GLOBAL WARMING AND ACID RAIN: EFFECTS ON RAINBOW TROUT

ENVIRONMENTAL ACIDIFICATION AND GLOBAL WARMING: EFFECTS ON THE GROWTH AND PHYSIOLOGY OF JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM)

By

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Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) were chronically exposed (90 days) in synthetically softened water ($[Ca^{2+}]$=50, $[Na^{+}]$=100 $\mu equiv \cdot L^{-1}$), to sublethal low pH (5.2) and a simulated global warming scenario (+2°C), added to the natural summer thermal cycle of inshore Lake Ontario. Two studies were conducted over the periods June - September 1993 and 1994 in order to examine the effects of the sublethal stressors under conditions of unlimited food ration (satiation feeding twice daily = 10% dry body weight$\cdot day^{-1}$), and a limited food ration (4% dry body weight$\cdot day^{-1}$) respectively. The addition of 2°C and sulphuric acid was designed to result in four treatment conditions: i) Control conditions; ambient water temperature and pH; ii) ambient water temperature and pH 5.2; iii) simulated global warming at ambient pH; iv) a combination of simulated global warming and pH 5.2. Year to year variation in temperature provided the trout in the satiation-fed study with an ambient temperature range of 13-24°C, while those in the limited ration study experienced a range of 16.5-21°C. Consequently, the trout in the treatments with an additional 2°C experienced temperatures close to the upper incipient lethal level, particularly the trout in the satiation feeding study. Apparent specific dynamic action raised routine metabolic rates in all treatments to ~75% and 55% $MO_{2(max)}$ in the satiation and limited ration studies respectively, the difference of 20% indicating the influence of an unlimited feeding regime on metabolism. Trout in the satiation feeding study increased in wet body mass by 30-50 g, while trout in the limited ration study increased by only 3-4 g. Whole body proximate composition of the trout fed to satiation changed over time with large increases in lipid content, small increases in protein content, and compensating decreases in water content in all treatments. No such changes occurred in the limited ration trout, although whole body lipid and carbohydrate were highly variable. In both studies,

the addition of 2°C resulted in decreased growth, with an accompanying depression in appetite in the satiation fed trout, especially at peak temperatures. Surprisingly, trout exposed to low pH alone exhibited improved growth in both treatments. Energy budgets indicated that the addition of 2°C reduced gross energy intake and increased fecal (and unidentified) energy losses resulting in lowered conversion efficiencies, while in limited ration trout, energy expenditure was slightly higher. Trout exposed to low pH exhibited higher gross energy intake and gain, and more efficient energy conversion under unlimited food conditions while trout with limited rations expended the least metabolic energy and exhibited lower nitrogen energy losses. The surprising lack of ionoregulatory disturbance in these pH 5.2 exposed trout in both studies suggests that the availability of NaCl in the diet was compensating for branchial ion losses, and perhaps driving appetite in the satiation fed trout. Where ration was limited, reductions in activity level may have contributed to energy conservation and consequently improved growth. A $^{22}Na^{+}$ flux experiment conducted at the end of the limited ration exposure, in which the fish were exposed to a challenge concentration of H^{+} (pH 4.2), provided evidence for improved recovery of ionoregulatory balance in trout which had been chronically exposed to low pH. Overall, the combination of increased global temperatures and sublethal low pH results in increased physiological costs for juvenile rainbow trout, most noticeably when summer temperatures peak. Ration level is of integral importance when considering the degree of impact of such environmental conditions.

Acknowledgements

First I'd like to thank Chris Wood, Gord McDonald, and Pat Chow Fraser for their advice and guidance along the way. Aaah, those cozy committee meetings in Rm 119. Thanks also to Mike O'Donnell, for the last minute examining committee duty.

On arrival from England, I thought I would do what normal people do when moving countries; find somewhere to live. The day after my flight to Toronto, I walked into Chris Wood's lab to say hello and meet everybody before setting off in search of a place to call home. I learned quickly that introductions can be very brief, and B121 is at least a warm place to sleep. The next thing I knew, I was down in B112 attaching standpipes to the tanks intended for the "Global Warming" experiments, scrubbing tanks to provide a nice clean home for our fish (not me), and feeding the tiny unfortunates, who would soon become my best buddies, because I spent practically every minute of every hour of every day of every week down there with them. At least I had friends.

In those first months I forged a friendship with two absolutely fantastic people, without whom, I wouldn't have survived. Scott Reid (the project coordinator and more importantly, my teacher) made all this overwhelming physiology stuff, and the many early mornings in the dungeon (not another #$@!*#$ lightning storm) seem bearable. Tyler Linton, my 'pardner' in crime, buoyed up my spirits with a constant infusion of chocolate, won, of course, as a result of his atrocious basketball skills, and his seemingly constant desire to bet and lose. That Day 0, 3 o'clock morning didn't seem quite so bad with the added music and song (memories dull with time).

Soon, I became aquainted with the (huge) Wood lab. Space was at a premium, and I rapidly learned to avoid Rm 203, and stay down in the basement (not that I had much choice), just me, my fish, and my earplugs. I also quickly learned, that everyone was incredibly helpful, particularly Yuxiang (Xiang-man) Wang, and Randy (Lauffer) Lauff. Those squillions of assays were made easier by their input and professional guidance, and of course the laughs. All the other seasoned Woodites helped at various times and provided a shoulder to lean on occasionally: Marjorie Patrick (Mama-mia), Mike Wilkie, Annika Salama, Steve Munger, and Fernando Galvez. I also appreciated the computer guidance from Shaheen Zia, and the amazingly organized and helpful Vandy Thomas.

Neither Tyler, nor I could have managed to get through all of the sampling periods, assays, and twice daily feedings (the fish, not us), without the incredibly efficient assistance of Teresa Banka, Matthew Norton, and Kristen Young. Teresa, who stuck with

us for 2 years, deserves a gold medal for all she did, and always with a smile. Sara Croke provided us with a break from feeding the fish so we could feed ourselves occasionally, and Stephanie Pendry was a saviour in the wee hours, volunteering (you must be crazy) to grind fish.

In my second year, Scott was called away to the Okanagan Valley (how could you leave all this?) to work. Poor, unsuspecting post-doc, Ian Morgan, found himself thrown into the role of mentor and mechanic, plumber and electrician - welcome to the warmer, more polluted world we live in. Under the prevailing circumstances, he managed to roll with the waves, and provide a beacon of safety and common sense when we were practically drowning in it. More importantly, he provided me with a constant supply of fiction, and an opportunity to forget the realities of 'mechanical failure', and RO woes.

An equally sensible and kind person, as well as an exceptional scientist, is Katie Gilmour. At the eleventh hour she sorted out my grammar and missed spelling mistakes, and gave a smile that was very much needed. Those Scots are gaining in a big way!

Finally, those brand new Woodites deserve mention, and I wish them all the luck in the world with everything they do: Leela de Cruz (the newest Global Warmer), Jody Vandeputte, Nathan Webb, Mary Fletcher (only half a Woodite), Derek Alsop, Ruban Kanagaratnam, Jim Kieffer, and Darryl Burgess.

The people outside of the lab who have always supported me, and gave me the alternative view on life during this experience, were most important. My Mum and Dad, amazing people, who have constantly encouraged me in my educational pursuits, and contributed everything they could to my many goals. My wonderful friends, David Johnston, Connie Russell, John Ankenman, Chris Brown, Glenn Pedersen, Robin Willcocks, Ruth Waldick and Martin Gerrits, each a good laugh and incredibly supportive.

But without doubt, the person who saw me through everything - listened to all of my lab adventures, both good and bad; gave me a kick in the butt when I needed it; took me away from it all when I needed it; joined me on very long, evening bike rides; laughed at me when I couldn't, and with me most of the time; and always, always remained positive, was Brent Murray. Thank you.

Finally, three important things to remember in order to be successful in a fish physiology lab: 1) Never believe anyone who tells you that grinding fish takes only 20 minutes; add, at the very least, an hour to that estimate. 2) Do not get sucked in to feeding the Muskey's. 3) Teach the American you're working with to speak English so you're not left wondering why he's gone to that room to rest again...

Thesis Format

This thesis is organized as three chapters, the first of which is a general introduction, providing a general overview of the study. The second and third chapters have been written as manuscripts for submission to a peer-reviewed scientific journal. Abstracts have been included for each of the two chapters prepared as papers for submission. Chapter 2 has been submitted to a scientific journal, and we are awaiting a response from the editor. A Concluding Remarks section summarizes the results and interpretation thereof, of each chapter, and an appendix describes a Na^+ flux experiment that is pertinent to the overall study. The literature cited has been amalgamated into one section at the end of the thesis for the ease of the reader.

Chapter 1: General Introduction.

Chapter 2: The effects of chronic exposure to a 2°C elevation in temperature over the natural summer thermal cycle, in combination with sublethal low pH and unlimited food supply, on juvenile rainbow trout (*Oncorhynchus mykiss*).

Authors: J.J. Dockray, S.D. Reid and C.M. Wood.

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Chapter 3: Responses of juvenile rainbow trout, under food limitation, to chronic low pH and a summer global warming scenario, alone and in combination.

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

Comments: These data are pertinent to this thesis, and were generated by J.J.D. in close association with I.J.M., under the supervision of C.M.W. Advice on methods was provided by S.D.R.

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Chapter 1

General Introduction

Environmental Change - The Issues

Few dispute the fact that humankind has drastically altered the face of the planet we call home (see Vitousek 1994 for review). The numbers of humans now residing on earth have brought with their expansion a multitude of ecological problems, mostly as a result of the production of varied chemicals that are usually by-products of industrial processes (Kemp 1994). These by-products we now term pollutants, for reasons that are becoming clearer as the impacts of these chemicals on our environment are realized.

Two issues that have been the focus of a great deal of research in recent years, are the threat of the enhanced greenhouse effect, commonly termed global warming, and the significant effects of acidic precipitation on our environment (Likens et al. 1979; Schindler et al. 1980; Patrick et al. 1981; Schindler 1988; Rogers 1990; Kemp 1994; Vitousek 1994). Research into the impact of acidic precipitation on the world's freshwater resources has been conducted for at least three decades (Likens and Bormann 1974; Beamish 1974b; Leivestad and Muniz 1976; Fromm 1980; Lacroix and Townsend 1987; Kelso et al. 1990). On the other hand, the effects of climatic change on freshwaters has rarely been addressed in major global change programmes (Schindler et al. 1990). However, the potential impact of climate warming on aquatic environments necessitates consideration of the effects of a combination of increased temperature with aquatic pollutants such as increased acidity (Parsons 1990).

Global Climate Change

The threat of global climate change, due to anthropogenic production of

"greenhouse" gases such as carbon dioxide, methane, nitrous oxide, and chlorofluorocarbons, has recently stimulated research into the effects of increased temperatures on important resources such as the Great Lakes fishery (Regier and Meisner 1990). Predictions of increased air temperatures have been made using General Circulation Models, and range from 1.3-4.5°C over the next 50 to 100 years (Hansen et al. 1988; Mohnen and Wang 1992). Such air temperature increases would result in increased water temperatures, thereby altering the biological organization of aquatic ecosystems (Frank et al. 1990). For example, many native fishes in the river systems of the southern Great Plains, and the Southwest United States, already exist at summer temperatures that are near, or exceeding, their thermal limits (Matthews and Zimmerman 1990). Further increases in temperature may result in the extirpation of a number of the existing species, unless they are able to adapt behaviourally or genetically. In the boreal ecosystems of northwestern Ontario, a study at the Experimental Lakes Area has shown that air temperatures have increased by 2°C over the last 20 years, resulting in increased evaporation rates, decreased precipitation, and consequently, decreased water renewal rates. Stenothermal fish species have been directly affected by the decrease in available cold water habitats (Schindler et al. 1990).

Acidic Precipitation

Human activity has substantially changed the atmospheric sulphur cycle, with smelter operations and increased fossil fuel combustion resulting in acidic precipitation. Although the amounts of sulphur dioxide (SO_2), nitrogen oxides (NO_x), and volatile organic compounds emitted today are much lower than in the early 1970's, levels are still almost twice that in 1900 (Irving 1991). The resultant acidic precipitation has reduced the pH of many softwater lakes in North America and Europe by between 0.5 and 1.5 units over the last 140 years (Kemp 1994). Photo-oxidants such as ozone, hydrogen peroxide

and the hydroxyl radical, produced by other atmospheric pollutants, facilitate oxidation of SO_2 and NO_x to their respective acids (Cocks and Kallend 1988), and may contribute to the greater acidity of summer precipitation (Mason 1990). The ecological impact of freshwater acidification on fish has been studied extensively (Beamish 1972; Leivestad et al. 1976; Gunn and Keller 1981; Kelso and Gunn 1984). It is evident from these studies that different species exhibit different tolerances to low pH, with particularly sensitive species such as the fathead minnow providing an early warning signal, much like the miners canary (Kelso et al. 1990). Also, early life history periods, especially the interval from hatching to the start of exogenous feeding, are particularly sensitive in most fish species (Peterson et al. 1982).

The Rainbow Trout

The fish species utilized in the following studies is the rainbow trout (*Oncorhynchus mykiss* Walbaum). This salmonid is the reference coldwater species of fish physiology and toxicology experiments, and is representative of other salmonid species. It is generally recognized as one of the most sensitive of the salmonids to acid stress (Grande et al. 1978). It is also of economic importance around the world as a food source, and as a provider of 'sport' to fishing enthusiasts.

The rainbow trout was originally a native of the eastern Pacific Ocean and fresh waters west of the Rocky Mountains (Migdalski and Fichter 1983). It has been widely introduced to many other parts of the world, including eastern Canada. Like other salmonids, it prefers cold, well-oxygenated water, and lays its eggs in cold, gravel-based tributaries of their home rivers, or inlet or outlet streams of their lakes. It is usually a spring spawning species, although it may also spawn in the early winter, as do Great Lake occupants (Scott and Crossman 1990). The young hatch after about two months, and have usually absorbed their yolk after another week. The fry may move directly to the lake, or

remain in the streams for up to three years, while those born of river-resident parents will remain in the river (Scott and Crossman 1990).

As poikilotherms, the body temperature of fish is set by the temperature of the water in which they live. An increase in temperature generally accelerates the rates of most physiological and biochemical processes (Schmidt-Nielsen 1987). There is an upper limit to the thermally induced increases in rate however, and once this limit is reached, biological processes become deleteriously affected, and the rate begins to drop off (Cossins and Bowler 1987). At even higher temperatures, thermal damage begins to occur, rates are severely decreased, and eventually, the fish will die at an absolute boundary for temperature tolerance, the lethal temperature. For example, the upper incipient lethal temperature of rainbow trout is 26.2°C, while the ultimate lethal temperature is 30°C (Elliott 1981).

Juveniles are probably the life-stage that would be most affected by increased water temperatures, because they feed and reside in the shallows of lakes, or in shallow river areas. Some relief may be sought by retreating to deeper pools. However, the need to avoid predation by larger fish, and the availability of suitable food, may limit the forays of these juveniles to the shallows until an appropriate size is reached.

Physiological Damage Caused by Acid Stress

Rainbow trout are the most sensitive of the salmonids to acid stress (Grande 1978). A pH of 4.2 is ultimately lethal to this species (Graham and Wood 1982). Physiological studies of the effects of increased $[H^+]$ have shown that hydrogen ions exert their toxic effect at the gill by disturbing electrolyte balance (Leivestad et al. 1976; Howells et al. 1983; McDonald 1983; Wood 1989). Lethal concentrations of H^+ have been shown to result in ionoregulatory failure and subsequent fluid shifts, hemoconcentration, and eventual circulatory collapse (Milligan and Wood 1982). Exposure to chronic sublethal

low pH, however, has been shown to result in stabilization of electrolyte balance and blood plasma parameters at reduced levels, although recovery to control plasma ion concentrations does not occur (Audet et al. 1988). Further, low pH may either limit, or have no effect on growth in fish (Mount 1973; Leivestad et al. 1976; Menendez 1976; Jacobsen 1977). Resting oxygen uptake has also been shown to increase in fish exposed to low pH (Butler et al. 1992).

As described above, chronic sublethal exposure to low pH conditions has been shown to cause ionoregulatory disturbance as indicated by decreases in plasma or whole body Na^+ and Cl^-. These disturbances have been shown to occur as a result of a transient net loss of Na^+ and Cl^- across the gills. Furthermore, sublethal low pH exposure increases plasma protein concentration but does not appear to affect hematocrit. Therefore, measurements of plasma and whole body ion concentrations, plasma protein levels, and hematocrit are important indicators of stress under conditions of low pH, and may indicate the causes of any increased metabolic costs associated with this environment. Warmer temperatures may also contribute to changes in each of these factors due to rate effects, or loss of cell membrane integrity at the upper limits for temperature tolerance.

Assessing Cost

In order to assess the effects of increased temperature and decreased pH on rainbow trout, it is important to have a measure of the physiological cost of living in such a changed environment. Cost suggests payment in some form of currency. It is reasonable to suppose that a trout exposed to warmer temperatures (which increase biological rate processes) and higher concentrations of H^+ (which may require the adoption of protective or adaptive measures), may have to expend a greater amount of energy. The universal energy currency in all animals is ATP (adenosine triphosphate), and the production of ATP requires the breakdown and oxidation of foodstuffs or energy reserves. Aerobic

metabolism, as it suggests, requires oxygen for the production of ATP. Therefore, the measurement of oxygen consumption provides an indirect measure of energy production. This energy is produced for, and expended during, many biochemical and physiological activities. These include locomotion, feeding and digestion, the deposition of protein, fat, and carbohydrates as newly synthesized body materials resulting in growth, and the maintenance of appropriate ion levels in plasma and cells, to name a few. Each of these processes contributes to an overall rate of energy consumption that is generically referred to as the metabolic rate. As is evident from the above description, metabolic rate is a composite measure of the cost of living for a fish in a particular environment.

To determine how a fish, such as the rainbow trout, is partitioning its expenditure of energy, it is necessary to measure the separate energy requirements of each of the contributing processes. To measure every single ATP-requiring process would be quite an endeavour. Instead, an assessment of those processes that may compromise production or success in terms of predator-prey relationships, or the fish's exploitation of the available resources, for example, would give a good indication of any change in the costs associated with living in a warmer, more acidic environment.

Growth requires the input of energy in the form of food. Feeding behaviour may provide information regarding related stress in the changed environment. Reduced growth may be a function of a loss of appetite, rather than an increase in metabolism that requires more energy than is provided via the diet. In order to measure appetite in fish, it is necessary to provide them with as much food as they want/need. This can only be achieved by feeding the fish until they are satiated, and will no longer accept food. This allows measurement of food intake rates, and provides a measure of increased energy requirements related to the more challenging environment. Knowledge of both growth rates and feeding rates allows a relative measure of the efficiency of conversion of weight of food to body weight, giving another index of the cost of living in the altered

environment.

Body compositional analysis provides us with information regarding the anabolic process of growth. The utilization of existing stores of lipid, carbohydrate or protein, or reduced deposition of the latter, may indicate that the trout have to rely on another source of energy for daily maintenance other than their food source, thereby providing evidence of increased cost.

Measurement of quantities of food consumed, growth, proximate body composition, rates of energy expenditure, and waste excretion rates (ammonia and urea), provides information for the construction of energy budgets. Utilizing the energy equivalents of the components of the food and body, and of the energy expended and lost, it is possible to determine a reasonable assessment of the anabolic and catabolic processes occurring within the fish (Weatherley and Gill 1987). Since protein, lipid, and carbohydrate have different energy values and are stored with different amounts of water, expression of growth as weight gain is unsatisfactory when compositional changes cause the gain in weight. Conversion to energy equivalents allows examination of the various inputs and outputs to the animal in common transferable units (Soofiani and Hawkins 1985).

The basic energy budget equation is $C=P+M+U+F$, where C represents the total energy intake, P is the total energy gained, M is metabolic expenditure, U is excretory nitrogen energy loss, and F is fecal energy loss. Other energy losses, for which we are unable to account, include that contained in the production of mucous and sloughed epidermal cells (Weatherley and Gill 1987), and the production of mucous tubes that envelop the feces (Shehadeh and Gordon 1969). Fecal production was not measured in the following studies, and energy losses associated with it were determined by difference. The value F, therefore, includes the unaccounted energy losses described above.

In determining the allocation of ingested energy as gains, expenditures and losses in

fish, energy budgets can provide another source of information regarding the level of stress and consequent costs associated with pollution and altered thermal regimes (Calow 1991; Mehner and Wieser 1994).

The Study

The present study was designed to establish some baseline physiological, toxicological and bioenergetic data concerning the effects of warmer environmental temperatures and sublethal low pH on a reference coldwater species. Only one other study combining temperature and acidic water has been conducted (Kwain 1975), however, it was based on different environmental issues, and encompassed embryonic development and constant temperatures. The present study employs the *natural* fluctuating summer thermal regime of inshore Lake Ontario, and photoperiod that closely follows that of the summer season. A conservative increase in temperature of 2°C above the natural ambient temperature was used to simulate a global warming scenario. It was important to expose the experimental fish to an environmentally realistic low pH environment. Since aquatic acidification is essentially a softwater problem, water of relevant hardness was produced by removing Ca^{2+} through the use of Na^{+} exchange resins in a softening column, and subsequent removal of Na^{+} and Cl^{-} via reverse osmosis. The desired concentrations of Ca^{2+}, Na^{+}, and Cl^{-} were achieved by adding back appropriate amounts of dechlorinated Hamilton tapwater. Sulphuric acid was added by titration to achieve the sublethal low pH of 5.2.

Two 90 day exposures were conducted over the summer season encompassing the months of June to September in 1993 and 1994. The design of the experiments was such that they included an overall control treatment of naturally varying ambient temperature and ambient pH, a sublethal pH 5.2 treatment at ambient temperature, the same thermal regime with an additional 2°C at ambient pH, and a combination treatment of an additional 2°C and

sublethal pH 5.2. These four treatment conditions were carried out simultaneously, thereby allowing for direct comparison between treatments.

The two parallel studies were carried out to address the important issue of food ration. As stated above, satiation feeding is required when establishing the link between the effects of the environmental stressors on appetite, thereby contributing to effects on growth. The difficulty associated with feeding these fish to satiation is in attempting to relate the findings to the natural situation. Feeding rates in the wild are difficult to measure (Elliott 1982), and are often based on estimates from growth rates in laboratories or stomach content analysis (Soofiani and Hawkins 1985). However, it is unlikely that fish in the wild are able to satiate themselves on a daily basis. Therefore, the second study was conducted over the same time period the following summer, but with a limited ration. The size of limited ration was selected as one which is considered a maintenance ration but that still allows for some growth. Furthermore, the ration was calibrated to that of the overall control treatment to ensure that food intake rates were controlled equally.

Chapter 2 describes the results of the first exposure in which juvenile rainbow trout were fed to satiation twice daily, and exposed to the conditions described above. Peak temperatures in the summer of 1993 were unusually high, and resulted in distinct thermal effects, especially in the trout exposed to an additional 2°C. The results of this study provided further reasons for carrying out the second limited ration study, and led to a hypothesis relating feeding level to ionoregulatory capabilities.

Chapter 3 compares the results from the first satiation feeding exposure with the second limited ration exposure. Year to year variation resulted in a much lower temperature peak, removing the extent of the effects that were observed in the first exposure. Nevertheless, this experiment provided further evidence to suggest that feeding level is an important variable in studies such as this.

A brief appendix addresses the question of whether prolonged exposure to sublethal

acidity and/or elevated temperature significantly affects the ability of the fish to deal with a more severe acidic challenge. As branchial Na^+ exchange is known to be the key site of damaging impact of external H^+ ions, unidirectional flux rates of Na^+ across the gills were measured radioisotopically, using $^{22}Na^+$.

It is hoped that the information contained herein will provide a basis for, and stimulation of, further questions and research into the combined effects of environmental pollutants, particularly those that affect as valuable a resource as Canada's fresh waters.

Chapter 2

Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) were chronically exposed to a simulated global warming/acidic water scenario over a 90 day summer period (ambient temp. range 13-24°C). The addition of 2°C to the natural summer thermal cycle of inshore Lake Ontario, and sulphuric acid to synthetic soft water (nominally $[Ca^{2+}] = 50$ and $[Na^{+}] = 100$ $\mu equiv \cdot L^{-1}$) resulted in four treatment conditions: 1) Control conditions; ambient water temperature at pH 6.3 (0/6.3); 2) acidification of the control conditions (0/5.2); 3) simulated global warming alone (+2/6.3); 4) a combination of simulated global warming and environmental acidification (+2/5.2). Trout were fed to satiation twice daily. Metabolic rates were ~75% of $MO_{2(max)}$ in all treatments, indicating the influence of an unlimited feeding regime on metabolism. Proximate composition changed over time with large increases (2.5 fold) in lipid content, small increases (3%) in protein content, and compensating decreases in water content in all treatments (78-72%). The addition of 2°C resulted in depressed appetites and growth, particularly during the period of peak temperature (Day 60-90; 26°C). Metabolic rate and nitrogenous waste excretion were also depressed at this time in the +2°C treatments. Overall, exposure to low pH resulted in increased appetites and growth, significantly so in the 0/5.2 treatment. Energy budgets indicated that the addition of 2°C reduced gross energy intake and increased fecal energy losses, while exposure to low pH resulted in increased energy intake and gain, and better conversion efficiency. The surprising lack of ionoregulatory disturbance in trout chronically exposed to pH 5.2 suggests that the availability of NaCl in an unlimited food supply was compensating for branchial ion losses, and perhaps driving appetite. Overall, increased global temperatures, in combination with environmental acidification, result in

increased physiological costs for juvenile trout in the summer, particularly when temperatures are at their peak.

Introduction

The Intergovernmental Panel on Climate Change (IPCC; 1991) has forecast that with a doubling of atmospheric CO_2, which will occur over the next 50 to 100 years if current production levels continue, and increases in other "greenhouse" gases such as methane, nitrous oxide, and chlorofluorocarbons, mean global air temperatures may increase 1.3-4.5°C. These predictions are based on General Circulation Models (GCMs) such as those used by the Goddard Institute for Space Studies (Hansen et al. 1988) and described by Mohnen and Wang (1992).

Increased air temperatures will result in increased temperatures of fresh water bodies which will directly impact the fisheries associated with these habitats. Regier and Meisner (1990) discuss the effects of increased temperature on water quantity and quality in the Great Lakes using an iterative assessment process. Schindler et al. (1990) describe an increase in air temperature at the Experimental Lakes Area in northwestern Ontario of 2°C over the last 20 years, and provide data that indicate that evaporation has increased, precipitation has decreased, and consequently, water renewal rates have decreased. These changes have directly impacted stenothermal fish species by decreasing available cold water habitats. From these observations, they concluded that fresh waters should be considered in major global change programmes due to the fisheries' importance as a food source, and their scarcity worldwide.

Anthropogenic production of pollutants has many other environmental impacts which should be considered in concert with projected climate change. For example, the burning of coal and oil, and the smelting of metallic ores, results in the production of sulphur dioxide (SO_2), while internal combustion engines produce nitrogen oxides (NO_x). Release of SO_2 and NO_x into the atmosphere results in acid precipitation, which has

decreased lake pH's between 0.5 and 1.5 units in many softwater lakes in North America and Europe, over the last 140 years (Kemp 1994). Particularly sensitive lakes, with alkalinities below 50 $\mu equiv \cdot L^{-1}$, include those found in the eastern provinces of Canada (Schindler 1988). In Ontario, lakes found on the Canadian Shield have a highly resistant granitic base which results in low water buffering capacity. Acidification of these lakes has been extensively studied by Scheider et al. (1979), Schindler et al. (1980), Kelso et al. (1982; 1986), and the deleterious effects on fish described by Beamish (1974b), Beamish et al. (1975), Kelso and Gunn (1984) and Kelso et al. (1986; 1990).

Because fish are poikilotherms, their body temperature is set by the external temperature of the water they inhabit. Of all the environmental factors that affect fish, temperature is considered to be the ecological master factor (Brett 1971a), and has direct influence on metabolic rates, feeding rates, and activity levels. Metabolic rate, as measured by oxygen consumption, is a composite measure of the cost of living for a fish in a particular environment. Important costs include maintenance metabolism, energy expended in feeding, nutrient assimilation, subsequent waste excretion, growth, and locomotion. All of the latter requirements for living have a requisite cost associated with them, with the external temperature setting the billing rate.

Exposure to low pH has been shown to either limit, or have no effect, on growth in fish (Mount 1973; Leivestad et al. 1976; Menendez 1976; Jacobsen 1977). Decreased growth may be a consequence of decreased feeding under acid conditions (Beamish 1972; Swarts et al. 1978). Low pH has also been shown to increase resting oxygen uptake ($\dot{M}O_2$), indicating increased metabolic cost, and decrease critical swimming speeds (U_{crit}), thereby reducing the scope for activity (Butler et al. 1992).

Protons exert their toxic effect at the gills by disturbing electrolyte balance. Ionoregulatory failure and subsequent fluid shifts, hemoconcentration, and circulatory collapse are the result of lethal levels of low pH (Milligan and Wood 1982; Wood 1989).

Chronic sublethal levels of low pH, however, have been shown to result in stabilization of electrolyte balance and blood plasma parameters, but recovery does not occur (Audet et al. 1988). The physiological changes associated with the original ionoregulatory disturbance, and adjustment to the new conditions, are considered deleterious, and do not result in acclimation to more severe acid stress in rainbow trout (Audet and Wood 1988).

The physiological adjustments required by fish exposed to low pH, and the rate increase caused by higher temperatures, presumably would result in increased metabolic costs. Our goal in the present study was to assess whether or not chronic exposure to sublethal low pH (5.2), a small elevation (+2°C) in water temperature over the natural thermal cycle, and the combination of these two stressors, would in fact raise metabolic costs. The experiment was carried out over the summer season (June - September 1993) using juvenile rainbow trout (*Oncorhynchus mykiss*) that were fed to satiation. Hamilton dechlorinated tap water, which follows the natural thermal regime of inshore Lake Ontario, was synthetically softened to achieve water of an environmentally relevant hardness ($[Ca^{2+}] \approx 50$; $[Na^{+}] \approx 100$ $\mu equiv \cdot L^{-1}$]. Water was sequentially split, and 2°C and sulphuric acid added to achieve four treatments: Ambient temperature/Ambient pH (6.3), Ambient temperature/pH 5.2, Ambient +2°C/pH 6.3, and Ambient +2°C/pH 5.2. A suite of cost indicators was measured including appetite, growth, metabolic rate and the partitioning of exogenous energy as protein, lipid, and carbohydrate. Other potential indicators of increased cost and stress that were monitored include whole body and plasma ion levels, hematocrit, plasma protein, and lactate levels. The exposure was conducted over a 90 day period during which the summer peak in temperature occurred.

Materials and Methods:

Animal Holding

Experiments were conducted on 1146 juvenile rainbow trout (2-3 g) which were

transported from Rainbow Springs hatchery, Thamesville, Ontario on April 19, 1993. At McMaster University, they were placed in a 400 L polypropylene tank, and acclimated to and maintained in, continuously flowing Hamilton dechlorinated tap water ($[Ca^{2+}]$ = 1.95 ± 0.22, $[Na^{+}]$ = 0.556 ± 0.33 mequiv·L^{-1}; pH = 7.4-7.8) at 11°C for 1 week after arrival. The water hardness was reduced gradually over a second 1 week period by introducing increasing amounts of artificially softened water generated by reverse osmosis (Anderson Water Conditioning Equipment, Dundas, ON). Once the desired water quality was achieved, the trout were further acclimated for another 7 weeks. Fish were fed ~ 4% (dry weight of food) of their dry body weight·day^{-1} of Zeigler Trout Starter #3 (for composition, see Table 1), and maintained on this diet for the 8 week acclimation period prior to experiment initiation. Photoperiod was controlled and adjusted to mimic the natural photoperiod throughout the acclimation and experimental periods.

Exposure System

The exposure system utilized is illustrated in Figure 1. The feed water was dechlorinated Hamilton City tap water which closely follows the natural thermal regime of the inshore region of Lake Ontario, from which it was collected (Figure 2). Passage through the reverse osmosis unit unavoidably added 2°C to the water temperature, so the "ambient" regime was consistently 2°C above the natural regime. After softening and deionization by reverse osmosis, the product water was pumped to a main head tank where hardwater was added in a ratio of 1:40 to achieve $[Ca^{2+}]$ and $[Na^{+}]$ of ~50 and 100 μequiv·L^{-1}, respectively. This synthetic softwater (pH 6.3) 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 a further 2°C was added to the ambient temperature. The water from each of these two tanks was further split into two more sub-head tanks, resulting in four treatment head tanks. H_2SO_4 (0.2 N) was metered into one each of the ambient and +2°C

Table 1. Zeigler Trout Starter #3 & 4 feed composition. Water content was determined by drying at 80°C to a constant weight.

Component	Content
Water	11.66%
Protein	50%
Fat	15%
Fibre	2%
Sodium	0.50%
Calcium	2.30%
Phosphorus	1.80%

Figure 1.

Experimental system used to expose juvenile rainbow trout to increased temperatures and sublethal low pH. Synthetic soft water filled the head tank and was sequentially split in two stages. H_2SO_4 (0.2 N) was added at the first stage to produce two pH 5.2 treatments, and a heat exchanger was used to add 2°C to half of the exposure water. The resulting 4 treatments, to which 142 trout were exposed for 90 days, were: Ambient temperature/pH 5.2 (0/5.2), ambient temperature/ambient pH (6.3) (0/6.3), ambient +2°C/pH 6.3 (+2/6.3), and ambient +2°C/pH 5.2 (+2/5.2).

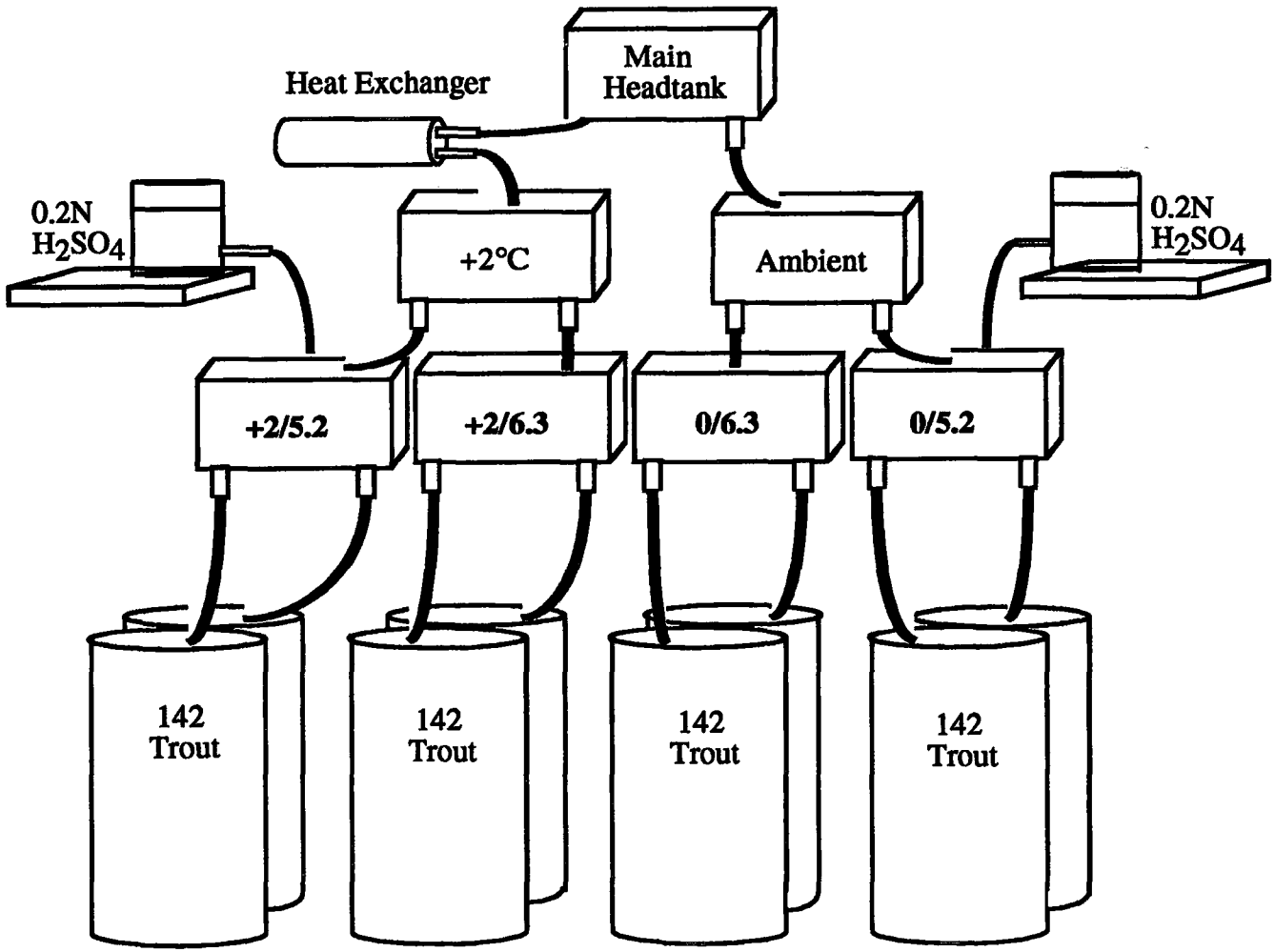
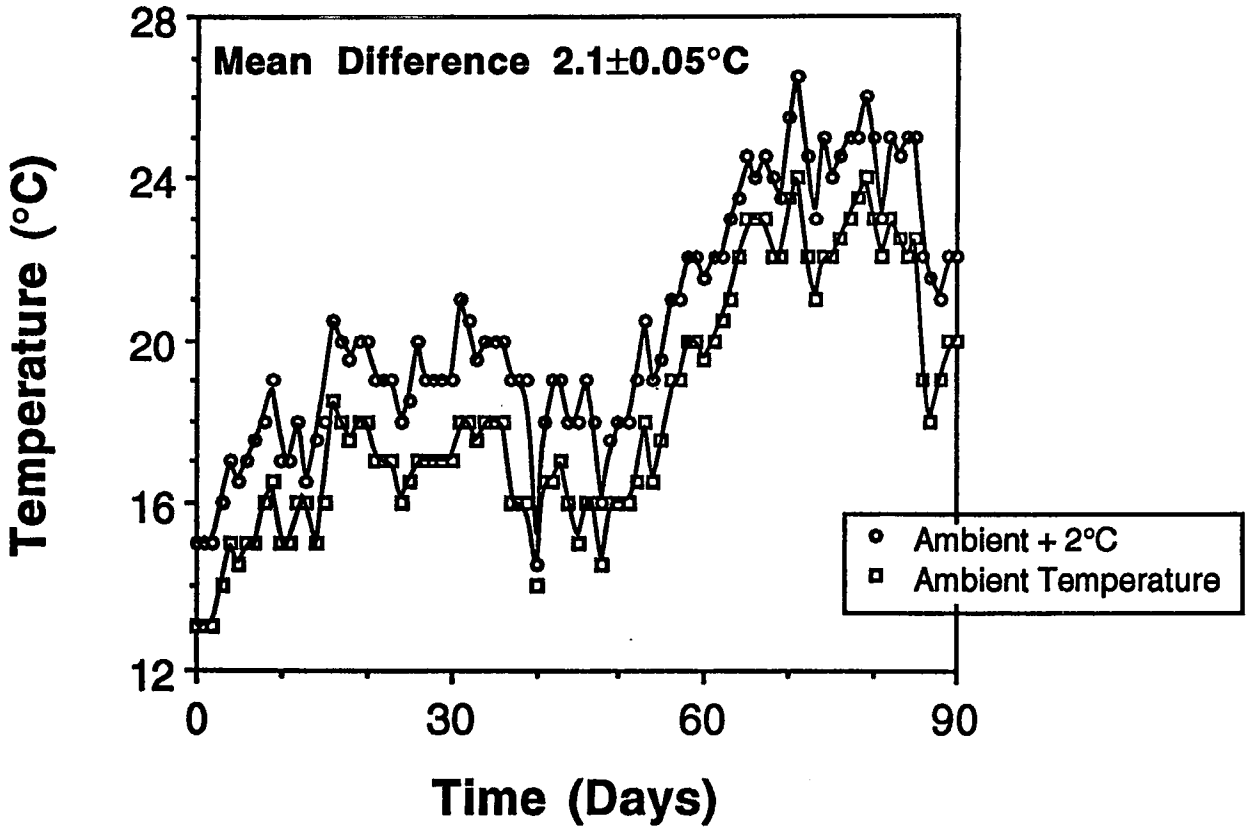

Heat Exchanger
Main Headtank
0.2N H_2SO_4
+2°C
Ambient
0.2N H_2SO_4
+2/5.2
+2/6.3
0/6.3
0/5.2
142 Trout
142 Trout
142 Trout
142 Trout

Figure 2.

Summer thermal cycle (June - September, 1993) experienced by juvenile rainbow trout over the 90 day experimental period. Temperatures peaked between days 60 and 90 at 24°C (26°C in the +2°C treatments).


Mean Difference 2.1±0.05°C
Temperature (°C)
28
24
20
16
12
0
30
60
90
Time (Days)
Ambient + 2°C
Ambient Temperature

temperature head tanks, resulting in the following four treatments: Ambient temperature/ambient pH 6.3 (0/6.3); ambient temperature/sublethal pH 5.2 (0/5.2); ambient temperature +2°C/ambient pH 6.3 (+2/6.3); ambient temperature +2°C/sublethal pH 5.2 (+2/5.2). The water from the treatment head tanks flowed into duplicate 211 L polypropylene exposure tanks at an average rate of 1.2 $L \cdot min^{-1}$ which allows for 90% replacement in 7 h. Sublethal pH 5.2 was maintained by monitoring the pH in the treatment tanks using a Leeds & Northrup Meredian II® Combination industrial pH electrode. The electrode reading controlled a Cole Parmer Instrument Co. solenoid valve (CP#01367-70) that opened and closed according to the tank pH and the high and low pH control points, adding H_2SO_4 to the treatment head-tank. Temperature and low pH were monitored via a Ladder Logic and Texas PLC Model TI315 programmable controller which was connected to an alarm and Safe House Model 49-433A automatic message dialer system. The alarm system was triggered by a drop or increase in temperature of 1.0 °C and/or pH of 0.8 pH units. All tanks and head tanks were aerated, and water partial pressure of O_2 (PO_2) determined once a week using a thermostatted Radiometer/Copenhagen E5046 PO_2 electrode connected to a Cameron Instrument Co. oxygen meter. PO_2's averaged 115 Torr, and did not drop below 80 Torr. Temperature and pH were measured daily. Ambient pH averaged 6.27±0.03, while low pH averaged 5.27±0.03. $[Na^+]$ and $[Ca^{2+}]$ were monitored twice a week using atomic absorption spectroscopy (Varian AA-1275), and averaged 97.5±2.23 and 57.7±2.28 $\mu equiv \cdot L^{-1}$, respectively. Titratable alkalinity was determined once a week, by titration to an endpoint of pH 4.2, using 0.02 N HCl. Mean values were 269.5±18.8 and 120.3±15.3 $\mu equiv \cdot L^{-1}$ for the ambient pH and low pH treatments, respectively. Water chloride (69.9±14.5 $\mu equiv \cdot L^{-1}$; spectrophotometric determination; Zall et al. 1956) and total chlorine (10-12 $\mu g \cdot L^{-1}$; Hach; Colorado, USA) were measured every 30 days.

Feeding Regime

At the end of the 8 weeks of acclimation, 142 trout were selected at random and placed in each of the 8 treatment tanks, with 152 fish being placed in the 0/6.2 treatment tank to allow 10 extra fish for Day 0 sampling. Fish were hand-fed Zeigler's Trout Starter #3 (Table 1) to satiation twice daily, at 8:30 am and 4:30 pm. The food was changed to Trout Starter #4 (of identical composition) halfway through the exposure because of fish growth. An aliquot of food from pre-weighed bags was sprinkled on the water surface of each replicate tank. If all the food was consumed within 1 min, more food was placed on the water surface. When food was left on the surface at the end of the 1 min period, a second minute was allowed. If food remained after this 2 min period, the fish were assumed to be satiated and feeding was stopped. If not, the process was repeated until feeding stopped. At the end of each day, the bag was reweighed, to determine the amount of food consumed. Fish consumed ~10-11% of their dry body weight·day^{-1} of dry food on this feeding regime. Tanks were siphoned every day and cleaned once a week.

Physiological Measurements

Sampling Protocol

Sampling and physiological measurements were conducted over a 4 day period every 30 days, including Day 0 (experiment initiation). Trout were not fed on the third and fourth days of the sampling period, when whole body and blood samples were collected.

Nitrogenous Waste Excretion

Day 0 (experiment initiation) - In-tank routine nitrogenous waste excretion was measured over a 24 h period on the first day of each 30 day exposure period, by stopping water flow to the tank. Aeration ensured mixing, and tests demonstrated no loss of ammonia due to aeration. Initial and final water samples were taken at time 0 and 1 h. The

flow to the tank was then resumed for 1 h, and the sampling and closure continued at times 3 h and 4 h. This cycle was continued over the 24 h period. Samples were frozen at -20°C for later analysis of ammonia by the salicylate-hypochlorite assay (Verdouw et al. 1978), and urea by the diacetyl monoxime method (Rahmatullah and Boyd 1980) as modified by T.P. Mommsen (see Lauff and Wood 1995). For the latter, the samples were first freeze-concentrated 5-fold by lyophilization. Nitrogen production (N: total ammonia and urea nitrogen) was determined by the difference between N concentration at the beginning and end of each closed tank period, factored by time, volume, and total fish weight.

Days 30, 60, 90 - Nitrogenous waste excretion was measured on a flow-through basis on the first day of each 30 day exposure period, by taking an inflow and outflow water sample for each treatment every 4 h over a 24 h period. Inflow water rate was measured for each sampling period. Samples were frozen and later analyzed for urea and ammonia nitrogen as above. The difference between inflow and outflow total N-concentration, factored by flow rate and total fish weight, gave the nitrogen production rate in $\mu mol \cdot g^{-1} \cdot h^{-1}$. For each of the six sampling periods, data were graphed against time, and the area under the curve determined for each treatment, to give an overall daily nitrogen production rate.

Metabolic Rates

Day 0 (experiment initiation) - Metabolic rates were determined on the second day of each 30 day exposure period, by measuring in-tank routine oxygen consumption rates (MO_2) every other hour (alternating open and closed periods) over a 10 h period, from 7:00 am to 5:00 pm. Tanks were first siphoned to remove fecal matter. Aeration and inflow water were removed, and the tanks were then sealed with a clear, air-tight lid, and water recirculated within each tank using a submersible pump (Little Giant, 1 EUAA-MD). A water sample was removed from mid-tank every 20 min using a 5 ml syringe, and this

sample was injected into a jacketed Radiometer/Copenhagen E5046 PO_2 electrode with ambient temperature water circulating through the jacket. The PO_2 was recorded on a Cameron Instrument Co. oxygen meter. PO_2 was never allowed to decrease below 80 Torr. Mean MO_2 values, determined for each hour using oxygen solubility coefficients from Boutilier et al. (1984), were graphed against time. The curve produced was integrated, for each treatment, to give an overall mean MO_2 value for that 10 h period.

Days 30, 60, 90 - The same methods as above were used, except the O_2 electrode was suspended directly in the tank rather than in an external thermostatted cuvette. All nitrogen excretion and MO_2 data were corrected for size differences using the weight exponent 0.824, determined for rainbow trout by Cho (1992).

Blood Analysis

Day 0 (experiment initiation) - Ten fish were randomly chosen from the control tank. Each fish was quickly killed by a blow to the head and blotted dry, and weight and total length were determined. Blood was collected from the dorsal aorta by caudal severance into ammonium heparinized capillary tubes. The fish were then freeze-clamped using aluminum tongs immersed in liquid nitrogen, wrapped individually in labelled aluminum foil, and stored at -80°C.

The blood samples were spun in an IEC MB micro hematocrit centrifuge (Damon/IEC Division) for 5 min at 10,000 x g. Hematocrit was determined as the ratio of the length of packed red blood cells to the total sample length, and multiplied by 100 to give a percentage value. Due to the small size of the fish, it was important to conserve blood; the plasma in the capillary tube was removed using a 50 µl Hamilton syringe, and frozen for subsequent ion analysis. When volume permitted, plasma protein was measured using a hand-held refractometer (American Optical) (Alexander and Ingram 1980).

Days 30, 60, 90 - Ten fish were randomly netted from each duplicate treatment

tank, and blood collected as above. A portion of the white muscle was removed from the dorsal section posterior to the operculum, and anterior to the dorsal fin, for water content determination. The skin and fin rays were removed, and the white muscle frozen in liquid N_2, wrapped in labelled aluminum foil, and stored at -80°C for further analysis.

Hematocrit and plasma protein were determined as above. The remaining plasma was frozen in liquid N_2 and stored at -80°C for later analysis.

Whole Body Samples

Day 0 - See Day 0 Blood Analysis above.

Days 30, 60, 90 - Ten fish were randomly selected from each duplicate treatment tank and placed in terminal anaesthetic (1.0 g l^{-1} MS222 and 2.0 g l^{-1} $NaHCO_3$). Each fish was blotted dry, weight and total length were determined, and the fish was rapidly frozen using aluminum tongs frozen in liquid N_2. Fish were wrapped individually in labelled aluminum foil, and stored at -80°C for later analysis.

Sample Processing and Analysis

Plasma Na^+ and Ca^{2+} levels were determined by atomic absorption spectroscopy, and Cl^- levels by coulometric titration (Radiometer CMT10).

White muscle samples were weighed and then dried at 80°C to a constant weight to determine water content.

Whole fish were ground frozen, using an IKA (M10/M20) grinding mill cooled to ~-72°C. A sample of the tissue was weighed, and then dried to constant weight at 80°C, to determine water content. The remainder of the tissue was lyophilized (Labconco Lyph-Lock 6), desiccated, and stored at -20°C. Whole body Na^+, Ca^{2+}, K^+ and Cl^- levels were determined as above for plasma ions, after digestion of 100 mg of tissue, for 48 h, in 900 µl of 1 N H_2SO_4 at 80°C. Subsamples of freeze-dried tissue were used to determine

whole body protein levels, using the Lowry assay, as modified by Miller (1959). Lipids were extracted and quantified using the chloroform/methanol (2:1) method (Folch et al. 1957). Glycogen, glucose and lactate levels were determined as an estimate of whole body carbohydrate using standard enzymatic analyses (Bergmeyer 1985). Percentage inorganic content (ash) was determined by burning a subsample of whole body tissue at 550°C until a constant weight was achieved.

Statistical Analysis

Values are given as the mean ± SEM. The design of the experiment was such that each treatment was considered unique. As a result, interactive effects between temperature and pH were not statistically tested. Therefore mean values for each treatment were compared using one-way ANOVA. In cases where the F-value indicated significance, the Tukey-Kramer comparison of all pairs test was applied, to determine treatment differences within a sampling period. The accepted probability level for significance was $p<0.05$.

Results

Few mortalities occurred due to the treatments over the 90 day period; they amounted to less than 10% overall. Unfortunately, due to a laboratory mishap, the +2/5.2 treatment duplicate tank was lost at day 23.

Figure 2 illustrates the thermal cycle experienced by the trout over the period June 18 to September 15, 1993. The ambient temperature ranged from 13 to 24°C, and therefore the +2°C treatments ranged from 15 to 26°C. The upper incipient lethal temperature for rainbow trout has been determined by Elliott (1982) to be 26.2°C. Ambient temperature rose from 13°C to 16-18°C over the first 20 days, after which it was fairly stable until Day 55. Thereafter, the temperature rose to a maximum of 24°C at Day 70, and fluctuated

around this temperature until Day 82. At this point, the temperature started to decrease, and reached 20°C on Day 90.

From Day 0 (experiment initiation), the appetites of the trout in all treatments averaged ~10% body weight·day^{-1} (dry food/dry weight) up until the period Day 60-90 (Figure 3). After the peak in temperature at ~Day 70, appetites started to drop off, and trout were consuming about 5% body weight·day^{-1}. Overall, the trout exposed to ambient temperatures tended to consume more than the trout exposed to +2°C, and those exposed to low pH tended to eat more than their respective controls. Evidence of appetite differences began to appear at about Day 55, where temperatures started to increase.

Growth was also greater in the ambient temperature treatments, and again in the low pH treatments (Figure 3). The 0/5.2 fish grew significantly more than either of the +2°C treatments, and the 0/6.3 treatment trout grew significantly more than the +2/6.3 trout. Again, the differences became apparent after Day 55.

The condition factor (weight/length^3) of the trout in all treatments increased over the 90 day exposure period from ~0.9 to 1.2, while the efficiency with which dry weight of food was converted to dry weight of tissue decreased over the 90 days from ~40 to 25% (Table 2). There were no consistent differences among treatments in these variables.

Proximate Analysis

Whole body analysis indicated that the percentage of protein by weight increased from ~12.0 to 15.5% over the 90 day period (Figure 4). Protein content was significantly greater in the 0/5.2 treatment than in its control at Day 30, and significantly greater than in the +2°C treatments at Day 60. However, this trend was not maintained at Day 90.

Figure 4 shows the substantial increase (~2.5 fold) in whole body lipid content, over the 90 day period, from ~4.5 to 11.5%. Among treatments, lipid content was

Figure 3.

Cumulative appetites, expressed on a dry weight of food basis ($g \cdot fish^{-1}$), and absolute growth (g), expressed as wet body mass, over each 30 day period. Appetites began to differ at ~ Day 45, with the ambient temperature treatments tending to consume more than the +2°C treatments. By Day 90, it was apparent that the trout exposed to sublethal low pH were consuming more than their respective controls. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.

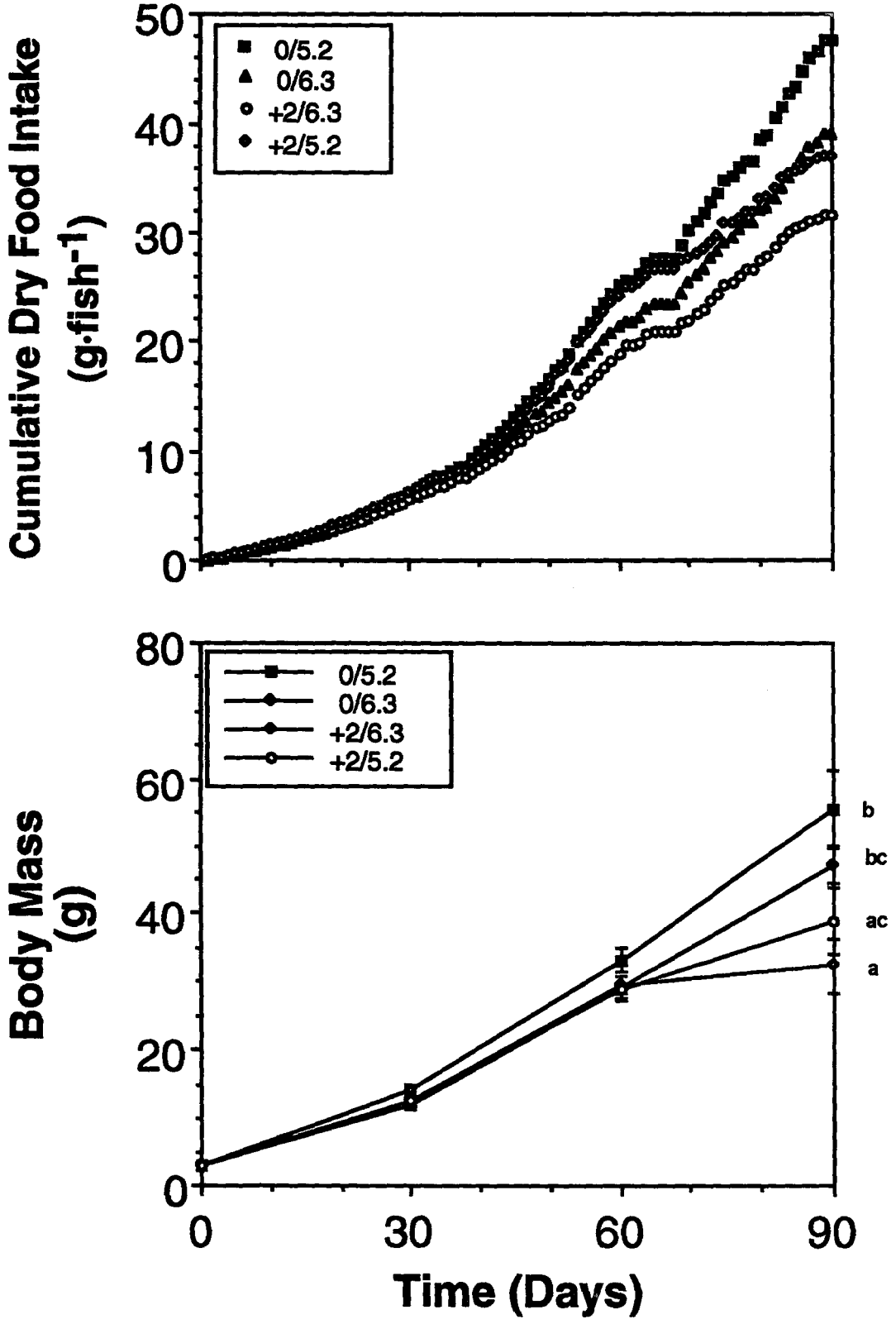
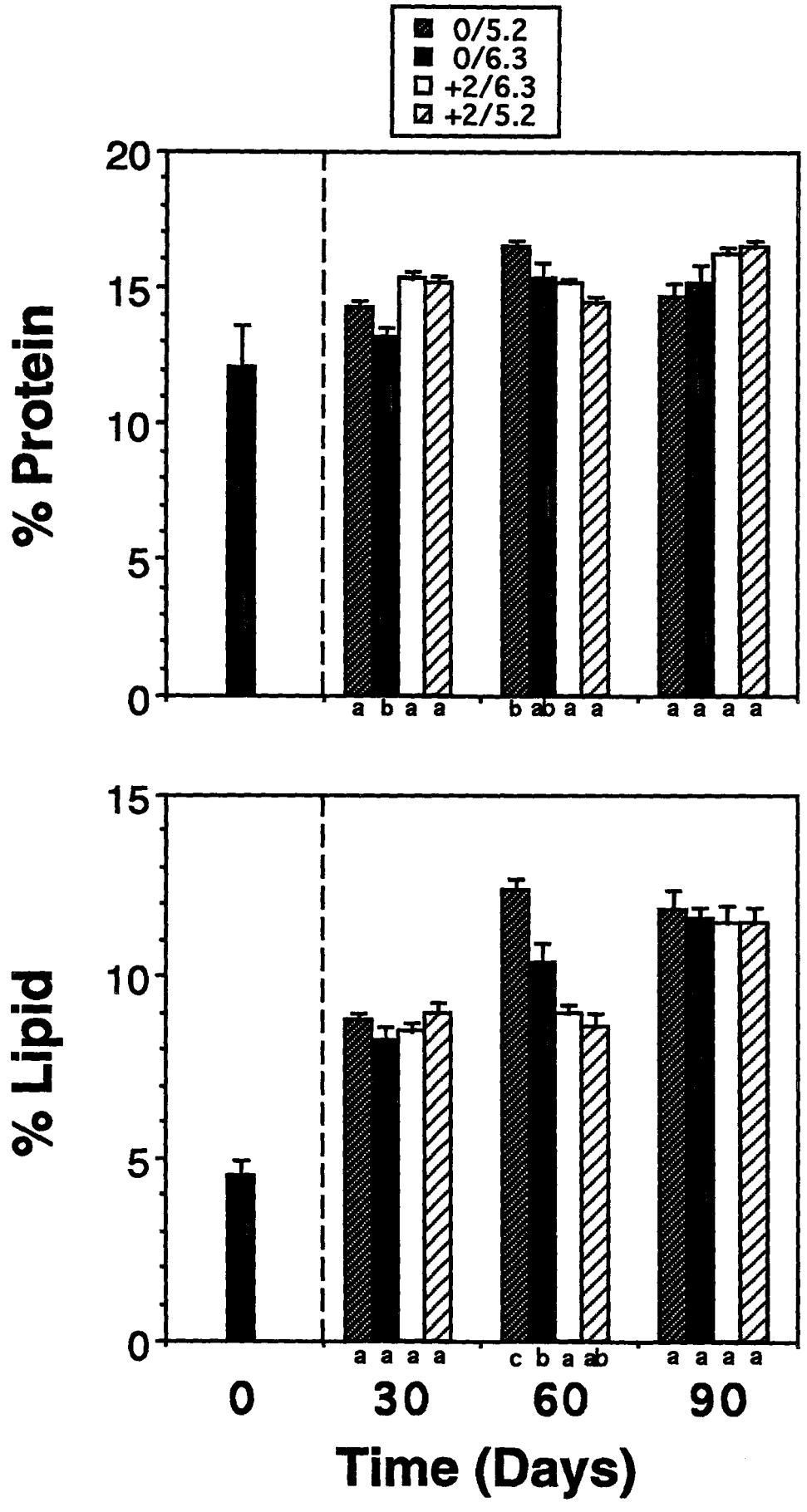

Cumulative Dry Food Intake
(g·fish-1)
0/5.2
0/6.3
+2/6.3
+2/5.2
0
10
20
30
40
50
Body Mass
(g)
0/5.2
0/6.3
+2/6.3
+2/5.2
0
20
40
60
80
b
bc
ac
a
0
30
60
90
Time (Days)

Table 2. Food conversion efficiencies (dry weight of food: dry weight of fish) (%·day^{-1}) calculated for each 30 day period.

Time Period	Treatment			
	0/5.2	0/6.3	+2/6.3	+2/5.2
0-30	46.6	42.4	45.6	40.3
30-60	27.2	29.5	33.8	24.5
60-90	28.3	27.3	6.2	20.9

Figure 4.

Whole body protein and lipid (%). Ambient temperature treatments are represented by the two shaded bars on the left, while the +2°C treatments are represented by the two light bars on the right. The hatched bars represent low pH (5.2) treatments, while the solid bars represent the ambient pH (6.3) treatments. Values given are means ± SEM. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.


0/5.2
0/6.3
+2/6.3
+2/5.2
% Protein
20
15
10
5
0
a b a a
b ab a a
a a a a
% Lipid
15
10
5
0
a a a a
c b a ab
a a a a
0
30
60
90
Time (Days)

significantly greater in the 0/5.2 treatment, at Day 60 only.

Total carbohydrate (estimated as glucose+glycogen+lactate) did not show any consistent trend among treatments, or over time, and constituted only ~0.36% of whole body composition (Table 3). Table 3 also shows that percent whole body water decreased over time in all the treatments, from 78 to 72%. This compensated for the large increase in lipid.

Whole body Na^+, Cl^-, Ca^{2+} and K^+ levels indicated no treatment effects whatsoever (Table 4). Na^+ and Cl^- tended to decrease over the 90 day period, while Ca^{2+} increased, and K^+ was not affected. There was no change in total inorganic content (ash; Table 3).

Blood Analysis

Initial values of hematocrit on Day 0 were quite high at 45% (Table 5), perhaps reflecting the difficulty we experienced in obtaining blood from such small fish (3 g). On Days 30, 60, and 90, hematocrits were between 35 and 40%, and no treatment differences were evident. Plasma protein levels remained unchanged at ~5.5 $g \cdot 100ml^{-1}$ until Day 90 (Table 5). At this point, plasma protein decreased significantly in both +2°C treatments.

Plasma Na^+ increased over time from the initial value of ~105 to 150 $mequiv \cdot l^{-1}$ (Figure 5). After the peak in temperature between Days 60 and 90, the +2/5.2 treatment had significantly lower levels of plasma Na^+ than both ambient temperature treatments. Plasma Cl^- did not mirror this trend (Figure 5). Since plasma was limited at Day 0, there is no initial value for plasma Ca^{2+}. At subsequent samplings, plasma Ca^{2+} was ~4.5 $mequiv \cdot l^{-1}$ with no apparent influence of treatment (data not shown).

Metabolic and Nitrogenous Waste Excretion Rates

Oxygen consumption measurements indicated that routine values for these satiation

Table 3. Whole body carbohydrate, water and total inorganic (=ash) contents, as percentages, for each 30 day period. Mean values ± SEM and sample size (n) are shown. There were no significant differences ($p<0.05$) amongst treatments at each time, but the overall decrease in % Water with time was significant.

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
% Carbohydrate	0.34	0.47	0.44	0.43	0.38	0.38	0.34	0.34	0.39	0.36	0.45	0.29	0.36
±SEM	0.032	0.030	0.031	0.018	0.043	0.017	0.022	0.018	0.020	0.028	0.014	0.021	0.049
n	10	19	10	10	5	10	10	9	5	7	18	12	6
% Water	77.29	74.18	75.47	74.14	73.59	72.22	71.79	71.64	71.89	71.49	71.46	71.27	71.15
±SEM	0.54	0.33	1.04	0.23	0.20	0.40	0.31	0.24	0.48	0.52	0.026	0.37	0.30
n	10	17	17	18	9	19	14	19	10	9	17	12	5
% Inorganics	1.95	2.15	1.50	1.52	2.08	1.52	2.09	2.16	2.10	2.11	1.97	1.53	2.02
±SEM	0.11	0.10	0.17	0.18	0.46	0.14	0.17	0.19	0.28	0.16	0.11	0.23	0.27
n	9	11	10	10	10	10	10	10	8	10	11	9	6

Table 4. Whole body [Na^+], [Cl^-], [Ca^{2+}] and [K^+] (mequiv·kg^{-1}) for each treatment at each 30 day period. Mean values ± SEM and sample size (n) are shown. Significant differences occurred between treatments at Day 90 only. During this period, treatments that share a letter are not significantly different from each other ($p<0.05$).

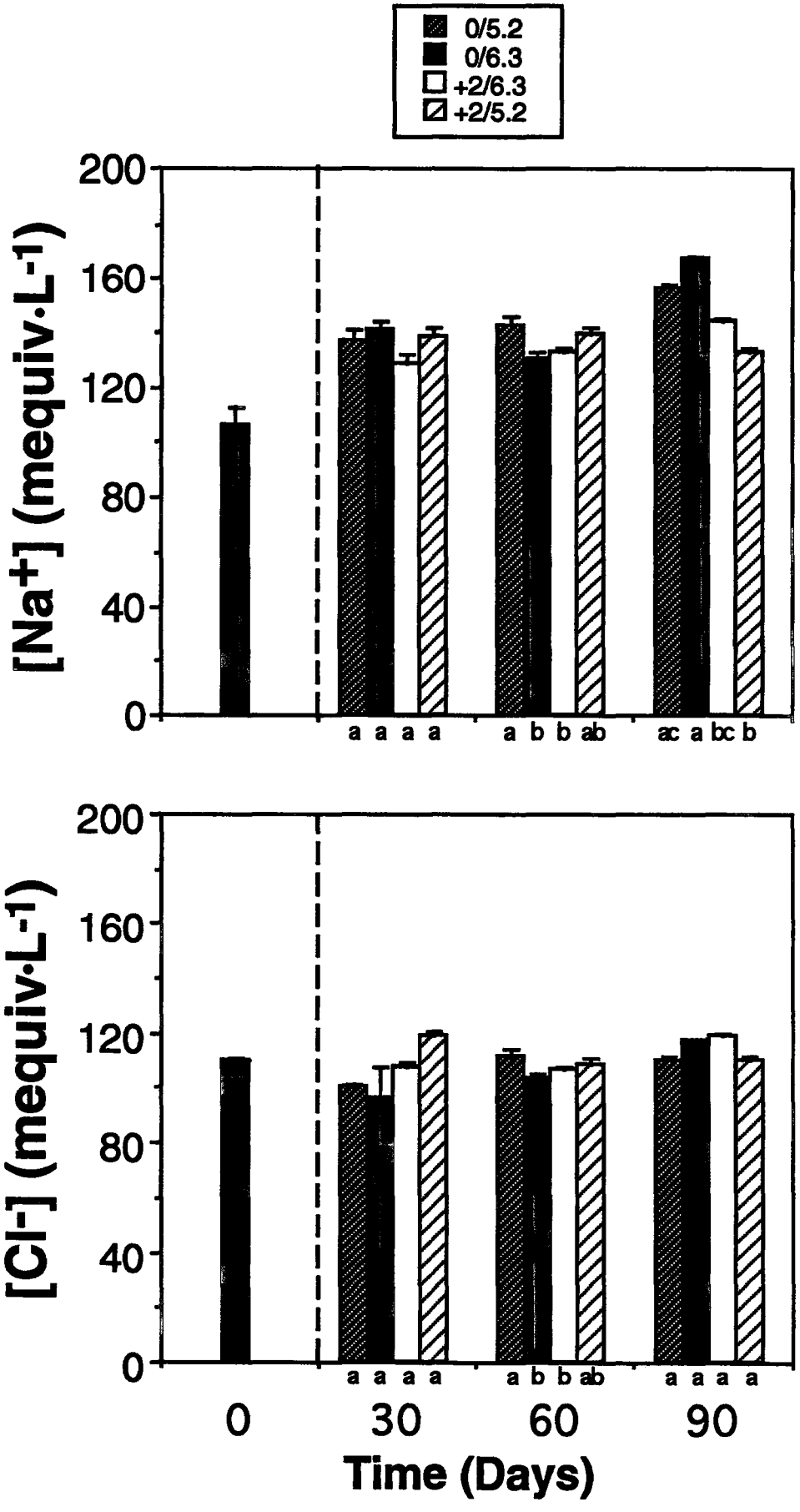
Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
n	10	19	20	18	10	20	18	19	10	12	18	12	6
[Na^+]	45.4	43.6	46.5	42.2	44.3	37.5	37.8	39.7	38.5	36.2a	40.9ab	48.7b	37.7ab
±SEM	0.4	2.8	4.0	0.7	0.6	1.1	0.9	0.6	1.1	2.0	0.7	5.9	1.1
[Cl^-]	40.1	35.8	38.7	33.8	35.7	30.2	29.7	30.2	29.6	26.5b	31.9a	33.7a	27.9ab
±SEM	0.6	1.8	3.4	0.6	0.4	0.9	0.6	0.5	1.0	2.0	0.6	1.1	1.1
[Ca^{2+}]	73.3	85.2	84.9	76.8	77.3	84.6	86.1	88.7	87.5	81.4a	84.6ab	79.3a	92.0b
±SEM	1.4	4.7	6.9	1.0	1.9	1.7	1.2	1.1	2.1	1.9	1.3	2.6	1.9
[K^+]	83.2	86.6	105.1	92.9	87.1	87.4	93.5	88.9	87.1	84.9a	85.8a	84.5a	84.6a
±SEM	2.3	4.3	9.6	1.3	1.7	2.3	2.0	1.7	3.0	1.8	1.2	1.7	2.9

Table 5. Measured hematocrit (%) and plasma protein ($g \cdot 100ml^{-1}$) for each treatment at 30 day periods. Mean values ± SEM and sample size (n) are shown. Significant differences between treatments occurred at Day 90 only, for plasma protein, and at this time period, treatments that share a letter are not significantly different from each other ($p<0.05$).

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
Hematocrit (%)	46.2	33.1	35.5	33.1	32.8	39.0	38.8	41.2	38.7	33.1	33.2	31.8	30.9
±SEM	1.5	1.2	1.4	2.1	1.2	0.8	1.1	0.9	1.2	1.9	1.4	0.7	1.1
n	20	20	19	19	10	20	17	18	10	9	12	11	6
Plasma Protein ($g \cdot 100ml^{-1}$)	5.6	5.6	5.3	5.7	5.5	5.6	5.2	5.5	5.6	5.3b	4.6ab	4.4a	3.8a
±SEM	0.1	0.1	0.1	0.2	0.3	0.1	0.1	0.2	0.2	0.3	0.1	0.2	0.3
n	10	10	13	10	5	10	10	12	8	9	12	11	6

Figure 5.

Plasma Na^+ and Cl^- levels (mequiv·L^{-1}). Other details as in legend of Figure 4. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.


0/5.2
0/6.3
+2/6.3
+2/5.2
[Na+] (mequiv·L-1)
200
160
120
80
40
0
a a a a a b b ab ac a bc b
[Cl-] (mequiv·L-1)
200
160
120
80
40
0
a a a a a b b ab a a a a
0
30
60
90
Time (Days)

fed fish were about 7.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Figure 6), which is about 75% of maximal MO_2's determined for juvenile rainbow trout swimming at maximum speed (U_{crit}) (Wilson et al. 1994b). Among treatments, there was a trend towards a higher MO_2 in the +2/5.2 treatment until Day 60, after which point the temperatures reached their peak of 26°C in the +2°C treatments, and MO_2's were subsequently depressed. The depression was particularly severe in the +2/5.2 treatment.

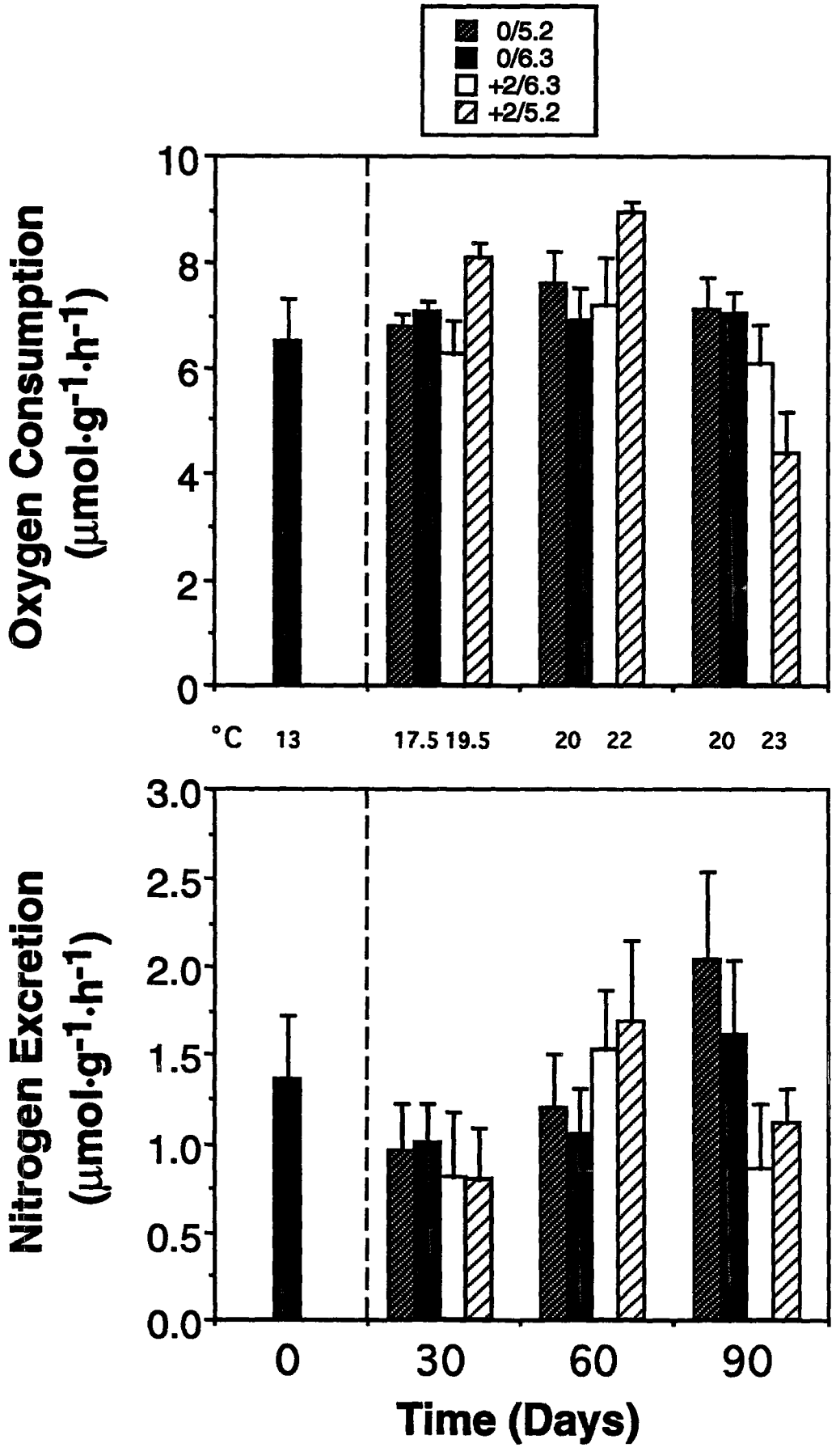
Nitrogenous waste excretion rates increased from the Day 30 levels of ~0.75 $\mu mol \cdot g^{-1} \cdot h^{-1}$ to 1.75 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at Day 90 (Figure 6). About 10-20% of this nitrogenous excretion was represented by urea nitrogen. Over the 90 day period, this fraction decreased from ~20 to 10%, as a result of increased ammonia nitrogen excretion, and relatively constant urea nitrogen excretion. At Days 60 and 90, the low pH treatments tended to excrete more nitrogen than their respective controls, and again, excretion rates were depressed in the +2°C treatments at Day 90, after the peak in temperature between Days 60 and 90.

Discussion

The present study is the first to assess the effects of a combination of chronic sublethal low pH, and the natural or slightly elevated summer thermal cycle, on freshwater fish. Our original hypothesis was that the cost of living in a polluted environment, such as anthropogenically acidified soft water, would be further increased by the addition of 2°C to the natural thermal cycle. It is also one of the few studies (Kwain 1975; Menendez 1976; Leino and McCormick 1992; Wilson et al. 1994a) to examine the effects of these environmental stressors on fed fish. Often, studies investigating environmental stressors, such as pollutants, are carried out on starved or weight-maintained fish (Neville 1985; Audet et al. 1988; Booth et al. 1988; Butler et al. 1992). In the wild, and particularly in the

Figure 6.

Routine in-tank oxygen consumption and nitrogen excretion (total ammonia + urea nitrogen) rates ($\mu mol \cdot g^{-1} \cdot h^{-1}$). Rates were determined for the total number of fish in one duplicate tank, over an 8 h period during the day, including feeding periods. Numbers between the graphs are the temperatures at which the rates were determined. Error bars represent measurement standard error only, and thus no statistical comparisons can be carried out.


0/5.2
0/6.3
+2/6.3
+2/5.2
Oxygen Consumption
(µmol·g-1·h-1)
10
8
6
4
2
0
°C 13 17.5 19.5 20 22 20 23
Nitrogen Excretion
(µmol·g-1·h-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 30 60 90
Time (Days)

summer months, fish actively feed as a consequence of both increased temperatures and food availability.

Indicators of Cost - Temperature Effects

Metabolic rate is a measure of the integrated cost of whole-body physiological and biochemical processes. In fish, it is affected by temperature, size, and food intake and assimilation [the heat increment of feeding, (Cho and Kaushik 1990) also known as apparent specific dynamic action (SDA) (Beamish 1974a)]. After correcting for weight increases, the most striking aspect of the routine MO_2 data in the present study, was that they were about 75% of $MO_{2(max)}$ values (Figure 6). This across-the-board effect was likely due to the feeding regime employed. The increased cost of metabolism due to feeding is well documented (Beamish 1974a; Brett 1976; Soofiani and Hawkins 1982; Cho and Kaushik 1990). Ration size, composition, and temperature determine the extent of the increase in oxygen consumption (Cho et al. 1982). Soofiani and Hawkins (1982) found that for cod fed to satiation, MO_2 almost equalled the active metabolic rate. This dramatic increase has important consequences for the scope for activity (difference between routine and active metabolic rates; Brett 1956) in fish, and was found, by the latter authors, to reduce the scope for activity in *Gadus morhua* L by 83-97%, depending on the temperature. However, Brett (1976) found that the increase in MO_2 due to feeding had less of an impact on the scope for activity in fingerling sockeye salmon.

Another interesting observation was the lack of a substantial increase in MO_2 with increasing temperatures, over the summer exposure. The rates of most physiological processes increase with increasing temperature (Schmidt-Nielsen 1987), the rate increase often expressed as a Q_{10}. For example, Mehner and Wieser (1994) found that MO_2's in juvenile perch acclimated to 20°C had a Q_{10} of 2.43 when compared to perch acclimated to 15°C. Claireux et al. (1995) found that resting cod, acclimated to 5°C, exhibited a Q_{10} of 2

in MO_2 when the temperature was increased to 7.5°C. In the present study however, Q_{10}'s averaged only ~ 1.0. Fish acclimate more rapidly to increases in temperature than to decreases in temperature (Jobling 1994). Since the temperatures gradually increased over the 90 day test period, the fluctuating and increasing thermal regime involved in the present study may have allowed for continuous acclimation, thereby permitting metabolic compensation, and thus no apparent increase in metabolic rates. Thermal history therefore, is an important parameter to be considered when determining metabolic expenditures, especially when predictions of growth are important.

The addition of 2°C did not affect metabolic rates in the first 60 days, until the peak temperatures were reached. Following this peak, metabolic rates were somewhat depressed in the +2°C treatment. Temperatures approaching the upper lethal temperature for salmonids have been shown to depress appetite and metabolic rates (Brett 1971a; Elliott 1982), and, subsequently, growth (Brett et al. 1969). The upper incipient lethal temperature for rainbow trout (26.2°C; Elliott 1982) was reached in the +2°C treatments. Temperatures in the +2°C treatments then dropped to, and remained at, 24°C for 10 days (Figure 2). However, temperatures had been increasing for the 60 days prior to peak temperatures, allowing for gradual acclimation. This may well have increased thermal tolerance, as no mortalities occurred over the 10 day peak temperature period. Figure 3 indicates that appetite and growth were depressed in the +2°C treatments, notably at Day 90. Gross conversion efficiency was also decreased in the +2°C treatment at Day 90 (Table 2). It appears from these results that 2°C added to the ambient thermal cycle significantly affected trout at, and after, peak temperatures, resulting in an overall loss of appetite, poor conversion of food into weight gain, and thus, decreased growth. A 20% reduction in protein turnover was also evident in these fish during this period of peak temperatures (Reid et al. 1995a), and is further evidence of the suppressive effects of high temperature

on metabolic processes.

Proximate analyses indicated that the addition of 2°C had little effect on whole body protein, lipid, or carbohydrate levels (Figure 5; Table 3). This suggests that even though food intake was reduced in these treatments, energy intake throughout the 90 day exposure period was still sufficient to satisfy energy demands at the higher temperature, and did not necessitate endogenous energy catabolism. However, as discussed above, the effect of the peak temperatures on these trout was to suppress appetite and metabolic rates. The adaptive significance of this response may be to conserve energy, by reducing the energy expended on SDA. This metabolic compensation would likely also affect endogenous energy catabolism, reducing any use of internal protein and lipid stores.

Addition of 2°C tended to increase nitrogen excretion rates, until the peak in temperature was reached (Figure 6). At Day 90, nitrogen excretion rates in the +2°C treatments were reduced. Since endogenous nitrogen utilization did not increase, illustrated by the maintenance of whole body protein levels, this reduction was due to the suppression of appetite during the 60-90 day period. A reduction in exogenous protein intake, and no reliance on endogenous protein sources, is further supported by the reduction in use of protein, as indicated by the low fractional protein utilization (FPU) value in the +2/6.3 treatment (Table 6). FPU is an index of the fraction of MO_2 that supports protein metabolism. It 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. In effect, the FPU represents the degree to which the fish depend upon protein as an aerobic fuel source.

In general, addition of 2°C to the ambient thermal cycle of a fresh water body appeared beneficial to rainbow trout until their optimum temperature for growth was surpassed. This temperature has been determined by Cho and Kaushik (1990) to be 15°C.

Table 6. Fractional protein utilization (moles N produced/moles O_2 consumed)/0.27. The denominator is the theoretical maximum nitrogen quotient as determined by Kutty (1972).

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
FPU	0.78	0.52	0.52	0.48	0.37	0.59	0.56	0.78	0.70	1.07	0.85	0.52	0.96

Ambient temperatures, for the first 50 days, fluctuated between 15 and 18°C, and a separation in appetites was evident at Day 50, just as the temperature began its incline to the peak temperature at Day 70. It was over this period that the inhibitory effects of the additional 2°C on appetite and growth became most evident.

Low pH Effects

No increase in metabolic cost was evident as a consequence of exposure to sublethal low pH alone (Figure 6). However, trout exposed to sublethal acidity exhibited greater appetite and better growth than their respective controls, significantly so at Day 90. These responses to low pH were somewhat surprising, in light of many previous studies showing deleterious effects of acidic pH. For example, some investigators have reported that sublethal low pH increases MO_2 (Hargis 1976; Waiwood and Beamish 1978; Butler et al. 1992), indicating increased metabolic cost, and reduces growth in fish. Menendez (1976) and Sadler and Lynam (1987) found that growth in brook trout and brown trout, respectively, was impaired at pH levels below 5.2. The important difference in the present study was that trout were fed to satiation. In this regard, Wilson and Wood (1992) reported no difference in growth for rainbow trout fed 1% body weight·day^{-1}, and concluded that chronic exposure to pH 5.2 did not deleteriously impact the energy budget of fed fish. Indeed, in a separate study, Wilson et al. (1994a) reported increased growth in trout that were exposed to pH 5.2 and fed to satiation for 35 days, a result similar to that of the present study. Conversion efficiencies of acid-exposed trout (Table 2) were not, in general, different from the control, indicating that the subsequent increased growth was purely a result of increased appetite. It is interesting then, that chronic exposure to pH 5.2 should apparently stimulate appetite. This was also evident in the +2/5.2 treatment discussed below, although growth was not significantly different from control growth.

Proximate analysis indicated that increased appetites in the 0/5.2 treatment resulted

in deposition of more protein and lipid at Days 30 and 60 (Figure 4). This trend was not maintained at Day 90, as a result of the peak in temperature over the Day 60 - 90 period. Also, a tendency for higher nitrogen excretion rates (Figure 6) denoted increased protein catabolism (as a result of increased food intake rates).

It is clear from the above discussion that chronic exposure to low pH in trout fed to satiation does not deleteriously affect growth.

Combination Treatment Effects

A greater metabolic price was paid by trout exposed to the combination of low pH and increased temperature (Figure 6). Likewise, after the peak in temperature, the Day 90 MO_2 was depressed much more in the +2/5.2 treatment than in the +2/6.3 treatment, indicating greater physiological impact of the peak temperatures on the former. Although there was apparently a greater metabolic cost, these trout had greater appetites than the fish exposed to +2°C alone, and equal to those of the 0/6.3 treatment (Figure 3). However, they did not exhibit better growth than the trout in the +2/6.3 treatment. This result implies that the increased cost of living in this +2/5.2 environment was being paid in the form of greater immediate use of the exogenous energy, resulting in less energy available for deposition as growth. This is supported by the lower conversion efficiencies exhibited by these fish at all periods (Table 2).

Proximate composition shows that there was no difference in protein, fat, or carbohydrate deposition (Figure 5; Table 3) from the controls. The amount of energy taken in was thus sufficient to maintain endogenous energy stores. Again, at Day 90, after the period of peak temperatures, it is evident that the trout in the +2/5.2 treatment were reducing both food intake (Figure 3) and MO_2 (Figure 6). Consequently, nitrogen excretion rates were reduced (Figure 6), arguing that endogenous sources were not utilized. Energy conservation was clearly of paramount importance during this period.

Indicators of Stress

Exposure to chronic sublethal low pH did not result in decreases in plasma or whole body Na^+ and Cl^- (Figure 5; Table 4) (cf. Audet et al. 1988), nor did it cause fluid shifts, increased hematocrit, or increased plasma protein concentrations (Table 5). This lack of evidence for ionoregulatory disturbance was surprising in light of the fact that chronic studies, such as that carried out by Leino and McCormick (1992) on juvenile largemouth bass, demonstrated that mean blood osmolalities declined at pH 5.0 and 4.5. Audet et al. (1988) showed conclusively, that the main toxic effect of chronic exposure to sublethal low pH (pH 4.8) in adult rainbow trout was ionoregulatory disturbance, manifested by a partial inhibition of Na^+ and Cl^- influx, and a reduction in Na^+ and Cl^- efflux. In their study, plasma Na^+ and Cl^- concentrations were reduced, but stabilized at a lower level, while plasma protein concentration was increased, and hematocrit was not affected. However, the fish in their study were fed only once per week. Wilson et al. (1994a) demonstrated that juvenile rainbow trout, exposed to pH 5.2 for 35 days, recovered whole body [Na^+] and [Cl^-] to control levels, after a nadir at day 17. This recovery was attributed to "enough time"; these fish were fed to satiation twice a day, a feeding regime duplicated in the present study.

As Smith et al. (1989) have pointed out, very little research has been directed at accounting for ions taken in as part of the diet, and the role they play in ionoregulation. We have calculated the dietary ion budget for our fish based on food ion concentrations (Table 1), and whole body ion pools. Trout in the low pH treatments were consuming, on average, 10% of their body pool of Na^+ per day. If indeed there was an initial loss of ions, as described by Wilson et al. (1994a), the availability of ions in the food in combination with enough time might have allowed the observed recovery in their study, and accounts for the observed lack of ionoregulatory disturbance in the trout exposed to low pH in the present study. Sadler and Lynam (1987) found similar effects in yearling

brown trout that were exposed to low pH conditions (4.4-5.2) and fed 2% of their body weight per day. In brown trout that were starved, however, reductions in plasma chloride concentration, increased muscle water content, and losses of body sodium and potassium were significant. The authors suggested that the fed trout may have made use of dietary ions to ameliorate the effects of low pH.

Energy Budget

Since we determined the quantity of food consumed by trout in each treatment, the weight gain and the proximate components of that weight gain (protein, lipid and carbohydrate), metabolic expenditure and excretory losses, we have calculated a crude energy budget for each treatment over the 90 day period (Table 7). It is crude in that these measurements were made on whole tanks of fish rather than on individuals. However, the energy budget allows for examination of each of the inputs and outputs of energy in common units (Soofiani and Hawkins 1985). The transformation of each of the inputs and outputs into energy units (kJ) allows a more accurate assessment of growth and energy conversion as it relates to energy gain, since each of the components of new tissue (protein, lipid, carbohydrate) have different energy values. The energy budget, therefore, should show the effects of treatments on the allocation of energy in the present study, and give a composite measure of the cost of living for trout in each treatment.

The basic energy budget equation is $C=P+M+U+F$, where C equals the total energy intake, P is the total energy gained, M is metabolic expenditure, U is excretory nitrogen energy loss, and F is fecal loss. It is important to note that there are other energy losses for which we are unable to account, such as energy lost in the excretion of mucous sloughed from the surface of the body, and the production of mucous tubes that envelop the feces (Shehadeh and Gordon 1969). Since fecal losses were estimated by difference, these unaccountable energy losses are included in the estimated value for fecal energy

Table 7. Energy budget for each treatment for the 90 day period. The general energy budget equation is: $C = P+M+U+F$ where C is the energy consumed, P is the energy gained, M is metabolic expenditure, U is excretory energy loss, and F is fecal and unaccounted energy loss. Fecal and unaccounted losses are estimated by difference $[C-(P+M+U)]$. Each component of the budget is expressed as a percentage of C to determine allocation differences. K is the efficiency with which food energy is converted into total energy gain ($C/P*100$).

	C		P	(K)	M		U		F	
Treatment	Total Energy Consumed		Total Energy Gained	Conversion Efficiency	Total Metabolic Expenditure		Total N Energy Lost		Fecal/Unaccounted Energy Lost	
	kJ	%	kJ	%	kJ	%	kJ	%	kJ	%
0/5.2	963.9	100	480.6	49.9	288.7	30.0	51.8	5.4	142.9	14.8
0/6.3	837.1	100	369.7	44.2	249.5	29.8	38.3	4.6	179.6	21.5
+2/6.3	785.8	100	254.4	32.4	218.9	27.9	29.0	3.7	283.5	36.1
+2/5.2	723.0	100	283.6	39.2	224.0	31.0	34.5	4.8	180.9	25.0

losses. We refer to F, then, as fecal and unaccounted energy losses (Table 7). The sum of the energy gains, expenditures and losses should equal the energy consumed. If the energy source is not adequate to meet the expenses of daily living, then endogenous stores will be mobilized, and the fish should have a negative *P* value. To determine the energy equivalences of the food and fish, conversion factors from Jobling (1994) were used.

The data in Table 7 support the preceding discussion of greater appetites and thus weight gain in the 0/5.2 treatment. Energy intake (*C*) was greater in both the ambient temperature treatments than in their respective +2°C treatments. The value beside each gross energy value indicates the percentage of the total energy intake allocated to each component of the energy budget. It is clear that the fish in the 0/5.2 treatment not only took in more energy, but also allotted a greater percentage of that energy intake to growth (energy gain; 49.9%). There were almost equal metabolic energy expenditures over the 90 day period, and the 0/5.2 trout lost more energy in the form of nitrogen than the other treatments, particularly the +2/6.3 treatment. However, lower estimated fecal and unaccounted energy losses (14.8%) indicated that not only were the 0/5.2 trout taking in a greater gross amount of energy, but they were also converting that energy more efficiently into gross energy gain (*K*). In contrast, and in agreement with the previous discussion, the +2/6.3 trout showed the least energy gain and the least energy expenditure, but the greatest fecal and unaccounted energy losses. It is also apparent from the data that the +2/6.3 treatment had a greater gross energy intake over the 90 day period than the +2/5.2 treatment. This is explained by a slightly greater absolute food intake over the period Day 60-90 than in the +2/5.2 treatment, although appetites at all other periods were less than those in the +2/5.2 treatment fish. Nonetheless, the trout in the +2/6.3 treatment converted food less efficiently into energy gain than all other treatments. In general, the two ambient temperature treatments consumed more food, gained more energy as growth, and converted the energy taken in to energy gain, more efficiently than the +2°C treatments. Also, both

low pH treatments exhibited better conversion efficiency and greater overall energy gain than their respective controls.

Concluding Remarks

The results of this 90 day exposure of juvenile rainbow trout to sublethal low pH, +2°C, and a combination of these two treatments indicate that, averaged over the entire exposure period, those trout exposed to +2°C exhibited a greater cost of living than those exposed to the ambient thermal regime. This is supported by the decreased food intake rates of trout exposed to higher temperatures, lower conversion efficiencies, and subsequent depressions in growth (energy gain). The billing rate, as set by the environmental temperature, was especially costly for these treatments over the Day 60-90 period when temperatures peaked, close to the incipient lethal temperature. It is evident then, that a global temperature increase of 2°C, as a consequence of "greenhouse gas" production, may be deleterious to temperate coldwater fish species, particularly when temperatures reach the summer high.

Surprisingly, exposure of juvenile rainbow trout to sublethal levels of acidity resulted in greater food intake and growth rates (energy gain), notably at ambient temperatures. This may be an indication that sublethal levels of this particular toxicant act as a stimulus to increase the energy intake to compensate for increased costs associated with living in an acidic environment. These higher costs could be a consequence of ionoregulatory disturbance. If so, appetite might be driven by the requirement for NaCl through the diet. This stimulus, coupled with an unlimited food supply, may result in an over-compensation for the increased cost of living in an acidic environment, in the form of increased growth. Smith et al. (1989) state that under conditions of impairment of branchial sodium uptake, such as under acidic conditions, dietary ions may play a critical role in body ion homeostasis. Sadler and Lynam (1987) indicate that compensation for

increased energy expenditure and ionoregulatory imbalance, due to moderately low levels of pH, may occur when fish are given sufficient food. It is uncertain how much food juvenile salmonids consume in the wild, but an unlimited ration is probably not environmentally realistic. It is also uncertain how much energy a fish expends searching for food and avoiding predators. However, it is unlikely that the unlimited food supply available to the trout in the present study is representative of food availability in the wild, since MO_2 values were at 75% of $MO_{2(max)}$, as a consequence, we propose, of SDA. We suggest that future studies on global warming/low pH scenarios should examine the effects of food limitation on trout exposed to these conditions. Limiting food may elucidate whether there are actually increased costs associated with living in a sublethally stressful environment, and what role food intake rates play in compensation.

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Chapter 3

Abstract

Juvenile rainbow trout were chronically exposed (90 days) in synthetically softened water to sublethal low pH (5.2) and a simulated global warming scenario (+2°C above the *natural* ambient summer daily temperature range of 16.5 to 21°C), alone and in combination, under conditions of limited food rations (~4% dry body weight·day^{-1}). A previous study (Chapter 2) in which trout were fed to satiation (~11% dry body weight·day^{-1}) indicated that the addition of 2°C depressed appetite and growth, while exposure to pH 5.2 improved appetite and growth, and did not cause ionoregulatory disturbance. We suggested that the unlimited ration was allowing trout to "eat their way out of danger" by providing a replacement salt load in the diet. However, in the present study, with limited ration, similar results were found. Trout exposed to +2°C had reduced growth rates, while trout chronically exposed to pH 5.2 alone, exhibited significantly greater growth than all other treatments, and again ionoregulatory disturbance did not occur. Metabolic rates and nitrogen energy losses were reduced in the low pH exposed trout at ambient temperatures, possibly as a result of reduced activity levels. The consequent higher conversion efficiencies resulted in better growth in these low pH exposed trout. Metabolic rates in all treatments were ~55% of $MO_{2(max)}$ in contrast to ~75% of $MO_{2(max)}$ found in the unlimited ration trout of our previous study. The reduction in quantity of food and feeding activity levels accounts for this decrease. Very small changes in proximate composition occurred, with no change in whole body protein, but a great deal of variability in whole body lipid and carbohydrate. No treatment trends were apparent, and we suggest that a need to utilize endogenous energy stores at times, on this limited ration, accounts for the variability. Energy budget comparisons with our first

study indicate that the limit on food intake in the present study resulted in a lowered optimum temperature for growth, and a greater proportion of the energy intake allotted for metabolic expenditure. These factors combined resulted in reduced conversion efficiencies. Fecal and unaccounted energy losses were reduced at least 4-fold in the present study, indicating that absorption efficiencies were much higher than in the satiation-fed trout. Trout in the 0/5.2 treatment expended the least metabolic energy and exhibited lower nitrogen energy losses, supporting our suggestion of energy conservation by reduced activity levels.

Introduction

Acidification of poorly buffered soft waters throughout the northern hemisphere by acidic precipitation is now well documented (Kemp 1994). At the same time, climate appears to be changing. General circulation models now predict increases in mean air temperature of 1.3-4.5°C over the next 50-100 years (Hansen et al. 1988; Mohnen and Wang 1992), with corresponding effects on the thermal regimes of freshwater bodies (Regier and Meisner 1990). Indeed, the mean annual water temperature in one well-studied softwater ecosystem, the Experimental Lakes Area of northwestern Ontario, Canada, has already increased by 2°C over the past 20 years (Schindler et al. 1990). With this background in mind, we have embarked on a research programme to understand the chronic impacts of sublethal acidity and global warming, alone and in combination, on the physiology and energetics of a model freshwater salmonid, the rainbow trout *Oncorhynchus mykiss* (cf. Reid et al. 1995a,b). The studies employ an acidic pH of 5.2 in synthetic soft water, and a global warming scenario of +2°C above the ambient daily temperature, which in turn reflects the natural thermal cycle of in-shore Lake Ontario.

In our first study (Chapter 2), juvenile trout were exposed to these regimes over a 90 day summer period from mid-June to mid-September, 1993. To allow measurements of appetite, the fish were fed to satiation twice per day, i.e. the food supply was unlimited. In accord with predictions, trout chronically exposed to low pH in combination with +2°C did suffer greater metabolic costs, although these were not particularly marked. However, several of the findings were surprising. Overall, the addition of 2°C alone depressed appetite and growth, while chronic low pH exposure alone resulted in improved appetite and growth. Most surprisingly, the fish exposed to chronic low pH exhibited no ionoregulatory disturbance, in contrast to a vast literature which indicates that low pH

interferes with ionic regulation at the gills (cf. Fromm 1980; Wood and McDonald 1982; McDonald 1983; Audet et al. 1988; Wood 1989).

These findings raised the possibility that the unlimited diet (up to ~11%·day^{-1} on a dry weight food/dry weight fish basis) was confounding interpretation. Specifically, we suspected that the availability of unlimited food: (i) might result in the expression of negative feeding and growth effects at +2°C which would not be seen or would be reversed at lower ration level; (ii) might allow fish chronically exposed to low pH to "eat their way out of ionoregulatory disturbance", resulting in increased appetite and growth driven by the need for NaCl acquisition from the diet (Sadler and Lynam 1987; Smith et al. 1989); and (iii) as a result of these opposing effects, might mitigate the physiological costs of the combined stressors. The objective of the present study was to test these ideas using a similar exposure regime, in the summer of 1994, but with a limited ration.

Ration levels in the field are difficult to measure (Elliott 1982) and are usually estimated by indirect methods. Estimates for predaceous fish in the wild at summer temperatures vary greatly (Brett 1971b; Fortunatova and Popova 1973; Elliott 1975) , and are higher for young growing fish. More difficult is the determination of rates of energy expenditure in the wild (Brett and Groves 1979; Boisclair and Tang 1993), further adding to the difficulty of assessing natural food consumption rates. However, it is clear that in the wild, a continuous, unlimited supply of food is unlikely, and food consumption rates are probably far lower than the satiation levels (~11%·day^{-1}) used in the previous study. We therefore elected to use 4%·day^{-1} (equivalent to our standard holding ration of 1% per day on a wet weight food/wet weight fish basis) in the present study. This was selected as a ration level which was less than 40% of maximum, yet one which would still allow some growth to occur based on a previous study under very similar softwater conditions in our laboratory (Wilson and Wood 1992).

Materials and Methods

Animal Holding

Experiments were conducted on 1240 juvenile rainbow trout (4-5 g), which were transported from Rainbow Springs hatchery, Thamesville, Ontario to McMaster University on May 13, 1994 . They were placed in a 400 L polypropylene tank, and acclimated to, and maintained in, continuously flowing Hamilton dechlorinated tap water ($[Ca^{2+}]$=1.90, $[Na^{+}]$=0.60 mequiv·L^{-1}; pH=7.6) at 13°C for 2 days. Artificially softened water, generated by reverse osmosis (Anderson Water Conditioning Equipment, Dundas, Ontario), was gradually introduced to the holding tank in increasing amounts over the following week. The trout were further acclimated for another 4 weeks to this soft water, once the desired water quality was achieved. Trout were fed ~4% (dry weight of food) of their dry body weight·day^{-1} of Zeigler Trout Starter #3 (for composition, see Table 1 of Chapter 2), and maintained on this diet for the 5 week acclimation period, prior to experiment initiation. Photoperiod was controlled and adjusted to mimic the natural photoperiod throughout the acclimation and experimental periods.

Exposure System

The exposure system utilized has been described previously (see Figure 1 of Chapter 2). Briefly, Hamilton City dechlorinated tap water, synthetically softened by reverse osmosis, was pumped to a main head tank where hard water was added, in a ratio of 1:40, to achieve nominal $[Ca^{2+}]$ and $[Na^{+}]$ of ~50 and 100 μequiv·L^{-1}, respectively. The water flowed by gravity, and was split into two sub-head tanks, where 2°C was added to the water of one tank via a heat-exchanger. The water from each of these two sub-head tanks was further split, and H_2SO_4 (0.2 N) was metered into one each of the ambient and +2°C temperature sub-head tanks. This resulted in four treatments: Ambient temperature/ambient pH 6.2 (0/6.2); ambient temperature/sublethal pH 5.2 (0/5.2); ambient

temperature +2°C/ambient pH 6.2 (+2/6.2); ambient temperature +2°C/sublethal pH 5.2 (+2/5.2). The water from the treatment head tanks flowed into duplicate 211 L polypropylene exposure tanks, at an average rate of 1.2 $L \cdot min^{-1}$, which allows for 90% replacement in 7 h. Sublethal pH 5.2 was maintained using a Leeds & Northrup Meredian II® Combination industrial pH electrode, in combination with the automatic control and alarm system described in chapter 2. All tanks and head tanks were aerated, and the partial pressure of oxygen (PO_2) determined once a month. PO_2's averaged 147 Torr, and pH, measured daily, averaged 6.23±0.04 in the ambient pH treatments, and 5.19±0.01 in the low pH treatments. Temperatures were measured daily. Other water chemistry parameters were monitored as described previously (Chapter 2), yielding the following mean values: $[Na^+]$=64.1±1.93, $[Ca^{2+}]$=53.3±3.20, and $[Cl^-]$=42.6±2.24 $\mu equiv \cdot L^{-1}$; and titratable alkalinity = 185.2±0.01 and 100.8±0.02 $\mu equiv \cdot L^{-1}$ for the ambient pH and low pH treatments, respectively.

Growth and Feeding Regime

After the 5 week acclimation period, 150 trout were randomly selected and placed into each treatment tank, with 160 fish placed in the 0/6.2 treatment tanks to allow 10 fish for Day 0 sampling. To ensure that all treatments received the same ration, the feeding amount was standardized to that of the 0/6.2 treatment. The fish in each tank were hand-fed 1% per day (~4% dry food:dry mass) of the measured average wet biomass of the fish in the 0/6.2 treatment tanks, adjusted for the number of fish in each tank. Biomass was measured in each tank once a week (see below), and the amount of food required for each tank determined at this time. All mortalities were recorded, and feeding amounts were adjusted on a daily basis whenever mortalities occurred. Half the feeding amount of Zeigler's Trout Starter #3 was fed to the fish twice daily, at 8:30 a.m. and 4:30 p.m. Tanks were cleaned once a week.

In view of the limited ration used in this study, we anticipated much greater inter-individual variability in growth due to in-tank feeding hierarchies (Jobling 1994). We also anticipated much lower absolute growth rates, which would be impractical to measure by the 30 day terminal subsampling method of the previous study (Chapter 2). In order to monitor growth more accurately and more frequently, a bulk weighing technique was employed to weigh all the trout in each tank once per week, with a minimum of disturbance. Fish were quickly netted *en masse* into a 10 L bucket filled with water of the appropriate pH and temperature, and lined with a tared plastic sieve. The bucket and contents were weighed on a GSE 450 Scale Systems (Michigan, U.S.A.) balance, and then the sieve and fish briefly lifted free of the water and the fish replaced in the tank. The bucket and water were then reweighed, so as to yield the weight of the trout by difference.

Physiological Measurements

Unless otherwise noted, methods were identical to those described in detail in chapter 2, and are only briefly summarized here.

Sampling protocol

Sampling and physiological measurements were conducted over a 4 day period every 30 days, including Day 0 (experiment initiation). Trout were not fed on the third and fourth days of the sampling period until after sampling was completed, at which time they received the full 4% ration.

Nitrogenous Waste Excretion

In-tank nitrogenous waste excretion (M_N) was measured on the first day of each 30 day sampling period by flow-through respirometry. Every 2 h over a 10 h period, starting at 7:30 a.m. and ending at 5:30 p.m., water inflow rate was measured, inflow and outflow

water samples were collected for ammonia (Verdouw et al. 1978), and urea analyses (Rahmatullah and Boyd 1980), and excretion rates were calculated by the Fick principle. For each of the five sampling periods, data were graphed against time, and the area under the curve determined for each treatment, to give an overall mean M_N for that 10 h period.

Metabolic Rates

Metabolic rate was determined on the second day of each 30 day sampling period over a similar time frame, using closed-system respirometry to measure in-tank routine oxygen consumption (MO_2). Rates were determined every other hour (alternating open and closed periods) over the 10 h period from 7:00 a.m. to 5:00 p.m. Tanks were first siphoned to remove fecal matter, then sealed and recirculated as described in chapter 2, while MO_2 was measured with a Radiometer/Copenhagen E5046 O_2 electrode suspended in the tank. PO_2 did not fall below 113 Torr. Data were again integrated over time to yield an overall mean MO_2 for that 10 h period. All nitrogen excretion and metabolic rate data were corrected for size differences using the weight exponent 0.824, determined for rainbow trout by Cho (1992).

Blood Analysis

Ten fish were randomly chosen from each treatment tank. Each fish was quickly killed by a blow to the head, blotted dry, weighed and the total length measured. Blood was collected by caudal severance into ammonium heparinized capillary tubes. Hematocrit was determined by centrifugation for 5 min at 10,000 x g. Due to the small size of the fish, it was important to conserve blood; the plasma in the capillary tube was removed using a 50 µl Hamilton syringe and frozen for subsequent ion analysis. When volume permitted, plasma protein was measured using a hand-held refractometer (American Optical)

(Alexander and Ingram 1980).

Whole Body Samples

Ten fish were randomly netted from each treatment tank, and placed in terminal anaesthetic (1.0 $g \cdot l^{-1}$ MS222 and 2.0 $g \cdot l^{-1}$ $NaHCO_3$). Each fish was blotted dry, weighed and measured, freeze-clamped using aluminum tongs chilled in liquid N_2, and stored at -80°C for later analysis.

Sample Processing and Analysis

Since limited volumes of plasma were available, only plasma Na^+ levels were determined by atomic absorption spectroscopy. Whole fish were ground frozen, using an IKA (M10/M20) grinding mill cooled to ~-72°C with a dry ice/methanol mixture, and a sample of the tissue weighed and then dried to constant weight at 80°C, to determine water content. The remainder of the tissue was lyophilized (Labconco Lyph-Lock 6), desiccated, and stored at -20°C. Whole body Na^+ and Ca^{2+} levels were determined by atomic absorption spectroscopy, and Cl^- levels by coulometric titration (Radiometer CMT10) after digestion of 100 mg of tissue, for 48 h, in 900 µl of 1 N H_2SO_4 at 80°C. The Lowry assay, as modified by Miller (1959), was used to determine whole body protein. Lipids were extracted and quantified using the chloroform/methanol (2:1) method (Folch et al. 1957). Glycogen, glucose and lactate levels were determined as an estimate of whole body carbohydrate, using standard enzymatic analyses (Bergmeyer 1985). Percentage inorganic content (ash) was determined by burning a subsample of whole body tissue at 550°C until a constant weight was achieved.

Statistical Analysis

Values are given as the mean ± SEM. Growth curves were compared using analysis of covariance. The design of the experiment was such that each treatment was

considered unique. As a result, interactive effects between temperature and pH were not statistically tested. Therefore, mean values for all other measurements were compared using one-way ANOVA, and in cases where the F-value indicated significance, the Tukey-Kramer comparison of all pairs test was applied, to determine treatment differences within a sampling period. The accepted probability level for significance was $p<0.05$.

Results

Few mortalities occurred over the 90 day period due to the treatments and amounted to 14% overall. Unfortunately, due to equipment failure, the entire +2/5.2 treatment was lost at day 77.

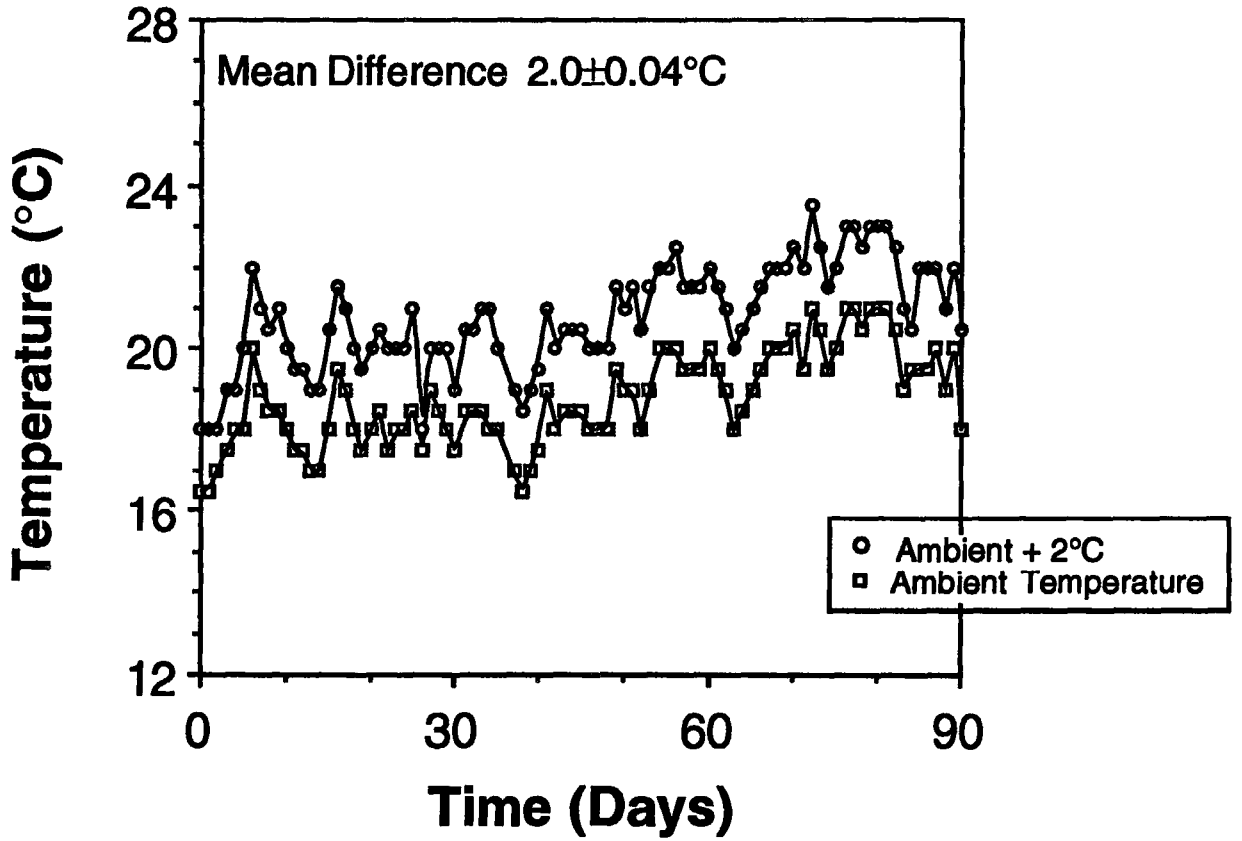
Figure 7 illustrates the thermal cycle experienced by the trout over the period June 20 to September 18, 1994. The ambient temperature ranged from 16.5 to 21.0°C, and therefore the +2°C treatment fish experienced temperatures ranging from 18.5 to 23°C. Ambient temperatures fluctuated around 18°C for the first 50 days, and then increased slightly to 19 - 21°C between Days 60 - 90, with a slight drop in temperature to 18°C again, at Day 90. During the peak in temperature, between Days 60 - 90, the trout at +2°C experienced temperatures of ~23°C for 10 days.

Because our experimental design employs the *natural* thermal regime of in-shore Lake Ontario, year-to-year variation is expected. In general, the 1994 regime until Day 60 was broadly similar to that seen in our 1993 study (Chapter 2), but differed thereafter because only small further increases of temperature occurred between Days 60 and 90 in 1994. In contrast, in 1993, ambient temperature rose to 24°C, and +2°C temperature to 26°C, elevations which were sustained over days 70 to 82. The present fish, therefore, did not come as close to the upper lethal temperature late in the summer.

Trout were limited to a daily ration of 1% on a wet weight basis (~4% on a dry weight basis), calibrated to the mean weight of the fish in the 0/6.2 treatment. As all food

Figure 7.

Summer thermal cycle (June - September, 1994) experienced by juvenile rainbow trout over the 90 day experimental period. Temperature reached the summer peak between Days 60 and 90 at 21°C (23°C in the +2°C treatments) and decreased again over the last 10 days of the exposure period.


Mean Difference 2.0±0.04°C
Temperature (°C)
28
24
20
16
12
0
30
60
90
Time (Days)
Ambient + 2°C
Ambient Temperature

offered was eaten in all tanks, there were no differences in consumption rates among treatments. Over 90 days, cumulative food consumption was ~4.5 g per fish (Figure 8) in contrast to the 32-48 g dry weight per fish consumed in the unlimited ration exposures (Chapter 2).

As a result of this limited ration, trout increased in wet body weight by only 3-4 g per fish over 90 days (Figure 8), in contrast to the 30-50 g increases seen under unlimited ration (compare Figure 8 with Figure 2 in chapter 2). Nevertheless, there were small but significant differences in growth rates among all treatments in the present study, as detected by analysis of covariance ($p<0.05$), except between the +2/6.2 and +2/5.2 treatments. Trout in the ambient temperature treatments grew significantly faster than in the +2°C treatments. The 0/5.2 trout grew significantly faster than the 0/6.2 trout.

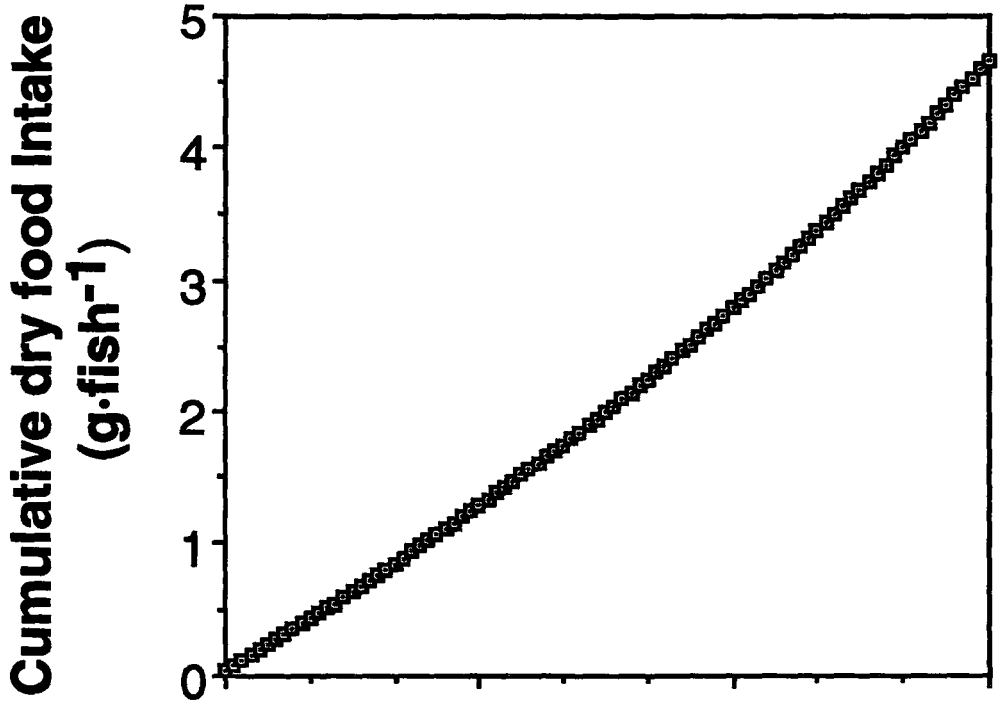
The condition factor (weight/length^3) of the trout in all treatments remained the same over the 90 day period at the starting value of ~0.9, in contrast to the increase to ~1.2 seen previously with unlimited feeding (data not shown). Gross conversion efficiencies (the efficiency with which dry weight of food was converted to dry weight of tissue) remained unchanged over the 90 days at about 16% in the 0/6.2 treatment, whereas it increased in all other treatments from ~13 to 16% (Table 8). The +2/6.2 treatment exhibited the lowest conversion efficiencies overall, while the 0/5.2 treatment had the highest conversion efficiencies ranging from ~18-20%. These values may be contrasted with conversion efficiencies in the range of 25-40% seen in the previous unlimited ration experiments (Chapter 2).

Proximate Analysis

Changes in whole body proximate composition over time in these fish on limited ration were very different from those seen previously with unlimited food supply. Indeed, composition exhibited only very small changes over 90 days in the present fish in contrast

Figure 8.

Cumulative appetite of the 0/6.2 treatment trout only, expressed on a dry weight basis ($g \cdot fish^{-1}$), and growth curves (g) expressed as wet body mass. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.


Cumulative dry food Intake
(g·fish-1)
0
1
2
3
4
5

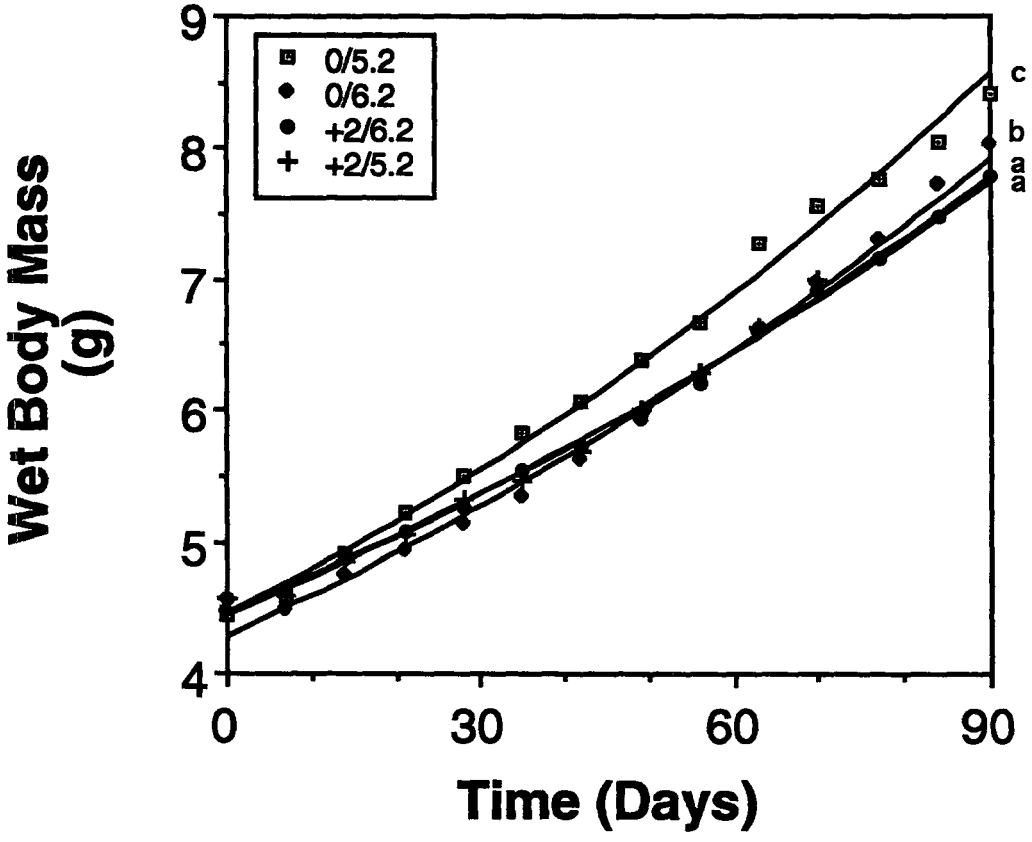

Wet Body Mass
(g)
4
5
6
7
8
9
0
30
60
90
Time (Days)
0/5.2
0/6.2
+2/6.2
+2/5.2
c
b
a
a

Table 8. Food conversion efficiencies (dry weight of food: dry weight of fish) (%·day^{-1}) calculated for each 30 day period.

Time Period	Treatment			
	0/5.2	0/6.2	+2/6.2	+2/5.2
0-30	17.8	16.0	13.7	16.2
30-60	18.0	15.7	14.4	15.1
60-90	19.8	16.2	15.9	n/a

to the large increases in lipid content, small increases in protein content, and compensating large decreases in water content occurring over 90 days in the previous study (Chapter 2).

On a whole body basis, the percentage of protein by weight decreased from ~14.5 to 13% from Day 0 to 60 in all treatments (Figure 9). Protein then increased again to ~14.5% at Day 90. No treatment effects were evident, and no significant differences were maintained.

Initial whole body lipid levels were determined to be ~4%. A great deal of variability in lipid levels, among treatments, was evident at each subsequent time period. In general, all levels increased to about 5% over the 90 day test period, with levels in each of the two ambient pH treatments increasing significantly at Days 30 and 60 (+2/6.2) and at Day 90 (0/6.2). No treatment trends were maintained over the 90 day period, although it is evident that lipid levels in the low pH treatments were maintained at a lower level than in the ambient pH treatments.

Total carbohydrate (estimated as glucose+glycogen+lactate) was also variable over the 90 day exposure period in all treatments, and showed no consistent trends among treatments or over time. Carbohydrate constituted about 0.8% of whole body composition (Table 9). Whole body water content increased from the initial value of 78% to ~80% over time. No consistent trends were evident among treatments. There was no change in total inorganic content (ash: Table 9). It is noteworthy, however, that carbohydrate contents, expressed as percentages of fish body weight, were approximately twice those of the previous study (Chapter 2). Ash content was also up to 1.5 fold higher. These differences likely reflected, at least in part, the lower lipid and protein contents of the present fish.

Whole body Na^+ and Cl^- concentrations were similarly higher by about 33 and 22%, respectively, relative to fish fed an unlimited ration, but Ca^{2+} concentrations were about 26% lower. Whole body Na^+, Cl^-, and Ca^{2+} levels indicated no effect of low pH on ion balance, and no other consistent treatment effects (Table 10). $[Na^+]$ and $[Cl^-]$

Figure 9.

Whole body protein and lipid (%). The two shaded bars on the left represent ambient temperature treatments, while the two light bars on the right represent the +2°C treatments. The hatched bars indicate low pH (5.2) treatments, and the solid bars indicate the ambient pH (6.3) treatments. Values are given as means ± SEM. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.

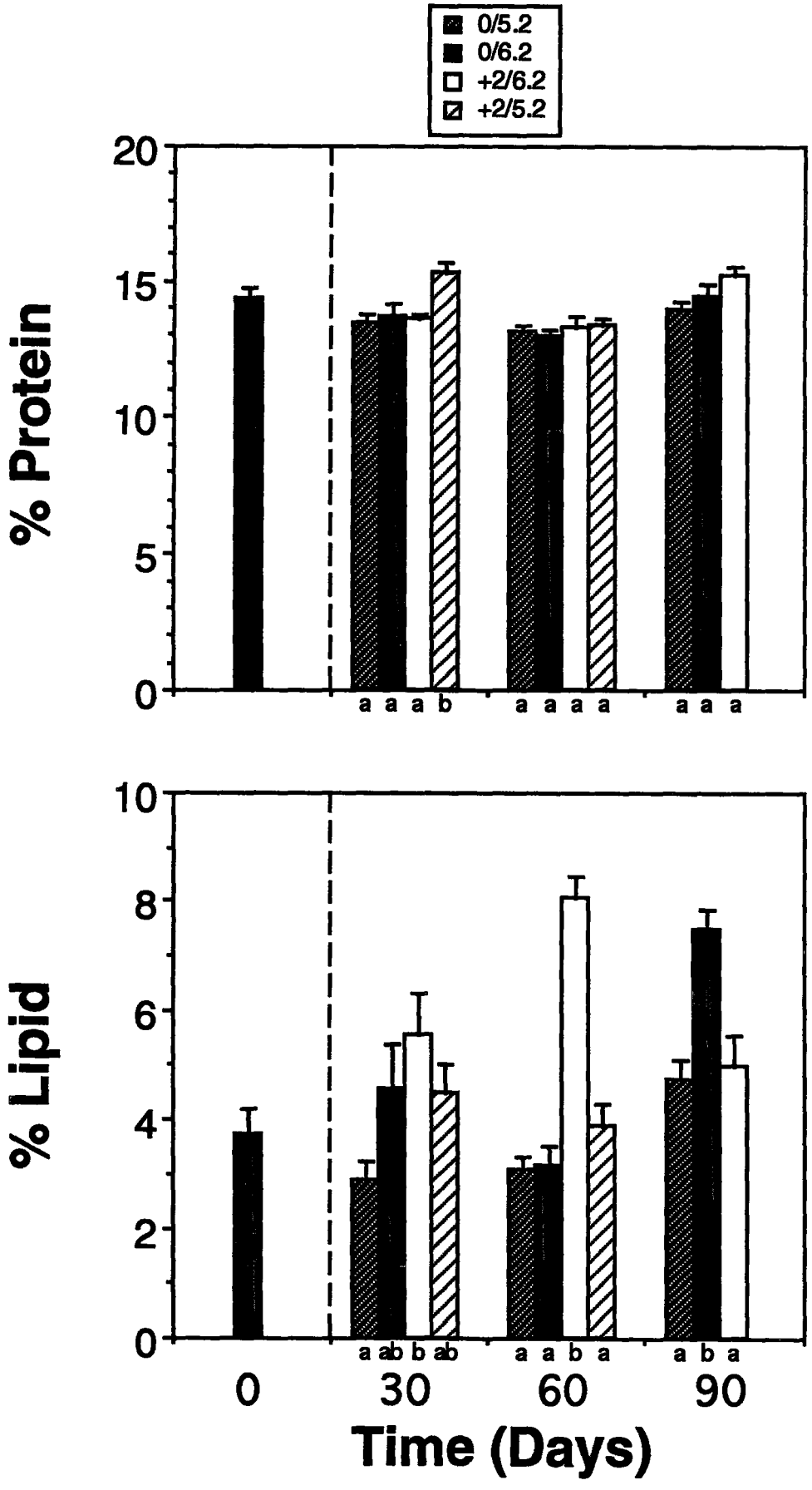

0/5.2
0/6.2
+2/6.2
+2/5.2
% Protein
20
15
10
5
0
a a a b
a a a a
a a a
% Lipid
10
8
6
4
2
0
a ab b ab
a a b a
a b a
0
30
60
90
Time (Days)

Table 9. Whole body carbohydrate, water and total inorganic (=ash) contents, as percentages, for each 30 day period. Mean values ± SEM and sample size (n) are shown. There were no significant differences for % carbohydrate or % inorganics. For % water, significant differences ($p<0.05$) are indicated by treatments that do not share a letter.

Day	0	30				60				90			
Treatment	0/6.2	0/5.2	0/6.2	+2/6.2	+2/5.2	0/5.2	0/6.2	+2/6.2	+2/5.2	0/5.2	0/6.2	+2/6.2	+2/5.2
% Carbohydrate	0.84	0.86	0.67	0.75	0.71	1.07	0.77	0.85	0.92	0.76	0.74	0.85	n/a
±SEM	0.14	0.07	0.04	0.07	0.06	0.13	0.15	0.16	0.11	0.04	0.08	0.04	n/a
n	6	6	6	6	6	6	6	6	6	6	6	6	n/a
% Water	78.1	79.2ab	78.9ab	80.4a	77.2b	80.3	80.4	79.6	79.5	78.2ab	79.4a	76.5b	n/a
±SEM	0.5	0.5	0.6	0.3	0.7	0.4	0.5	0.5	0.5	0.5	0.6	0.8	n/a
n	10	14	16	16	16	15	14	16	16	16	16	16	n/a
% Inorganics	2.30	2.45	2.55	1.96	2.77	2.00	2.32	2.68	2.60	2.78	2.54	3.23	n/a
±SEM	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.3	n/a
n	9	10	10	9	9	10	10	9	10	8	10	10	n/a

Table 10. Whole body [Na^+], [Cl^-] and [Ca^{2+}] (mequiv·kg^{-1}) for each treatment at each 30 day period. Mean values ± SEM and sample size (n) are shown. At each sample time, significant differences between treatments are indicated by treatments that do not share a letter ($p<0.05$).

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
[Na^+]	52.6	55.5ab	57.1a	51.0b	60.7a	53.3a	54.1a	54.3a	56.3a	55.2a	53.9a	57.1a	n/a
±SEM	0.9	1.1	1.9	1.1	2.5	1.1	1.5	1.3	1.1	1.6	1.2	2.4	n/a
n	10	14	16	16	15	16	16	16	16	16	16	15	n/a
[Cl^-]	38.7	40.8ab	41.9a	37.4b	42.7a	38.5a	38.0a	39.6a	39.8a	40.1a	38.5a	41.5a	n/a
±SEM	0.6	0.8	1.2	0.9	1.7	0.8	1.1	1.1	0.6	1.2	1.1	1.9	n/a
n	10	14	16	16	16	16	16	16	16	16	16	16	n/a
[Ca^{2+}]	70.8	62.3ab	60.7ab	57.5b	66.3a	57.1a	55.1a	57.9a	58.7a	63.7ab	60.2a	70.1b	n/a
±SEM	2.0	2.2	2.2	2.2	2.1	1.3	1.6	1.9	1.5	2.3	1.5	3.5	n/a
n	10	14	16	16	15	16	16	16	16	16	16	15	n/a

remained the same over the 90 day period at ~55 and 40 mequiv·kg^{-1}, while [Ca^{2+}] decreased from the initial value of 70.8 mequiv·kg^{-1} to ~60 mequiv·kg^{-1} at Day 30, and remained at this concentration over the rest of the exposure period.

Blood Analysis

Hematocrit remained at ~37% over the entire exposure period, and no treatment differences were evident (Table 11). Plasma protein values were variable due to the small sample size, and no treatment trends were obvious (Table 11).

Plasma [Na^+] remained the same at ~140 mequiv·L^{-1} over the 90 day exposure period, and no effects of low pH or temperature were evident (Figure 10).

Metabolic and Nitrogenous Waste Excretion Rates

Oxygen consumption rates were ~5 μmol·g^{-1}·h^{-1} in all treatments until Day 60 (Figure 11). This value is about 55% of $MO_{2(max)}$ as determined by Wilson and Wood (1992), and may be contrasted with the 75% of $MO_{2(max)}$ value seen in trout fed to satiation (Chapter 2). At Day 60, MO_2 values in the +2/5.2 treatment trout increased to ~6 μmol·g^{-1}·h^{-1}. This MO_2 would likely have been maintained, had this treatment survived at Day 90, judging by the pattern of MO_2 in the other 3 treatments. The slight increase in MO_2 over the peak temperature period (Day 60-90) may have been the result of the increased temperatures at this time (maximum 23°C in the +2°C treatments). Overall, oxygen consumption remained relatively constant over time, with no major treatment differences.

Nitrogen excretion rates tended to mimic the treatment patterns observed for MO_2 (Figure 11). Rates varied from 0.45 - 0.75 μmol·g^{-1}·h^{-1} over the 90 day treatment period, or approximately 50% of those seen in fish on an unlimited ration (Chapter 2). Urea

Table 11. Measured hematocrit (%) and plasma protein ($g \cdot 100ml^{-1}$) for each treatment at 30 day periods. Mean values ± SEM and sample size (n) are shown. Significant differences between treatments occurred at Day 90 only for hematocrit, and at this time period, treatments that share a letter are not significantly different from each other ($p<0.05$).

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
Hematocrit (%)	38.1	36.0	35.8	33.0	36.9	37.4	36.3	35.8	36.9	32.1a	34.9ab	37.6b	n/a
±SEM	2.3	1.2	1.3	1.1	1.0	1.2	1.1	1.4	0.8	2.4	1.0	0.8	n/a
n	10	14	16	16	16	16	16	15	16	16	16	17	n/a
Plasma Protein ($g \cdot 100ml^{-1}$)	4.1	3.7	3.8	2.9	3.5	3.7	3.8	3.3	4.3	3.5	3.9	4.0	n/a
±SEM	0.2	0.5	0.5	0.4	0.2	0.1	0.1	0.5	0.1	0.3	0.2	0.1	n/a
n	5	5	5	5	5	5	5	5	5	5	5	5	n/a

Figure 10.

Plasma Na^+ levels (mequiv·L^{-1}). See legend for Figure 3 for other details. Significant differences ($p<0.05$) are indicated by treatment groups that do not share a common letter.

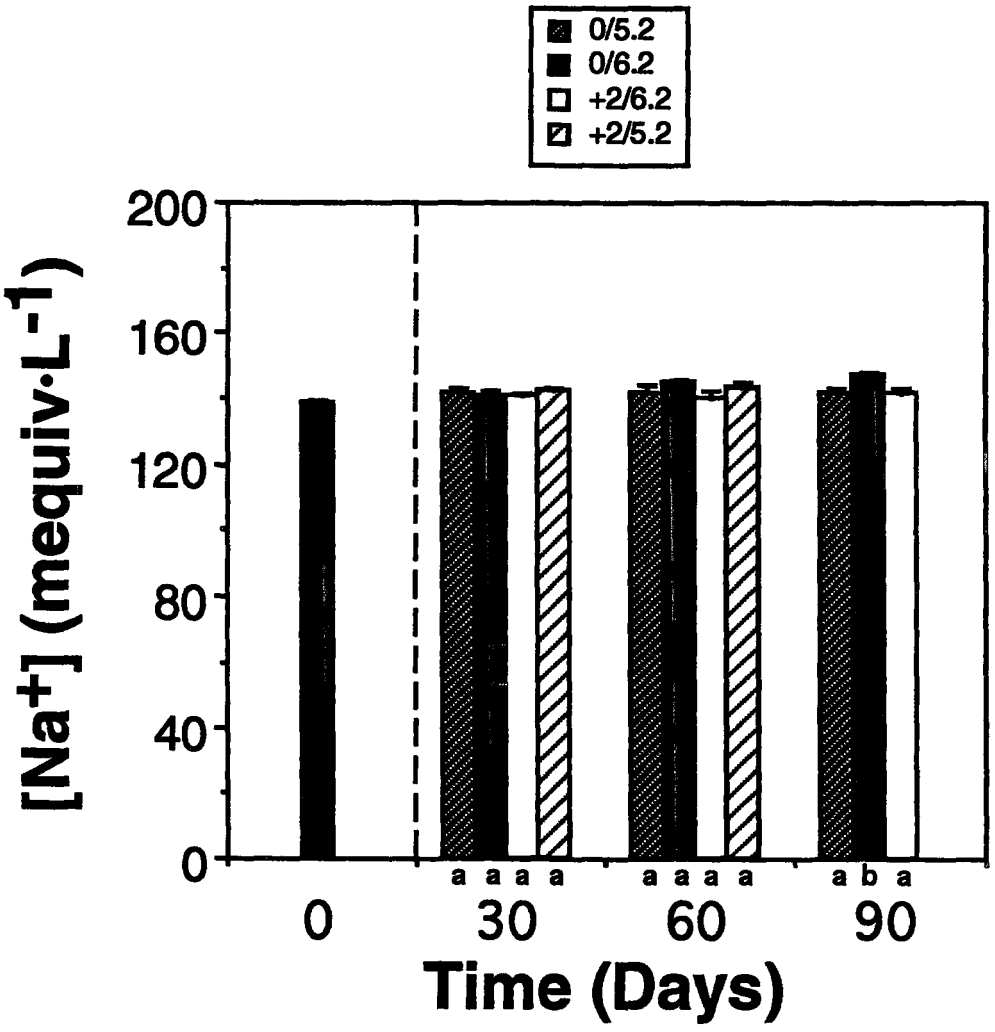
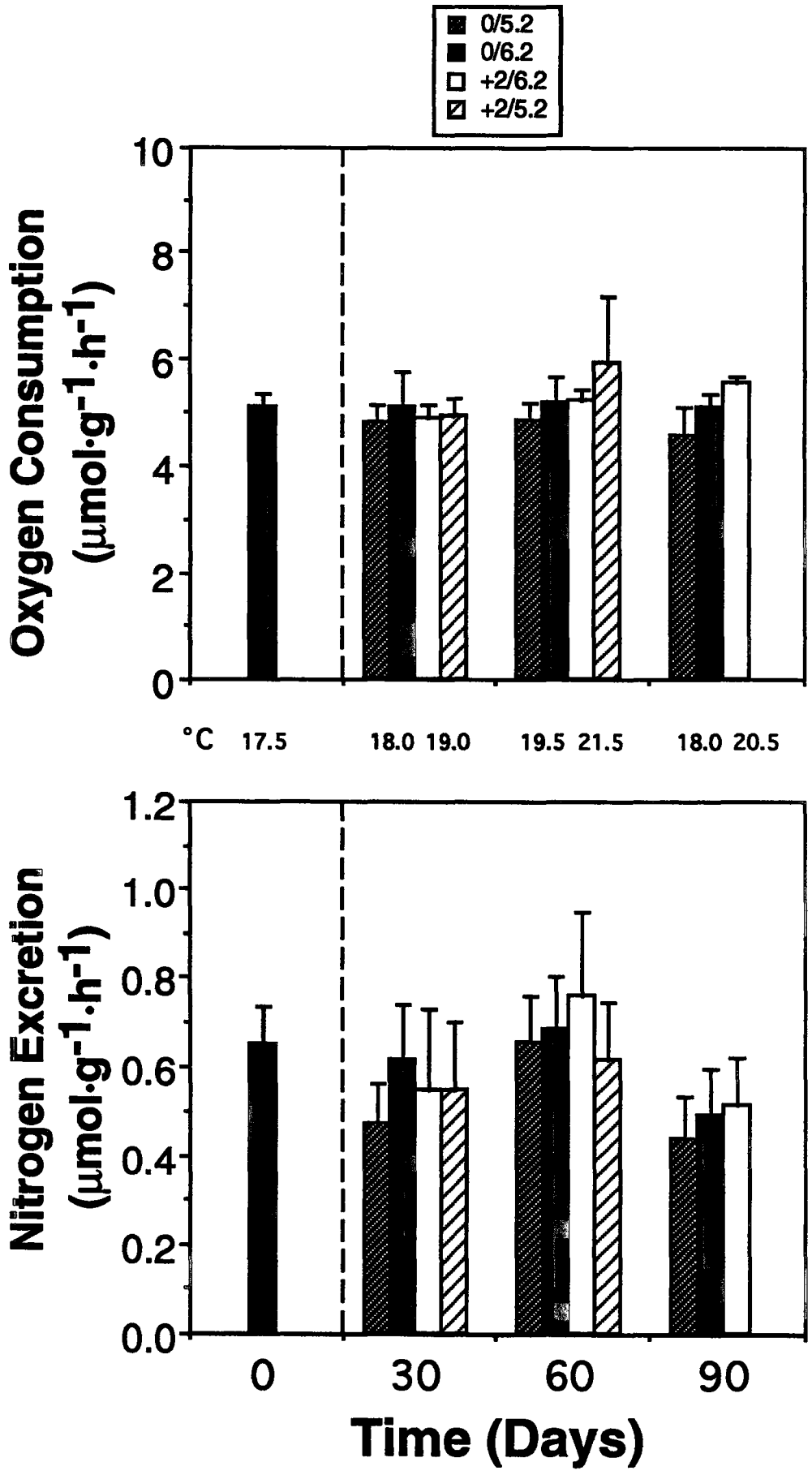

0/5.2
0/6.2
+2/6.2
+2/5.2
200
160
120
80
40
0
[Na+] (mequiv·L-1)
a a a a a a a a a b a
0
30
60
90
Time (Days)

Figure 11.

Routine in-tank oxygen consumption and nitrogen excretion (total ammonia + urea nitrogen) rates ($\mu mol \cdot g^{-1} \cdot h^{-1}$). Rates were determined for the total number of fish in one duplicate tank, over an 8 h period during the day, including the feeding periods. The temperatures at which the rates were determined appear between the graphs. Error bars represent measurement standard error only, and thus, no statistical comparisons can be made.


0/5.2
0/6.2
+2/6.2
+2/5.2
Oxygen Consumption
(µmol·g-1.h-1)
10
8
6
4
2
0
°C 17.5 18.0 19.0 19.5 21.5 18.0 20.5
Nitrogen Excretion
(µmol·g-1.h-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 30 60 90
Time (Days)

nitrogen excretion remained relatively constant over the 90 day period, and varied between 10 and 20% of the total nitrogen excreted (data not shown). At Day 90, the fraction of urea nitrogen excreted increased to 17-20% of the total nitrogen excreted, in all treatments. This occurred as a result of decreased ammonia production in all treatments at this period.

Discussion

In an effort to further elucidate the effects of chronic sublethal exposure of rainbow trout to low pH and warmer environmental temperatures, the results of the present study have been directly compared with the results from our previous study (Chapter 2). We suggested that by limiting the food intake of these juvenile rainbow trout, greater insight into the compensatory effects of unlimited diet on ionoregulatory balance and the impact of warmer temperatures would be gained.

Indicators of Cost - Temperature Effects

Temperature is generally considered a controlling factor that governs metabolic rate in ectotherms (Fry 1947; Schmidt-Nielsen 1987). However, the very similar metabolic rates, both over time and among treatments, throughout the 90 day exposure period, indicated that there was no substantially increased cost of living in a warmer environment (Figure 11). The thermal regime experienced by the trout followed the natural summer thermal cycle of inshore Lake Ontario. The gradual increase in temperature over the 90 day exposure period, in combination with daily fluctuations, and the fact that fish are able to acclimate more rapidly to increases than to decreases in temperature (Brett 1944), allowed for continuous acclimation, resulting in metabolic compensation. This effect was also evident in our first exposure (Chapter 2).

Nevertheless, the slight trend towards higher MO_2's in the +2°C treatments at Days 60 and 90 was enough to reduce growth rates in the limited ration fish in these treatments

(Figure 8). Similar effects of temperature on growth were obtained in our first study, although the effects were most obvious in the period during which temperatures rapidly increased towards incipient lethal levels (Days 55-90; Chapter 2). In the present study, the effects of the additional 2°C on metabolism and subsequently, growth were, in part, a consequence of higher (+2°C) temperatures at the beginning of the study (18°C vs. 15°C Chapter 2) which exceeded the optimal temperature for growth (15°C; Cho and Kaushik 1990) throughout the study. Also, the limit on energy intake under the +2°C conditions, meant that metabolic energy expenditure accounted for a greater percentage of the overall energy budget (Table 13 (discussed below); cf. Table 7 in chapter 2), thereby increasing the sensitivity of fish growth to metabolic differences. Gross conversion efficiencies were slightly lower in the +2°C treatments also (Table 9), contributing to the lower growth rates observed.

The feeding regime in the present study, a limited ration of 4%, resulted in routine MO_2 values that were ~55% of $MO_{2(max)}$ in contrast to ~75% of $MO_{2(max)}$ in fish on an unlimited feeding regime (~10% body weight·day^{-1}). Overall, reducing feeding rates by 60% resulted in routine MO_2 values that were reduced by almost 30%. As Cho et al. (1982) explain, ration size, food composition, and temperature determine the extent of the increase in MO_2. Since food composition was the same, and temperatures only slightly lower overall, the reduction in MO_2 was clearly a consequence of the reduction in food intake known as apparent specific dynamic action (Beamish 1974a). Activity levels were also likely reduced due to less time spent feeding. The MO_2 values determined for fish in the present study are the equivalent of MO_2's determined for starved juvenile rainbow trout swum at 3 body lengths·sec^{-1} at 15°C (U_{crit}=4.5 body lengths·sec^{-1}; D. Alsop, J.J. Dockray and C.M. Wood, McMaster University, unpublished data).

The addition of 2°C did not significantly affect protein content in these trout,

although lipid and carbohydrate levels fluctuated somewhat over the 90 day period (Figure 9; Table 9). These results indicate that a limited 4% body weight·day^{-1} ration was sufficient to meet the energy demands of every day life under the treatment conditions. However, endogenous lipid stores were utilized at times to supplement the increased energy requirements as a result of the slightly warmer temperatures, while the costs were not great enough to affect endogenous protein stores. 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. Lipid levels in the present fish were only about half of those in trout on an unrestricted ration (Chapter 2), and water contents were higher. These low, fluctuating, whole body lipid levels suggest that an increased dependence on endogenous lipid stores occurred when the warmer temperatures resulted in increased energy requirements.

Nitrogen excretion rates were affected little by the addition of 2°C. Overall, nitrogen excretion rates in trout fed a 4% ration were almost 60% lower than excretion rates in trout fed to satiation in the first exposure. This 60% reduction in nitrogen excretion is equivalent to the 60% reduction in food intake rates discussed above. Endogenous protein utilization did not increase, as indicated by the stable whole body protein levels (Figure 9), and the relatively stable fractional protein utilization (FPU) indices (Table 12). The present FPU values were lower than in trout on an unrestricted ration (Chapter 2). The FPU represents the degree to which the fish depend upon protein as an aerobic fuel source. As explained previously (Chapter 2), it 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=0.27 (Kutty 1972) in which protein supports all aerobic metabolism. These data suggest that the present feeding regime, and exogenous protein supply, was enough to compensate for any increased dependence on endogenous protein that may have occurred as a consequence of the increased temperatures.

Table 12. Fractional protein utilization (moles N produced/moles O_2 consumed)/0.27. The denominator is the theoretical maximum nitrogen quotient as determined by Kutty (1972).

Day	0	30				60				90			
Treatment	0/6.3	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2	0/5.2	0/6.3	+2/6.3	+2/5.2
FPU	0.48	0.33	0.44	0.41	0.41	0.44	0.48	0.56	0.41	0.30	0.37	0.33	n/a

Low pH Effects

Surprisingly, trout exposed to ambient temperatures and pH 5.2 grew significantly faster than trout in all other treatments (Figure 8). Better growth was also evident in the 0/5.2 exposed trout in our previous study, but these unlimited ration fish also ate more (Chapter 2). Increased food intake was not a complication in the present study. Some previous studies have demonstrated growth impairment at higher $[H^+]$ (pH<5.2) (Menendez 1976; Cleveland et al. 1986) , and reduced growth has been related to reduced food consumption under low pH conditions (Brown et al. 1984; Lacroix and Townsend 1987; Tam et al. 1988). However, neither of these effects were observed in this, or our previous study. Wilson and Wood (1992) reported no difference in growth for juvenile rainbow trout fed 1% body weight·day^{-1} and exposed to pH 5.2, while Wilson et al. (1994a) reported increased growth with unchanged appetite in trout fed to satiation and exposed to the same conditions. Clearly, regardless of feeding rate, pH 5.2 does not negatively impact growth rate in juvenile rainbow trout.

Metabolic rates in the 0/5.2 treatment, tended to be slightly lower than MO_2's in all other treatments (Figure 11). Sublethal low pH has been shown by other researchers to increase MO_2 (Hargis 1976; Waiwood and Beamish 1978; Butler et al. 1992), the increased energy expenditure resulting in reduced growth. The slightly lower MO_2's in the present study suggest a reduction in energy expenditure, perhaps as a conservation measure, thereby allowing this energy to be deposited as growth. Also, gross conversion efficiencies indicate that food was converted more efficiently to growth in the 0/5.2 treatment than in all other treatments (Table 8). These results suggest that the trout exposed to low pH were somehow reducing their energy expenditure, thereby allowing them to utilize their food more efficiently. We suggest that the food ration, although 60% less than the amount consumed by the satiation fed fish in our first study, was sufficient to combat any increased costs associated with living in this challenging environment. It is likely that

these trout reduced their activity levels. Reduced activity levels would result in more energy available for deposition as growth, and thereby increase gross conversion efficiencies. Reduced spontaneous activity has been reported in larval brook trout exposed to pH 4.5, and reduced duration of activity observed at pH 5.5 (Cleveland et al. 1986).

Combination Treatment Effects

Trout exposed to a combination of increased temperature and pH 5.2 exhibited a slight increase in MO_2 at Day 60, which, we speculate, would have been maintained at Day 90. The trout in this exposure also exhibited significantly reduced growth rates when compared to the ambient temperature treatment trout. This reduction in growth was not significantly different from the +2/6.2 treatment. It appears then, that the observed reduction in growth rates was a consequence of exposure to increased temperatures, rather than to low pH. Temperatures were above the optimum temperature for growth (15°C) over the entire 90 day exposure period. Gross conversion efficiencies were similar in both +2°C treatments, and slightly lower than for the trout exposed to ambient temperatures (Table 8). This further supports the assertion that warmer temperatures, above the optimum range for growth, depress growth rates. Brett (1971a) describes the decline in conversion efficiency evident as temperatures increase past the optimum temperature. The optimum temperature for growth shifts progressively to a lower temperature as the food quantity is restricted. The reduction in growth in the trout exposed to an additional 2°C then, is likely a consequence of both the warmer temperatures, and the restricted ration. Nevertheless, in our previous exposure on unlimited ration (Chapter 2), growth was also depressed in the trout exposed to an additional 2°C.

The maintenance of whole body protein levels in the trout of the +2/5.2 treatment (Figure 9) indicates that the 4% ration was adequate to maintain endogenous protein stores.

Lipid and carbohydrate levels (Figure 9; Table 9) fluctuated somewhat in this treatment, as in the other treatments, suggesting that endogenous lipid stores were utilized at times. However, no clear treatment effect on endogenous energy sources was evident.

From these results, low pH in the +2/5.2 treatment did not cause the trout to conserve energy, as was suggested above for the 0/5.2 exposed trout. The trend towards increased metabolic rates in the +2/5.2 treatment trout at Day 60 suggests greater metabolic cost, unlike the slightly lower MO_2 values exhibited by the 0/5.2 treatment trout at all times. As temperatures were starting to increase at Day 50 towards the summer peak of 23°C at Day 75, decreased activity levels, suggested above as an effort to reduce metabolic expenditure in the 0/5.2 exposed trout, may have been just offset by the additional 2°C in the +2/5.2 exposed trout at these higher temperatures.

Indicators of Stress

There was no evidence of increased stress as a consequence of the treatments at any time throughout the 90 day exposure. Those trout exposed to sublethal low pH did not exhibit any evidence of ionoregulatory disturbance as described by Audet et al. (1988) and Wilson et al. (1994a). Plasma and whole body [Na^+] remained stable throughout the exposure period, as did whole body [Cl^-] (Figure 10; Table 10), plasma protein and hematocrit (Table 11).

A calculation of the dietary ion budget for the trout in the first exposure (Chapter 2) showed that trout were consuming about 10% of their body pool of Na^+ per day. In the present study, trout consumed about 4% of their body pool of Na^+ per day. This 60% reduction in ions available to the trout through the diet did not result in decreased plasma or whole body Na^+. This suggests that the restricted diet in the present study was still sufficient to replace ions lost as a result of ionoregulatory disturbance in the low pH treatments. The results of the present and previous study agree with those of Sadler and

Lynam (1987), who fed yearling brown trout 2% of their body weight·day^{-1} (wet or dry basis unstated) and exposed them to low pH conditions (4.4-5.2). If the water contents of food and fish were similar to those in the present study, these trout would actually have consumed about 8% dry body weight·day^{-1}, a consumption rate between those of the present and previous studies. Ionoregulatory disturbance did occur in starved trout exposed to the same conditions in Sadler and Lynam's (1987) study. We suggest that quite low levels of dietary ion intake are sufficient to compensate for losses of ions due to the acidic exposure.

Energy Budget

The use of energy budgets to determine the allocation of ingested energy as gains, expenditures, and losses in fish, has led to a greater understanding of the influence of factors such as fish size, water temperature, and ration size on each of these components (Elliott 1982). These variables are considered to be the most important independent variables that affect growth in fish. Energy budgets can also provide useful information on the level of stress associated with pollution and altered thermal regimes (Calow 1991; Mehner and Wieser 1994).

Table 13 is a comparison of the energy budgets for the satiation fed fish from our first exposure, and the limited ration fish in the present study. The basic energy budget equation is $C=P+M+U+F$, where C is the total energy intake, P is the total energy gained, M is metabolic expenditure, U is excretory nitrogen energy loss, and F is fecal and unaccounted energy loss. Fecal and unaccounted energy loss includes undigested food, mucous, sloughed epithelial cells from the intestine, catabolized digestive enzymes, and bacteria (Soofiani and Hawkins 1985). Each energy equivalence is expressed as a percentage of the total energy consumed, in order to determine allocation differences. The energy equivalences of the food and the fish were calculated using conversion factors from

Table 13. Energy budget comparison between the satiation-fed fish of our first study (Chapter 2) and the limited ration trout of the present study. The budget covers the period Day 0-60. The general energy budget equation is: $C = P+M+U+F$ where C is the energy consumed, P is the energy gained, M is metabolic expenditure, U is excretory energy loss, and F is fecal energy loss. Fecal and unaccounted losses are estimated by difference [$C-(P+M+U)$]. Each component of the budget is expressed as a percentage of C to determine allocation differences. K is the efficiency with which food energy is converted into total energy gain (C/P*100).

Treatment	C Total Energy Consumed		P Total Energy Gained	(K) Conversion Efficiency	M Total Metabolic Expenditure		U Total N Energy Lost		F Fecal/Unaccounted Energy Lost	
	kJ	%	kJ	%	kJ	%	kJ	%	kJ	%
Satiation Fed										
0/5.2	516.8	100	266.4	51.6	140.9	27.3	17.5	3.4	92.0	17.8
0/6.3	474.5	100	230.9	48.7	119.9	25.3	14.3	3.0	109.5	23.1
+2/6.3	438.2	100	198.9	45.4	122.5	27.9	18.0	4.1	98.9	22.6
+2/5.2	423.5	100	168.6	39.8	150.3	35.5	19.3	4.6	85.3	20.2
4% Ration										
0/5.2	55.6	100	7.9	14.2	40.6	73.0	3.9	7.0	3.2	5.8
0/6.2	56.4	100	6.7	11.9	41.4	73.4	4.3	7.6	4.0	7.1
+2/6.2	56.7	100	18.8†	33.1	41.8	73.7	4.4	7.8	-8.3†	-14.6†
+2/5.2	56.0	100	8.5	15.1	43.7	78.0	3.8	6.8	0.1	0.1

† The high P value in this treatment reflects an unusually high lipid content at Day 60, which is possibly aberrant. The F values are negative in consequence.

Jobling (1994). The energy budgets have been calculated up to, and including, Day 60. This allows for a comparison of trout that have been exposed to a *similar* thermal history. Furthermore, a comparison at Day 60 also allows consideration of the +2/5.2 treatment trout, which were lost at Day 77 as a result of equipment failure in the present study.

In the satiation-fed study, the trout in the ambient temperature treatments consumed a greater absolute amount of energy, and converted that energy more efficiently into weight gain than the trout exposed to an additional 2°C. The greatest energy gain and conversion occurred in the 0/5.2 treatment, in which appetite was the greatest. The greatest metabolic expenditure and excretory energy losses occurred in the +2/5.2 treatment, suggesting that the combination of increased temperature and low pH was indeed more costly than either of the stressors alone. Fecal and unaccounted energy losses were similar in all treatments, although they were lowest in the 0/5.2 treatment. In general, it is clear that the 0/5.2 treatment trout were consuming proportionately more energy, and making better use of that energy by converting it more efficiently to energy gain. However, metabolic expenditure was high in this treatment, perhaps as a consequence of prolonged feeding activity. Thus the increased energy intake was necessary to ensure energy deposition (growth) in the face of this increased metabolic expenditure.

As stated above, the three most important variables influencing fish growth are water temperature, fish size, and energy intake (Elliott 1982). It has been demonstrated by Brett et al. (1969) that as temperature increases past optimum, the efficiency with which food energy is converted to energy gain decreases. Likewise, as ration decreases, the optimum temperature for growth decreases. Therefore, as temperature increases past an optimum temperature for growth, fish have to consume more to achieve a conversion efficiency similar to that at the optimum temperature. In the satiation fed juveniles in our first exposure (Chapter 2), growth was achieved over the entire temperature range (13-24°C) by increasing appetites, since ration was unlimited. In the present limited-ration

experiment, juvenile trout were exposed to similar temperature conditions, but they were not able to increase their food intake. Since temperatures were higher than the optimum temperature for growth throughout the duration of the present study, and noting that the optimum temperature would be lower in these limited ration fish than for those in the satiation feeeding exposure, conversion efficiency was lower.

Metabolic expenditure in the limited ration trout (though 20% lower than in satiation fed trout) accounted for ~70% of the total energy intake. This is contrasted with metabolic expenditures in the unlimited ration trout of ~30% of the total energy intake. Mehner and Wieser (1994) explain that high temperature causes a greater proportion of the metabolizable energy (*M+P*) to be allocated to *M* (Total metabolic expenditure) than to *P* (Total energy gained). Thus, at higher temperatures, when rations are restricted, conversion efficiencies are reduced (Elliott 1976a).

The differences between treatments in the limited ration exposure were very slight. However, the +2/5.2 treatment trout tended to expend more energy metabolically than trout in the other treatments, while trout in the 0/5.2 treatment expended the least metabolic energy. This, in combination with lower nitrogen energy losses in the 0/5.2 treatment trout, supports our previous suggestion that these fish may be conserving energy by reducing activity levels. This strategy in fish has been described by Forstner and Wieser (1990), Mehner and Wieser (1994), in response to warmer temperatures, and Wilson et al. (1994a) to low pH + Al exposure. The conversion efficiencies indicate that the +2/6.2 trout made substantially better use of their energy consumed than all other treatments. Lipid levels in these treatments fluctuated substantially, and reached particularly high levels in the +2/6.2 treatment at Days 30 and 60. This may be a consequence of the increased temperatures temporarily improving energy deposition, or it may be a transient effect due to the fluctuating nature of the endogenous stores of lipid. It may also be a random sampling aberration. At day 90 the lipid level in this treatment decreased again.

The fecal and unaccounted energy losses comprised 7% or less of the energy intake in the limited ration trout, and ~20% in the satiation fed fish. Fecal losses in carnivorous fish have been determined as 2-31% of the energy intake (Elliott 1979). Trout in the present study, therefore, made very efficient use of the metabolizable energy they consumed, although that energy, as described above, was expended metabolically under the summer temperature conditions, rather than put towards growth. This observation is supported by Elliott (1982), who found that as temperature increased, and ration decreased, absorption efficiency increased. He explains that the "physiologically useful energy" (Elliott 1976b), that energy available for growth and metabolism, decreases with temperature at higher temperatures, and increases with decreasing ration level.

Unlike the energy budget for the trout in our first unlimited ration exposure, the evidence for improved conversion efficiency in the 0/5.2 treatment trout is not clear. Metabolic energy expenditure was slightly lower in this treatment, as was excretory energy loss, perhaps associated with a decrease in spontaneous activity. It may be that growth rates were greater in these trout simply because they did not mobilize endogenous energy stores from time to time for activity, as was evident in the other three treatments.

Concluding Remarks

Chronic exposure of juvenile rainbow trout to warmer environmental temperatures and sublethal low pH under limited ration conditions of 4% dry body weight·day^{-1} did not result in more clear-cut treatment effects on their physiology and energetics than previously seen under unlimited ration (Chapter 2). The trout exposed to an additional 2°C had depressed growth rates, and those exposed to pH 5.2 exhibited no evidence of ionoregulatory disturbance. These results are similar to the results of our first study (Chapter 2), and indicate that the 4% ration in the present study was sufficient to ameliorate the effects of acidic exposure. This reduced ration still provides an adequate salt load for

the replacement of any ion losses caused by low pH exposure. High summer temperatures, above the optimum temperature for growth, reduce conversion efficiency. These effects are more evident in limited ration fish, because they are unable to increase their food intake to combat the subsequent high metabolic rates (relative to energy intake), resulting in low conversion efficiencies. Absorption efficiencies ($P+M$), however, are high, indicating very efficient use of the energy consumed.

The combination of warmer temperatures and sublethal low pH appears to be slightly more costly than either stressor alone, in trout that have an unlimited or limited ration. The limited ration used in the present study, as previously suggested, appears to be sufficient to compensate for the increased physiological costs associated with these conditions. This is an important consideration when assessing the impact of a marginal environment, such as the one examined here, on freshwater fish. It appears then, that feeding trout would withstand low pH conditions, and to some degree, warmer environmental temperatures, more effectively than starved trout. A further reduction in ration to the maintenance level may uncover treatment effects. Trout in the wild would have such limitations on their diet, particularly under winter conditions. Future studies, relevant to the wild situation, should be aimed at determining the seasonal effects of these environmental stressors on fish.

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Concluding Remarks

The studies contained herein were designed to consider the combined effect of an environmental toxicant and climate change on freshwater fish. Research directed at assessing the impact of environmental change on the natural world should attempt to do so using realistic conditions. The reality today is that pollution comes in many forms, and the toxicants and/or climate change will combine to result in effects that may not be evident, or are less significant when these factors are examined separately. Moreover, the nature of many environmental issues is such that acute and lethal conditions are not the norm, and chronic sublethal exposure of organisms to pollutants, the reality. Longterm exposures, therefore, are of environmental relevance to the situation today. Completely realistic conditions, however, are extremely difficult to achieve in the laboratory. Attempting to study the effects of just two environmental factors on trout proved to be exceptionally challenging.

As a study proceeds, it often becomes evident that factors aside from those that are under direct observation must also receive attention. Ration level was such an issue in the present work. As it turned out, ration level was perhaps the most important variable in determining the degree to which the stressors affected the trout. In chapter 2 it appeared that the low pH exposed trout were "eating their way out" of ionoregulatory disturbance, while in the process, improving growth through increased appetite. Chapter 3 indicated that a 60% reduction in food intake rates still provided the trout with sufficient replacement salt through the diet, while it was suggested that reduced activity levels may have contributed to the improved growth rates in these trout.

Food intake rates have a significant effect on apparent specific dynamic action (Beamish 1974a). This was illustrated by the reduction in routine metabolic rates in the

trout fed the limited ration (see Table 14 for study comparison). Whereas trout fed to satiation consumed oxygen at a rate equivalent to 75% of $MO_{2(max)}$, trout fed 60% less (the limited ration exposure), consumed oxygen at a rate equivalent to only 55% of $MO_{2(max)}$. Likewise, nitrogen excretion rates were 50% lower in the limited ration trout.

As expected, reduced ration drastically reduced absolute growth (Table 14). Trout fed to satiation increased in wet body mass by 30-50 g, while trout fed the limited ration increased by only 3-4 g. Proximate composition revealed that lipid content increased substantially in the satiation fed trout, while protein content increased slightly, and compensating water content reductions occurred. Lipid and carbohydrate content were highly variable in the trout fed a limited ration. This variation may be a consequence of the utilization of endogenous stores at times over the 90 day exposure period. Overall, no significant changes occurred in proximate composition in these fish.

The present studies utilized the natural fluctuating temperature cycle of inshore Lake Ontario over the three month summer exposure period. Year to year variation resulted in small but important differences between the thermal profiles experienced by the fish. In the first study (Chapter 2), the fish experienced an eleven degree range of temperature (13-24°C) compared to four and a half (16.5-21°C) the following summer. Clearly, the trout in the first exposure were exposed to higher temperatures at the summer peak (26°C in the +2°C treatments) than trout in the subsequent experiment (23°C in the +2°C treatments). Consequently, appetites and growth were depressed in the satiation-fed trout exposed to an additional 2°C, particularly during and after peak summer temperatures.

Energy budgets (see Table 13) revealed the difference in allocation of energy between trout fed an unlimited versus limited ration. A greater proportion of the consumed energy was expended metabolically in the limited ration trout (70% vs. 30% in the satiation fed fish). At high temperatures, a greater proportion of the metabolizable energy is allocated to metabolism (Mehner and Wieser 1994). In addition, the restricted ration results

Table 14. A comparison of the main components measured for rainbow trout in the satiation feeding and limited ration exposures. Values presented represent a summary of those obtained over the entire 90 day exposure period, and overall trends are indicated.

Exposure	Cumulative Dry Food Consumption (g)	Growth Wet Mass (g)	Gross Food Conversion Efficiency (%·day^{-1})	Whole Body Protein (%)	Whole Body Lipid (%)	Whole Body [Na^+] (mequiv·Kg^{-1})	Oxygen Consumption (μmol·g^{-1}·h^{-1})	Nitrogenous Waste Excretion (μmol·g^{-1}·h^{-1})
Satiation Feeding (11%)	32.0-48.0	30.0-50.0	25-40	increased 12.0-15.0	increased 4.5-12.0	maintained ~45.0	~7.0	1.0-1.5
Limited Ration (4%)	4.5	3.0-4.0	16-20	maintained 14.5	maintained 4.0	maintained ~55.0	~5.0	0.45-0.65

in an increased relative proportion of energy required for metabolism. Consequently, conversion efficiencies in the limited ration trout were reduced. However, absorption efficiency (i.e. the energy available for growth and metabolism) was higher in the limited ration trout. This was reflected in the much lower fecal and unaccounted energy losses in these fish (7% vs. 20% in the fish fed to satiation). In general, the trout fed a limited ration utilized their energy source more efficiently than trout fed to satiation.

Energy budgets in the first exposure clearly indicated that not only were the trout in the 0/5.2 treatment taking in more gross energy than trout in the other treatments, they were converting that energy more efficiently to growth. The greater appetites and improved conversion efficiency resulted in greater growth in these trout. This is not clear in the trout fed a limited ration. However, metabolic expenditure was slightly lower in the 0/5.2 treatment trout in the limited ration study, as was excretory energy loss. This may have been a consequence of reduced spontaneous activity, leading to the improved growth rates in this treatment.

The Na^+ flux experiment conducted at the end of the limited ration exposure, in which fish were exposed to pH 4.2 for 24 h, provided evidence for improved recovery of ionoregulation, in trout which had been chronically exposed to pH 5.2 (see Appendix 1). While it appears that trout chronically exposed to pH 5.2 were able to replace branchial ion losses with salt provided in the diet, the Na^+ flux experiment data suggest that some physiological adaptation to acidic exposure may also have occurred at the gill.

The intent of the data produced and the interpretation presented is to provide some baseline information concerning freshwater fish, with respect to the potential conditions that may prevail in the face of climate change. It is also hoped that this study will provide the impetus for future work in this area, perhaps stimulating continued research into the effects of the present scenario conditions, as well as consideration of the multitude of other anthropogenically produced pollutants which are presently threatening the natural world.

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Appendix 1

Sodium Flux Experiment

Introduction

A flux experiment was conducted at the end of the limited ration 90 day exposure (September 30, 1994), to determine whether prior chronic exposure of juvenile rainbow trout to sublethal low pH (5.2) and/or increased natural fluctuating temperatures, imparted any increased ability to maintain branchial sodium transport in the presence of a challenge concentration of external hydrogen ions (pH 4.2). Trout with prior exposure to low pH exhibiting a lack (or reduction) of ionoregulatory disturbance in the face of a more severe exposure to low pH would provide evidence for acclimation to low pH conditions. On the other hand, trout exhibiting more severe disruption of Na^+ transport upon exposure to increased concentrations of H^+ would suggest that sensitization to low pH had occurred.

Previous studies have suggested that adaptation to sublethal low pH environments is possible based on histological studies (Leino and McCormick 1984), examination of interrenal activation and ion loss (Balm and Pottinger 1993), and ionic composition and growth studies on fed and unfed trout (Sadler and Lynam 1987). Other investigations have indicated that exposure to sublethal low pH is deleterious, with the effects of low pH manifested as abnormal behaviour and growth deformities (Mount 1973), reductions in viable eggs and growth (Menendez 1976), chronic ionoregulatory disturbance (Lacroix 1985), and ionoregulatory disturbance with eventual stabilization of ion flux rates below control levels (Audet et al. 1988). A follow-up experiment to investigate whether acclimation had occurred in the latter study indicated that long-term sublethal exposure to low pH had, in fact, sensitized these adult rainbow trout, rather than conferring increased tolerance (Audet and Wood 1988).

The results of our 90 day exposure of trout to sublethal low pH did not provide evidence of ionoregulatory disturbance, a consequence, we suggested, of salt replacement through the diet. The present experiment was conducted in light of these interesting findings.

Materials and Methods

Six fish were selected randomly from each treatment tank, and placed in individual 1 L flux chambers. As a result of equipment failure on Day 77 of the 90 day exposure, the +2/5.2 treatment was lost, and was thus not represented in the flux experiment. To allow the fish to acclimate to the flux chambers, the respective treatment water flowed through each chamber for 17 h prior to initiation of the experiment (i.e. ambient temperature/pH 6.2 or 5.2, and +2°C/pH 6.2). Ambient or +2°C temperatures were maintained by running the appropriate water around the chambers in a wet table. Each chamber was fitted with an airstone to ensure adequate aeration and mixing, and chambers were first sealed with lids, and then covered with black plastic sheets to minimize disturbance during the acclimation and experimental periods.

An initial 1 h control flux experiment was conducted by removing the water supply to each chamber, and adding 1 μCi $^{22}Na^+$ to each 1 L chamber. Four 5 ml water samples were removed after a 10 min mixing period for $^{22}Na^+$ activity, $[Na^+]$ and total ammonia (NH_3/NH_4^+) determinations. The water sampling was repeated after 1 h to allow measurement of the disappearance, i.e. the uptake, of $^{22}Na^+$. The chambers were then flushed twice, and re-filled with pH 4.2 water in order to carry out a challenge flux experiment. Again, $^{22}Na^+$ was added to each chamber, a 10 min mixing period allowed, and water samples removed immediately after the 10 min mixing period, and at the end of the 1 h challenge period.

After this initial challenge, water of pH 4.2 was returned to the chambers on a flow-through basis, and the flux determinations were repeated after 6-7 h and 23-24 h of exposure to pH 4.2. The four flux periods are referred to as control, 1 h, 7 h, and 24 h. In all periods, acidic pH's (5.2, 4.2) were maintained by manual pH statting with 0.2 N H_2SO_4. Typically pH was maintained within 0.05 units of the desired level.

At the end of the fourth period, trout were killed by a blow to the head, weight and length were determined, and blood was collected using ammonium heparinized capillary tubes after caudal severance. Blood was centrifuged for 5 min at 10,000 x g, and plasma collected and frozen at -80°C for backflux corrections. All water samples for total ammonia determination were frozen at -20°C, and later analyzed for NH_3/NH_4^+ using the indophenol method of (Verdouw et al. 1978). Total [Na^+] measurements were carried out by atomic absorption spectroscopy (Varian AA-1275) on the remainder of the samples collected for ^{22}Na gamma count determination

Calculations

The remaining water samples and plasma sample were analysed for $^{22}Na^+$ activity by measuring gamma activity on a Canberra Packard Minigamma 5000 gamma counter. Sodium influx rates were calculated using the standard unidirectional sodium influx calculation,

$$J^{Na+}_{in} = \frac{(cpm_i - cpm_f) \times V}{MSA \times T \times W}$$

where J^{Na+}_{in} = rate of sodium influx, cpm_i = initial counts per minute in ml (f = final), V= volume of the bath (ml), MSA = mean specific activity (cpm $^{22}Na^+$/Total μmol Na^+), T= time (h), and W = weight of fish (kg) (Maetz 1956). Net flux rates were determined using,

$$J^{Na+}_{net} = \frac{Na^+_f - Na^+_i \times V}{T \times W}$$

where J^{Na+}_{net} = net rate of sodium flux, and Na^+ = total sodium concentration. J^{Na+}_{out} (sodium efflux) was calculated by the conservation equation:

$$J^{Na+}_{out} = J^{Na+}_{net} - J^{Na+}_{in}$$

Corrections were made for water volumes removed. A backflux correction was not required since internal (i.e. plasma) specific activity at the end of the experiment was less than 2.1% of the external specific activity.

Values were expressed as mean ± SEM. Comparisons of means with the control over time, and between treatments within a flux period, were made using one-way ANOVA. Where the F-value indicated significance, Dunnett's test was employed to determine differences over time, and Tukey-Kramer comparison of all pairs test was applied to determine treatment differences, with an acceptance level of $p<0.05$.

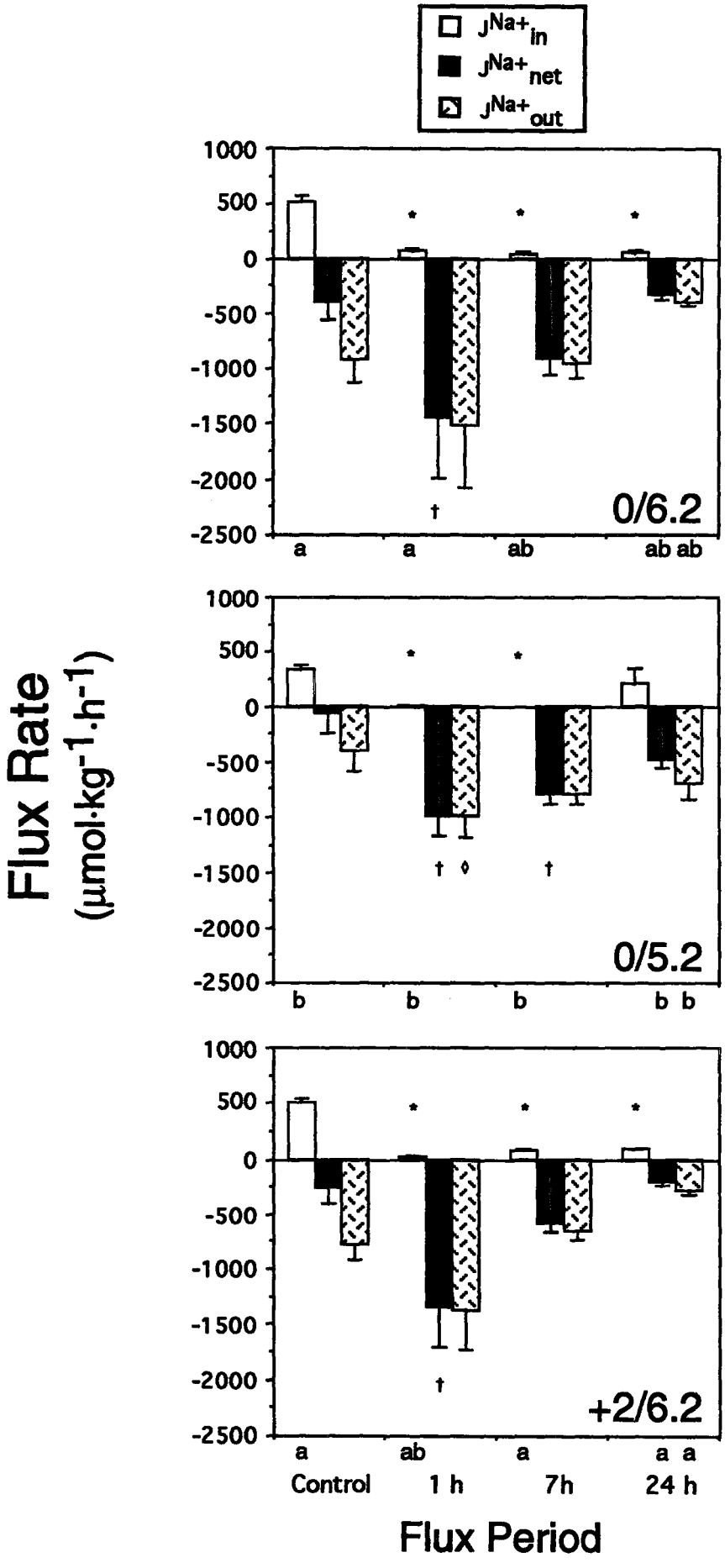
Results

Figure 12 illustrates the results of the flux experiment for each treatment over time. In the control period, Na^+ influx (J^{Na+}_{in}) was significantly lower in the 0/5.2 treatment than in the 0/6.2 and +2/6.2 treatments (Figure 12). However, net Na^+ fluxes (J^{Na+}_{net}) were not significantly different among treatments, and neither were Na^+ efflux rates (J^{Na+}_{out}). After 1 h and 7 h of exposure to pH 4.2, all treatments exhibited significantly reduced influx rates by about 90%. This inhibition was maintained in both the 0/6.2 and +2/6.2 treatments after 24 h of exposure to pH 4.2, whereas the 0/5.2 treatment trout exhibited recovery of Na^+ influx to the lower control levels representative of this treatment.

Control net flux rates indicate a net loss of Na^+ in all treatments. These net losses increased significantly in all treatments after 1 h of pH 4.2 exposure, due to both significant inhibition of Na^+ uptake, and increases in rates of Na^+ efflux, the latter significant only in the 0/5.2 treatment. Significant net loss was maintained in the 0/5.2 treatment after 7 h of

Figure 12.

Na^+ flux rates in trout exposed to a challenge concentration of H^+ (pH 4.2). Flux rates are displayed over time for each treatment, and are expressed as mean ± SEM. Significant differences from control rates are indicated by a * for influx rates, a ◊ for efflux rates, and a † for net flux rates. Significant differences among treatments at a flux period are indicated by treatments that do not share a letter.


J_{Na+in}
J_{Na+net}
J_{Na+out}
Flux Rate
(µmol·kg⁻¹·h⁻¹)
1000
500
0
-500
-1000
-1500
-2000
-2500
0/6.2
a a ab ab ab
0/5.2
b b b b b
+2/6.2
a ab a a a
Control
1 h
7h
24 h
Flux Period

exposure to pH 4.2, although efflux rates were no longer significantly greater than control efflux rates. At 7 h in the 0/6.2 and +2/6.2 treatments, net flux and efflux rates were no longer significantly different from control rates, and this was maintained after 24 h of pH 4.2 exposure. In the 0/5.2 treatment trout at 24 h, the efflux and net flux rates were no longer significantly different from control flux rates.

In general, after 24 h of pH 4.2 challenge exposure, the trout pre-exposed to ambient pH recovered and further reduced Na^+ efflux and net flux rates from control rates, but did not recover Na^+ influx rates from the significant inhibition observed after 1 h of pH 4.2 exposure. In contrast, the trout that had experienced pre-exposure to pH 5.2 for 90 days recovered all components of the Na^+ flux to levels not significantly different from control.

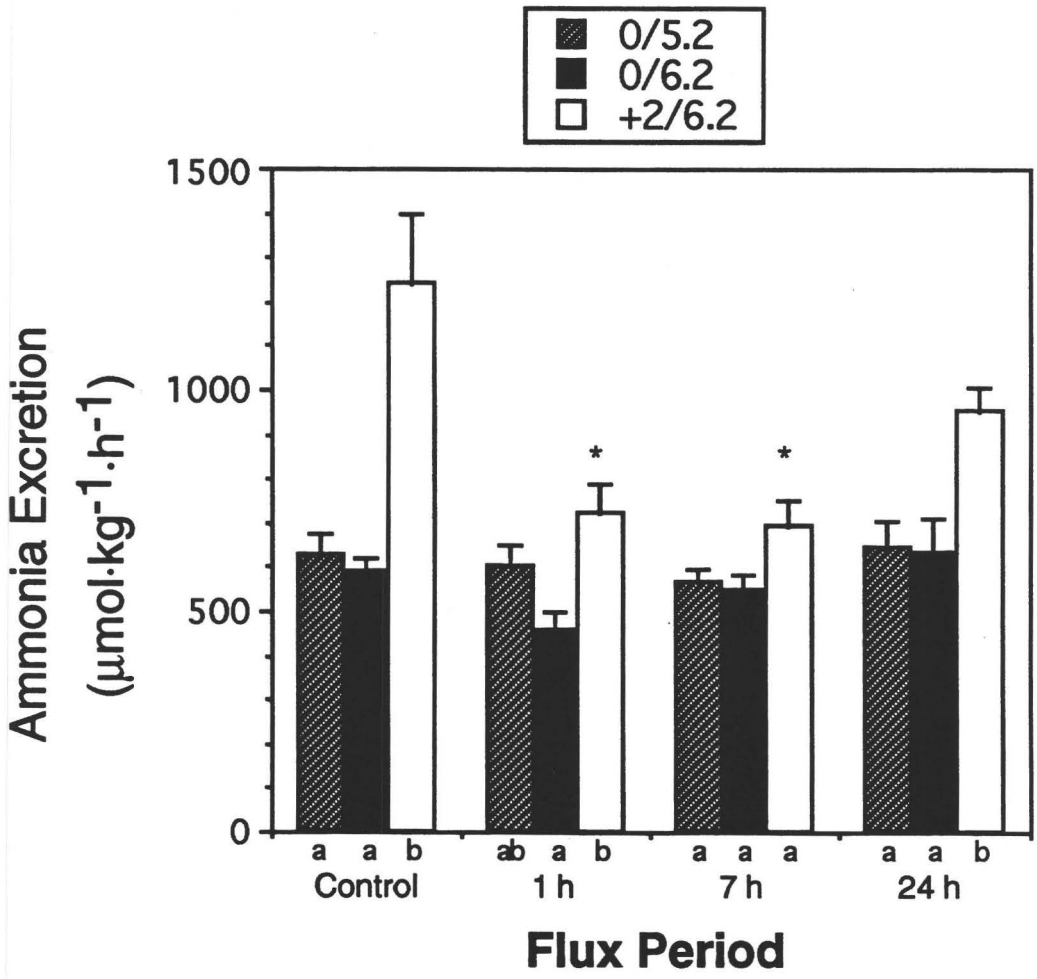
Ammonia excretion rates were higher in the +2/6.2 treatment trout than the trout in the 0/5.2 or 0/6.2 treatments throughout the experiment, a difference which was significant in the control and 24 h flux periods (Figure 13). Over time, while ammonia excretion in the ambient temperature treatments was unaffected by the low pH 4.2 exposure, excretion rates in the +2/6.2 treatment were significantly lower than the control rate after both 1 h and 7 h of pH 4.2 exposure. After 24 h, excretion rates were no longer significantly different.

Discussion

The results of the present challenge Na^+ flux experiment indicate that long term (90 days) exposure of juvenile rainbow trout to sublethal low pH (5.2) increased their ability to recover their branchial sodium transport rates to control levels after 24 h of exposure to a challenge concentration of external hydrogen ions (pH 4.2). These results differ from the findings of Audet and Wood (1988) who chronically exposed (3 months) adult rainbow trout to a pH of 4.8 in artificial softwater, and then challenged both acid pre-exposed and naive fish (pH 6.5) to a pH of 4.0 for 4.5-5 h. Audet and Wood (1988) found that

Figure 13.

Ammonia excretion rates in trout exposed to challenge pH 4.2. Rates are expressed as mean ± SEM, and are displayed over time. Flux rates that were significantly different from control values are indicated by a *. Significant differences among treatments at a flux period are indicated by treatments that do not share a letter.


0/5.2
0/6.2
+2/6.2
Ammonia Excretion
(μmol·kg-1·h-1)
1500
1000
500
0
*
*
a a b
ab a b
a a a
a a b
Control
1 h
7 h
24 h
Flux Period

although significant ionoregulatory disturbance occurred in both naive and acid pre-exposed trout, rates of net Na^+ loss were 2 fold larger in the acid pre-exposed trout. In the present study, net Na^+ loss rates in all of the treatments increased by about 1000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ after 1 h of exposure to pH 4.2, as a result of similar reductions in Na^+ uptake and increases in Na^+ efflux rates. After 7 h of exposure to pH 4.2, Na^+ influx was still inhibited in all treatments, although the net Na^+ losses had decreased as a result of reductions in Na^+ efflux rates. After 24 h of exposure to pH 4.2, it was apparent that while the trout in the 0/6.2 and +2/6.2 treatments still exhibited significant inhibition of Na^+ uptake, the trout in the 0/5.2 treatment had recovered all components of the Na^+ flux mechanism to control levels.

This recovery of Na^+ flux rates to control levels suggests that these previously low pH exposed trout were better equipped to deal with further insult from even higher concentrations of H^+. However, it is important to note that the trout with prior exposure to pH 5.2 already had significantly lower influx rates under control conditions. Furthermore, they were challenged with a reduction of only 1 pH unit (a 10-fold increase in $[H^+]$), whereas those with prior exposure to pH 6.2 suffered a reduction of 2 pH units (a 100-fold increase in $[H^+]$). It may be that given more time, the trout previously exposed to pH 6.2 may also have recovered from this challenge level of acidity.

Ammonia excretion rates were not significantly reduced in either of the ambient temperature exposed groups over the 24 h challenge exposure period, even though Na^+ influx rates were inhibited in both of these treatments. However, ammonia excretion rates were significantly reduced in the +2/6.2 treatment trout after 1 and 7 h of pH 4.2 exposure. Excretion rates recovered in this treatment after 24 h of exposure to pH 4.2. Wright and Wood (1985) observed a drop in ammonia excretion in trout exposed to pH 4.0 in hard water. Audet and Wood (1988) found that naive trout exhibited a 50% reduction in ammonia excretion, while trout previously exposed to low pH maintained chronically

elevated levels of ammonia efflux. The reduction in ammonia efflux was attributed to the inhibition of Na^+ uptake, since it has been suggested (McDonald and Prior 1988) that the uptake of Na^+ across the gills is coupled to NH_4^+ efflux via a Na^+/NH_4^+ exchange mechanism.

The present data are difficult to interpret since although there is an indication of a reduction in ammonia excretion in the 0/6.2 treatment trout, the decreases are not significantly different from control excretion rates. The trout from the +2/6.2 treatment may have been producing ammonia at a higher rate as a result of the higher temperature, resulting in a more pronounced reduction in ammonia excretion rate. It should be noted, however, that there was no evidence of higher ammonia excretion rates in this treatment during the 90 day exposure. An argument similar to that used by Audet and Wood (1988) may be used for the trout with prior exposure to pH 5.2. These trout exhibited significantly lower Na^+ uptake rates in the control experiment than the other two treatments. Although ammonia production rates were not chronically elevated, as they were in the study by Audet and Wood (1988), these trout may, nonetheless, have been relying on NH_3 diffusion to a greater extent as a result of an improved diffusion gradient at pH 5.2. The subsequent inhibition of Na^+ uptake after exposure to pH 4.2 may have made little difference to the overall efflux of ammonia if diffusional loss of NH_3 was the major contributer to the overall rate of ammonia excretion.

Prior exposure of juvenile rainbow trout to increased temperature did not impart any obvious increased or decreased ability of trout to combat ionoregulatory disturbance in the face of challenge levels of low pH.

Overall, the present data suggest that trout with prior long-term exposure to sublethal low pH (5.2) do not suffer any greater deleterious impact of exposure to a challenge level of low pH, namely pH 4.2, than do naive trout, without prior exposure to low pH. In fact, there is some suggestion of an improved ability to recover from the

ionoregulatory disturbance caused by pH 4.2. The difference in the present findings relative to those of Audet et al. (1988) and Audet and Wood (1988) may be due to the less severe, more environmentally realistic levels of pH used in the present chronic exposures (pH 5.2 vs. 4.8) and acute challenge (pH 4.2 vs. 4.0), as well as the fact that the present trout were monitored over a longer challenge period (24 h vs. 5 h). Our previous experiments indicated no ionoregulatory disturbance in these low pH exposed trout (Chapters 2 & 3). We suggested that the diet was supplying a sufficient replacement salt load for ions lost across the gills. The present data suggest that additional physiological adaptation may have taken place in the gills as well, to aid ion transport. Future studies should investigate the differences between responses of fed and unfed trout chronically exposed to sublethal low pH (5.2), to challenge concentrations of H^+.