RESPONSE OF RAINBOW TROUT (*Oncorhynchus mykiss*) TO SIMULATED CLIMATE WARMING AND SUBLETHAL AMMONIA

By

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ABSTRACT

The earth's temperature is predicted to increase roughly 1 - 5°C over the next 50 years, against a backdrop of increasing environmental pollution. This is expected to have a profound effect on fish, organisms whose life processes are intimately linked to their environmental temperature. The present investigation quantified, in the laboratory, the basic bioenergetic, physiological, and toxicological responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to a naturally fluctuating water temperature cycle (characteristic of inshore Lake Ontario), and to this cycle + 2°C (simulated warming scenario) in the presence or absence of 70 μM total ammonia (a common environmental pollutant).

This thesis provides concrete evidence that small temperature increase and low-level environmental pollution substantially alter the growth, feeding, physiology, and metabolism of juvenile rainbow trout, especially at the temperature extremes: positively during minimum winter temperatures (≈ 42% increase in growth rate and energy conversion efficiency), and negatively during summer maximum temperatures (≈ 38% decrease in growth rate and 11% decrease in conversion efficiency). Their responses to sublethal ammonia can be quite different based on seasonal temperature, but within the optimal temperature for growth (16 - 20°C), +70 μM ammonia appears to stimulate nitrogen retention and metabolism. Significant decreases during late summer in the activity of some of the enzymes involved in nitrogen metabolism indicated that protein systems of juvenile trout are potentially sensitive to the combination of chronic temperature increase in combination with sublethal ammonia pollution. An important factor influencing the responses of trout to a warmer, more polluted environment is nutritional status (i.e., 5 - 7 fold increase in oxygen consumption per unit protein growth). It is apparent from this

investigation that juvenile trout in a better nutritional state will exhibit at least some adaptation to chronic temperature increase. However, this adaptation, either alone or in the face of sublethal ammonia pollution, may require a significant increase in the overall cost of living (10 - 17%).

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Well, this is it. This is where I am supposed to acknowledge all those people who have made significant contributions to my graduate work and life. Good grief, how can I possibly expect to do any justice to them in just a few short paragraphs? I will do my best.

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God bless Canada and keep her cool, clean, and wild!

THESIS ORGANIZATION AND FORMAT

This thesis is presented in the "open-faced" format approved by McMaster University upon the recommendation of my supervisory committee. It is comprised of a total of six chapters. The first chapter provides a general introduction together with the specific objectives and findings of the studies. The remaining chapters (2-6) are the separate manuscripts that have been published, accepted for publication, or submitted for publication in scientific journals.

Chapter 1: General introduction, objectives of the investigations, and summary of major findings.

Chapter 2: The metabolic costs and physiological consequences to juvenile rainbow trout of a simulated summer warming scenario in the presence and absence of sublethal ammonia.

Authors: T.K. Linton, S.D. Reid, and C.M. Wood.

Date accepted: July 1996.

Journal: *Transactions of the American Fisheries Society* 126: 259-272 (1997).

Comments: This study was conducted by T.K.L. under the supervision of C.M.W. Considerable logistical support was provided by S.D.R.

Chapter 3: The metabolic costs and physiological consequences to juvenile rainbow trout of a simulated winter warming scenario in the presence and absence of sublethal ammonia.

Authors: T.K. Linton, S.D. Reid, and C.M. Wood.

Date submitted: April 1997.

Journal: *Transactions of the American Fisheries Society*

Comments: This study was the sequel to Chapter 2, and was again performed by T.K.L. with support and direction provided by S.D.R. and C.M.W. respectively.

Chapter 4: Effects of restricted ration on the physiology, growth, and energetics of juvenile rainbow trout exposed to a summer of simulated warming and sublethal ammonia.

Authors: T.K. Linton, S.D. Reid, and C.M. Wood.

Date submitted: May 1997.

Journal: *Environmental Biology of Fishes*

Comments: The work was accomplished by T.K.L. with assistance provided by S.D.R. and supervision by C.M.W.

Chapter 5: Long-term exposure to small temperature increase and sublethal ammonia in hardwater acclimated rainbow trout: does acclimation occur?

Authors: T.K. Linton, I.J. Morgan, S.D. Reid, and C.M. Wood.

Date accepted: June 1997.

Journal: *Aquatic Toxicology*

Comments: This study was conducted by T.K.L. with logistical assistance provided by I.J.M. and S.D.R. regarding sodium flux measurements and analysis. Supervision was provided by C.M.W.

Chapter 6: Chronic exposure of rainbow trout to simulated warming and sublethal ammonia: a year-long study of their appetite, growth, and metabolism.

Authors: T.K. Linton, I.J. Morgan, P.J. Walsh, and C.M. Wood.

Date submitted: May 1997.

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Comments: The results of this study have just recently been submitted for publication in CJFAS. It was conducted by T.K.L. under the supervision of C.M.W. Assistance was provided by I.J.M., and instruction and advice on nitrogen enzyme methodology was provided by P.J.W.

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CHAPTER 1

SCOPE, OVERVIEW, AND IMPLICATIONS OF THE STUDY

"Our knowledge of fisheries can no better be tested than by meeting the challenge of predicting how fish populations are likely to change with warming of the earth's atmosphere."

Beverton - 1989

I. BACKGROUND

Temperature is one of the most important environmental factors affecting animal activity and nowhere is its influence more pervasive than in aquatic ectotherms (e.g. fish). Tissue temperatures of most fish are at, or within, 1°C of the ambient water temperature (Hazel 1993). This is due to the rapid transfer of heat from their bodies to a surrounding medium of high thermal conductance and heat capacity (Crawshaw 1979). As a consequence, their physiological rate processes, activity levels, and even geographic distributions are inextricably linked to their ability to adapt to diel and seasonal fluctuations in environmental temperature.

Natural temperature variation, as influenced by factors such as solar radiation, wind convection, shading, etc., can be large in surface waters (Wetzel 1983). Wide fluctuations in surface water temperature are especially pronounced in northern temperate climates (Houston and Gingras-Bedard 1994). It is not uncommon for trout streams and littoral areas of lakes to vary seasonally by >14°C (Crisp and Le Cren 1970, Blumberg and DiToro 1990) and diurnally, by as much as 12°C (Needham and Jones 1959). Thus, in their natural setting, fish are never really acclimated to one particular temperature, but to a dynamic range of temperatures. Anthropogenic activities, such as thermal plumes from industrial discharge, water diversion for crop irrigation, and removal of riparian vegetation from forest clear cutting (Talmage and Coutant 1978, Sprague 1985, Thomas et al. 1986),

add to the natural variation in water temperature, thereby affecting the physiological and behavioral responses of fish to their immediate environment. Because the temperature of the environment plays such a critical role in the life processes of fish, its physiological impacts have been extensively studied for the past several decades (see Fry and Hart 1948, Fry 1967, 1971, Brett 1952, 1971, Elliott 1981, 1982, Hazel 1993). A resurgence of study in this area is currently underway due to growing concerns about global warming (Wood and McDonald 1997).

The principle tenet of global warming is that the recent acceleration in the rates of release of carbon dioxide, chlorofluorocarbons, methane, and nitrous oxide into the atmosphere will warm the earth's temperature through an increase in the greenhouse effect (Hengeveld 1990). Models predict that a doubling of the atmospheric CO_2 concentrations will increase the earth's average temperature anywhere from 1.0 to 5.0°C in the next several decades (Hansen et al. 1988, Mohnen and Wang 1992). The implications of this 'predicted warming' are that such increases in ambient air temperature will not only exert a direct effect on the biological structure of terrestrial and aquatic ecosystems (Dawson 1992), but may indirectly alter geophysical processes, i.e. change wind patterns and intensity, evaporation rates, stream flows, water levels of lakes, etc. (Coutant 1981, Cohen 1986). Empirical evidence suggests a strong relationship between the temperature of air and the temperature of streams and surface waters of lakes (Crisp and Howson 1982, Shuter et al. 1983). Indeed, the pioneering work of Schindler and co-authors (1990) has already indicated that air and lake temperatures of northwestern Ontario increased by 2°C over the last 30 years. As a result of the increase in ambient temperature, the ice-free season increased by 3 weeks and the ratio of total dissolved nitrogen to total dissolved phosphorus (N:P) doubled, via increased evapotranspiration and decreased water renewal,

from 25:1 to 50:1. This process can be expected to increase the concentration of many pollutants in the environment (Coutant 1981, Vitousek 1994).

It is now generally accepted that the earth's temperature regime is changing, and on a geologically rapid time scale. This change in ambient air temperature is also occurring against a backdrop of increasing environmental pollution (Kennedy and Walsh 1997). Despite the enormous body of scientific literature linking temperature and fish physiology, large uncertainties still exist about the extent to which physiological and biochemical processes in fish may be affected by a warmer and more polluted environment (DeAngelis and Cushman 1990; Lin and Regier 1995), in part, because of our inability to adequately assess sublethal responses under the appropriate conditions (see Reid et al. 1997). Data from field studies that incorporate annual and seasonal temperature variation (e.g., Wrenn 1980, 1984), or from laboratory studies in which fish are exposed to a simulation of the natural regime, but where other influential parameters can be controlled (Wurtsbaugh and Davis 1977), are particularly useful. However, more data using these types of approaches are urgently required (Jobling 1997, McCarthy and Houlihan 1997).

II. OVERVIEW OF THESIS

This thesis employs a 'controlled laboratory approach' to examine the biochemical, physiological, and toxicological responses of a reference cold-water fish species, rainbow trout (*Oncorhynchus mykiss*), to: i) the natural fluctuating water temperature cycle representative of the inshore region of Lake Ontario, ii) this cycle +2°C to simulate a global warming scenario, and iii) these cycles in the presence of an environmentally realistic level of a pollutant (+70 μM total ammonia). It is comprised of 5 papers from four long-term experiments. In all but one experiment (Chapter 4), the fish were hand-fed to satiation each day following a strict protocol so as to accurately monitor appetite. Appetite proved to

be an extremely important factor influencing the observed responses. There were two specific objectives in this thesis. The first was to characterize and quantify key physiological responses of trout to the warmer more polluted scenario, and the second was to determine if these responses increased the metabolic costs of living.

The first two papers, chapters 2 and 3 respectively, contain results from two 90-d exposures (see treatments above) conducted on juvenile trout fed to satiation over summer and winter respectively. These experiments showed, for the first time (see also Reid et al. 1995), that an increase in water temperature of only +2°C markedly affects the appetite, growth, and energetics of juvenile trout (negatively during maximum summer temperatures, positively during minimum winter temperatures). These experiments also showed for the first time that environmentally realistic levels of sublethal ammonia actually stimulate nitrogen metabolism and retention in juvenile trout, resulting in a beneficial effect on growth.

Because of the dramatic negative effects exhibited by fish exposed to the additional +2°C in summer without food limitation, a parallel experiment was conducted the following summer, but this time, imposing a restricted ration (chapter 4). The expectations were that food limitation would exacerbate the negative effects observed at peak summer water temperatures, and possibly impede the stimulatory effect of ammonia on nitrogen retention. Surprisingly, restricting the ration, which had the effect of markedly increasing the cost of living for all treatments of fish, did not further impair the ability of juvenile trout to cope with a small chronic temperature increase relative to the base temperature controls. Moreover, the sublethal ammonia appeared beneficial under these circumstances, even at +2°C. Thus, nutritional status appeared to play a major role in the response of fish to the warmer more polluted scenario.

The influences of prior exposure and nutritional history were examined in greater detail in chapter 5. Lethal challenges were conducted after each 90-d experiment to determine whether or not chronic pre-exposure to additional temperature and ammonia conferred any increased tolerance to elevated temperature or ammonia. In addition, acute sublethal ammonia challenges, together with undirectional Na^+ flux measurements, were conducted after the two summer exposures to gain further insight into the effects of prior sublethal ammonia exposure on Na^+ regulation, as influenced by ration. Trout fed unlimited ration and chronically exposed to +2°C over summer did indeed exhibit signs of temperature acclimation. However, those trout previously exposed to sublethal ammonia did not exhibit ammonia acclimation *per se*. It was clear from this work that previous exposure history does play a significant role in the ability of trout to tolerate acute temperature increase. The level of tolerance seemed to be directly correlated with the energetic costs required to achieve the acclimated state, which in this regard, is tied very closely with the nutritional status of the animal.

The last paper, chapter 6, was designed to integrate and confirm the findings of the previous chapters by exposing trout to the warmer, more polluted scenario for more than an entire year. During this period the trout grew from approximately 10 g to approximately 350 g. The results of this study successfully captured the 'dual influence' of small temperature increase on feeding, growth, and metabolism of juvenile trout. Trout exposed to +2°C exhibited the potential to secure great gains in growth from the additional +2°C during the majority of the year, but lost these gains during the upper critical temperature periods in late summer. Their physiological responses to sublethal ammonia were quite different at seasonal temperature extremes. Perhaps most importantly, the data indicated that small temperature increase, together with low level ammonia pollution, substantially altered the protein dynamics in juvenile freshwater fish at high temperature. The cost of

living, expressed as oxygen consumption per unit protein growth, for juvenile rainbow trout coping with a warmer more polluted environment was estimated to have increased 17%.

The implications of this work revolve around 3 main findings: 1) a warming scenario of a mere +2°C presents a genuine problem for freshwater rainbow trout (i.e., coldwater fish) during late summer. Until now, no one has been able to confirm this with hard experimental evidence; 2) 70 μM ammonia, which is a concentration slightly below the water quality criterion set to protect freshwater life, stimulates nitrogen metabolism and retention. This has important implications for environmental legislators, toxicologists, and aquaculturists alike and warrants much further attention, especially in the context of maximum 'safe' limits for fish; 3) nutritional status, as a function of feeding level (ration), has great influence on the cost of living, and hence tolerance of freshwater fish to a warmer more polluted environment. This factor, largely disregarded in many biochemical, physiological, and toxicological studies, can no longer be ignored in the study of cause/effect relationships.

III. KEY ISSUES AND INFLUENCES RELEVANT TO THIS STUDY

A. Temperature as an Environmental Stimulus

1) Thermal Relations

An increase in water temperature has a marked effect on the metabolic rate processes of fish, usually causing a 2-3 fold increase in metabolic rate for every 10°C rise in temperature (Sprague 1985, Hazel 1993). This generality, derived from empirical observations, complies with the van't Hoff rule. The van't Hoff rule describes the relationship between the rates of biological reactions measured at two different body temperatures, usually 10°C apart. The factorial increase (or decrease) in rate over this

increment in temperature is defined as the temperature coefficient or Q_{10}, and is expressed as:

$$Q_{10} = (R_2/R_1)^{10/(t_2-t_1)}$$

where R_2 is the rate constant at any temperature t_2 (measured in °C) and R_1 is the rate constant at any lower temperature t_1 (°C) (Hill and Wyse 1989). The magnitude of Q_{10} reflects the thermal sensitivity of the biotic process (i.e., metabolic rate). Although there are many problems associated with the use of mathematical expressions to explain temperature related phenomena in fish, e.g. the analysis assumes that the only parameter changing in the system is temperature - an often erroneous assumption (Ricker 1979, Regier et al.1990, Clarke 1987), the van't Hoff rule is useful for its descriptive capabilities, and therefore, is used for such purposes in this thesis.

One of the main strengths of the experimental approach employed in this thesis is that it allowed detection of large changes in thermal sensitivity at such a small degree of temperature separation. This is an observation which other studies have failed to achieve, and was likely successful in the present study because of the chronic nature of the exposures. The stimulatory effect of a +2°C elevation in temperature on the feeding, growth, and metabolism of trout at low temperature (noted by a very high Q_{10}), versus the opposite effect at high temperature (reduced sensitivity or very low Q_{10}), are central to the investigation of environmental warming. These effects lie at the extremes of the thermal range for coldwater fish, which are clearly the most vulnerable to temperature change (see Wood and McDonald 1997).

2) Acute Temperature Interactions

Due to the wide fluctuations of temperature in natural surface waters, fish have developed elaborate compensatory mechanisms that allow constant activity (i.e., homeokinesis) over a considerable range of environmental temperatures (Prosser and Nelson 1981). Within this range, fish are capable of temperature acclimation. Beyond certain thresholds in body temperature however, regulation of constant activity fails, and survival is dependent on more rapid, short-term responses that permit the extension of tolerable limits (i.e., heat-hardening, see Maness and Hutchinson 1980). These responses include phenotypic changes such as enzyme concentration, membrane fluidity, and the production of heat shock proteins, etc. (Fields et al.1993, Hazel 1993). Phenotypic adaptation is expected to play a major role in the tolerance of fish during climate change (Somero and Hofman 1997), but to date, this has not been easily tested. An approach such as the one used in this thesis could prove very useful.

Thermal tolerance alone is one of the defining characteristics of a fish's geographical boundaries (Brett 1952, Fry 1971, Murawski 1993). Therefore, an assessment of the physiology of fish at temperature extremes is imperative for comparing temperature effects between species (Elliott 1991). Moreover, knowledge of a fish's tolerable temperature range is also important for providing a reference for the comparison of the effects of sublethal temperature exposures (Beitinger and McCauley 1990).

There are two primary experimental methods employed to determine thermal tolerance in fish: 1) fish are held at constant acclimation temperature and then abruptly transferred to a higher constant temperature - a method used to determine the upper and lower incipient lethal temperature, or the temperature at which fish cannot survive indefinitely (in Fry 1971, Elliott 1981, 1991), and 2) fish acclimated at constant temperature are heated at a constant rate (usually 1°C/min) to their 'critical thermal

maximum,' or the temperature at which the fish loses the ability to escape from lethal conditions (Cowles and Bogert 1944, Cox 1974, Beitinger and McCauley 1990). In this thesis (Chapter 5), a version of this latter method was employed because it permits the quantitative measurement of the capacity of trout to tolerate lethal temperature, i.e., the extent of phenotypic adjustment made during prior chronic exposure (see above).

3) Chronic Temperature Interactions

Within the zone of tolerance (constant activity), temperature change directly affects the life of fish at all levels. The feeding, growth, and metabolic rates of fish are proportional to, and actually set by, the temperature of their surroundings (Fry and Hart 1948, Fry 1967, 1971, Elliott 1976, Brett 1979, Brett and Groves 1979). Since most fish are incapable of effectively regulating their body temperatures by circulatory-metabolic means (i.e., fish are thermal conformers), they must thermoregulate behaviorally or alter many aspects of their physiology and biochemistry to compensate for a change in temperature. Adjustment requires the coordinated activity of the whole animal in selecting or creating a microenvironment where optimal body temperature may be achieved (see Crawshaw and O'Connor 1997). Consequently, many fish are largely dependent on behavioral control over their body temperature (Jobling 1997). The design of the present study prevented behavioral thermoregulation by the fish, thereby allowing assessment of the physiological responses alone of fish to chronic temperature change.

Under controlled laboratory conditions, there are four main responses of fish that increase in magnitude continuously as acclimation temperature is increased up to a maximum: gastric evacuation, energy losses of fish when deprived of food, standard metabolic rate, and maintenance energy intake (Brett 1971, Elliott 1976, Fange and Grove 1979). There are also a number of responses that increase with temperature up to a

maximum near optimum temperature, then subsequently decrease with further temperature elevation. These include: appetite (to be discussed in depth in a separate section below), growth, active metabolic rate, maximum energy intake, and energy lost in feces and excretory products (Brett 1971, Brocksen and Bugge 1974, Elliott 1976).

The optimum temperatures for feeding and growth of salmonids in particular, have received considerable attention because of their importance in aquaculture (see Cho 1990). Optimum temperature ranges for these processes differ between life stages (Rombough 1997); juveniles are generally more tolerant of higher temperature than adults (Coutant 1977). The thermal optimum for growth and food utilization in adult rainbow trout is ordinarily around 15°C (Cho and Kaushik 1990). Recent evidence suggests that the optimum temperature for growth is slightly lower than that of feeding and slightly higher than that of conversion efficiency (see Jobling 1997), and coincides with the preferred temperatures selected by fish in behavioral studies (Jobling 1981, Kellogg and Gift 1983, also see Crawshaw and O'Connor 1997). Of the three major components of metabolism - swimming, maintenance, and growth - that make the greatest demands on net energy derived from foodstuffs, growth has the lowest immediate priority, but eventually dictates the long-term survival of fish (Brett and Groves 1979).

B. Ammonia as an Environmental Stimulus

1) Environmental Ammonia

Ammonia is considered one of the most important pollutants in the aquatic environment because of its highly toxic nature and ubiquity in surface water systems (Russo 1985). As a result, the toxicity of ammonia to a variety of fish has been extensively studied over the past quarter century (Wuhrmann and Woker 1948, Fromm and Gillette 1968, Smart 1976, Arillo et al. 1981, Thurston et al. 1984, Beamish and Tandler

1990, Twitchen and Eddy 1994). Ammonia enters aquatic environments naturally via direct means such as nitrogen fixation, and indirect means such as the excretion of nitrogenous wastes from the animal. Previous studies have shown that elevated water levels of ammonia may induce severe physiological problems in a number of freshwater fish species (Tomasso et al. 1980, Thurston and Russo 1983, Broderius et al. 1985, Miller et al. 1990, Knoph 1992, Wajsbrot et al. 1993). While much of the information regarding lethal concentrations of ammonia seems to have arisen from the processes of ammonia buildup in aquaculture (i.e. fish culture ponds, hatchery raceways, and fish holding and transporting tanks), the introduction of large amounts of ammonia into surface water systems from industrial processes, agricultural run-off, and sewage effluents has also received considerable attention (Alabaster and Lloyd 1980, EPA 1985).

The chemical form of ammonia in water consists of two species, a larger component which is the ammonium ion (NH_4^+) and a smaller component which is the non-dissociated or un-ionized ammonia (NH_3) molecule; their ratio in a given aqueous solution is dependent upon both pH and temperature (Emerson et al. 1975, Erickson 1985, Thurston 1988, Wood 1993). In general, the ratio of un-ionized ammonia to ammonium ion increases by 10-fold for each rise of a single pH unit and by approximately 2-fold for each 10°C rise in temperature over the 0-30°C range (Erickson 1985).

Chemically, ammonia in an aqueous medium behaves as a moderately strong base with pK values ranging from approximately 9 to slightly above 10 as a function of temperature and ionic strength (Emerson et al. 1975). Therefore, the total ammonia in solution will consist of the gas NH_3 and the cation NH_4^+, the sum of which is usually expressed as total ammonia-nitrogen (T_{Amm}), the expression most commonly employed (Thurston et al. 1984). Each separate fraction of T_{Amm} can be calculated in freshwater

(note: this relationship changes with salinity, see Armstrong et al. 1978) from the Henderson-Hasselbach equation if the pH and appropriate pK are known:

$$NH_4^+ = \frac{T_{Amm}}{1 + \text{antilog}\,(pH-pK)} \qquad \text{(Wood 1993)}$$

and,

$$pK = 0.09018 + \frac{2729.92}{(273.2 + T)} \qquad \text{(Emerson et al. 1975)}$$

where T is temperature in °C. In order to avoid confusion about which form of ammonia is being measured, some researchers have begun to report ammonia toxicity values in terms of the respective T_{Amm} components, the un-ionized form of which is expressed as NH_3 (molecular weight=17) and the ionized form as NH_4^+ (molecular weight=18). The concentration of sublethal ammonia examined in this thesis is reported on the basis of T_{Amm}, which is both more practical and more relevant to the real world, where heating of natural water leaves T_{Amm} unchanged, but alters the NH_3/NH_4^+ distribution ratio. However, in view of the fact that early evidence indicated that the un-ionized form NH_3 was the more toxic species, and the resultant situation that legislated water quality criteria are still expressed in terms of the NH_3 fraction only (see below), the latter fraction has also been reported where appropriate.

2) Acute Influences

Early investigations of the toxicity of ammonia to aquatic organisms indicated that the toxicity of total ammonia increases with increasing pH (Wuhrmann and Woker 1948, Downing and Merkens 1955), which would increase the relative amount of NH_3. Since the uncharged NH_3 molecule penetrates tissue membranes much more readily than the larger, charged, NH_4^+ molecules, toxicity to aquatic life was initially thought to arise

largely from the free base NH_3 (EIFAC 1973, Alabaster and Lloyd 1980, EPA 1985). However, Armstrong et al. (1978), and Tomasso et al. (1980) among others, have shown that ammonia is more toxic as the hydrogen ion concentration $[H^+]$ increases (pH decreases), at least below a pH of 7.3. In this case, more ammonia in the form of NH_4^+ would be present. This suggests that at low pH, NH_4^+ may also be toxic. This hypothesis was further substantiated by Thurston et al. (1981a), who have shown that over the pH range of 7.8 - 9.0, the acutely toxic effects of NH_3 on rainbow trout appear to be relatively consistent, but below this range of pH, apparent NH_3 toxicity increases markedly. This may in fact be due to the associated higher level of NH_4^+ present simultaneously.

The temperature-dependent toxicity of ammonia to freshwater fish was also initially reported to increase with elevations of temperature when expressed on the basis of NH_3 alone (Wuhrmann and Woker 1953). However, later investigations have consistently proven otherwise; an increase in LC_{50} value (concentration of NH_3 lethal to 50% of the organisms) occurs with increasing temperature, reflective of a decrease rather than an increase in toxicity (Colt and Tchobanoglous 1976, Thurston and Russo 1983, Knoph 1992). The obvious uncertainties associated with which particular component of T_{Amm} contributes most to ammonia toxicity still exist (see review by Meade 1985), and yet, both European and United States water-quality criteria are stated in terms of the NH_3 fraction only.

Despite the considerable efforts expended to elucidate the pathophysiological and biochemical mechanisms of ammonia toxicity to fish, the actual toxic effects associated with a particular concentration of ambient ammonia in water under a prescribed set of conditions remain unclear. The acute toxicity of ammonia is thought to be primarily neurological in origin resulting from severe metabolic alterations of the central nervous

system (Smart 1978, Levi et al. 1974), as evidenced by the hyperexcitability, coma, convulsions, and hyperventilation observed in fish acutely exposed to ammonia, $T_{Amm} \approx$ 1300 µmol/l, NH_3 = 35 µmol/l or 0.6 mg/l (Smart 1978). The general consensus is that acute ammonia intoxication decreases intermediary carbohydrate metabolism, critically supportive of ATP production (Buckley et al. 1979), which results in depletion of tissue energy stores, especially in the brain. It has recently been suggested that the depletion of energy stores in fish brain is linked with ammonia detoxification via glutamine synthetase (Korsgaard et al. 1995). Lloyd and Orr (1969), however, noted an increase in the rate of urine excretion by rainbow trout with an increase in the concentration of ambient un-ionized ammonia over the range of 5.29 to 26.5 µmol/l NH_3 (0.09 to 0.45 mg/l, T_{Amm} = 280 - 1400 µmol/l). They suggested that the diuresis was caused by an increase in the permeability of the fish gill to water, which implies an acute effect of ammonia on water uptake and turnover. Another possibility is that NH_3 converted to NH_4^+ can displace potassium (K^+) on the cell membrane (Binstock and Lecar 1969, Hille 1973). Theoretically, competition between NH_4^+ and K^+ at the K^+ channels and Na^+/K^+ ATPases in neurons could account for the toxic neural effects observed in fish and mammals, and therefore, explain the origin of the convulsions associated with acute ammonia toxicity. This mechanism of acute ammonia toxicity needs further study, especially in relation to the acute effects exhibited by fish at extreme temperature (see Chapter 5).

3) Chronic Influences

Unlike the vast majority of environmental pollutants, the effects of prolonged exposure of fish to ammonia are fairly well documented. However, they are not well understood at a mechanistic level. The list of sublethal effects is quite extensive, such as:

reduced food uptake and growth inhibition (Larmoyeux and Piper 1973, Robinette 1976, Rice and Bailey 1980), diuresis and ion imbalance (Lloyd and Orr 1969, Twitchen and Eddy 1994), inflammation and degeneration of the gills and other tissues (i.e. kidney) (Flis 1963, Smart 1976, and Thurston et al. 1984), changes in oxgyen-carrying capacity of the blood (Sousa and Meade 1977, Buckley et al. 1979, Knoph and Thorud 1996), and increased susceptibility to disease (Smart 1976). The European Inland Fisheries Advisory Commission (EIFAC 1973) recommended an ammonia criterion of 1.47 μmol/l NH_3 (0.025 mg/l) at temperatures above 5 °C and pH's below 8.5. The United States Environmental Protection Agency (EPA 1985) had recommended a similar criterion of 1.17 μmol/l NH_3 (0.020 mg/l). These values are approximately 4 times lower than reported median lethal concentrations of NH_3 from 96-h LC_{50} tests which range from 4.88 to 64.7 μmol/l NH_3 (0.083 to 1.1 mg/l, respectively) for salmonids, and approximately 5.5 times lower than the values reported for non-salmonids, 8.2 to 270.6 μmol/l NH_3 (0.14 to 4.6 mg/l, respectively) (Thurston et al. 1984, Russo 1985).

There is still some question as to whether these criteria are truly representative of the maximum acceptable concentration of NH_3, or T_{Amm}, for fish (see Meade 1985). The recommended 'safe' concentrations of NH_3, primarily derived from observed gill damage and growth reduction in hatchery fish (e.g., see Burrows 1964, and Smith and Piper 1975), have been contradicted in a number of studies (Smart 1981, Mitchell and Cech 1983, Dauost and Ferguson 1984, Thurston et al. 1984). Of particular interest here is the apparent reduction of NH_3 toxicity and prevention of gill damage in fish exposed in freshwater with higher ionic (e.g. Na^+) compositions (Tomasso et al. 1980, Bradley and Rourke 1985, Ruffier et al. 1981). In addition to passive diffusion of ammonia (see review by Wilkie 1997), ammonia excretion is associated with the active exchange of Na^+ for NH_4^+ (Maetz and Garcia-Romeu 1964). This mechanism could contribute to a

reduction in the accumulation of ammonia in the blood (see below). Based on the results of this thesis, the current water quality criterion are probably 'over-protective' and a concerted effort should be made to report toxicity on the basis of T_{Amm}.

The adverse effects of an elevation of ambient ammonia in general are thought to stem primarily from an increase in plasma NH_3 to toxic levels (Hillaby and Randall 1979, Cameron and Heisler 1983, Claiborne and Evans 1988) following the inhibition or reversal of ammonia excretion (Wilson and Taylor 1992, Wilson et al. 1994a). Fromm and Gillette (1968) conducted a classic study aimed at determining the effects of increased ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout. Blood NH_3 increased linearly with elevations in ambient levels of ammonia in water. Similarly, as the water NH_3 levels were increased from 0 - 2.8 µmol/l NH_3 (0.05 mg/l), values for total nitrogen excretion decreased. At the highest ambient ammonia level (2.8 µmol/l NH_3), the total nitrogen excreted as ammonia was greatly reduced, but total nitrogenous waste excretion remained the same. This suggests excretion of some other form of nitrogenous waste (i.e. amino acids, urea) which may ultimately play an important role in the ammonia acclimation responses previously observed (Vamos 1963, Lloyd and Orr 1969, Redner and Stickney 1979, Thurston et al. 1981b). This compensation, or acclimation, could contribute to an increase in energetic costs. In this thesis, urea excretion has been measured in addition to ammonia excretion, and in chapter 6, total nitrogen excretion, to provide a more accurate assessment of the N-budget of the fish.

C. Ration as an Environmental Stimulus

1) Ration and Environment

In nature, water temperature, day length, prey availability, fish activity, intra- and inter-species competition, water quality, etc., are among the many variables that affect the

amount of food consumed by fish (Windell 1978, Mann 1978). In the lab however, the freedom of the fish to fulfill the need to eat (appetite) is also affected by the duration of feeding, individual meal size, feeding time, and quality of feed (Brett 1979, Cho 1990), as well as social interactions (Jobling 1993). Taken together, a fish's appetite is a function of its maintenance energy requirements, of which protein synthesis accounts for 20 - 40% (Houlihan et al. 1993), plus an additional demand dictated by the fish's capacity for growth (Brett 1979). For instance, since young fish have very high growth requirements (Brett and Groves 1979), their maximum food intake can be expected to be greater as a percentage of body weight. On the other hand, it has become increasingly evident that certain environmental conditions (i.e., extremes in water temperature, pollution) have a dramatic effect on the appetite of fish (Wilson et al. 1994b, Dockray et al. 1996, D'Cruz 1997, Jobling 1997), which implies that ration could be governed by some other intrinsic need other than growth (i.e. metabolic regulation, NaCl balance).

2) Ration and Temperature

Ration and water temperature are intimately linked. As water temperature rises, maximum ration increases (Brett et al. 1969, Elliott 1976, Brett 1979). This occurs in parallel with increases in maintenance energy requirements (Brett 1979, Elliott 1982). The increased metabolic demand of temperature and ration has important ramifications on the respiratory and circulatory systems in fish. Oxygen consumption is known to increase up to several fold between fish that have been starved versus those fed to satiation (Soofiani and Hawkins 1982, Jobling 1981, Alsop and Wood 1997). At low temperature, an increase in ration is much less of a problem than at very high temperature, i.e., the oxygen content of the water is generally high and the maintenance requirements of the animal are low (Brett and Groves 1979). However, as water temperature increases the maintenance

requirements increase as well as the activity and appetite of the fish (Elliott 1982). Above a certain temperature threshold (which varies among species and probably within species according to size), the ability of the fish to take up and circulate oxygen becomes limited and cannot meet the energy demands of the tissues (Alsop and Wood 1997). As a consequence, appetite, and hence maximum ration, decreases in order for self-preservation. Indeed, it is a well known fact in aquaculture that already stressed fish should not be fed as this will invariably result in mortality.

3) Ration and Toxicants

The effects of nutritional status on the response of fish to pollutants has been largely disregarded in toxicology studies (Sprague 1985, Lanno et al. 1989). This is paradoxical given the marked effects that both quantity and quality of the diet has on the physiology and metabolism of fish (see above and also Cowey and Sargent 1979). Ration, in particular, influences the metabolism of fish (see above) which changes the dynamics of chemical uptake, metabolism, and depuration (Jiminez et al. 1987). Ration also changes the proximate composition of the fish (Brett et al. 1969, Jobling 1980, Cho and Kaushik 1990). For example, when more protein is available than can be stored from exogenous sources, excess amino acids in the body fluids are degraded and used for energy or are stored as fat (Brett and Groves 1979, Wood 1993). A higher lipid content is conducive to the bioaccumulation of lipophilic substances (Lech and Vodicnick 1985). In this context, feeding regime is extremely important.

Unlike acute toxicity studies during which fish are usually not fed, nearly all chronic toxicity testing protocols provide guidelines for some sort of feeding regime. Unfortunately, these feeding regimes may vary widely, and depending on the toxicant, may alter substantially the magnitude of responses observed (Sprague 1985, Lanno et al.

1989). This, of course, is a major concern for any chronic toxicity study. The sublethal endpoints of chronic studies (physiology, growth, energetics) are highly influenced by the nutritional status of the animal. Ammonia provides an excellent example of how the toxicity of a common pollutant could be dramatically altered by ration.

Fish exhibit much higher rates of nitrogen ingestion, turnover, and excretion than most other animals (Wood 1993). The amount excreted under steady-state conditions is heavily influenced by the rate of dietary protein uptake (Ramnarine et al. 1987). For example, actively growing fish fed a balanced diet excrete about 5 - 8% of the total nitrogen ingested as ammonia (Atherton and Aitken 1970, Soderberg 1995). It is generally assumed that the amount of net ammonia produced is equal to the amount of ammonia excreted (Wood 1993). Ammonia accounts for most (≈ 80%) of the nitrogenous waste excreted by teleost fish (Wood 1993), and is transported from the major production site (liver) to the excretion site (gills) by the blood (Forester and Goldstein 1969, Randall and Wright 1987). The combination of an increased ration with an effective environmental ammonia blockade (see above), could greatly increase the internal ammonia load in the blood. If levels of plasma T_{Amm} reach acutely toxic levels, considerable energy must be expended in the detoxification of ammonia via glutamine and possibly protein synthesis and/or urea (Korsgaard et al. 1995). On the other hand, plasma T_{Amm} levels are also raised in fish during starvation via the degradation and mobilization of structural protein (Hillaby and Randall 1979). The dual role of ammonia as both pollutant and nutrient warrants our investigation into the role of ration (appetite) in studies of ammonia toxicity.

D. Living in a Warmer, More Polluted World

At the present rate of industrial production and human population increase there is no reason to suspect an immediate decline in greenhouse gas emissions or environmental

pollution, even with 'tougher' environmental legislation. Superimposed on top of this is an extraordinarily high rate of natural habitat destruction and water use (Cairns and Lackey 1992). These unnatural disturbances are expected to increase the energetic costs of living for terrestrial and aquatic biota (Parsons 1990), and consequently, their requirements for energy and other materials such as protein and oxygen (Dawson 1992). Thus, energy flow should be affected at all levels of biological organization. It is beyond the scope of this thesis to address all the complex relationships between chronic temperature change and fish physiology and ecology, but its unique approach may provide valuable insight into the likely impacts as well as some of the mechanisms responsible for them.

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CHAPTER 2

THE METABOLIC COSTS AND PHYSIOLOGICAL CONSEQUENCES TO JUVENILE RAINBOW TROUT OF A SIMULATED SUMMER WARMING SCENARIO IN THE PRESENCE AND ABSENCE OF SUBLETHAL AMMONIA

ABSTRACT

Quantitative bioenergetic and physiological measurements were made on juvenile rainbow trout *Oncorhynchus mykiss* exposed over summer (June to September 1993) to a simulated summer warming scenario of +2°C in the presence and absence of 70 µmol/L total ammonia (nominal; equivalent to 0.013 mg/L NH_3-N, at 15°C, pH = 7.6) to determine the metabolic costs and physiological consequences associated with their growth in a warmer, more polluted environment. With unlimited food, fish exposed to an additional +2°C show better energy conversion efficiency and increased nitrogen retention at a metabolic cost equivalent to the base temperature group. Metabolic fuel use appears to have been optimized to support the bioenergetic demands imposed during maximum summer water temperatures. Low level ammonia enhances nitrogen and energy conversion efficiency by stimulating protein retention which ultimately results in the most cost-effective growth. However, in the +2°C ammonia treatment, the stimulatory effect of low level ammonia is lost during mid to late summer due to the greater energy demands when fish are forced to cope with the additional stress of a small further increase in temperature.

INTRODUCTION

Temperature is the single most important environmental factor affecting animal activity and nowhere would the effects of temperature change on animals be more pronounced than in aquatic ectotherms, such as fish. Aside from natural diel and seasonal elevations in water temperature, fish face increased water temperatures from the discharge of cooling water from power plants, water diversion for crop irrigation, and canopy removal from forest clear cutting (Sprague 1985; Thomas et al. 1986). Water temperatures are also likely to rise in association with global warming. Simulation modeling suggests that a doubling of the atmospheric CO_2 concentration will increase the earth's average temperature by 1.5 to 4.5°C in the next 50 years (Hansen et al. 1988). Schindler et al. (1990) have already reported a 2°C increase in mean air and lake temperatures over the past 20 years in northern Ontario, Canada. Despite a large body of literature addressing the effects of elevated temperature on fish, large uncertainties in the magnitude and timing of potential warming impacts still exist (Rubin et al. 1992). To date relatively few studies have addressed the effects of chronic small temperature elevations over the annual cycle on fish.

The metabolic and physiological effects of small temperature elevations above present, naturally fluctuating water temperatures could be large in fish. Many species inhabit waters encompassing a wide range of temperatures, but each typically has a narrow range at which growth and survival are maximized (Fry 1967; Magnuson et al. 1979; Elliot 1982; Trippel et al. 1991). Even small changes in local temperature may be enough to alter fishes' energetic requirements (Dawson 1992; Mehner and Wieser 1994).

The goal of the present paper is to quantify the metabolic costs and physiological consequences to juvenile trout associated with living in warmer, marginally polluted environments. The thermal optimum for growth and food utilization in rainbow trout is

ordinarily 15°C (Cho and Kaushik 1990), with an upper lethal limit of approximately 26°C (Bidgood and Berst 1969; Kaya 1978). In an earlier paper, Reid et al. (1995) compared juvenile rainbow trout (*Oncorhynchus mykiss*) held in hard- and softwater during the summer of 1993 to those held at 2°C above the *natural* water temperature cycle (characteristic of the inshore region of Lake Ontario) and found an average 20% reduction in growth, appetite, gross conversion efficiency and protein turnover during peak summer water temperatures (20 - 24°C). In this paper, we address the energetic and physiological effects of the above thermal regime as well as the effect of a low, but environmentally realistic, level of ammonia [70 μmol/L of total ammonia ($NH_4^+ + NH_3$) equivalent to 0.013 mg/L un-ionized ammonia at 15°C and pH = 7.6, respectively]. The thermal and elevated ammonia regimes are assessed alone and in combination, which has necessitated repetition of a small amount of data (growth, appetite, and conversion efficiency for the ammonia-free treatments only) originally reported in Reid et al. (1995).

Ammonia is an important pollutant in aquatic environments. It is highly toxic to fish and ubiquitous in surface waters (Thurston and Russo 1983; Erickson 1985; Russo 1985). More molecules of ammonia are manufactured each year than any other industrial chemical (Atkins 1987). Ammonia is also a problem in fish culture where high-density stocking may result in an accumulation of ammonia via excretion from the fish. Larmoyeux and Piper (1973) found that for juvenile rainbow trout raised in hatchery water-reuse systems, growth was significantly reduced in tanks with un-ionized ammonia loads greater than 0.01 mg/L. The lowest lethal concentration of un-ionized ammonia found for salmonids is 0.083 mg/L (Thurston et al. 1984), but sublethal effects have been reported at concentrations even lower than 0.01 mg/L (Rice and Bailey 1980; EPA 1985). This low chronic value is often exceeded in many urban and industrial areas including Hamilton Harbor and its surrounding tributaries (Lake Ontario, Canada) where summer total

ammonia concentrations have been recorded as high as 66 μmol/L at pH = 7.3, equivalent to 0.012 mg/L unionized ammonia (B. Crosbie, McMaster University, personal communication).

Our approach is novel in that it employs a number of physiological indices including food intake, growth, metabolic rate, nitrogen balance, and proximate composition to assess the long-term consequences and metabolic costs for juvenile trout exposed to a small increase in temperature and an environmentally relevant concentration of priority pollutant, alone and in combination. This study asks three questions: (i) what physiological changes occur in juvenile rainbow trout growing under the normal, fluctuating summer thermal regime?; (ii) what is the effect of +2°C superimposed on this regime?; (iii) what is the effect of 70 μmol/L ammonia on both of the above? The data suggest that additional temperature (2°C) and ammonia (70 μmol/L) are beneficial up to a threshold temperature above which loss of appetite and increased maintenance costs greatly reduce growth and nitrogen transformation efficiencies.

METHODS

Animals

Juvenile rainbow trout (2-5 g) were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario on 19 April 1993 and allowed to acclimatize for 6 weeks prior to testing. The trout were held in a 600 L aerated polyethylene tank receiving 2.5 $L \cdot min^{-1}$ dechlorinated Hamilton tap water ($[Ca^{2+}] = 0.98 \pm 0.11$ mmol/L, $[Na^{+}] = 0.56 \pm 0.03$ mmol/L, $[Cl^{-}] = 0.73$ mmol/L; pH = 7.57 ± 0.26) at an ambient water temperature of 12 ± 1°C. They were fed a maintenance ration equivalent to 1% body weight per day (wet basis) of Zeigler's Trout Starter #3 (50% protein, 15% lipid, 12% moisture) and kept under natural photoperiod for Hamilton, Ontario throughout the experiment.

Experimental

Groups of approximately 150 fish were randomly distributed to 8 exposure tanks (270 L) representing 4 treatment conditions (N = 300 fish per treatment). The tanks received 2.5 L/min (95 % replacement = 4.2 h) of either ambient temperature water (base = City of Hamilton tap water taken from the inshore region of Lake Ontario) or ambient temperature water plus 2°C (base+ΔT), each with or without an additional 70 µmol total ammonia/L ($T_{Amm} = NH_4^+ + NH_3$; base+Am and base+ΔT+Am, respectively). Water at base +2°C was achieved by a heat exchanger. The actual mean difference between temperature treatments was 1.90 ± 0.03°C (Fig. 1). The desired T_{Amm} concentration was achieved by delivering the required amount of $(NH_4)_2SO_4$ stock solution via mariotte bottles (Mount and Brungs 1967). The mean concentrations of total ammonia in the water of each tank along with the pH and partial pressure of oxygen are listed in Table 1.

During the experiment, the fish were fed to satiation twice daily (0830 h and 1630 h) from pre-weighed bags of Zeigler Trout Starter, so as to monitor appetite, following the methods of Wilson et al. (1994a). Samples of fish from each exposure were taken immediately after starting the test and approximately every 30 days thereafter.

Each sampling period began by measuring routine "in-tank" oxygen (O_2) consumption and nitrogenous (N) waste excretion (T_{Amm} + urea) rates in one tank per treatment simultaneously. Fish were fed at their regular times throughout the sampling period. To measure O_2 consumption, samples were taken every other hour over an 8 h period beginning at 0700 and ending at 1500 h. The tank water and air supplies were closed, the water surface was sealed with a transparent lid fitted snugly to the tank walls, and magnetic drive pumps (Little Giant, 1-EUAA-MD) were activated to recirculate and mix the water. Water samples were taken every 10 to 20 min for a total of 30 min to 1 h.

The partial pressure of O_2 (P_{O2}) of the water samples was measured with a temperature equilibrated Cameron O_2 electrode connected to a Cameron OM 200 oxygen meter. After the final P_{O2} measurement, the water and air supplies were re-opened for 1h prior to the next sampling period to allow flushing and re-aeration. The O_2 consumption rates for each closed sample period were calculated from the mean decline in P_{O2} over time (10 to 20 min readings) using the water oxygen solubility coefficients of Boutilier et al. (1984). The P_{O2} of the water for any one tank was not allowed to drop below 90 torr. Mean O_2 consumption rates were plotted for each closed cycle and the area under the curve determined with a digitizing tablet (Sigma Scan) to give a single integrated O_2 consumption rate representative of the 8 h interval between 0700 and 1500 h.

N-waste excretion was measured the next day. Tank flow and aeration were maintained. Approximately 50 ml of influent and effluent water were collected simultaneously every 4 h for 24 h and the samples were frozen immediately for future analysis of total ammonia-N and urea-N. The area under the curve was determined as above. All O_2 consumption and N-waste excretion data were corrected for differences in fish size using the weight exponent 0.824 determined for rainbow trout by Cho (1990). The contribution of bacteria to "in-tank" routine O_2 consumption and N-waste excretion was not estimated during the experiment, however, an estimate ("worst case scenario") was obtained following the experiment. Here, 25 g rainbow trout (which was the approximate size of the fish after 60 days of exposure) held at 21°C (equivalent to the highest temperature reached in the ambient temperature treatments over summer) were removed from their tank (270 L) just after feeding to satiation and the rates of bacterial O_2 and T_{Amm} consumption measured over 24 h. The contribution of bacterial respiration to O_2 consumption was determined to be no greater than 5%. The influence of bacteria on T_{Amm} consumption was negligible.

Following the "in-tank" measurements of routine O_2 consumption and N-waste excretion, food was withheld from the fish for the next 48 h during which blood and tissue sampling were conducted. Ten fish from each tank were killed for determination of whole body proximate analysis (protein, lipid, carbohydrate, and ash) and blood ions (plasma T_{Amm} and sodium) and another 10 were sacrificed for the estimation of T_{Amm}, urea, and glutamine in white muscle and liver. The fish were sampled by netting a number of fish from which one was randomly taken; the procedure was repeated 10 times at 5 min. intervals. With this approach, there was no effect of sample order on any of the measured parameters. For whole body composition and blood sampling, the fish were killed by a quick blow to the head, blotted dry, and their wet weight and total length were measured. A terminal blood sample was collected via caudal severance, the plasma collected, and the carcass freeze-clamped with aluminum tongs pre-cooled in liquid N_2. Both plasma and carcass were stored at -70°C until further analysis.

The fish sampled for T_{Amm}, urea, and glutamine in white muscle and liver were handled in a similar manner, but were sacrificed in a bucket of water containing an overdose of MS-222 (1 g/L) buffered with $NaHCO_3$ (2 g/L) to avoid T_{Amm} build-up in muscle due to struggling (Wang et al. 1994). The liver and a small portion of white muscle anterior to the dorsal fin and above the lateral line were excised and freeze-clamped immediately in liquid N_2. Each tissue was individually wrapped in aluminum foil and stored at -70°C for further analysis.

Analytical

Ammonia concentrations in water were determined by the salicylate-hypochlorite method of Verdouw et al. (1978), and in plasma by a commercial enzymatic kit (Sigma no. 170-UV). The two assays were cross-validated. Water urea samples were freeze-

concentrated 5-fold by lyophilization before assay by the modification of the diacetyl monoxime method described by Lauff and Wood (1996). The concentration of Na^+ in water and plasma was determined using atomic absorption spectrophotometry (Varian AA 1275).

For the determination of proximate composition, frozen whole bodies kept at -70°C were ground into a fine powder with a grinding mill (IKA - M10/M20) cooled to -40°C by a methanol/dry ice mixture and then the powder was lyophilized for 72 h at -55°C. A small portion of the frozen ground tissue was withheld and dried in an oven at 80°C for 48h to obtain the water content. The protein content of the lyophilized tissue was measured using the Lowry method as modified by Miller (1959); glucose, glycogen, and lactate (carbohydrate) as in Bergmeyer (1985); and lipid, after extraction in a 2:1 chloroform:methanol mixture, as described by Folch et al. (1957).

Frozen white muscle and liver were ground into a fine powder in an insulated mortar cooled with liquid N_2. Subsamples (100 mg) were deproteinized in 1 ml of 8 % perchloric acid (PCA) and measured for ammonia as described by Kun and Kearney (1971). Similar deproteinizing procedures were used to measure white muscle and liver urea as described by Crocker (1967). Conversely, for glutamine measurement we used approximately 50 mg of frozen white muscle and liver in 250 µL of 3% PCA and assayed as described by Bergmeyer (1985).

Calculations

Daily food intake (g) was determined as the difference in food bag weight at the beginning and end of feeding divided by the number of fish in the tank (grams per fish per day). Cumulative food intake was calculated using the sum of the daily food intake over each 30 day period.

Specific growth rates (SGR) were determined from 30 fish removed from each tank for monthly sampling (N = 60 per treatment) and calculated following the standard formula:

$$SGR = 100(\ln_e Y_2 - \ln_e Y_1)/ (t_2-t_1)$$

where Y_1 and Y_2 are the mean wet weights of fish at times t_1 and t_2. Condition factors were determined as the quotient of the wet weight of the fish and its total length cubed then multiplied by 100 percent.

The nitrogen quotient (NQ), or the extent of aerobic protein catabolism, was calculated as the ratio of moles of N produced to moles of O_2 consumed (Kutty 1972). The proportion of oxygen consumption dedicated to protein catabolism was measured as the ratio of the NQ at each respective time period to the maximum aerobic value (0.27; Kutty 1972).

The nitrogen budget was constructed following the equation summarized by Birkett (1969):

$$I - F_N = A = R + E$$

where I is the total N consumed, F_N is the N lost in fecal and unaccounted material (mucus, etc....), A is the N absorbed from food, R is the nitrogen retained in body materials, and E is the nitrogen lost via branchial and urinary excretion. Measurements of N consumption, retention, and excretion allowed for estimation of N lost through feces (and unaccounted N) and the N absorbed. Protein was measured as a surrogate for N; the percentage N by weight in the protein of the food consumed and in the protein of the fish was taken as the standard value, 16% (Jobling 1980; Soderberg 1995).

The energy budget was derived from the equation:

$$C = F_E + U + \Delta B + R_{tot}$$

where C is the total energy consumed from food, F_E is the energy lost in feces, U is the energy lost via excretion (branchial and urinary), ΔB is the energy stored in body materials, and R_{tot} is the total metabolic energy lost as heat. The following energy values were assumed: 23.6, 39.5, and 17.2 kJ/g for protein, lipid, and carbohydrate, respectively (Braefield and Llewellyn 1982), 24.9 kJ/g for non-fecal nitrogen (Cho and Kaushik 1990), and 13.6 kJ/g for O_2 (Elliot and Davison 1975).

A new and relatively simple index, the N-cost index (the total moles of O2 consumed per mole of nitrogen stored), was used to compare the total metabolic expenditure associated with the incorporation of nitrogen into body material under the stated conditions.

Statistics

The values for the nitrogen and energy budget are all reported on a per fish basis and from the one experimental tank in each treatment where O_2 consumption and N-waste excretion were measured (see legend Fig. 3). Since N = 1 for these parameters, no statistical comparisons could be employed. In addition, cumulative food consumption was calculated on a per fish basis. Here, N = 2 (two tanks per treatment). and again, statistics were not employed. All other data are expressed as means ± 1 SEM from individual samples pooled together from the two tanks per treatment. One-way analysis of variance using SAS Jmp (SAS Institute Inc., Version 2.0.5) followed by Tukey-Kramer HSD multiple means comparison test was used to distinguish statistically significant differences amongst the four separate treatment groups within each sample period (30, 60, and 90 days respectively). The level of statistical significance for all analyses was $P \leq 0.05$.

RESULTS AND DISCUSSION

Physiological Responses Due to the Base Summer Thermal Regime

Juvenile rainbow trout (2 - 5 g) exposed to the natural, or base, thermal regime and fed to satiation twice a day for 90 days exhibited an overall specific growth rate of 3.02 %/d. Despite several days of fluctuating water temperature about a mean of 20.5°C between days 60 and 90 (Fig. 1), our fish continued to feed and grow with no apparent loss in appetite (Fig. 2A and B). Thus, maximum energy intake was greater than the maintenance energy used during this period. In contrast, Elliott (1982) showed that brown trout (*Salmo trutta*) do not grow at water temperatures above 19.5°C when fed maximum rations of *Tubifex* sp.

The metabolic cost associated with growth in our trout was high. Their routine O_2 consumption rate (Fig. 3A), after size correction (see methods), was approximately 75% of the maximum O_2 consumption ($MO2_{max}$) determined for juvenile rainbow trout held in our laboratory - also size corrected and comparison made at 15°C (Wilson et al. 1994b). These high values were most likely associated with the high protein (50%) and lipid (15%) diet fed to them as well as the intensive feeding regime (2 x day to satiation). Soofiani and Hawkins (1982) showed that in cod, elevation in metabolic rate associated with feeding depends on ration size, increasing linearly as food intake increases. They also showed that the costs of food assimilation were highest in fish fed to satiation, and that the cost increases with increasing temperature.

The type of food and feeding regime also have considerable impact on the rate of N-waste excretion in fish. Wood (1995), in his review of the routes and rates of excretion in salmonids, concluded that the single most important factor affecting N-waste excretion is feeding. He reported that in rainbow trout fed to satiation, total N excretion rates generally range from 1.0 to 2.0 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (0.6 to 1.4 when scaled as above for size; see

methods), but pointed out from Fromm's (1963) data that it may drop to a low of 0.1 to 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (or 0.07 to 0.35; see above) after 5 days of starvation. The scaled N-waste excretion rates in our satiation fed fish dropped from an initial 1.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ to 0.8 $\mu mol \cdot g^{-1} \cdot h^{-1}$ by day 90 (see Fig 3B). Since appetite was not inhibited in this study, the decline in N-waste excretion rate may be the consequence of the metabolic partitioning of food energy into growth. For example, our results are consistent with the responses of other salmonids fed high lipid diets; i.e., increased lipid in the diet acts to spare protein for growth (Atherton and Aitken 1970), thus reducing the amount of exogenous N-waste excretion (Jayaram and Beamish 1992, Agradi et al. 1995) and ultimately increasing the proportion of ingested N retained as growth (Beamish and Tandler 1990). This would explain the observed decrease in protein catabolism. Our fish showed a 39 - 55% decrease in aerobic protein catabolism (NQ) between the initial value and days 30, 60, and 90 (see Fig. 3C), and hence, comparable reductions in the use of protein for energy (see methods for calculation).

The large increase of whole body lipid, from initial values, indicates an excess of dietary energy above and beyond what was needed for maintenance and metabolism, the exception occurring at the highest summer temperatures (Table 2). During the 90 day exposure period, condition factors increased from 0.87 ± 0.008 (Initial) to 1.22 ± 0.019 by day 90 in accord with this observed increase in whole body lipid level (Table 2). It is believed that deposition of energy into lipid may be one of the most critical components of survival in fish (Adams and Breck 1990). Swift (1955) found that fish typically accumulate lipid in the summer and fall and use lipid in the winter when food consumption is minimal. Conversely, the percentage of whole body carbohydrate (very small in comparison to protein and lipid) showed the opposite trend (Table 2). After the first 30 days, there was no change from the initial value. By day 60, when temperature had

increased dramatically (see Fig. 1), carbohydrate decreased to less than half the previous amount. Energy in excess of maintenance requirements can be stored as lipid or glycogen (Brett and Groves 1979; Wood 1993). However, during times of increased energy demand, such as stress, these energy stores are mobilized (Mayer et al. 1992).

Liver T_{Amm} levels were initially 4 times higher than in white muscle (Figs. 4 and 5A). However, over time, less and less ammonia was present in the liver, while more and more glutamine accumulated there (Fig. 5A and B). This may be a result of increased glutamine synthetase production and the incorporation of ammonia into protein synthesis. In an earlier paper, Reid et al. (1995) reported an increase in liver protein synthesis rates (K_S) over the duration of the experiment, with the most dramatic increase occurring over the first 30 days.

Crude nitrogen and energy budgets were constructed to assess the costs of growth incurred under these conditions. Nitrogen retention efficiency, for the period of growth between June and September, was 25.9% (Table 3). This value is comparable to, but somewhat lower than, those reported by Birkett (1969), who found that for plaice, sole, and perch fed to excess, gross N conversion efficiency (synonymous here with N retention efficiency) ranged from 27.9 to 49%. Our estimated fecal N losses, regarded as undigested or unabsorbed diet and all N not accounted for, are quite high (over 48%) (Table 3). Subsequent analysis of similar water samples in later experiments for N-waste excretion using the Kjeldahl method (Skoog and West 1982) indicates that total N-waste excretion may be as much as 34% greater than that measured here by T_{Amm} and urea only. The missing nitrogenous waste components probably include mucoproteins and amino acids (Olson and Fromm 1971). Nevertheless, feeding to satiation may have also contributed to the low N absorption as there was a strong correlation in the present study

between the amount of N consumed and the amount of N lost in feces before the dramatic temperature increase at day 60 ($R^2 = 0.92$; DF = 7; P = 0.0001).

The energy budget (kJ) tended to correlate with the results of the nitrogen budget (Table 4). Gross conversion efficiency over the 90 day study period was 38%. This was marked by high food intake, relatively high energy loss via excretion (fecal (F) 22.5%, non-fecal (U) 2.9%), and a high loss in heat production (R), 36%. The results reported here are consistent with previous reports under comparable conditions (Brett and Groves 1979). In fact, Cho et al. (1982) report a conversion of energy into body materials of 46%, an energy loss in excreta of 23% (fecal 15% and non-fecal 8%), and a final 30% loss via heat production. To summarize, for juvenile rainbow trout fed to satiation and growing under the base thermal regime, a relatively high proportion of gross energy intake was being stored in body materials (especially lipid), but at the expense of nitrogen retention efficiency and energy conversion efficiency. Their estimated cost of growth, or N-cost index, was 9.66 moles of O_2 consumed per mole of N stored.

Effects of an Additional +2°C

The high temperatures reached in the last 30 days approached the upper thermal limit of juvenile rainbow trout, i.e. 26°C (Bidgood and Berst 1969; Kaya 1978). Despite the higher temperature, the 90 day growth rate of fish held at +2°C (3.01 ± 0.05 %/d) was nearly identical to that of the base temperature group. However, this disguised the fact that during the first 60 days and before the sharp temperature increase, the growth rate of the base+ΔT fish was actually higher, as indicated by the statistically significant increase in wet weight (see Fig. 2A). It was not until the last 30 days that growth rate declined. This stemmed in part from the decrease in food intake (See Fig. 2B), and from a concomitant change in energy metabolism.

The 30% reduction in food intake during days 60-90 (mean temperature = 22°C) was perhaps the most significant effect of the 2°C warming on juvenile rainbow trout. This effect is not uncommon for salmonids; as water temperature nears the upper thermal tolerance of a species, food consumption declines precipitously (Jobling 1993). However, up until day 60, additional temperature had little effect on food consumption. Wurtsbaugh and Davis (1977) observed that for fish fed higher ration levels, elevated temperature up to 17°C increased growth rate, but that when the temperature reached 22.5°C in their summer experiment, the fish ate less than those at 19.5°C.

The additional 2°C did not affect routine O_2 consumption rates even when food intake was suppressed (Fig. 3A). However, N-waste excretion was markedly reduced at this time (Fig 3B). Protein utilization is dependent on water temperature (Steffens 1981). Reid et al. (1995) reported a 20% decrease in protein turnover in these last 30 days and proposed that metabolic fuels (i.e. protein, lipid, and carbohydrate) may have been diverted from growth to supply energy for maintenance and other homeostatic processes. Since N-waste excretion is reduced at this time, it is doubtful that protein was being used as an energy source. In fact, comparison of the whole body composition data between days 60 and 90 (Table 2) suggests that for trout exposed to an additional 2°C, more N was retained for growth (Table 3) and whole body protein increased (Table 2). However, whole body lipid decreased (Table 2). It is possible that lipid was being used to meet the increased energetic demands above 20°C as argued earlier. We suggest that during the last 30 days, change in fuel use allowed the fish exposed +2°C to meet the increased maintenance costs associated with the higher water temperature. As a result, the overall cost associated with N gain in base+ΔT fish (N-cost index = 9.94 moles of O_2 consumed per mole of N stored) was comparable to the base temperature group (i.e., 9.66).

Effects of 70 μmol/L T_{Amm}

The physiological effects of low environmental ammonia are not well documented. The vast majority of studies concerning the chronic effects of ammonia on freshwater fish were conducted at levels considerably higher than the concentration used here. Effects of sublethal ammonia toxicity to fish may include reduced growth (Robinette 1976; Rice and Bailey 1980; Beamish and Tandler 1990), reduced feeding (Beamish and Tandler 1990), gill damage (Smart 1976), and plasma ion disturbance (Buckley et al. 1979; Thurston et al. 1984; Twitchen and Eddy 1994). With respect to this last effect, the fish exposed to ammonia in the present study not only exhibited a significant increase in plasma ammonia, but also a significant elevation in plasma Na^+ by day 90 (Fig. 6A and B). Although the mechanisms of branchial ammonia excretion in freshwater teleosts remain controversial (Randall and Wright 1987), two mechanisms are thought to be responsible for the majority of the ammonia excreted under most environmental conditions: 1) passive NH_3 diffusion along the P_{NH3} gradient from blood to water, and 2) electroneutral exchange of NH_4^+ for Na^+ (Wood 1993). Since the P_{NH3} gradients calculated here for the ambient control and ammonia group at 14°C were +262 and +305 μtorr respectively using measured plasma and water T_{Amm} levels, measured water pH, arterial blood pH values from Randall and Cameron (1973), water and plasma pK values from Cameron and Heisler (1983), and equations from Wright and Wood (1985), and since a significant increase in plasma $[Na^+]$ was observed over time in these fish, it is likely that both mechanisms contributed to their excretion of ammonia to water.

One effect that has not been reported previously, however, is the stimulation of growth in fish exposed to such a low level of ammonia. In the present study, juvenile rainbow trout exposed over summer to 70 μmol/L T_{Amm} at base water temperature (base+Am) had significantly greater weight gain (Fig 2A), better N and energy conversion

(Tables 3 and 4), and higher N retention efficiency at a lower metabolic cost (Fig. 3A). The N-cost index for this group was only 6.58 moles of O_2 consumed per mole of N stored. Thus more N, in the form of protein, was being retained and put towards growth (Tables 2 and 3). These results are in contrast to those of Beamish and Tandler (1990) who reported no change in either carcass protein or growth of juvenile lake trout (*Salvelinus namaycush*) exposed for 60 days to approximately 330 μmol/L T_{Amm} at 11°C. The increase in growth can not be explained by increased N consumption because food intake was not affected by ammonia exposure (Fig. 2B). Instead, the ammonia appears to have stimulated protein turnover (S. D. Reid[1], J.J. Dockray[2], T.K. Linton[2], D.G. McDonald[2], and C.M. Wood[2]; [1]Okanagan University College, [2]McMaster University, unpublished data) resulting in better N conversion efficiency. In just 30 days, liver-somatic indices were elevated, and by day 60, liver protein synthesis, degradation and accretion rates were significantly increased. This increase in protein turnover may be the indirect result of ammonia detoxification mechanisms acting in response to the higher levels of plasma ammonia (Fig. 6A). For example, ammonia is incorporated into glutamine (Levi et al. 1974; Walton and Cowey 1977) and other amino acids (Iwata et al. 1981; Dabrowska and Wlasow 1986) which may in turn be used as substrates for protein synthesis, ultimately, improving growth. Indeed, the concentration of glutamine in the livers of the ammonia exposed fish does appear to be elevated in comparison to the base temperature group (see Fig 5B).

The above arguments also hold true for those fish exposed to the ammonia at +2°C (base+ΔT+Am) up to day 30; beyond this period (above 16°C), the stimulatory effect was lost and the combination became deleterious. This stems in part from a dramatic decrease in N retention and absorption efficiency and an increase in amount of energy lost as heat (R; see Table 4) between days 30 and 90, which, undoubtedly contributed to the high N-

cost index (11.06 moles of O_2 consumed per mole of N stored) calculated for the 90 days of exposure. From these results, it is apparent that the increased metabolic cost associated with both elevated temperature and ammonia detoxification was manifest in the high N-cost index estimated for these fish. We conclude that juvenile rainbow trout fed to satiation and exposed over summer to a simulated warming scenario of +2°C can make the necessary metabolic adjustments necessary to maintain growth. However, in the presence of sublethal ammonia, the cost of growth increases and growth may be compromised.

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Wilson, R. W., H. L. Bergman, and C. M. Wood. 1994b. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 2: Gill morphology, swimming performance and aerobic scope. Canadian Journal of Fisheries and Aquatic Sciences 51:536-544.

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Figure. 1. Daily water temperature profile as measured each day over the 90 day exposure from 17 June to 15 September 1993. Juvenile rainbow trout were exposed to either ambient laboratory water temperatures (base = dark solid line) or to ambient water temperatures + 2°C (base+ΔT = light solid line).

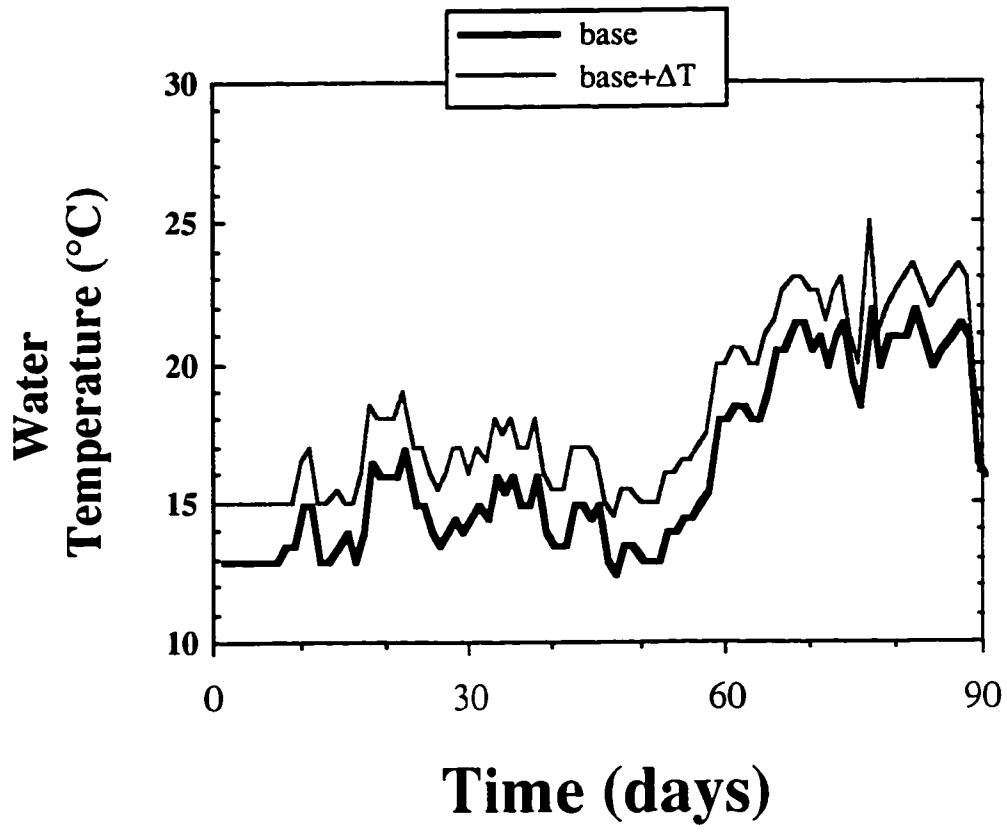
base
base+ΔT
Water Temperature (°C)
30
25
20
15
10
0
30
60
90
Time (days)

Figure. 2. Effects of +2°C and 70 µmol/L ammonia on (A) growth and (B) cumulative food intake of juvenile rainbow trout fed to satiation twice daily. Growth was measured as the wet weight of the fish sampled initially (N = 20) and after 30, 60, and 90 days of exposure (N = 60 per treatment per time period). Cumulative food intake is presented on a per fish basis and represents the cumulative food eaten over the respective time periods; each value represents the mean of the two replicate tanks per treatment (N = 2). Treatments with the same letters are not significantly different at the $P \leq 0.05$ level.

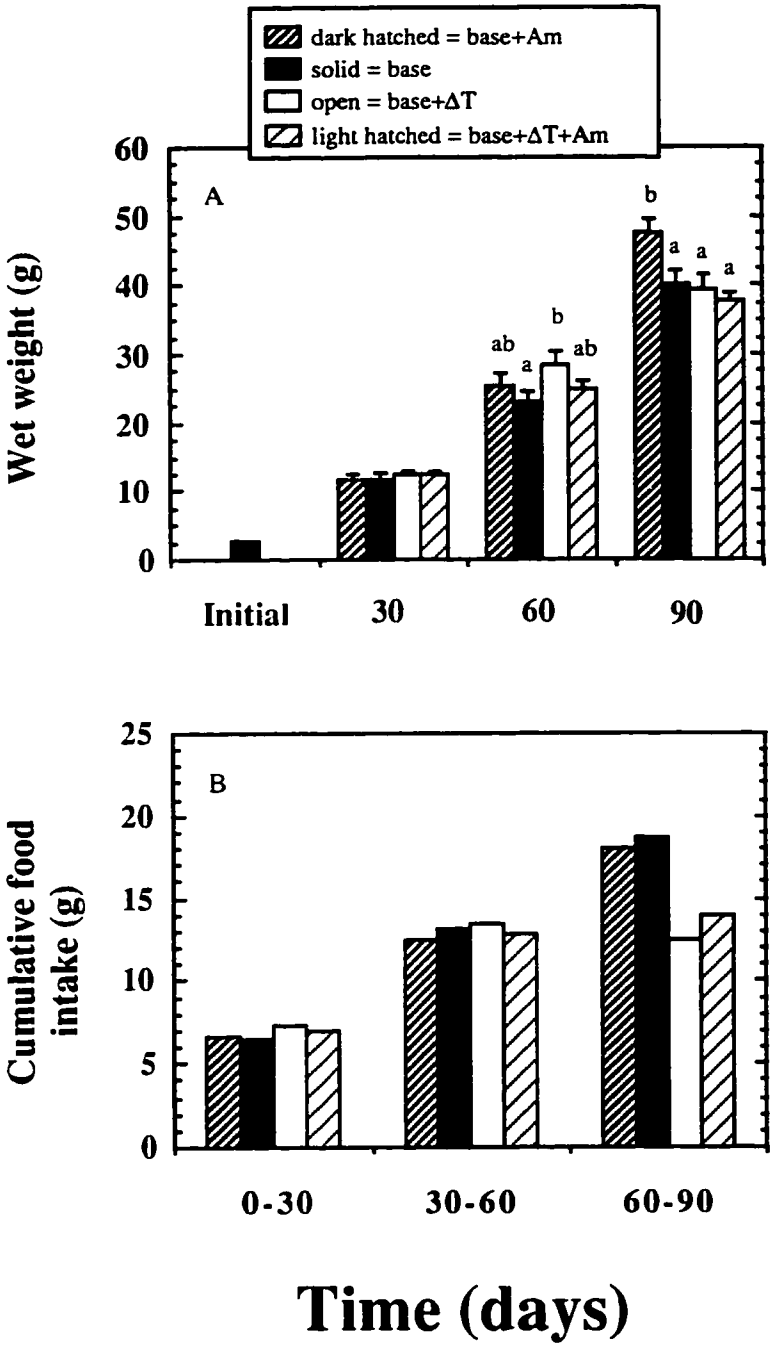
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
Wet weight (g)
60
50
40
30
20
10
0
ab
a
b
ab
b
a
a
a
Initial
30
60
90
B
Cumulative food intake (g)
25
20
15
10
5
0
0-30
30-60
60-90
Time (days)

Figure. 3. Effects of +2°C and 70 µmol/L ammonia on (A) routine "in-tank" oxygen consumption (N = 5 measurements on the same tank), (B) routine "in-tank" N-waste (ammonia + urea N) excretion (N = 6 measurements on the same tank), and (C) the nitrogen quotients of juvenile rainbow trout fed to satiation twice daily. In (A) and (B) the data have been scaled for weight, as explained in the text. Measurements were made on one tank of individuals (N = 1) per treatment. Error bars represent measurement SEM (the mean O2 consumption and N-waste excretion rates calculated over the respective sample intervals-see methods), and cannot be used for statistical comparisons between treatments.

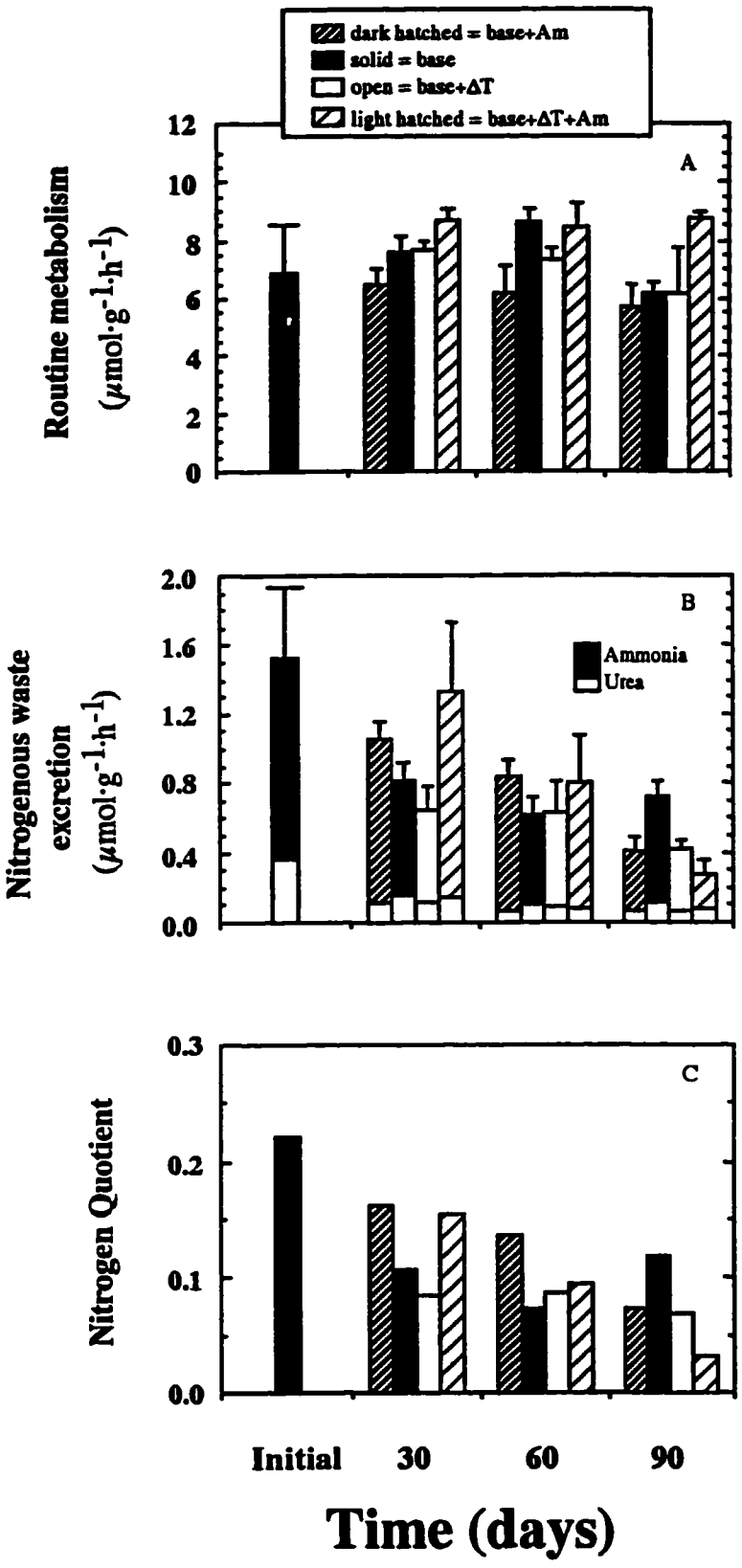
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
Routine metabolism
(μmol·g-1·h-1)
12
10
8
6
4
2
0
B
Ammonia
Urea
Nitrogenous waste
excretion
(μmol·g-1·h-1)
2.0
1.6
1.2
0.8
0.4
0.0
C
Nitrogen Quotient
0.3
0.2
0.1
0.0
Initial
30
60
90
Time (days)

Figure. 4. Effects of +2°C and 70 μmol/L ammonia on white muscle (A) total ammonia (N = 6 - 20), and (B) glutamine (N = 5 - 10) concentration of juvenile rainbow trout fed to satiation twice daily. Columns with the same letter on the same sampling day are not significantly different at the $P \leq 0.05$ level.

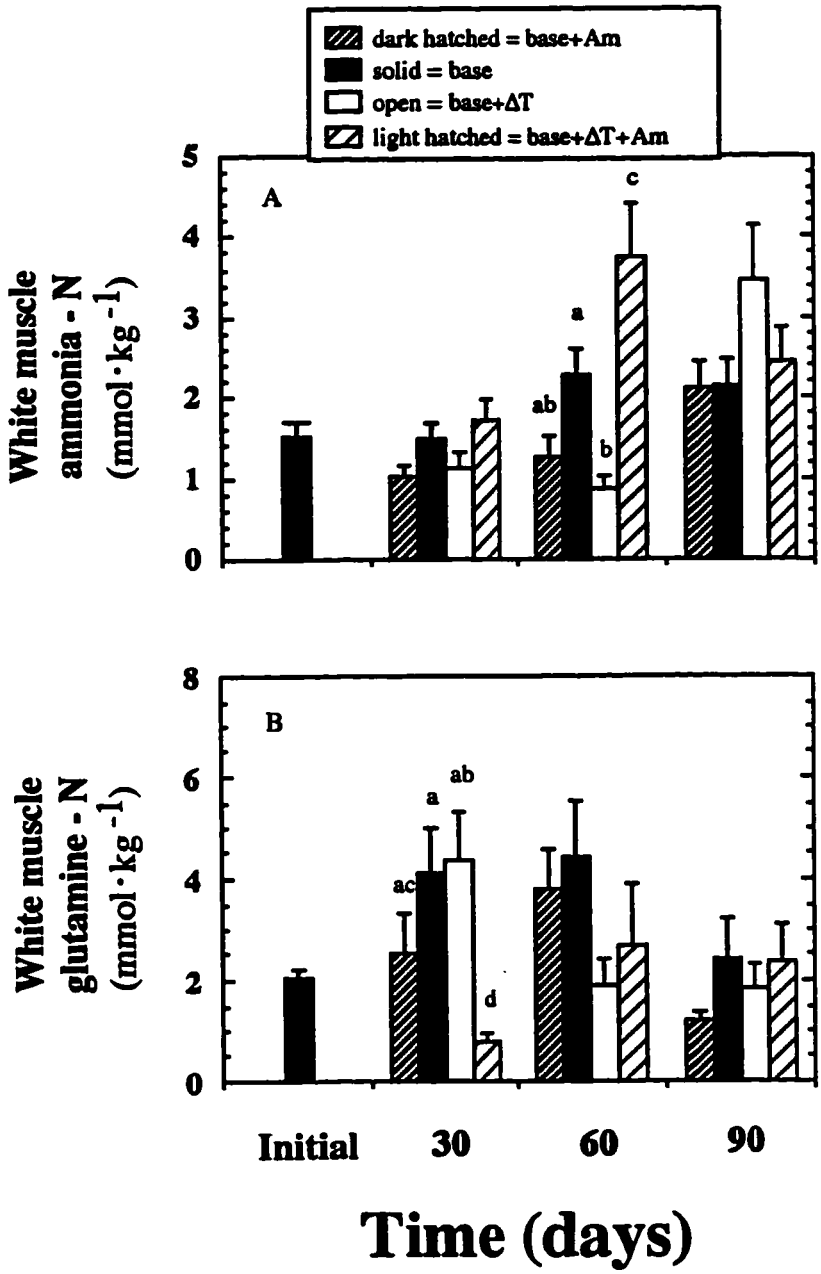
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
White muscle ammonia - N (mmol·kg-1)
0
1
2
3
4
5
ab
a
b
c
B
White muscle glutamine - N (mmol·kg-1)
0
2
4
6
8
ac
a
ab
d
Initial
30
60
90
Time (days)

Figure. 5. Effects of +2°C and 70 μmol/L ammonia on liver (A) total ammonia (N = 6 - 20), and (B) glutamine (N = 5 - 10) concentration of juvenile rainbow trout fed to satiation twice daily. Columns with the same letter on the same sampling day are not significantly different at the $P \leq 0.05$ level.

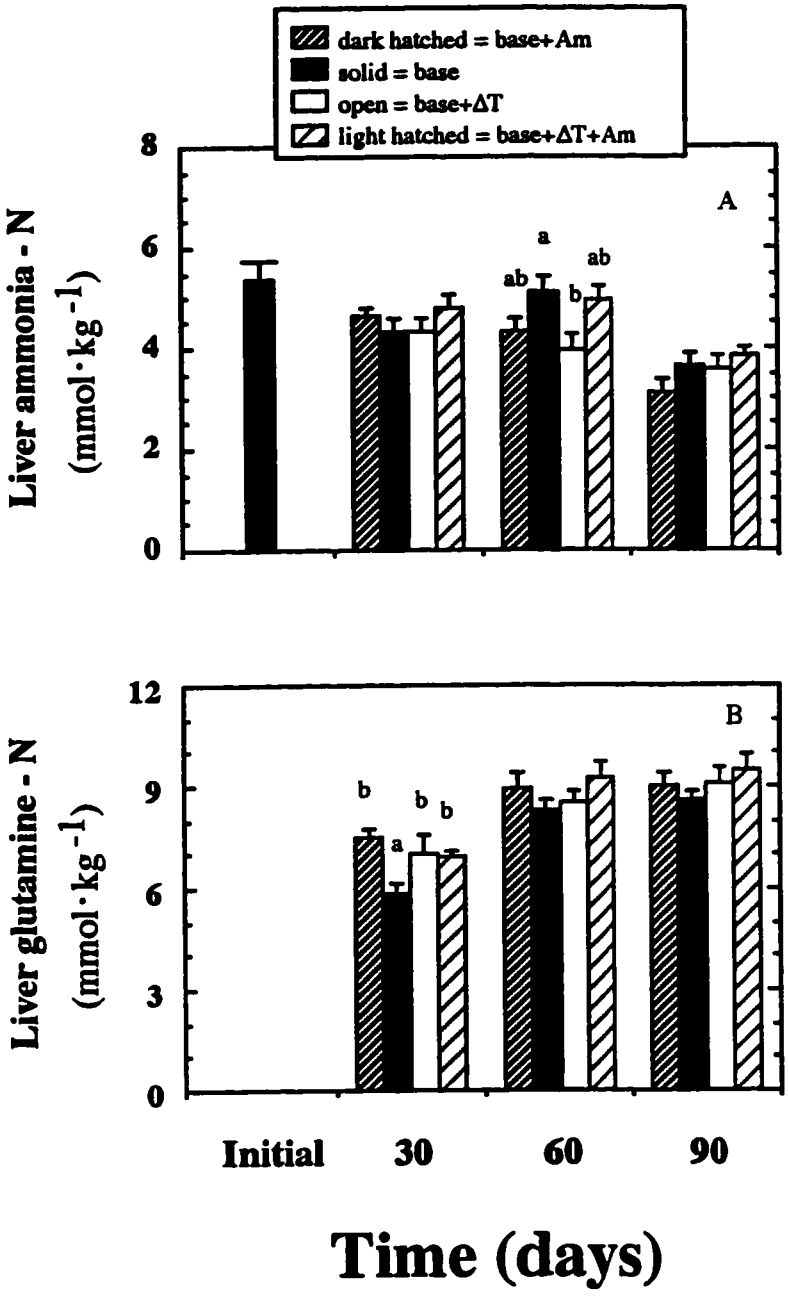
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
Liver ammonia - N
(mmol·kg-1)
A
a
ab
ab
b
Liver glutamine - N
(mmol·kg-1)
B
b
a
b
b
Initial
30
60
90
Time (days)

Figure. 6. Effects of +2°C and 70 µmol/L ammonia on (A) total ammonia in plasma (N = 10 - 20), and (B) plasma Na^+ (N = 5 - 10) concentration of juvenile rainbow trout fed to satiation twice daily. Columns with the same letter on the same sampling day are not significantly different at the $P \leq 0.05$ level. Note, the high initial plasma ammonia value is probably artificially elevated due to the fishes small size at sampling and the invasive procedure used to collect the sample (caudal severance).

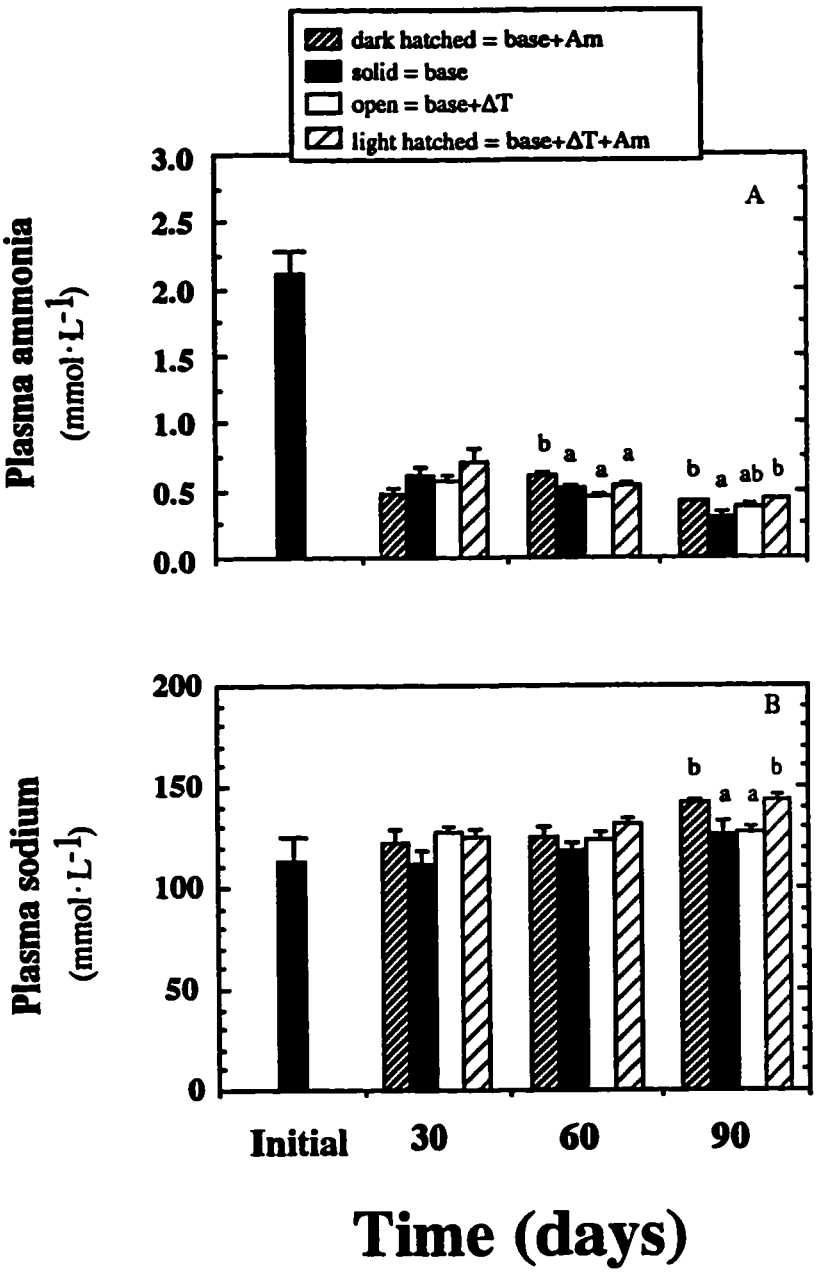
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
Plasma ammonia
(mmol·L-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
b a a a
b a ab b
Plasma sodium
(mmol·L-1)
200
150
100
50
0
B
b a a b
Initial
30
60
90
Time (days)

Table 1. Ammonia concentrations and water quality parameters of the 90-day exposure to +2°C and 70 µmol/L ammonia.

Treatment	Rep	Total Ammonia (µmol/L)	N	Total Ammonia treatment mean	pH	N	PO_2 (torr)	N
base+Am	1	65.8 ± 6.3	10	61.5 ± 3.8	7.6 ± 0.1	14	131.9 ± 4.5	14
	2	58.2 ± 5.6	10		7.6 ± 0.1	14	134.3 ± 4.1	14
base	1	6.2 ± 1.6	9	6.0 ± 0.2	7.6 ± 0.1	14	132.9 ± 3.5	15
	2	5.8 ± 0.9	8		7.6 ± 0.1	13	134.6 ± 3.9	15
base+ΔT	1	4.5 ± 1.0	9	5.8 ± 1.3	7.6 ± 0.1	14	132.2 ± 3.5	15
	2	7.1 ± 1.5	9		7.5 ± 0.1	13	124.3 ± 4.7	15
base+ΔT+Am	1	78.6 ± 7.6	10	75.7 ± 2.9	7.5 ± 0.1	14	121.3 ± 4.1	15
	2	72.8 ± 6.6	10		7.6 ± 0.1	14	123.3 ± 4.3	15

Table 2. Proximate chemical composition and condition factors of juvenile rainbow trout exposed to a +2°C warming scenario and 70 µmol/L ammonia. Fish were sampled initially and after 30, 60, and 90 days of exposure. Results are expressed as means ± SEM. All values are based on wet weight. Numbers with the same letter on the same sampling day are not significantly different at the $P \leq 0.05$ level. (n) = sample size.

Sampling Day and Treatment	Protein (mg/100 mg)	Lipid (mg/100 mg)	Carbo-hydrate (mg/100 mg)	Condition factor
Initial	10.7 ± 0.3 (9)	4.7 ± 0.2 (10)	0.45 ± 0.03 (10)	0.87 ± 0.01 (20)
Day 30				
base+Am	14.3 ± 0.2 b (20)	9.0 ± 0.2 a (20)	0.40 ± 0.02 a (20)	1.20 ± 0.01 a (20)
base	13.1 ± 0.2 a (20)	8.4 ± 0.4 a (20)	0.43 ± 0.04 a (10)	1.17 ± 0.01 ab (20)
base+ΔT	13.5 ± 0.2 a (20)	9.0 ± 0.3 a (20)	0.36 ± 0.03 ab (20)	1.18 ± 0.02 a (20)
base+ΔT+Am	14.6 ± 0.3 b (19)	9.4 ± 0.2 a (18)	0.30 ± 0.02 b (19)	1.13 ± 0.01 b (20)
Day 60				
base+Am	13.8 ± 0.2 b (20)	10.4 ± 0.2 ab (19)	0.20 ± 0.01 a (20)	1.11 ± 0.01 a (20)
base	12.8 ± 0.3 ab (20)	9.4 ± 0.2 a (20)	0.20 ± 0.01 a (20)	1.14 ± 0.02 a (20)
base+ΔT	12.2 ± 0.3 a (20)	10.8 ± 0.2 b (19)	0.25 ± 0.01 b (10)	1.12 ± 0.01 a (20)
base+ΔT+Am	15.5 ± 0.6 c (20)	10.6 ± 0.4 b (20)	0.23 ± 0.02 ab (6)	1.12 ± 0.01 a (20)
Day 90				
base+Am	15.5 ± 0.4 a (20)	8.8 ± 0.2 a (18)	0.35 ± 0.03 b (10)	1.24 ± 0.02 a (20)
base	15.6 ± 0.4 a (19)	8.8 ± 0.3 a (19)	0.27 ± 0.01 a (13)	1.22 ± 0.02 a (20)
base+ΔT	13.8 ± 0.3 b (15)	9.8 ± 0.3 b (16)	0.28 ± 0.02 a (4)	1.16 ± 0.02 a (20)
base+ΔT+Am	15.0 ± 0.4 a (20)	10.5 ± 0.2 b (20)	0.25 ± 0.01 a (10)	1.19 ± 0.04 a (20)

Table 3. Nitrogen budget for juvenile rainbow trout fed to satiation for 90 days and exposed to a +2°C warming scenario and 70 µmol/L ammonia.

Interval and Treatment	Nitrogen (mmol/fish) I	E	R	F_N	A	Retention Efficiency (%)	Absorption Efficiency (%)
Days 0 - 30							
base+Am	34.9	14.0	15.5	5.5	29.5	44.4	84.3
base	36.6	11.7	14.5	10.5	26.2	39.5	71.4
base+ΔT	41.8	10.0	15.3	16.6	25.3	36.5	60.4
base+ΔT+Am	40.4	16.4	17.5	6.5	33.9	43.4	83.8
Days 30 - 60							
base+Am	61.9	23.6	17.6	20.8	41.2	28.3	66.5
base	76.6	17.9	17.2	41.5	35.1	22.4	45.8
base+ΔT	66.8	17.8	19.0	30.0	36.8	28.4	55.1
base+ΔT+Am	74.8	26.2	12.7	35.9	38.9	16.9	52.0
Days 60 - 90							
base+Am	84.8	24.7	48.5	40.4	73.2	57.2	86.4
base	110.3	27.6	26.3	56.5	53.9	23.9	48.8
base+ΔT	68.1	22.0	29.6	16.6	51.5	43.4	75.7
base+ΔT+Am	83.1	20.4	21.9	40.9	42.3	26.3	50.9
Days 0 - 90							
base+Am	181.7	62.3	81.6	37.8	143.9	44.9	79.2
base	223.5	57.1	58.0	108.5	115.1	25.9	51.5
base+ΔT	176.8	49.8	63.8	63.1	113.6	36.1	64.3
base+ΔT+Am	198.3	63.0	52.1	83.3	115.0	26.3	58.0

I = total N consumed; E = N lost via branchial and urinary excretion; R = N retained in body materials; F = N lost in fecal material and all unaccounted N; A = N absorbed from food. Retention efficiency = R/I * 100. Absorption efficiency = A/I * 100.

Table 4. Energy budget for juvenile rainbow trout fed to satiation for 90 days and exposed to a +2°C warming scenario and 70 µmol/L ammonia.

Interval and Treatment	Energy (kJ/fish) C	U	ΔB	R	F_E	Conversion efficiency (%)
Days 0 - 30						
base+Am	108.5	4.9	80.0	35.2	-11.6	73.8
base	113.6	4.1	68.6	38.7	2.2	60.4
base+ΔT	129.7	3.5	79.8	40.7	5.8	61.5
base+ΔT+Am	125.3	5.7	86.0	44.7	-11.1	68.6
Days 30 - 60						
base+Am	192.1	8.2	83.8	70.2	29.9	43.6
base	237.7	6.2	94.6	93.5	43.3	39.8
base+ΔT	207.4	6.2	105.9	90.8	4.4	51.1
base+ΔT+Am	232.0	9.1	108.3	99.0	15.5	46.7
Days 60 - 90						
base+Am	263.0	8.6	160.0	105.3	-10.8	60.8
base	342.3	9.6	101.5	120.6	110.6	29.7
base+ΔT	211.3	7.7	95.2	120.6	-12.2	45.1
base+ΔT+Am	257.9	7.1	91.4	148.0	11.4	35.4
Days 0 - 90						
base+Am	563.7	21.7	323.8	210.7	7.5	57.4
base	693.6	19.9	264.7	252.8	156.1	38.2
base+ΔT	548.4	17.4	280.9	252.0	-1.9	51.2
base+ΔT+Am	615.2	22.0	285.7	291.8	15.8	46.4

C = total energy consumed from food; U = energy lost via excretion (branchial and urinary); ΔB = energy stored in body materials; R = total metabolic energy lost as heat; F = energy lost in feces. Conversion efficiency = ΔB/C * 100%.

CHAPTER 3

THE METABOLIC COSTS AND PHYSIOLOGICAL CONSEQUENCES TO JUVENILE RAINBOW TROUT OF A SIMULATED WINTER WARMING SCENARIO IN THE PRESENCE OR ABSENCE OF SUBLETHAL AMMONIA

ABSTRACT

Juvenile rainbow trout *Oncorhynchus mykiss* (5.50 ± 0.03 g) were fed twice daily to satiation in 90-d exposures (January to April 1994; temperature range 4 -7 °C) to a simulated warming scenario of +2°C in the presence or absence of a nominal 70 μmol total ammonia/L (equivalent to 0.005 mg/L NH_3 at 5°C and pH 7.5) to determine the metabolic costs and physiological consequences associated with their growth and energetics in a warmer, more polluted winter environment. In a previous summer experiment, trout exposed to the additional +2°C, despite a metabolic suppression at peak summer water temperatures (21-23°C), achieved a specific growth rate over the entire summer that was equivalent to that of the ambient 'base' temperature control group (3.0 %/d). The growth achieved by the fish in the current study at the lower temperature (6 - 9°C) was approximately 3 times lower. Moreover, during this winter exposure, wet weights and total lengths were roughly 30% higher in the 'warmed' fish than in the base temperature group. The enhanced growth for trout in the warmer winter thermal regime was due to a combination of greater appetite and higher energy conversion efficiency. Oxygen consumption and nitrogenous (ammonia + urea) waste excretion rates, though overall less than a third of those recorded in the summer, were also 30-40% higher for these 'warmed' fish in correspondence with a similar increase in their food intake, the latter associated with

elevations in whole body protein and lipid, but not carbohydrate. On the other hand, the addition of 70 μmol ammonia/L elevated nitrogenous waste excretion much like in the previous summer exposure, but over this winter period, did not result in increased weight gain. Plasma total ammonia was significantly elevated in the ammonia-exposed fish, whereas plasma Na^+ and hematocrit were both slightly reduced. Although nitrogen retention efficiency was much lower for over-wintering juvenile trout fed to satiation, the metabolic cost of nitrogen retention (growth) was similar to that which was achieved by juvenile trout exposed over summer. We conclude that over-wintering juvenile trout fed unlimited ration and subjected to simulated warming, both alone and in combination with elevated environmental ammonia, will exhibit increased growth with only a slight elevation in energetic cost.

INTRODUCTION

Mean annual air temperature is predicted to rise in response to an increase in atmospheric greenhouse gases (Hansen et al. 1988), and with it, mean annual water temperatures (Meisner et al. 1987). Such changes are expected to have profound effects on fish populations (Healey 1990), shifting species geographic ranges (Meisner 1990; Shuter and Post 1990; Keheler and Rahel 1996) and altering population dynamics (Magnuson et al. 1990; DeAngelis and Cushman 1990). Yet despite attempts to predict the overall response of fish to climate change (Magnuson et al. 1990: Regier et al. 1990), a considerable amount of uncertainty still exists. In particular, those predictions at or near the extreme minimum and maximum survival temperatures remain "not fully satisfactory" (Lin and Regier 1995).

We have designed a laboratory-based system that allows us to test the effects of a global warming scenario of +2°C to a reference cold water fish species, rainbow trout *Oncorhynchus mykiss*, over a naturally occurring thermal regime (representative of near-shore Lake Ontario) that includes seasonal temperature extremes (see Reid et al. 1997). In addition, we have incorporated chronic pollutant exposure (i.e., elevated ambient ammonia) in anticipation of the likely increase in the concentrations of aquatic pollutants which may accompany CO_2 enrichment of the atmosphere (Coutant 1981). Our goal has been to quantify the basic long-term bioenergetic, physiological, and toxicological responses of juvenile trout to a warmer and more polluted aquatic environment. Although this work is often considered in an ecological context, it is also particularly relevant to hatchery conditions. In our earlier study (see Linton et al. 1997), we discovered that trout exposed to +2°C and fed unlimited ration over summer exhibited enhanced energy conversion efficiency and increased nitrogen retention at a metabolic cost equivalent to trout grown at the base thermal regime. Although the appetite of these 'warmed' fish was

surpressed during maximum summer water temperatures, fuel use, and in particular lipid, appeared to have been optimized in order to meet the increased bioenergetic demands. Low level ammonia exposure, on the other hand, enhanced nitrogen and energy conversion efficiency resulting in increased nitrogen retention at a reduced metabolic cost. The beneficial ammonia effect was not exhibited in ammonia-exposed trout coping with the additional stress of +2°C.

The present report presents an entirely parallel study conducted in winter. We posed the following questions: (i) will the additional +2°C induce greater physiological effects due to increased thermal sensitivity (Q10s) of trout at lower temperatures?, and (ii) are the beneficial effects of +70 µmol ammonia/L on nitrogen retention observed by juvenile trout over summer still likely to occur at such low water temperatures where ammonia is considerably more toxic (see Brown 1968; EIFAC 1973; Knoph 1992)? We have adopted the same approach used in our earlier study, employing a number of physiological indices to assess the long-term consequences and metabolic costs for juvenile trout exposed to a small increase in temperature and an environmentally relevant concentration of priority pollutant, alone and in combination. These include food intake, growth, metabolic rate, nitrogen balance, and proximate composition.

In this winter study, relatively large increases in oxygen consumption and nitrogenous waste production due to increased food intake occurred in the presence of +2°C. As a result, trout exposed to additional temperature, alone and in combination with 70 µmol ammonia/L, exhibited increased growth at only slightly higher energetic costs. Moreover, a metabolic depression similar to that of the 'warmed' fish fed maximally during peak summer water temperatures was exhibited by maximally fed trout grown at the 'base' thermal regime, indicating higher thermal sensitivity at these low winter water temperatures. Although nitrogen absorption efficiency was elevated in ammonia-exposed

fish, they generally did not retain more nitrogen than fish grown in the absence of the additional ammonia.

METHODS

Animal Acclimation

Juvenile rainbow trout (2-5 g) were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario in November 1993, approximately six weeks prior to testing. The trout were held in a 600 L polyethylene tank receiving approximately 2.5 L/min of dechlorinated and aerated Hamilton tap water ($[Ca^{2+}]$ = 0.88 mmol/L, $[Na^{+}]$ = 0.50 mmol/L, $[Cl^{-}]$ = 0.73 mmol/L; pH = 7.5) at an ambient water temperature of 4-6°C. They were fed a maintenance ration equivalent to 1% body weight (wet basis) every other day of Zeigler's Trout Starter #3 (50% protein, 15% lipid, 12% moisture), and kept under natural photoperiod for Hamilton, Ontario throughout the experiment.

Experiments

Groups of approximately 130 trout were randomly distributed among 8 tanks representing 4 treatments (260 fish per treatment). The tanks (270 L) received 2.1 - 2.3 L/min of either ambient temperature water (de-chlorinated City of Hamilton tap water drawn from the inshore region of Lake Ontario which from now on is referred to as base water temperature) or this water plus 2°C (base+ΔT), each with or without the addition of 70 µmol total ammonia/L (base+Am and base+ΔT+Am, respectively). The additional water temperature and T_{Amm} concentrations were achieved using heat exchangers and mariotte bottles respectively, as described in Linton et al. (1997). The mean difference between base and base+ΔT thermal profiles was 2.01 ± 0.21°C (Fig. 1). Mean tank water T_{Amm} concentrations were 76.6 ± 1.9, 8.3 ± 0.3, 9.5 ± 0.3, and 88.8 ± 1.8 µmol/L

(n=16) for base+Am, base, base+ΔT and base+ΔT+Am treatments, respectively, while tank water pHs were 7.50 $\pm$ 0.02 and partial pressures of oxygen were 144.7 $\pm$ 1.1 torr (approximately 90% saturation).

During the experiment, appetite was monitored by hand-feeding the fish in each tank to satiation twice daily (0830 h and 1630 h) from pre-weighed bags of trout feed, following the methods of Wilson et al. (1994). Food was distributed at 1 min. intervals. If uneaten food remained on the water surface for more than 2 min., feeding was discontinued. Routine in-tank rates of oxygen (O_2) consumption and nitrogenous (N) waste excretion (T_{Amm} + urea) were measured at 0, 30, 60, 75 and 90 days as detailed by Linton et al. (1997). The additional measurement (day 75) was made just as the water temperature was beginning to rise (see thermal profile in Figure 1).

Following the routine in-tank O_2 consumption and N-waste excretion measurements, blood and tissue samples were collected at days 0 (initial), 75 (at the end of the constant temperature phase), and 90 (during temperature rise) . Food was withheld from the fish 24 h before sampling. All fish were sampled according to the randomization procedure described in Linton et al. (1997). Twenty fish were netted from each tank and sacrificed. Ten of these fish were sampled for determination of whole body proximate composition (lipid, protein, and carbohydrate) and hematocrit and plasma composition (protein, T_{Amm}, and Na^+), while the other ten fish were sampled for T_{Amm} in white muscle.

For whole body proximate composition and blood sampling, fish were killed by a quick blow to the head, blotted dry, and their wet weight and total length were measured. A terminal blood sample was collected via caudal severance, the plasma saved, and the carcass freeze-clamped with aluminum tongs pre-cooled in liquid N_2. Both plasma and carcass were stored at -70°C until further analysis. Fish sampled for determination of

T_{Amm} in white muscle were handled in a similar manner, but were sacrificed by an overdose of MS-222 (1 g/L, buffered with 2 g/L $NaHCO_3$) to avoid T_{Amm} build-up in muscle due to struggling (Wang et al. 1994). A small portion of white muscle anterior to the dorsal fin and above the lateral line was excised and freeze-clamped immediately in liquid N_2, and stored at -70°C for further analysis.

Analysis

Ammonia concentrations in water were determined by the salicylate-hypochlorite method of Verdouw et al. (1978), and in plasma by a commercial enzymatic kit (Sigma no. 170-UV). The two assays were cross-validated. Water urea samples were freeze-concentrated 5-fold by lyophilization before assay by the modification of the diacetyl monoxime method described by Lauff and Wood (1996). The concentration of Na^+ in water and plasma was determined using atomic absorption spectrophotometry (Varian AA 1275).

Whole body proximate composition was obtained by grinding to a fine powder with a grinding mill (IKA - M10/M20) cooled to -40°C by a methanol/dry ice mixture, lyophilizing the powder for 72 h at -55°C, and determining: (i) protein content using a modification of the Lowry method (Miller 1959), (ii) lipid, after extraction in a 2:1 chloroform:methanol mixture, as described by Folch et al. (1957), and (iii) carbohydrate, as glucose plus glycogen plus lactate, following Bergmeyer (1985). A small portion of the frozen ground tissue was withheld and dried in an oven at 80°C for 48h to obtain the water content.

Frozen white muscle was ground into a fine powder in an insulated mortar cooled with liquid N_2. Subsamples (100 mg) were deproteinized in 1 ml of 8 % perchloric acid (PCA) and measured for ammonia as described by Kun and Kearney (1971).

Calculations

Cumulative food intake, expressed on a per fish basis for each treatment, was calculated daily on the basis of food consumed by each tank divided by the number of fish in the tank.

Specific growth rates (SGR) were determined for the interval between sampling days using the weights of the 40 fish removed from each treatment and calculated as:

$$SGR = 100(\ln_e Y_2 - \ln_e Y_1)/(t_2 - t_1)$$

where Y_1 and Y_2 are the mean wet weights of fish at times t_1 and t_2. Condition factors were determined as the quotient of the wet weight of the fish and its total length cubed, multiplied by 100.

The nitrogen quotient (NQ), or the extent of aerobic protein catabolism, was calculated from the O_2 consumption and N-waste excretion rates obtained from the in-tank measurements and expressed as the ratio of moles of N produced to moles of O_2 consumed (Kutty 1972). The proportion of oxygen consumption dedicated to protein catabolism was measured as the ratio of the NQ at each respective time period to the maximum aerobic value (0.27; Kutty 1972).

The nitrogen budget, or partitioning of consumed N (I) into N retained for growth (R_N) after excretion (E), was constructed following the equation summarized by Birkett (1969):

$$I - F_N = A = R_N + E$$

where F_N is the N lost in fecal and unaccounted material (mucus, etc.), and A is the N absorbed from food. Measurements of N consumption, retention, and excretion allowed for estimation of N lost through feces (and unaccounted N) and the N absorbed. Fecal and branchial and urinary N losses as well as N absorption and retention efficiencies are expressed as percentages of N consumed. The N content of food and whole body was determined from protein concentration measurements assuming that the N content of protein was 16% (Jobling 1980; Soderberg 1995).

The energy budget was derived from the equation:

$$C = F_E + U + \Delta B + R_E$$

where C is the total energy consumed from food, F_E (estimated from the measured components) is the energy lost in feces, U is the energy lost via branchial and urinary excretion, ΔB is the energy stored in the body (from measured whole body protein, lipid, and carbohydrate contents), and R_E is the total energy lost in metabolism. The values used to determine the energy content of food and body composition were 23.6, 39.5, and 17.2 kJ/g for protein, lipid, and carbohydrate, respectively (Braefield and Llewellyn 1982). Values of 24.9 kJ/g for non-fecal nitrogen (Cho and Kaushik 1990), and 13.6 kJ/g for O_2 (Elliott and Davison 1975), were used to determine energy lost from N-waste excretion (U) and O_2 consumption (R_E), respectively.

The N-cost index, representing the total moles of O2 consumed per mole of nitrogen stored (Linton et al. 1997), was used to compare the total metabolic expenditure associated with the incorporation of nitrogen into growth (as protein). The equation is given by:

$$\text{N-cost index} = R_E\ (t_1 \text{ to } t_2)/R_N\ (t_1 \text{ to } t_2)$$

where R_E and R_N are as defined above for the time interval between t_1 and t_2.

Statistics

The values for the nitrogen and energy budget are reported on a per fish basis from the single experimental tank in each treatment where O_2 consumption and N-waste excretion were measured. Cumulative food consumption was also calculated on a per fish basis (n = 2 tanks per treatment). For these values statistical procedures were not applicable. All other data are expressed as means ± 1 SE from individual samples pooled together from the two tanks per treatment. One-way analysis of variance using SAS Jmp (SAS Institute Inc., Version 2.0.5) followed by Tukey-Kramer honestly significant difference multiple-means comparison test was used to distinguish statistically significant differences amongst the four separate treatment groups within each sample period (75 and 90 days, respectively), while multiple factor analyses with leverage plots were employed to distinguish statistically significant temperature (+2°C) and ammonia (+70 µmol ammonia/L) effects. The level of statistical significance for all analyses was $P \leq 0.05$.

RESULTS

The thermal profile from the period of 20 January to 30 April 1994 was characterized by a relatively constant 4°C over the first 75 days, followed by a slow but gradual rise to 7 °C during the last 15 days (Figure 1). Warming the water by +2°C during winter greatly stimulated the appetite of juvenile trout (Figure 2). The total amount of food consumed per fish at ambient water temperatures was 7.7 and 7.4 g for base+Am and base treatments, respectively, whereas their +2°C counterparts were 11.2 and 11.0 g. As a

consequence, wet weight, total length, and SGR were significantly greater in the +2°C groups throughout the exposure (Figures 3A, 3B). SGRs (0-90 days) were 0.78 ± 0.07, 0.73 ± 0.05, 1.10 ± 0.06, and 1.08 ± 0.06 %/d for the base+Am, base, base+ΔT, and base+ΔT+Am groups, respectively. The addition of 70 μmol/L T_{Amm}, therefore, had no significant effect on appetite or growth in either temperature regime (Figures 2, 3).

Condition factors were significantly higher in the +2°C treatments after the first 75 days of exposure (Figure 3C), but not at day 90. The condition factors correlated well with whole body lipid content (Figure 4A). Protein content, which increased marginally in the first 75 days, was significantly elevated in fish exposed to +2°C (Figure 4B). During this time, the base+ΔT+Am group gained the most protein. Following the temperature rise, protein content fell to near initial values in fish from all treatments, which corresponded to the general increase in white muscle ammonia concentration (Figure 5). There were no differences in carbohydrate until day 90 (Figure 4C), when fish exposed to +2°C had significantly lower carbohydrate content. The greatest carbohydrate content was measured in fish sampled from the base+Am group. There were no significant differences in whole body water content throughout the exposure, although mean water content decreased to 73% during the exposure from an initial 76.0 ± 0.5%.

Like food consumption, routine O_2 consumption and N-waste excretion rates were 30-40% higher in fish exposed to +2°C throughout the exposure (Figure 6A and B), and in general, ammonia exposed fish oxidized more protein (as indicated by higher NQs; see Figure 6C). Urea comprised only a small portion of the total N-waste produced averaging a mere 13%. The proportion of the oxygen consumed that was dedicated to protein catabolism averaged 84 and 69% for base+Am and base+ΔT+Am groups, respectively, whereas the corresponding non-ammonia exposed groups averaged 43 and 47%. Yet despite the elevated protein catabolism, ammonia exposed fish retained the same amount of

N relative to the amount of N consumed due to a 14-21% higher N absorption efficiency (Table 1). An initial deficit in N retention in fish exposed to base+Am when compared with the base group between 0 and 75 d suggests at least some inhibition by ammonia at 4°C (Table 1).

Similar to the situation for N retention, fish at +2°C gained twice as much energy in body materials (ΔB), though there were no marked differences in conversion efficiency after 90 d of exposure (see 0-90; Table 2). This lack of difference in energy conversion efficiency appears to be related to trade-offs in fecal (F_E) and metabolic (R_E) energy loss. However, at the very low water temperatures to which the base group's were exposed between days 0 and 75 (4°C), energy conversion efficiency was noticeably reduced (Table 2), and more so in the base+Am group. As a result, this groups metabolic cost of N gain (N-cost index) was initially higher, but after base temperatures rose (from 4.5 to 6.5°C, see days 75-90 Fig. 1), it was markedly reduced (Table 2). The additional +2°C, in general, only slightly increased the metabolic costs of N gain (growth).

The effect of additional temperature and ammonia on hematocrit and plasma composition varied with the fluctuation in thermal regime, but were characterized by significantly higher plasma T_{Amm} and significantly lower hematocrit in fish exposed to ammonia, and significantly higher plasma protein in fish exposed to an additional 2°C (Table 3). Plasma Na^+ also tended to be lower in ammonia exposed fish, whereas additional temperature had little effect (Table 3).

DISCUSSION

Effects of an Additional +2°C

The impact of climate change on fish is a particular concern because of its potential influence on physiological and ecological rate processes, especially growth, which may

contribute to large-scale events such as geographic shifts and local extinction (Lin and Regier 1995). It is predicted that if warming should actually occur, ice-free time on the Great Lakes will increase (Shuter et al. 1983), and subsequently, the annual growth of yearling trout, providing that prey availability remains adequate (Hill and Magnuson 1990). In the present exposure over winter, trout exposed to the base thermal regime and fed to satiation twice daily achieved a SGR of only 0.7 %/d, whereas their +2°C counterparts achieved a rate of 1.1 %/d. By comparison, trout of equivalent age and fed to satiation over summer (base thermal regime rising from 13-21°C) grew at a rate of approximately 3.0 %/d, as did the trout at +2°C in summer (Linton et al. 1997). In this latter study, we concluded that the most significant effect of the +2°C warming scenario over summer exposure was the 30% reduction in food intake experienced by the warmed fish in the last 30 days, i.e., when water temperatures were approaching their upper lethal maximum (Bidgood and Berst 1969). A significant temperature-dependent feeding effect was also experienced by the warmed fish of the present study, however this time, their food consumption increased by over 30%. It is evident here that simulated winter warming promotes the growth of juvenile rainbow trout on unlimited ration, which is in agreement with earlier predictions (Hill and Magnuson 1990). In contrast, Wurtsbaugh and Davis (1977) examined the effect of temperature and ration level on growth and conversion efficiency in rainbow trout during 25 d seasonal experiments. Simulated warming of 3 and 6°C were superimposed on the natural fluctuating thermal regime of a small trout stream. The authors reported very little change in the winter (January to February 1972) growth rates of warmed trout (2 g) consuming rations equivalent to those used in the present study (4-5 % /day-dry basis). However, the water temperatures in their study were much more variable (e.g., range of 3.9 to 10.1°C around the mean base temperature of 6.9°C) and the exposure period much shorter (by 65 d) in comparison to the present study. Our results

were more in line with those of Elliott (1976), who reported a 5 fold increase in stored energy (ΔB) between maximally fed brown trout *Salmo trutta* weighing 50 g and exposed to constant temperatures of either 3.8 or 5.6°C.

The +2°C warming scenario, though stimulating growth in juvenile trout, did not give rise to an appreciable change in whole body proximate composition. Lipid and protein content (Figure 4) were significantly different only at 75 d following the period of relatively constant temperatures at 4 and 6°C, respectively (Figure 1). Differences in carbohydrate, on the other hand, did not occur until after the temperature began to rise (days 75 - 90). Whole body proximate composition was considerably altered in comparison to juvenile trout exposed during summer (see Linton et al. 1997); in particular, fat content was less than about half the values previously reported. This last observation supports earlier findings that trout preferentially use lipid as metabolic fuel over winter (Swift 1955), thus conserving valuable protein for growth. In addition, the whole body proximate compositions and water contents of juvenile rainbow trout in the present study were similar to fingerling sockeye salmon *Oncorhynchus nerka* fed equivalent rations and acclimated to 5°C (Brett et al. 1969). However, lipid content in the present study was approximately 30% lower, most likely due to differences in dietary composition. The SGR of these salmon was only 0.6 %/d.

Elevated rates of O_2 consumption and N-waste excretion were associated with the increased feeding of warmed fish, which may also explain the increase in plasma protein (Nose 1972). Soofiani and Hawkins (1982) showed that the metabolic rate of Atlantic cod *Gadus morhua* increased linearly with ration size. Jobling (1983) suggested that the magnitude of the increase in O_2 consumption observed after feeding (SDA) is reflective of the cost of growth. This hypothesis was later confirmed through an ingenious experiment

designed by Brown and Cameron (1991) using channel catfish, *Ictalurus punctatus*. Blockade of growth associated protein synthesis eliminated the SDA effect.

Along with the elevated O_2 consumption and N-waste production over the first 75 d, fish exposed to the additional +2°C also exhibited substantial increases in both N absorption and energy conversion efficiencies (Tables 1, 2). As a consequence, the temperature coefficients (Q_{10}s) for appetite and other related physiological processes (growth, oxygen consumption, and N-waste excretion) were extraordinarily high, averaging 19.6. The differences in response may be related to temperature-dependent metabolic depression (Hazel 1993) of fish at these extreme low temperatures, which is parallel to the metabolic suppression observed in warmed fish at peak summer temperatures (Reid et al. 1995; Linton et al. 1997). Similarly, the Q_{10} for rates of oxygen consumption in American eels *Anguilla rostrata* increased dramatically at temperatures below 10°C (Walsh et al. 1983). During the last 15 days of the present experiment, the water temperatures rose 2°C from approximately 4.5 and 6.5°C to 6.5 and 8.5°C in the base and base+ΔT thermal regimes, respectively (see Figure 1). Net energy conversion efficiency in warmed fish was actually lower than in base fish during this period, and the mean temperature coefficient (Q_{10}) for all the physiological parameters measured dropped to 6.6. We suspect that the changes were due to physiological adjustments to rising water temperature that allowed the colder acclimated fish (base) to compensate for the imposed metabolic depression, possibly even promoting a compensatory growth response after relief from a long period of depressed feeding (Kim and Lovell 1995).

Effects of Elevated Ammonia

Over-wintering juvenile trout exposed to additional ammonia, although exhibiting a similar elevation in N-waste excretion and NQ (protein turnover) as in summer (see Linton

et al. 1997), did not show greater growth (Figure 3), and their appetite, again, was unaffected (Figure 2). In the summer exposure, with the exception of fish exposed to the combination of +2°C and ammonia at peak water temperatures, the stimulated N retention (as protein) was associated with higher rates of protein synthesis (unpublished data from our laboratory). At the very low winter temperatures of the present study, ammonia exposed fish exhibited greater N absorption (Table 1), but protein synthesis was not elevated. Houlihan et al. (1995) observed that trout with higher nitrogen retention efficiencies were found to have reduced rates of protein degradation, and consequently, a lower cost of growth per mole of N gain. Tomas et al. (1991) suggested that protein degradation rates are genetically determined, while protein synthesis rates were more responsive to ration and environmental factors such as temperature.

The ammonia loading effect exhibited by fish exposed to +70 μmol ammonia/L in the present study (Table 3) is similar to rainbow trout exposed to high external pH, i.e., there is an initial high-pH-induced blockade of ammonia excretion at the gill that, together with a possible up-regulation of hepatic ammonogenesis, increases plasma T_{Amm} concentrations until a favorable outward P_{NH3} gradient is achieved (Wilkie and Wood 1995). Such a mechanism was proposed by Linton et al. (1997) as a plausible explanation for the elevated plasma T_{Amm} levels in juvenile trout exposed to 70 μmol ammonia/L during summer. In contrast to Wilkie and Wood (1991, 1995), however, the elevated plasma T_{Amm} in trout exposed during summer did not lead to increased storage of ammonia in white muscle. It is interesting to note that in the present study as water temperature increased (days 75 - 90), white muscle ammonia increased in accord with a decrease in whole body protein content, suggesting a possible link between white muscle ammonia concentration and protein turnover. The concentration of ammonia in the white muscle of trout fed to satiation and exposed over winter in general is comparatively high

(see Linton et al. 1997) which may be a reflection of the overall decrease in ammonia diffusion similar to other metabolites and respiratory gases within tissues at low temperature (Sidell and Hazel 1987).

We suggest that ammonia-induced detoxification may be linked with the increased protein turnover in rainbow trout. The slight anemic response and elevated plasma T_{Amm} in ammonia exposed fish are indicative of mild ammonia intoxication (Buckley et al. 1979; Thurston et al. 1984). Carp exposed to sublethal ammonia concentrations that were approximately 10-fold higher than the concentrations reported here exhibited a 24% increase in the level of free amino acids in muscles (Dabrowska and Wlasow 1986). Moreover, when mudskipper *Periophthalmus cantonensis* were loaded internally (via infusion) with ammonia, amino acid production was stimulated (Iwata et al. 1981) - a mechanism which could serve for ammonia detoxification via enzymes such as glutamine synthetase (Levi et al. 1974, Arillo et al. 1981).

In contrast to summer exposure, the elevated plasma T_{Amm} in ammonia-exposed trout over winter was not correlated with a simultaneous increase in plasma Na^+, but rather, a slight decrease (Table 3). Twitchen and Eddy (1994) recently showed that juvenile trout exposed to acute sublethal ammonia exhibited a Na^+ imbalance that was characterized by an increase in Na+ efflux, however, the external T_{Amm} concentration that first caused the imbalance in their study was approximately 40 times greater than the one used here.

The focus of this study was to provide hard experimental data on the metabolic and physiological effects of chronic small increases (+2°C) in water temperature with or without sublethal pollutant exposure (70 μM ammonia) on over-wintering rainbow trout as compared to a similar summer exposure (see Linton et al. 1997). The results clearly show a simulated warming of +2°C superimposed on the base temperature regime over winter

stimulates the appetite of juvenile rainbow trout, eventually leading to greater growth. Although some additional metabolic cost was incurred as temperature rose, the extra energetic cost over the 90 d period was negligible. The overall cost of growth for juvenile trout fed to satiation in winter was similar to the costs incurred by maximally feeding trout in summer owing to stoichiometrically equivalent reductions in the amounts of oxygen consumed and N retained. The low winter temperatures of the present exposure also acted to moderate the ammonia effects experienced by trout during summer, resulting in a lack of growth stimulation at base temperatures and no elevation of metabolic costs at +2°C. We conclude that an increase in ambient water temperature of only +2°C over winter, both in the presence or absence of sublethal ammonia, will promote the growth of juvenile trout at no additional metabolic cost, placing these fish at an ecological advantage leading into the summer growth period.

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Figure. 1. Daily water temperature profile as measured each day over 90-d exposures from January to April 1994. Juvenile rainbow trout were exposed to either ambient laboratory water temperatures (base = solid line) or to this water + 2°C (base+ΔT = dashed line).

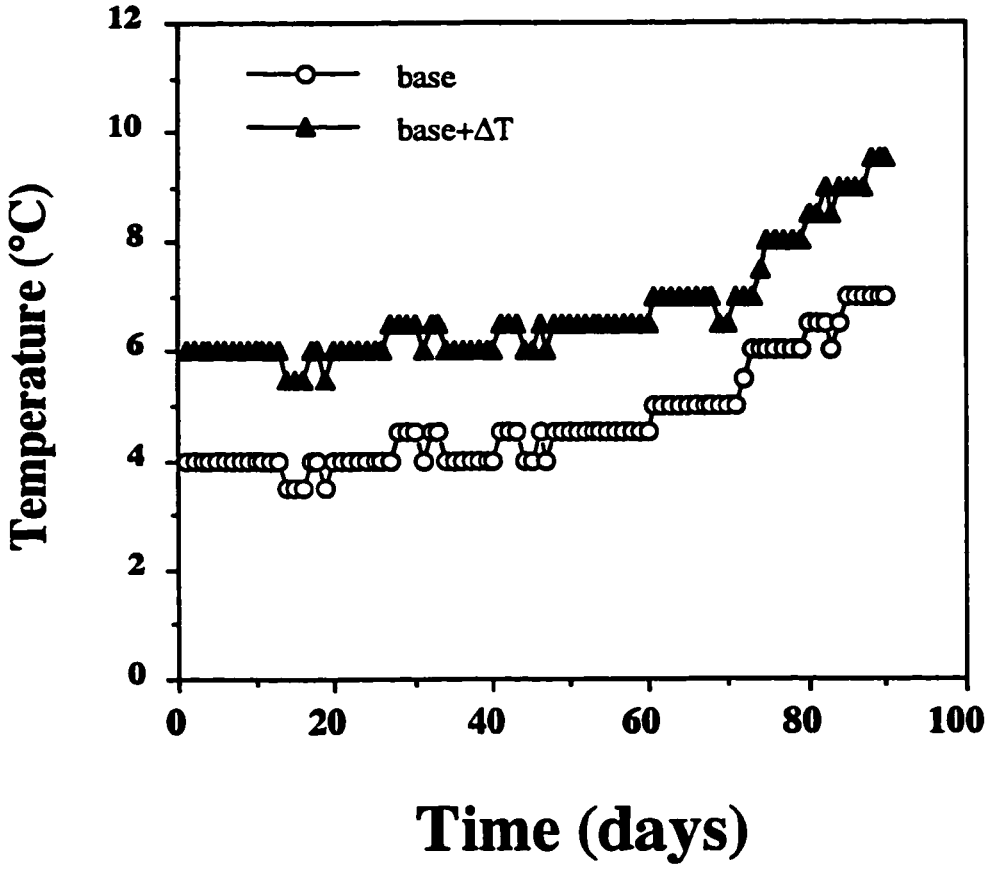
12
10
8
6
4
2
0
base
base+ΔT
Temperature (°C)
0
20
40
60
80
100
Time (days)

Figure. 2. Effects of +2°C (ΔT) and 70 μmol ammonia/L (Am) on appetite of juvenile rainbow trout fed to satiation twice daily. Appetite was measured as cumulative food intake over the 90-d exposures. The data are presented on a per fish basis. Each value represents the mean of the two replicate tanks per treatment (N = 2).

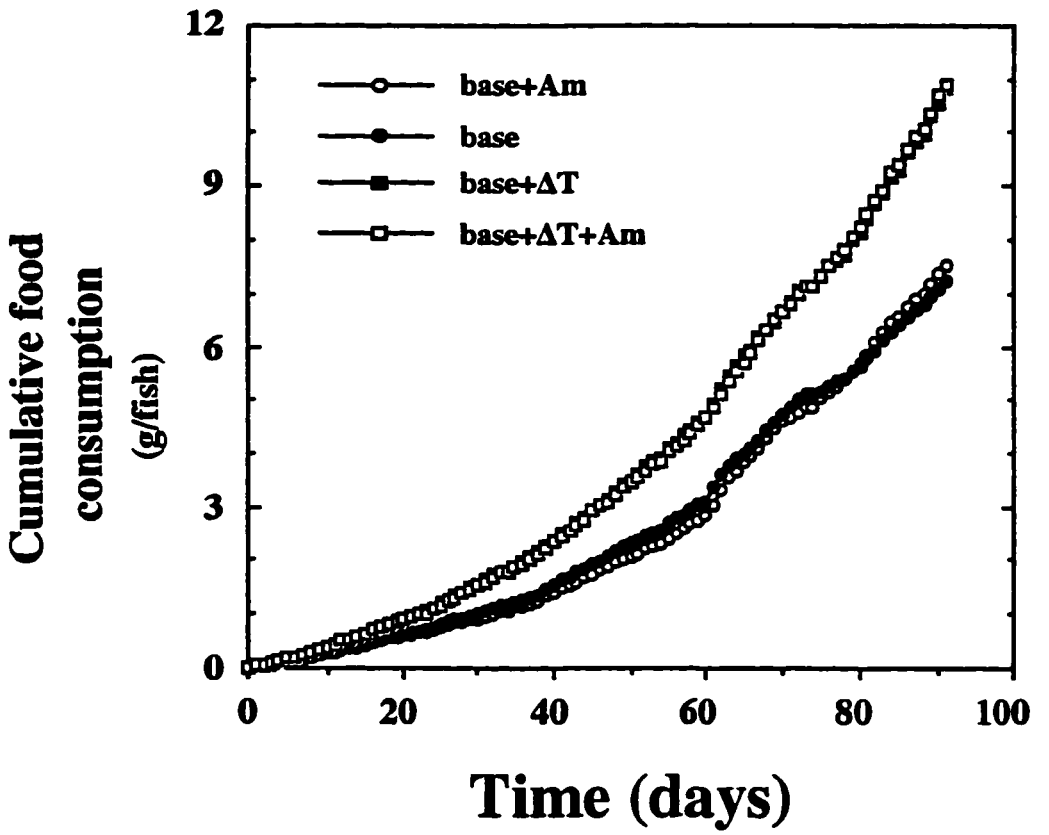
Cumulative food consumption (g/fish)
12
9
6
3
0
0
20
40
60
80
100
Time (days)
base+Am
base
base+ΔT
base+ΔT+Am

Figure. 3. Effects of +2°C (ΔT) and 70 µmol ammonia/L (Am) on (A) wet weight, (B) total length, and (C) condition factor of juvenile rainbow trout fed to satiation twice daily. The data are from groups of fish sampled initially (N = 20) and after 75 and 90 days of exposure (N = 40). Each value represents the mean + SE of fish sampled from the two duplicate tanks per treatment. Columns with the same letter on the same sampling day are not significantly different ($P > 0.05$).

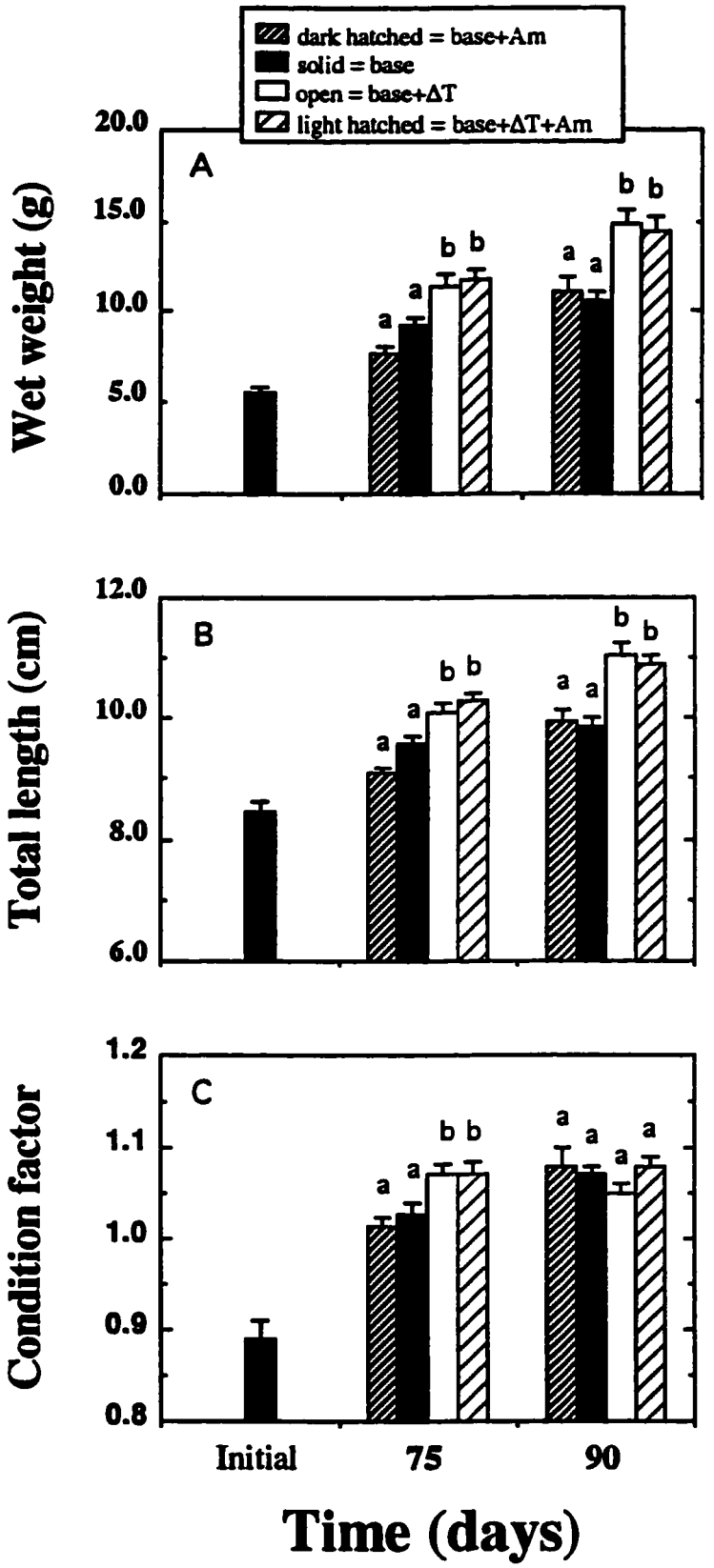
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
Wet weight (g)
20.0
15.0
10.0
5.0
0.0
B
Total length (cm)
12.0
10.0
8.0
6.0
C
Condition factor
1.2
1.1
1.0
0.9
0.8
Initial
75
90
Time (days)

Figure. 4. Effects of +2°C (ΔT) and 70 μmol ammonia/L (Am) on lipid (A), protein (B), and carbohydrate (C) content of juvenile rainbow trout fed to satiation twice daily. The data are from groups of fish sampled initially (N = 10) and after 75 and 90 days of exposure. Each value represents the mean + SE of fish sampled from the two duplicate tanks per treatment (N = 10 per treatment per time period). All values are reported as percentage composition (mg/100 mg) of wet tissue. Columns with the same letter on the same sampling day are not significantly different ($P > 0.05$).

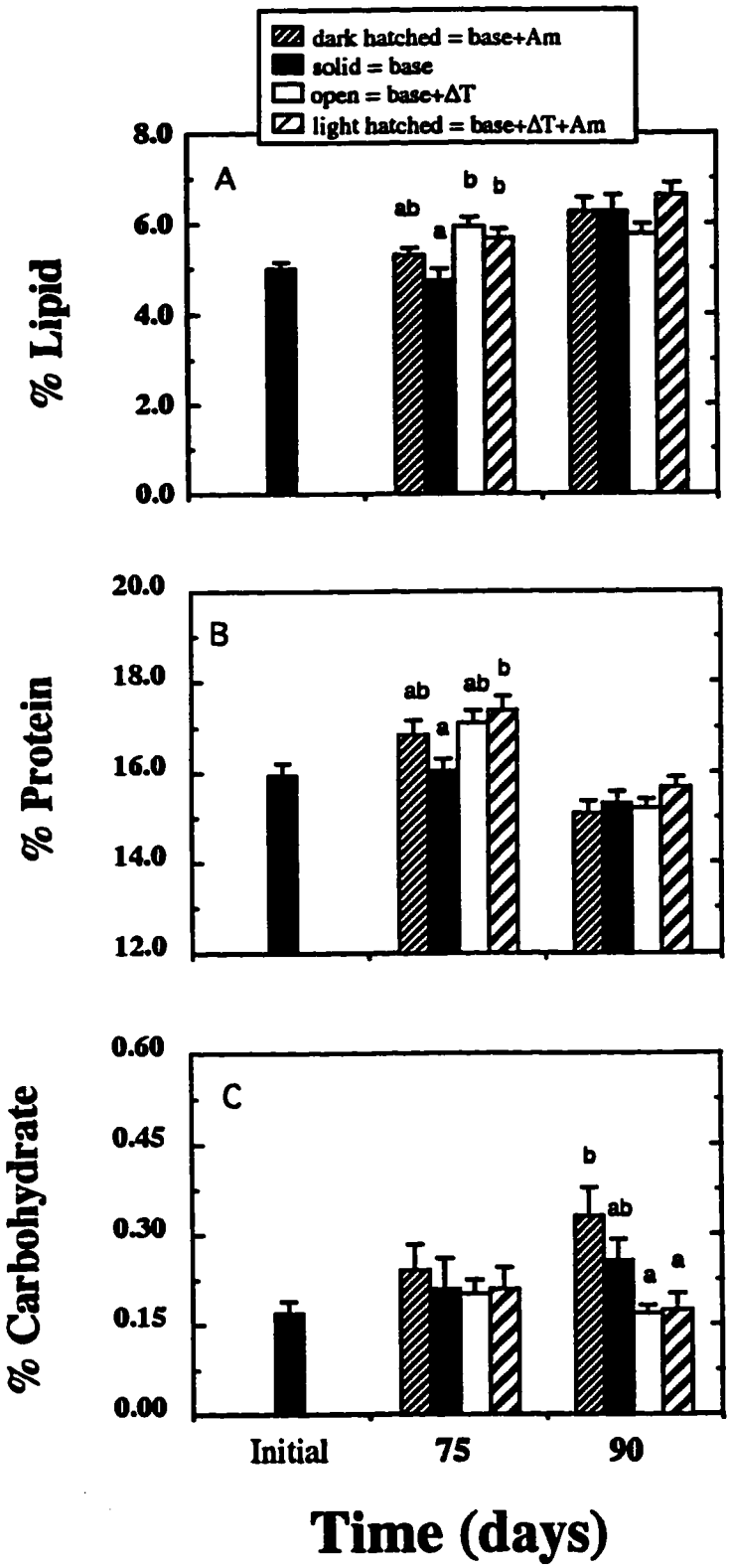
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
B
C
% Lipid
% Protein
% Carbohydrate
8.0
6.0
4.0
2.0
0.0
20.0
18.0
16.0
14.0
12.0
0.60
0.45
0.30
0.15
0.00
ab
a
b
b
ab
a
ab
b
b
ab
a
a
Initial
75
90
Time (days)

Figure. 5. Effects of +2°C (ΔT) and 70 μmol ammonia/L (Am) on white muscle total ammonia concentration of juvenile rainbow trout fed to satiation twice daily. The data are from groups (N = 10) of fish sampled initially and after 75 and 90 days of exposure. Each value represents the mean + SE of fish sampled from the two duplicate tanks per treatment. Columns with the same letter on the same sampling day are not significantly different ($P > 0.05$).

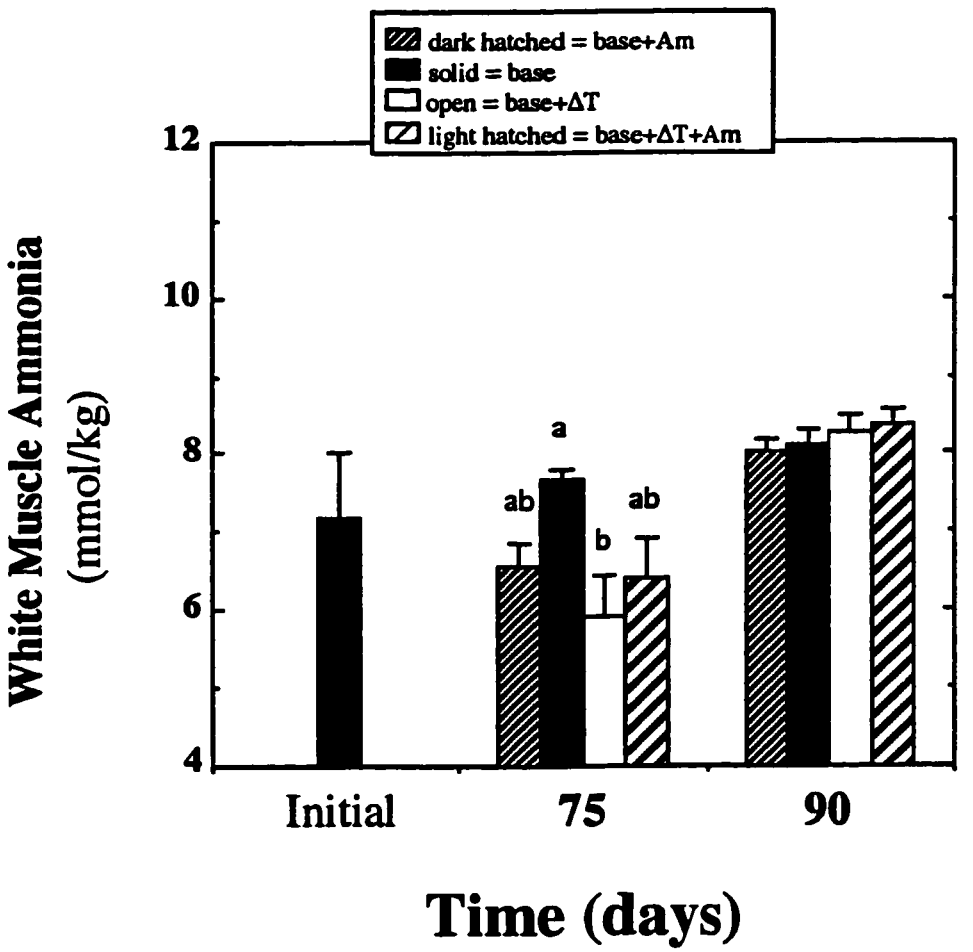
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
White Muscle Ammonia
(mmol/kg)
12
10
8
6
4
a
ab
b
ab
Initial
75
90
Time (days)

Figure. 6. Effects of +2°C (ΔT) and 70 μmol ammonia/L (Am) on (A) routine in-tank oxygen consumption (N = 5 measurements on the same tank), (B) routine in-tank N-waste excretion (ammonia + urea N; N = 6 measurements on the same tank), and (C) the nitrogen quotients of juvenile rainbow trout fed to satiation twice daily. In (A) and (B) the data have been scaled for weight as detailed in Linton et al. (1997). Measurements were made on one tank of individuals per treatment. Error bars represent measurement SE (of the mean O_2 consumption and N-waste excretion rates calculated over the respective sample intervals) and cannot be used for statistical comparisons between treatments.

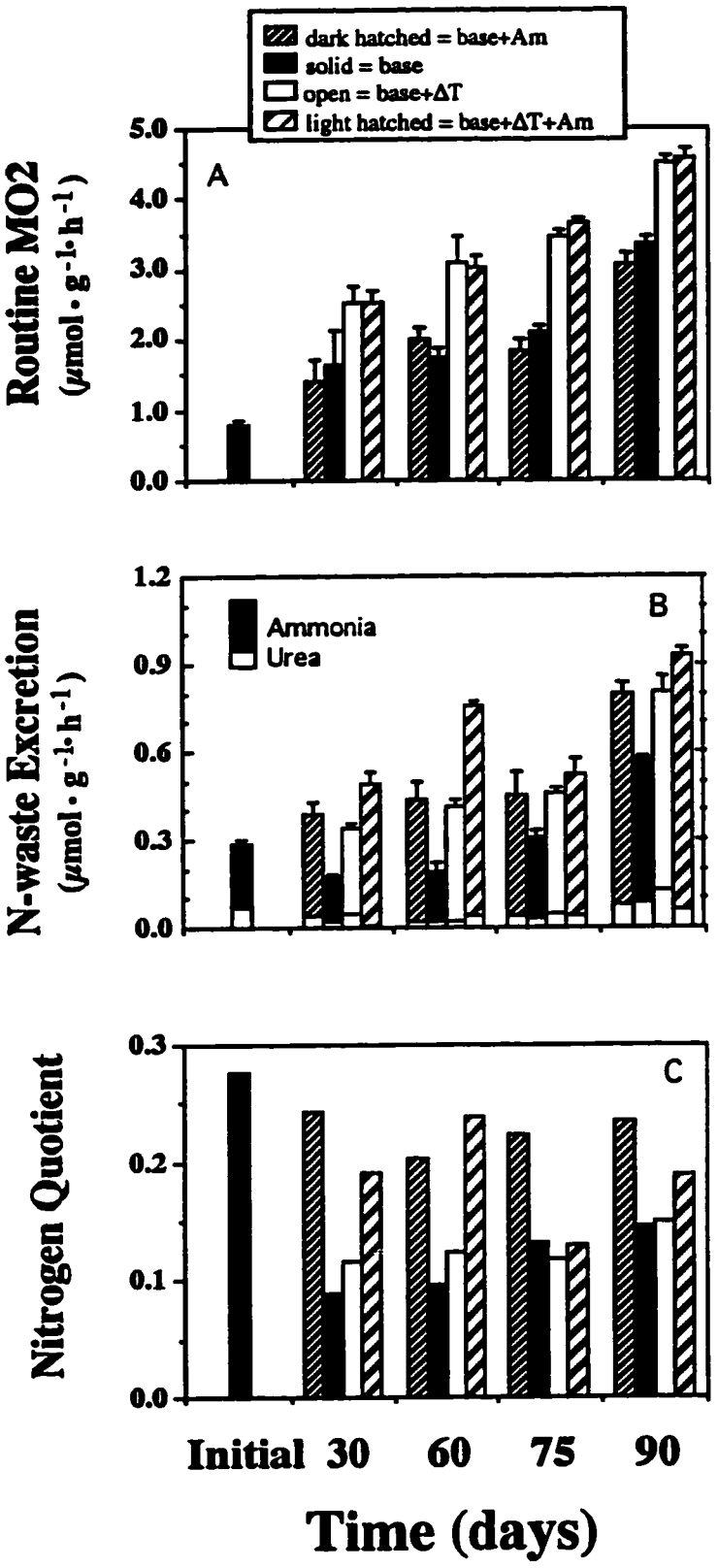
dark hatched = base+Am
solid = base
open = base+ΔT
light hatched = base+ΔT+Am
A
Routine MO2
(μmol • g-1• h-1)
5.0
4.0
3.0
2.0
1.0
0.0
B
Ammonia
Urea
N-waste Excretion
(μmol • g-1• h-1)
1.2
0.9
0.6
0.3
0.0
C
Nitrogen Quotient
0.3
0.2
0.1
0.0
Initial
30
60
75
90
Time (days)

Table 1. Nitrogen budgets for juvenile rainbow trout fed to satiation for 90 d (January to April 1994) and exposed to a +2°C warming scenario (ΔT) and 70 μmol/L ammonia (Am).

Interval and treatment	Nitrogen (mmol/fish)[a]					Retention Efficiency[b] (%)	Absorption Efficiency[c] (%)
	I	E	R_N	F_N	A		
Days 0 - 75							
base+Am	27.7	10.6	4.9	12.2	15.5	17.5	55.9
base	29.1	5.3	7.0	16.9	12.3	23.9	42.1
base+ΔT	40.9	11.6	12.2	17.0	23.9	29.9	58.4
base+ΔT+Am	40.7	19.4	13.5	7.8	32.9	33.1	80.9
Days 75 - 90							
base+Am	16.2	4.7	4.4	7.1	9.1	27.2	56.2
base	13.2	3.0	1.6	8.6	4.6	11.9	34.6
base+ΔT	22.2	6.1	3.5	12.6	9.6	15.6	43.3
base+ΔT+Am	23.2	7.8	3.7	12.9	10.3	16.0	44.2
Days 0 - 90							
base+Am	44.0	15.4	9.3	19.3	24.6	21.1	56.0
base	42.3	8.3	8.5	25.5	16.8	20.2	39.7
base+ΔT	63.1	17.8	15.7	29.6	33.5	24.9	53.1
base+ΔT+Am	63.9	27.2	16.0	20.7	43.2	25.0	67.6

[a]I = total N consumed; E = N lost via branchial and urinary excretion; R_N = N retained in body materials; F_N = N lost in fecal material and all unaccounted N; A = N absorbed from food.

[b]Retention efficiency = 100R/I.

[c]Absorption efficiency = 100A/I.

Table 2. Energy budgets and metabolic costs of growth (N-cost index) for juvenile rainbow trout fed to satiation for 90 d (January to April 1994) and exposed to a +2°C warming scenario (ΔT) and 70 µmol/L ammonia (Am).

Interval and Treatment	Energy (kJ/fish)[a]					Conversion efficiency[b] (%)	N-cost index
	C	U	ΔB	R_E	F_E		
Days 0 - 75							
base+Am	86.0	3.7	15.6	21.2	45.4	18.1	10.0
base	90.4	1.8	21.1	21.1	46.3	23.4	7.0
base+ΔT	126.8	4.1	41.4	40.3	41.0	32.7	7.6
base+ΔT+Am	126.2	6.8	43.8	41.3	34.3	34.7	7.0
Days 75 - 90							
base+Am	50.4	1.6	20.7	10.0	18.0	41.1	5.2
base	41.2	1.1	12.2	9.4	18.5	29.7	13.8
base+ΔT	69.1	2.1	14.3	19.2	33.5	20.7	12.7
base+ΔT+Am	71.9	2.7	16.6	20.3	32.5	23.1	18.8
Days 0 - 90							
base+Am	136.4	5.4	36.3	31.3	63.4	26.6	7.6
base	131.3	2.9	33.4	30.5	64.6	25.4	8.2
base+ΔT	195.7	6.2	55.7	59.4	74.4	28.5	8.7
base+ΔT+Am	198.0	9.5	60.4	61.5	67.7	30.5	8.8

[a]C = total energy consumed from food; U = energy lost via excretion (branchial and urinary); ΔB = energy stored in body materials; R_E = total metabolic energy lost as heat; F_E = energy lost in feces and unaccounted energy.
[b]Conversion efficiency = 100ΔB/C.

Table 3. Hematocrit and plasma protein, total ammonia (T_{Amm}), and sodium (Na^+) of juvenile rainbow trout fed to satiation and exposed to a +2°C warming scenario (ΔT) and 70 µmol/L ammonia (Am) from January to April 1994. Fish were sampled initially and after 75 and 90 days of exposure. Results are expressed as means ± SE of 10 fish (5 fish from each duplicated tank per treatment) per sample period. For each sampling day separately, values within a column without a letter in common are significantly different ($P \leq 0.05$).

Sampling day and Treatment	Protein (g/dL)	Hematocrit (% RBC)	T_{Amm} (µmol/L)	Na^+ (mmol/L)
Initial	3.9 ± 0.20	37.5 ± 0.9	784 ± 51	-
Day 75				
base+Am	4.4 ± 0.3 ab	31.8 ± 1.5 a	712 ± 82 ab	161 ± 3 a
base	4.2 ± 0.2 a	36.1 ± 2.3 a	539 ± 36 a	162 ± 4 ab
base+ΔT	5.0 ± 0.2 b	36.6 ± 3.4 a	606 ± 29 a	169 ± 6 a
base+ΔT+Am	5.1 ± 0.1 b	30.9 ± 1.9 a	774 ± 56 b	155 ± 5 b
Day 90				
base+Am	4.6 ± 0.2 b	35.6 ± 2.6 a	598 ± 56 a	154 ± 2 a
base	5.1 ± 0.2 a	34.0 ± 1.5 a	495 ± 27 a	157 ± 2 a
base+ΔT	5.2 ± 0.1 a	33.4 ± 1.5 a	338 ± 32 b	158 ± 3 a
base+ΔT+Am	5.3 ± 0.1 a	35.5 ± 1.6 a	490 ± 30 a	153 ± 2 a

CHAPTER 4

EFFECTS OF RESTRICTED RATION ON THE PHYSIOLOGY, GROWTH, AND ENERGETICS OF JUVENILE RAINBOW TROUT EXPOSED TO A SUMMER OF SIMULATED WARMING AND SUBLETHAL AMMONIA

SYNOPSIS

Juvenile rainbow trout (*Oncorhynchus mykiss*) were fed a fixed restricted ration equivalent to 1% wet body weight·d^{-1} to determine their physiological and bioenergetic responses to a simulated global warming scenario of +2°C superimposed above the ambient water temperature while in the presence or absence of 70 μM total ammonia. The 90-d exposures lasted from June to September 1994, and were designed to mimic an earlier study (Linton et al. 1997a) where juvenile trout were fed to satiation. The restricted ration markedly increased (4 - 7 fold) the metabolic costs of nitrogen gain for juvenile trout growing over summer. This dramatic increase in 'cost of growth' was a result of high maintenance energy requirements in the face of minimal fuel supply. For instance, fish from the present study exhibited O_2 consumption and specific growth rates that were 50 - 70% and 15 - 20%, respectively, of the rates of the fish fed to satiation. Whole body protein content was generally maintained throughout the experiment, but lipid content was reduced within the first 30 days by as much as 33%. Total body carbohydrate, on the other hand, nearly doubled by day 60. This latter effect was concurrent with a reduction in liver ammonia, although there was negligible reduction in liver urea and white muscle ammonia and urea. Overall, trout exposed to the additional +2°C managed to retain more nitrogen for growth but at a slightly increased energetic cost. Fish exposed to +70 μM ammonia also exhibited higher energetic costs, but this was accompanied by a

comparatively large increase in nitrogen retention efficiency. Thus, their 'costs of growth' were reduced. We conclude that a restricted ration of $1\%\cdot d^{-1}$ will not further impair the ability of juvenile rainbow trout to cope with a chronic small temperature increase. Moreover, sublethal ammonia may even be beneficial under these circumstances.

INTRODUCTION

Global warming forecasts predict a mean rise in ambient air temperature from 1 - 2 °C over the next 30 years (Mohnen and Wang 1992), with a concurrent increase in water temperature (Meisner et al. 1987). The implications for fish production and distribution are profound (Christie and Regier 1988), because the body temperatures of fish, and hence, their metabolic and feeding rates, are set by the temperature of their environment (Brett et al. 1969, Elliott 1976). Thus, small changes in local temperature could have important consequences on physiological and ecological processes of fish, such as growth, energetics, and ultimately geographical distribution (Lin and Regier 1995).

An important factor influencing chronic temperature effects is ration. Prey abundance and availability is likely to be dramatically altered as a direct result of warming which could pose a serious problem for fish already adjusting to a small temperature increase (Hill and Magnuson 1990). Food availability is a powerful temperature-dependent selective force affecting growth of aquatic ectotherms (Baldwin 1957; Paloheimo and Dickie 1966). For any given fixed ration, as water temperature increases, maintenance energy requirements also increase (Elliott 1982) so that at higher water temperatures, less energy is available for synthesis of new body materials (Mehner and Wieser 1994). Therefore, growth under a fixed ration will reflect the individual's ability to repartition the resources available from ingested fuel rather than to increase fuel consumption (Jobling 1997).

The present study was conducted to determine the potential effects of chronic temperature change and marginal water quality on the physiology and energetics of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a restricted ration (1 $\%\cdot d^{-1}$ wet). This work differs from most previous studies in that the fish were exposed under controlled laboratory conditions to a naturally fluctuating summer water temperature regime. The

warming scenario of +2°C above the natural summer thermal regime was chosen to mimic predictions based on current climate models (see above), but it also has relevance to other warming scenarios such as elevations in stream temperatures after the removal of riparian vegetation (Holtby 1988, Crawshaw and O'Connor 1997). The approach is similar to our previous experiments under different conditions (see Linton et al. 1997a) in which we monitored food intake, growth, metabolic rate, proximate composition, and tissue and blood metabolites to assess the long-term physiological consequences and metabolic costs to juvenile trout of simulated warming in conjunction with environmentally realistic levels of a common pollutant, 70 μM total ammonia (equivalent to 0.013 mg NH_3-N/l at 15°C and pH = 7.6).

Climate warming is expected not only to increase water temperature, but also to increase the concentration of contaminants such as ammonia in the physical environment (Coutant 1981, Vitousek 1994). Ammonia is highly toxic to fish and ubiquitous in surface waters (Russo 1985); more molecules of this pollutant are manufactured each year than any other industrial chemical (Atkins 1987). Since ammonia is also a natural biological degradation product of nitrogenous organic matter, it is a particularly good candidate for examining sublethal environmental pollutant effects in conjunction with temperature. The lowest lethal concentration of un-ionized ammonia found for salmonids is 0.083 mg NH_3-N/l (Thurston et al. 1984), but several sublethal effects have been reported at concentrations as low as 0.010 mg NH_3-N/l (Meade 1985).

In the wild, a fish's natural feeding regime usually lies somewhere between maintenance and satiation rations (Boisclair and Marchand 1993). Comparison of the results of the present study with those of our previous satiation feeding experiment (Linton et al. 1997a) provides an excellent opportunity to quantify the metabolic cost of living for trout coping with environmental change under these extremes in food availability. Based

on our previous results (Linton et al. 1997a), where appetite, oxygen consumption, and protein metabolism were seriously impaired in satiation-fed trout during maximum summer water temperatures, we predicted that food limitation might impose further restriction on the ability of juvenile trout to compensate for small chronic temperature change. It is also reasonable to predict that, under food restriction, growth may not be stimulated by sublethal ammonia as seen in juvenile trout fed to satiation over summer (Linton et al. 1997a). Finally, in this earlier experiment, the +2°C and +70 μM ammonia combination increased the overall metabolic cost of living. We predicted that this effect might become even more pronounced under food constraint at high summer temperature.

MATERIALS AND METHODS

Experimental Animals and Protocol

Juvenile rainbow trout (approximately 4.5 g) were acquired from Rainbow Springs Trout Farm, Thamesford, Ontario in the spring of 1994. The trout were held in a 600 l aerated polyethylene tank receiving approximately 2.5 $l \cdot min^{-1}$ dechlorinated Hamilton tapwater ($[Ca^{2+}] = 0.94 \pm 0.11$ mM, $[Na^{+}] = 0.60 \pm 0.03$ mM, $[Cl^{-}] = 0.75 \pm 0.01$ mM; pH = 7.5 ± 0.02) for 5 weeks prior to commencing the exposures. During this period water temperatures ranged from 11 to 13°C. The fish were fed approximately 1% wet body $weight \cdot d^{-1}$ of Zeigler's Trout Starter #3 (50% protein, 15% lipid, 8% moisture). Simulated natural photoperiod for Hamilton, Ontario was used throughout the acclimation and experimental periods.

On 20 June 1994, groups of 150 fish were randomly distributed to 8 tanks comprising 4 treatments (i.e., 2 replicates per treatment, 300 fish per treatment). The tanks (270 l) received 2.0 $l \cdot min^{-1}$ of water at either ambient temperature (referred to as the 'base' water temperature treatment) or at this temperature plus 2°C (base+2°C), each with or

without an additional 70 μM total ammonia ($T_{Amm} = NH_4^+ + NH_3$; base+Am and base+2°C+Am, respectively). Water at +2°C was achieved by a heat exchanger while water containing +70 μM T_{Amm} was achieved by delivering the required amount of $(NH_4)_2SO_4$ stock solution via mariotte bottles (Mount and Brungs 1967). The base water temperature reflects the natural thermal profile of in-shore Lake Ontario. Water quality and ammonia concentrations are shown in Table 1.

During the exposure, biomass was measured weekly by bulk weighing to gain frequent measures of the weight increment of fish on low ration. Fish were netted from the tanks into a bucket containing 10 l of the appropriate water and a removable plastic sieve. The bucket and contents were weighed on a tared scale (GSE 450 Scale Systems, Michigan, U.S.A.). The fish were seined and immediately placed back into their original tank. The bucket and contents without the fish was reweighed, the difference yielding total fish biomass. Each group was subsequently fed a restricted ration calculated on a per fish basis equivalent to 1% of the base treatment group's mean wet body weight·d^{-1} (0.5% at 0830 h and 0.5% at 1630 h).

Immediately after commencing the test, and every 30 days thereafter, routine 'in-tank' oxygen (O_2) consumption rates were estimated in one of the two replicate tanks per treatment (see Linton et al. 1997a for details). In brief, in order to obtain daily routine O_2 consumption, five measurements were made over an 8 h period beginning at 0700 and ending at 1500 h. To begin these measurements, the water and air feeding the holding tanks were turned off. Each tank was sealed, a recirculation pump was started, and water samples were withdrawn at 10 min. intervals up to total of 30 min. The mean decline in partial pressure of O_2 was obtained with a Cameron O_2 electrode and OM 200 oxygen meter. Water PO_2 was not allowed to drop below 100 torr. Between measurements (approximately 1.5 h) tanks were re-aerated and flushed. The O_2 consumption rates

reported were derived from the integrated O_2 consumption rates measured between 0700 and 1500 h (Sigma Scan). This mean O_2 consumption rate was then corrected for differences in fish size (standardized to a 1 kg fish) using the weight exponent 0.824 determined for rainbow trout by Cho (1990). Fish were fed at their regular time in the morning (0830 h) in between O_2 measurements.

Food was withheld from the fish the afternoon (1630 h) following the routine O_2 consumption measurements prior to sampling whole bodies, blood, and tissues, i.e., days 1, 31, 61, and 91. All fish were sampled according to the randomization procedure described in Linton et al. (1997a). Twenty fish were netted from each tank and sacrificed. Ten of these fish were sampled for determination of whole body proximate composition (lipid, protein, and carbohydrate) and hematocrit and plasma composition (protein, total ammonia, and Na^+), while the other ten fish were sampled for total ammonia (T_{Amm}) and urea in liver and white muscle. For whole body proximate composition and blood sampling, fish were killed by a quick blow to the head, blotted dry, and their wet weight and total length were measured. A terminal blood sample was collected via caudal severance, the plasma saved, and the carcass freeze-clamped with aluminum tongs pre-cooled in liquid N_2. Both plasma and whole bodies were stored at -70°C until further analysis. Fish sampled for determination of T_{Amm} and urea in tissue were handled in a similar manner, but were sacrificed rapidly by an overdose of MS-222 (1 $g \cdot l^{-1}$) buffered with $NaHCO_3$ (2 $g \cdot l^{-1}$) to avoid T_{Amm} build-up in muscle due to struggling (Wang et al. 1994). The liver and a small portion of white muscle anterior to the dorsal fin and above the lateral line were excised and freeze-clamped immediately in liquid N_2, and stored at -70°C for further analysis.

Analyses

Ammonia concentrations in water were determined by the salicylate-hypochlorite method of Verdouw et al. (1978), and in plasma by a commercial enzymatic kit (Sigma no. 170-UV). The two assays were cross-validated. The concentration of Na^+ in water and plasma was determined using atomic absorption spectrophotometry (Varian AA 1275).

Whole body proximate composition was obtained from frozen tissue which was ground to a fine powder with a grinding mill (IKA - M10/M20) cooled to -40°C by a methanol/dry ice mixture, and then lyophilized for 72 h at -55°C. A small portion of the frozen ground tissue was withheld before the lyophilization procedure and dried in an oven at 80°C for 48h to obtain the water content. Protein content was measured using a modification of the Lowry method (Miller 1959). Lipids, after extraction in a 2:1 chloroform:methanol mixture, were measured as described by Folch et al. (1957). Finally, carbohydrate content was measured as the combination of glucose, glycogen, and lactate following methods outlined in Bergmeyer (1985).

Frozen livers and white muscles were ground into a fine powder in an insulated mortar cooled with liquid N_2. Subsamples (100 mg) were deproteinized in 1 ml of ice-cold 8 % perchloric acid and measured for ammonia as described by Kun and Kearney (1971). Similar deproteinizing procedures were used to prepare the tissues for urea determination as in Crocker (1967).

Calculations

Food consumption, expressed on a per fish basis for each treatment, was calculated on the basis of food consumed by each tank divided by the number of fish in the tank.

Specific growth rates (SGR) were determined over the 90-d interval using the formula:

$$SGR = 100(\ln_e Y_2 - \ln_e Y_1)/ (t_2-t_1)$$

where Y_1 and Y_2 are the mean wet weights of fish estimated from bulk weighing (see above) at times t_1 and t_2. Condition factors were determined as the quotient of the wet weight of the fish and its total length cubed, multiplied by 100.

Estimates of the N content in food and whole body were calculated from the protein concentration measurements assuming a protein-N content of 16% (Jobling 1980; Soderberg 1995).

The energy budget was derived using the equation:

$$C = (F+U) + \Delta B + R$$

where C is the total energy consumed from food, (F + U) are the combined energies lost in feces and excreta, respectively, ΔB is the energy stored in the body (from measured whole body protein, lipid, and carbohydrate contents), and R is the total metabolic energy lost as heat, as determined from the O_2 consumption measurements. Fecal (F) and excreted energy (U), not measured in this study, were derived by back calculation. The values used to determine the energy content of food and body composition were 23.6, 39.5, and 17.2 $kJ \cdot g^{-1}$ for protein, lipid, and carbohydrate, respectively (Braefield and Llewellyn 1982). A value of 13.6 $kJ \cdot g^{-1}$ was assumed for O_2 (Elliott and Davison 1975).

The N-cost index, representing the total moles of O_2 consumed per mole of nitrogen stored (Linton et al. 1997a), was used to compare the total metabolic expenditure associated with the incorporation of nitrogen into growth (as protein-N).

Statistics

The values for the energy budget are reported on a per fish basis from the single experimental tank in each treatment where O_2 consumption was measured. For these values statistical procedures were not employed. All other data are expressed as means ± 1 SE from individual samples pooled together from the two replicate tanks per treatment. One-way analysis of variance (using SAS Jmp, SAS Institute Inc., Version 2.0.5) followed by Tukey-Kramer's multiple-means comparison test was used to distinguish significant differences amongst the four separate treatment groups within each sample period (30, 60, and 90 days, respectively). Multiple factor analyses with leverage plots were used to distinguish statistically significant differences due to temperature or ammonia. Finally, analysis of covariance was employed to determine differences in growth slopes derived from tank biomass measurements. The level of statistical significance for all analyses was $P \leq 0.05$.

RESULTS

The thermal profile for near-shore Lake Ontario from 20 June to 18 September 1994 was characterized by a slow but gradual rise in 'base' water temperature from 13 to 18°C which amounted to 1419 degree days of exposure (Figure 1). Those fish exposed to an additional +2°C experienced water temperatures ranging from 15 to 20°C, or a total of 1585 degree days of exposure. During this period trout consumed on average 0.060 g of food·d^{-1} resulting in a mean SGR of only 0.57 %·d^{-1}. There was little difference in wet weights, as estimated from tank biomass measurements (Figure 2), or total lengths, which increased from a mean of 7.89 ± 0.14 to 8.94 ± 0.16 cm, between treatment groups, and condition factors did not change appreciably from an initial value of 0.94 ± 0.01 obtained at the start of the experiment. However, analysis of covariance did indicate a significant

difference in growth profiles between treatments. This difference was characterized by a 'crossing-over' of growth slopes between base and base+2°C groups at day 60 (Figure 2).

Subtle differences existed in the partitioning of food energy into body materials. All trout exhibited a small increase in whole body water content (Figure 3a) and substantial reduction in lipid content (>30%) following the first 30 days of exposure (Figure 3b). The percentage whole body water was significantly elevated in those trout exposed to additional ammonia during this period. After 90 days of exposure, however, water contents fell to near initial values in all fish while their lipid levels remained depressed. With a single exception at day 30, there was little effect of additional +2°C or ammonia on whole body carbohydrate content (Figure 3c), but by day 60, carbohydrate content in all fish had approximately doubled. Note, however, total carbohydrate content remained very low relative to lipid and protein content. Whole body protein tended to be conserved in fish exposed to +70 μM total ammonia (Figure 3d); protein contents were significantly elevated in these fish compared to their temperature controls (non-ammonia exposed) by day 60. Nitrogen retention efficiencies (as a percentage of the total N consumed) were a striking 4 - 5% higher for fish exposed to elevated ambient ammonia (Table 2). However, there were no significant differences in muscle ammonia (Figure 4a), which remained relatively stable throughout the experiment, as did liver and muscle urea, approximately 325 and 225 $\mu mol \cdot kg^{-1}$, respectively (data not shown). A large reduction in liver ammonia, however, occurred at day 60 in all treatment groups (Figure 4b).

Routine O_2 consumption rates were elevated in fish exposed to either the additional +2°C, ammonia , or their combination (Figure 5). The highest metabolic losses (that proportion of energy consumed required to fuel metabolism, R/C in Table 2) were recorded for the base+2°C+Am group, 80%, followed by 67% for the base+Am group, and 64% and 62% for the base+2°C and base groups, respectively. Despite their increased

energetic demands, fish exposed to the additional +2°C or ammonia stored proportionately more energy into body materials (Table 2). Thus, net energy conversion efficiencies were 2.5 to 4.5 fold higher than for fish grown at base temperatures alone (Table 2). The period of greatest energy conversion occurred between days 60 and 90 when water temperatures were maximal.

After an initial decrease, plasma T_{Amm} remained relatively constant throughout the exposures (Table 3). An exception was the base+2°C+Am group, which exhibited significantly elevated plasma T_{Amm} at day 60. Conversely, plasma Na^+, which was significantly depressed in the ammonia treated fish after 60 days, increased markedly between days 60 and 90 (Table 3). Plasma hematocrit and protein, though variable, were in general decline throughout the exposures (Table 3). At day 90, however, hematocrit was significantly elevated in the ammonia-treated fish.

DISCUSSION

The present study was designed to assess whether a restricted ration caused juvenile trout to respond differently to small chronic elevations in water temperature and sublethal environmental ammonia when compared to fish fed to satiation (see Linton et al. 1997a). Although the thermal profiles between summer exposures were slightly different, the degree days of exposure were similar; 1419 degree days in the present study versus 1445 degree days during the summer of 1993 (see Linton et al. 1997a).

Compared to the satiation experiment, the greatest effect of the restricted ration was the marked increase (4 - 7 fold) in the metabolic costs of N retention (N-cost index, Figure 6a). For instance, the total O_2 consumed by fish in the present exposure was 50 - 70% of the O_2 consumed by fish during the previous summer (see Figure 6b), but their specific growth rates were a mere 15 - 20% (Figure 6c). Moreover, the 90-d metabolic

expenditures (R/C in Table 2) of fish in the present study comprised 62 to 80% of the total energy consumed as opposed to only 36 to 47% for juvenile trout fed to satiation (Linton et al. 1997a). As a result, net energy conversion efficiencies were reduced to as little as one fifteenth of those of the maximally fed trout. This large reduction in energy conversion efficiency of salmonids fed restricted rations is not uncommon (see Wurtsbaugh and Davis 1977; Brett and Groves 1979) given that greater rations are required to achieve better conversion efficiency during temperature increase (Brett et al. 1969; Elliott 1982; Soofiani and Hawkins 1982). With food restriction, however, trout exposed to an additional +2°C exhibited higher energy conversion efficiencies, but their cost of N retention was slightly increased compared to the base temperature group (Figure 6a). This latter effect was not observed in the previous summer satiation feeding experiment where trout appeared to optimize fuel utilization to meet the increased energetic demands of the additional +2°C (Linton et al. 1997a). By comparison, trout exposed to the combination of temperature and ammonia (base+2°C+Am), despite greater energy demands, had a much reduced cost of N retention (Figure 6a).

These findings support the conclusions surmised from a separate set of experiments with these and other fish designed to determine whether this chronic exposure conferred any increased tolerance of rainbow trout to lethal temperature and ammonia (see Linton et al. 1997b). For example, higher temperature tolerance was not observed after the current exposure implying that the increased energetic demands placed on the fish held at +2°C precluded the metabolically expensive physiological adjustments necessary. However, trout fed restricted ration and exposed to ammonia over summer, at either temperature regime, were better able to withstand lethal temperature and ammonia challenge compared to non-ammonia exposed trout (see Linton et al. 1997b). Thus, nutritional status is an important factor influencing the observed responses.

The subtle changes in energy partitioning may also explain some of the apparent discrepancy between responses of trout fed restricted versus unlimited ration to the warmer and more polluted scenario over summer. Juvenile trout consuming a restricted ration exhibited a large reduction, rather than increase, in lipid content which suggests increased utilization of lipid for energy (Jobling 1980; Morata et al. 1982), and perhaps less utilization of dietary precursors, i.e. amino acids or glucose, for lipogenesis (Pandian 1967). It has been shown that fish oxidize lipid for energy, thus conserving protein for other activities such as growth and maintenance (Atherton and Aitken 1970; Beamish and Tandler 1990; Jayaram and Beamish 1992; Agradi et al. 1995). This may explain the general preservation of whole body protein exhibited by fish in the present study. However, the stimulatory effect of 70 μM ammonia on protein-N retention of fish fed restricted rations warrants further investigation. Linton et al. (1997a) proposed a mechanism whereby protein was retained in fish exposed to +70 μM ammonia due to an increase in liver protein synthesis induced as an ammonia detoxifying process.

Whole body carbohydrate content of trout in the present study was initially less than a third of that reported during the satiation feeding experiment (Linton et al. 1997a), but by day 60, had nearly doubled, suggesting increased gluconeogenesis. Gluconeogenesis in rainbow trout may be influenced by hormonal and dietary manipulation (Cowey et al. 1977 a,b), and indirect evidence suggests that gluconeogenesis plays a significant role in maintaining blood sugar levels in fasting fish (Cowey and Sargent 1979; Morata et al. 1982). Although the fish were growing slowly (0.57 vs. 3.0 $\%\cdot d^{-1}$ with satiation feeding) during the course of the present experiment, the large decrease in lipid reserve and already low carbohydrate content suggest changes in energy metabolism similar to that which might occur in fasting fish (Zammit and Newsholme 1979; Jobling 1980; Black and Love 1986). Moreover, the reduction in both hematocrit and plasma

protein levels suggest that the fish were experiencing a degree of starvation (Kawatsu 1974).

The changes in protein and carbohydrate metabolism of fish during the exposure correspond with an overall reduction in liver ammonia concentration (Figure 4b). These liver ammonia concentrations were only 40% of those reported earlier for the juvenile trout fed to satiation (Linton et al. 1997a) which is reflective of an equivalent reduction in protein turnover rates of this tissue (unpublished data from our laboratory). Conversely, total ammonia concentrations in white muscle and plasma were similar between studies. When fish are faced with inadequate food supply, lipid deposits are preferentially used for fuel (see above) and gluconeogenesis is activated to meet the continuous glucose requirement of some tissues (Morata et al. 1982). Cortisol was not measured during the present exposures, but it may be related to the stimulation of gluconeogenesis in these fish (see Barton and Iwama 1991 for review). For example, cortisol has recently been shown to be elevated in juvenile trout fed restricted rations (D'Cruz 1997). Cortisol elevation is also associated with chloride cell proliferation (Froskett et al. 1983), which may explain the increase in plasma Na^+ between days 60 and 90.

The present study clearly shows that changes in metabolic fuel utilization play a significant role in the growth and energetics of juvenile trout fed restricted ($1\%\cdot d^{-1}$) ration over summer. Moreover, restricting the ration during a naturally fluctuating summer temperature regime will invoke physiological responses similar to those seen in fasting fish at constant temperatures. Despite the gross changes in energy partitioning associated with this restricted ration, the addition of +2°C did not cause juvenile trout to respond unfavorably to the warmer environment, although food limitation probably did inhibit the ability of juvenile trout to acclimate to this small chronic temperature increase (see Linton et al. 1997b), due to less energy being available for any necessary physiological adjustments

(i.e. enzymatic stability, membrane fluidity, heat shock proteins, etc.). Trout exposed to +70 μM ammonia managed to incorporate comparatively more N into body materials, thereby reducing their overall metabolic costs of nitrogen retention. However, growth was not stimulated, as it was in juvenile trout fed to satiation. The 'cost of growth' for trout exposed to +2°C and ammonia was similarly reduced. Thus, we conclude that restricted ration will not further impair the ability of juvenile trout to cope with a warmer and polluted environment.

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Figure. 1. Daily water temperature profile as measured each day over 90-d exposures from 20 June to 20 September 1994. Juvenile rainbow trout were exposed to either ambient laboratory water temperatures (base, solid line) or to this water temperature + 2°C (base+2°C, dashed line). The mean difference between temperatures was 1.9 ± 0.3°C.

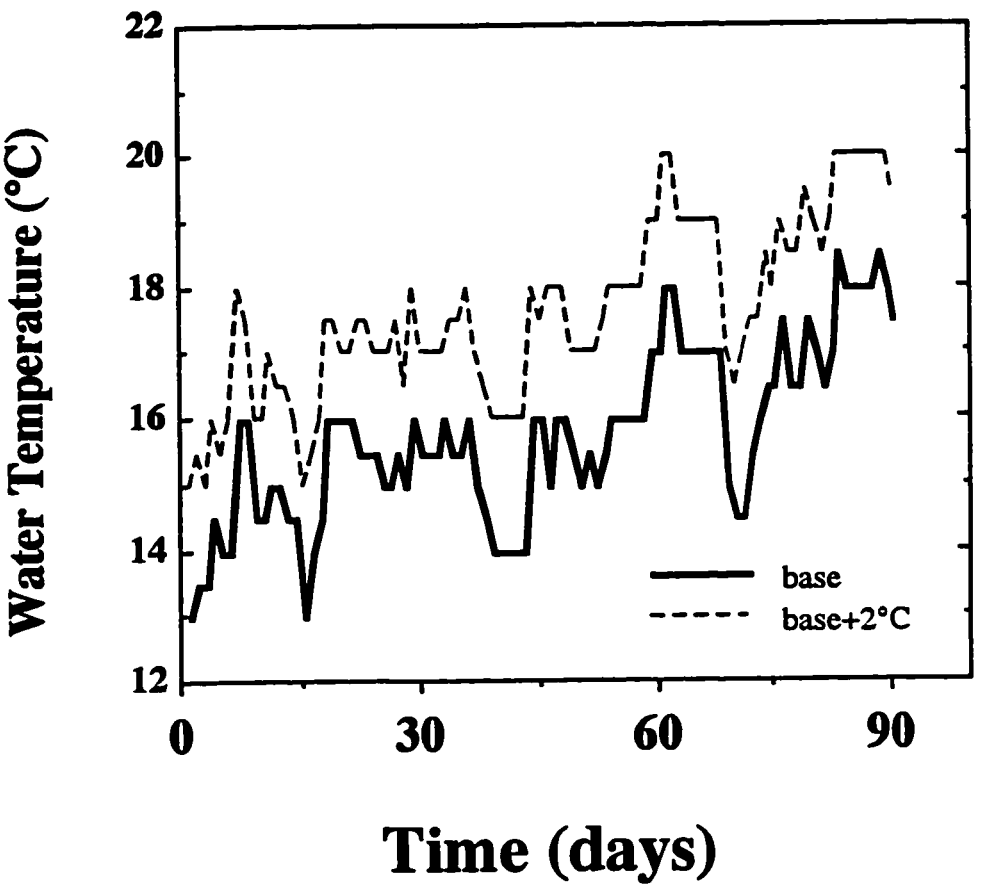
Water Temperature (°C)
22
20
18
16
14
12
0
30
60
90
Time (days)
base
base+2°C

Figure. 2. Effects of +2°C and 70 μM total ammonia (Am) on the growth profile (wet body mass) of individual juvenile rainbow trout fed a restricted ration equivalent to $1\%\cdot d^{-1}$ for 90 d (June to September 1994). Individual body mass was estimated from weekly tank biomass measurements. Solid lines represent growth slopes of fish exposed to the base temperature and dashed lines represent fish exposed to base+2°C. Data are means pooled from the two replicate tanks per treatment. Note the crossing slopes between the temperature groups at day 60.

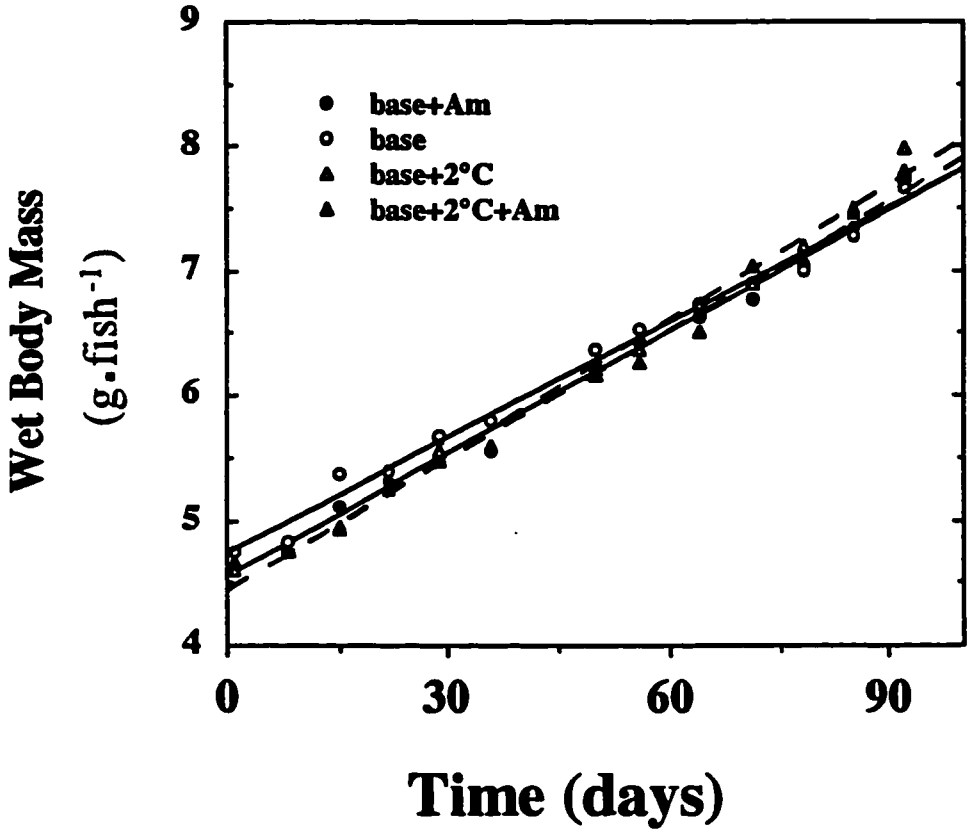
Wet Body Mass
(g.fish-1)
9
8
7
6
5
4
0
30
60
90
Time (days)
base+Am
base
base+2°C
base+2°C+Am

Figure. 3. Effects of +2°C and 70 µm total ammonia (Am) on whole body (a) water, (b) lipid, (c) carbohydrate (glucose, glycogen, and lactate) and (d) protein content of juvenile rainbow trout fed a restricted ration equivalent to $1\%\cdot d^{-1}$ for 90 d (June to September 1994). Data from groups (N = 10) of fish sampled initially and after 30, 60 and 90 days of exposure. Each value represents the mean +SE of fish sampled from the two replicate tanks per treatment (N = 10 per treatment per time period). All values are reported as percentage composition (g/100 g*100%) of wet tissue. Columns with the same letter on the same sampling day are not significantly different ($P > 0.05$). Dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure). solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and ammonia). Within each time period, a capital A represents a significant ammonia (Am) pre-exposure effect (two-way ANOVA; significance at $P \leq 0.05$).

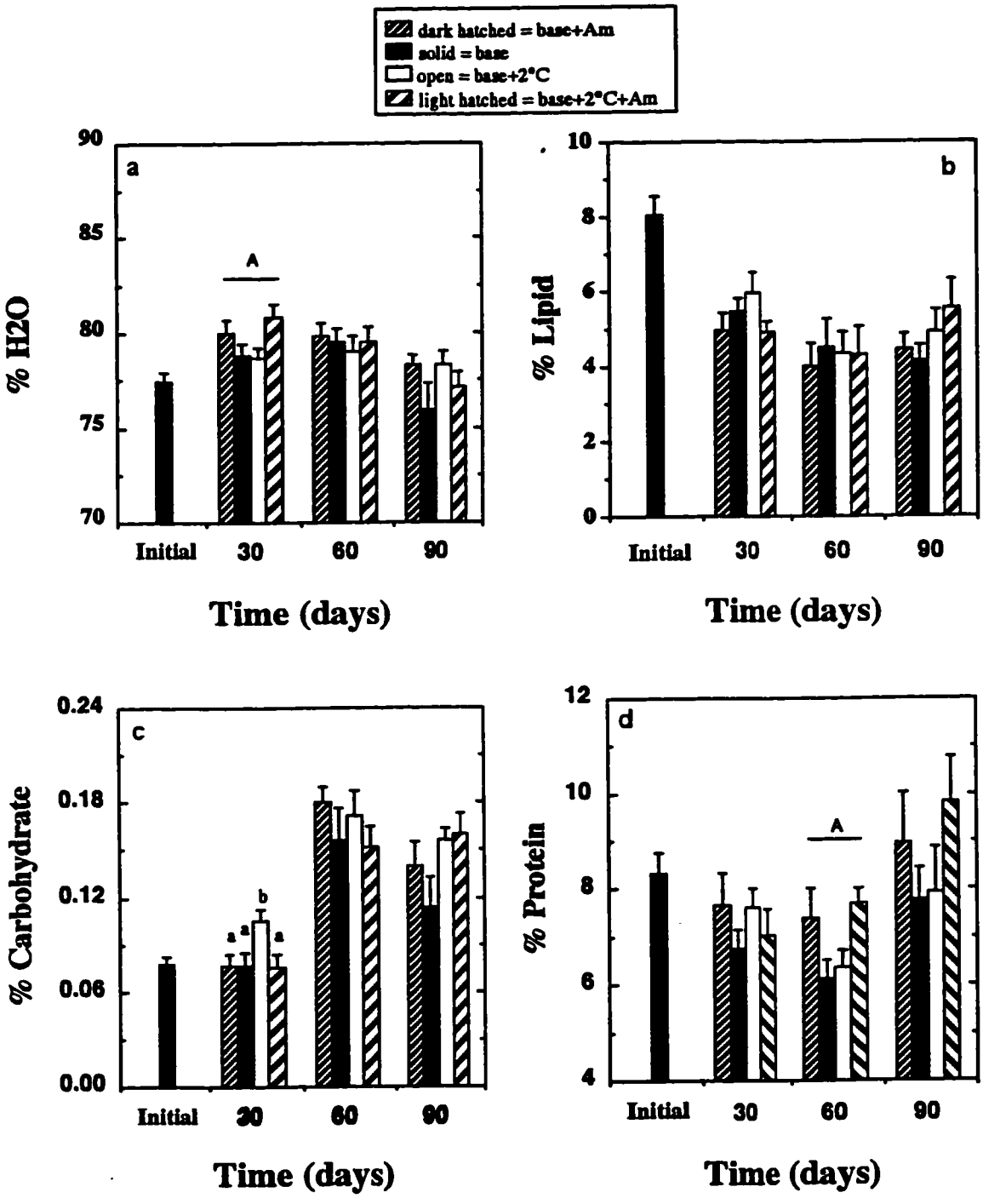
dark hatched = base+Am
solid = base
open = base+2°C
light hatched = base+2°C+Am
a
% H2O
90
85
80
75
70
A
Initial
30
60
90
Time (days)
b
% Lipid
10
8
6
4
2
0
Initial
30
60
90
Time (days)
c
% Carbohydrate
0.24
0.18
0.12
0.06
0.00
a a b a
Initial
30
60
90
Time (days)
d
% Protein
12
10
8
6
4
A
Initial
30
60
90
Time (days)

Figure. 4. Effects of +2°C and 70 μM total ammonia (Am) on (a) white muscle and (b) liver ammonia concentration in juvenile rainbow trout fed a restricted ration equivalent to 1%·d^{-1} for 90 d (June to September 1994). Data from groups (N = 10) of fish sampled initially and after 30, 60 and 90 days of exposure. Each value represents the mean +SE of fish sampled from the two replicate tanks per treatment (N = 10 per treatment per time period). All concentrations are reported per kg of wet tissue. Dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure), solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and ammonia). There were no significant differences between treatment groups on the same sampling day ($P > 0.05$).

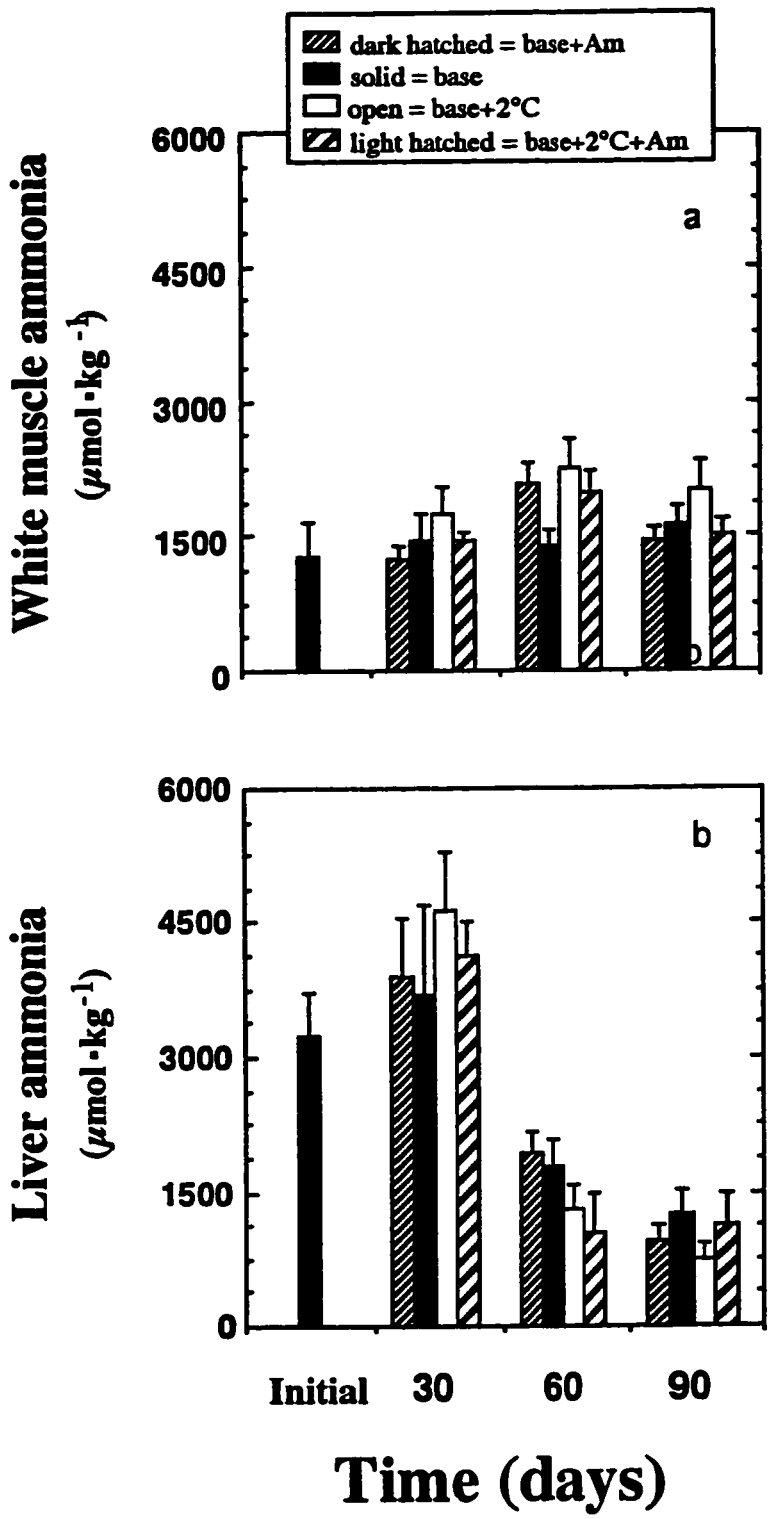
dark hatched = base+Am
solid = base
open = base+2°C
light hatched = base+2°C+Am
White muscle ammonia
(μmol·kg⁻¹)
6000
4500
3000
1500
0
a
Liver ammonia
(μmol·kg⁻¹)
6000
4500
3000
1500
0
b
Initial
30
60
90
Time (days)

Figure. 5. Effects of +2°C and 70 μM total ammonia (Am) on routine "in-tank" oxygen consumption of juvenile rainbow trout fed a restricted ration equivalent to 1%·d^{-1} for 90 d (June to September 1994). Measurements were made on one tank of individuals per treatment per time period. Dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure), solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and ammonia). As only one tank per treatment was monitored and error bars represent measurement SEMs, no statistical analyses were conducted.

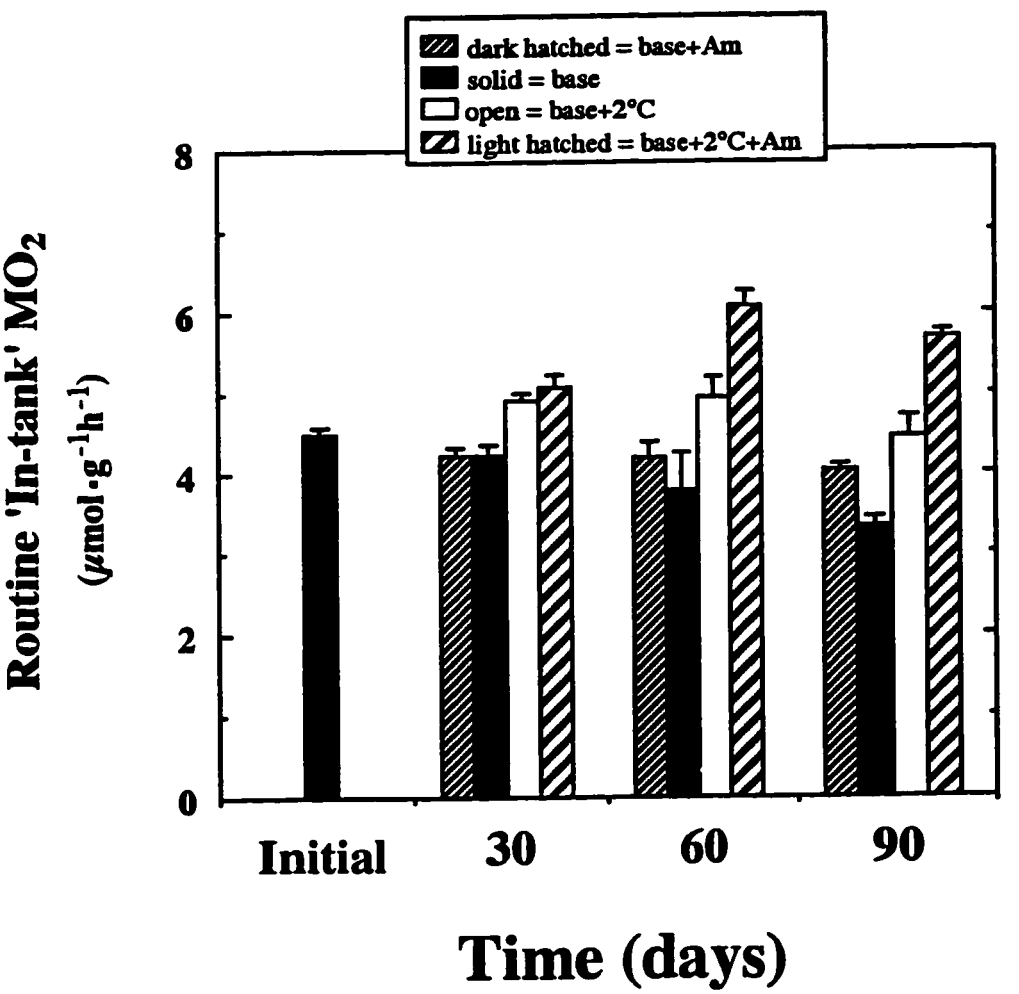
dark hatched = base+Am
solid = base
open = base+2°C
light hatched = base+2°C+Am
Routine 'In-tank' MO_2
(μmol·g^{-1}h^{-1})
0
2
4
6
8
Initial
30
60
90
Time (days)

Figure. 6. Comparison of (a) N-cost indexes (see Materials and Methods - *Calculations* for definition), (b) oxygen consumption, and (c) SGRs of juvenile rainbow trout fed to satiation for 90 d over the summer of 1993 (summer satiation; data from Linton et al. 1997a), or a restricted ration equivalent to 1%·d^{-1} for 90 d over the summer of 1994 (summer restricted; present study). Dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure), solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and ammonia).

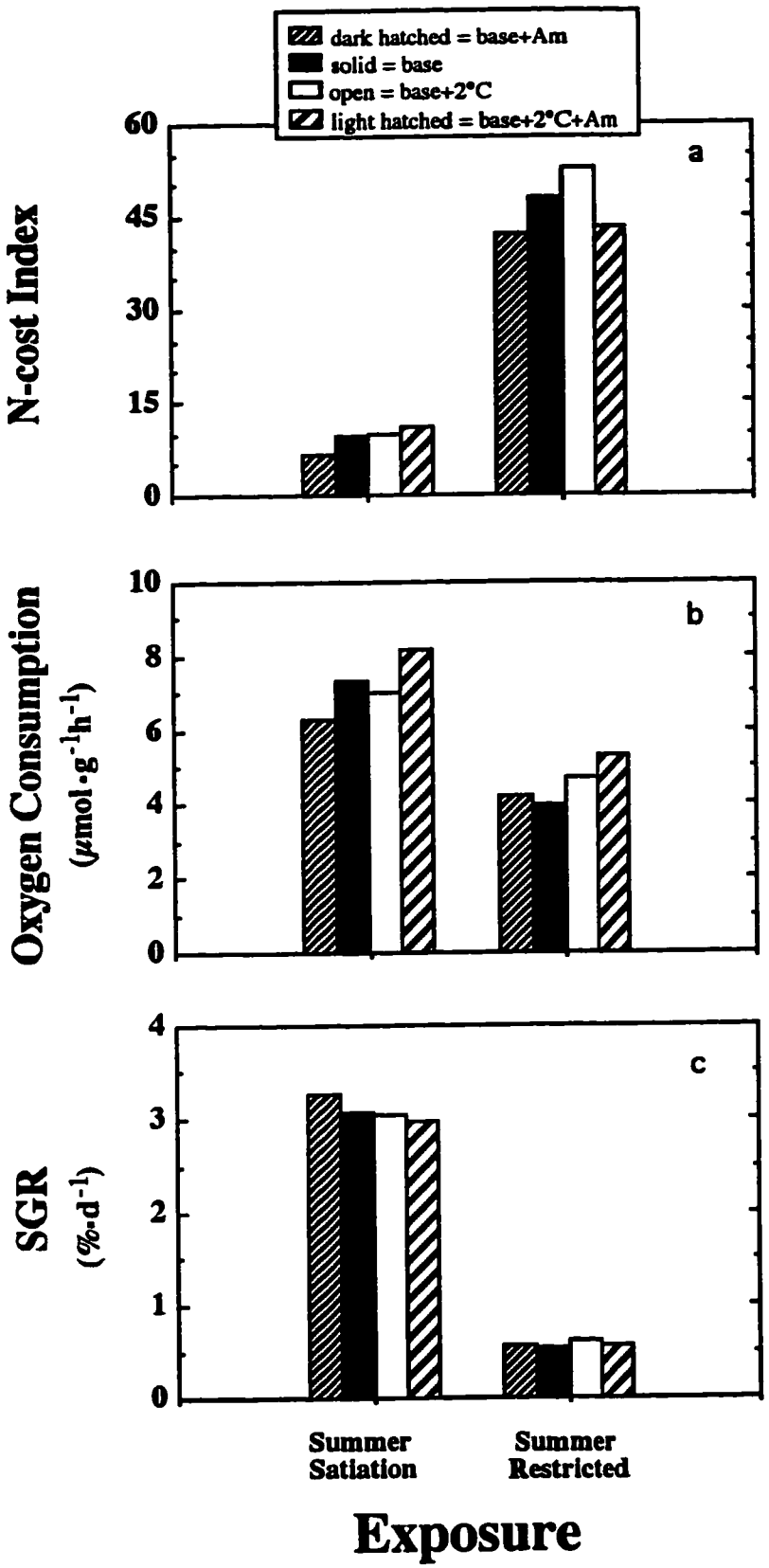
dark hatched = base+Am
solid = base
open = base+2°C
light hatched = base+2°C+Am
N-cost Index
60
45
30
15
0
a
Oxygen Consumption
(μmol•g-1h-1)
10
8
6
4
2
0
b
SGR
(%•d-1)
4
3
2
1
0
c
Summer
Satiation
Summer
Restricted
Exposure

Table 1. Mean (± SE) water total ammonia concentrations (N = 18), pH (N = 5), and partial oxygen pressures (N = 4) in tanks during 90-d exposures (20 June to 18 September 1994) of juvenile rainbow trout to +2°C and 70 μM total ammonia (Am) at a fixed ration equivalent to 1 %·d^{-1}.

Treatment	Rep	Total Ammonia (μM)	pH	$P0_2$ (torr)
base+Am	1	66.3 ± 2.3	7.60 ± 0.04	154.0 ± 2.8
	2	66.3 ± 2.1	7.60 ± 0.03	154.6 ± 1.4
base	1	7.8 ± 0.8	7.40 ± 0.10	151.0 ± 3.2
	2	8.4 ± 0.9	7.30 ± 0.16	156.5 ± 2.4
base+2°C	1	8.1 ± 0.8	7.20 ± 0.22	155.9 ± 1.0
	2	8.6 ± 0.9	7.40 ± 0.11	159.9 ± 1.9
base+2°C+Am	1	66.1 ± 3.6	7.40 ± 0.13	154.6 ± 1.5
	2	63.1 ± 3.2	7.40± 0.10	157.4 ± 1.7

Table 2. Energy budgets and N retention efficiencies for juvenile rainbow trout fed a restricted ration equivalent to 1 %·d^{-1} for 90 d (20 June to 18 September 1994) and exposed to a +2°C warming scenario and 70 µM total ammonia (Am).

	Energy (kg·fish^{-1})[a]					
Treatment and interval	C	ΔB	R	(F + U)	Net energy conversion efficiency[b]	N retention efficiency[c]
Days 0-30						
base+Am	26.4	-2.5	17.7	11.2	-9.3	6.7
base	25.9	-3.0	17.6	11.3	-11.6	-1.4
base+2°C	27.4	-0.7	18.3	9.8	-2.4	5.0
base+2°C+Am	27.6	-4.3	19.3	12.6	-15.5	-0.4
Days 30-60						
base+Am	30.5	0.9	21.7	7.9	2.9	6.5
base	30.6	0.4	20.6	9.6	1.4	3.3
base+2°C	32.5	-1.3	22.6	11.3	-4.1	0.4
base+2°C+Am	32.9	3.3	27.7	1.8	10.1	12.8
Days 60-90						
base+Am	38.9	7.9	25.0	6.0	20.2	18.4
base	38.8	4.9	21.2	12.7	12.7	16.6
base+2°C	42.3	8.8	20.1	9.5	20.7	17.3
base+2°C+Am	42.7	12.5	34.7	-4.5	29.2	22.9
Days 0-90						
base+Am	95.7	6.3	64.3	25.1	6.6	11.4
base	95.3	2.4	59.3	33.6	2.5	7.5
base+2°C	102.2	6.8	64.9	30.5	6.6	8.6
base+2°C+Am	100.9	11.5	81.7	7.7	11.4	13.4

[a]C = energy consumed; ΔB = energy stored in body material; R = total metabolic energy lost as heat (from routine 'in-tank' oxygen consumption measurements); (F+U) = unaccounted energy lost in feces (F) and by urinary and branchial excretion (U) as derived from back-calculation of measured budget parameters (see Materials and Methods - *Calculations*).

[b]Net energy conversion efficiency = 100ΔB/C.

[c]N retention efficiency = 100N retained/N consumed (as determined from whole body and food protein measurements assuming N content of 16%).

Table 3. Hematocrit and plasma protein, total ammonia (T_{Amm}), and sodium (Na^+) of juvenile rainbow trout fed a restricted ration equivalent to 1 $\%\cdot d^{-1}$ for 90 d (20 June to 18 September 1994) and exposed to a +2°C warming scenario and 70 µM total ammonia (Am). Fish were sampled initially and after 30, 60, and 90 days of exposure. Results are expressed as means ± SE of 10 fish (5 fish from each replicate tank per treatment) per sample period. For each sampling day separately, values within a column without a letter in common are significantly different ($P \leq 0.05$). Where there are no letters, there were no significant differences.

Sampling day and Treatment	Protein ($g\cdot dL^{-1}$)	Hematocrit (% RBC)	T_{Amm} (µM)	Na^+ (mM)
Initial	4.1 ± 0.1	37.8 ± 2.0	926.3 ± 55.6	154.5 ± 7.8
Day 30				
base+Am	3.2 ± 0.4	35.7 ± 3.1	537.1 ± 15.9	149.5 ± 3.8
base	3.3 ± 0.2	33.8 ± 1.9	543.7 ± 45.6	147.9 ± 2.4
base+2°C	3.9 ± 0.4	31.5 ± 1.8	620.9 ± 25.9	147.6 ± 3.0
base+2°C+Am	2.9 ± 0.4	30.1 ± 2.3	579.3 ± 26.6	147.3 ± 3.0
Day 60				
base+Am	3.0 ± 0.3	29.4 ± 0.9 ab	581.4 ± 35.8 a	149.4 ± 1.5 ab
base	3.1 ± 0.3	27.3 ± 1.8 a	629.0 ± 81.5 a	162.5 ± 5.9 a
base+2°C	2.9 ± 0.4	33.5 ± 1.2 b	622.3 ± 99.1 a	154.4 ± 3.6 ab
base+2°C+Am	2.4 ± 0.3	27.5 ± 1.3 a	1025.7 ± 68.2 b	145.5 ± 1.0 b
Day 90				
base+Am	3.1 ± 0.4	35.5 ± 1.0 *	501.8 ± 43.1	180.3 ± 2.6
base	2.7 ± 0.4	30.1 ± 1.8	631.7 ± 84.3	169.7 ± 4.2
base+2°C	3.3 ± 0.3	31.5 ± 1.3	545.6 ± 53.1	178.0 ± 3.6
base+2°C+Am	3.4 ± 0.3	32.1 ± 1.4 *	689.7 ± 93.0	176.3 ± 3.0

* significant ammonia effect (Two-way ANOVA; $P<0.05$).

CHAPTER 5

LONG-TERM EXPOSURE TO SMALL TEMPERATURE INCREASE AND SUBLETHAL AMMONIA IN HARDWATER ACCLIMATED RAINBOW TROUT: DOES ACCLIMATION OCCUR?

ABSTRACT

Juvenile rainbow trout (Oncorhynchus mykiss; initially 2-5 g) were exposed for 90 days to either ambient water temperature (natural thermal regime) or to +2°C superimposed above the ambient water temperature (simulated global warming scenario) in the presence or absence of a nominal 70 μM total ammonia (1,290 μg/L ionized (NH_4^+), 10 μg/L un-ionized (NH_3) ammonia). The exposures were conducted in moderately hard de-chlorinated water from Lake Ontario ($[Ca^{2+}] = 0.96 \pm 0.02$ mM, $[Na^+] = 0.55 \pm 0.01$ mM, $[Cl^-] = 0.737 \pm 0.004$ mM) on three occasions: over summer (temperature range: 13.0 - 21.0°C; pH = 7.57 ± 0.26) and winter (temperature range: 3.5 - 7.0°C; pH = 7.46 ± 0.02) without food limitation (satiation feeding), and during summer (temperature range: 13.0 - 18.5°C; pH = 7.38 ± 0.09) with food limitation (1% daily, or restricted ration). Lethal temperature, lethal ammonia (1.8 mM total ammonia; approximately 31,700 μg/L NH_4^+, 900 μg/L NH_3), and lethal temperature plus ammonia challenges were conducted after each 90-day exposure to determine whether or not chronic pre-exposure conferred any increased tolerance to elevated temperature or ammonia. In addition, acute sublethal ammonia challenges (1.0 mM total ammonia; approximately 17,800 μg/L NH_4^+, 200 μg/L NH_3), together with unidirectional Na^+ flux measurements, were conducted after the two summer exposures to gain further insight into the effects of prior sublethal ammonia exposure on Na^+ regulation, as influenced by ration. The juvenile trout on unlimited ration

and exposed to a warming scenario of +2°C exhibited a slight, but significant elevation in lethal temperatures in both summer and winter, but the effect was not observed in fish fed a restricted ration. Challenge to lethal temperature and ammonia in combination reduced the lethal temperature anywhere from 3-7°C for fish from all treatments; pre-exposure to ammonia offered some protective effect. However, prior ammonia exposure did not prolong survival times ($LT_{50}s$) during lethal ammonia challenge, and there was no evidence of acclimation to elevated external ammonia with respect to Na^+ balance. These results suggest that juvenile trout are likely to adapt to a small temperature increase such as could be associated with a global warming scenario, but their potential for doing so may be restricted by sublethal ammonia and by nutritional status.

1. INTRODUCTION

The impact of human activity on the aquatic environment has been such that the survival of fish may very well be dependent on their ability to acclimatize to changing environmental conditions. Two such changing conditions are small increases in temperature over the annual water temperature profile, characteristic of global warming, and reduced water quality due to low level contamination with common pollutants such as acidity and ammonia (Vitousek 1994). At present, physiological data assessing the ability of fish to acclimatize to these environmental scenarios are scarce when investigated alone, and virtually non-existent when studied in combination. For this reason, our laboratory began studying the physiological and toxicological effects of chronic, small temperature increases together with sublethal levels of priority pollutants on a reference cold water fish species, the rainbow trout (Oncorhynchus mykiss; see Reid et al. 1995, 1996, 1997; Dockray et al. 1996; Linton et al. 1997). The present paper focuses on the interactive effects of temperature and ammonia.

Previous research with other salmonids indicates that temperature tolerance increases with increasing acclimation temperature (Fry et al. 1946; Brett 1952; Elliott 1981). For example, juvenile salmon (Salmo salar) held at 27°C reached a critical thermal maximum (CTM; Cowles and Bogert 1944) of 32.9°C, whereas those acclimated to 5°C only reached 29.9°C (Elliott 1991). Likewise, prior exposure to sublethal ammonia has been shown to increase the tolerance of freshwater fish to further elevations of ammonia (Vamos 1963; Lloyd and Orr 1969; Malacea 1968; Redner and Stickney 1979; Thurston et al. 1981a; Alabaster et al. 1983). However, these studies lack environmental relevance because they do not take into account the fact that water temperature fluctuates both on a diel and seasonal basis, and that these natural water temperature fluctuations can alter the

acclimation responses of fish to both temperature (Thomas et al. 1986; Konecki et al. 1995) and ammonia (Thurston et al. 1981a).

In the present study, juvenile rainbow trout were chronically exposed for 90 days over summer and winter without food limitation and over summer with food limitation (as an additional stress) to a natural fluctuating water temperature cycle (13.0 - 21.0°C in summer, 3.5 - 7.0 °C in winter; characteristic of inshore Lake Ontario), or to +2°C superimposed above this cycle, in the presence or absence of an additional 70 μM total ammonia. They were subsequently challenged (see Wedemeyer et al. 1990) with lethal temperature, lethal ammonia, or a combination of lethal temperature and ammonia to determine: (1) the physiological 'cost' of previous long-term exposure to elevated temperature and ammonia, and (2) whether or not previous long-term exposure increased the ability of juvenile rainbow trout to tolerate higher levels of temperature or ammonia. We also challenged juvenile trout with acute sublethal ammonia (1.0 mM total ammonia) to determine if prior low ammonia exposure imparted any increased ability to maintain sodium regulation. Previous evidence suggests that exposing rainbow trout to high external ammonia initiates a sodium regulatory disturbance via stimulation of sodium efflux (Twitchen and Eddy 1994). Correction of the disturbance would require a subsequent increase in sodium uptake (Wilson et al. 1994a) or a reduction of gill permeability to Na^+ (as seen in the later phases of acid stress - e.g. McDonald et al. 1983; Audet et al. 1988), both of which could be associated with an acclimation response. For the purposes of this paper, acclimation is defined as the increased tolerance of an elevated, usually lethal, concentration of a toxicant arising from chronic exposure to a sublethal concentration of that toxicant (McDonald and Wood 1993).

2. MATERIALS AND METHODS

Experimental Animals and Protocol

Juvenile rainbow trout (initially 2-5 g) were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario, and held in flowing de-chlorinated Hamilton tapwater (moderately hard water from Lake Ontario) at seasonal temperature for at least 5 weeks prior to test. The fish were then exposed for 90 days in 270 L aerated polyethylene tanks (95% replacement every 4.2 - 5.2 h) to either ambient temperature water (control group = base) or ambient temperature water plus 2°C (warming group = base+2°C), each with or without an additional 70 μM total ammonia (T_{Amm} = ionized (NH_4^+) + un-ionized (NH_3) ammonia), base+Am and base+2°C+Am groups, respectively. This protocol was carried out 3 times over the period of June 1993 to September 1994 (for detailed water composition - ions, pH, T_{Amm}, and ranges of NH_4^+ and NH_3, see Table 1). During exposures 1 (June to September 1993) and 2 (January to April 1994), the trout were hand-fed to satiation twice daily (0830 h and 1630 h) with Zeigler Trout Starter #3 (50% protein, 15% lipid, 12% moisture) following the methods of Wilson et al. (1994b). In exposure 3 (June to September 1994), the fish were hand-fed twice per day as before but with a restricted ration equivalent to 1% of the mean body weight of the base group. All fish were kept under natural photoperiod for Hamilton, Ontario throughout the experiments. The ambient water temperature regime 'base' over the 90-day period was that of Hamilton tap water, which was that of its source, near-shore Lake Ontario (Fig. 1). Thus natural summer and winter water temperature regimes were used. The differences between exposures 1 and 3 reflect real year-to-year variation in the natural regime. In each exposure, the chosen warming scenario added +2°C to the natural regime.

Immediately following the 90-day exposures, 6-10 fish from each of the 4 original treatments were challenged with either lethal temperature, lethal ammonia, or the

combination of lethal temperature and ammonia. A further group of fish were tested for their ability to maintain sodium regulation in the face of acute sublethal ammonia. This last challenge was performed only after exposures 1 (summer-satiation) and 3 (summer - restricted ration).

Each lethal challenge was conducted in a circular tank containing approximately 200 L of vigorously aerated dechlorinated tap water. Oxygen saturation remained greater than 75% throughout all tests. A perforated insert was constructed to divide the challenge tank into 4 separate quadrants where fish representing each exposure treatment could be allocated and tested simultaneously. For the lethal temperature challenge (Lethal Challenge I), the tank water was heated at a rate of 1°C every 2 h from 24°C after exposures 1 (summer - satiation) and 3 (summer - restricted ration) and at a rate of 1°C per 1 h from 9.5°C after exposure 2 (winter - satiation) until all fish were dead. The temperature at which each fish died was recorded. For the lethal temperature and ammonia challenge (Lethal Challenge II), the fish were heated as above, but this time in water containing a nominal 1.8 mM T_{Amm} - equivalent to the 48 h LC_{50} as determined from a 48 h static range finding toxicity test using techniques recommended by ASTM (1980). Although pH was not measured over the course of each temperature and ammonia challenge (see Table 2 for initial values), a later simulation demonstrated that neither T_{Amm} concentration nor pH (7.50) changed over the temperature range of 20.5 to 30.5 °C; however, the un-ionized ammonia (NH_3) concentration doubled from 1.3 to 2.5% of the T_{Amm}, respectively. Following this second lethal challenge, a third challenge (Lethal Challenge III) was conducted. Here, the fish were exposed to the nominal 1.8 mM T_{Amm} as in the second lethal challenge, but without the heating. Mean water temperatures, T_{Amm} concentrations, pHs, and fractions of NH_4^+ and NH_3 together with their corresponding ranges were as in Table 2.

To gain a better understanding of the effect of prior ammonia exposure on Na^+ regulation, a fourth and final test (Sublethal Challenge IV) was conducted which involved measuring net ammonia and unidirectional Na^+ fluxes during exposure to an elevated, but non-lethal, level of T_{Amm} (1.0 mM). Individual fish were placed in 1L flux chambers 24 h prior to the initiation of the experiment. During this acclimation period, the chambers were supplied with a constant supply of the appropriate treatment water at the appropriate temperature. Each flux chamber was fitted with an airstone to ensure adequate aeration and mixing. To begin the experiment, the water supply to each chamber was removed and 1μCi/L ^{22}Na was added and allowed to equilibrate for 10 min. after which an initial water sample (3 x 5 ml) was taken . Water samples were taken hourly over a 3 h period for determination of ^{22}Na activity, total Na^+, and T_{Amm} before the water flow to the flux chamber was re-established. This flux protocol was carried out under control (pre-challenge) conditions, and at 0-3, 3-6, and 21-24 h of continuous exposure to 1.0 mM T_{Amm}. This T_{Amm} concentration was equivalent at a representative flux pH of 7.6 and water temperature of 16°C to an NH_4^+ concentration of 17,800 μg/L, and an NH_3 concentration of 200 μg/L. A later simulation experiment demonstrated that the effect of the fish on water pH during any one 3h flux period was an elevation of about +0.26 pH units, or a 1.9 fold increase in the NH_3 fraction. Appropriate temperatures were maintained throughout as each flux chamber sat in a water bath. Immediately following the challenge exposure (i.e., at 24 h), the fish were sacrificed, weighed, and a blood sample collected via caudal severance.

Measurements, Calculations, and Statistics

Ammonia concentrations in water were determined by the salicylate-hypochlorite method of Verdouw et al. (1978), and in plasma by a commercial enzymatic kit (Sigma no.

170-UV). The two assays were cross-validated. The concentration of Na^+ in water was determined using atomic absorption spectrophotometry (Varian AA 1275). ^{22}Na gamma-radioactivity was counted in triplicate on 5 ml water samples in a Canberra-Packard A5000 Minaxi gamma counter or in liquid scintillation for beta activity on an LKB beta counter.

Net flux rates of total ammonia (J^{Amm} net) were calculated as:

$$J^{Amm}\ net = \frac{(Initial\ TAmm - final\ TAmm) \times V}{t \times W}$$

where T_{Amm} is the total ammonia concentration in μmol/L, V is the volume of the flux chamber (L), t is the elapsed time (h), and W is the weight of the fish (kg) (Wright and Wood 1985). Net Na^+ flux rates (J^{Na} net) were calculated from an equation analogous to J^{Amm} net, and influx rates (J^{Na} in) from:

$$J^{Na}\ in = \frac{(Initial\ R - final\ R) \times V}{MSA \times t \times W}$$

where R is the ^{22}Na radioactivity of water in c.p.m./L, MSA is the mean specific activity of the water (c.p.m./μeq) over the 1 h flux period, and the other symbols are as above (Maetz 1956). Inasmuch as final internal specific activity was <10% of external specific activity, backflux correction was not necessary. Unidirectional efflux rates (J^{Na} out) were calculated by difference:

$$J^{Na}\ out = J^{Na}\ net - J^{Na}\ in$$

For all fluxes, losses by the animal are denoted by a negative sign and gains by a positive sign.

Data are expressed as means ± 1 SEM except in the case of median survival times (LT_{50}), where data are expressed as means ± 95% confidence limits. Multiple factor analyses with leverage plots (SAS Jmp, SAS Institute Inc., Version 2.0.5) were employed

to distinguish statistically significant effects of temperature (+2°C, marked by T in Figures) and ammonia (+70 μM, marked by A in Figures) amongst treatment groups in the lethal temperature and lethal temperature plus ammonia challenges. Mean lethal temperatures were calculated as arithmetic averages of the temperatures at each death from the timed record of mortality during the 1°C/2 h and 1°C/1 h temperature increase protocols. In the lethal ammonia challenges, log probit analyses and nomographic methods were used to compare survival curves and to determine median survival times (LT_{50}), 95% confidence limits, slope functions, and LT_{50} significance (Litchfield 1949). One-way analysis of variance followed by the Tukey-Kramer HSD test for all pairs was used to compare exposures for seasonal (marked by S in Figures) and ration (marked by R in Figures) effects, and to compare plasma total ammonia concentrations. One-way analysis of variance followed by Dunnett's test (Dunnett 1955) was used to compare sodium and ammonia flux rates at 0-3, 3-6, and 21-24 h to the control (-3-0 h) values during the sublethal high ammonia challenge. Finally, Student's t tests were used to compare unidirectional flux rates between the naive and ammonia pre-exposed fish. The level of statistical significance for all analyses was $P \leq 0.05$.

3. RESULTS

Overview of Lethal Challenges (Figures 1 and 2)

Challenge with lethal temperature alone was clearly much less damaging for juvenile trout than the combination of lethal temperature and ammonia regardless of prior exposure. Upper lethal temperatures were reduced by 3-7°C when lethal temperature and lethal ammonia were applied in combination. Temperature acclimation (from prior exposure to +2°C) was only apparent in the lethal temperature challenges while the protective influence of prior exposure to ammonia (+70 μM TAmm) was only apparent in

the combination lethal temperature and ammonia challenges. Both of these effects, not surprisingly, were much more pronounced in winter challenges, where lethal temperatures were consistently lower than during summer challenges. Trout fed restricted ration over summer were better able to withstand lethal temperature and ammonia challenge than their satiation fed counterparts. Prior sublethal ammonia exposure did not prolong median survival times (LT_{50}s) during lethal ammonia challenge, although overall, LT_{50}s of juvenile trout were elevated after winter satiation exposure.

Challenge I - (Lethal Temperature)

Within individual challenge trials, the temperatures associated with lethality in juvenile rainbow trout fed to satiation and grown over summer and winter were dependent on previous thermal exposure (significant effect of +2°C) but independent of previous ammonia (base+Am) or ammonia + 2°C (base+2°C+Am) exposure (Fig. 2a). The differences were slight (0.2-1.0°C) but significant. In addition, when comparing the 3 different challenges (after exposures 1, 2, and 3 respectively), the mean lethal temperatures attained after exposure 1 (summer-satiation) ranged from 1.2 - 1.9°C higher (a significant difference) than the corresponding lethal temperatures reached after exposure 2 (winter-satiation). When trout grown over summer on restricted ration were tested (exposure 3), the significant +2°C effect disappeared and the lethal temperatures, relative to those determined after exposure 1, were slightly depressed (0.1-0.7°C; Fig. 2a), a difference which was not statistically significant.

Challenge II - (Lethal Temperature and Ammonia)

The protective effect of +2°C was entirely lost when lethal temperature challenge was applied in the presence of lethal ammonia (Fig. 2b). Moreover, overall temperature

tolerance was reduced in the presence of lethal ammonia for summer exposures (1 and 3). Mean lethal temperatures were 3 - 4°C lower overall in the presence of 1.8 mM T_{Amm} (mean = 26 - 27°C) than those reached when temperature was elevated without the additional ammonia (Fig. 2a), a highly significant difference. This effect was even more pronounced in winter (exposure 2), where the lethal temperatures in the presence of high ammonia averaged around 22°C. These temperatures were approximately 6 - 7°C lower than in the lethal temperature challenge alone.

Prior ammonia exposure did offer some protection in these challenges. In exposure 2, ammonia pre-exposed fish, both at base and base+2°C, survived to significantly higher water temperatures (Fig. 2b). A similar effect was observed in exposure 3 (summer - restricted ration) in trout with prior ammonia experience, but not in exposure 1 (summer - satiation) (Fig. 2b). Overall, the mean lethal temperatures attained by trout after exposure 3 (summer - restricted ration) were slightly, but significantly, higher than those reached after exposure 1 (summer - satiation) (Fig. 2b). This is opposite to the trend observed with temperature challenge alone (Fig. 2a).

Challenge III - (Lethal Ammonia)

The most dramatic differences in the survival of trout exposed to lethal ammonia occurred between challenge trials. Exposing juvenile trout for 90 days to the four treatments over summer, both with and without food restriction, led to significantly lower LT_{50}'s (increased toxicity) than when fish were fed to satiation and exposed over winter (Fig. 3), in seemingly close association with the NH_3 concentration in the challenge test (see Table 2). Within respective challenges, the trout that had been previously exposed to ammonia (base+Am), +2°C (base+2°C), or their combination (base+2°C+Am) showed little difference in their ability to tolerate acutely lethal ammonia (Fig. 3).

Challenge IV - (Sublethal High Ammonia - Net Ammonia and Unidirectional Na^+ Fluxes)

This challenge was conducted only after the two summer exposures, 1 (satiation) and 3 (restricted ration). In both trials, the addition of +2°C had negligible effect on the net ammonia and unidirectional Na^+ flux rates. Therefore, within each trial, the two temperature groups of fish not exposed to ammonia (henceforth naive fish) were combined, and the two temperature groups that were exposed to ammonia (henceforth ammonia pre-exposed fish), were combined.

The pre-challenge net ammonia flux rates after exposure 1 (summer-satiation) were similar in naive and ammonia pre-exposed fish (Fig. 4a). Net ammonia flux (J^{Amm}_{net}) remained unchanged in both groups throughout the 24 h challenge with 1.0 mM total ammonia, with the exception of a significant 2.5 fold increase in J^{Amm}_{net} in the naive fish from 3-6 h. Pre-challenge plasma ammonia levels (which may have been elevated by the caudal severance sampling procedures - see Wood, 1993) were significantly different at 334 ± 19 and 414 ± 10 μM T_{Amm} between the naive and ammonia pre-exposed groups, respectively (not shown). The plasma T_{Amm} levels following the challenge were elevated >4 fold to 1731 ± 68 and 1897 ± 68 μM T_{Amm} respectively, but were no longer significantly different between the two treatment groups.

The results from the high external ammonia challenge following exposure 3 again indicated that J^{Amm}_{net} in both groups did not change from pre-challenge levels over most of the test period. However, the ammonia pre-exposed group exhibited a significant increase in J^{Amm}_{net} over 0-3 h of exposure to 1.0 mM T_{Amm} (Fig. 4b). Plasma T_{Amm} levels before the 1.0 mM T_{Amm} challenge were not significantly different at 584 ± 48 and 623 ± 58 μM T_{Amm} for the naive and ammonia pre-exposed groups, respectively (data not

shown). After the challenge, plasma T_{Amm} had doubled to 1327 ± 100 and 1376 ± 136 μM T_{Amm}.

Following exposure 1 (summer-satiation), and just prior to challenge, Na^+ turnover was greater in ammonia pre-exposed fish than in naive fish (Fig. 5a). Pre-challenge J^{Na}_{in} was significantly greater in this group when compared with the naive fish. During the challenge, J^{Na}_{in} was only slightly and transiently reduced by high external ammonia in the naive group (0-3 h) and unaffected in the ammonia pre-exposed group (Fig. 5a). However, at this same time, J^{Na}_{out} was stimulated in the ammonia pre-exposed group resulting in significant net loss of Na^+ (Fig. 5a).

There was no difference in Na^+ turnover prior to challenge after exposure 3 (summer - restricted ration). There was also little effect of 1.0 mM ammonia on J^{Na}_{in} and J^{Na}_{out} with the exception of a significant reduction in J^{Na}_{out} and increase in net Na^+ to positive values for the ammonia pre-exposed group between 3-6 h. Both groups showed a significant decrease from pre-challenge levels in J^{Na}_{in} between 21- 24 h (Fig. 5b).

4. DISCUSSION

General

Despite the controversy over which experimental method best describes upper temperature tolerance of fish (Kilgour and McCauley 1986), we found the method of progressively heating fish to a lethal maximum particularly applicable for the purposes of this present study. The heating rates used were similar to those of Elliott (1991), and were chosen because they not only resemble natural diurnal rates of temperature increase in a variety of salmonid streams (Crisp and Le Cren 1970; Holtby 1988; Li et al. 1994), but also allow time for toxicants (e.g. ammonia) to exert their effect (Wedemeyer et al. 1990).

There is also some question concerning the interpretation of ammonia effects to fish based on the concentration of NH_3 alone, because un-ionized ammonia is probably not the only form of ammonia toxic to fish (Armstrong et al. 1978; Hillaby and Randall 1979; Tomasso et al. 1980; Thurston et al. 1981b; Haywood 1983). We chose to examine ammonia toxicity on the basis of T_{Amm}, which is both more practical and more relevant to the real world, where heating of natural waters leaves T_{Amm} unchanged, but alters the NH_3/NH_4^+ distribution ratio. This ratio, among other variables, is highly dependent upon the pH and temperature of the environment, and thus, undoubtedly influences the toxicity of ammonia to fish (Erickson 1985). The overall effect of ammonia depends on the concentration of ammonia at the site of uptake, the gills. This is a function not only of the ambient water ammonia level, but also the ammonia produced and excreted by the fish themselves (Meade 1985). Therefore, to express ammonia toxicity on the basis of only the one moiety (NH_3) may lead to erroneous conclusions, especially since the milieu (e.g. pH, ionic composition) at the gills is different from the bulk water in which the fish lives (Playle and Wood 1989, Lin and Randall 1990).

Temperature Acclimation

Testing juvenile trout at the end of the summer versus winter exposure regimes gave rise to substantial differences in temperature tolerance, in general agreement with previous literature (see Introduction). More interestingly, our results indicate that the ability of juvenile rainbow trout to tolerate a higher lethal temperature will increase slightly but significantly with a chronic 2°C elevation above ambient water temperature provided there are no additional metabolic costs such as those associated with sublethal toxicant stress (i.e. ammonia) or food limitation. The 5°C drop in temperature experienced by fish in the last few days of the first 90-d exposure (summer-satiation) may have actually

subverted some of their increased temperature tolerance. For example, two short-term physiological strategies invoked by fish to help cope with a rapid reduction in water temperature include regulating acid-base balance to minimize the possible reduction in enzyme activity, and altering membrane composition (i.e. viscosity) to maintain a favorable enzyme environment (see Clarke 1987). Either strategy could be costly, and therefore, might be expected to impede the acclimation process.

The increased tolerance that was exhibited by trout with prior chronic temperature exposure is probably related to the fact that these trout had been exposed for 90 days to 2°C above the natural fluctuating water temperature profile (which incorporates normal seasonal and daily variation in water temperature for the near-shore region of Lake Ontario) under the natural photoperiod regime (normal daily photoperiod for the region) prior to the lethal temperature challenges. Under such regimes, and with unlimited food supply, these fish may have made the necessary physiological adjustments to help cope with the warming - e.g. enzymatic stability, membrane fluidity, heat shock proteins, etc. (Fields et al. 1993). That higher temperature tolerance was not observed after exposure 3 (summer - restricted ration) is not surprising given that increasing energetic demands were probably being placed on those fish held at +2°C as water temperature rose over summer (Brett 1971; Elliott 1982).

The implication is that the response of juvenile rainbow trout to a very small increase in temperature (+2°C) indicative of a warming scenario may be adaptive, but requires an additional metabolic cost. Previous 'thermal history' is a critical parameter dictating thermal tolerance in fish (Beitinger and McCauley 1990), and since wild fish are typically acclimatized to a natural range of temperature rather than a particular absolute temperature (Fields et al. 1993), their survival is strongly dependent on past thermal adaptation (Fry 1971). In the present study, the differences in thermal tolerance that were

found in comparisons of trout of similar origin must be representative of acquired properties due to the altered thermal regime (the additional +2°C), and thus, reflect modification through phenotypic adjustment (see Lagerspetz 1987). This is probably related to the thermal regime employed. For example, while most other lethal temperature studies have been conducted on fish held at a constant, prescribed water temperature prior to challenge (Cox 1974; Maness and Hutchinson 1980; Watenpaugh et al. 1985; Elliott 1991), our fish had undergone slow, but continual acclimation to both the diel (max. daily variation 5°C) and seasonal (max. monthly variation 9°C) temperature change. Up until now, very few studies have examined the effects of naturally fluctuating water temperature regimes on the thermal tolerance of fishes, although, some studies have used sinusoidal fluctuations (Hokanson et al. 1977; Threader and Houston 1983; Thomas et al. 1986). Based on the present results, we predict that 'pre-warmed' trout will be better equipped to survive chronically elevated summer water temperatures should they arise with global warming. However, as Parsons (1990) points out, the diversion of energy for stress (e.g. temperature) tolerance could be expected to increase under these circumstances, leaving less energy available for other processes such as growth and reproduction.

Combination Temperature and Ammonia Acclimation

Challenge to high water temperature and lethal ammonia (1.8 mM T_{Amm}) in combination imposed an additional burden to juvenile rainbow trout from all treatments reducing their upper lethal temperature by 3-7 °C, the magnitude of which was dependent upon season. This was not surprising given that reductions in the temperature tolerance of fish forced to cope with aquatic pollutants are common (Watenpaugh et al. 1985, also see review by Beitinger and McCauley 1990). More intriguing, however, was the observation that juvenile trout with prior ammonia exposure over winter or with restricted ration were

better suited to tolerate this lethal combination. The mechanism(s) is unclear. The acclimation response may be related to changes in gill permeability or ammonia detoxification (see below).

Ammonia Acclimation

There are a number of studies that address the question of whether or not fish acclimate to ammonia, and all report increased tolerance to acutely lethal ammonia concentrations following prior sublethal exposure (see Introduction). However, the ammonia levels used for acclimation prior to these acute ammonia exposures were much higher than the level reported here, suggesting a possible threshold concentration for the acclimation response. Unfortunately, the actual mechanism(s) of the acclimation process is poorly understood. Lloyd and Orr (1969) suggested a modification of gill permeability, while Redner and Stickney (1979) attributed the acclimation response to metabolic adjustments such that the high water ammonia levels inhibited nitrogen metabolism, thereby reducing or even eliminating endogenous ammonia production and inducing an ammonia detoxification mechanism.

In the present study, prior exposure to low environmental ammonia (70 μmol/L T_{Amm}) had negligible effect on juvenile rainbow trout subsequently challenged with concentrations equivalent to a pre-determined 48 h LC_{50} (1.8 mM T_{Amm}). However, median survival times (LT_{50}'s) were affected by both previous thermal history and food limitation (see Fig. 3). To date, there has been very little study on the influence of either previous thermal history or food limitation on ammonia toxicity to fish. While there are some reports of higher ammonia toxicity at higher temperature (see EIFAC 1973), ammonia, in general, is considered to be less toxic to fishes near the upper end of the normal environmental temperature range (Colt and Tchobanoglous 1976; Haywood 1983;

Thurston 1988). For example, the acute toxicity of ammonia to rainbow trout decreased as temperature increased between 10-19°C (Thurston and Russo 1983), and Brown (1968) observed that the threshold LC_{50} of ammonia for rainbow trout at 3°C was approximately half that at 10°C. The present results show that LT_{50}'s of trout challenged with lethal ammonia after the winter-satiation exposure (exposure 2) were highest, suggesting reduced, or at least slower toxicity, whereas LT_{50}'s of trout challenged with ammonia after the summer - restricted ration exposure (exposure 3) tended to be lowest (see Fig. 3). The results may simply reflect the difference in ammonia toxicity associated with the lower NH_3 concentration in the winter challenge (Downing and Merkens 1955; Armstrong et al. 1978; Tomasso et al. 1980; Thurston et al. 1981b), but this does not explain the effects of high external ammonia challenge (1 mM T_{Amm}) on net ammonia and unidirectional Na+ fluxes in trout fed different rations.

The toxicity of ammonia to fish in general is thought to stem from an increase in plasma NH_3 to toxic levels (Fromm and Gillette 1968; Cameron and Heisler 1983; Claiborne and Evans 1988;) following the inhibition or reversal of ammonia excretion (Hampson 1976; Wilson and Taylor 1992; Wilson et al. 1994a). In the present study during challenge with high ambient ammonia (1.0 mM T_{Amm}; approximately 200 µg/L NH_3) there was a 4 fold increase in plasma T_{Amm} level after exposure 1 (summer - satiation) and a 2 - 2.5 fold increase after exposure 3 (summer - restricted ration), but no mortalities were seen. Although ammonia in freshwater fish appears to be excreted primarily by NH_3 diffusion, electroneutral ionic exchange of Na^+ for H^+ or NH_4^+ on the apical surface of the gill epithelium is also a possibility (Wood 1993). Our results tend to support the results of Wilson et al. (1994a) in that we did not observe an increase in unidirectional Na^+ influx during 24 h of high external ammonia exposure. However, during the challenge after exposure 1 (summer - satiation), the ammonia pre-exposed fish

did lose significantly more Na^+ via stimulated Na^+ efflux. Why the effect was not observed after exposure 3 (summer - restricted ration) has yet to be explained, but reduced gill permeability associated with a lower metabolic rate could be a contributing factor (Gonzalez and McDonald 1992). In this regard, feeding regime may be important and clearly deserves further study. Twitchen and Eddy (1994) showed no increase in Na^+ efflux of 'naive' juvenile rainbow trout challenged with a similar T_{Amm} concentration (pH 7.0) when starved 48 h prior to, and during, experimentation.

5. CONCLUSIONS

Predicting effects associated with low level environmental changes on fish are difficult because the effects are not always apparent. Often, fish are able to adapt to the stress rendering the assessment of the stressor on the fish's health impossible. In the present study, the physiological tolerance limits of juvenile rainbow trout previously exposed to small, chronic temperature increase (+2°C), sublethal ammonia (70 μM T_{Amm}), or their combination were purposely exceeded in order to identify adverse physiological effects as well as the presence or absence of acclimation. Prior exposure to just +2°C above the natural fluctuating water temperature profile did indeed result in fish with greater temperature tolerance, and prior sublethal ammonia exposure afforded some protection against the combination of lethal temperature and ammonia. However, the median survival times (LT_{50}) of the 'ammonia experienced' fish did not increase during lethal ammonia challenge indicating the lack of an acclimation response per se. These results imply that previous exposure history is an important parameter influencing the ability of trout to tolerate chronic temperature and pollutant increase. Moreover, the level of tolerance appears to be correlated with the energetic costs required to achieve this 'acclimated' state.

Nutritional status appears to be an important factor influencing the observed responses and deserves further study.

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Figure 1. Water temperature profiles for three 90-day exposure periods which were conducted (a) over summer feeding the trout to satiation (Summer Satiation - June to September 1993), (b) over winter again feeding to satiation (Winter Satiation - January to April 1994), and (c) over summer feeding the fish a restricted (1% daily) ration (Summer Restricted - June to September 1994). Daily measurements were made on test tanks receiving ambient temperature water (base = large solid line) or ambient temperature water +2°C (base+2°C = small solid line). Included are the mean daily water temperature differences ± 1 SE.

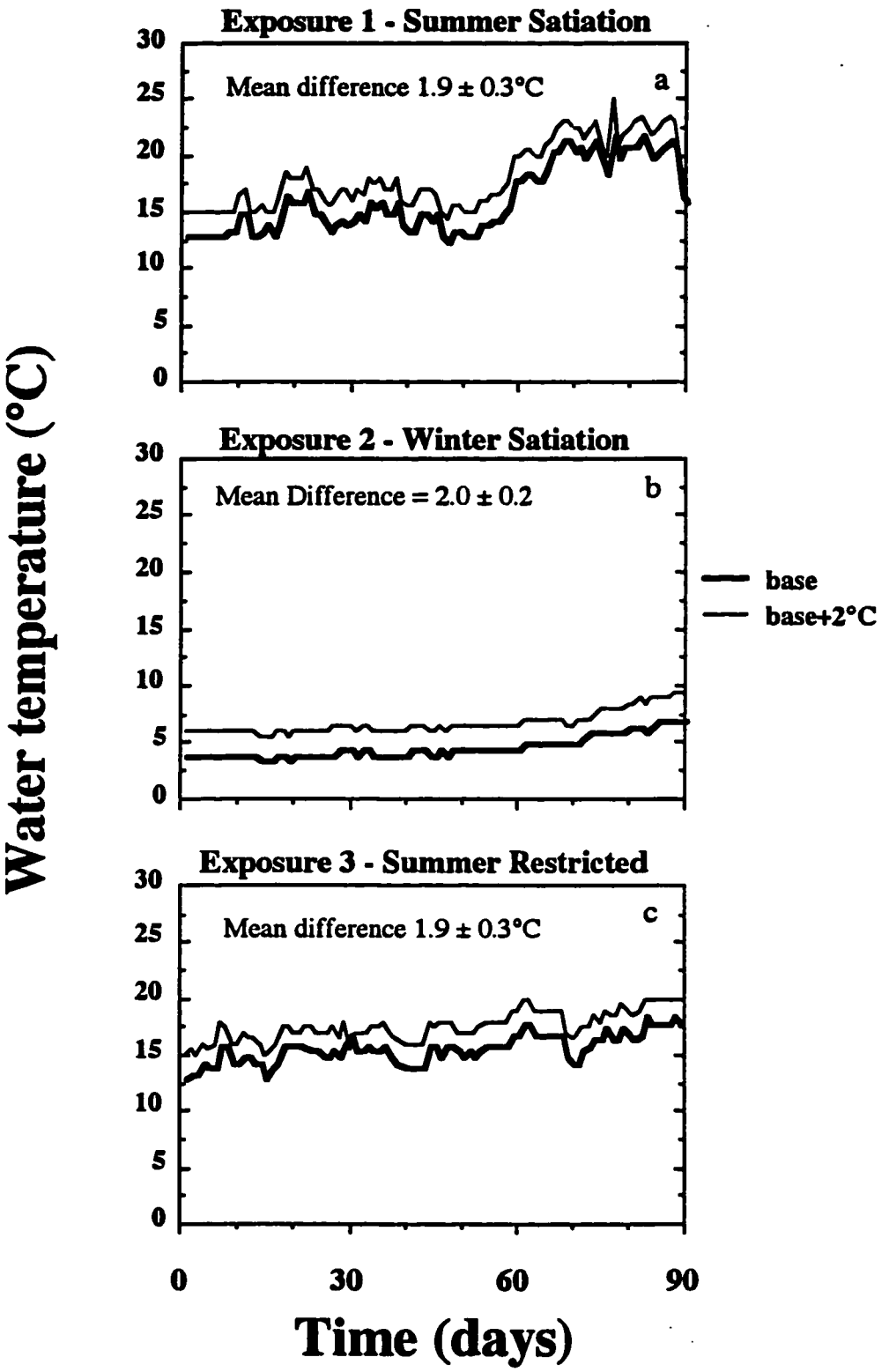
Exposure 1 - Summer Satiation
Mean difference 1.9 ± 0.3°C
a
Exposure 2 - Winter Satiation
Mean Difference = 2.0 ± 0.2
b
Exposure 3 - Summer Restricted
Mean difference 1.9 ± 0.3°C
c
Water temperature (°C)
Time (days)
base
base+2°C
0
5
10
15
20
25
30
0
30
60
90

Figure 2. Mean lethal temperatures of juvenile rainbow trout challenged by either (a) heating normal tap water (see Table 1 for ionic composition) at a rate of 1°C every 1 h (Exposure 2 - winter satiation) or 2 h (Exposures 1 and 3 - summer satiation and summer restricted, respectively) and recording the temperature at death for each fish (Lethal Temperature challenge), or (b) heating water containing 1.8 mM T_{Amm} under a similar fashion (Lethal Temperature and Ammonia challenge). Means (+SE) represent the lethal temperature of 6 to 10 trout originating from the 90-day chronic exposures (see caption Fig. 1) to one of four pre-challenge treatments: dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure), solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and ammonia). Within each exposure, a capital T represents a significant temperature effect exhibited in trout pre-exposed to +2°C, and a A represents a significant ammonia (Am) pre-exposure effect. Among exposures, a capital S represents a significant seasonal effect, and a R represents a significant ration effect (one-way ANOVA - see Methods). Significance at $P \leq 0.05$ in all cases. Note that challenging juvenile rainbow trout to a combination of lethal temperature and ammonia (panel b) reduced their upper lethal temperature by 3-7°C (compare with panel a).

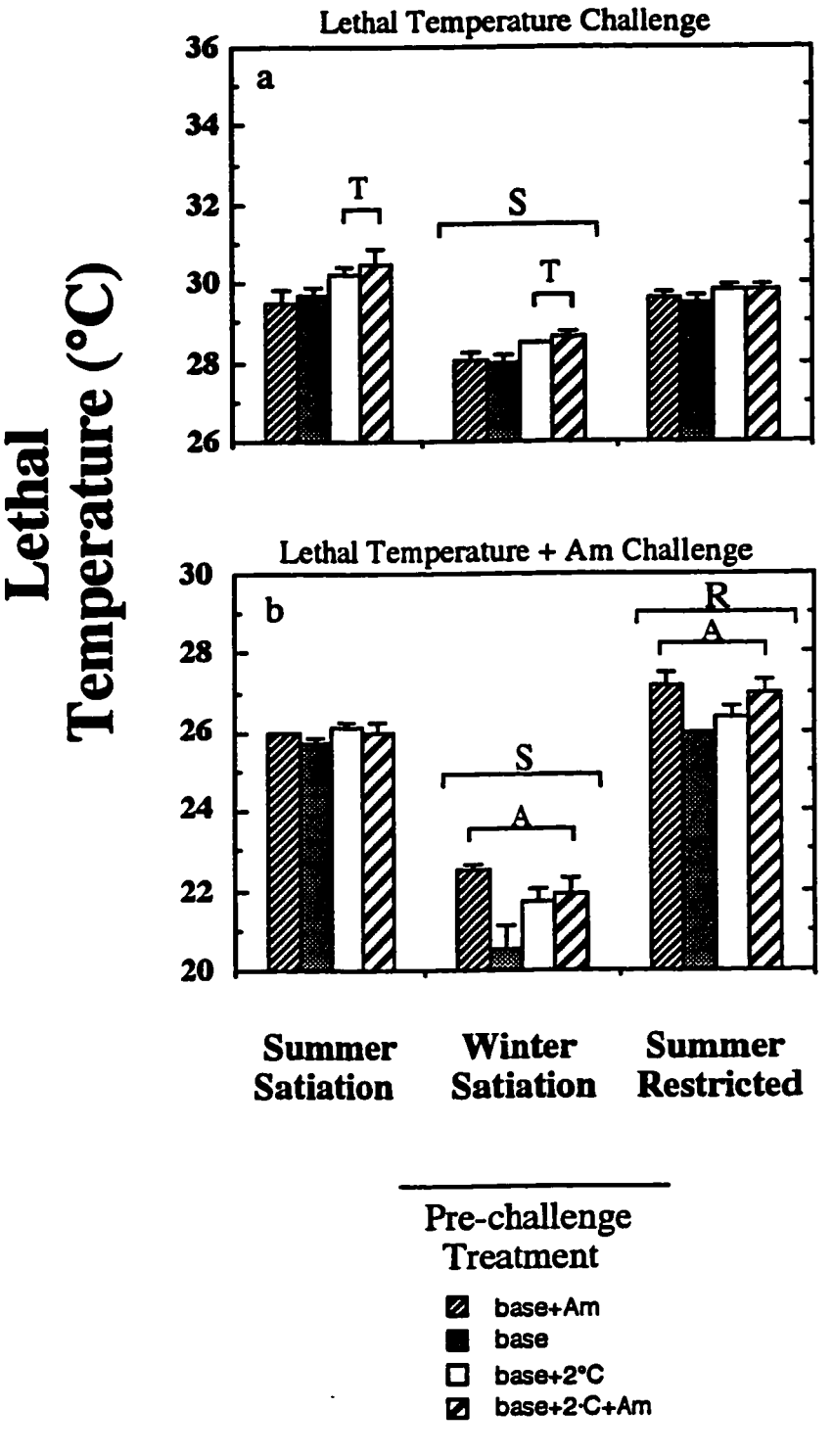
Lethal Temperature Challenge
a
T
S
T
Lethal Temperature + Am Challenge
b
R
A
S
A
Lethal Temperature (°C)
36
34
32
30
28
26
30
28
26
24
22
20
Summer Satiation
Winter Satiation
Summer Restricted
Pre-challenge Treatment
base+Am
base
base+2°C
base+2°C+Am

Figure 3. Time to 50% mortality ($LT_{50} \pm 95\%$ C.L.) of fish from all four pre-challenge treatment groups (see caption Fig. 2) during a static 1.8 mM T_{Amm} challenge following the three 90-day exposure periods (see caption Fig. 1). The LT_{50}'s and their 95% confidence limits were calculated and compared using log/probit analysis (Litchfield 1949). The capital S represents a significant seasonal effect (one-way ANOVA; $P \leq 0.05$).

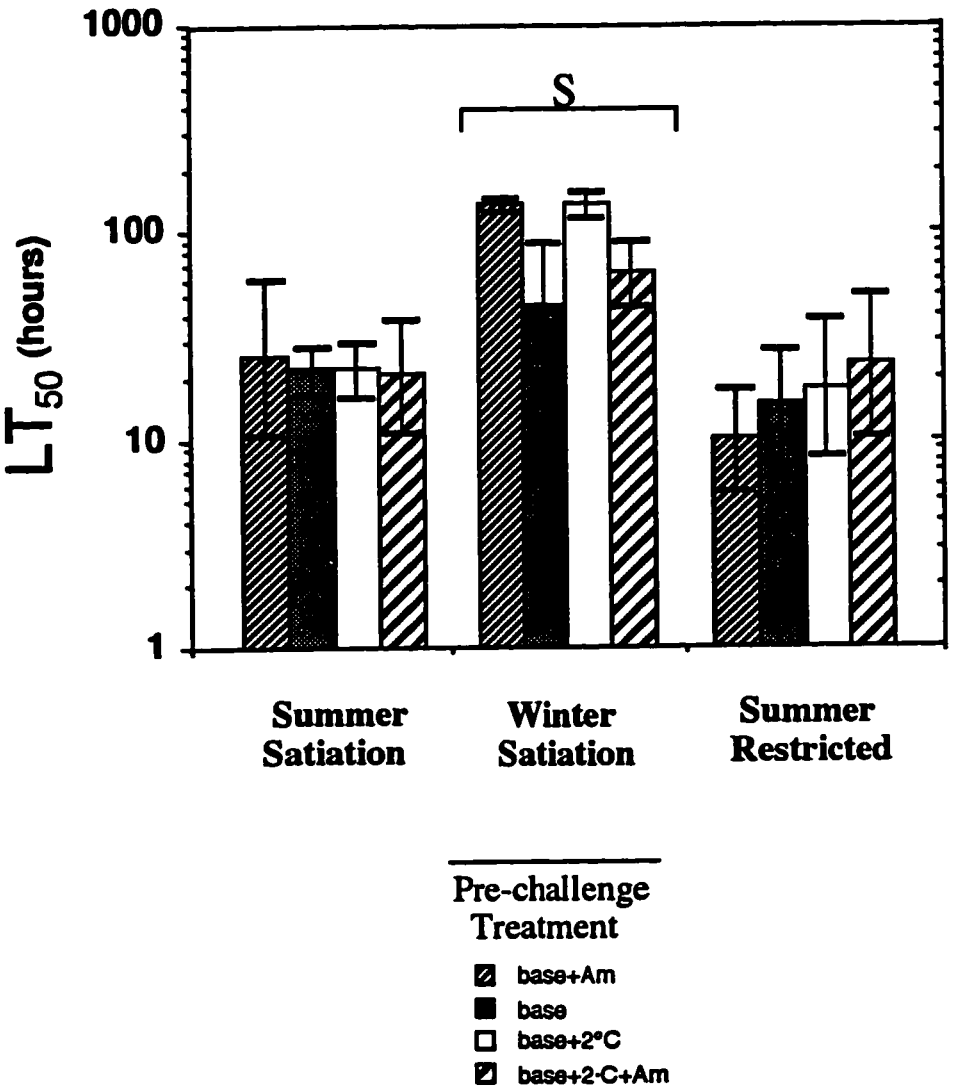
1000
100
10
1
LT50 (hours)
S
Summer Satiation
Winter Satiation
Summer Restricted
Pre-challenge Treatment
base+Am
base
base+2°C
base+2-C+Am

Figure 4. The effect of 24 h of exposure to high external ammonia (1 mM T_{Amm}) on net ammonia fluxes (J^{Amm}_{net}) in juvenile rainbow trout (means± SE, N = 16) after: (a) exposure 1 - Summer Satiation, and (b) exposure 3 - Summer Restricted (see caption Fig. 1). Naive fish (with no prior exposure to ammonia, base and base+2°C, respectively) are represented by open columns and the ammonia pre-exposed fish (Am) by hatched columns. Asterisks (*) indicate values significantly different ($P \leq 0.05$) from the (pre)-high external ammonia challenge control value and daggers (†) indicate values significantly different between naive and ammonia pre-exposed fish at each respective challenge interval thereafter.

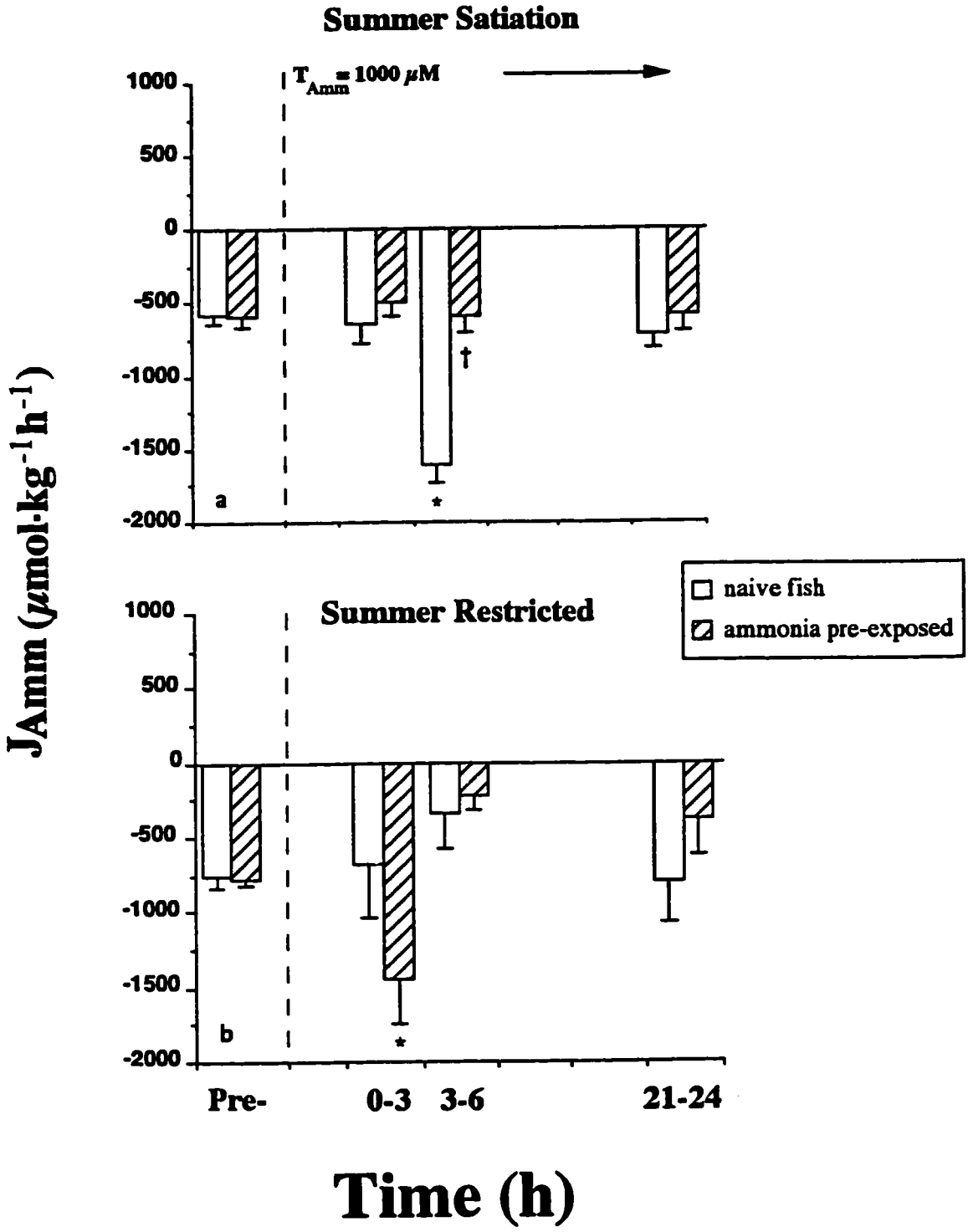
Summer Satiation
T_{Amm} = 1000 μM
1000
500
0
-500
-1000
-1500
-2000
a
†
*
Summer Restricted
b
*
naive fish
ammonia pre-exposed
JAmm (μmol·kg⁻¹h⁻¹)
Pre-
0-3
3-6
21-24
Time (h)

Figure 5. The effect of 24 h of exposure to high external ammonia on net (dark solid columns) and unidirectional sodium uptake (J^{Na+}_{in}; upward bars) and efflux (J^{Na+}_{out}; downward bars) rates from trout (means± SE, N = 16) after: (a) exposure 1 - Summer Satiation, and (b) exposure 2 - Summer Restricted ration. Columns and symbols indicating significance ($P \leq 0.05$) are as in Fig. 4.

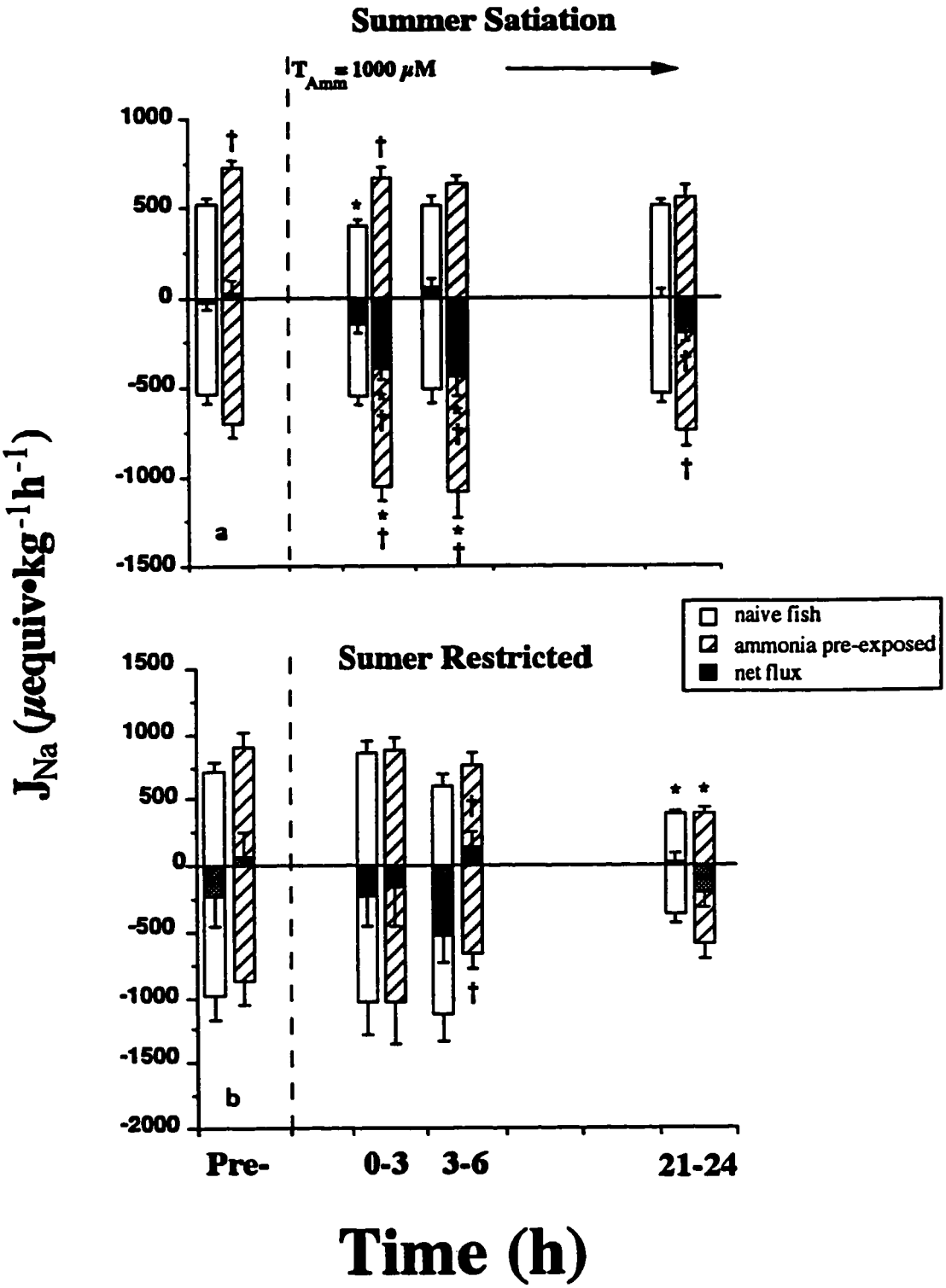
Summer Satiation
T_{Amm} = 1000 μM
1000
500
0
-500
-1000
-1500
a
Sumer Restricted
1500
1000
500
0
-500
-1000
-1500
-2000
b
naive fish
ammonia pre-exposed
net flux
J_{Na} (μequiv•kg^{-1}h^{-1})
Pre-
0-3
3-6
21-24
Time (h)

Table 1. Mean (± SE and/or range) water temperature, total ammonia (T_{Amm}) concentration, pH, and ammonia speciation in tanks during 90-day exposures of juvenile rainbow trout to +2°C and 70 μM T_{Amm} (nominal = Am) in de-chlorinated Hamilton tap water ($[Ca^{2+}]$ = 0.956 ± 0.019 mM, $[Na^+]$ = 0.546 ± 0.008 mM, $[Cl^-]$ = 0.737 ± 0.004 mM).

Exposure and Treatment	Mean Temperature °C (Range)	T_{Amm} (μM)	pH	$[NH_4^+]^{a,b,c}$ μg/L (Range)	$[NH_3]^{a,b,c}$ μg/L (Range)
Exposure 1 (summer satiation)					
base+Am	16.3 ± 0.3 (13.0 - 21.5)	61.5 ± 3.8	7.59 ± 0.26	1094.1 (717 - 1795)	11.9 (4 - 74)
base	16.3 ± 0.3 (13.0 - 21.5)	6.0 ± 0.2	7.57 ± 0.26	106.8 (34 - 180)	1.2 (0 - 8)
base+2°C	18.1 ± 0.3 (15.0 - 23.5)	5.8 ± 1.3	7.55 ± 0.22	103.1 (30 - 249)	1.2 (0 - 11)
base+2°C+Am	18.1 ± 0.3 (15.0 - 23.5)	75.7 ± 2.9	7.55 ± 0.25	1346.0 (671 - 2238)	15.3 (4 - 101)
Exposure 2 (winter satiation)					
base+Am	4.7 ± 0.1 (3.5 - 7.0)	76.6 ± 1.9	7.51 ± 0.02	1373.3 (1149 - 1565)	5.2 (3 - 13)
base	4.7 ± 0.1 (3.5 - 7.0)	8.3 ± 0.3	7.46 ± 0.02	148.9 (128 - 195)	0.5 (0 - 1)
base+2°C	6.7 ± 0.1 (5.5 - 9.5)	9.5 ± 0.3	7.47 ± 0.02	170.3 (134 - 184)	0.7 (0 - 1)
base+2°C+Am	6.7 ± 0.1 (5.5 - 9.5)	88.8 ± 1.8	7.56 ± 0.02	1590.1 (1371 - 1771)	7.9 (4 - 15)
Exposure 3 (summer restricted)					
base+Am	15.8 ± 0.1 (13.0 - 18.0)	66.3 ± 1.5	7.56 ± 0.03	1180.7 (957 - 1581)	12.0 (6 - 27)
base	15.8 ± 0.1 (13.0 - 18.0)	8.1 ± 0.6	7.38 ± 0.09	148.3 (61 - 274)	1.0 (0 - 5)
base+2°C	17.6 ± 0.1 (15.0 - 20.0)	8.4 ± 0.6	7.30 ± 0.12	150.2 (59 - 240)	0.9 (0 - 4)
base+2°C+Am	17.6 ± 0.1 (15.0 - 20.0)	64.6 ± 2.4	7.40 ± 0.08	1153.2 (748 - 1881)	9.1 (1 - 34)

[a] The equilibrium expression: $K'_a = [NH_3][H^+]/[NH_4^+]$ was adjusted for temperature dependence following the equation: $pK_a = 0.09018 + 2729.92/(273.2+T°C)$ from Emerson et al. (1975).

[b] The separate fractions of T_{Amm} in the solution were calculated from the Henderson-Hasselbach equation: $NH_4^+ = T_{Amm}/ 1 + \text{antilog}(pH - pKa) = T_{Amm} - NH_3$, as reported by Wood (1993).

[c] Note that fractions of NH_4^+ and NH_3 are expressed in traditional toxicological units of μg/L rather than units of μM more normally used in physiological studies.

Table 2. Initial and final water temperature, total ammonia (T_{Amm}) concentration, pH, and ammonia speciation in tanks during lethal temperature and ammonia (Challenge II) and lethal ammonia challenges (Challenge III), respectively. The separate fractions of T_{Amm} were calculated and expressed as in Table 1.

	Mean Temperature (°C)		T_{Amm} (μM)		pH		$[NH_4^+]$ (μg/L)		$[NH_3]$ (μg/L)	
Lethal challenge and exposure	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Temperature + Ammonia										
Exposure 1 (summer satiation)	24.0 - 28.0[a]		1,898 - 1,887		7.87[c]		32,884 - 32,272		1,207 - 1,598	
Exposure 2 (winter satiation)	9.5 - 23.5[b]		1,612 - 1,597		7.83		28,663 - 27,783		340 - 918	
Exposure 3 (summer restricted)	24.0 - 29.0[a]		1,731 - 1,452		7.85		30,042 - 24,804		1,054 - 1,258	
Ammonia										
Exposure 1 (summer satiation)	16.0 - 14.0		2,138 - 3,860		7.87 - 8.11		37,641 - 67,302		799 - 2,057	
Exposure 2 (winter satiation)	10.0 - 15.0		1,788 - 1,989		7.83 - 8.13		31,784 - 34,519		377 - 1,207	
Exposure 3 (summer restricted)	18.0 - 19.5		1,834 - 1,870		7.85 - 8.10		32,221 - 32,123		748 - 1,445	

[a] Highest temperature reached after heating at a rate of 1°C/2 h.
[b] Highest temperature reached after heating at a rate of 1°C/1 h.
[c] Only initial pH values were recorded for lethal temperature and ammonia challenges (see Methods).

CHAPTER 6

CHRONIC EXPOSURE OF RAINBOW TROUT TO SIMULATED WARMING AND SUBLETHAL AMMONIA: A YEAR-LONG STUDY OF THEIR APPETITE, GROWTH, AND METABOLISM

ABSTRACT

This study was conducted to assess over the thermal cycle of an entire year, the potential effects (on appetite, growth, and metabolism) of a chronic small temperature increase (+2°C) and sublethal levels (+70 $\mu mol \cdot l^{-1}$) of ammonia (a common pollutant) on rainbow trout (*Oncorhynchus mykiss*). Juvenile trout (≈ 11 g initially) were exposed for 14 months to four treatment regimes: the *natural* water temperature cycle representative of the inshore region of Lake Ontario, this cycle +2°C to simulate a global warming scenario, and these temperature cycles in the presence of an additional 70 $\mu mol \cdot l^{-1}$ total ammonia (NH_3 range: 0.005 to 0.013 $mg \cdot l^{-1}$). The fish were hand-fed to satiation each day to quantify appetite while being exposed to a natural photoperiod. The additional +2°C substantially increased appetite over winter, significantly elevating specific growth rates. These gains were lost, however, over summer due to high temperature related suppression of appetite and growth. Within the growth zone, ammonia had little adverse effect on the above parameters and perhaps a slight stimulating effect on protein accretion. Additional ammonia at low base temperature in winter decreased growth performance suggesting threshold-induced toxicity. At high summer temperatures, the combination of +2°C and ammonia resulted in a general decrease of nitrogen enzyme (alanine and aspartate aminotransferase, glutamate dehydrogenase, and glutamine synthetase) activity that was

not exhibited by fish exposed to +2°C alone. Overall, the metabolic cost of nitrogen accretion (i.e., oxygen consumption per unit protein growth) for juvenile trout was lowest for the 'base' temperature group (16.8), slightly increased with the addition of 70 $\mu mol \cdot l^{-1}$ total ammonia (18.2) or +2°C (18.7), and highest in the +2°C+70 $\mu mol \cdot l^{-1}$ total ammonia combination group (19.6). Confirming previous predictions, these results document the dramatic influence of a +2°C warming scenario on the growth and feeding metabolism of juvenile rainbow trout. Moreover, the data indicate that this small increase in temperature, together with low level ammonia pollution, could substantially alter protein dynamics in juvenile freshwater fishes. The implications of this work are such that juvenile trout without thermal refuge will experience an increase in their cost of living in a warmer more polluted environment.

INTRODUCTION

Growing concern over the incremental increase in ambient air temperature in the past few decades, whether from natural climatic variation or anthropogenic emissions of 'greenhouse gases', has prompted an international initiative to gain a better understanding of how temperature increases of only 1.5-4.5°C could affect the basic physiological and biochemical functions of aquatic organisms (McDonald and Wood 1997). Fish are particularly susceptible to environmental temperature changes because their body temperatures, and hence their feeding, metabolic, growth, and excretion rates are governed by the temperature of the water in which they live (Savitz 1969, Fry 1971, Elliott 1976, Weatherley 1990). Based on studies conducted at constant acclimation temperatures, increased temperatures within the preferred thermal range of temperate fish species lead to greater growth rates provided that food is not limiting (Elliott 1976, Wurtsbaugh and Davis 1977). Beyond these boundaries, however, growth potential decreases (Brett et al. 1969, Elliott 1982). This is due to the influence of a number of factors, particularly temperature and dissolved oxygen, on appetite, growth, and energetics (Fry and Hart 1948, Brett 1979). Therefore, understanding the effect of temperature change on growth and food consumption is fundamental to predicting the potential effects of climate change on the growth performance of fish (McCarthy and Houlihan 1997).

The implication of the temperature influence on individual growth has population relevance. The majority of temperate fish species feed and grow at temperatures much narrower in range than those which permit short-term survival (Elliott 1981, Jobling 1981). Although growth under certain circumstances may have low 'immediate' priority (Mehner and Wieser 1994), satisfying the growth requirement is normally a high priority for fish in nature (Brett and Groves 1979). There may be a selective advantage for those fish that thermoregulate behaviorally to maximize growth under different conditions of

temperature and food availability (Jobling 1997). Historical records indicate that annual temperature fluctuations of a smaller magnitude to those projected by most global warming scenarios have indeed had significant short-term impacts on species distribution and abundance (Murawski 1993). Clearly small temperature increases, particularly those that cause a decline in growth and energetic efficiency, have the potential to define fish distribution (Rombough 1997).

Temperature and food availability, however, are not the only factors affecting fish distribution patterns. The effective zone of temperature tolerance in fish can be restricted in nature by other environmental factors such as pollution (Rombough 1997). Temperature change will almost certainly occur against a backdrop of increasing pollution (Kennedy and Walsh 1997). This could alter the response of fish to increases in ambient temperature. For example, juvenile rainbow trout challenged with high temperature in the presence of elevated ammonia exhibit considerably reduced temperature tolerance (Linton et al. 1997a).

Ammonia is not only ubiquitous in the aquatic environment, but its origins are rather unique. Elevated ambient water ammonia levels stem not only from anthropogenic sources (e.g. agricultural run-off, industrial discharge, sewage effluent, etc.), but also from ammonia produced and released into the environment by aquatic organisms themselves. This has great importance for the actual exposure of fish to ammonia because its overall effect depends on the concentration at the site of exchange, the gills. In intensive fish culture, ammonia concentrations lower than the maximum allowable concentration of 0.02 $mg \cdot l^{-1}$ as NH3-N set by the EPA to protect aquatic life (EPA 1985) have been reported to cause gill damage (Burrows 1964) and reduced growth (Smith and Piper 1975). However, in an extensive review on this subject, Meade (1985) concluded that differences in water quality are likely to account for most of the adverse sublethal effects caused by NH_3 at such low levels. We have, in fact, just recently reported growth

stimulation in juvenile rainbow trout feeding to satiation and exposed to +70 $\mu mol \cdot l^{-1}$ total ammonia (NH_3 approximately 0.013 $mg \cdot l^{-1}$) over summer (see Linton et al. 1997b), suggesting the elevated ambient ammonia may contribute to N-retention.

To date, most data regarding the response of fish to temperature have been collected under conditions of controlled temperature (usually 5 to 10°C apart) and food availability. There is currently a need to examine how short-term and seasonal fluctuations in water temperature affect the basic physiology and biochemistry of fish, especially in the face of low-level environmental contamination. The present study was conducted to determine the potential effects of a warmer (+2°C) and polluted (+70 $\mu mol \cdot l^{-1}$ total ammonia) scenario on juvenile rainbow trout (*Oncorhynchus mykiss*) exposed under naturally fluctuating temperature throughout the entire year. The feeding regime (hand-fed once a day to satiation) was not one in which the fish were fed a controlled or equal ration, but rather one which was allowed to fluctuate in correspondence with the needs of the fish.

Our goal was to estimate the physiological and metabolic costs of growth for freshwater fish coping with the warmer more polluted environment. The study corroborates and extends results from three previous experiments (90 d), conducted over summer and winter without food limitation (Reid et al. 1997, Linton et al. 1997b and c) and during summer with food limitation (Linton et al. 1997d), and substantiates two predictions about the likely impacts of small chronic temperature increase on a freshwater fish species. First, we show clearly the profound effects of only +2°C superimposed above the natural fluctuating thermal regime on the feeding physiology, growth and energetics of juvenile rainbow trout, especially at the seasonal temperature extremes, where the temperature elevation exerts opposite effects. Second, we show that the influence of this small temperature increase, under environmentally realistic water quality scenarios, is large enough to alter the protein dynamics in a reference cold water fish species, the

rainbow trout. Together, these data confirm that the levels of temperature increase predicted by some global warming models will have direct and significant effects on the metabolic costs associated with the growth of freshwater fish. Moreover, these costs will be increased in the face of low-level environmental ammonia pollution.

METHODS AND MATERIALS

Experimental Animals

Juvenile rainbow trout (approximately 11 g) were obtained from Humber Springs Trout Club, Orangeville, Ontario in June 1995 and allowed to acclimatize for 2 weeks prior to testing. The trout were held in a 600 l aerated polyethylene tank receiving 2.5 $l \cdot min^{-1}$ dechlorinated Hamilton tap water ($[Ca^{2+}]$ = 0.95 $mmol \cdot l^{-1}$, $[Na^{+}]$ = 0.60 $mmol \cdot l^{-1}$, $[Cl^{-}]$ = 0.75 $mmol \cdot l^{-1}$; pH = 7.65) at an ambient water temperature of 17-19°C. They were fed a maintenance ration equivalent to 1% body $weight \cdot d^{-1}$ of Zeigler Bros., Inc (Gardner, PA, USA) Floating Fish Nuggets (40% protein, 10% lipid, manufacturer's specification) and kept under simulated natural photoperiod for Hamilton, Ontario throughout the experiment.

Chronic Exposure to Temperature and Elevated Ammonia

Sixteen groups of approximately 80 fish each were randomly distributed to 16 exposure tanks (270 l) representing 4 treatment conditions (N = 320 fish per treatment). Within each group, 11 fish were marked with a Panjet Fish Marker (Wright Medical Inc., Dundee, Scotland) for individual identification. The tanks received an average 1.8 $l \cdot min^{-1}$ (95 % replacement = 5.8 h) of either ambient temperature water (base = City of Hamilton tap water representative of near-shore water temperatures of Lake Ontario) or ambient temperature water plus 2°C (base+2°C), each with or without an additional 70 $\mu mol \cdot l^{-1}$ total ammonia ($T_{Amm} = NH_4^+ + NH_3$; base+Am and base+2°C+Am, respectively). Water

at base+2°C was achieved by using a heat exchanger while water containing +70 $\mu mol \cdot l^{-1}$ T_{Amm} was achieved by delivering the required amount of $(NH_4)_2SO_4$ stock solution via mariotte bottles (Figure 1). The mean concentrations of T_{Amm} in the water of each tank along with the pH and partial pressure of oxygen are listed in Table 1. The background levels of T_{Amm} in the base and base+2°C treatments of 15 - 20 $\mu mol \cdot l^{-1}$ resulted from the metabolism of the fish themselves. Thus, the exposure levels in the nominal 70 $\mu mol \cdot l^{-1}$ T_{Amm} base+Am and base+2°C+Am treatments were 3 - 4 times background levels. During the experiment, the fish were hand-fed to satiation daily (0900 h) from pre-weighed bags of food, so as to monitor appetite, following the methods of Wilson et al. (1994).

Measurement of Oxygen Consumption and Nitrogenous Waste Excretion

Routine oxygen (O_2) consumption rates were measured initially (-2 d), and then monthly thereafter, on whole tanks of fish using a stop-flow method similar to that of Brett and Zala (1975). Rates were determined for all 4 tanks per treatment, 2 each on consecutive days, and the fish were fed at their regular time during the measurements. Samples were taken over a 24 h period beginning at 1000 (just after feeding), then at 1500, 2000, 0100, 0600, and 0900 h (just before feeding). The tank water and air supplies were closed, the water surface was sealed with a transparent lid fitted snugly to the tank walls, and magnetic drive pumps (Little Giant, 1-EUAA-MD) were activated to recirculate and mix the water. For each sampling, tank water was extracted every 10 min over 40 min to obtain an mean rate of oxygen depletion for that period. The partial pressure of O_2 (P_{O2}) of each water sample was measured with a temperature equilibrated Cameron O_2 electrode connected to a Cameron OM 200 oxygen meter. After the final P_{O2} measurement, the air supplies were re-opened prior to the next sampling period to allow re-aeration, and water

flow was resumed for 2-3 h. The P_{O2} of the water for any one tank was not allowed to drop below 90 torr. Mean rates of O_2 consumption were determined for each period using oxygen solubility coefficients of Boutilier et al. (1984), factored by time, volume and total fish biomass. Biomass was measured by netting the fish from the tanks into a bucket containing 10 l of the appropriate water and a removable plastic sieve. The bucket and contents were weighed on a tared scale (GSE 450 Scale Systems, Michigan, U.S.A.). The fish were seined and immediately placed back into their original tank. The bucket and contents without the fish were reweighed, the difference yielding total fish biomass. All six O_2 consumption rates were averaged to give a single O_2 consumption rate representative of the full 24 h interval.

Routine nitrogenous (N) waste excretion was measured at the same time as O_2 consumption initially (-2 d), and on days 60 (September 1995), 150 (December 1995), 240 (March 1996), 360 (June 1996), and 420 (August 1996). An initial 50 ml water sample was collected just after the water flow was turned off for the O_2 consumption measurements. After 3 h, a final sample was collected and the water flow turned back on to allow flushing (approximately 2 h) before the next O_2 consumption measurement. The water samples were frozen immediately for future analysis of total N on a Antek 7000 Total N Analyzer. The mean N-waste excretion rate was determined from the difference in N concentration of the water, factored as above. All O_2 consumption and N-waste excretion data were corrected for differences in fish size using the weight exponent 0.824 determined for rainbow trout by Cho (1990).

Measurements of the contribution of bacteria to O_2 consumption and N-waste excretion were estimated on a tank of fish that had been receiving additional temperature and ammonia (base+2°C+Am treatment) in September (water temperature = 23°C, biomass = 1.8 kg) representing a 'worst case scenario'. The fish were removed and placed in a

separate tank while measurements were made over the next hour. Bacteria were estimated to contribute only 7% to the total oxygen consumed. However, they could reduce total N-waste excretion based on ammonia-N and urea-N measurements by up to 38%, mainly by converting excreted ammonia into nitrate or nitrite. Therefore we elected to use the Antek Nitrogen Analyzer which measures all the N in the water sample, e.g., 100% recovery of ammonia-N, urea-N, nitrate- and nitrite-N. We assumed that all N accumulated over the 3 h closed period (see above) came originally from the fish, regardless of the form of N measured.

Measurement of Growth Increment

Several times throughout the year marked fish were measured for their growth increment. Fish were netted from the tanks, and each marked fish was identified, anaesthetized with MS222 (0.1 $g \cdot l^{-1}$ adjusted to pH 7.5 with 1N NaOH), and their wet weights and total lengths measured. The fish were then allowed to recover in fresh water and returned to their original holding tanks. At test termination, the marked fish were handled in a similar manner but were sacrificed with an over-dose of MS-222 (1 $g \cdot l^{-1}$, pH = 7.5). Growth was measured as before, but afterwards, the peritoneal cavity was opened for sex determination.

Whole Body, Blood, and Tissue Sampling

At days 0 (initial sample), 25 (August 1995) and 422 (August 1996) whole bodies, individual tissues, and blood samples were collected 24 h after the O_2 consumption and N-waste excretion rates were measured. Food was withheld from the fish during this period. Five non-marked fish per tank (i.e. 20 per treatment) were randomly selected for determination of whole body protein and water content and plasma Na^+, while another 5

were selected for the determination of N-enzyme activity: alanine aminotransferase (Alat), aspartate aminotransferase (Aspat), glutamate dehydrogenase (GDH), and glutamine synthetase (GNS), in liver, gill, and white muscle.

For whole body protein content and blood sampling, the fish were killed by a quick blow to the head and a terminal blood sample was collected via caudal severance. The plasma was separated by centrifugation and the body freeze-clamped with aluminum tongs pre-cooled in liquid N_2. Both plasma and whole body were stored at -70°C until further analysis. The fish sampled for tissue N-enzyme activity were handled in a similar manner, but sacrificed with an overdose of MS-222 (1 $g \cdot l^{-1}$) at pH=7.5 to minimize metabolic disturbance due to struggling (Wang et al. 1994). The liver, gill basket, and a small portion of white muscle anterior to the dorsal fin and above the lateral line were excised and freeze-clamped immediately in liquid N_2. Each tissue was individually wrapped in aluminum foil and stored at -70°C for further analysis.

Analytical

Whole body protein was determined on fish blended into a homogenous mixture in 3 parts distilled water and assayed using a modification to the Lowry method (Miller 1959). A small portion of this mixture was withheld and dried in an oven at 80°C for 48h to obtain the water content. The concentration of Na^+ in plasma was determined using atomic absorption spectrophotometry (Varian AA 1275).

Frozen livers, gills, and white muscle were ground into a fine powder in an insulated mortar cooled with liquid N_2. A volume (mls) equal to 4 times the tissue weight, i.e., 100 mg of liver and gill or 200 mg of white muscle, of homogenization buffer (50% glycerol, 20 mM K_2HPO_4, 10 mM HEPES, 0.5 mM EDTA, and 1 mM dithiothreitol; pH = 7.5) was added to each sample of frozen tissue in 1.5 ml microcentrifuge tubes. The

tissue samples were then sonicated (Fisher Sonic Dismembrator Model 300) for 30 s (35% maximum) and kept on ice before centrifugation (Eppendorf Centrifuge 5415C) for 2.5 min at 14,000 rpm at 5°C. The Alat, Aspat, and GDH assays were performed on supernatants of the three tissue homogenates by continuous measurement of the decrease in absorbance at 340 nm as NADH was converted to NAD using a LKB Biochrom Ultrospec Plus spectrophotometer. Specific cocktails (1 ml·tissue sample^{-1}) consisted of the following made in 50 mM HEPES and adjusted to pH 7.5:

Alat - 0.12 mM NADH, 200 mM alanine, 0.025 mM pyridoxyl 5' phosphate, 12 units/ml of lactic dehydrogenase (in glycerol), 10.5 mM alpha-ketoglutarate;

Aspat - 0.12 mM NADH, 40 mM aspartate, 0.025 mM pyridoxyl 5' phosphate, 8 units/ml malic dehydrogenase (in glycerol), 7 mM alpha-ketoglutarate;

GDH - 0.12 mM NADH, 250 mM ammonium acetate, 0.1 mM EDTA, 1 mM ADP, 14 mM alpha-ketoglutarate. Control activity without alpha-ketoglutarate was less than 1% of activity with alpha-ketoglutarate. The millimolar extinction coefficient of NADH (6.22) was used to calculate rates of substrate disappearance.

GNS was measured by the arsenolysis of glutamine to gamma glutamyl hydroxamate at 540 nm. One milliliter of cocktail (60 mM glutamine, 15 mM hydroxylamine, 0.4 mM ADP, 20 mM KH2AsO4, 50 mM HEPES, 3 mM MnCl2, pH = 6.7) was required to convert 50 µl of sample supernatant. Liver and gill tissue samples were incubated for 1 h, while muscle samples were incubated for 4 h. A 300 µl aliquot of ferric chloride reagent (50% HCl: 24% trichloroacetic acid: 10% FeCl3 in 0.2 N HCl) was added to control samples (i.e., a duplicate sample spiked with ferric chloride reagent at time t=0) and incubated samples to stop the reaction. Control and incubated samples were then centrifuged for 1 min (14,000 rpms), transferred to cuvettes, and read at 540 nm. Micromoles product were calculated using the slope of gamma glutamyl hydroxamate

standards similarly prepared. All enzymatic assays were conducted at room temperature (23°C), and all reagents were from Sigma Chemical, Co. (St. Louis, MO.). Enzyme activities are expressed as units (μmols product/min) per gram tissue wet weight.

Calculations

Appetite was the daily average amount of food consumed per fish by the four replicate tanks as fish were fed to satiation. Conversion efficiency was calculated as the ratio of the weight gain (marked fish) to food consumed, both in wet weight. Cumulative food intake was calculated using the sum of the daily food intakes over each specified time period.

Specific growth rates (SGR) were determined from the 11 marked fish measured for growth increment (N ≈ 44 per treatment). The growth rates were calculated following the standard formula:

$$SGR = 100(\log_e Y_2 - \log_e Y_1)/ (t_2-t_1)$$

where Y_1 and Y_2 are the mean wet weights of fish at times t_1 and t_2. Condition factors were determined as the quotient of the wet weight of the fish and its total length cubed then multiplied by 100 percent.

The nitrogen quotient (NQ), or the extent of aerobic protein catabolism, was calculated as the ratio of moles of N produced to moles of O_2 consumed (Kutty 1972). The N-cost index, or the total moles of O2 consumed per mole of nitrogen stored, was used to compare the total metabolic expenditure associated with the incorporation of nitrogen into body material, i.e., the metabolic 'cost of growth' (see Linton et al. 1997b). Protein was measured as a surrogate for N. The percentage N by weight in the protein of the fish was taken as the standard value, 16% (Soderberg 1995).

Statistical Analyses

All whole body, blood and tissue enzyme data are expressed as means ± 1 SE from individual samples pooled together from the 4 tanks per treatment. There were no substantial differences amongst tanks within a treatment. One-way analysis of variance using SAS Jmp (SAS Institute Inc., Version 2.0.5) followed by Tukey-Kramer HSD multiple comparison test was used to distinguish statistically significant differences in food intake, wet weights, total lengths, and condition factors amongst treatment groups within each sample period. Multiple factor analyses with leverage plots were used to distinguish statistically significant temperature, ammonia, and interactive effects on whole body protein and water content, plasma Na^+, and N-enzyme activity amongst treatment groups within each sample period. One-way analysis of variance followed by Dunnett's test (Dunnett 1955), where N represented the number of tanks per treatment, was used to compare differences in appetite, gross conversion efficiencies, O_2 consumption rates, N-waste excretion rates, and NQs of the respective treatment groups to the 'base' control group; an exception was with the analysis of SGRs, where N represented the total number of marked fish. The level of statistical significance for all analyses was $P \leq 0.05$.

RESULTS

Temperature Profiles

The temperature profiles for the base and base+2°C temperature regimes (characteristic of near-shore Lake Ontario) show that the yearly water temperature cycle varies by more than just a few °C (Figure 2). For example, the mean monthly water temperature during August 1995 was 23°C whereas in August 1996 it was only 18°C. It is also evident from Figure 1 that trout grown under the base thermal regime spent at least 6

months at temperatures below 10°C (November to mid-May) compared to only about 5 months (mid-November to May) for trout exposed to base+2°C. Rates of fall cooling and spring warming were about 5.5 and 4.5°C per month, respectively.

Mortality

During the first 25 days of exposure, mortality rates were 0.19, 0.45, 0.58, and 0.37 %·day^{-1} for base, base+Am, base+2°C, and base+2°C+Am groups respectively. By day 90, these mortality rates had decreased to 0.03, 0.05, 0.12, and 0.15 %·day^{-1} respectively. Mortality was virtually non-existent after these initial 3 months (September 1995).

Growth and Feeding

Over the course of the entire exposure, juvenile trout fed to satiation daily had gained 30 - 35 times their original wet weight, and approximately 3 times their total length (Table 2). Those fish exposed to the combination of +2°C and ammonia (base+2°C+Am) bested their 3 other cohort groups by at least 30 g in weight and an extra 1 - 1.5 cm in length. Substantial gains in growth increment were achieved by trout exposed to the additional +2°C (base+2°C) over winter and spring, only to be lost in the final 30 days (mean water temperatures at base+2°C = 20°C) of the experiment (Table 2). The increased growth was associated with an elevation in food intake through winter and spring which ultimately contributed to the consumption of an additional 60 and 75 g of food, for the base+2°C and base+2°C+Am groups respectively, compared to the base group (Table 2). Condition factors fluctuated seasonally over the course of the year, but in general, rose steadily from an initial mean of 0.88 to 1.26 (Table 2). An overall sex ratio

(males:females) of 1.7 existed among marked fish at test termination. Gender had no significant influence on final weight of the fish.

Specific Treatment Effects

Base vs. Base+Am - The addition of +70 $\mu mol \cdot l^{-1}$ T_{Amm} to the base thermal regime did not affect appetite, specific growth rate, or gross food conversion efficiency of trout with an exception between 15 December 1995 and 25 January 1996 (Figure 3a,b, and c respectively). Here, appetite and SGR were significantly depressed in the trout exposed to the additional ammonia compared to the base group. Oxygen consumption, however, tended to be elevated in the ammonia-exposed fish, especially during fall and spring (Figure 4a). Little difference in N-waste excretion existed throughout much of the year (Figure 4b), although N-waste excretion of the base+Am group tended to fall late in the second summer (1996) which was apparent in the significantly lower NQs (see Figure 4c).

Base vs. Base+2 °C - The effects of an additional +2°C on growth and metabolism of rainbow trout were much more dramatic owing to substantial temperature-dependent changes in appetite (Figures 5 and 6). The extraordinarily high water temperatures reached during August 1995 resulted in a significantly depressed appetite (Figure 5a), and a greater than 50% reduction in SGR of the warmed fish (25°C+) in comparison to the base group (Figure 5b). This was also reflected in a lower gross food conversion efficiency, but the effect was not significant (Figure 5c). A similar, and even more pronounced, negative temperature-related effect on appetite and growth occurred the next summer when the fish were larger; this despite lower mean temperatures (Figure 5a,b, and c). In fact, prior to August (from June to July 1996), the warmed fish had consumed significantly more food than the base group, but were less able to convert that food into body materials.

Through winter and spring, the additional +2°C stimulated appetite leading to slightly greater growth. However, the greater appetites and growth were also associated with significant elevations in the rates of oxygen consumed (Figure 6a). There was little effect of the additional +2°C on N-waste excretion (Figure 6b), except toward the end of fall where an elevated NQ indicated the greater reliance of these fish on protein as a metabolic fuel (Figure 6c).

Base vs. Base+2 °C+Am - As the effects of +70 $\mu mol \cdot l^{-1}$ T_{Amm} were relatively small, the combination of +2°C and +70 $\mu mol \cdot l^{-1}$ T_{Amm} added to 'base' water led to similar types of effects as with the addition of +2°C alone (Figures 7 and 8). However, there were subtle differences. For instance, appetite was less dramatically altered in the presence of +70 $\mu mol \cdot l^{-1}$ T_{Amm} (Figure 7a) despite very pronounced reductions in SGRs during the high water temperatures of late summer (Figure 7b). The additional temperature and ammonia also helped stimulate growth through fall as well as winter, though the effect did not appear to give rise to changes in gross food conversion efficiency (Figure 7c).

The growth stimulation during fall, winter, and spring were again at an additional metabolic cost. Oxygen consumption rates were significantly elevated in October, and from February through April, compared to the base group (Figure 8a). On the other hand, high temperatures in combination with the additional ammonia during late summer tended to suppress N-waste excretion leading to lower NQs (Figures 8b and c). The opposite effect was observed at the low winter temperatures, indicating a greater reliance on protein as a metabolic fuel at this time.

Whole Body Protein, Water Content, and Plasma [Na^+]

Aside from an initial decline (5%) in whole body protein content, which fell to 10% after the first 25 days (August 1995), the initial whole body protein content of 15% was maintained at the end of the experiment. However, whole body protein contents of fish grown at +2°C were significantly lower (two-way ANOVA; $P \leq 0.05$) than fish grown at base water temperatures at this time (data not shown). Similarly water content, which fell from an initial 77 to approximately 70% (data not shown), was also reduced in the base+2°C fish after 420 d of exposure. Plasma [Na^+] increased nearly 25% from 110 to 130 mequiv·l^{-1}, but no clear treatment effects were exhibited (data not shown).

Tissue N-enzymes

The activity of alanine aminotransferase (Alat) in liver was initially 30 times greater than in the gill tissue, but only 7 times greater than in white muscle (Table 3). After 25 days of exposure, Alat activity remained fairly constant in all three tissues. By the end of the experiment, there was an approximate 5-fold increase in liver Alat activity (Table 3). Alat activity in the gills had only increased about 2-fold by this time, whereas the muscle activity was reduced by > 50%. A significant interactive effect was apparent in the base+2°C+Am group which exhibited lower overall liver Alat activity. In muscle, significantly lower Alat activity was exhibited by the ammonia-exposed fish at both temperature regimes.

Initial levels of aspartate aminotransferase (Aspat) activity in liver were similar to Alat. However, activities in gill and muscle were comparatively higher representing almost 50 and 75% of liver Aspat activity, respectively (Table 3). Almost a month into the exposure, Aspat activity was largely unchanged in all three tissues. However, at the end of the experiment (Table 3), there was a large increase in Aspat activity (3-fold) in the liver,

and interactive effects such that overall, Aspat activities in liver and muscle were depressed in the base+2°C+Am treated fish. These effects were similar to those of Alat. In gills, significantly lower Aspat activity was exhibited by the ammonia-exposed fish at both temperature regimes.

The highest levels of glutamate dehydrogenase (GDH) activity were exhibited in the liver of trout, but high levels were also present in the gills which were double the activities in muscle (Table 4). The addition of +2°C caused the rapid (25 d) stimulation of GDH activity in muscle, and interacted with +70 $\mu mol \cdot l^{-1}$ T_{Amm} in the gills to enhance activity even further. Levels of GDH activity in liver were relatively unaltered at this time. After 420 days, however, a dramatic increase in the activity of liver GDH, similar in magnitude to Alat and Aspat, was measured (Table 4). GDH activities in liver, gill and muscle were reduced in the ammonia exposed fish (Table 4). A significant interactive effect was observed in the liver such that GDH activity was depressed in the base+Am group, but this may be an artifact due to the low sample size. On the other hand, additional temperature depressed GDH activity in the gills at this time.

Out of the 4 enzymes measured, glutamine synthetase (GNS) was by far the lowest in activity (approximately 50 times). Initial GNS activities in livers and gills were the same, but GNS activity in muscle was two orders of magnitude lower (Table 4). A significant negative effect after 25 days of exposure was seen in the livers and gills of fish exposed to an additional +2°C. The activity of this enzyme increased the least in trout by the end of the experiment, e.g., liver GNS activity increased only about 2 fold. As with the other enzymes, liver GNS activity was significantly depressed in fish coping with the interaction of +2°C and ammonia. The activity of GNS in gills, which had doubled in the first 25 days, had tripled by the end of the experiment. Gill GNS activity was significantly

lower in the ammonia-treated groups at this time. Muscle GNS activity changed very little with time, but the base+2°C group had significantly higher GNS activity overall.

DISCUSSION

The prospect of global warming in today's marginalized environments requires innovative research in order to accurately assess potential effects on fish. Until recently, the effects of temperature have not been approached from a global warming perspective (see Wood and McDonald 1997). This study employed the natural variation of water temperature and photoperiod characteristics of near-shore Lake Ontario to examine the effects of simulated global warming and sublethal ammonia on the appetite, growth, and energetics of juvenile trout growing over an entire year.

Effects of +2°C During Summer Maximum Temperatures

One of the most dramatic effects of the additional +2°C was on the appetite of 'warmed' fish during late summer. Suppression of appetite induced by high temperature ($Q_{10} \approx 0.4$) caused a cascade of whole-animal responses that tended to decrease oxygen consumption, N-waste excretion, and the NQ (extent of protein used to fuel metabolism). This ultimately led to a significant impairment of gross food conversion efficiency ($Q_{10} \approx 0.05$) and a substantial decline (> 50%) in growth rate of warmed fish compared to those at base temperature. These results confirm our earlier findings that +2°C superimposed above the annual cycle depresses appetite and protein turnover of trout fed an unlimited ration during peak summer water temperatures (see Reid et al. 1995 and Linton et al. 1997b).

The implications of appetite suppression induced by high temperature in juvenile fishes are profound. Appetite and growth of fish in general are normally bound by progressively narrowing thermal limits within which these processes are maximized (Brett

1979, Elliott 1982, Jobling 1997). Optimal growth, in particular, takes place within a narrow range of the growth zone up to a threshold temperature above which feeding ceases, and survival time becomes dependent on the amount of stored energy available to resist the ever increasing stimulatory effect of temperature on metabolic rate processes and the ever decreasing availability of oxygen in the water (Jobling 1997, Rombough 1997).

The threshold temperatures for the cessation of feeding, and subsequently growth, differed between summers (> 20 *versus* < 20°C), and thus, with fish size and age (Figure 5 a and b). This is in apparent contradiction to previous work. Elliott (1981) found no difference in the highest temperature for normal feeding in brown trout weighing 10-12, 37-40, and 175-185 g, and a recent compilation of data suggest that the thermal sensitivities for other processes such as metabolism and growth usually decrease with age (Rombough 1997). These studies, however, were conducted on fish acclimated to constant temperatures.

Despite a considerable negative impact on gross food conversion efficiency in trout in late summer (Figure 5c), the additional +2°C had little effect on the efficiency with which food is converted to body material throughout the rest of the year (below 15°C), in agreement with the results of Wurtsbaugh and Davis (1977). At a very young stage, fish on maximum ration can grow (i.e., energy storage) at rates that exceed rates of oxygen consumption (i.e. energy expenditure; Brett and Groves 1979). This can be seen in the present results between July and August 1995 (mean temperature ≈ 17°C). However, as fish get older and larger, their growth and metabolic rates slow down (Elliott 1982), but at different rates such that eventually the rate of metabolic energy expenditure is considerably higher than the amount deposited as energy (Brett and Groves 1979). This serves as the basis for the generality that conversion efficiency decreases with increasing fish size. Aside from the initial month of the present exposure (mean water temperatures

approximately 17°C), little effect of fish size, and possibly age, on gross food conversion efficiency was observed. The discrepancy may again be related to the use of a natural thermal regime, and perhaps variable feeding regime, rather than constant temperature and fixed ration.

The mechanism(s) of appetite suppression in fish is(are) largely unknown. In brown trout (*Salmo trutta*), feeding is discontinued at approximately 19°C (Elliott 1982), whereas in juvenile sockeye salmon (*Oncorhynchus nerka*), feeding ceases above 23°C (Brett 1995). Brett et al. (1969) have shown that a large increase in ration is required to meet the defined growth parameters as temperature rises. This process can be complicated, however, by the increased metabolic demands of feeding fish at high temperature (Jobling 1997). The metabolic rates of feeding fish are up to several-fold higher than those of fish on low ration (Brett 1979, Jobling 1993, Soofiani and Hawkins 1982, Alsop and Wood 1997). The increase, along with the increased energetic demands at higher temperatures (compare Linton et al. 1997b with Linton et al. 1997c for instance), impose greater demands on the respiratory and circulatory system. In the present study, routine oxygen consumption rates of fish at base temperature late in the second summer approached 90% of maximum oxygen consumption (VO_2 max) measured at maximum swimming speeds (Ucrit) in fasting juvenile rainbow trout (Alsop and Wood 1997), whereas oxygen consumption rates were only 75% of VO_2 max in the base+2°C fish. Alsop and Wood (1997) also show that although there is no ration effect on VO_2 max at Ucrit, Ucrit is reduced in satiation-fed fish as compared to fasted fish. Thus, the authors concluded that VO_2 max "is limited by the capacity to take up O_2 at the gills and/or deliver O_2 through the circulatory system, rather than the capacity to consume O2 at the tissues." Jobling (1997) recently used Brett's (1995) data to show that appetite suppression may be related to

limitations in the capacity of the respiratory and circulatory systems to deliver oxygen to respiring tissues at temperatures above threshold.

The obvious influence of temperature over the control of appetite and growth in fish deserves further study, especially in the context of climate warming. Under these circumstances growth should have the least priority. However, Mehner and Wieser (1994) found little indication that perch (*Perca fluviatilis*) fed submaximal rations at 15 and 20°C used behavioral measures (voluntary reduction in activity) to reduce metabolic expenditures. It is evident in this study, where ration fluctuated in correspondence with the needs of the fish, that a general down-regulation ($Q_{10} \approx 0.65$) of metabolic processes at temperatures above threshold (22-24°C during August 1995, 17-19°C August 1996), whether invoked behaviorally or physiologically, was employed as a necessary method of surviving environmental hardship.

Effects of +2°C During Winter Minimum Temperatures

The effects of the +2°C warming scenario were equally noticeable at low temperatures. The period from January to April 1996 averaged about 4°C at 'base' temperature (Figure 2). During this time, fish exposed to +2°C consumed approximately 8 g more food and gained an extra 20 g in weight; conversion efficiencies were elevated approximately 15%. The increased metabolism associated with feeding, defined as the apparent heat increment of feeding (see Beamish and Trippel 1990), had a large influence on oxygen consumption rate (Figure 6a). Linton et al. (1997c), who showed similar results, attributed the phenomenon to the increased metabolic costs of protein synthesis (Reid et al. 1995). The present results are perhaps not surprising given the known influence of temperature on appetite and growth. However, it was unexpected that the difference in growth and metabolic response to feeding of juvenile trout at 4 and 6°C would

be so large (mean $Q_{10} = 5.2$), especially in view of the fact that during spring (see April through June 1996, Figure 5a), appetite was significantly elevated in fish exposed to +2°C, but growth and metabolic rates were not different from the 'base' fish.

Growth and metabolic modulation in relation to temperature need not only be imperative for fish at the upper thermal extreme. Decreased temperature directly affects protein content and rates of enzyme catalyzed reactions (Clarke 1987, Nathanailides 1996). It might be that rainbow trout down-regulate metabolism below a certain threshold temperature, or that this process is triggered by the falling temperature itself. This would explain the lower N-waste excretion rate and NQ in base fish leading into winter (see December, Figure 6b and c). Patterns of temperature compensation such as this (type 5 - inverse compensation; see Hazel and Prosser 1974) may be induced at extreme lows in an animal's thermal range to conserve energy reserves, which would increase the length of time the animal could survive winter. A similar cold-induced temperature compensation has been observed in American eel (*Anguilla rostrata*), although the mechanisms are probably quite different (Walsh et al. 1983). Down-regulation has great advantages for fish because all of the other major adaptive strategies for counteracting the effects of extreme low temperature, i.e., increase in enzyme activity, production of new enzyme variants, and alteration in cellular environment (Clarke 1987), impose additional cost. Thus, for juvenile temperate fishes such as rainbow trout, survival during a severe winter could be compromised if metabolic energy conservation did not occur.

It is apparent from the present results that a +2°C warming scenario has a substantial effect on the appetite, growth and metabolism of juvenile rainbow trout at the temperature extremes. The consequences of these effects were considerable gains in growth of fish exposed to +2°C during winter, which were eventually lost towards the end of summer. This could be explained largely by ration, which closely followed the thermal

cycle (Figure 9). The total moles of oxygen consumed for fish exposed to +2°C was estimated to be about 5% higher than the base group (Figure 10a), but the total moles of N they retained was about 5% lower (Figure 10b). This resulted in an increase in cost of living (i.e. oxygen consumption per unit protein growth or N-cost index) of approximately 10% (Figure 10c).

Influence of Environmental Ammonia

The low levels of ammonia used in the present study had more of a stimulatory effect rather than an inhibitory effect on the overall appetite, growth and metabolism of juvenile trout, except at the temperature extremes (Figures 3, 4, 7, and 8). In fact, the fish exposed to both additional ammonia and temperature achieved the greatest gain in weight by the end of the experiment (Table 2). This occurred despite a considerable decline in growth rate in the last month (August 1996) due to high temperature suppression of appetite as discussed above.

There is little precedence for the stimulatory effect of sublethal ammonia on fish growth in the literature. In the majority of cases, fish, and salmonids in particular, exposed to ammonia at equivalent concentrations exhibit gross physiological or even growth impairment, but these effects may have been complicated by other biotic and abiotic factors (see Meade 1985 for review). Linton et al. (1997b), in an earlier study, reported a stimulation of growth in juvenile trout exposed to +70 $\mu mol \cdot l^{-1}$ T_{Amm} at base temperatures during summer. These fish also exhibited higher rates of protein synthesis and turnover in their livers (unpublished data). By the end of the present experiment, trout exposed to ammonia had retained up to 10% more nitrogen than fish not exposed to ammonia (Figure 10b). This phenomenon has been reported on several different occasions in feeding

juvenile trout (see Linton et al. 1997 a,b, and c) and was one of the major reasons for the measurement of N-enzyme activity in the present study.

Although appetite and growth were generally depressed in trout exposed to additional ammonia at base temperatures during winter and fall relative to the base group, they were stimulated in the ammonia-exposed fish at +2°C. Oxygen consumption, on the other hand, tended to be elevated in both groups throughout most of the year (see Figure 10a). This latter response resulted in elevated N-cost indexes, 8% and 17% for the base+Am and base+2°C+Am groups respectively, as compared to the base group (Figure 10c). Brown (1968) was able to demonstrate that ammonia was twice as toxic to rainbow trout at 3°C than at 10°C, and in an earlier study (Linton et al. 1997c), growth of juvenile trout exposed to +70 $\mu mol \cdot l^{-1}$ T_{Amm} was slightly reduced at 4°C (though not significantly) as was their efficiency of N retention. These data confirm that nitrogen retention is not stimulated by sublethal ammonia at very low temperature (< 5°C), which could be a threshold temperature below which ammonia effects are negative or non-existent. Above this threshold temperature, ammonia appears to directly stimulate nitrogen retention, either through a detoxification mechanism or as additional substrate (also see Linton et al. 1997b, c). Since most enzymes function optimally within narrow temperature and pH ranges, it is entirely possible that ammonia acts to stimulate nitrogen retention only within the temperature range necessary for optimal growth.

Temperature, Ammonia, and Nitrogen Enzymes

The stimulation of nitrogen retention by fish exposed to very low levels of ammonia over summer (see Linton et al. 1997b) prompted our investigation of some of the key enzymes involved in nitrogen metabolism and ammonia detoxification in freshwater teleost fish. Although the same pronounced stimulation of nitrogen retention did not

appear to take place during the present experiment (see discussion above), the change in activities of some of these enzymes (albeit small changes) did indicate the potential sensitivity of nitrogen metabolism to environmental change. This was particularly evident in those fish exposed to the combination of +2°C and 70 $\mu mol \cdot l^{-1}$ T_{Amm}, base+2°C+Am (see Tables 3 and 4).

The additional temperature itself had negligible effect on N-enzyme activity. However, in nearly all cases, fish exposed to ammonia at +2°C had significantly reduced enzyme activities. On the other hand, the activities of at least Alat and Aspat in fish exposed to ammonia at base temperature tended to be elevated. The exception was GDH, where enzyme activity was significantly reduced in all tissues of fish exposed to additional ammonia. This may explain the reduced N-waste excretion rates and NQs of these fish at this time (Figures 4b and 8b, respectively). Taken together, these results suggest that the added cost of maintaining high energy levels through amino acid catabolism at higher temperature may be too great for fish already coping with an additional demand (i.e., ammonia) on metabolism.

Metabolic "down-regulation", particularly in biosynthesis and macromolecular turnover, is an effective energy conservation mechanism induced by animals facing environmental insult (Hand and Hardewig 1996). For example, amino acid deamination is reduced in tilapia (*Tilapia mossambica*) exposed to atrazine, possibly in order to minimize extra energy expenditure through protein catabolism (Prasad et al. 1991). The large increase, after 415 days, in the activities (in the oxidative direction) of Alat, Aspat, and GDH, which are all highly involved in aerobic ammonia production in fish (van Waarde 1981), are indicative of fish catabolizing excess amino acids for energy (Walton and Cowey 1977, Wood 1993). Transamination of alanine and aspartate by Alat and Aspat generate oxaloacetate and pyruvic acid respectively (Hill and Wyse 1989), as well as

copious amounts of glutamate. All three of these can enter the Krebs Cycle for direct energy production, or act as precursors for lipid and carbohydrate synthesis (Wood 1993). However, the large difference in tissue enzyme activity levels between sampling periods observed here, especially in the liver, is counter to the scaling of oxidative enzyme activity (e.g., citrate synthetase) with size noted previously in rainbow trout (Somero and Childress 1990). In fact, Somero and Childress (1990) observed only 'positive' scaling for lactate dehydrogenase and creatine phosphokinase, which are enzymes indicative of potential for anaerobic glycolysis or to help maintain stable ATP concentration during muscular activity, respectively. The examination of changes in N enzyme activity with temperature, size, water quality, and feeding activity is a relatively unexplored area in fish physiology and deserving of much further attention. The present data suggest the potential sensitivity of these pathways to environmental change.

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Figure 1. A schematic diagram of the exposure system and holding tanks employed to assess the effects of chronic small temperature increase (+2°C) and sublethal levels of ammonia (+70 $\mu mol \cdot l^{-1}$) on juvenile trout over the period from 8 July 1995 to 30 August 1996.

Exposure System

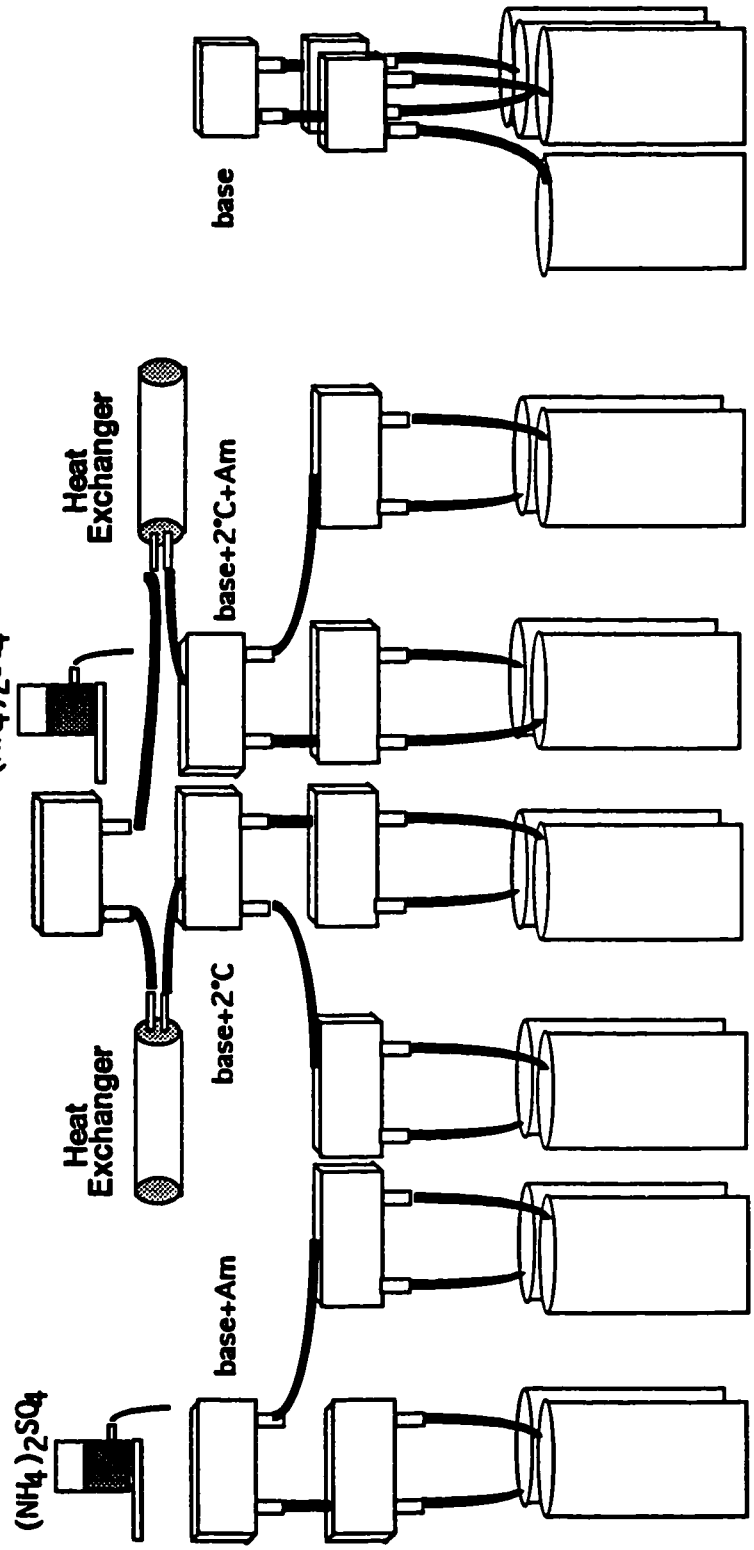


Figure 2. Water temperature profile as measured each day over 420-d exposures from 8 July 1995 to 30 August 1996. Values represent the mean monthly temperature. Juvenile rainbow trout were exposed to either ambient laboratory water temperatures (base = solid line) or to this water + 2°C (base+2°C = dashed line).

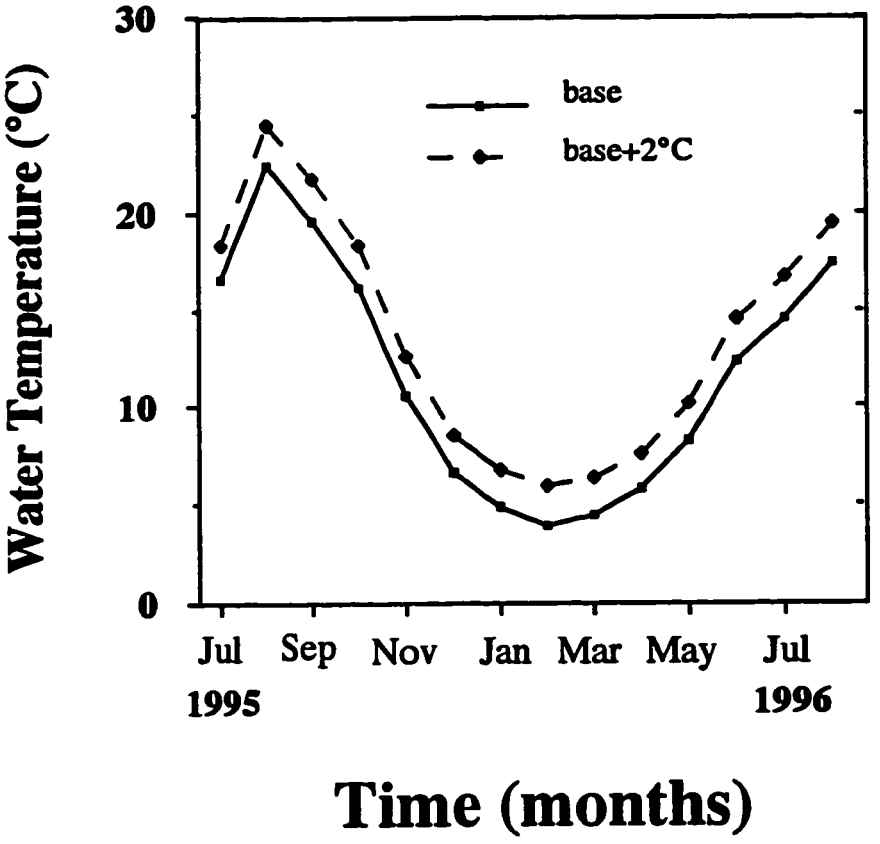

30
20
10
0
Water Temperature (°C)
base
base+2°C
Jul
Sep
Nov
Jan
Mar
May
Jul
1995
1996
Time (months)

Figure 3. Comparison of effects on (a) appetite, (b) specific growth rate (SGR), and (c) gross food conversion efficiency of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or base + 70 $\mu mol \cdot l^{-1}$ T_{Amm} (shaded columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. Appetite was the daily average amount of food consumed by the four replicate tanks of fish as fish were fed to satiation. Gross food conversion efficiency was calculated as the ratio of the weight gain (marked fish) to food consumed, both in wet weight. Specific growth rates were determined from the 11 marked fish measured for growth increment (N = 44 per treatment). Columns with an asterisk (*) are significantly different from the 'base' control group ($P > 0.05$). The base thermal regime is depicted at the top on a monthly basis.

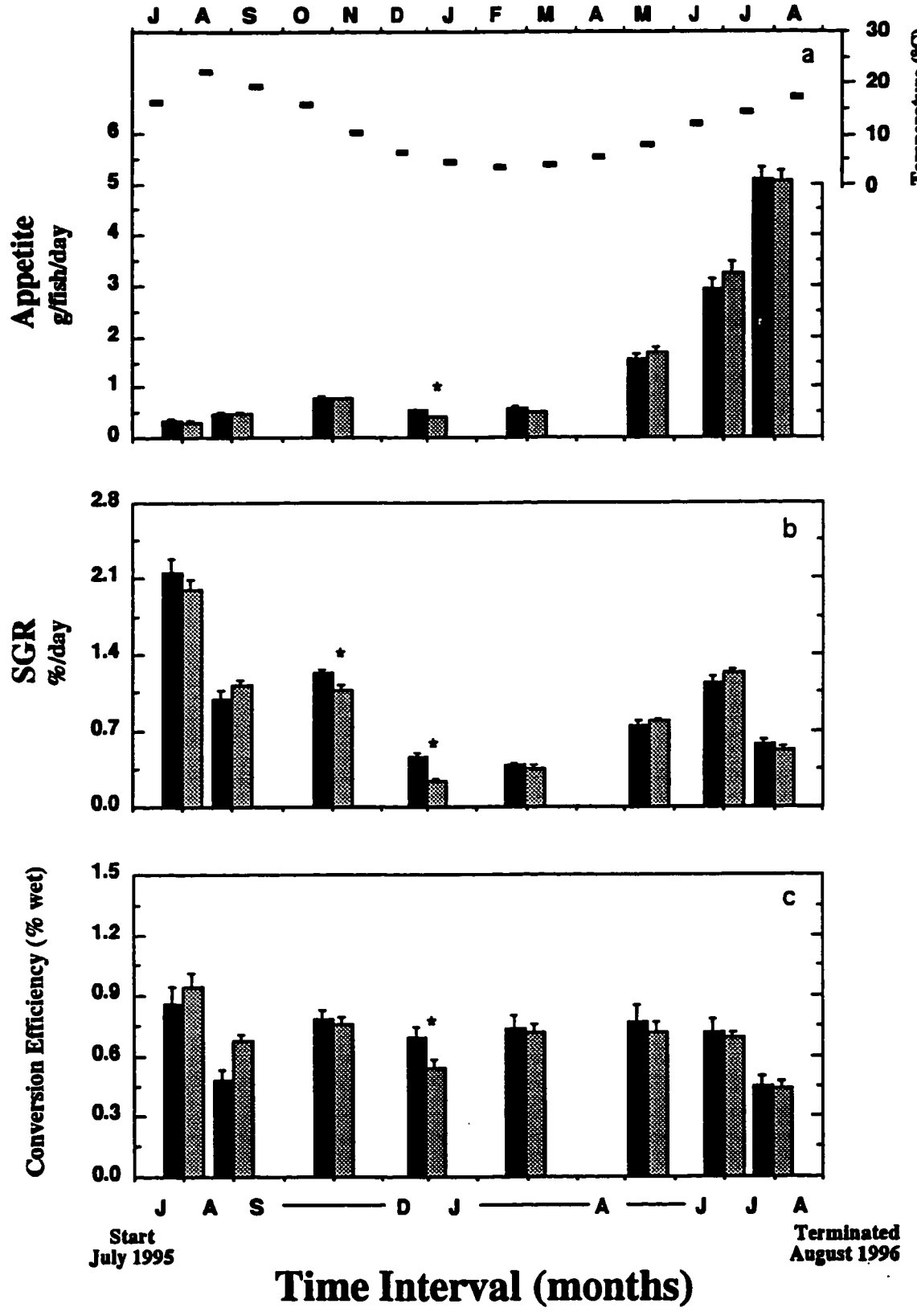

J A S O N D J F M A M J J A
a
Appetite
g/fish/day
Temperature (°C)
30
20
10
0
6
5
4
3
2
1
0
b
SGR
%/day
2.8
2.1
1.4
0.7
0.0
c
Conversion Efficiency (% wet)
1.5
1.2
0.9
0.6
0.3
0.0
J A S D J A J J A
Start
July 1995
Terminated
August 1996
Time Interval (months)

Figure 4. Comparison of effects on (a) routine oxygen consumption, (b) total N-waste excretion, and (c) the nitrogen quotients of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or base + 70 $\mu mol \cdot l^{-1}$ T_{Amm} (shaded columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. In (a) and (b) the data have been scaled for weight using the weight exponent 0.824 (Cho 1990). Measurements were made over a 24 h period on all 4 tanks per treatment. Columns with an asterisk (*) are significantly different from the 'base' control group ($P > 0.05$). The base thermal regime is depicted at the top on a monthly basis.

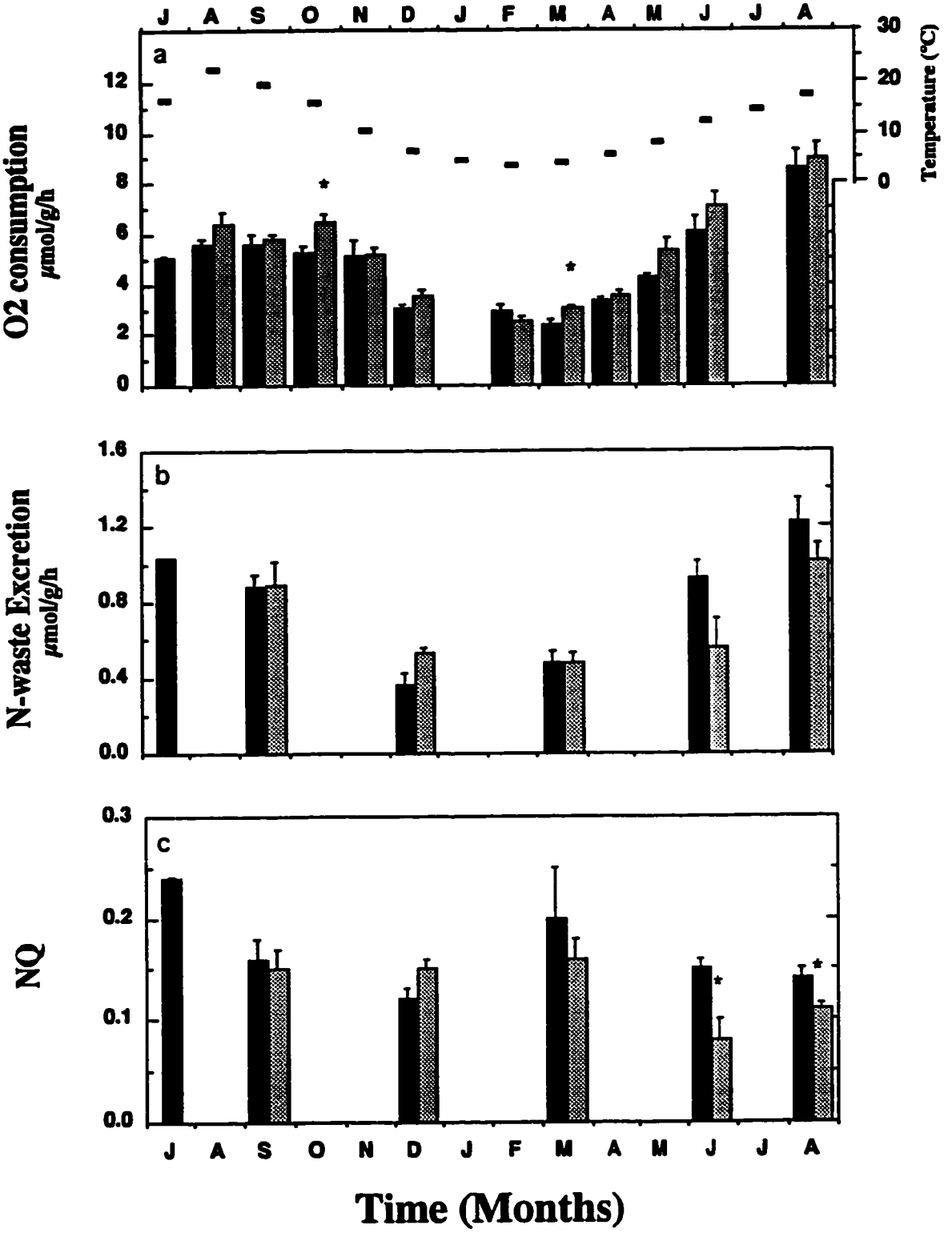

J A S O N D J F M A M J J A
a
O2 consumption
µmol/g/h
Temperature (°C)
b
N-waste Excretion
µmol/g/h
c
NQ
Time (Months)

Figure 5. Comparison of effects on (a) appetite, (b) specific growth rate (SGR), and (c) gross food conversion efficiency of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or this regime + 2°C (open columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. Other details as in Figure 3.

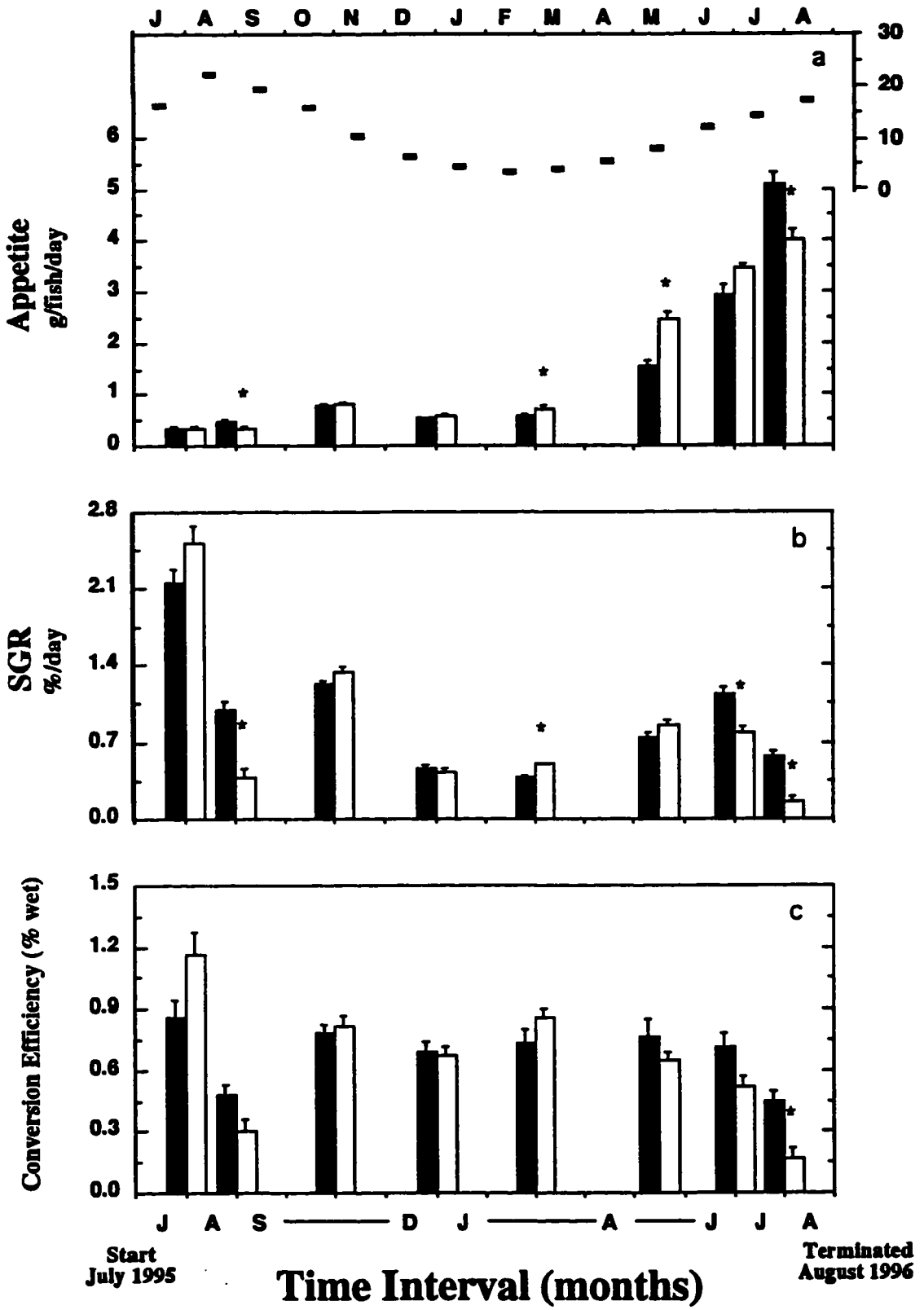

J A S O N D J F M A M J J A
a
Appetite
g/fish/day
Temperature (°C)
30
20
10
0
6
5
4
3
2
1
0
b
SGR
%/day
2.8
2.1
1.4
0.7
0.0
c
Conversion Efficiency (% wet)
1.5
1.2
0.9
0.6
0.3
0.0
J A S D J A J J A
Start
July 1995
Terminated
August 1996
Time Interval (months)

Figure 6. Comparison of effects on (a) routine oxygen consumption, (b) total N-waste excretion, and (c) the nitrogen quotients of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or this regime + 2°C (open columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. Other details as in Figure 4.

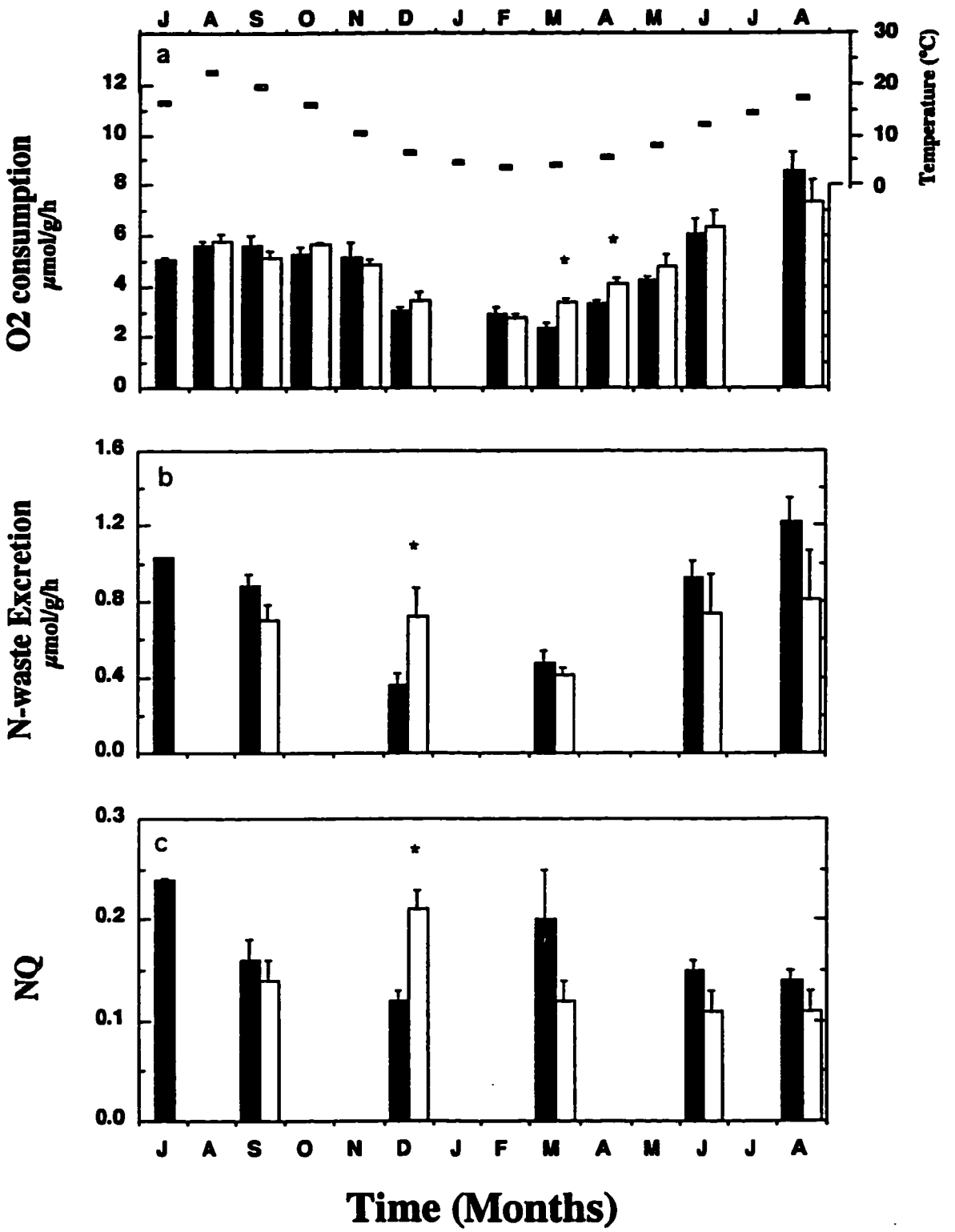

J A S O N D J F M A M J J A
a
O2 consumption
µmol/g/h
Temperature (°C)
b
N-waste Excretion
µmol/g/h
c
NQ
Time (Months)

Figure 7. Comparison of effects on (a) appetite, (b) specific growth rate (SGR), and (c) gross food conversion efficiency of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or this regime + 2°C+70 μmol·l^{-1} T_{Amm} (hatched columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. Other details as in Figure 3.

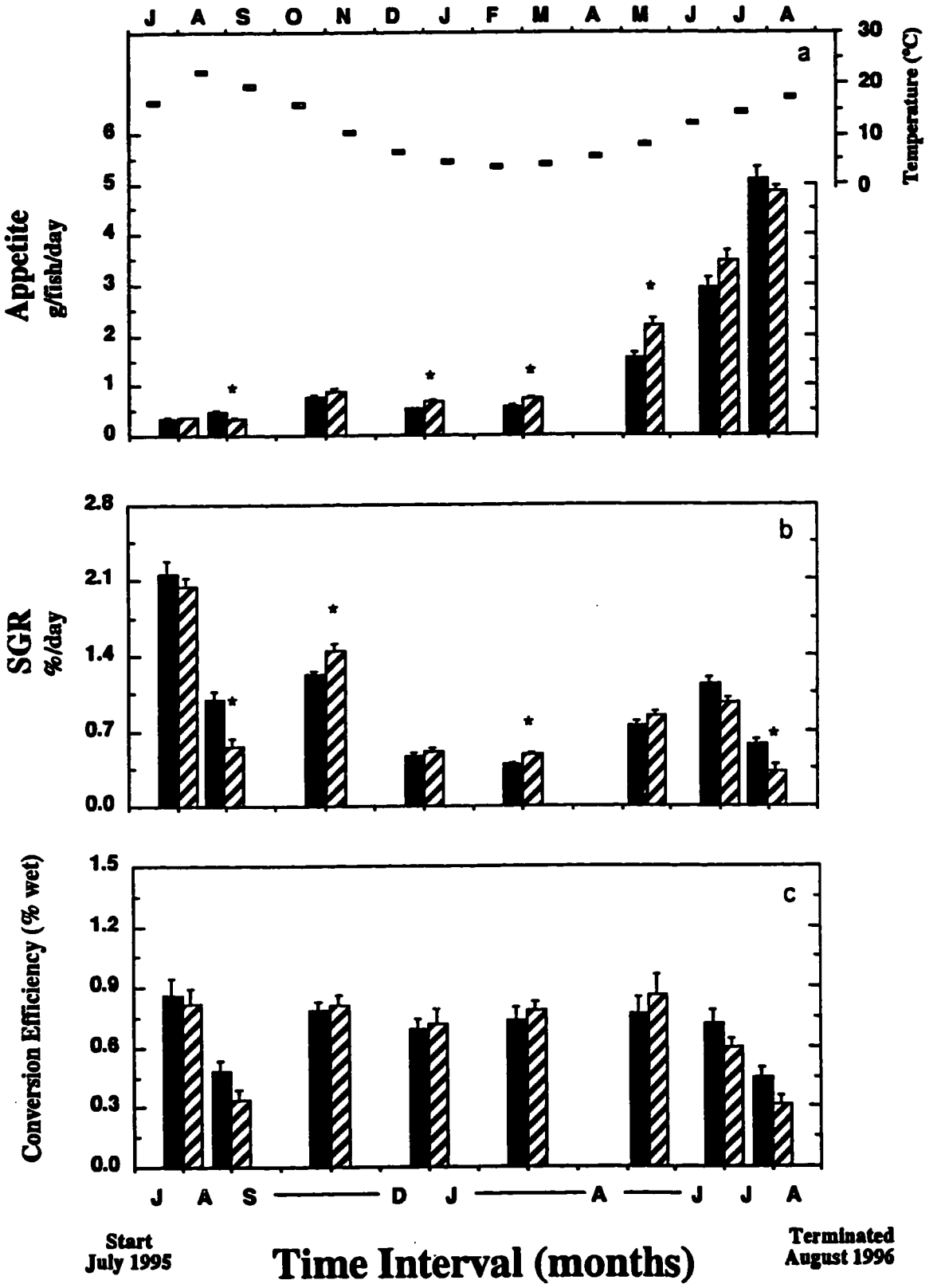

J A S O N D J F M A M J J A
a
Appetite
g/fish/day
Temperature (°C)
30
20
10
0
6
5
4
3
2
1
0
b
SGR
%/day
2.8
2.1
1.4
0.7
0.0
c
Conversion Efficiency (% wet)
1.5
1.2
0.9
0.6
0.3
0.0
J A S D J A J J A
Start
July 1995
Time Interval (months)
Terminated
August 1996

Figure 8. Comparison of effects on (a) routine oxygen consumption, (b) total N-waste excretion, and (c) the nitrogen quotients of juvenile rainbow trout fed to satiation daily and exposed to base temperature and ammonia (solid columns) or this regime + 2°C+70 μmol·l^{-1} T_{Amm} (hatched columns). Exposures lasted 420-d from 8 July 1995 to 30 August 1996. Other details as in Figure 4.

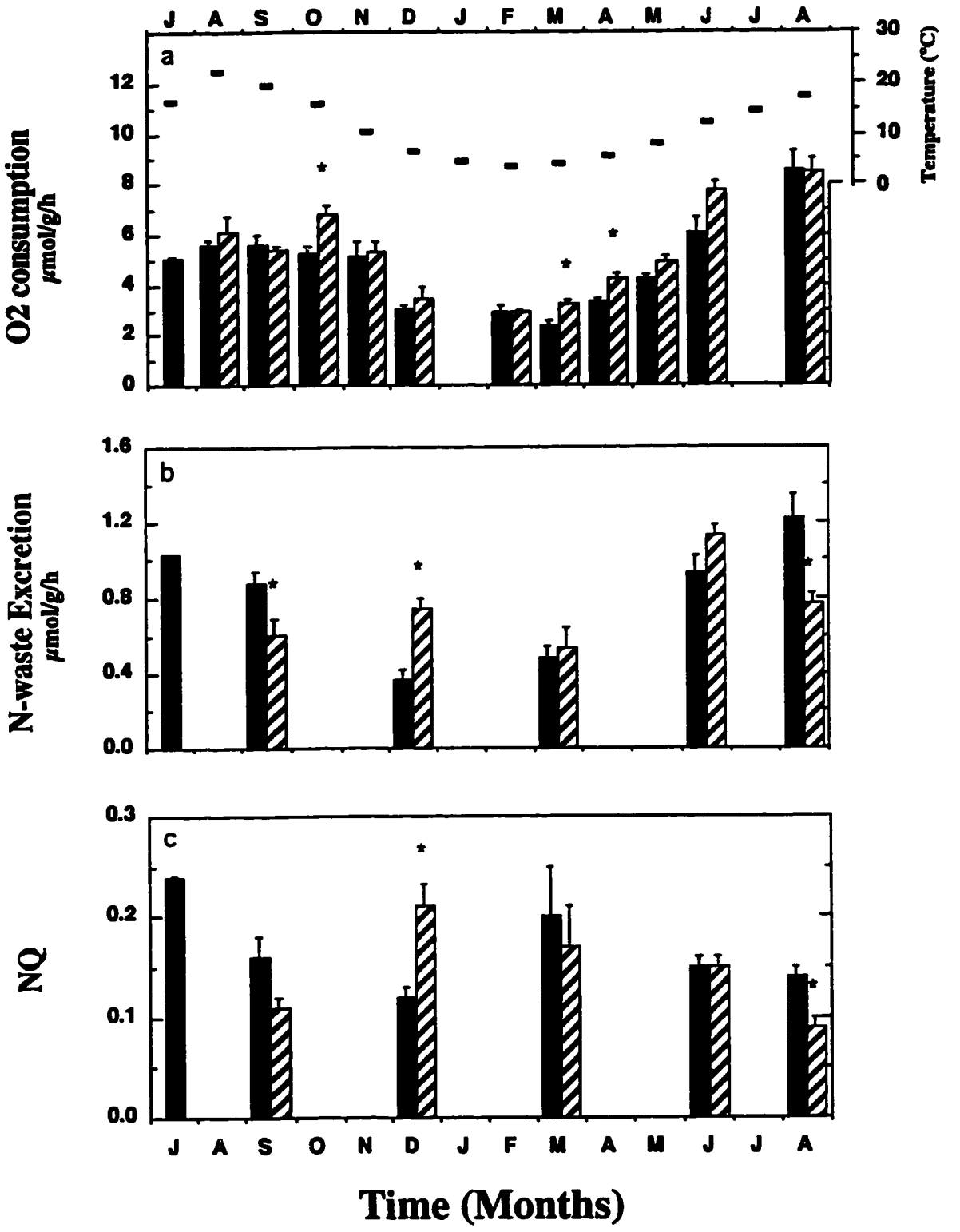

J A S O N D J F M A M J J A
a
O2 consumption
µmol/g/h
Temperature (°C)
b
N-waste Excretion
µmol/g/h
c
NQ
Time (Months)

Figure 9. Comparison of rations, expressed as % wet body weight per day, consumed by juvenile rainbow trout fed to satiation daily and exposed to either: the *natural* water temperature cycle representative of the inshore region of Lake Ontario (base), this cycle +2°C to simulate a global warming scenario (base+2°C), and these temperature cycles in the presence of an additional 70 $\mu mol \cdot l^{-1}$ total ammonia (base+Am and base+Am+2°C, respectively). Exposures lasted 420-d from 8 July 1995 to 30 August 1996.

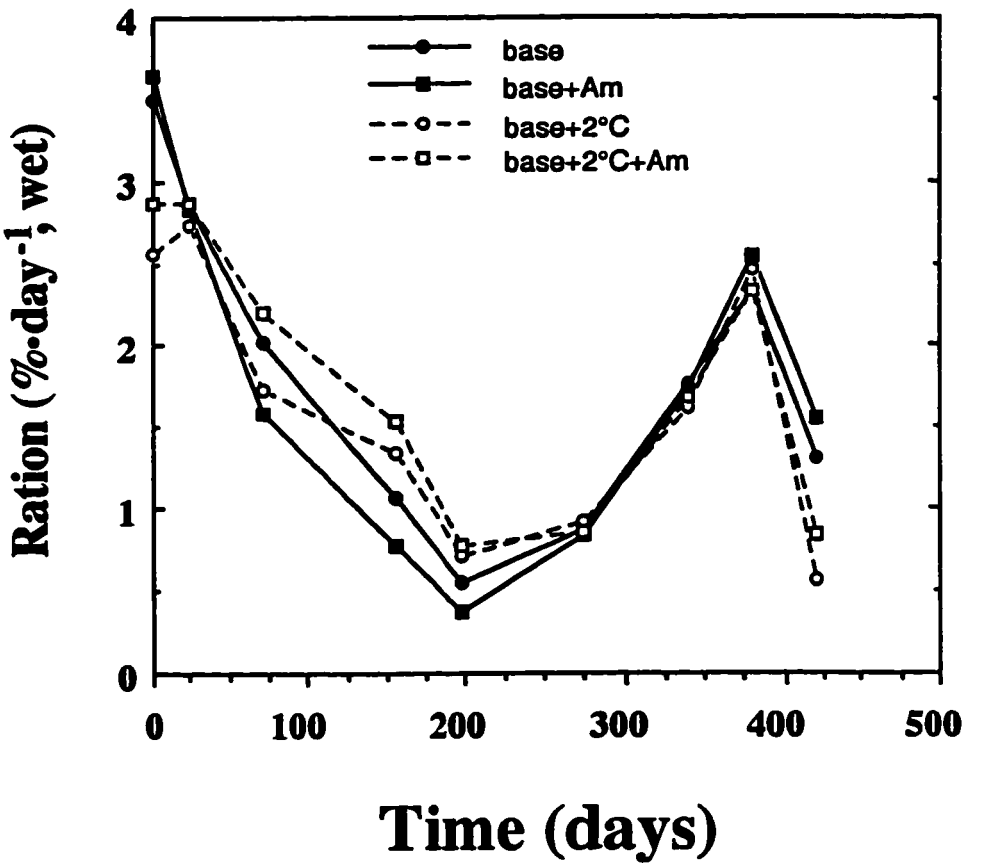

base
base+Am
base+2°C
base+2°C+Am
Ration (%·day-1, wet)
0
1
2
3
4
0
100
200
300
400
500
Time (days)

Figure 10. Comparison of (a) total moles of oxygen consumed, (b) total moles of nitrogen retained from whole body protein measurements and factored by 6.25, and (c) N-cost indexes (see Materials and Methods - *Calculations* for definition) of juvenile rainbow trout fed to satiation for 420 d from 8 July 1995 to 30 August 1996. Values are expressed on a per fish basis. Dark hatched = base+Am (ambient thermal regime and sublethal ammonia exposure), solid = base (ambient thermal regime only), open = base+2°C (a simulated warming scenario only), light hatched = base+2°C+Am (the combination +2°C and sublethal ammonia exposure).

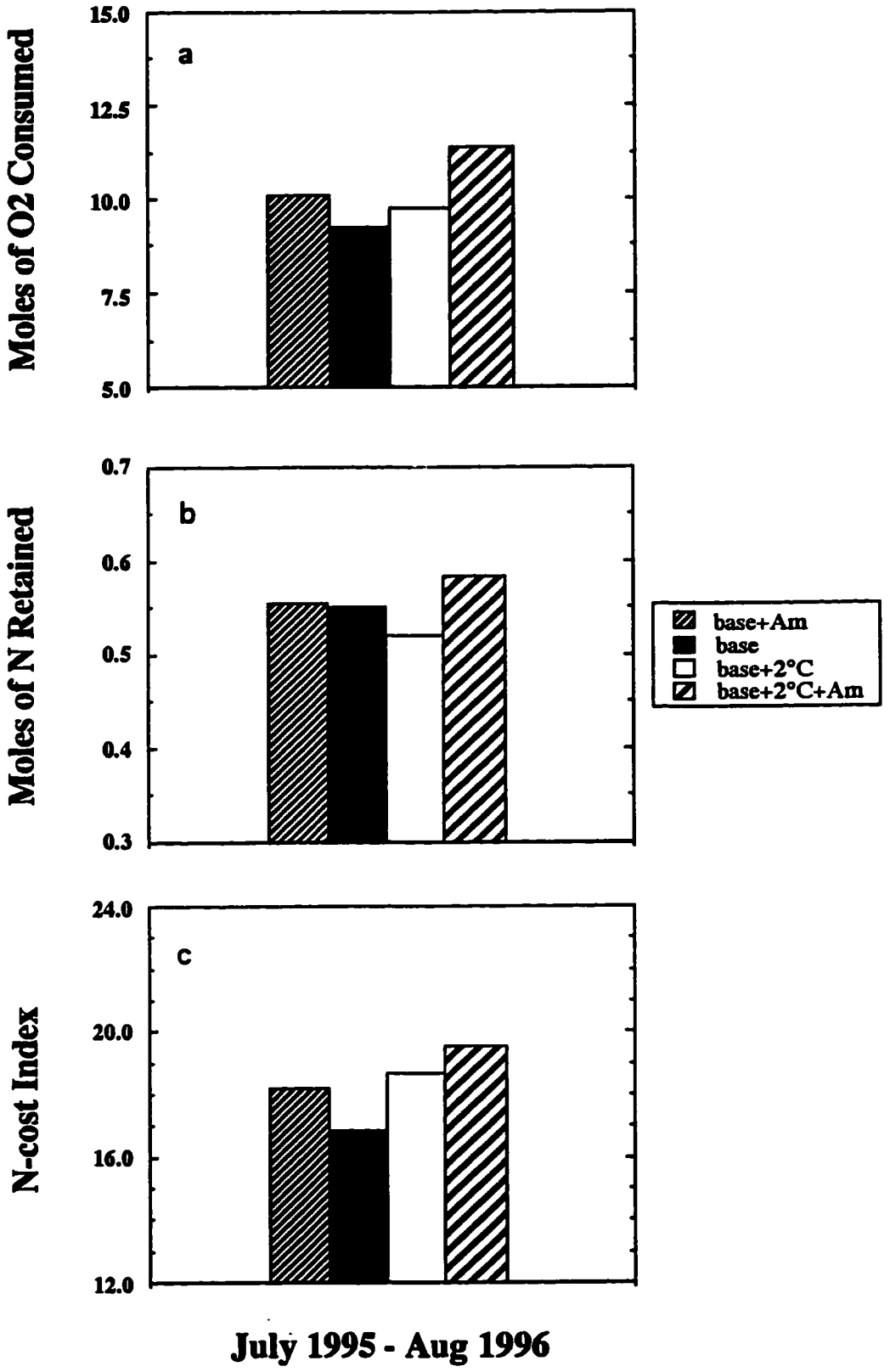

Moles of O2 Consumed
15.0
12.5
10.0
7.5
5.0
a
Moles of N Retained
0.7
0.6
0.5
0.4
0.3
b
N-cost Index
24.0
20.0
16.0
12.0
c
base+Am
base
base+2°C
base+2°C+Am
July 1995 - Aug 1996

Table 1. Mean (± SE) ammonia concentrations, pH, and partial oxygen pressures in treatments during 420-d exposures (July 1995 to August 1996) of juvenile rainbow trout fed to satiation and exposed to +2°C and 70 $\mu mol \cdot l^{-1}$ total ammonia (Am).

Treatment	Tank	Total Ammonia ($\mu mol \cdot l^{-1}$)	N	pH	N	P_{O2} (torr)	N
base	1	15.1 ± 1.4	38	7.70 ± 0.04	5	126.4 ± 5.4	13
	2	17.1 ± 2.5	13	7.71 ± 0.04	6	131.3 ± 4.6	12
	3	22.0 ± 3.1	13	7.65 ± 0.04	5	120.5 ± 4.1	10
	4	20.8 ± 2.6	13	7.59 ± 0.02	12	123.9 ± 4.5	13
base+Am	1	79.0 ± 4.9	38	7.62 ± 0.07	6	119.5 ± 4.2	13
	2	81.2 ± 6.3	18	7.66 ± 0.03	6	127.9 ± 4.5	12
	3	81.9 ± 7.2	14	7.52 ± 0.04	12	122.8 ± 4.7	12
	4	88.1 ± 5.7	15	7.64 ± 0.03	17	129.0 ± 4.4	13
base+2°C	1	17.6 ± 2.7	37	7.64 ± 0.05	10	117.2 ± 4.9	13
	2	19.0 ± 4.0	12	7.62 ± 0.06	6	121.4 ± 4.2	13
	3	19.2 ± 3.2	9	7.73 ± 0.16	2	120.7 ± 3.4	11
	4	17.3 ± 3.2	14	7.62 ± 0.04	9	123.5 ± 3.5	12
base+2°C+Am	1	68.8 ± 3.6	39	7.55 ± 0.06	5	123.2 ± 2.9	13
	2	73.6 ± 6.9	21	7.62 ± 0.05	6	120.8 ± 3.1	12
	3	61.1 ± 6.3	13	7.66 ± 0.05	5	123.1 ± 2.8	12
	4	60.8 ± 7.1	14	7.57 ± 0.04	11	124.0 ± 3.6	13

Table 2. Food intake (total consumed per fish between each period), wet weight, total length, and condition factor during 420-d exposures of juvenile rainbow trout (N = 20 to 40) to +2°C warming scenario and 70 $\mu mol \cdot l^{-1}$ T_{Amm} (Am). For each sampling day separately, values within a column without a letter in common are significantly different ($P \leq 0.05$). Where there are no letters, there were no significant differences.

	Time (days) and Calendar Date								
Parameter Measured and Treatment	0 8 Jul 1995	25 4 Aug 1995	71 18 Sep 1995	155 13 Dec 1995	198 25 Jan 1996	275 11 Apr 1996	339 14 Jun 1996	380 15 Jul 1996	420 29 Aug 1996
Food Intake (g/fish)									
base+Am	8.0 ± 0.4	21.6 ± 0.7[a]	65.3 ± 1.5	16.7 ± 0.5[c]	39.3 ± 1.1[a]	108.7 ± 5.1[a]	132.8 ± 9.3	177.6 ± 7.2[a]	
base	8.7 ± 0.2	21.4 ± 1.1[a]	67.2 ± 1.5	23.2 ± 0.5[a]	42.9 ± 2.3[a]	99.8 ± 5.7[a]	119.6 ± 8.6	178.4 ± 8.3[a]	
base+2°C	8.8 ± 0.7	15.6 ± 0.5[b]	68.4 ± 2.2	24.6 ± 1.7[ab]	54.9 ± 2.4[b]	156.7 ± 8.7[b]	142.4 ± 2.9	140.1 ± 7.5[b]	
base+2°C+Am	9.0 ± 0.4	15.6 ± 0.1[b]	75.5 ± 3.9	28.7 ± 1.2[b]	57.6 ± 2.4[b]	139.4 ± 8.3[b]	142.1 ± 8.6	169.5 ± 3.9[ab]	
Wet Weight (g)									
base+Am	11.8 ± 0.6	19.8 ± 1.1	35.3 ± 1.8[b]	83.9 ± 3.5	93.6 ± 3.9	123.0 ± 4.8	196.2 ± 7.4[a]	284.7 ± 9.8[a]	361.3 ± 13.0
base	10.3 ± 0.7	18.1 ± 1.3	27.7 ± 2.1[a]	81.3 ± 5.5	95.1 ± 6.1	128.1 ± 8.7	198.2 ± 15.1[a]	281.2 ± 22.3[a]	361.2 ± 28.8
base+2°C	12.1 ± 0.6	22.3 ± 1.4	27.1 ± 1.7[a]	82.9 ± 4.1	99.5 ± 4.9	147.9 ± 7.2	254.8 ± 11.4[b]	337.1± 16.6[ab]	364.9 ± 17.5
base+2°C+Am	11.2 ± 0.8	18.9 ± 1.4	24.5 ± 1.8[a]	84.6 ± 5.6	102.5 ± 6.5	147.7 ± 9.2	253.4 ± 12.9[b]	338.3 ± 16.5[b]	391.8 ± 18.6
Total Length (cm)									
base+Am	10.8 ± 0.2	12.1 ± 0.2	14.1 ± 0.3[b]	19.4 ± 0.2	20.1 ± 0.3	21.9 ± 0.2[a]	25.6 ± 0.3[a]	27.9 ± 0.3[ab]	30.3 ± 0.4
base	10.6 ± 0.3	11.8 ± 0.3	13.5 ± 0.3[a]	19.3 ± 0.5	20.0 ± 0.4	21.7 ± 0.5[a]	25.3 ± 0.5[a]	27.7 ± 0.6[a]	30.1 ± 0.8
base+2°C	10.9± 0.2	12.4 ± 0.2	13.3 ± 0.3[a]	18.8 ± 0.3	20.0 ± 0.3	23.1 ± 0.3[b]	27.0 ± 0.4[b]	29.5 ± 0.5[b]	30.5 ± 0.4
base+2°C+Am	10.7 ± 0.2	11.8 ± 0.3	12.8 ± 0.3[a]	19.1 ± 0.4	20.2 ± 0.4	23.0 ± 0.4[ab]	27.3 ± 0.4[b]	29.4 ± 0.5[b]	31.5 ± 0.5
Condition Factor									
base+Am	0.89 ± 0.01	1.09 ± 0.01	1.11 ± 0.01	1.13 ± 0.01[ab]	1.14 ± 0.02[b]	1.16 ± 0.02	1.21 ± 0.03	1.29 ± 0.02	1.28 ± 0.02
base	0.84 ± 0.02	1.07 ± 0.02	1.10 ± 0.03	1.12 ± 0.03[a]	1.17 ± 0.01[a]	1.22 ± 0.02	1.19 ± 0.03	1.28 ± 0.03	1.28 ± 0.02
base+2°C	0.93 ± 0.04	1.14 ± 0.03	1.10 ± 0.02	1.22 ± 0.04[b]	1.21 ± 0.01[ab]	1.18 ± 0.02	1.28 ± 0.02	1.31 ± 0.04	1.26 ± 0.02
base+2°C+Am	0.87 ± 0.01	1.09 ± 0.02	1.09 ± 0.02	1.17 ± 0.02[ab]	1.21 ± 0.02[ab]	1.18 ± 0.02	1.20 ± 0.02	1.32 ± 0.03	1.22 ± 0.02

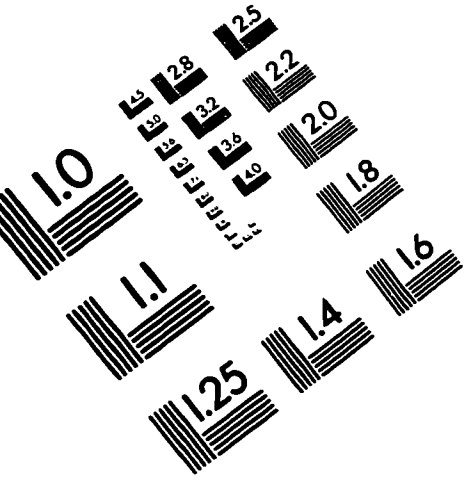
Table 3. Activities of alanine aminotransferase (Alat) and aspartate aminotransferase (Aspat) in liver, gill, and muscle of juvenile rainbow trout initially and after approximately 1 month and 14 months of exposure to a +2°C warming scenario and 70 $\mu mol \cdot l^{-1}$ T_{Amm} (Am). For each sampling day separately, statistically significant temperature (+2°C treatments = T), ammonia (+70 $\mu mol \cdot l^{-1}$ T_{Amm} treatments = A), and interactive effects (I) are denoted underneath the respective columns (two-way ANOVA; $P \leq 0.05$). Where there are no letters, there were no significant differences.

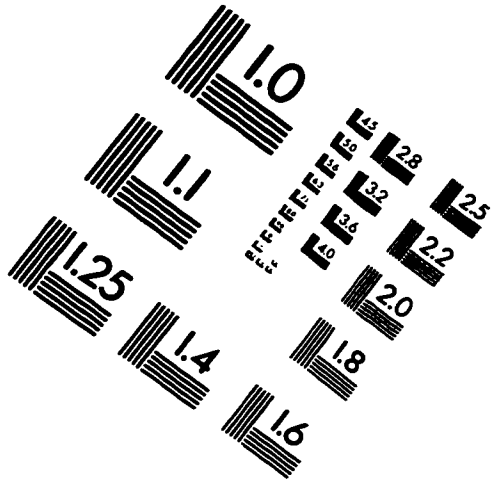
	ALAT			ASPAT		
Test day and enzyme	Liver (µmol/min/g)	Gill (µmol/min/g)	Muscle (µmol/min/g)	Liver (µmol/min/g)	Gill (µmol/min/g)	Muscle (µmol/min/g)
Initial	17.86 ± 2.23 (4)	0.57 ± 0.05 (3)	2.58 ± 0.22 (9)	15.66 ± 1.64 (7)	6.45 ± 0.19 (10)	12.61 ± 0.38 (8)
Day 25						
base+Am	14.02 ± 1.24 (11)	0.57 ± 0.10 (5)	2.53 ± 0.19 (18)	15.47 ± 1.51 (13)	8.08 ± 0.89 (16)	11.07 ± 1.00 (8)
base	11.07 ± 0.81 (14)	0.62 ± 0.03 (12)	2.60 ± 0.23 (16)	13.95 ± 1.43 (18)	8.83 ± 0.80 (19)	10.50 ± 0.72 (11)
base+2°C	13.27 ± 1.38 (12)	0.68 ± 0.04 (8)	2.83 ± 0.32 (16)	13.79 ± 1.84 (17)	7.62 ± 0.41 (18)	10.80 ± 0.55 (7)
base+2°C+Am	14.16 ± 1.18 (16)	0.72 ± 0.08 (7)	2.61 ± 0.24 (18)	12.28 ± 1.11 (15)	7.12 ± 0.34 (18)	12.22 ± 0.65 (11)
Day 420						
base+Am	61.35 ± 2.95 (18)	1.13 ± 0.09 (11)	0.87 ± 0.04 (18)	51.16 ± 3.50 (19)	7.75 ± 0.38 (19)	16.61 ± 0.56 (15)
base	59.03 ± 3.28 (20)	0.91 ± 0.08 (14)	1.12 ± 0.09 (14)	39.63 ± 2.15 (20)	7.99 ± 0.37 (20)	15.32 ± 0.41 (11)
base+2°C	75.68 ± 5.16 (10)	0.93 ± 0.10 (9)	1.16 ± 0.07 (12)	43.49 ± 2.69 (11)	8.56 ± 0.67 (13)	17.34 ± 0.97 (8)
base+2°C+Am	51.96 ± 3.51 (11)	0.79 ± 0.09 (4)	0.97 ± 0.06 (15)	31.22 ± 3.00 (14)	6.91 ± 0.35 (17)	15.32 ± 0.28 (13)
	I		A	I	A	I

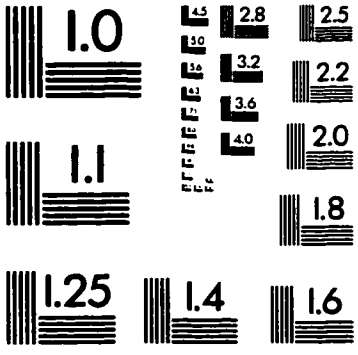
Table 4. Activities of glutamate dehydrogenase (GDH) and glutamine synthetase (GNS) in liver, gill, and muscle of juvenile rainbow trout initially and after approximately 1 month and 14 months of exposure to a +2°C warming scenario and 70 $\mu mol \cdot l^{-1}$ T_{Amm} (Am). For each sampling day separately, statistically significant temperature (+2°C treatments = T), ammonia (+70 $\mu mol \cdot l^{-1}$ T_{Amm} treatments = A), and interactive effects (I) are denoted underneath the respective columns (two-way ANOVA; $P \leq 0.05$). Where there are no letters, there were no significant differences.

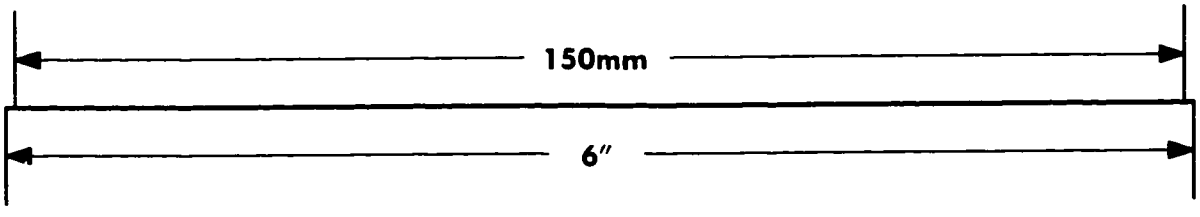
	GDH			GNS		
Test day and enzyme	Liver (μmol/min/g)	Gill (μmol/min/g)	Muscle (μmol/min/g)	Liver (μmol/min/g)	Gill (μmol/min/g)	Muscle (μmol/min/g)
Initial	13.83 ± 1.48 (7)	4.02 ± 0.30 (9)	2.65 ± 0.26 (10)	0.27 ± 0.05 (10)	0.30 ± 0.02 (10)	0.0038 ± 0.0014 (10)
Day 25						
base+Am	13.53 ± 0.88 (15)	5.14 ± 0.36 (20)	3.95 ± 0.21 (17)	0.23 ± 0.02 (18)	0.45 ± 0.03 (19)	0.0023 ± 0.0003 (20)
base	12.26 ± 0.89 (16)	6.05 ± 0.40 (20)	3.31 ± 0.31 (19)	0.19 ± 0.01 (16)	0.44 ± 0.02 (17)	0.0018 ± 0.0002 (20)
base+2°C	11.03 ± 0.65 (13)	5.20 ± 0.25 (17)	4.40 ± 0.36 (17)	0.15 ± 0.07 (15)	0.39 ± 0.02 (16)	0.0017 ± 0.0003 (18)
base+2°C+Am	12.53 ± 0.84 (12)	5.77 ± 0.31 (19)	4.06 ± 0.28 (18)	0.13 ± 0.01 (14)	0.38 ± 0.03 (14)	0.0022 ± 0.0003 (20)
		I	T	T	T	
Day 420						
base+Am	34.56 ± 0.84 (3)	7.72 ± 0.58 (19)	2.62 ± 0.21 (19)	0.73 ± 0.05 (19)	0.84 ± 0.07 (19)	0.0011 ± 0.0001 (17)
base	54.97 ± 2.67 (20)	8.68 ± 0.33 (20)	2.88 ± 0.13 (20)	0.60 ± 0.03 (20)	1.10 ± 0.03 (20)	0.0020 ± 0.0003 (19)
base+2°C	40.46 ± 1.28 (6)	8.44 ± 0.51 (13)	3.10 ± 0.26 (12)	0.57 ± 0.06 (13)	1.00 ± 0.05 (13)	0.0038 ± 0.0006 (13)
base+2°C+Am	44.61 ± 4.26 (13)	5.73 ± 0.41 (17)	2.50 ± 0.12 (17)	0.34 ± 0.02 (16)	0.70 ± 0.03 (17)	0.0015 ± 0.0004 (16)
	I	T, A	A	I	T, A	T, A

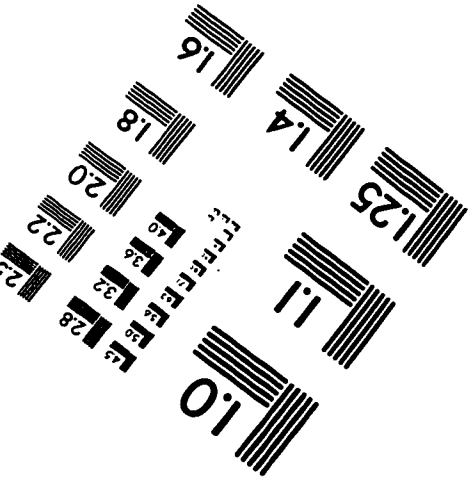
IMAGE EVALUATION TEST TARGET (QA-3)

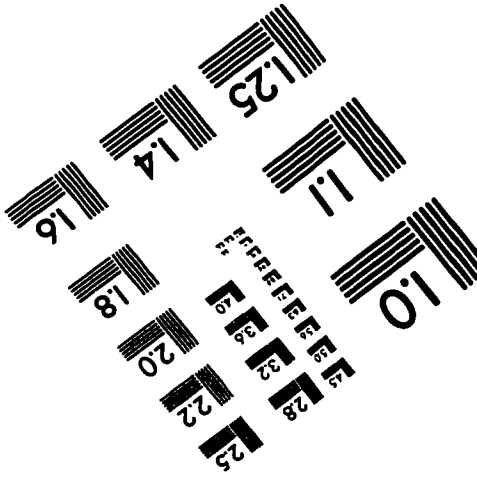












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