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

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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^{-1} ionized (NH_4^+), 10 μg l^{-1} 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^{-1} NH_4^+, 900 μg l^{-1} 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^{-1} NH_4^+, 200 μg l^{-1} 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. A 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

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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. © 1998 Elsevier Science B.V.

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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; Reid et al., 1996; Reid et al., 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 whether 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 per-

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 ($[Ca^{2+}] = 0.956 \pm 0.019$ mM, $[Na^{+}] = 0.546 \pm 0.008$ mM, $[Cl^{-}] = 0.737 \pm 0.004$ mM)

Exposure and treatment	Mean temperature °C (range)	T_{Amm} (μM)	pH	$[NH_4^+]$ [a,b,c] μg l^{-1} (range)	$[NH_3]$ [a,b,c] μg l^{-1} (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	7.3 ± 0.12	150.2 (59-240)	0.9 (0-4)
Base+2°C+Am	17.6 ± 0.1 (15.0-20.0)	64.6 ± 0.6	7.40 ± 0.08	115.32 (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: $NH_4^+ = T_{Amm}/1 + \text{antilog}(pH - pK_a) = T_{Amm} - NH_3$, as reported by Wood (1993).
[c] Note that fractions of NH_4^+ and NH_3 are expressed in traditional toxicological units of μg l^{-1} rather than units of μM, more normally used in physiological studies.

meability to Na^+ (as seen in the later phases of acid stress (see, for example, McDonald et al., 1983; Audet and Wood, 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

2.1. Experimental animals and protocol

Juvenile rainbow trout (initially 2–5 g) were obtained from Rainbow Springs Trout Farm, Thamesford, Ont., and held in flowing de-chlorinated Hamilton tap water (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 three 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–September 1993) and 2 (January–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–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 the natural photoperiod for Hamilton, Ont., 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 four original treatments were challenged with either lethal temperature, lethal ammonia,

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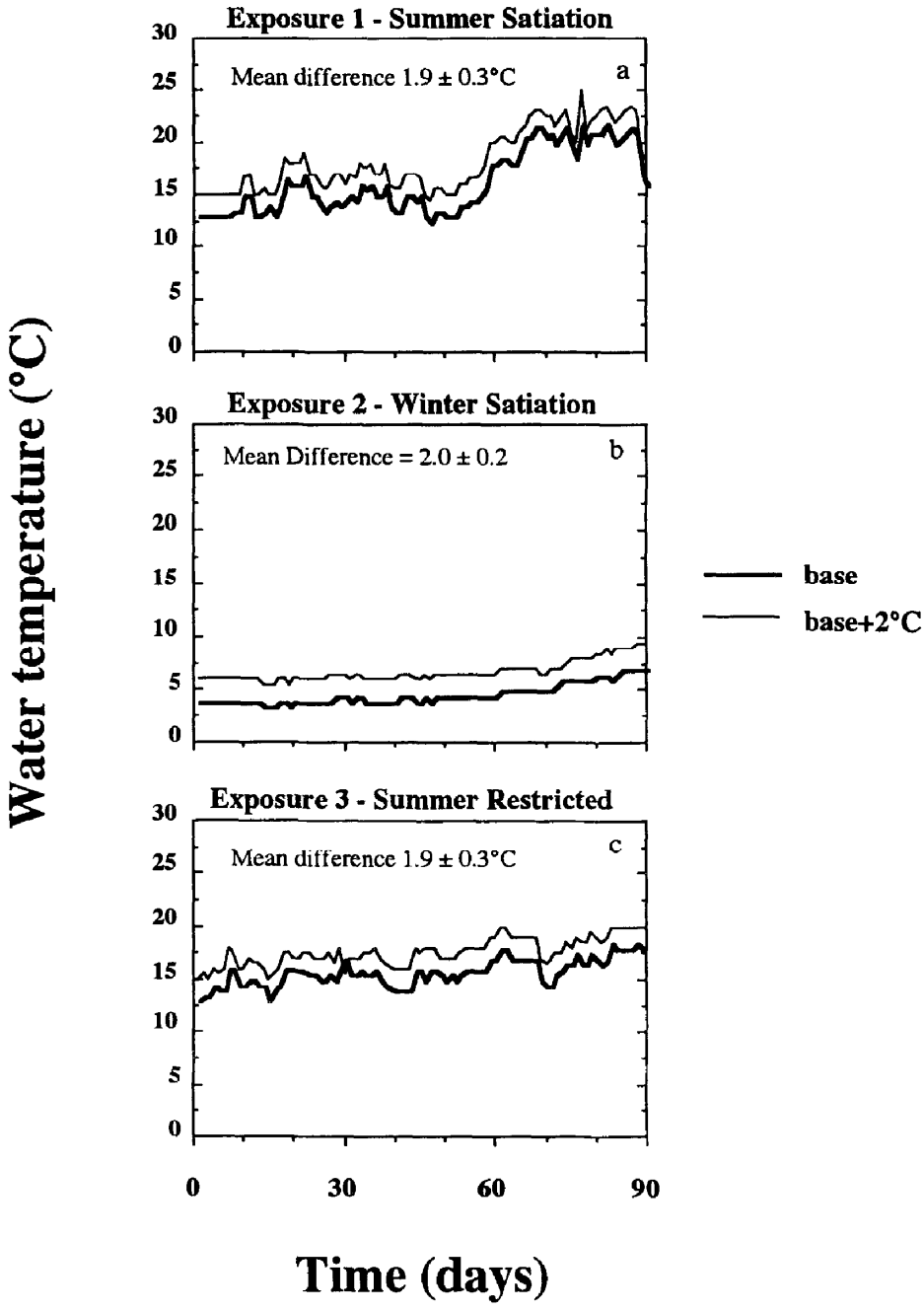
Fig. 1. Water temperature profiles for three 90-day exposure periods which were conducted (a) over summer feeding the trout to satiation (summer satiation, June–September 1993), (b) over winter again feeding to satiation (winter satiation, January–April 1994), and (c) over summer feeding the fish a restricted (1% daily) ration (summer restricted, June–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
30
25
20
15
10
5
0
0
30
60
90
Water temperature (°C)
Time (days)
base
base+2°C

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 four 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},

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

Lethal challenge and exposure	Mean temperature (°C) Initial–final	T_{Amm} (μM) Initial–final	pH Initial–final	$[NH_4^+]$ (μg l^{-1}) Initial–final	$[NH_3]$ (μg l^{-1}) 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 Section 2).

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, pH values, and fractions of NH_4^+ and NH_3, together with their corresponding ranges, were as presented 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 1 l 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 1 μCi l^{-1} ^{22}Na was added and allowed to equilibrate for 10 min, after which an initial water sample (3×5 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 0–3 h, 3–6 h, 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^{-1}, and an NH_3 concentration of 200 μg l^{-1}. A later simulation experiment demonstrated that the effect of the fish on water pH during any one 3 h 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.

2.2. 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_{net}^{Amm}) were calculated as:

$$J_{net}^{Amm} = \frac{(\text{Initial } T_{Amm} - \text{final } T_{Amm}) \times V}{t \times W}$$

where T_{Amm} is the total ammonia concentration in μmol l^{-1}, 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_{net}^{Na}) were calculated from an equation analogous to J_{net}^{Amm}, and influx rates (J_{in}^{Na}) from:

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

where R is the ^{22}Na radioactivity of water in c.p.m. l^{-1}, MSA is the mean specific activity of the water (c.p.m. μeq^{-1}) 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_{out}^{Na}) were calculated by difference:

$$J_{out}^{Na} = J_{net}^{Na} - J_{in}^{Na}$$

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 the figures) and ammonia (+70 μM, marked by A in the 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 the figures) and ration (marked by R in the 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 h, 3–6 h, and 21–24 h to the control (−3 to 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

3.1. Overview of lethal challenges (Figs. 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

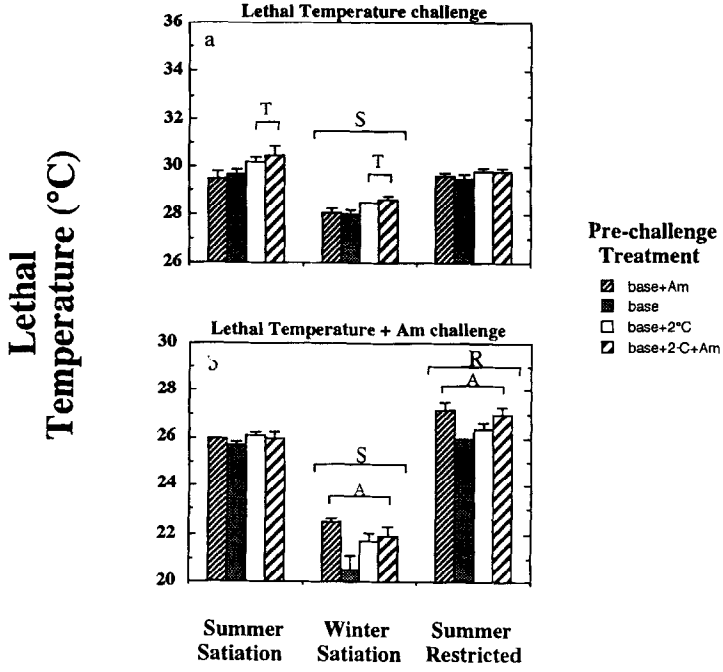


Fig. 2. Mean lethal temperatures of juvenile rainbow trout challenged by either (a) heating normal tap water (see Table 1 for ionic composition) at a rate of 1°C every 1 h (exposure 2, winter satiation) or 2 h (exposures 1 and 3, summer satiation and summer restricted, respectively) and recording the 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 6–10 trout originating from the 90-day chronic exposures (see caption of 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 an A represents a significant ammonia (Am) pre-exposure effect. Among exposures, a capital S represents a significant seasonal effect, and an R represents a significant ration effect (one-way ANOVA, see Section 2). 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)).

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 T_{Amm}) was only apparent in the combination lethal temperature and ammonia challenges. Both 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.

3.2. 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. 2(a)). The differences were slight (0.2–1.0°C) but significant. In addition, when comparing amongst the three different challenges (after exposure 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. 2(a)), a difference which was not statistically significant.

3.3. 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. 2(b)). 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. 2(a)), 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 tem-

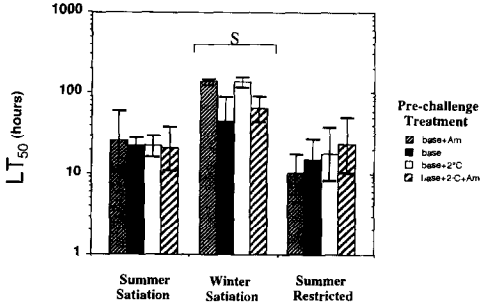


Fig. 3. Time to 50% mortality ($LT_{50} \pm 95\%$ C.L.) of fish from all four pre-challenge treatment groups (see caption of Fig. 2) during a static 1.8 mM T_{Amm} challenge following the three 90-day exposure periods (see caption of Fig. 1). The LT_{50}'s and their 95% confidence limits were calculated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

peratures 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. 2(b)). 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. 2(b)). Overall, the mean lethal temperatures attained by trout after exposure 3 (summer-restricted ration) were slightly, but

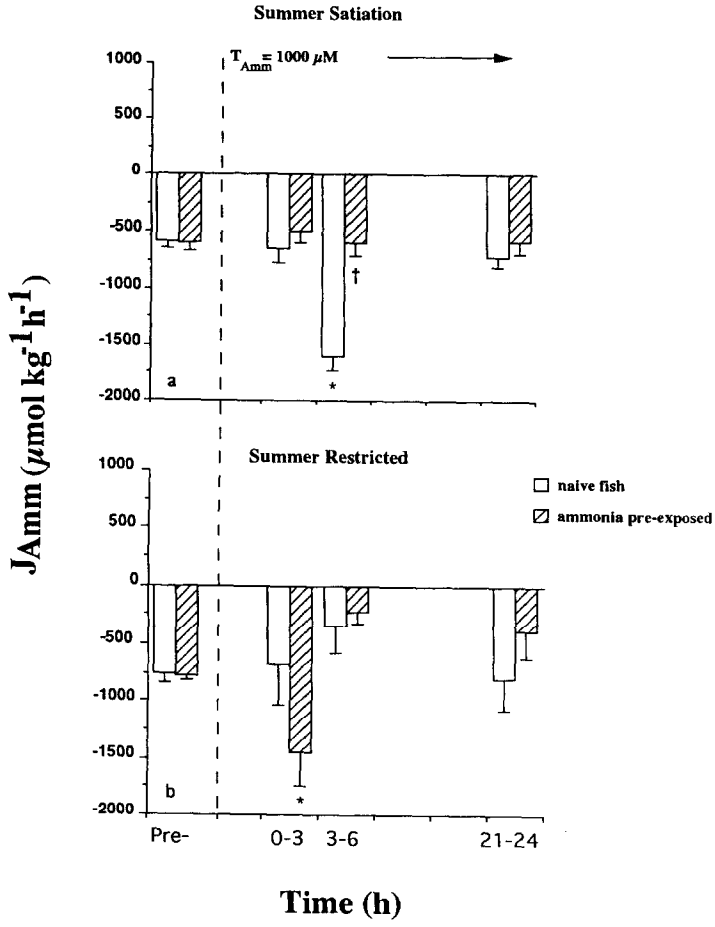


Fig. 4. The effect of 24 h of exposure to high external ammonia (1 mM T_{Amm}) on net ammonia fluxes (J^{Amm}_{net}) in juvenile rainbow trout (means ± SE, $N = 16$) after: (a)exposure 1, summer satiation; and (b) exposure 3, summer restricted (see caption of Fig. 1). Naive fish (with no prior exposure 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.

significantly, higher than those reached after exposure 1 (summer-satiation) (Fig. 2(b)). This is opposite the trend observed with temperature challenge alone (Fig. 2(a)).

3.4. 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).

3.5. 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. 4(a)). Net ammonia flux (J_{net}^{Amm}) 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_{net}^{Amm} 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_{net}^{Amm} 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_{net}^{Amm} over 0–3 h of exposure to 1.0 mM T_{Amm} (Fig. 4(b)). 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^+ turn-

over was greater in ammonia pre-exposed fish than in naive fish (Fig. 5(a)). Pre-challenge J_{in}^{Na} was significantly greater in this group when compared with the naive fish. During the challenge, J_{in}^{Na} 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. 5(a)). However, at this same time, J_{out}^{Na} was stimulated in the ammonia pre-exposed group resulting in significant net loss of Na^+ (Fig. 5(a)).

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_{in}^{Na} and J_{out}^{Na} with the exception of a significant reduction in J_{out}^{Na} and increase in net Na^+ to positive values for the ammonia pre-exposed group between 3–6 h.

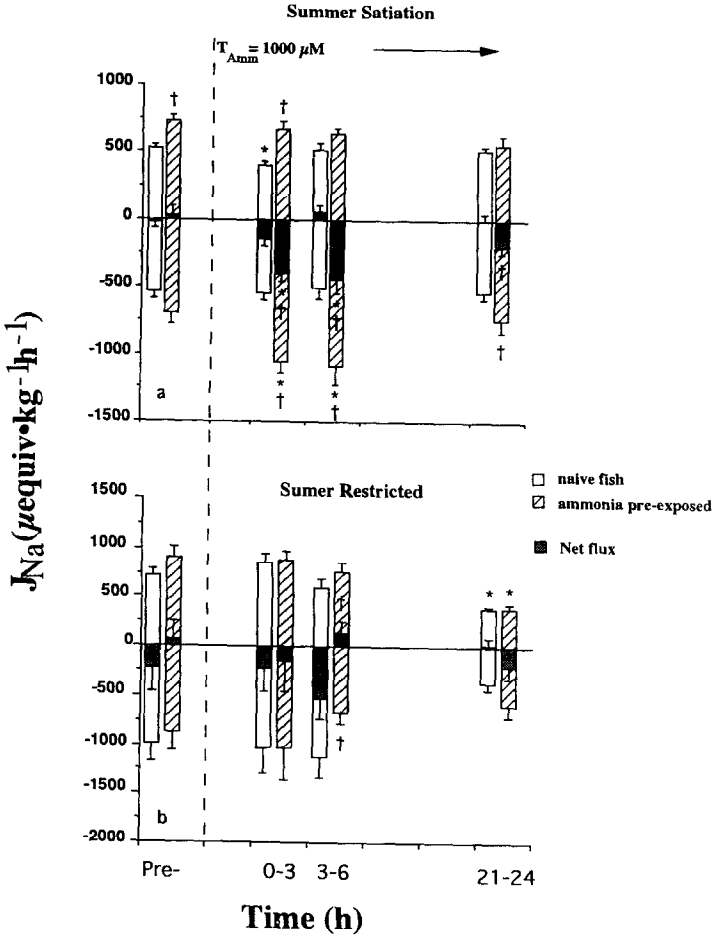


Fig. 5. The effect of 24 h of exposure to high external ammonia on net (dark solid columns) and unidirectional sodium uptake ($J^{Na+}{}_{in}$; upward bars) and efflux ($J^{Na+}{}_{out}$; downward bars) rates from trout (means ± SE, $N = 16$) after: (a)exposure 1, summer satiation; and (b) exposure 2,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

Both groups showed a significant decrease from pre-challenge levels in J_{in}^{Na} between 21–24 h (Fig. 5(b)).

4. Discussion

4.1. 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 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).

4.2. 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-day 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 favourable 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.

4.3. 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).

4.4. 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 Section 1). 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 are 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^{-1} 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 the 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 the 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^{-1} NH_3) there was a 4 fold increase in plasma T_{Amm} level after exposure 1 (summer-satiation) and a 2- to 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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