown and Cameron 1991), and not the mechanical work. For example, the same increased in M_{O2} occurs if amino acids are delivered intravenously rather than ingested (reviewed in Cho et al. 1982; Brown and Cameron 1991) . Similarly, Smith et al (1978 a,b) showed that sham feeding in rainbow trout did not increase metabolic resting rate. In the present study, feeding with low energy diets high in cellulose, similarly caused no SDA effect.

SDA causes an increase in oxygen consumption and as the gills of fish are the site for both respiration and ionoregulation, an increase in oxygen transfer is associated with an increase in ion flux (Randall et al. 1972; Wood and Randall 1973; Gonzalez and McDonald 1992). The ability to balance the two processes, respiration and ionoregulation, has been coined the "osmorespiratory compromise" (Randall et al. 1972). For example, Wood and Randall (1973) and Gonzalez and McDonald (1992) showed that increases in M_{O2} in exercising fish resulted in increased ion loss across the gills. We suggest that fish fed the regular energy/low salt diets may have had greater branchial ion losses due to the SDA response evoked by the regular energy diets. This, of course, would be more detrimental in fish fed the regular energy/low salt diets where SDA would be accompanied by a loss of ions through the gills without replacement from the diet.

Starvation and Acid Exposure

In the present study, starved fish exhibited the lowest M_{O2} ~1.4 umol/g/hr (Day 15). Beamish (1964) and Dickson and Kramer (1971) reported a decrease in the standard

metabolism of fish during the first 2 or 3 days of starvation. Similarly, Jobling (1980) and Lauff and Wood (1996) showed a steady decline in M_{O2} over time during starvation. Food deprivation leads to a reduction in ribosomal number and a fall in protein synthetic capacity. This decrease in protein synthesis capacity is thought to give rise to an overall reduction in protein turnover and the net result may be a reduction in metabolic activity and conservation of energy reserves (Jobling, 1994). Protein was also conserved in the starved fish (similar to Stirling 1976, Jobling 1980, Lauff and Wood 1996) and decreased catabolism of this stored energy reserve may have also aided in maintaining a low M_{O2}. This low M_{O2} probably minimized diffusive loss of ions at the gills and maintained ionoregulatory needs at a minimal rate.

Low mortality in acid exposed starved fish was also reported by Sadler and Lynam (1986), though they attributed this to decreased aggression between fish. However, in their exposure, starved fish suffered typical ionoregulatory disturbances, perhaps due to the longer exposure to low pH.

Indicators of Stress

Several studies have reported prolonged elevation of cortisol during low pH exposure (Brown et al. 1989; Audet and Wood 1993; Chapter 2), in contrast, other studies have found that the pituitary interrenal axis in acid exposed fish was not substantially activated (Brown et al. 1984; Adams et al. 1985; Goss and Wood 1988; Balm and Pottinger 1993; and Chapter 2). The present study suggests that dietary factors may explain the apparent dichotomy.

The mobilization of cortisol coincided with two interrelated factors regarding feeding. First, when fish were fed daily either to satiation (Chapter 2) or 2% of their body weight (Balm and Pottinger 1993) no cortisol elevation occurred and there were no ionoregulatory disturbance. Additionally, when fish were starved, no cortisol response

was evoked (Goss and Wood 1988; Brown et al. 1984) similar to results reported by Anderson et al. 1991 in non-acid exposed fish. However, when diet was limited or feeding was periodic (Chapter 2; Audet and Wood 1993;), cortisol levels were elevated and ionoregulatory disturbances occurred. Hence, elevated cortisol levels could be a result of a restricted ration or ionoregulatory disturbances. One benefit of increased cortisol production is chloride cell proliferation (Foskett et al. 1983; Perry and Laurent 1990). Thus, the regular energy/regular salt fed fish may have enhanced ion uptake aiding in maintaining ionoregulation, despite losses due to the osmorespiratory compromise. Moreover, when fish were in a catabolic state, the cortisol response was not as pronounced, with low levels not only in the starved fish but also in the fish fed low energy diets. This may be due to atrophy of the adrenotrophic tissue (Love, 1970) or because food deprivation reduces the normal stress response of fish (Anderson et al. 1991). The stress response may not be elicited during satiation feeding (Balm and Pottinger 1993; Chapter 2) because dietary salts are compensating for branchial loss.

Summary

Dietary salts clearly aid in maintaining ion balance during acid stress as suggested in several papers (Smith et al. 1995, Dockray et al. 1996, Chapter 2). In fact, pH 5.2 proved to be quite detrimental when fish were presented with a regular energy/low salt diet (mortality rate of 4.13%/day). Rainbow trout were able to make up for any impairment in sodium influx due to low pH exposure with dietary salts. Surprisingly, it was not the energy expended to maintain ionoregulation, but the relationship between energy expended during feeding and branchial ion loss that played a larger role during acid stress. Clearly, when metabolic rate was depressed due to starvation or a low energy diet, fish could recover ion balance. The most detrimental situation occurred when fish were fed a regular energy/ low salt diet, elevating M_{O2} by the SDA effect, thus losing ions due to the

disturbance of the "osmorespiratory compromise" but with no method of replacing these branchial ion losses with dietary sources.

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Table 3-1: Crude analysis of the diets used to feed juvenile rainbow trout. Zeigler trout starter was used as feed during acclimation while the four other diets were used as feed during the pH 5.2 exposure.

	Regular Energy/ Low Salt	Low Energy/ Low Salt	Regular Energy/ Regular Salt	Low Energy/ Regular Salt	Zeigler Trout Starter
% Digestible Protein	39	23	39	23	50
% Lipids	18	11	18	11	15
*Energy Content (MJ/kg)	16.3	9.8	16.3	9.8	17.7
** Na^+ mmol/kg	78	44	283	243	217
** Cl^- mmol/kg	79	43	254	254	215

* Approximate energy content is based on the percentage of proteins and lipids in the food. Values were determined using 23.6 MJ/kg for protein, and 39.5 MJ/kg for lipid (Braefield and Llewellyn 1982).

** Determined from food digested in 8% perchloric acid in a ratio of 1:9.

Table 3-2: Food Conversion Efficiencies (change in fish weight/food consumed), determined over the 28 days of exposure to pH 5.2.

Regular Energy/ Low Salt	Low Energy/ Low Salt	Regular Energy/ Regular Salt	Low Energy/ Regular Salt
0.05	0.06	0.77	-0.28

Table 3-3: Measured hematocrit (%) and plasma protein (g/100mL) for each dietary regime. Mean values ± SEM and sample size are shown. Significant differences between treatments occurred on Days 11 and 28 only, for plasma protein and at these periods, treatments that share a letter are not significantly different from each other ($p<0.05$).

Day and Treatment		**Plasma Analysis**	
	Sample size	**Hematocrit (%)**	**Plasma Protein (g/100mL)**
Pre-Acid Challenge	6	28.2 ± 4.0	3.6 ± 0.4
Post Acid Challenge	10	39.9 ± 4.4	4.6 ± 0.5
Day 5			
Regular Energy/ Low Salt	15	35.6 ± 2.8	4.4 ± 0.3
Low Energy/Low Salt	17	31.9 ± 2.1	3.7 ± 0.2
Regular Energy/Regular Salt	14	33.9 ± 1.5	4.5 ± 0.3
Low Energy/Regular Salt	16	39.8 ± 2.5	4.1 ± 0.2
Starved	19	31.7 ± 2.1	3.6 ± 0.1
Day 11			
Regular Energy/ Low Salt	10	34.9 ± 2.5	4.7 ± 0.30^{a}
Low Energy/Low Salt	9	35.7 ± 3.2	3.7 ± 0.2^{ab}
Regular Energy/Regular Salt	10	29.3 ± 2.4	3.8 ± 0.3^{ab}
Low Energy/Regular Salt	10	28.4 ± 2.9	3.5 ± 0.2^{b}
Starved	10	25.4 ± 1.8	3.6 ± 0.2^{ab}

Table 3-3 continued

Day 20			
Regular Energy/ Low Salt	7	29.8 ± 1.8	3.9 ± 0.2
Low Energy/Low Salt	8	30.0 ± 3.5	3.1± 0.4
Regular Energy/Regular Salt	10	33.0 ± 2.2	4.0± 0.4
Low Energy/Regular Salt	11	28.4 ± 1.9	3.4 ± 0.2
Starved	11	34.4 ± 2.6	3.3 ± 0.1
Day 28			
Regular Energy/ Low Salt	12	43.0 ± 2.3	5.8 ± 0.4^{a}
Low Energy/Low Salt	18	35.7 ± 1.8	5.0 ± 0.4^{ab}
Regular Energy/Regular Salt	20	36.7 ± 1.7	5.1 ± 0.2^{ab}
Low Energy/Regular Salt	17	38.2 ± 1.5	4.2 ± 0.2^{b}
Starved	17	34.9 ± 2.5	2.8 ± 0.1^{c}

Figure 3-1

Mortality rate for each sampling period. Numbers represent the number of fish that died per day between each sampling time as a percentage of the number of fish remaining at the beginning of the time period. The overall treatment means ± SEM (n=8) are shown by the last 5 bars. Significant differences ($p<0.05$) among the overall means were determined by the Tukey Kramer test for all pairs, and are indicated by an *. Fish maintained on the low salt diets are represented by the two bars on the left. The regular energy/low salt fish are represented by the light stippled bar while the low energy/low salt fed fish are represented by the heavily stippled bar. The next two bars represent the fish fed the regular salt diets. The broken cross hatched bars represent the regular energy/regular salt fed fish while the full cross hatched bar represents the low energy/regular salt fed fish. The starved fish are represented by the far right bar with the broken, alternating cross hatched bars.

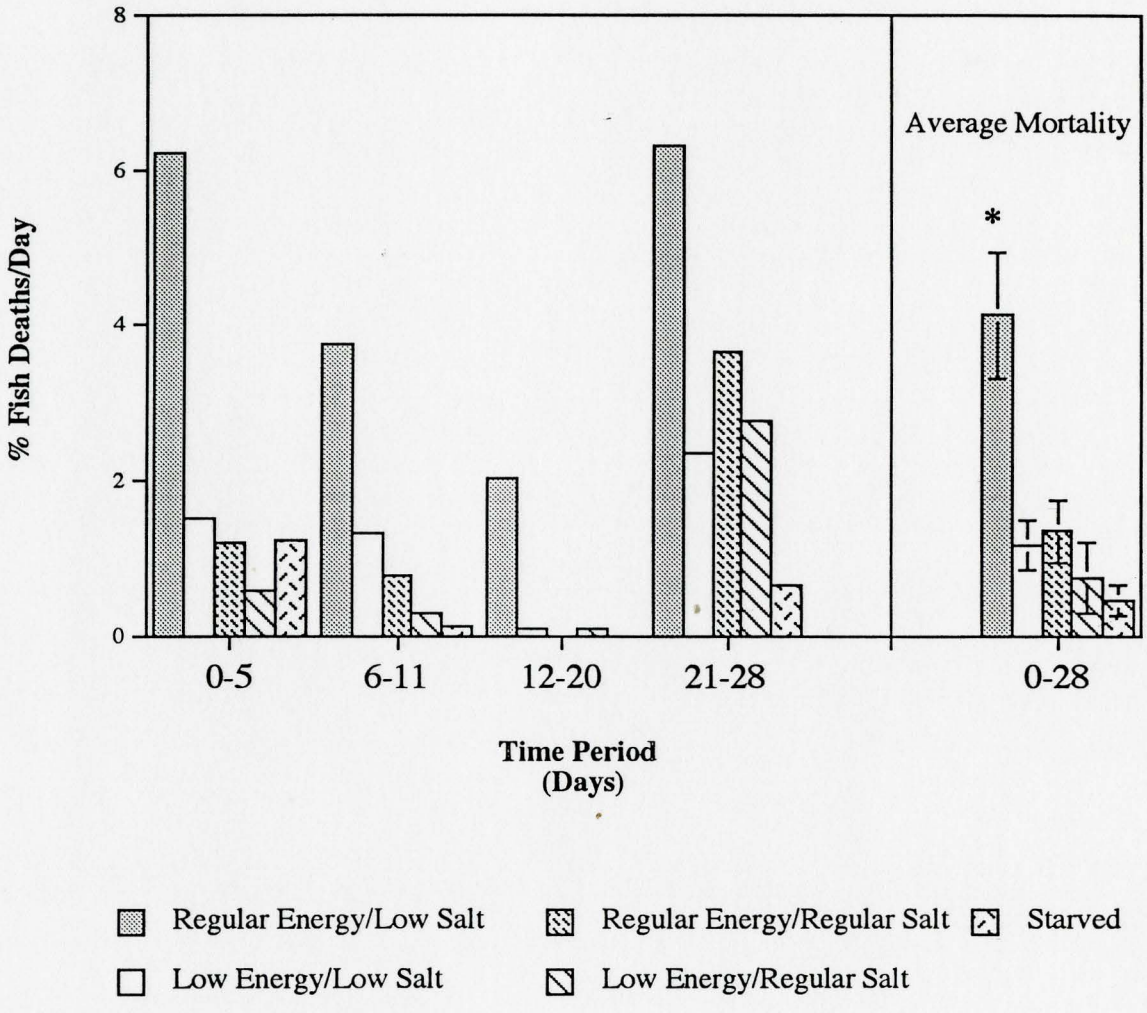
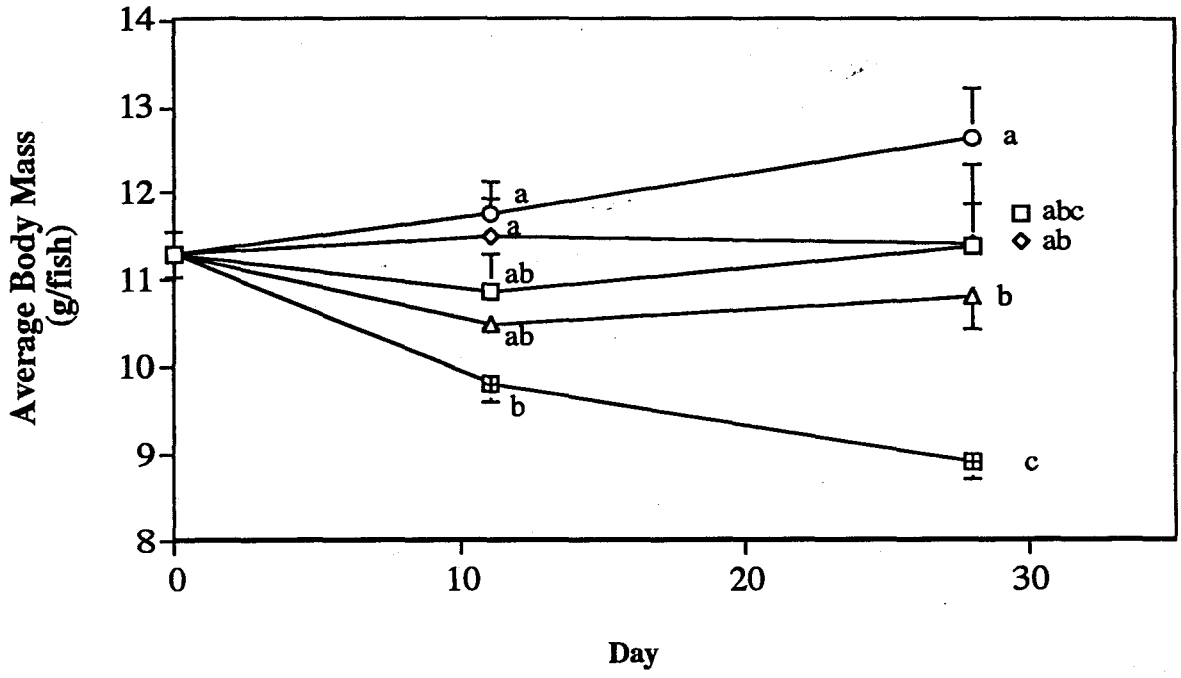

8
6
4
2
0
% Fish Deaths/Day
Average Mortality
*
0-5
6-11
12-20
21-28
0-28
Time Period
(Days)
Regular Energy/Low Salt
Regular Energy/Regular Salt
Starved
Low Energy/Low Salt
Low Energy/Regular Salt

Figure 3-2

Wet body mass (g) on days 0 (n=150), 11 (n=60-80), and 28 (n=20-80). The means ± SEM. By day 11, there were significant differences in growth between the starved fish and the fish maintained on the regular energy diets. By day 28, differences in growth were even more apparent, with the fish fed the regular energy/regular salt diets growing the most. Significant differences ($p<0.05$) are indicated among treatment means that do not share a common letter.



Regular Energy/Low Salt — Regular Energy/Regular Salt — Starved

Low Energy/Low Salt — Low Energy/Regular Salt

Figure 3-3

In-tank oxygen consumption on day 15, corrected for size differences using the weight exponent determined by Cho (1992). Values (μmol/g/hr) for each treatment are averages of the replicate tanks (bars represent ranges) over the sampling periods. The white bar represents the in-tank oxygen consumption for the four hours before feeding while the cross hatched bars represent the 3 one hour periods after feeding.

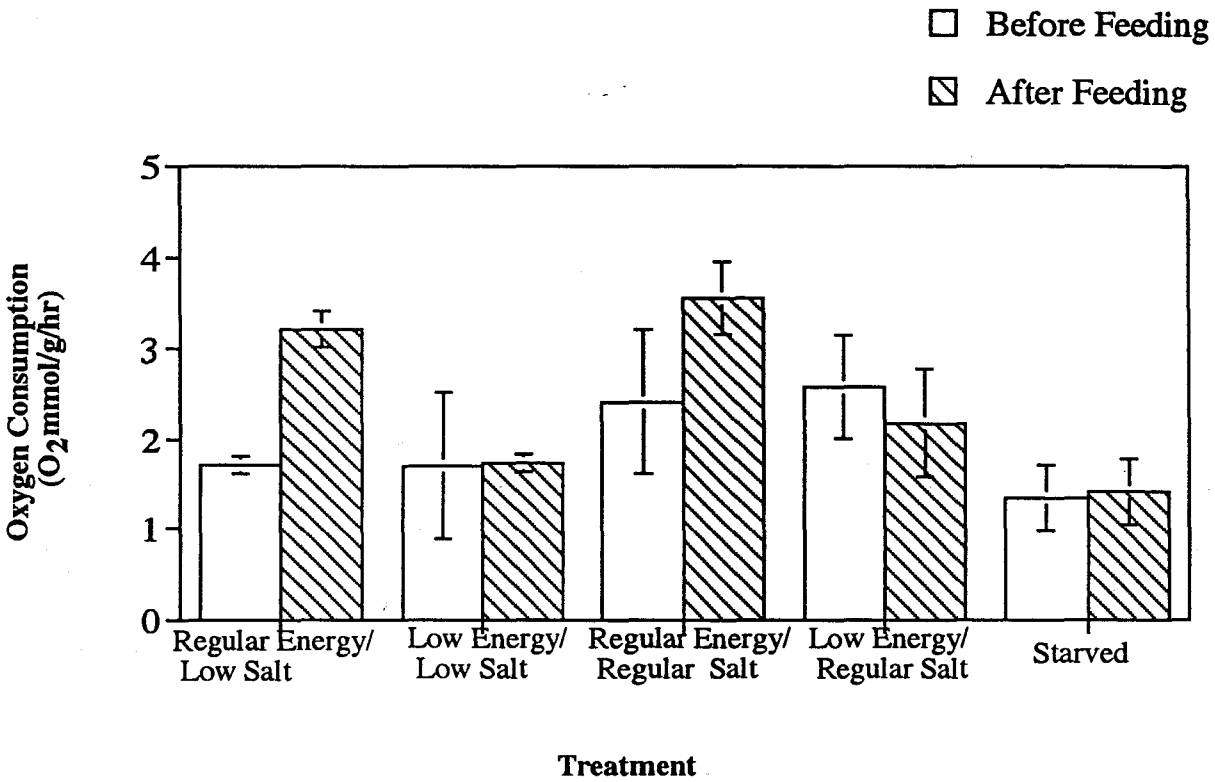

Before Feeding
After Feeding
Oxygen Consumption (O_2mmol/g/hr)
5
4
3
2
1
0
Regular Energy/ Low Salt
Low Energy/ Low Salt
Regular Energy/ Regular Salt
Low Energy/ Regular Salt
Starved
Treatment

Figure 3-4

Plasma cortisol (ng/mL)(means ± SEM (n=10 to 12)). Measurements prior to the commencement of the feeding regime are represented by the solid bars. The white solid bar represents plasma cortisol levels prior to the pH 4.0 challenge, while the black solid bar represents plasma cortisol levels after the pH challenge. Fish maintained on the low salt diets are represented by the two bars on the left. The regular energy/low salt fish are represented by the light stippled bar while the low energy/low salt fed fish are represented by the heavily stippled bar. The next two bars represent the fish fed the regular salt diets. The broken cross hatched bars represent the regular energy/regular salt fed fish while the full cross hatched bar represents the low energy/regular salt fed fish. The starved fish are represented by the far right bar with the broken, alternating cross hatched bars. Values are not available for Day 20. Significant differences ($p<0.05$) were found in plasma cortisol before and after the acid challenge and are denoted by an *. Significant differences ($p<0.05$) were also found on day 30 and are indicated by treatment groups that do not share a common letter.

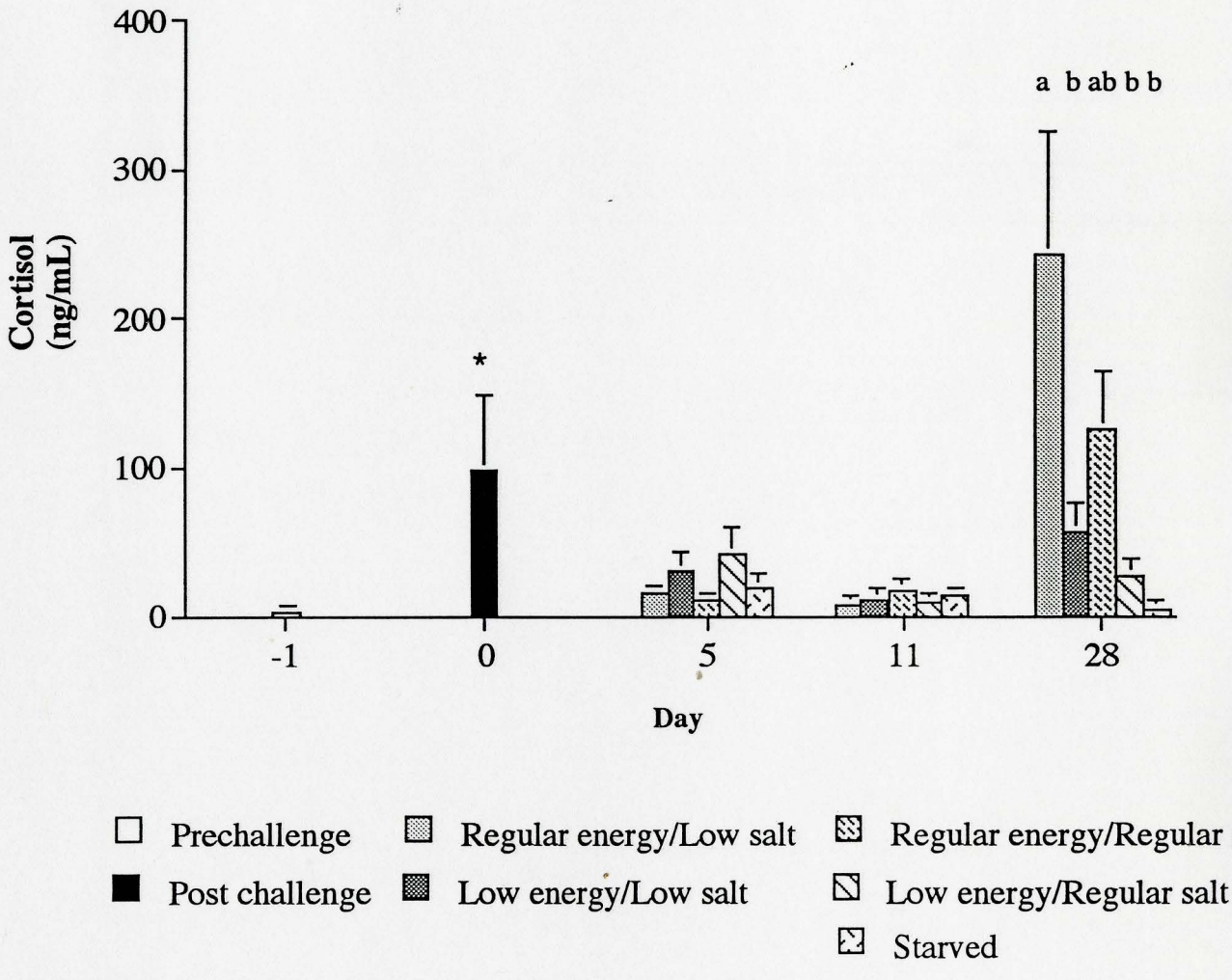
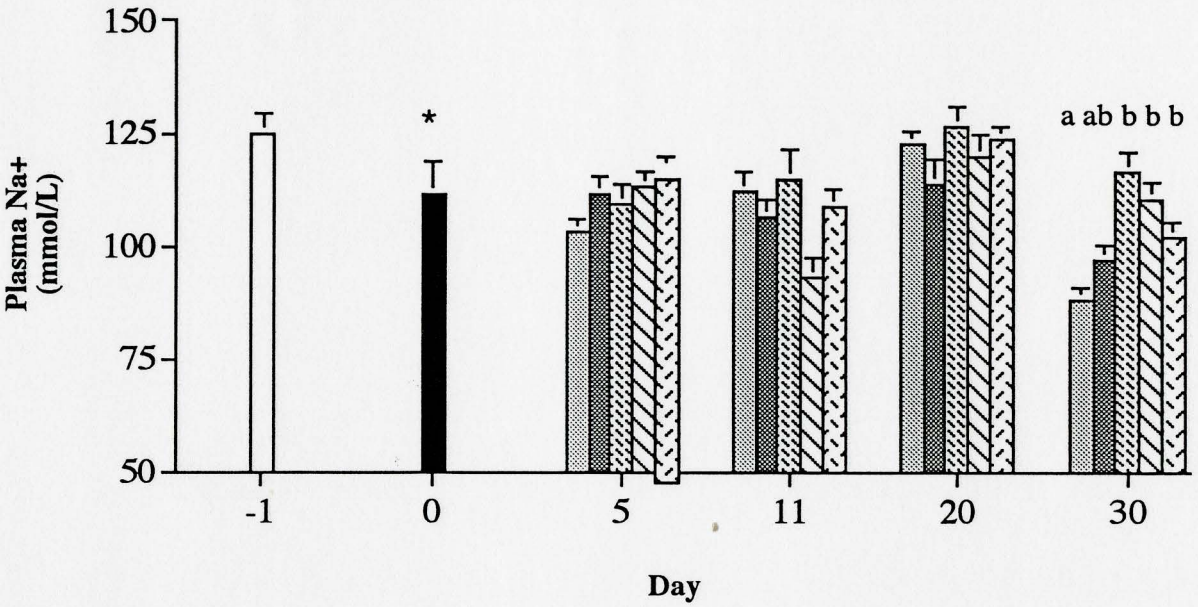

400
300
200
100
0
Cortisol (ng/mL)
*
a b ab b b
-1
0
5
11
28
Day
Prechallenge
Post challenge
Regular energy/Low salt
Low energy/Low salt
Regular energy/Regular sa
Low energy/Regular salt
Starved

Figure 3-5

Plasma Na^+ (mmol/L). Other details are in the legend of Figure 3-4 Values are given as means $\pm$ SEM (n=10 to 20). Significant differences ($p<0.05$) were found in plasma Na^+ before and after the acid challenge and are denoted by an *. Significant differences ($p<0.05$) amongst treatments were found on day 28 and are indicated by treatment groups that do not share a common letter.



Prechallenge
Post challenge
Regular energy/Low salt
Low energy/Low salt
Regular energy/Regular salt
Low energy/Regular salt
Starved

Figure 3-6

Whole body ions (mmol/kg): (A) $[Na^+]_{wb}$, (B) $[Cl^-]_{wb}$ and (C) $[K^+]_{wb}$. Other details are in the legend of Figure 3-4. Values are given as means $\pm$ SEM (n = 20) except for the Days -1 and 0 in which n=10. Significant differences ($p<0.05$) were found only in $[K^+]_{wb}$ before and after the acid challenge and are denoted by an *. Significant differences ($p<0.05$) are indicated among treatment means that do not share a common letter. If no letters are present for the sampling period, there are no significant differences between means.

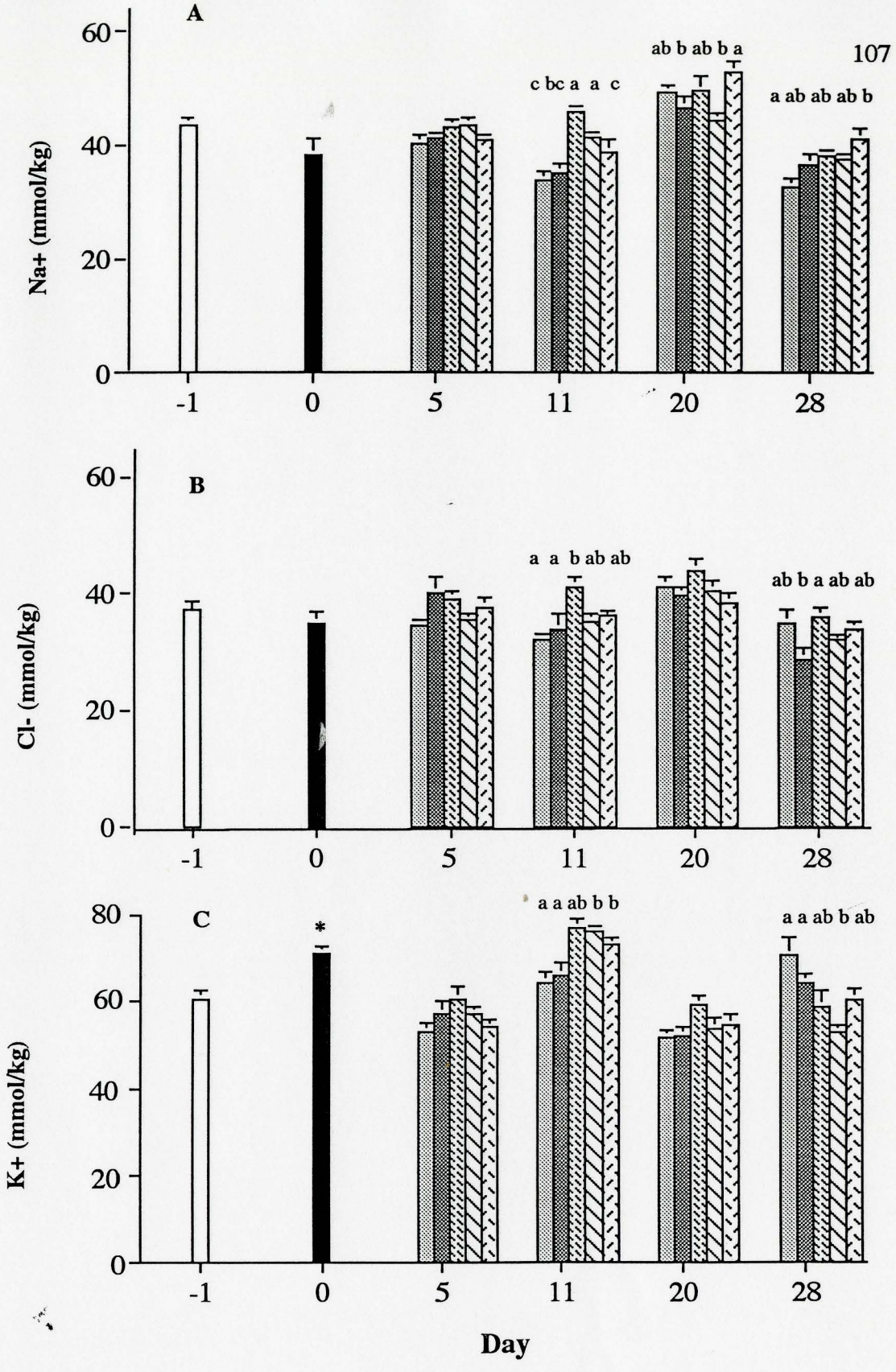
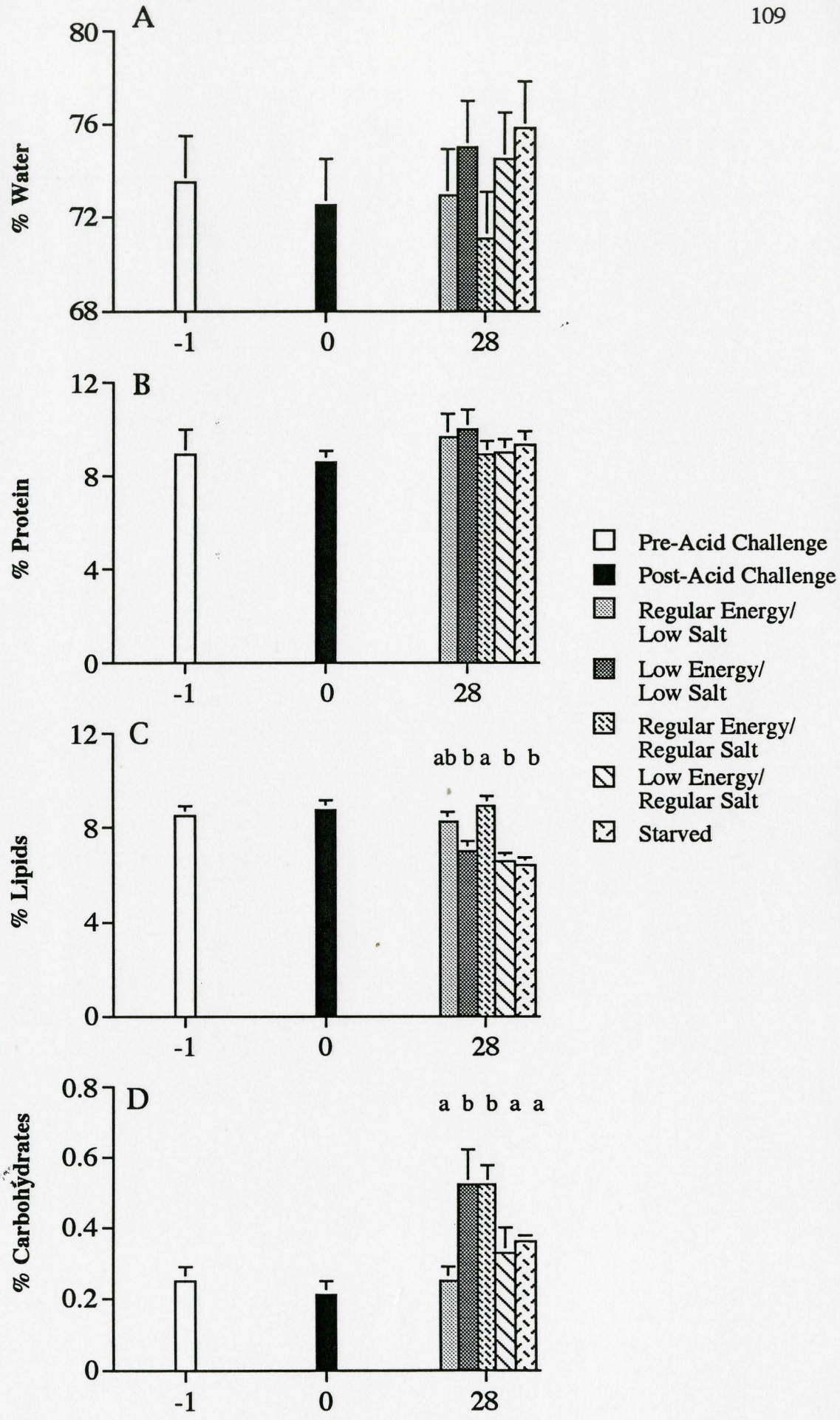

A
Na+ (mmol/kg)
60
40
20
0
c bc a a c
ab b ab b a
a ab ab ab b
-1
0
5
11
20
28
B
Cl- (mmol/kg)
a a b ab ab
ab b a ab ab
C
K+ (mmol/kg)
80
*
a a ab b b
a a ab b ab
Day
Prechallenge
Post challenge
Regular energy/Low salt
Low energy/Low salt
Regular energy/Regular sa
Low energy/Regular salt
Starved

Figure 3-7

Whole body water, protein, lipids and carbohydrates (%) for fish before and after the acid challenge and after the 28 day exposure to pH 5.2 Other details are in the legend of Figure 3-4. Values are given as means $\pm$ SEM (n=10-20). Significant differences ($p<0.05$) are indicated among treatment means that do not share a common letter. If no letters are present for the sampling period, there are no significant differences between means.


A
80
76
72
68
% Water
-1
0
28
B
12
8
4
0
% Protein
-1
0
28
C
12
8
4
0
% Lipids
ab b a b b
-1
0
28
D
0.8
0.6
0.4
0.2
0
% Carbohydrates
a b b a a
-1
0
28
Pre-Acid Challenge
Post-Acid Challenge
Regular Energy/ Low Salt
Low Energy/ Low Salt
Regular Energy/ Regular Salt
Low Energy/ Regular Salt
Starved

Concluding Remarks

Laboratory research in environmental physiology tries to understand the natural world. Therefore, to accurately assess the impact of environmental change, realistic and relevant experiments must be designed. When the "Global Warming Project" commenced, it was designed to mimic realistic conditions as closely as possible. This included using water of ambient temperature, natural photoperiod and large numbers of fish. However, an issue which became quite pressing, quite rapidly, was food ration. This aspect of research became the major focus of the present thesis. The results expand our ideas on the importance of feeding in physiological experiments, and also tentatively explain some of the discrepancies in the literature regarding the effects of chronic low pH exposure. These discrepancies include the presence and absence of ionoregulatory disturbances, and the presence or absence of a stress response involving the interrenal pituitary axis during chronic low pH exposure.

In previous studies, when fish were fed, ionoregulatory disturbances were small or did not occur, suggesting fish were eating their way out of trouble (Kwain 1984; Menendez 1976; Sadler and Lynam 1986; Balm and Pottinger 1993; Wilson et al. 1994a; Dockray et al. 1996). This thesis critically examined this hypothesis. As it turned out, not only could fish eat their way *out of trouble*; they could eat their way *into trouble.*

The results of Chapter 2 confirmed the first point. A comparison of fish fed to satiation with those fed a limited ration every four days, demonstrated that typical ionoregulatory disturbances occurred in fish maintained on the limited ration and not in fish fed to satiation. The next question asked was how dietary factors were important in reducing ion loss. The two possibilities were either food provided the necessary fuel to

meet the increased cost of living in a low pH environment, or dietary salts replaced branchial ion loss.

The results of Chapter 3 specifically indicated that rainbow trout were able to use dietary salts to compensate for elevated branchial losses during chronic low pH exposure. In other words, it is the salt in the food and not the energy in the food which is critical in allowing adaptation during long term acid stress. This experiment also showed that the costs associated with eating a protein rich food, specific dynamic action (SDA), can be detrimental to freshwater fish during chronic acid stress when the diet is deficient in salt.

Rainbow trout are able to use dietary salts to compensate for impairment of ion balance due to chronic low pH. It also suggests that increases in M_{O2} due to feeding can be costly, from the standpoint of ionoregulatory disturbances, especially if diets are not balanced or if feeding is periodic. It is hoped that this study will not only provide insight into conflicting results regarding acid stress in the literature, but also aid researchers in planning studies with more consideration about feeding regime and dietary quality, thus creating more environmentally relevant research.

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Addendum

Data from Chapter 3 were also analyzed using a two way ANOVA (SAS Jmp 2.0.5) to determine the effects of salt, energy and the interaction of salt and energy on the response of juvenile rainbow trout to low pH. Starved fish were not included in this analysis. The following section describes some of these findings and discusses the implication of the analysis on the overall results of Chapter 3.

Mortality

Salt and energy and the interactive effects of both had significant effects on mortality. Fish fed regular energy and low salt diets had increased mortality and the interaction of both led to increased mortality in the regular energy/low salt fed fish.

Growth

Interactive effects of energy and salt were significant with the regular energy/regular salt fed fish and the low energy/low salt fed fish having significantly increased growth at day 11. By day 28, the factor influencing growth was energy, with those fed the regular energy diets growing more.

Plasma

Plasma cortisol showed no effects due to either salt or energy until day 28, when fish fed the regular energy diets had elevated levels of cortisol compared to fish fed the low energy diets. The salt level of food did not influence cortisol elevation. $[Na^+]_p$ showed transient differences throughout the 28 days, on day 11, fish fed the regular energy diets had higher $[Na^+]_p$ however, by day 28 fish fed the low salt diets had significantly lower Na+ levels. Plasma protein was significantly higher in fish fed the regular energy diets throughout the 28 days while hematocrit showed transient differences. On days 5 and 28, energy and salt levels were interacting to influence hematocrit while on day 11, fish fed the low salt diets had higher hematocrit values. On day 20, there was not effect by either salt or energy.

Whole body ions and proximate composition

$[Na^+]_{wb}$, $[Cl^-]_{wb}$ and $[K^+]_{wb}$ all showed transient differences throughout the 28 days. Day 5 showed no effects of either salt or energy. Days 11 and 28 showed that $[Na^+]_{wb}$ was lower due to the low salt diets while on day 20, low $[Na^+]_{wb}$ was due to low energy in the diets. $[Cl^-]_{wb}$ was lower on day 5 due to interactive effects of salt and energy, by day 11, significant differences were due to low salt diets and by day 20 there were no differences in $[Cl^-]_{wb}$. By day 28, the differences were due to energy, with significantly lower levels in the low energy fed fish. $[K^+]_{wb}$ had no significant differences, day 11 and 12 indicated that low salt diets had lower $[K^+]_{wb}$ and by day 28, fish fed the low salt diets had higher $[K^+]_{wb}$ and fish fed the low energy diets had lower levels. Proximate composition revealed that water and protein were no different regardless of energy or salt content of the food, lipids were lower in the low energy fed fish while carbohydrate levels were influenced by both salt and energy.

The above analysis indicates that there is still quite an interaction between the energy and salt content of food. Fish fed the low salt diets suffered from typical acid stress with more ionoregulatory disturbances. Cortisol elevation during low pH exposure also seems to be linked more to the energy in food than the salt. However, the extent of the interactions between the energy and salt content are also shown with the regular energy/low salt combination being the most detrimental for fish.

Appendix 1

Sodium and Chloride Flux Measurements

Introduction

"Acclimation has typically been used to mean an increased tolerance of an elevated, usually lethal concentration of a toxicant arising from chronic exposure to a sublethal concentration of that toxicant"(McDonald and Wood 1993). Research has shown that wild fish found in low pH waters have increased resistance to acid stress in the laboratory (McWilliams 1980; Brown 1981); however, this may be due to genetic selection of more acid tolerant individuals and/or strains. Laboratory experiments with hatchery raised trout have shown varying results with respect to low pH tolerance (Audet and Wood 1988; Brown et al. 1990; Balm and Pottinger 1993; Dockray 1995). For example, Audet and Wood (1988) found that an 81 day sublethal exposure to pH 4.8 sensitized fish when challenged with pH 4.0. However, Dockray (1995) found that after 90 days of exposure to pH 5.2, fish were more tolerant to low pH, with increased ability to recover branchial ion losses during a 24 hour challenge with pH 4.2. Balm and Pottinger (1993) proposed that ultrastructural changes in the gills of fish previously exposed to an acidic medium conferred increased tolerance to pH 4.0. Audet and Wood (1993) reported no such changes.

These varying results may reflect the different physiological responses of these fish during the period of chronic exposure. In the Audet and Wood (1988; 1993) study, ionoregulatory parameters stabilized at a lower levels without recovery after the first few weeks of acid exposure. However, Dockray (1995) found no ionoregulatory disturbances throughout the 90 days prior to the pH challenge. Balm and Pottinger (1993) reported decreased plasma Cl^-, but few other physiological changes due to low pH exposure.

Differences may be due to different feeding regimes during the long term pH exposure which subsequently influence the responses to low pH challenge. In the Audet et al. study (1988) fish were fed to satiation only once per week, while in the other two studies, fish were fed 1% or 2% of their wet body weight daily.

In the present study which employed the fish of Chapter 2, trout were exposed to sublethal low pH (5.2) and/or increased natural fluctuating temperatures for 90 days, during which time they were fed a limited ration, 1% of their wet body weight once every four days (~0.25%, daily ration). Detailed results of this chronic exposure have been described in Chapter 2. Fish exposed to low pH showed typical ionoregulatory disturbances with increased perturbations in the fish held at a slightly elevated temperature. These trout were then challenged with higher concentrations of external hydrogen ions (pH 4.0) to determine if prior chronic exposure imparted an increased ability to maintain net branchial sodium and chloride balance.

Material and Methods

Fish used in these challenge fluxes had been maintained for 90 days and exposed to four treatment regimes: control temperature (8 - 10° C)/pH 5.2 (0/5.2), control temperature/control pH (0/6.1), +2° C above control temperatures (10-12° C)/ control pH (2/6.1) and +2° C above control/pH 5.2 (2/5.2). Four to five fish per tank were randomly chosen from each tank, nine to ten fish per treatment; and placed in ten 400mL plexi-glass flux chambers which were individually aerated. Fish were allowed to acclimate for 17 hours during which time their respective treatment waters flowed through each chamber. Water temperatures were 12°C for the control temperature fish and 14°C for the +2°C fish. Fish were not fed for 2 days prior to or during the challenge experiment. An initial control flux measurement was conducted, followed by a 24 hour pH 4.0 challenge, during which time unidirectional and net flux measurements were made for Na^+ and Cl^- between 0 - 1

hours, 3 - 4 hours, 9 - 10 hours, and 23 - 24 hours, henceforth referred to as 1, 3, 10 and 24.

Measurements were made by stopping the water flow to each chamber and adding 0.5uCi $^{22}Na^+$ and 1uCi $^{36}Cl^-$; individual aeration was maintained at P_{O2} >120 torr and ensured thorough mixing. After a ten minute equilibration period, 15mL water samples were taken, of which 5 mL were frozen at -20° C for subsequent analysis for ammonia using the salicylate-hypochlorite method (Verdouw et al. 1978). The other 10mL were used for determining $^{22}Na^+$ and $^{36}Cl^-$ activity, and $[Na^+]$ and $[Cl^-]$. A second sample for the same analyses was taken an hour later. The pH challenge started by lowering pH to 4.0 by running water of this H^+ concentration through the chambers for 5 minutes, after the control flux was completed. The pH was held during the one hour flux measurement periods by manually statting with 0.2N H_2SO_4; typically pH was within 0.1 units of the desired level. Control and +2°C temperatures were maintained during the flux by running water of the appropriate temperature around each chamber on the wet table.

Prior to each measurement period during the acid challenge, mortalities were noted and at the end of the 24 hour challenge, fish were killed by a blow to the head. Blood was collected by caudal severance into ammonium heparinized capillary tubes. Hematocrit was determined following centrifugation at 10 000 x g, and plasma protein was measured using a hand-held refractometer (American Optical). Remaining plasma was frozen at -70°C for subsequent analysis of $[Na^+]_p$ by atomic absorption spectroscopy (Varian AA-1275), and $[Cl^-]_p$ coulometric titration (Radiometer CMT10).

Calculations

Net fluxes (J_{net}) for Na^+ and Cl^- were calculated from net changes in their concentration in the water, factored by volume, fish weight and time. J_{in} for Na^+ and Cl^-

was measured by the disappearance of radioisotopes from the water while J_{out} was determined as the difference between J_{in} and J_{net}.

Water samples were analyzed for $^{22}Na^+$ radioactivity by measuring gamma activity on a Canberra Packard Minigamma 5000 gamma counter and total [Na^+] by atomic absorption spectroscopy (Varian AA-1275). $^{22}Na^+$ and $^{36}Cl^-$ radioactivities in combination were measured by liquid scintillation counting (LKB 1217 Rack beta); $^{36}Cl^-$ radioactivity alone was then determined by subtraction after correction for the differences in efficiency of $^{22}Na^+$ counting on the two instruments. Sodium and chloride influx rates were calculated using the standard unidirectional influx calculations, as there was no need to account for backflux of the isotopes, because the specific activity of the plasma after 24 hours of pH 4.0 exposure was less than 10% of the water specific activity (Kirschner 1970). Calculations were as follows:

J_{in}=Δ cpm x volume (mL) x time^{-1} (hours) x weight $^{-1}$ (kg) x 1/MSA,

where cpm= counts per minute and MSA is the mean specific activity (cpm ion/total [ion]). Net flux rates were determined using:

J_{net}=Δ[ion] x volume x time^{-1} x weight $^{-1}$ and

J_{out}= J_{net}-J_{in}.

Statistical Analysis

Values are expressed as mean $\pm$ SEM (n=3-10). A one way ANOVA was used to compare means (SAS JMP Version 5.0); if the F-value indicated significance, Dunnett's test was then used to compare the means during the 24 hour flux with the control. The Tukey Kramer comparison of all pairs was applied to determine differences among treatments at each flux period, if the F-value indicated significance. An unpaired Student's t-test was used to compare plasma values before and after the pH 4.0 challenge. The acceptance level was $p<0.05$. Throughout the pH challenge exposure, sample numbers

decreased due to mortality. This "natural selection process" may obviously bias the patterns of physiological response. If data are reported only for survivors, then only the physiological response of the most resistant fish is documented, a "survivor effect". If data from all fish are reported, then sequential mortality of weaker fish may distort temporal patterns. Recognizing these latter limitations, the latter approach was adopted, and is evaluated in the Results section. Data reported for each flux period (Figures A-2 and A-3) include measurements from all fish alive at the end of the flux period.

Results

Figure A-1 clearly illustrates that the 24 hour exposure to pH 4.0 was detrimental to fish previously exposed to the 2/5.2 treatment with only 3 of 9 fish alive for the last flux measurement (67% mortality). The 0/5.2 and 2/6.1 groups had only 20% of the fish die while the 0/6.1 group exhibited 40% mortality.

Comparison for the 2/5.2 treatment between all fish (the fish that died during the 24 hour flux as well as the survivors) versus the three survivors only are shown in Figure A-2. There were few substantial differences in J^{Na+}_{net} between the survivors and the fish that died before 24 hours, although J^{Na+}_{in} was slightly higher in the survivors. Thus, interpretation of the flux measurements during the 24 hour challenge does not seem biased by the "survivor effect".

Results for the Na^+ and Cl^- fluxes for each treatment over time (including data from all fish) are seen in Figures A-3 and A-4. Values for 0/6.1 at 10 hours are not available for Na^+ fluxes. During the control period, there were no significant differences in Na^+ movement among the four treatments, and they all appeared to have a J^{Na+}_{net} around 0, neither gaining nor losing sodium (Figure A-3). At 1 hour, there was a significant reduction in J^{Na+}_{in} from the control values, and J^{Na+}_{out} increased, resulting in a negative J^{Na+}_{net} for all treatments, regardless of acid pre-exposure. Comparison of J^{Na+}_{net}

at 1 hour among treatments indicated that the 0/6.1 treatment had a greater loss of ions than the 2/6.1 treatment, while 0/5.2 and 2/5.2 were no different from these two treatments. Inhibition of J^{Na+}_{in} remained throughout the 24 hours with no recovery in any of the treatments to the control values. J^{Na+}_{out} decreased over time, reaching control values for all treatment by 24 hours. However J^{Na+}_{net} remained significantly more negative for the 0/5.2 group while in the other treatments net movement of Na^+ had returned to control values.

During the control period, there were no significant differences in Cl^- movement among the four treatments, and they all appeared to have a J^{Cl-}_{net} around 0, neither gaining nor losing chloride (Figure A-4). J^{Cl-}_{in} showed similar inhibition as J^{Na+}_{in} (Figure A-3), however this inhibition of Cl^- influx was slightly delayed compared to the inhibition of Na^+ influx, with a more severe depression at 4 hours. Statistically, J^{Cl-}_{in} was not significantly less than the control flux in the 0/6.1 and 2/5.2 treatments throughout the 24 hours. The significant increase in J^{Cl-}_{in} in the 0/5.2 group during the first hour of the challenge is of unknown origin. J^{Cl-}_{in} was only significantly less than during the control period in the 2/6.1 treatment. J^{Cl-}_{out} increased but by the 10th hour of the pH 4.0 challenge, values were no longer significantly different from the control in most groups (except for the 0/5.2 treatment), and no significant difference persisted at 24 hours. J^{Cl-}_{net} showed a net loss of Cl^- after the initial pH 4.0 challenge but by 24 hours underwent recovery to control values, similar to J^{Cl-}_{out}. Among treatments the initial response to the pH 4.0 challenge was different. Both +2°C treatments had a lower net Cl^- loss than the 0/6.1 treatment, although the 0/5.2 treatment was not significantly different from the other three treatments. By the fourth hour, the 2/6.1 group still had the lowest net loss of Cl^-, while fish in the 0/6.1 and 2/5.2 treatments had significantly greater losses. By 10 hours there were no differences in J^{Cl-}_{net} among treatments.

Plasma $[Na^+]_p$ and $[Cl^-]_p$ both dropped significantly after the pH 4.0 challenge except in the 2/5.2 treatment (Table 1). Fish in the 2/6.1 had the highest $[Na^+]_p$ and $[Cl^-]_p$ among the other three treatments. In the 2/5.2 treatment, $[Na^+]_p$ and $[Cl^-]_p$ were already substantially lower prior to the challenge. Hematocrit increased significantly for all treatments except 2/5.2 while plasma protein also tended to increase. However, the latter change was only significant in the control temperature treatments (Table 1). Interpretation of the plasma data for the 2/5.2 treatment should be approached with caution as they are based on an n=3 which may not be representative of all 2/5.2 fish challenged with pH 4.0.

Throughout the control period and 24 hour challenge, ammonia excretion did not exhibit a pronounced difference among the four treatments, except at 24 hours when the 0/6.1 treatment was significantly lower than the 2/5.2 treatment (Figure A-5). However, 0/5.2 treatment fish had a significantly higher ammonia excretion rate throughout the challenge compared to their control value. The ammonia excretion rates of the 0/6.1 group were only significantly higher at 10 hours though it appeared that ammonia excretion was increased throughout the challenge. Ammonia excretion did not change during the challenge in either +2°C treatments.

Discussion

Typically, toxic effects of low pH exposure in softwater are attributed to branchial ion loss followed by circulatory distress (Wood 1989; Reid 1995). This rapid loss of ions causes osmotic pressure to fall in the extracellular fluid, which is compensated for by a shift of fluid into the intracellular fluid compartment causing an increase in hematocrit, and plasma protein concentration. Thus, blood viscosity is augmented, increasing arterial blood pressure leading to circulatory failure (Milligan and Wood 1982; Wood and McDonald 1988). Consequently, there are several parameters to use in deciding if acclimation to low pH occurs, including rate of mortality, level of ion loss, rate of recovery

of J^{ion}_{net}, or decreased secondary effects in relation to changes in ion and fluid shifts. Results from this present study highlight the difficulty in determining whether fish acclimate or sensitize to low pH because, depending on the parameter used, different conclusions can be drawn.

The rate of mortality varied among the treatments. The high rate in fish previously exposed to the 2/5.2 treatment suggests they did not acclimate to pH, however, the decreased mortality in the 0/5.2 treatment suggests otherwise. If the rate of ion loss is examined, initially all fish had a dramatic net loss, but the 2/6.1 had the lowest net loss, again suggesting that acclimation does not occur in previously acid exposed fish. If recovery is examined, a large inhibition of Na^+ uptake was seen among all treatments, with no subsequent recovery. By 24 hours, J^{Na+}_{net} had not returned to control values for the 0/5.2 fish while in the other treatments it had. However J^{Cl-}_{net} returned to control values for the acid exposed fish while it did not for the 2/6.1 fish suggesting that there is a temperature effect (different results between 0 vs. +2°C treatments) and/or an ion effect (different results between J^{Na+}_{net} and J^{Cl-}_{net}) thereby, compounding the confusion of an already hazy area.

Interpretation of the Cl fluxes goes no further in clearing up the picture about acclimation to low pH. All treatments resulted in an elevated efflux of Cl^- from the fish resulting in net loss of Cl^-, initially with a slightly delayed inhibition of Cl- uptake. By 24 hours , J^{Cl-}_{net} fluxes had returned to control levels for all fish except the 0/ 6.1 treatment. As well, fish in the 0/6.1 treatment had the greatest J^{Cl-}_{out}, though this was only significantly different from the fish at +2°C.

If blood plasma parameters (secondary effects) are considered, fish in the +2°C treatments did slightly better than fish at control temperatures (Table A-1). The fish in the 2/5.2 treatment did not have significantly different values from pre-challenge values,

inferring less dramatic secondary effects, and supporting the argument for acclimation to low pH. However, there is only an n of 3 in the 2/5.2 treatment and as death is rather an indisputable manifestation of stress, it can be concluded that fish exposed to the 2/5.2 treatment did not acclimate to pH.

Data from these experiments do indicate that there is no link between Na^+ and Cl^- transport because of the generally delayed inhibition of $J^{Cl^-}_{in}$ which was more pronounced at 4 hours. It seems more likely that the high [H+] resulted in depletion of HCO_3^- or OH^- from gill cells, decreasing the substrates available for coupled exchange in the epithelial cells, or that damage to a Cl^- carrier occurred over time (Wood 1989).

As mentioned, several issues do arise suggesting there may have been a temperature effect on acclimation or that these fish had been compromised physiologically and were not able to handle any increased stress. For example, fish previously exposed to the 0/5.2 treatment had a lower mortality compared to the 2/5.2 treatment. Although, J^{Na+}_{net} did not return to its control value in the 0/5.2 treatment while it did in the 2/5.2 treatment, the net loss from these fish were no different. When comparing the naive fish, the 0/6.1 treatment had a slightly higher mortality and lower $[Na^+]_p$ and $[Cl^-]_p$ compared to the 2/6.1treatment.

Although differences in the fish's response to pH 4.0 may have been temperature dependent, the previous health of these fish may also have played a role in their response to the challenge. The 2/5.2 fish were physiologically more compromised prior to the pH 4.0 acid challenge (Chapter 2), and therefore may not have been able to handle the acid challenge. Fish in the 0/5.2 treatment had a much higher lipid content compared to the other treatments, prior to the acid challenge. If energy is utilized to maintain homeostasis in a marginalized environment, than these fish may have been more capable of coping with this acid challenge (Sadler and Lynam 1986 Reid et al. 1995)

As mentioned, Dockray (1995) found that juvenile rainbow trout previously exposed to pH 5.2 improved ability to recover their net branchial sodium transport rates to control levels after 24 hours challenge to pH 4.2. Dockray's fish had been fed a four fold greater ration than the present fish and did not show any ionoregulatory disturbance prior to the acid challenge. However, in the present study, fish were kept on a maintenance ration of only 0.25%/day. The 2/5.2 fish in this present study exhibited typical ionoregulatory distress prior to the pH 4.0 challenge, suggesting that previous nutritional status may also play a role in decreasing tolerance to low pH. A second factor which may play a role is temperature, perhaps because of increased passive ion loss at higher temperatures due to thermodynamics. Results are similar to those of Audet and Wood (1988).

Inhibition of J^{Na+} was not accompanied by reduced ammonia excretion. In fact, ammonia excretion in the 0/5.2 fish was particularly elevated. These results are contrary to Wright and Wood (1985)who observed a drop in ammonia excretion during a pH 4.0 challenge in hardwater, and Audet and Wood (1988) who reported a 50% inhibition of ammonia excretion in naive fish exposed to pH 4.0 in softwater. Thus, these results do not support a Na^+/NH_4^+ coupled exchange as suggested by McDonald and Prior (1988). However, they do support results by Avella and Bornancin (1989) and Wilson et al. (1994). The latter studies suggested that ammonia excretion is passive and probably a function of internal ammonia concentration and that the gill boundary acidification layer plays a role in driving ammonia excretion. Lower gill boundary layer pH during pH 4.0 challenge would explain the tendency for greater ammonia excretion in the present study.

McDonald and Wood (1993) proposed that branchial mechanisms of acclimation are a function of the extent of gill damage. For acclimation to occur, structural damage to the gill epithelium must evoke an epithelial response. Sublethal pH is thought not to induce

significant enough damage to result in acclimation (Wood 1989; Reid 1995; but see Balm and Pottinger 1993), thus results from this experiment are probably due to the previous health of the fish. We predict that as fish in a global warming and low pH scenario are already in a poorer condition, they will be more sensitive to episodic acid surges during snowpack melt especially if food ration is limited, which is expected during winter.

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Table A-1: Measured Plasma Na^+, Plasma Cl^-, hematocrit (%) and plasma protein (g·100/ml) before and after the pH 4.0 challenge. Mean values ± SEM are shown (n=3 to 10). Treatments that share a letter are not significantly different from each other ($p<0.05$). * indicates significant differences between pre challenge and post challenge values.

	Pre-Challenge				**Post-Challenge (24 Hour pH 4.0)**			
	0/5.2	**0/6.1**	**2/6.1**	**2/5.2**	**0/5.2**	**0/6.1**	**2/6.1**	**2/5.2**
$[Na^+]_p$ (mM)	124.9^a	129.3^a	130.5^a	108.3^b	$*78.9^{ab}$	$*72.7^a$	$*97.4^b$	94.5^{ab}
	± 3.8	± 0.9	± 1.5	± 4.0	± 4.5	± 4.5	± 5.9	± 7.4
$[Cl^-]_p$ (mM)	125.0^a	119.4^a	125.6^a	106.8^b	$*61.9^a$	$*58.8^a$	$*104.4^b$	86.2^{ab}
	± 1.3	± 2.5	± 1.4	± 3.9	± 4.2	± 4.4	± 2.5	±13.7
Hematocrit (%)	33.2^a	30.0^{ab}	31.6^a	36.2^b	$*61.9^a$	$*56.7^a$	44.6^a	45.2^a
	±1.2	±1.0	±1.1	±1.2	± 6.1	± 2.5	± 3.2	± 8.3
Protein (g/100ml)	2.2^a	2.9^b	2.4^{ab}	3.0^b	$*5.7^{ab}$	$*4.7^a$	$*2.9^b$	3.1^{ab}
	±0.2	±0.1	±0.2	±0.2	± 0.6	± 0.8	± 0.4	± 1.4

Figure A-1:

The percentage of fish surviving during the 24 hours pH 4.0 challenge. Mortalities were counted prior to starting each flux. The highest percentage of mortalities occurred in the 2/5.2 treatment.

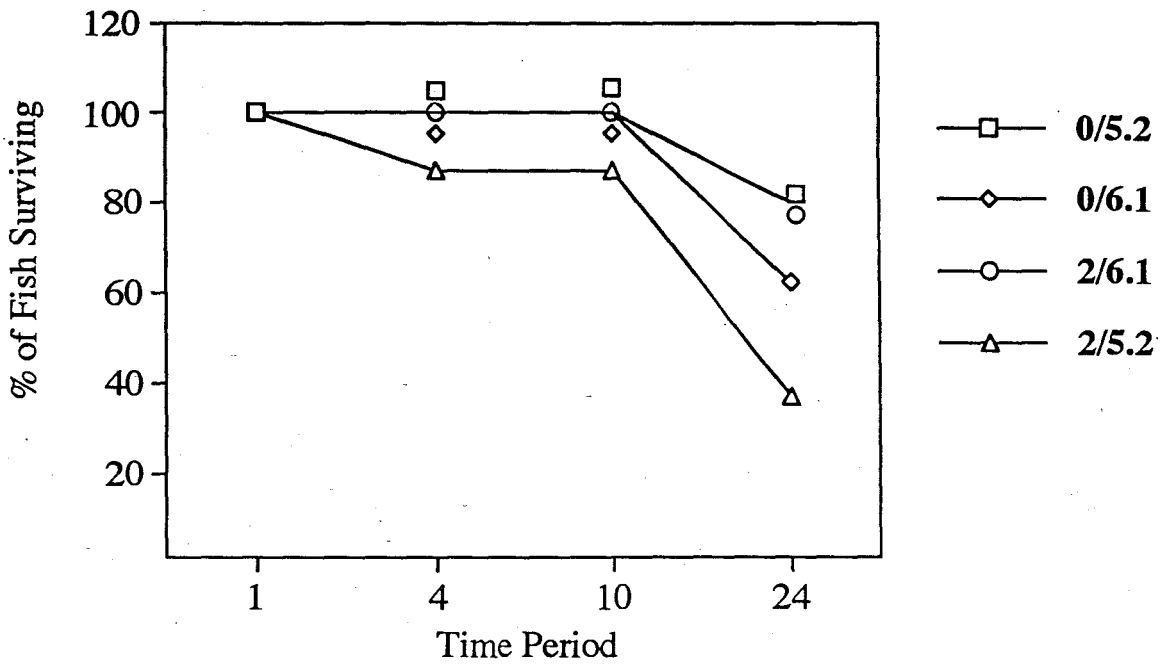
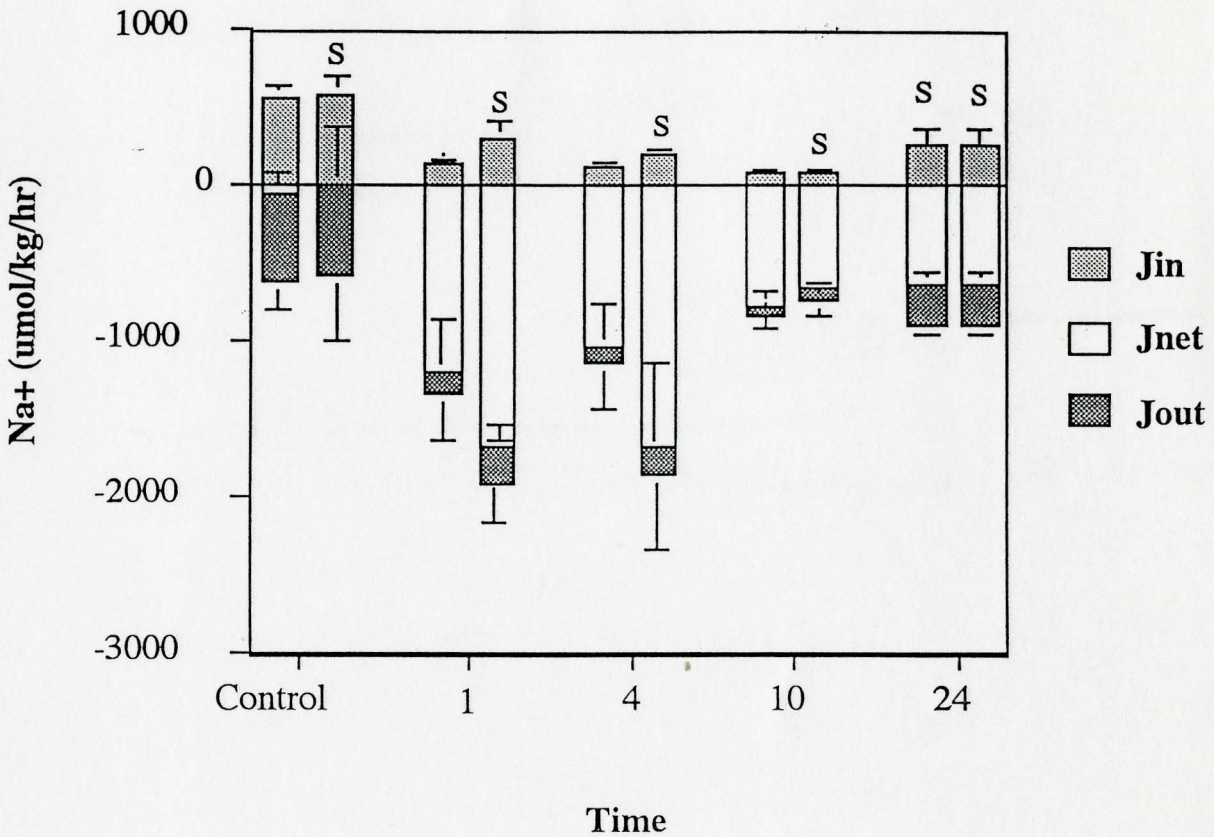

120
100
80
60
40
20
% of Fish Surviving
1
4
10
24
Time Period
0/5.2
0/6.1
2/6.1
2/5.2

Figure A-2

The unidirectional and net Na^+ flux rates for the 2/5.2 treatment, the first bar at each time period is the measurements for all fish alive during the time period, while the second bar is the measurements of only the 24 hour survivors (S) throughout the exposure. There were few noticeable differences between the survivors and the fish that died comparing flux rates.



S= the unidirectional and net Na_+ fluxes for the fish that survived the 24 hours pH 4.0 challenge.

Figure A-3

Unidirectional and net Na^+ flux rates in trout exposed to pH 4.0 for 24 hours. Flux rates are displayed over time for each treatment and are expressed as a mean ± SEM (n=3-10). Flux rates that are significantly different from the control period values are indicated by a * for influx, † for net flux, and Δ for efflux. Significant difference for net fluxes among treatments at a flux period are indicated by treatments that do not share a letter ($p<0.05$). Significant differences for influx and efflux are mentioned in the text.

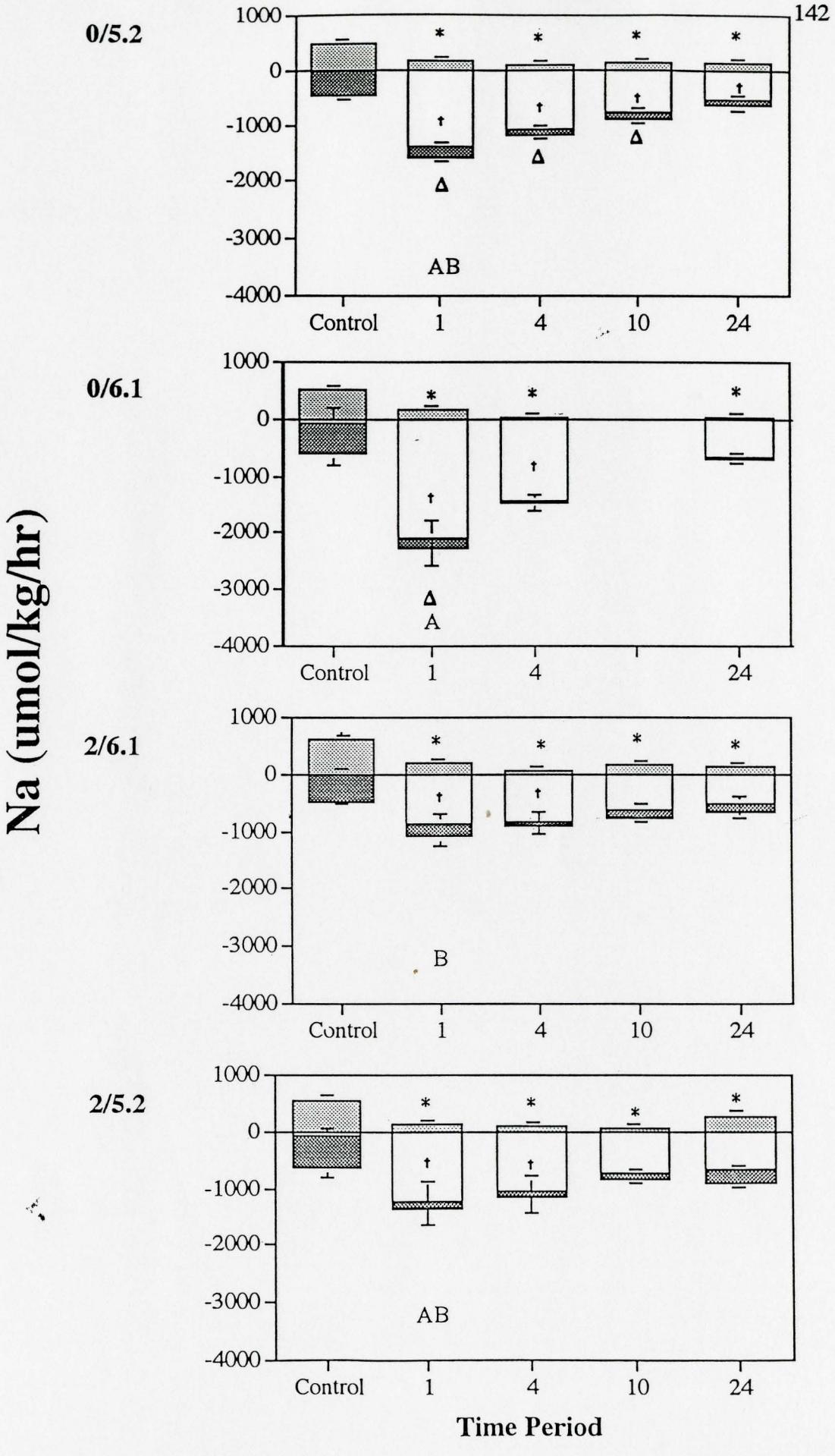

0/5.2
0/6.1
2/6.1
2/5.2
Na (umol/kg/hr)
1000
0
-1000
-2000
-3000
-4000
AB
A
B
AB
Control
1
4
10
24
Time Period
J_{in}
J_n
J_o

Figure A-4

Unidirectional and net Cl^- flux rates in trout exposed to pH 4.0 for 24 hours. Flux rates are displayed over time for each treatment and are expressed as a mean ± SEM (n=3-10). Flux rates that are significantly different from the control period values are indicated by a * for influx, † for net flux, and Δ for efflux. Significant difference for net fluxes among treatments at a flux period are indicated by treatments that do not share a letter ($p<0.05$). Significant differences for influx and efflux are mentioned in the text.

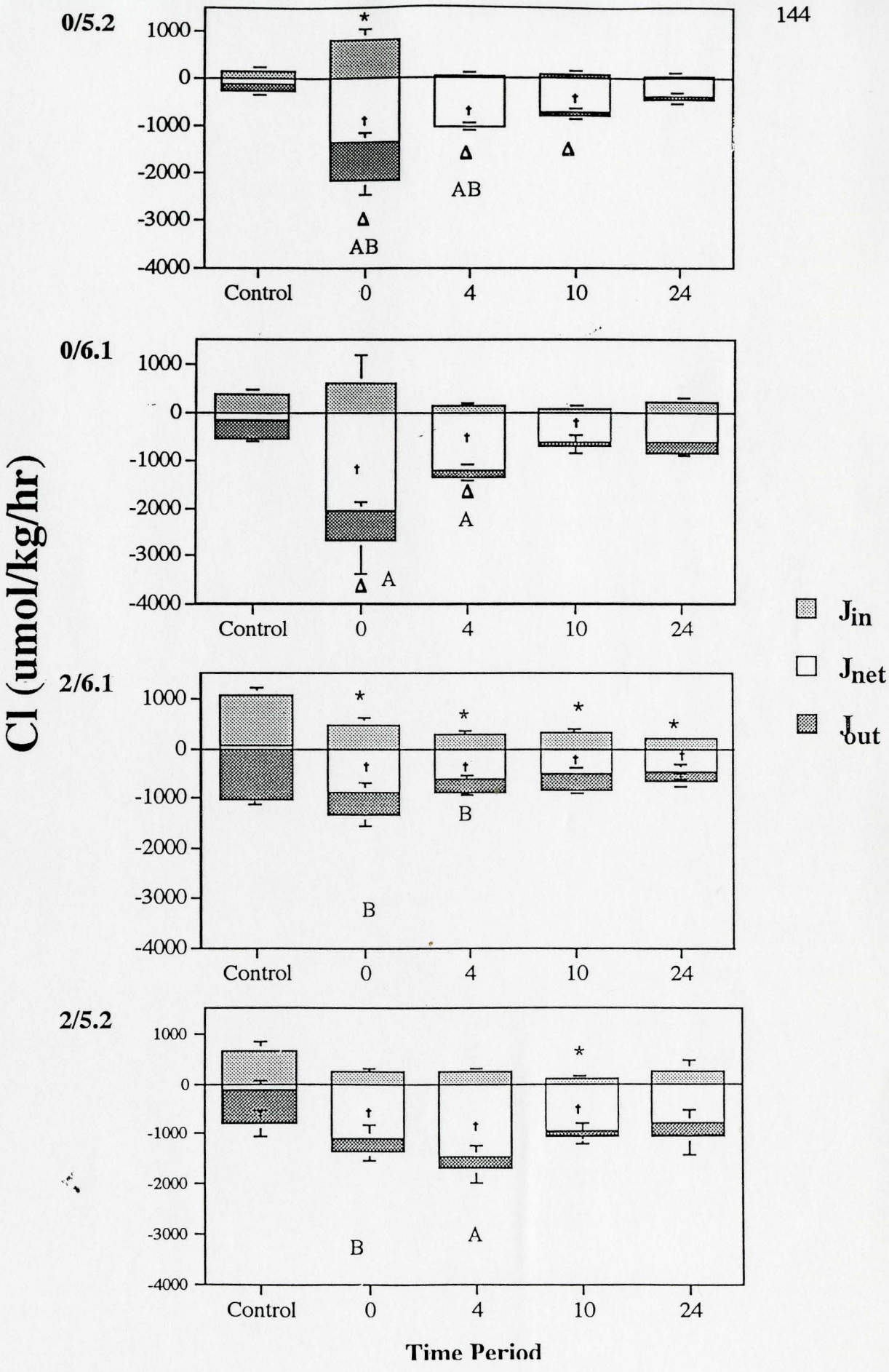
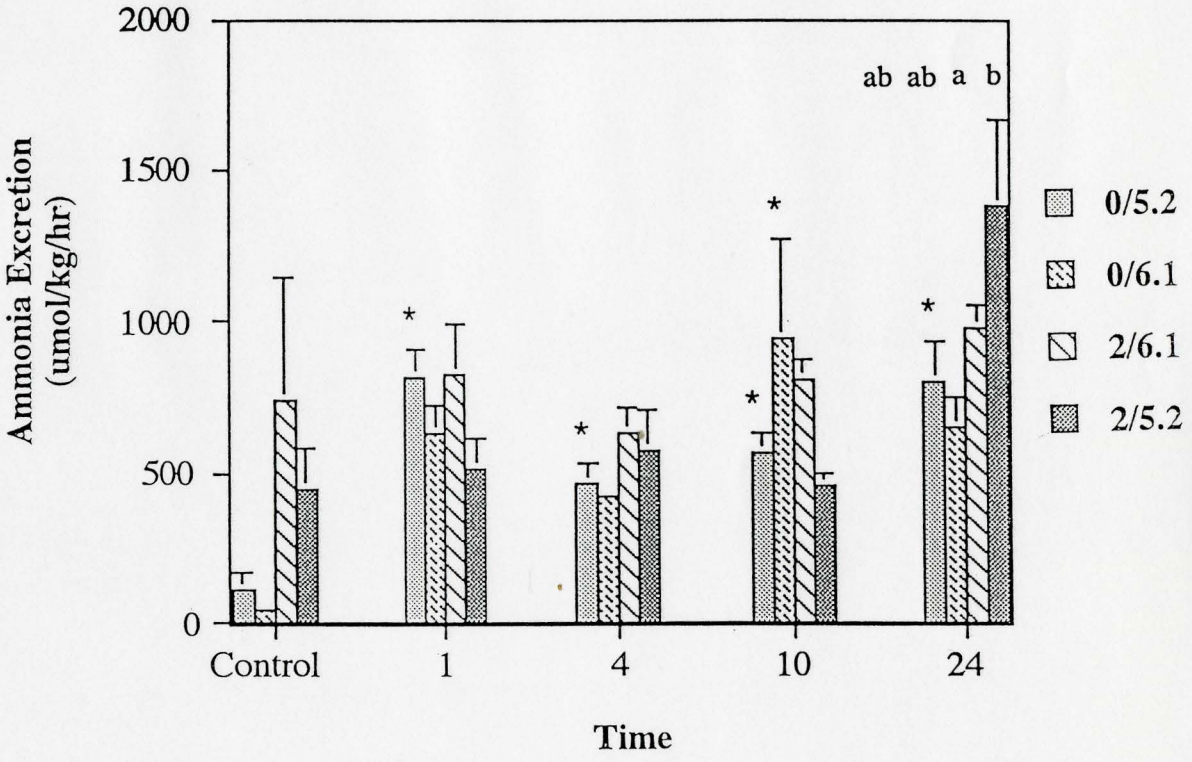

0/5.2
0/6.1
2/6.1
2/5.2
1000
0
-1000
-2000
-3000
-4000
Control
0
4
10
24
AB
A
B
Cl (umol/kg/hr)
Time Period
Jin
Jnet
Jout

Figure A-5

Ammonia excretion rates in trout exposed to the pH 4.0 challenge. Rates are expressed as mean ± SEM (n=3-10), and are displayed over time. Flux rates that are significantly different from the control period values are indicated by a *. Significant differences among treatments at a flux period are indicated by treatments that do not share a letter ($p<0.05$).


Ammonia Excretion
(umol/kg/hr)
2000
1500
1000
500
0
ab ab a b
*
Control
1
4
10
24
Time
0/5.2
0/6.1
2/6.1
2/5.2