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# Responses of juvenile rainbow trout, under food limitation, to chronic low pH and elevated summer temperatures, alone and in combination

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Rainbow trout were exposed (90 days) in synthetic soft water to sublethal low pH (5·2) and a simulated climate warming scenario (+2° C above the control summer temperature range of 16·5–21° C), alone and in combination, under conditions of limited food (~4% dry body weight day$^{-1}$). Weight specific oxygen consumption rates ($\dot{M}O_2$) were ~55% of $\dot{M}O_{2(max)}$, in contrast to ~75% of $\dot{M}O_{2(max)}$ found in trout fed an unlimited ration. This is likely due to a reduction in food quantity and thus feeding activity. However, the trout exposed to low pH at control temperatures exhibited higher conversion efficiencies and increased growth. In contrast, trout exposed to +2° C had reduced growth rates. No ionoregulatory disturbance occurred in any treatment, suggesting that this ration was sufficient to provide a replacement salt load in the diet. Energy budgets indicated that the limited ration resulted in a lowered optimum temperature for growth, with a greater proportion of the energy intake dissipated for metabolic expenditure, resulting in reduced conversion efficiencies. A fourfold reduction in faecal and unaccounted energy losses indicated higher absorption efficiencies than in satiation–fed trout.



Key words: rainbow trout; acidification; temperature; food limitation; energy budget.

## INTRODUCTION

Acidification of poorly buffered soft waters throughout the northern hemisphere by acidic precipitation is well documented (Kemp, 1994). Climate also appears to be changing. General circulation models predict increases in mean air temperature of 1·3–4·5° C over the next 50–100 years (Hansen *et al.*, 1988; Mohnen & Wang, 1992), with corresponding effects on the thermal regimes of freshwater bodies (Regier & Meisner, 1990). Indeed, the mean annual water temperature in one well-studied softwater ecosystem, the Experimental Lakes Area of north-western Ontario, Canada, has already increased by 2° C over the past 20 years (Schindler *et al.*, 1990). This research programme aims to understand the chronic impacts of sublethal acidity and global warming, alone and in combination, on the physiology and energetics of a model freshwater salmonid, the rainbow trout *Oncorhynchus mykiss* (cf. Reid *et al.*, 1995, 1996). The studies employ an acidic pH of 5·2 in synthetic soft water, and a warming scenario of +2° C above a control daily temperature which is based on the natural thermal cycle of inshore Lake Ontario, a site to which rainbow trout are

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now endemic. The domestic water supply used in these experiments is pumped directly from inshore Lake Ontario, and 5 years of temperature records demonstrate a virtual identity of weekly values between the lake site and the delivered water supply.

In our first study (Dockray *et al.*, 1996), juvenile trout were exposed to these regimes over a 90-day summer period from mid-June to mid-September 1993. To allow measurements of appetite, the fish were fed to satiation twice per day. Trout chronically exposed to low pH in combination with +2° C did suffer greater metabolic costs, although these were not marked. However, several of the findings were surprising. Overall, the addition of 2° C alone depressed appetite and growth, while chronic low pH exposure alone resulted in improved appetite and growth. Most surprisingly, the fish exposed to chronic low pH exhibited no ionoregulatory disturbance, in contrast to a vast literature which indicates that low pH interferes with ionic regulation at the gills (Fromm, 1980; Wood & McDonald, 1982; McDonald, 1983; Audet *et al.*, 1988; Wood, 1989).

These findings raised the possibility that the unlimited diet (up to ~11% $day^{-1}$ on a dry weight food/dry weight fish basis) was confounding interpretation. Specifically, it was suspected that the availability of unlimited food: (i) might result in lower feeding and growth at +2° C, effects which might be absent or reversed at lower ration level; (ii) might allow fish chronically exposed to low pH to eat their way out of ionoregulatory disturbance, resulting in increased appetite and growth driven by the need for NaCl acquisition from the diet (Sadler & Lynam, 1987; Smith *et al.*, 1989); and (iii) as a result of these opposing effects, might mitigate the physiological costs of the combined stressors. The objective of the present study was to test these ideas using a similar exposure regime, in the summer of 1994, but with a limited ration.

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

## MATERIALS AND METHODS

### ANIMAL HOLDING

Experiments were conducted on 1240 juvenile rainbow trout (4–5 g), which were transported from Rainbow Springs hatchery, Thamesville, Ontario to McMaster University on 13 May 1994. While not living in Lake Ontario itself, the parent stock encounters a very similar thermal regime to that of the inshore region of the lake throughout the year, and therefore that used during the exposures. The fish were placed

in a 400-l polypropylene tank served with continuously flowing tap water from inshore Lake Ontario [$Ca^{2+}$] = 1·90, [$Na^{+}$] = 0·60 mequiv $l^{-1}$; pH = 7·6 at 13° C for 2 days. Artificially softened water, generated by reverse osmosis (Anderson Water Conditioning Equipment, Dundas, Ontario, Canada), was gradually introduced to the holding tank in increasing amounts over the following week. Once the desired water quality was achieved, the trout were acclimated for another 4 weeks to this soft water. Trout were fed ~4% (dry weight of food) of their dry body weight $day^{-1}$ of Zeigler Trout Starter 3 (for composition, see Dockray *et al.*, 1996), and maintained on this diet for the 5-week acclimation period. Photoperiod was controlled to mimic the natural photoperiod throughout the acclimation and experimental periods.

## EXPOSURE SYSTEM

The exposure system has been described by Dockray *et al.* (1996). Hamilton City dechlorinated tap water was softened synthetically by reverse osmosis. The passage of the water through the high pressure reverse osmosis unit raised the control temperature 2° C higher than the true ambient temperature in Lake Ontario, as explained in Dockray *et al.* (1996). The water at control temperature was pumped to a main head tank where hard water was added, in a ratio of 1 : 40, to achieve nominal [$Ca^{2+}$] and [$Na^{+}$] of ~50 and 100 μequiv $l^{-1}$ respectively. The water flowed by gravity into two sub-head tanks, where a further 2° C was added to the water of one tank via a heat-exchanger. The water from each of these two sub-head tanks was split further, and $H_2SO_4$ (0·2 N) was metered into one each of the control and +2° C temperature sub-head tanks. This resulted in four treatments, each replicated in two exposure tanks: control temperature/ambient pH 6·2 (0/6·2); control temperature/sublethal pH 5·2 (0/5·2); control temperature +2° C/ambient pH 6·2 (+2/6·2); control temperature +2° C/sublethal pH 5·2 (+2/5·2). The water from the treatment head tanks flowed into duplicate 211-l polypropylene exposure tanks, at an average rate of 1·2 l $min^{-1}$, which allowed for 90% replacement in 7 h. Sublethal pH 5·2 was maintained using a Leeds & Northrup Meredian II® Combination industrial pH electrode, in combination with the automatic control and alarm system described by Dockray *et al.* (1996). All fish tanks and head tanks were aerated, and the partial pressure ($P_{O_2}$) of oxygen determined once a month. $P_{O_2}$ averaged 19·6 kPa (147 Torr) in the fish tanks, and pH, measured daily, averaged 6·23 ± 0·04 in the ambient pH treatments, and 5·19 ± 0·01 in the low pH treatments. Temperatures were measured daily. Other water chemistry parameters were monitored as described previously (Dockray *et al.*, 1996), yielding the following mean values: [$Na^{+}$] = 64·1 ± 1·93, [$Ca^{2+}$] = 53·3 ± 3·20, and [Cl] = 42·6 ± 2·24 μequiv $l^{-1}$; and titratable alkalinity = 185·2 ± 0·01 and 100·8 ± 0·02 μequiv $l^{-1}$ for the ambient pH and low pH treatments, respectively.

## GROWTH AND FEEDING REGIME

After the 5-week acclimation period, 150 trout were selected randomly, and placed into each treatment tank, with 160 fish placed in the 0/6·2 treatment tanks to allow 10 fish for day 0 sampling. Ration was standardized to that of the 0/6·2 treatment. The fish in each tank were hand-fed 1% $day^{-1}$ (~4% dry food : dry mass) of the measured average wet biomass of the fish in the 0/6·2 treatment tanks, adjusted for the number of fish in each tank. Biomass was measured in each tank once a week (see below), and the amount of food required for each tank determined at this time. All mortalities were recorded, and feeding amounts were adjusted on a daily basis whenever mortalities occurred. Half the ration of Zeigler's Trout Starter 3 was fed to the fish twice daily, at 0830 and 1630 hours. Tanks were cleaned once a week.

In view of the limited ration, much greater interindividual variability in growth was anticipated due to in-tank feeding hierarchies (Jobling, 1994). Also, much lower absolute growth rates were expected, which would be impractical to measure by the 30-day terminal sub-sampling method of the previous study (Dockray *et al.*, 1996). To monitor growth more accurately and frequently, a bulk weighing technique was employed in each tank once per week. All fish were netted quickly *en masse* into a 10-l bucket filled with water of the appropriate pH and temperature, and lined with a tared plastic sieve. The

bucket and contents were weighed on a GSE 450 Scale Systems (Michigan, U.S.A.) balance, and then the sieve and fish lifted free of the water briefly and the fish replaced in the tank. The bucket and water were then reweighed, so as to yield the weight of the trout by difference. Experiments in which all trout in the tank were bulk-weighed by this technique repetitively, then anaesthetized, dried, and weighed individually demonstrated that variability was extremely low (coefficient of variation 0·3%) and that bulk weighing overestimated true weight by only 1·6% due to adherent water. The deviation was independent of the number and size of fish weighed over the ranges encountered in this study; no correction was made for this small systematic error.

## PHYSIOLOGICAL MEASUREMENTS

Unless otherwise noted, methods were identical to those described in detail by Dockray *et al.* (1996), and are summarized here only briefly.

### *Sampling protocol*

Sampling and physiological measurements were conducted over a 4-day period every 30 days, including the first day (day 0 measurements) of the experiment. Trout were not fed on days 3 and 4 of the sampling period until after whole body and blood sampling was completed, at which time they received the full 4% ration.

### *Nitrogenous waste excretion*

In-tank nitrogenous waste excretion ($M_N$) was measured on the first day of each 30 day sampling period by flow-through respirometry. Every 2 h over a 10-h period, starting at 0730 hours and ending at 1730 hours, water inflow rate was measured, inflow and outflow water samples were collected for ammonia (Verdouw *et al.*, 1978), and urea analyses (Rahmatullah & Boyd, 1980), and excretion rates were calculated by the Fick principle. For each of the five sampling periods, data were graphed against time, and the area under the curve determined for each treatment, to give an overall mean $M_N$ for that 10-h period.

Fractional protein utilization (FPU) represents the degree to which the fish depend upon protein as an aerobic fuel source. FPU was calculated by dividing the nitrogen quotient (NQ = the ratio of the moles of nitrogen produced to moles of oxygen consumed) by the theoretical maximum NQ = 0·27 (Kutty, 1972) in which protein supports all aerobic metabolism.

### *Metabolic rates*

Metabolic rate was determined on day 2 of each 30-day sampling period over a similar time frame, using closed-system respirometry to measure in-tank routine oxygen consumption ($M_{O_2}$). Rates were determined every other hour (alternating open and closed periods) over the 10-h period from 0700 to 1700 hours. Tanks were first siphoned to remove faecal matter, then sealed and recirculated as described by Dockray *et al.* (1996), while $M_{O_2}$ was measured with a Radiometer/Copenhagen E5046 $O_2$ electrode suspended in the tank. $P_{O_2}$ did not fall below 15 kPa (113 Torr). Data were integrated over time to yield an overall mean $M_{O_2}$ for that 10-h period. All nitrogen excretion and metabolic rate data were corrected (to 1 kg) for size differences using the weight exponent 0·824, determined for rainbow trout by Cho (1992). Blank trials were carried out to determine the contribution of bacterial processes to measured rates of $M_{O_2}$ and $M_N$ by feeding a comparable group of trout for several days, then removing them from the tank, performing the normal siphoning, and making measurements on the empty tank. The measured $M_{O_2}$ in the absence of the fish amounted to <5% of the total, and measured $M_N$ was negligible; these values were considered within the error of the measurements, and corrections were not applied.

## GENERAL PROCEDURES FOR BLOOD AND TISSUE ANALYSIS

### *Blood analysis*

From each treatment tank, 10 fish were selected randomly, killed quickly by a blow to the head, blotted dry, weighed and measured for total length. Blood was collected by

caudal severance into ammonium heparinized capillary tubes. Haematocrit was determined by centrifugation for 5 min at 10 000 ***g***; the plasma in the capillary tube was removed using a 50-µl Hamilton syringe and frozen for subsequent ion analysis. Since limited volumes of plasma were available from these small fish, only plasma $Na^+$ levels were determined by atomic absorption spectroscopy. When volume permitted, plasma protein was measured using a hand-held refractometer (American Optical; Alexander & Ingram, 1980).

### *Whole body samples*

Ten fish were netted randomly from each treatment tank, and placed in terminal anaesthetic (1·0 g $l^{-1}$ MS-222, and 2·0 g $l^{-1}$ $NaHCO_3$). Each fish was blotted dry, weighed and measured, freeze-clamped using aluminum tongs chilled in liquid $N_2$, and stored at $-80^\circ$ C for later analysis. Whole fish were ground frozen, using an IKA (M10/M20) grinding mill cooled to $\sim -72^\circ$ C with a dry ice/methanol mixture, and a sample of the tissue weighed and then dried to constant weight at 80° C, to determine water content. The remainder of the tissue was lyophilized (Labconco Lyph-Lock 6), desiccated, and stored at $-20^\circ$ C. Whole body $Na^+$ and $Ca^{2+}$ levels were determined by atomic absorption spectroscopy, and $Cl^-$ levels by coulometric titration (Radiometer CMT10) after digestion of 100 mg of tissue, for 48 h, in 900 µl of 1 N $H_2SO_4$ at 80° C. The Lowry assay, as modified by Miller (1959), was used to determine whole body protein. Lipids were extracted and quantified using the chloroform/methanol (2 : 1) method (Folch *et al.*, 1957). Glycogen, glucose and lactate levels were determined as an estimate of whole body carbohydrate, using standard enzymatic analyses (Bergmeyer, 1985). Percentage inorganic content (ash) was determined, by burning a subsample of whole body tissue at 550° C until a constant weight was achieved.

### *Mortalities*

Mortalities due to the treatments over the 90-day period amounted to <14% overall. Unfortunately due to equipment failure, the entire +2/5·2 treatment was lost at day 77.

## ENERGY BUDGET

An energy budget was constructed as in Dockray *et al.* (1996). The basic energy budget equation is $C = P_G + M + U + F$, where $C$ is the total energy intake, $P_G$ is the total energy gained, $M$ is metabolic expenditure, $U$ is excretory nitrogen energy loss, and $F$ is faecal and unaccounted energy loss. Faecal and unaccounted energy loss includes undigested food, mucus, sloughed epithelial cells from the intestine, catabolized digestive enzymes, and bacteria (Soofiani & Hawkins, 1985). Each energy equivalence is expressed as a percentage of the total energy consumed, in order to determine allocation differences. The energy equivalences of the food and the fish were calculated using conversion factors from Jobling (1994).

## STATISTICAL ANALYSIS

Values are given as the mean ± S.E. ($n$ = number of fish). In no cases were there significant differences between replicate tanks; therefore individual data from the replicate tanks were combined in all analyses. Growth curves, using both linear and logarithmic models, were compared using analysis of covariance to determine differences between slopes for each treatment ($P<0·05$). At first, the experimental design seems ideal for two-way analysis of variance (temperature, pH) with a nested, repeated measures design for most parameters measured. However, there are theoretical reasons why this is not appropriate. Most importantly, temperature *per se* was not a factor, but rather temperature elevation (+2° C) above a fluctuating control level which changed over time. As seen in our previous study (Dockray *et al.*, 1996), the qualitative effect (stimulatory or inhibitory) of the temperature elevation on physiological parameters is expected to change dependent upon the magnitude of control temperature relative to the species optimum temperature. Furthermore since temperature in itself influences pH through its effect on the ionization constant of water, temperature and pH are not entirely

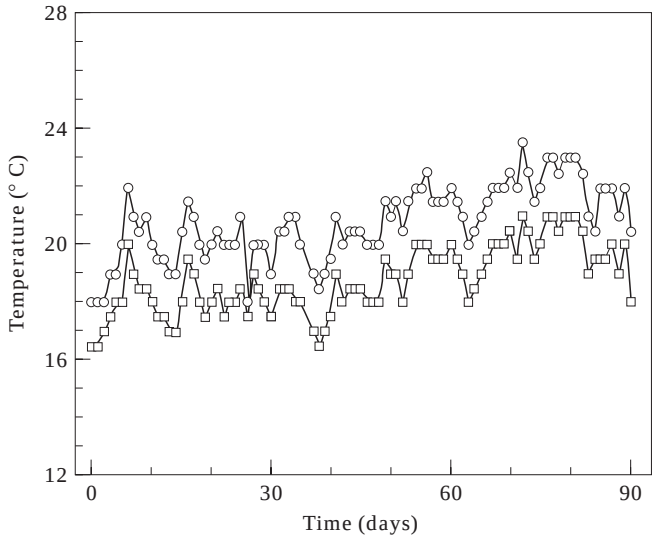


FIG. 1. Thermal cycle (June–September, 1994) experienced by juvenile rainbow trout over the 90-day experimental period. Temperature reached the summer peak between days 60 and 90 at 21° C (23° C in the +2° C treatments) and decreased again over the last 10 days of the exposure period. Mean difference 2·0 ± 0·04° C; ○, control +2° C; □, control temperature.

independent variables. Therefore each treatment at each time was considered unique, and the interactive effects between temperature and pH were not evaluated statistically. At each sampling period, a one-way ANOVA was employed, and in cases where the $F$ value indicated significance, the Tukey–Kramer comparison of all pairs test was applied, to determine treatment differences within a sampling period. The accepted probability level for significance was $P<0{\cdot}05$.

## RESULTS

### EXPERIMENTAL TEMPERATURE REGIME

Over the period 20 June to 18 September 1994, the control temperature ranged from 16·5 to 21·0° C and the elevated temperature from 18·5 to 23° C (Fig. 1). Control temperatures fluctuated around 18° C for the first 50 days, and then increased slightly to 19–21° C between days 60–90, with a slight drop to 18° C again, at day 90. During the peak between days 60–90, the trout at +2° C experienced temperatures of ∼23° C for 10 days.

Because our experimental regime was superimposed on the natural thermal regime of inshore Lake Ontario, year-to-year variation was expected. In general, the 1994 regime, until day 60, was broadly similar to our 1993 study (Dockray *et al.*, 1996), but differed thereafter because only small further increases of temperature occurred. In contrast, in 1993, the control temperature rose to 24° C, and +2° C temperature to 26° C, elevations which were sustained over days 70–82. The present fish, therefore, did not come as close to the upper lethal temperature late in the summer.

### CONSUMPTION, GROWTH AND CONDITION

Trout were limited to a daily ration of 1% (∼4% on a dry weight basis), calibrated to the mean weight of the fish in the 0/6·2 treatment, i.e. identical in all

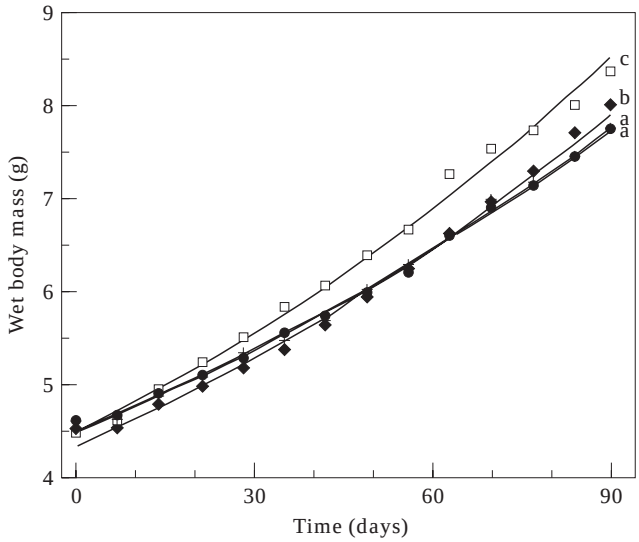


FIG. 2. Growth curves (g) expressed as wet body mass per individual fish. Significant differences ($P<0{\cdot}05$) are indicated by treatment groups that do not share a common letter. Logarithmic (as shown) and linear growth models yielded the same results statistically. Treatment: □, 0/5·2; ◆, 0/6·2; ●, +2/6·2; +, +2/5·2.

treatments because all food offered was eaten. Over 90 days, cumulative food consumption was ~4·5 g per fish in contrast to the 32–48 g dry weight per fish consumed in the unlimited ration exposures (Dockray *et al.*, 1996).

On this limited ration, trout increased in wet body mass by only 3–4 g per fish over 90 days (Fig. 2), in contrast to the 30–50 g increases seen under unlimited ration (Fig. 2 in Dockray *et al.*, 1996). Nevertheless, there were small but significant differences in growth rates among all treatments in the present study except between the +2/6·2 and +2/5·2 treatments. Trout in the control temperature treatments grew significantly faster than in the +2° C treatments. The 0/5·2 trout grew significantly faster than the 0/6·2 trout.

The condition factor (weight length$^{-3}$; Bagenal & Tesch, 1978) of the trout in all treatments remained the same over the 90-day period at the starting value of ~0·9, in contrast to the increase to ~1·2 seen previously with unlimited feeding (data not shown). While there were some fluctuations over time, gross conversion efficiencies (the efficiency with which dry weight of food was converted to dry weight of tissue) did not vary significantly in three of the four treatments, averaging 15·5 ± 0·2% (S.E.; $n=16$ determinations; range 13·7–16·2%) in the 0/6·2, +2/6·2, and +2/5·2 treatments. However, in the 0/5·2 treatment, conversion efficiency was significantly greater throughout the 90 days (18·5 ± 0·4%, $n=6$; range 17·8–19·8%). These values may be contrasted with conversion efficiencies in the range 25–40% seen in the previous unlimited ration experiments (Dockray *et al.*, 1996).

## METABOLIC AND NITROGENOUS WASTE EXCRETION RATES

Oxygen consumption rates were ~5–6 μmol g$^{-1}$ h$^{-1}$ and remained relatively constant over time, with no major treatment differences in all treatments

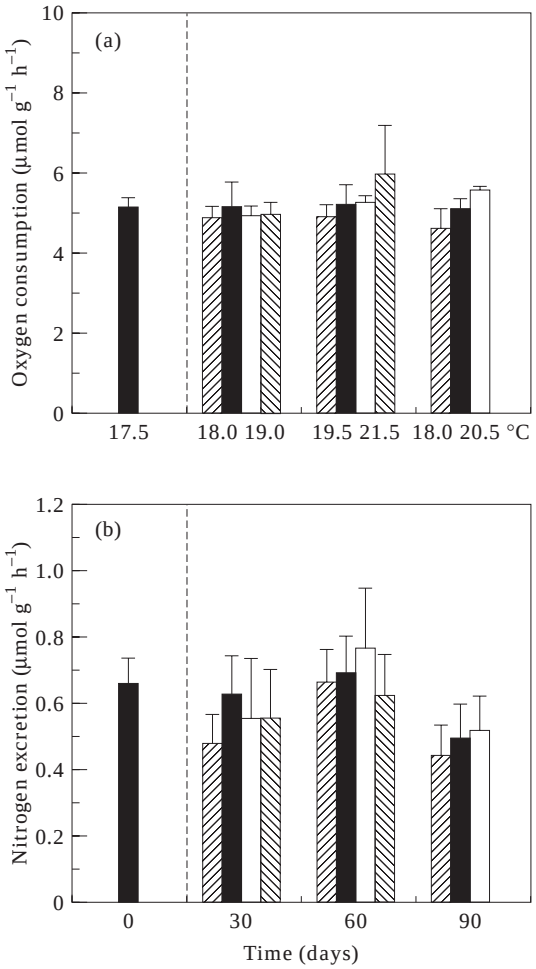


FIG. 3. (a) Routine in-tank oxygen consumption ($M_{O_2}$) and (b) nitrogen excretion (total ammonia + urea nitrogen; $M_N$) rates (μmol $g^{-1}$ $h^{-1}$). Rates were determined for the total number of fish in one of the duplicate tanks, over a 10-h period during the day, including the feeding periods. The temperatures at which the rates were determined are shown also. The vertical dashed line separates the day 0, pre-exposure values from the other measurements. Treatment: ▨, 0/5·2; ■, 0/6·2; □, +2/6·2; ▧, +2/5·2. Error bars represent measurement standard error only, and thus, no statistical comparisons can be made.

[Fig. 3(a)]. This value is about 55% of $M_{O_{2(max)}}$ as determined by Wilson & Wood (1992; corrected for weight differences), and may be contrasted with the 75% of $M_{O_{2(max)}}$ value seen in trout fed to satiation (Dockray *et al.*, 1996). Nitrogen excretion rates were more variable (0·45–0·75 μmol $g^{-1}$ $h^{-1}$) over time and amongst treatments, but no clear trends emerged apart from a tendency to fall at day 90 [Fig. 3(b)]. These rates corresponded to approximately 50% of those seen in fish on an unlimited ration (Dockray *et al.*, 1996). Urea $M_N$ remained relatively constant over time, and varied between 10 and 20% of the total nitrogen excreted (data not shown). At day 90, the fraction of nitrogen excreted as urea increased to 17–20% in all treatments. This occurred as a result of decreased ammonia production in all treatments at this time. FPU values

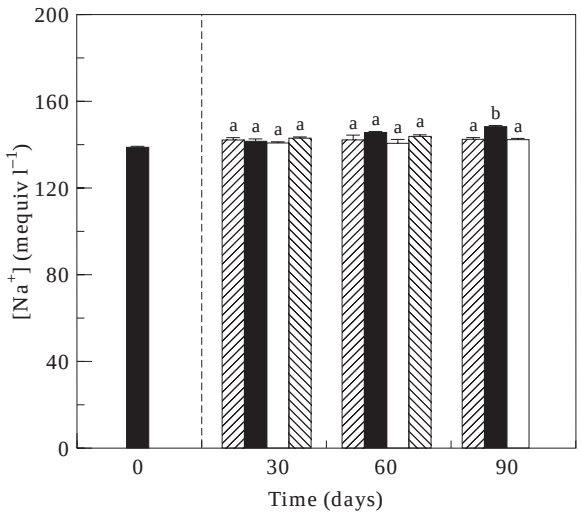


FIG. 4. Plasma $Na^+$ concentrations (mequiv $l^{-1}$). Values given are means ± S.E. ($n=10$). Significant differences ($P<0{\cdot}05$) are indicated by treatment groups that do not share a common letter. See Fig. 3 for other details.

were lower than in trout on unrestricted ration (Dockray *et al.*, 1996), and remained relatively stable over the first 60 days at ~0·45. At day 90, FPU values generally decreased to ~0·35 (data not shown).

## BLOOD ANALYSIS

Haematocrit was quite constant, remaining at ~37% over the entire exposure period, and no treatment differences were evident until day 90 (data not shown). At this time, the 0/5·2 treated fish exhibited significantly lower haematocrits (~32%) than the other groups. Plasma protein values averaged about 3·7 g 100 $ml^{-1}$ overall but were more variable (range = 2·9–4·3) amongst groups and over time, reflecting the relatively small sample size (typically $n=5$ group $^{-1}$ *v.* 16 for haematocrit), and no treatment or time effects were significant.

Plasma [$Na^+$] remained remarkably uniform at ~140 mequiv $l^{-1}$ over the 90-day exposure period, and no effects of low pH or temperature were evident (Fig. 4).

## PROXIMATE ANALYSIS

Changes in whole body proximate composition over time in these fish on limited ration were different from those seen previously with unlimited food supply. Indeed, composition exhibited only small changes over 90 days in the present fish in contrast to the large increases in lipid content, small increases in protein content, and compensating large decreases in water content occurring over 90 days in the study of Dockray *et al.* (1996).

On a whole body basis, the percentage of protein by weight decreased from ~14·5 to 13% from day 0 to 60 in all treatments [Fig. 5(a)]. Protein then increased again to ~14·5% at day 90. No treatment effects were evident, and no significant differences were maintained.

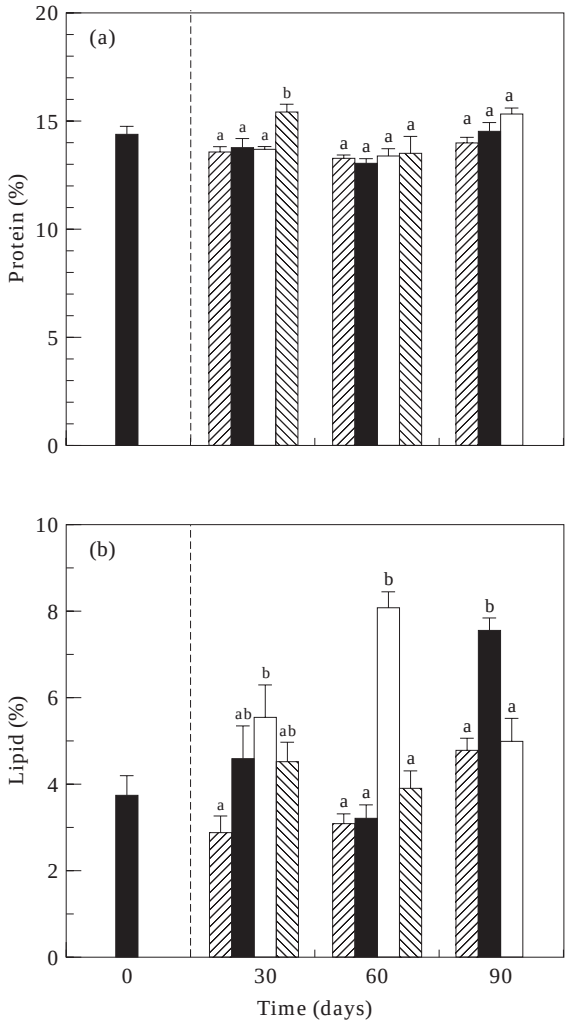


FIG. 5. (a) Whole body protein and (b) lipid (%). Values are given as means ± S.E. ($n$ = 10). Significant differences ($P$ < 0·05) are indicated by treatment groups that do not share a common letter. See Fig. 3 for other details.

Initial whole body lipid levels were ~4% (Fig. 5(b)]. A great deal of variability among treatments was evident at each subsequent time period. In general, all levels increased to about 5% over the 90-day test period, with levels in each of the two ambient pH treatments increasing significantly at days 30 and 60 (+2/6·2) and at day 90 (0/6·2). Throughout, lipid levels in the low pH treatments were lower than in the ambient pH treated fish, differences which were significant at some times.

Total carbohydrate (estimated as glucose + glycogen + lactate) was also variable over the 90-day exposure period in all treatments, and showed no consistent trends among treatments or over time. Carbohydrate constituted about 0·8% of whole body composition (data not shown). Whole body water content increased from the initial value of 78 to ~80% over time. No consistent trends were

evident among treatments (data not shown). There was no change in total inorganic content (ash: data not shown). Carbohydrate contents, expressed as percentages of fish body weight, were approximately twice those of the previous study (Dockray *et al.*, 1996) and ash content was also up to 1·5-fold higher. These differences reflected probably, at least in part, the lower lipid and protein contents of the present fish.

Whole body $Na^+$ and $Cl^-$ concentrations were similarly higher by about 33 and 22%, respectively, relative to fish fed an unlimited ration, but $Ca^{2+}$ concentrations were about 26% lower. Whole body $Na^+$, $Cl^-$, and $Ca^{2+}$ levels indicated no effect of low pH on ion balance, and no other consistent treatment effects (Table I). $[Na^+]$ and $[Cl^-]$ remained the same over the 90-day period at $\sim$55 and 40 mequiv $kg^{-1}$, while $[Ca^{2+}]$ decreased from the initial value of $\sim$71 mequiv $kg^{-1}$ to $\sim$60 mequiv $kg^{-1}$ at day 30, and remained at this concentration over the rest of the exposure period.

## ENERGY BUDGET

The energy budgets for the satiation–fed fish from Dockray *et al.* (1996) and the limited ration fish from the present study were calculated up to, and including, day 60 (Table II) allowing comparison of trout that have been exposed to a similar thermal history. Furthermore a comparison up to day 60 also allowed consideration of the +2/5·2 treatment trout, which were lost at day 77 as a result of equipment failure in the present study.

In the satiation–feeding study, the trout in the control temperature treatments consumed a greater absolute amount of energy, and converted that energy more efficiently into weight gain than the trout exposed to an additional 2° C. The greatest energy gain and conversion occurred in the 0/5·2 treatment, in which appetite was the greatest. The greatest metabolic expenditure and excretory energy losses occurred in the +2/5·2 treatment. Faecal and unaccounted energy losses were similar in all treatments, although they were lowest in the 0/5·2 treatment. The 0/5·2 treatment trout were consuming proportionately more energy, and making better use of that energy by converting it more efficiently to energy gain. However, metabolic expenditure was high in this treatment, perhaps as a consequence of prolonged feeding activity. Thus the increased energy intake fuelled both greater energy deposition (growth) and greater metabolic expenditure.

In the present limited-ration study, total energy consumed was the same in each treatment. Total energy gained and conversion efficiency was high in the +2/6·2 treatment trout. These high values, and the consequent negative $F$ values, reflect an unusually high lipid content at day 60 which may have been a consequence of the increased temperatures temporarily improving energy deposition, or a transient effect due to the fluctuating nature of the endogenous stores of lipid, or a random sampling aberration. At day 90 the lipid level in this treatment decreased again. Otherwise, total energy gained was higher in the two low pH treatments than in the 0/6·2 treatment, a fact reflected in the conversion efficiencies.

Metabolic expenditure in the limited-ration trout was 70% lower than in satiation–fed trout, yet accounted for $\sim$75% of the total energy intake. This

TABLE I. Whole body [$Na^+$], [$Cl^-$] and [$Ca^{2+}$] (mequiv $kg^{-1}$) for each treatment at each 30-day period

| | Day | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | | | | 60 | | | | 90 | | | |
| | 0/6·3 | 0/5·2 | 0/6·3 | +2/6·3 | +2/5·2 | 0/5·2 | 0/6·3 | +2/6·3 | +2/5·2 | 0/5·2 | 0/6·3 | +2/6·3 | +2/5·2 |
| [$Na^+$] | 52·6 | 55·5$^{ab}$ | 57·1$^{a}$ | 51·0$^{b}$ | 60·7$^{a}$ | 53·3$^{a}$ | 54·1$^{a}$ | 54·3$^{a}$ | 56·3$^{a}$ | 55·2$^{a}$ | 53·9$^{a}$ | 57·1$^{a}$ | NA |
| ± S.E. | 0·9 | 1·1 | 1·9 | 1·1 | 2·5 | 1·1 | 1·5 | 1·3 | 1·1 | 1·6 | 1·2 | 2·4 | NA |
| *n* | 10 | 14 | 16 | 16 | 15 | 16 | 16 | 16 | 16 | 16 | 16 | 15 | NA |
| [$Cl^-$] | 38·7 | 40·8$^{ab}$ | 41·9$^{a}$ | 37·4$^{b}$ | 42·7$^{a}$ | 38·5$^{a}$ | 38·0$^{a}$ | 39·6$^{a}$ | 39·8$^{a}$ | 40·1$^{a}$ | 38·5$^{a}$ | 41·5$^{a}$ | NA |
| ± S.E. | 0·6 | 0·8 | 1·2 | 0·9 | 1·7 | 0·8 | 1·1 | 1·1 | 0·6 | 1·2 | 1·1 | 1·9 | NA |
| *n* | 10 | 14 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | NA |
| [$Ca^{2+}$] | 70·8 | 62·3$^{ab}$ | 60·7$^{ab}$ | 57·5$^{b}$ | 66·3$^{a}$ | 57·1$^{a}$ | 55·1$^{a}$ | 57·9$^{a}$ | 58·7$^{a}$ | 63·7$^{ab}$ | 60·2$^{a}$ | 70·1$^{b}$ | NA |
| ± S.E. | 2·0 | 2·2 | 2·2 | 2·2 | 2·1 | 1·3 | 1·6 | 1·9 | 1·5 | 2·3 | 1·5 | 3·5 | NA |
| *n* | 10 | 14 | 16 | 16 | 15 | 16 | 16 | 16 | 16 | 16 | 16 | 15 | NA |

Mean values ± S.E. and sample size (*n*) are shown. At each sample time, significant differences between treatments are indicated by treatments that do not share a letter ($P < 0{\cdot}05$).

TABLE II. Energy budget comparison between the satiation–fed fish of Dockray *et al*. (1996) and the limited ration trout of the present study

| Treatment | Total energy consumed, $C$ | | Total energy gained, $P_G$ | Conversion efficiency, $K$ | Total metabolic expenditure, $M$ | | Total n energy lost, $U$ | | Faecal/unaccounted energy lost, $F$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | kJ | % | kJ | % | kJ | % | kJ | % | kJ | % |
| Satiation–fed | | | | | | | | | | |
| 0/5·2 | 516·8 | 100 | 266·4 | 51·6 | 140·9 | 27·3 | 17·5 | 3·4 | 92·0 | 17·8 |
| 0/6·3 | 474·5 | 100 | 230·9 | 48·7 | 119·9 | 25·3 | 14·3 | 3·0 | 109·5 | 23·1 |
| +2/6·3 | 438·2 | 100 | 198·9 | 45·4 | 122·5 | 27·9 | 18·0 | 4·1 | 98·9 | 22·6 |
| +2/5·2 | 423·5 | 100 | 168·6 | 39·8 | 150·3 | 35·5 | 19·3 | 4·6 | 85·3 | 20·2 |
| 4% ration | | | | | | | | | | |
| 0/5·2 | 55·6 | 100 | 7·9 | 14·2 | 40·6 | 73·0 | 3·9 | 7·0 | 3·2 | 5·8 |
| 0/6·2 | 56·4 | 100 | 6·7 | 11·9 | 41·4 | 73·4 | 4·3 | 7·6 | 4·0 | 7·1 |
| +2/6·2 | 56·7 | 100 | 18·8* | 33·1 | 41·8 | 73·7 | 4·4 | 7·8 | − 8·3* | − 14·6* |
| +2/5·2 | 56·0 | 100 | 8·5 | 15·1 | 43·7 | 78·0 | 3·8 | 6·8 | 0·1 | 0·1 |

The budget covers the period day 0–60. The general energy budget equation is: $C = P_G + M + U + F$ where $C$ is the energy consumed, $P_G$ is the energy gained, $M$ is the metabolic expenditure, $U$ is excretory energy loss, and $F$ is faecal energy loss. Faecal and unaccounted losses are estimated by difference $[C - (P_G + M + U)]$. Each component of the budget is expressed as a percentage of $C$ to determine allocation differences. $K$ is the efficiency with which food energy is converted into total energy gain $(C/P_G \times 100)$.

*The high $P_G$ value in this treatment reflects an unusually high lipid content at day 60, which is possibly aberrant. The $F$ values are negative in consequence.

contrasted with metabolic expenditures in the unlimited-ration trout of ~30% of the total energy intake. The differences between treatments in the limited-ration exposure were slight.

Nitrogen energy losses were comparable in all treatments for the limited-ration trout, amounting to about 7% of the total energy consumed. In the unlimited feeding study, these energy losses were four to fivefold higher on an absolute basis, but amounted to only ~3·5–4·0% of the total energy consumed in all treatments. The faecal and unaccounted energy losses comprised 7% or less of the energy intake in the limited ration trout, in contrast to ~20% in the satiation–fed fish, indicating higher absorption efficiencies in the former.

## DISCUSSION

The results of the present study have been compared directly with those from our previous study (Dockray *et al.*, 1996) to elucidate further the effects of chronic low pH and warmer environmental temperatures on juvenile trout. It was suggested that by limiting the food intake, greater insight would be gained into the compensatory effects of unlimited diet on ionoregulatory balance and the impact of warmer temperatures.

### TEMPERATURE AND RATION EFFECTS

Temperature is generally considered a controlling factor that governs metabolic rate in ectotherms (Fry, 1947; Schmidt-Nielsen, 1987). However, the similar metabolic rates, both over time and among treatments, throughout the 90-day exposure period, indicated that there was no substantially increased cost of living in a warmer environment. The gradual increase in temperature over the 90-day exposure period, in combination with daily fluctuations, and the fact that fish are able to acclimate more rapidly to increases than to decreases in temperature (Brett, 1944), resulted in metabolic compensation. This effect was also evident in our first exposure (Dockray *et al.*, 1996).

Nevertheless, the continuous +2° C elevation was sufficient to reduce growth rates in the limited ration fish in these treatments. Similar effects of temperature on growth were obtained in our first study, although the effects were most obvious in the period during which temperatures increased rapidly towards incipient lethal levels (days 60–90; Dockray *et al.* 1996). Limited energy intake means that metabolic energy expenditure accounts for a greater percentage of the overall energy budget (Table II, discussed below), thereby increasing the sensitivity of fish growth to very minor metabolic differences as might be caused by +2° C. Brett (1971*a*,*b*) described how the optimum temperature for growth shifts progressively to a lower temperature as the food quantity is restricted. The reduction in growth in the trout exposed to an additional 2° C then, was probably a consequence of both the warmer temperatures, and the restricted ration.

The limited ration feeding regime resulted in routine $M_{O_2}$ values that were ~55% of $M_{O_{2(max)}}$ in contrast to ~75% of $M_{O_{2(max)}}$ in fish on an unlimited feeding regime. Overall, reducing feeding rates by 60% resulted in routine $M_{O_2}$ values that were reduced by almost 30%. As Cho *et al.* (1982) explained, ration size, food composition, and temperature determine the extent of the increase in

$M_{O_2}$ associated with feeding, known as specific dynamic action. Since food composition was the same, and temperatures only slightly lower overall, the reduction in $M_{O_2}$ was clearly a consequence of the reduction in food intake (Beamish, 1974). Also, activity levels were probably reduced due to less time spent feeding.

The addition of 2° C did not affect protein content in these trout significantly, although lipid and carbohydrate levels fluctuated somewhat over the 90-day period. Jobling (1980), Miglavs & Jobling (1989) and Quinton & Blake (1990) have demonstrated that starvation or restricted feeding leads to reduced lipid content and a replacement of this lipid with water in fish tissues. Lipid levels in the present fish were only about half of those in trout on an unrestricted ration (Dockray *et al.*, 1996), and water contents were higher.

Nitrogen excretion rates were affected little by the addition of 2° C. Overall, $M_N$ in trout fed a 4% ration were almost 60% lower than excretion rates in trout fed to satiation, equivalent to the 60% reduction in food intake rates discussed above. Protein utilization did not increase, as indicated by the stable whole body protein levels, and the relatively stable fractional protein utilization (FPU) indices. The present FPU values were substantially lower than in trout on unrestricted ration.

## LOW pH EFFECTS

Trout exposed to control temperatures and pH 5·2 grew significantly faster than trout in all other treatments. Better growth was also evident in the 0/5·2 exposed trout in our previous study, but these unlimited ration fish also ate more (Dockray *et al.*, 1996). Increased food intake was not a complication in the present study. Some previous studies have demonstrated growth impairment at higher $[H^+]$ (pH < 5·2) (Menendez, 1976; Cleveland *et al.*, 1986), and reduced growth has been related to reduced food consumption under low pH conditions (Brown *et al.*, 1984; Lacroix & Townsend, 1987; Tam *et al.*, 1988). However, neither of these effects were observed in this, or our previous studies. Wilson & Wood (1992) reported no difference in growth for juvenile rainbow trout fed 1% body weight $day^{-1}$ and exposed to pH 5·2, while Wilson *et al.*, (1994) reported increased growth with unchanged appetite in trout fed to satiation and exposed to the same conditions. Clearly, regardless of feeding rate, pH 5·2 does not impact growth rate negatively in juvenile rainbow trout.

In other studies, sublethal low pH has been reported to increase $M_{O_2}$ (Butler *et al.*, 1992; Hargis, 1976; Waiwood & Beamish, 1978), the increased energy expenditure resulting in reduced growth. However, there was no detectable effect of sublethal acidity on $M_{O_2}$ in the present study. Also, gross conversion efficiencies indicated that food was converted more efficiently to growth in the 0/5·2 treatment than in all other treatments. These results suggest that the trout exposed to low pH may have reduced their overall energy expenditure, thereby allowing them to utilize their food more efficiently. It appears that the food ration, although 60% less than the amount consumed by the satiation–fed fish, was sufficient to combat any increased costs associated with living in this challenging environment. It is likely that these trout reduced their activity levels at low pH. Reduced activity levels would result in more energy available for deposition as growth, and thereby explain increased gross conversion efficiencies.

Reduced spontaneous activity has been reported in larval brook trout *Salvelinus fontinalis* (Mitchill) exposed to pH 4·5, and reduced duration of activity observed at pH 5·5 (Cleveland *et al.*, 1986).

## COMBINATION TREATMENT EFFECTS

Trout exposed to a combination of +2° C and pH 5·2 exhibited significantly reduced growth rates when compared to the control temperature treatment trout. This reduction in growth was not significantly different from the +2/6·2 treatment, indicating that the effect was a consequence of exposure to increased temperature, rather than to low pH. Low pH in the +2/5·2 treatment did not cause the trout to conserve energy. Decreased activity levels, suggested above as an effort to reduce metabolic expenditure in the 0/5·2 exposed trout, may have been just offset by the additional 2° C in the +2/5·2 exposed trout, especially at the higher temperatures later in the exposure.

The maintenance of whole body protein levels in the trout of the +2/5·2 treatment indicated that the 4% ration was adequate to maintain endogenous protein stores. Lipid and carbohydrate levels fluctuated somewhat in this treatment, as in the other treatments, suggesting that endogenous lipid stores were utilized at times. However, no clear treatment effect on endogenous energy sources was evident.

## INDICATORS OF STRESS

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

A calculation of the dietary ion budget for the trout in the first exposure (Dockray *et al.*, 1996) showed that trout were consuming about 10% of their body pool of $Na^+$ $day^{-1}$. In the present study, trout consumed about 4% of their body pool of $Na^+$ $day^{-1}$. This 60% reduction in ions available to the fish through the diet did not result in decreased plasma or whole body $Na^+$. This suggests that the restricted diet in the present study was still sufficient to replace any salts that may have been lost in the low pH treatments as a result of the probable inhibitory effects of $H^+$ on net ion uptake at the gills (see Introduction). The results of the present and previous study agree with those of Sadler & Lynam (1987) who fed yearling brown trout *Salmo trutta* L., 2% of their body weight $day^{-1}$ (wet or dry basis unstated) and exposed them to low pH (4·4–5·2) without inducing ionoregulatory disturbance. If the water contents of food and fish were similar to those in the present study, these trout would actually have consumed about 8% dry body weight $day^{-1}$, a consumption rate between the present and previous studies. However, ionoregulatory disturbance did occur when starved trout were exposed to the same conditions in Sadler & Lynam's (1987) study. The present results suggested that quite low levels of dietary salt intake are sufficient to compensate for losses of ions due to the acid exposure.

ENERGY BUDGET

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

Brett *et al.* (1969) demonstrated that as temperature increases past an optimum, the efficiency with which food energy is converted to energy gain decreases. Likewise, as ration decreases, the optimum temperature for growth decreases. Therefore, as temperature increases past the optimum, fish have to consume more to achieve a similar growth rate. In the satiation–fed juveniles of our first exposure (Dockray *et al.*, 1996), growth was achieved over the entire temperature range (13–24° C) by increasing appetites, since ration was unlimited. In the present, limited-ration experiment, juvenile trout were exposed to similar temperature conditions, however, they were not able to increase their food intake. Since temperatures were higher than the optimum temperature for growth throughout the duration of the present study, and since the optimum temperature would be lower in these limited ration fish than for those in the satiation–fed exposure, conversion efficiency was lower.

Mehner & Wieser (1994) explained that high temperature causes a greater proportion of the metabolizable energy ($M + P_G$) to be allocated to $M$ (total metabolic expenditure) than to $P_G$ (total energy gained). This was clearly evident in the limited ration trout of the present study with 70% of their total energy consumption expended metabolically in comparison with the 30% metabolic expenditures in the unlimited ration trout of our previous study. Thus, at higher temperatures, when rations are restricted, conversion efficiencies are reduced (Elliott, 1976*a*).

The slightly lower metabolic expenditure in the 0/5·2 treatment trout, in combination with lower nitrogen energy losses in this group, supports our previous suggestion that these fish may be conserving energy by reducing activity levels. This strategy in fish has been described by Forstner & Wieser (1990), Mehner & Wieser (1994), and Wilson *et al.* (1994) in response to warmer temperatures, and low pH + Al exposure respectively.

Faecal losses in carnivorous fish have been determined as from 2 to 31% of the energy intake (Elliott, 1979). Trout in the present study, therefore, made very efficient use of the metabolizable energy they consumed with a considerably higher absorption efficiency than in the unlimited-ration trout. Nevertheless that energy, as described above, was expended metabolically under the summer temperature conditions, rather than put towards growth. This observation is supported by Elliott (1982) who found that as temperature increased, and ration decreased, absorption efficiency increased. He explained that the physiologically useful energy (Elliott, 1976*b*), that energy available for growth and metabolism, decreased with temperature at higher temperatures, and increaased with decreasing ration level.

Unlike the energy budget for the trout in our first unlimited ration exposure, the evidence for improved conversion efficiency in the 0/5·2 treatment trout is not

clear. Metabolic energy expenditure was slightly lower in this treatment, as was excretory energy loss, perhaps associated with a decrease in spontaneous activity. It may be that growth rates were greater in these trout simply because they did not mobilize endogenous energy stores from time to time for activity, as was evident in the other three treatments.

## CONCLUDING REMARKS

Chronic exposure of juvenile rainbow trout to warmer environmental temperatures and sublethal low pH under limited ration conditions of 4% dry body weight $day^{-1}$ did not result in more clear-cut treatment effects on their physiology and energetics than previously seen under unlimited ration (Dockray *et al.*, 1996). The combination of warmer temperatures and sublethal low pH appeared to be slightly more costly than either stressor alone, in trout that have an unlimited or limited ration. The trout exposed to an additional 2° C had depressed growth rates, and those exposed to pH 5·2 exhibited no evidence of ionoregulatory disturbance. These results are similar to the results of our first study (Dockray *et al.*, 1996), and indicate that the 4% ration in the present study was sufficient to ameliorate the effects of acidic exposure. This reduced ration still provided an adequate salt load for the replacement of any ion losses caused by low pH exposure. High summer temperatures, above the optimum temperature for growth, reduce conversion efficiency. These effects were more evident in limited ration fish, because they were unable to increase their food intake to combat the subsequent high metabolic rates (relative to energy intake), resulting in low conversion efficiencies. Absorption efficiencies ($P_G + M$), however, were high, indicating very efficient use of the energy consumed.

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

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