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Author(s): Leela Marie D'Cruz and Chris M. Wood

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

Leela Marie D'Cruz
Chris M. Wood*
Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

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ABSTRACT

This study evaluated the role of diet, specifically the relative importance of salt content versus energy content, in the response of juvenile rainbow trout to environmental acid stress in soft water ($[Ca^{2+}] = 0.057$ mmol L^{-1}, $[Na^{+}] = 0.047$ mmol L^{-1}). Diets were formulated at two energy levels (regular, 16.3 MJ kg^{-1}, and low, 9.8 MJ kg^{-1}) and two levels of NaCl (regular, ~263 mmol kg^{-1}, and low, ~64 mmol kg^{-1}), yielding four treatment combinations, each fed at a ration of 0.6% body weight d^{-1}. A fifth group of fish was not fed during the experiment. All groups were subjected to an initial acid challenge (24 h at pH 5.0 plus 12 h at pH 4.0), followed by 28 d of exposure to pH 5.2. Following the initial acid challenge, typical ionoregulatory disturbances were seen, but most effects had attenuated or disappeared by day 20 of chronic low-pH exposure. However, after 28 d, fish fed the regular-salt diets maintained the restored ionic homeostasis, whereas those fed low-salt diets did not, regardless of the energy content of the diet. Growth and food conversion efficiency were greatest in trout fed the regular-energy/regular-salt diet, negative in fish fed the low-energy/regular-salt diet, and intermediate in trout on the other diets; starved fish lost weight. Fish maintained on the regular-energy/low-salt diet exhibited the most deleterious effects, including elevated cortisol levels and a 4.1% d^{-1} mortality rate. Fish fed the low-energy/low-salt diet, those fed regular-salt diets, and starved fish were not as adversely affected by the acid stress. Following a regular-energy meal, fish tended to exhibit an elevated rate of oxygen consumption, but this did not occur after a low-energy meal, regardless of its salt content. Elevated oxygen consumption may be accompanied by a loss of ions via the osmorespiratory compromise. We hypothesize that fish fed the regular-energy/low-salt diets were most deleteriously affected in an acidified environment because they were unable to replace increased branchial ion losses with dietary salts. These results indicate that it is the salt content of the food rather than the energy content that is critical in protecting against the deleterious effects of low pH.

*To whom correspondence should be addressed. E-mail: woodcm@mcmail.cis.mcmaster.ca.

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Introduction

Freshwater fish constantly lose ions to the external medium by diffusion across the gills and body surface and by excretion in the feces and urine. Ionic balance is maintained by active ion uptake at the gills (Evans 1993) and through the diet (Cowey and Sargent 1979; Salman and Eddy 1987, 1988; Smith et al. 1989, 1995). However, when fish are faced with a low-pH environment, ionoregulatory disturbance occurs. Na^{+} influx at the gills is depressed (H^{+} competes with Na^{+} for transport sites and/or access to channels), and there is enhanced paracellular ion loss (H^{+} permeabilizes tight junctions via Ca^{2+} displacement or inflammation), leading to greater whole-body Na^{+}, K^{+}, and Cl^{-} loss (McDonald and Wood 1981; Wood 1989; Reid 1995). Under these conditions, dietary salts may become much more important in maintaining homeostasis during acid stress. For example, when fish are starved or fed a very limited diet during exposure to low pH, typical ionoregulatory disturbances are seen (Fromm 1980; Neville 1985; Audet et al. 1988; Booth et al. 1988; Butler et al. 1992; D'Cruz et al. 1998). Conversely, when fish are fed adequately and exposed to low pH, ionoregulatory disturbances are much reduced or do not occur (Menendez 1976; Kwain et al. 1984; Sadler and Lynam 1986; Wilson et al. 1994; Dockray et al. 1996; D'Cruz et al. 1998). If feeding is important in overcoming ionoregulatory dysfunction during acid exposure, there are two possible explanations: food provides the necessary fuel to meet increased costs of living in a low-pH environment or dietary salt replaces branchial ion loss.

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

Table 1: Analysis of the diets used to feed juvenile rainbow trout

	Regular-Energy/ Low-Salt Diet	Low-Energy/ Low-Salt Diet	Regular-Energy/ Regular-Salt Diet	Low-Energy/ Regular-Salt Diet	Zeigler Trout Starter Diet
Digestible protein (%)	39	23	39	23	50
Lipids (%)	18	11	18	11	15
Energy content (MJ kg^{-1})[a]	16.3	9.8	16.3	9.8	17.7
Na^+ (mmol kg^{-1})[b]	78	44	283	243	217
Cl^- (mmol kg^{-1})[b]	79	43	254	254	215

Note. Zeigler Trout Starter was used as feed during acclimation, while the four other diets were used as feed during the pH 5.2 exposure.

[a] Approximate energy content is based on the percentage of proteins and lipids in the food. Values were determined using 23.6 MJ kg^{-1} for protein and 39.5 MJ kg^{-1} for lipid (Braefield and Llewellyn 1982).

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

Wilson et al. (1994), Dockray et al. (1996), and D'Cruz et al. (1998) found that chronic exposure to low pH seemed to stimulate appetite, suggesting a higher cost of living (Reid et al. 1996, 1997). Sadler and Lynam (1986) argued that food compensates for the increased energy expenditure needed to combat ionoregulatory imbalance caused by moderately low levels of pH. On the other hand, indirect evidence supporting dietary replacement of branchial losses comes from Smith et al. (1989, 1995), who found that dietary ions may play a critical role in maintaining whole-body ion homeostasis at circumneutral pH. Salman and Eddy (1987) reported that appetite may be stimulated by decreased whole-body levels of Na^+. Conceivably, this could explain the increased food intake of acid-exposed fish reported by Dockray et al. (1996) and D'Cruz et al. (1998). Thus, the present study was carried out to further elucidate whether it is the energy or the salt in the diet that aids fish in maintaining ionoregulatory balance when chronically exposed to an acidic environment.

Material and Methods

Preexposure Holding

Approximately 800 juvenile rainbow trout (10–12 g) were purchased from Humber Valley Springs Farm, Orangeville, Ontario, and held in two 600-L polypropylene tanks that were supplied with dechlorinated Hamilton tap water (Ca^{2+} = 1.02 ± 0.10 mmol L^{-1}; Na^+ = 0.60 ± 0.03 mmol L^{-1}; pH, 7.6–7.8; temperature, 18°C). After 2 wk, acclimation to soft water was started by progressively increasing the flow of soft water and decreasing the flow of tap water to the tank over a 1-wk period. Synthetic soft water was created by first deionizing tap water via reverse osmosis (Anderson Water Conditioning Equipment, Dundas, Ontario) and then titrating back small amounts of tap water to yield soft water with a Ca^{2+} concentration of 0.057 ± 0.010 mmol L^{-1} and a Na^+ concentration of 0.047 ± 0.007 mmol L^{-1}. During acclimation, fish were fed Zeigler Trout Starter no. 3 daily, at a ration of 1% of their wet body weight per day (see Table 1) for dietary composition. Photoperiod mimicked the seasonal conditions during the acclimation and exposure (September–November 1996). Fish were held in soft water for another 3 wk before experimentation.

Experimental Design and Exposure System

The 800 fish were divided among 10 205-L polypropylene tanks and held in these tanks for the last 7 d of the 3-wk soft water acclimation period before the acid challenge was started on October 23, 1996. Low pH was attained by titrating H_2SO_4 (0.2 N) into the synthetic soft water in a vigorously aerated primary head chamber. This water was then divided into five secondary head chambers, each feeding two replicate exposure tanks. This low pH was maintained by an automatic control system: pH in the treatment tanks was continually monitored with Leeds and Northrup Meridian II (Mississauga, Ontario) combination industrial electrodes that regulated the opening and closing of solenoid valves (Cole Parmer Instruments CP#01367-70, Niles, Ill.) delivering H_2SO_4 to the primary head chambers.

The goal of the experiment was to use an acute acid exposure to induce an initial ionoregulatory disturbance with minimal mortality, then to hold the fish for an extended period at otherwise sublethal low pH level, during which time the effect of different diets on mortality, recovery of ionic status, and other physiological parameters could be monitored. This might represent a sudden acid rainstorm event in a chronically acidified water body. At the start of the experiment, in-tank pH was lowered over 3 h to pH 5.0; fish were then held at this pH for 24 h, following which pH was lowered to 4.0 over 3 h, and then held at this level for another 12 h. The day 0 sampling (see below) was performed at the end of this period. A pH of

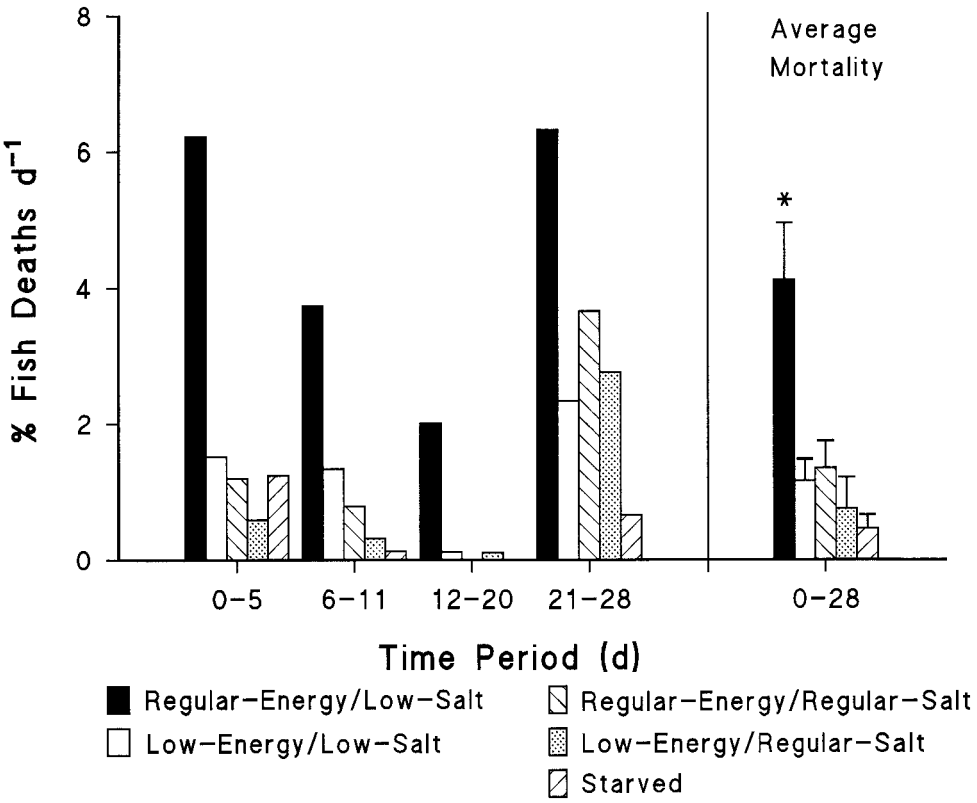


Figure 1. Mortality rate for each sampling period. Numbers represent the number of fish that died per day between each sampling time as a percentage of the number of fish remaining at the beginning of the time period. The overall treatment means ± SEM ($N = 8$) are shown by the last five bars. An asterisk indicates significant differences ($P < 0.05$) among the overall means (Tukey-Kramer test for all pairs).

4.0 is slightly more severe than the 4-d LC_{50} (Graham and Wood 1981) and is well known to cause severe ionoregulatory disturbance in rainbow trout (Wood 1989). Preliminary trials demonstrated that this two-step acidification induced the desired ionoregulatory disturbance while minimizing immediate mortality. After this challenge, pH was raised over 4 h and held at nominal pH 5.2 (actual pH = 5.27 ± 0.02, $N = 280$) for the next 28 d. Chronic exposure to pH 5.2 does not cause mortality when trout are fed to satiation (Dockray et al. 1996). Vigorous aeration maintained O_2 levels and prevented CO_2 buildup in the exposure tanks.

Before the acid challenge, all fish were fed the commercial diet (Zeigler Trout Starter no. 3) at a daily ration of 1% of body weight. Starting on day 1 of the 28-d period, fish were fed one of four diets at 0.6% daily ration: regular-energy/low-salt, low-energy/low-salt, regular-energy/regular-salt, or low-energy/regular-salt (Table 1). Each diet was fed to two tanks of fish, yielding eight (2×4) replicate treatment tanks; in addition, two tanks of fish were not fed throughout the 28 d of acid exposure.

Water temperatures decreased steadily from 18° to 13.5°C during the 28-d exposure, reflecting the normal seasonal pattern for this autumn period. Temperature and pH for each tank were measured daily, and $[Na^+]$ and $[Ca^{2+}]$ were monitored weekly using atomic absorption spectroscopy (Varian AA-1275, Mississauga, Ontario).

Diet and Feeding Regimes

Diets were formulated in the Department of Nutritional Science at the University of Guelph. Diets were developed to contain all essential nutrients but different amounts of energy, by manipulation of the digestible protein and lipid content. Proteins and lipids are generally considered to be the main dietary fuels for trout, as carbohydrates are poorly utilized (Cowey and Sargent 1979; Cho and Kaushik 1990). The values used to determine the energy content of food were 23.6 MJ kg^{-1} and 39.5 MJ kg^{-1} for protein and lipid, respectively (Braefield and Llewellyn 1982). On the basis of the percentage of protein and lipids (Table 1), the regular-energy diets had approximately 16.3 MJ kg^{-1} of food energy, while the low-energy diets had 9.8 MJ kg^{-1}. Thus, the energy content was reduced by 40% in the low-energy diets; cellulose, a poor metabolic

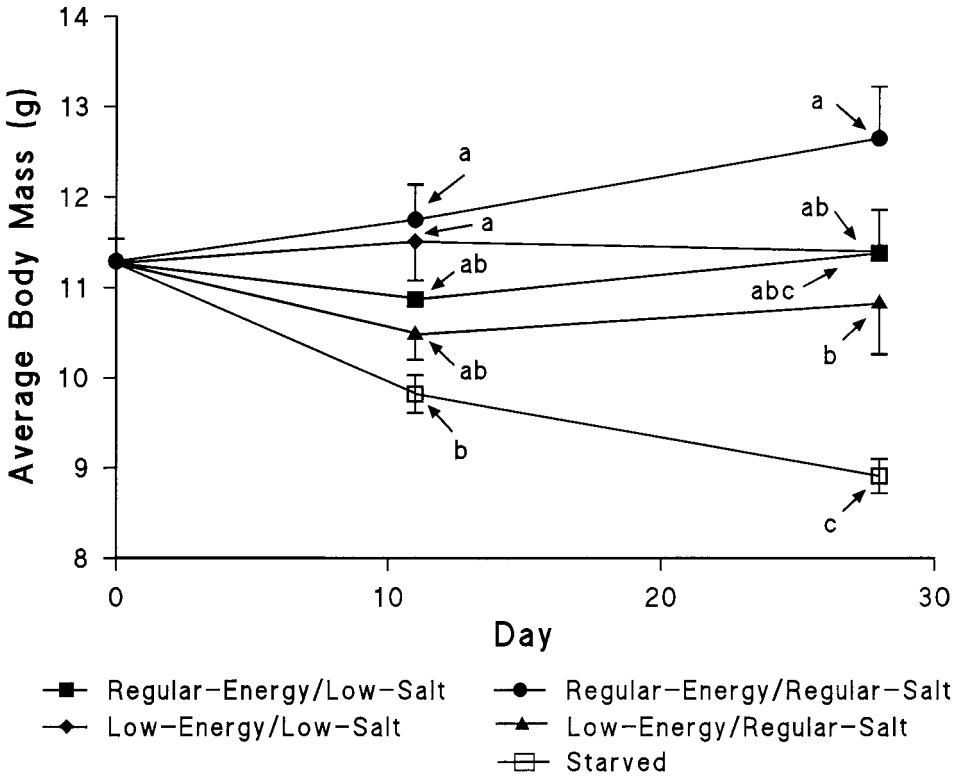


Figure 2. Wet body mass (g) on days 0 ($N = 150$), 11 ($N = 60–80$), and 28 ($N = 20–80$). The values are means ± SEM. For the regular-energy/low-salt group, the SEM bars (not shown) are 0.44 g and 0.95 g on days 11 and 28, respectively. By day 11, there were significant differences in growth between the starved fish and the fish maintained on the regular-energy diets. By day 28, differences in growth were even more apparent, with the fish fed the regular-energy/regular-salt diets growing the most. Significant differences ($P < 0.05$) are indicated among treatment means that do not share a common letter.

fuel, was used to make up the differences (Cho and Kaushik 1990).

Na^+ and Cl^- contents in the low-salt diets were about 80% lower than regular-salt diets (see Table 1 for the exact values), a difference achieved by decreasing the amount of NaCl added in the mineral premix. Note that these salt concentrations in the diets are expressed on a dry weight basis: the actual ratio of Na^+ and Cl^- contents to dry matter in the regular-salt diet is approximately equivalent to that found in natural prey organisms (e.g., other fish). All diets were steam-pelleted and crumbled to sizes readily consumed by the fish. Fish were fed a daily ration of 0.6% of their wet body weight, except on sampling days, when they were not fed. Following the sampling day, fish were fed 1.2% of their body weight to compensate for this missed feeding. All food was consumed. Feeding amount was determined from the whole-tank biomass. This was determined after each sampling day using the bulk-weighing technique described by D'Cruz et al. (1998).

Sampling Protocol

On day −1 and day 0 (immediately at the end of the 12 h at pH 4.0, and before the pH was raised to 5.2 again), 10 fish were sampled, one fish from each tank. Six fish were sampled from each tank on days 11 and 20, and 10 fish from each tank were sampled on days 5 and 28. Fish were rapidly killed by a blow to the head and blotted dry; weight and fork length were then measured. Blood was collected by caudal severance into ammonium-heparinized capillary tubes. Hematocrit (hct) was determined by centrifugation at 10,000 *g;* plasma was removed from the capillary tubes for measurement of plasma protein using a handheld refractometer (American Optical). The remaining plasma was frozen at −70°C for subsequent analysis of plasma $[Na^+]$ and cortisol. Plasma was not available for cortisol analysis on day 20 because of freezer failure. Fish were freeze-clamped using aluminum tongs that were chilled in liquid nitrogen, and then the fish were stored at −70°C for later analysis.

Following this whole-body sampling, whole-tank biomass was measured. In addition, fish were individually measured to determine changes in growth. In order to determine a mean starting fish size, before acid exposure 20 fish from each tank

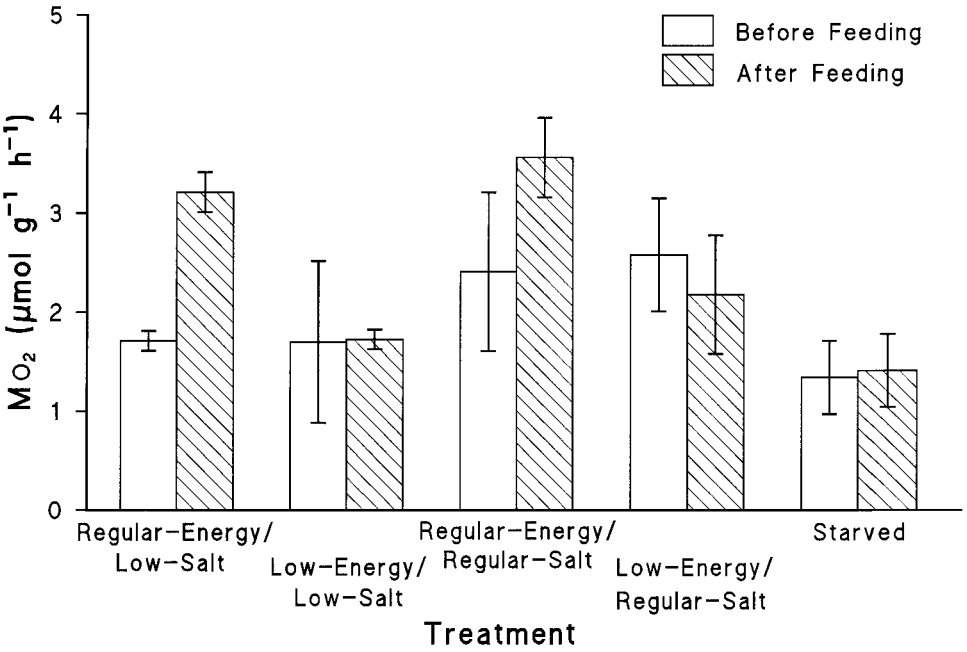


Figure 3. In-tank oxygen consumption (M_{O_2}) on day 15, corrected for size differences (to 1 kg) using the weight exponent determined by Cho (1992). Values (μmol g^{-1} h^{-1}) for each treatment are averages of the replicate tanks (bars represent ranges) over the four 1-h sampling periods before feeding (*open columns*) and the three 1-h sampling periods after feeding (*hatched columns*).

were randomly chosen and lightly anaesthetized with MS-222 that had been buffered with $NaHCO_3$, and weight and fork length were determined. On days 11 and 28, all fish in each tank were anaesthetized with MS-222, and the same parameters were measured.

Metabolic Rates

Whole-tank oxygen consumption was determined on day 15 over four 1-h periods before feeding and three 1-h periods after feeding. Both tanks for each treatment were measured. Tanks were sealed with airtight, transparent lids, and the water within each tank was recirculated using a pump (Little Giant, 1EUAA-MD, Oklahoma City, Okla.). For the 4-h prefeeding period, water samples were taken over each hour, and the partial pressure of oxygen (P_{O_2}) was measured by injecting water samples into a thermostatted Radiometer E5046 electrode (Copenhagen) connected to a Cameron Instrument oxygen meter (Port Aransas, Tex.). Fish were then fed with their respective diets, and 0.5 h later the same method was resumed to measure oxygen consumption (M_{O_2}) for a further 3 h. Measurement of M_{O_2} was stopped and re-aeration initiated if P_{O_2} decreased below 100 Torr (where 1 Torr = 133.322 Pa). M_{O_2} was calculated from the rate of oxygen depletion over each hour, using oxygen solubility coefficients from Boutilier et al. (1984), factored by time, volume, and total fish weight. Data for each hour were averaged from replicate tanks. Rates were adjusted for mean fish size differences (scaling to 1 kg) using the weight exponent 0.824 that was determined by Cho (1992) for rainbow trout. Blanks for microbial respiration were not run in the present investigation, because blanks measured in two other similar studies in our laboratory (on tanks from which all fish were removed immediately after feeding) revealed negligible values relative to measured M_{O_2}'s with fish present (Alsop and Wood 1997; Linton et al. 1997).

Sample Processing

Plasma [Na^+] was determined by atomic absorption spectroscopy. Cortisol was measured using a [^{125}I] radioimmunoassay (ICN Immunocorp, Montreal). Frozen whole bodies were ground using an IKA-Labortechnik M10/M20 grinding mill (Janke and Kunkel, Staufen, Germany) cooled to −70°C with a dry ice and methanol mixture. About 2 g of this ground tissue was oven-dried to a constant weight to determine water content. Remaining tissue was lyophilized (Labconco Lyph-Lock 6, Kansas City, Mo.) and stored at −20°C for proximate body analysis. Whole-body ions were determined from lyophilized tissue that was digested in 8% perchloric acid at a 9 : 1 ratio. Whole-body [Na^+], [Ca^{2+}], and [K^+] were measured using atomic absorption spectroscopy and whole-body [Cl^-] was determined by a colorimetric method (Zall et al. 1956). Proximate analysis was measured only for fish sampled on days −1, 0, and 28. Whole-body

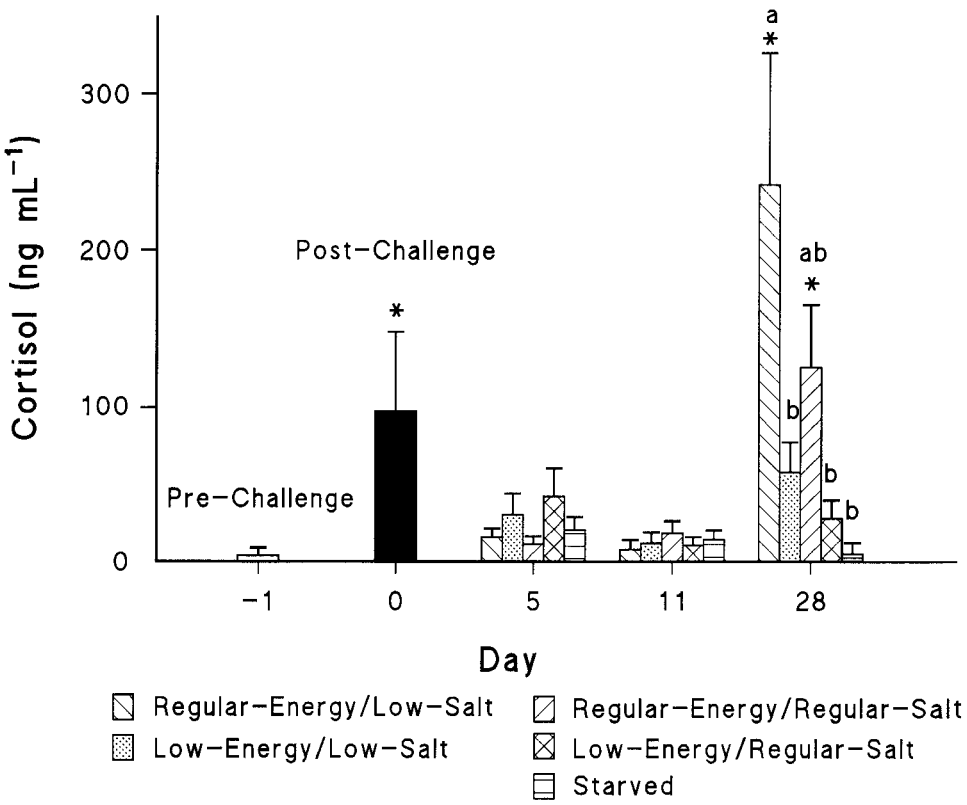


Figure 4. Plasma cortisol concentrations (ng mL^{-1}; means ± SEM, $N = 10–12$). Values are not available for day 20. An asterisk indicates significant differences ($P < 0.05$) between plasma cortisol concentrations before and after the acid challenge. Significant differences ($P < 0.05$) among treatments at one sampling period are indicated by treatment groups that do not share a common letter. If no letters are present for the sampling period, there were no significant differences between means.

protein and lipids were quantified using the Lowry assay as modified by Miller (1959) and chloroform/methanol method extraction (Folch et al. 1957), respectively. Standard enzymatic analyses (Bergmeyer 1985) were employed for glucose, glycogen, and lactate; the sum of the three was used as a measure of total carbohydrates.

Gross food conversion efficiencies were determined by dividing the amount grown (wet weight) by the weight of food consumed during the 28 d, and condition factors for each fish were determined as 100 × weight (g) per fork length (cm)^3 × 100. Mortalities were calculated for intervals between sampling periods, based on the number of fish remaining at the start of each period and expressed in percentages per day.

Statistical Analysis

Values are given as the mean ± standard error (SEM), except in the case of M_{O_2} where the mean and the range of replicate measurements are reported. There were no significant differences between replicate tanks, and therefore data were combined in all analyses. Mean values were compared using one-way ANOVA (SAS JMP, version 5.0); when the *F*-value indicated significance, the Tukey-Kramer comparison of all pairs was used to determine treatment differences within a sampling period and to compare mortality rate over the 28 d. To determine if fish recovered from the initial acid stress, a one-way ANOVA was used to compare means of whole-body ions (Na^+, Cl^-, and K^+) and plasma [Na^+]. If the *F*-value indicated significance, the Dunnett's test was then used to compare the means to the values at day −1. An unpaired Student's *t*-test was used to compare values on day −1 and day 0 and also to compare proximate composition values between day −1 and day 28. A 95% confidence level was chosen.

Results

Mortality

Mortalities were highest (1%–6% d^{-1}) for all treatments between days 0 and 5 (shortly after the pH 4.0 challenge), decreased progressively at days 6–11 and 12–20, and then increased again between days 21 and 28 (Fig. 1). The highest mortality rate throughout the exposure was experienced by

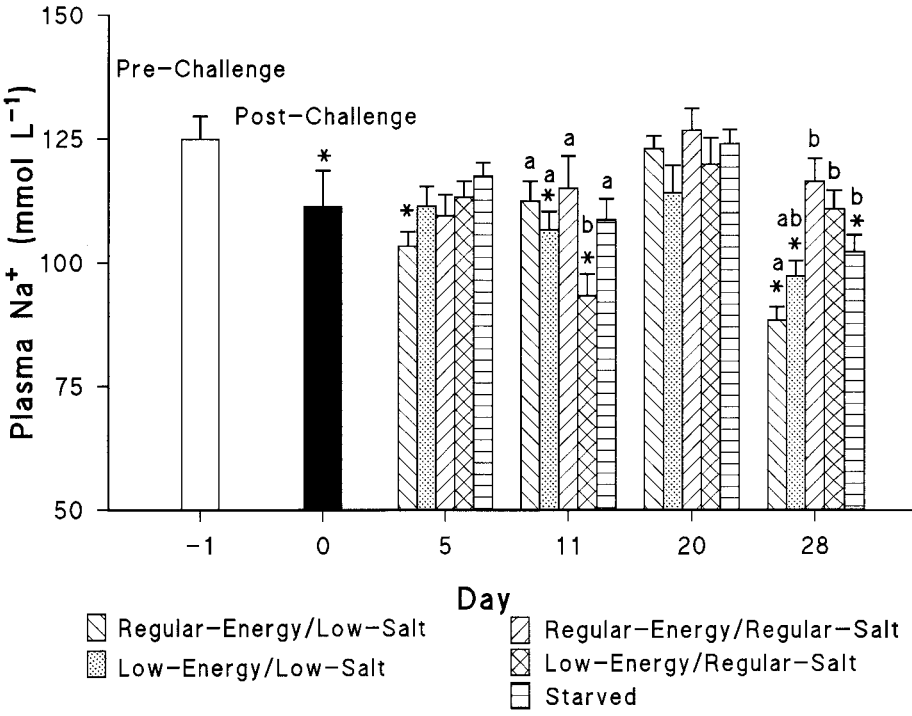


Figure 5. Plasma [Na^+] (mmol L^{-1}; means ± SEM, $N = 10–20$). An asterisk indicates significant differences ($P < 0.05$) between plasma [Na^+] before and after the acid challenge. Significant differences ($P < 0.05$) among treatments at one sampling period are indicated by treatment groups that do not share a common letter. If no letters are present for the sampling period, there were no significant differences between means.

fish fed the regular-energy/low-salt diet, with an overall mean of 4.1% d^{-1}, significantly different from the other four treatments. The mortality rate was lowest for starved fish, with a rate of 0.5% d^{-1}, while the rest of the treatments had rates varying from 0.7% to 1.3% d^{-1}. The differences in mortality rate among these other four treatments were not significant.

Growth and Food Conversion Efficiency

By day 11, differences in weight gain or loss were already noted, and by day 28, fish fed the regular-energy/regular-salt diet were significantly larger than either fish fed the low-energy/regular-salt diet or starved fish, both of which had lost weight (Fig. 2). Indeed, the starved fish were significantly lower in weight than fish in all the other treatments, except in the regular-energy/low-salt diet. The low-energy/regular-salt fed fish were larger than the starved fish, yet smaller than the fish fed the regular-energy/regular-salt diet.

Initial mean condition factor was 0.90 ± 0.01, and by day 11 this had not changed for any of the treatments. However, by day 28, condition factors for fish fed the low-salt diets were significantly greater than the mean initial value: the condition factor for fish on the regular-energy/low-salt diet was 1.20 ± 0.04 and 1.21 ± 0.02 for fish on the low-energy/low-salt diet, respectively. On the other hand, fish fed low-energy/regular-salt diets and starved fish had significantly lower condition factors (0.90 ± 0.02 and 0.87 ± 0.01, respectively). Condition factors for fish fed the regular-energy/regular-salt diet were intermediate at 1.11 ± 0.03. On the basis of a calculation of gross food conversion efficiency, the regular-energy/regular-salt diet was far better utilized (conversion efficiency averaged 0.77 over 28 d) than the three other diets. The regular-energy/low-salt and low-energy/low-salt treatments produced conversion efficiencies close to zero (0.05 and 0.06, respectively), while the low-energy/regular-salt diet produced a substantially negative efficiency (−0.28). The fish resorted to catabolism of internal stores during this latter exposure.

Metabolic Rate

As only replicate measures on two tanks per treatment were made, these data could not be evaluated statistically. Nevertheless, clear trends were apparent. Before feeding, fish fed the low-salt diets tended to have lower M_{O_2} values than fish fed the regular-salt diets (Fig. 3). After feeding, the similarities between similar salt levels ended, and changes in M_{O_2} generally corresponded with the energy content of the food. Fish fed the regular-energy diets tended to experience a postprandial increase (32%–47%) in M_{O_2} compared with the low-energy

Table 2: Measured hematocrit (%) and plasma protein (g [100 mL]$^{-1}$) for each dietary regime

Day and Treatment	*N*	Hematocrit (%)	Plasma Protein (g [100 ml]$^{-1}$)
Pre-acid-challenge	6	28.2 ± 4.0	3.6 ± .4
Post-acid-challenge	10	39.9 ± 4.4	4.6 ± .5
Day 5:			
Regular-energy/low salt diet	15	35.6 ± 2.8	4.4 ± .3
Low-energy/low-salt diet	17	31.9 ± 2.1	3.7 ± .2
Regular-energy/regular-salt diet	14	33.9 ± 1.5	4.5 ± .2
Low-energy/regular-salt diet	16	39.8 ± 2.5	4.1 ± .2
Starved	19	31.7 ± 2.1	3.6 ± .1
Day 11:			
Regular-energy/low salt diet	10	34.9 ± 2.5	4.7 ± .3[a]
Low-energy/low-salt diet	9	35.7 ± 3.2	3.7 ± .2[a,b]
Regular-energy/regular-salt diet	10	29.3 ± 2.4	3.8 ± .3[a,b]
Low-energy/regular-salt diet	10	28.4 ± 2.9	3.5 ± .2[b]
Starved	10	25.4 ± 1.8	3.6 ± .2[a,b]
Day 20:			
Regular-energy/low salt diet	7	29.8 ± 1.8	3.9 ± .2
Low-energy/low-salt diet	8	30.0 ± 3.5	3.1 ± .4
Regular-energy/regular-salt diet	10	33.0 ± 2.2	4.0 ± .4
Low-energy/regular-salt diet	11	28.4 ± 1.9	3.4 ± .2
Starved	11	34.4 ± 2.6	3.3 ± .1
Day 28:			
Regular-energy/low salt diet	12	43.0 ± 2.3	5.8 ± .4[a]
Low-energy/low-salt diet	18	35.7 ± 1.8	5.0 ± .4[a,b]
Regular-energy/regular-salt diet	20	36.7 ± 1.7	5.1 ± .2[a,b]
Low-energy/regular-salt diet	17	38.2 ± 1.5	4.2 ± .2[b]
Starved	17	34.9 ± 2.5	2.8 ± .1[c]

Note. Mean values ± SEM and sample size are shown. Significant differences between treatments occurred on days 11 and 28 only for plasma protein, and at these periods, treatments that share a letter are not significantly different from each other ($P < 0.05$).

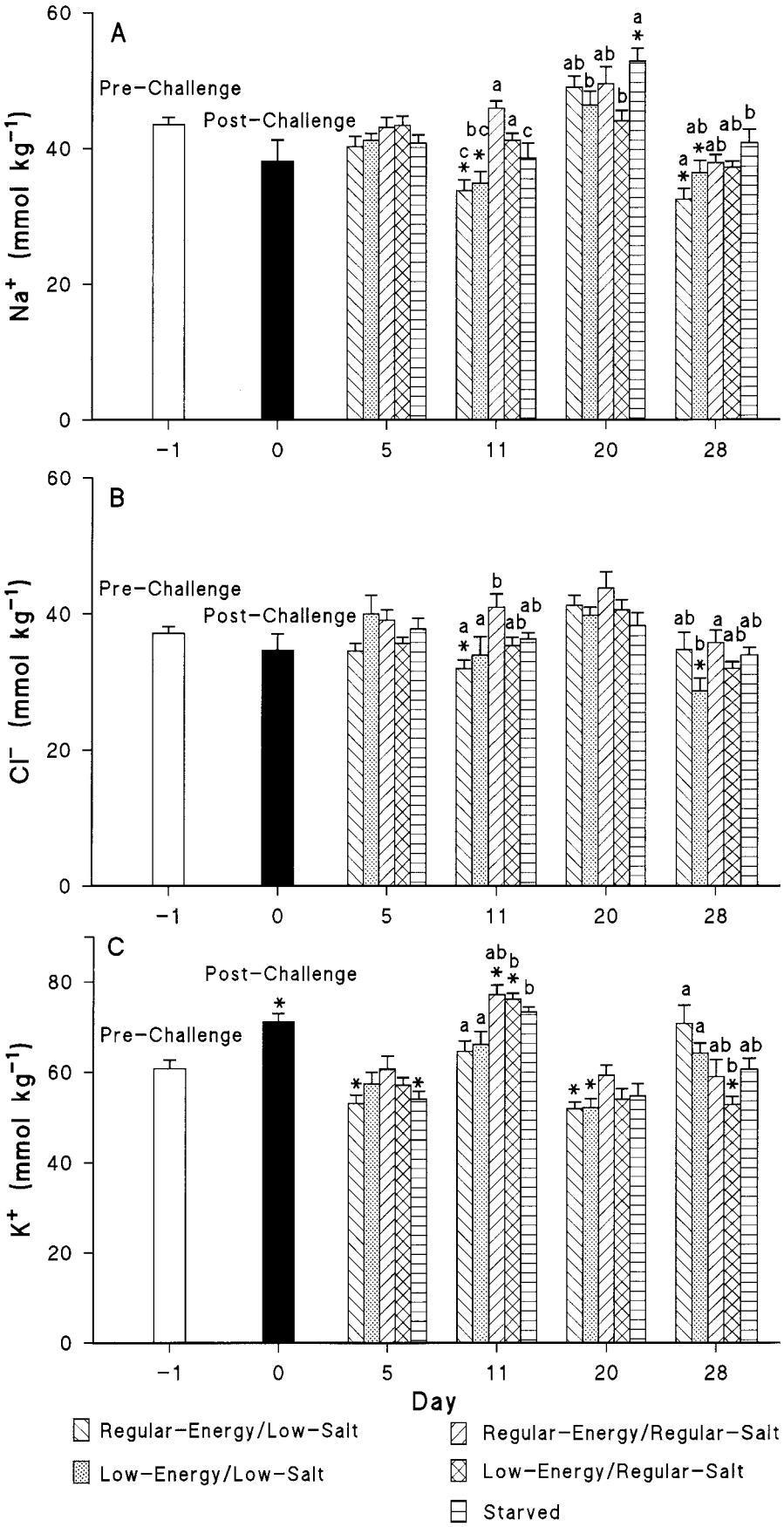
diets, in which M_{O_2} did not change from the preprandial value. Starved fish tended to have the lowest M_{O_2} of all treatments.

Plasma Analysis

Cortisol levels rose significantly immediately following the initial severe acid challenge (pH 4.0), then decreased in all five treatments until day 28, when the fish on regular-energy diets had higher levels, especially the fish fed a regular-energy/low-salt diet (Fig. 4). Cortisol levels remained relatively low in the fish fed the low-energy diets and starved fish.

Plasma [Na^+] showed a significant decrease following the initial severe acid challenge, and this effect persisted at days 5 and 11 in fish on regular-energy/low-salt diets and low-energy/low-salt diets, respectively (Fig. 5). There were no treatment effects at day 20, but after 28 d of exposure to pH 5.2, plasma [Na^+] was lower in fish fed low-salt diets than in those on regular-salt diets, and slightly below those in starved fish. Plasma [Na^+] was lowest in trout on the regular-energy/low-salt diet. Thus, fish fed the regular-salt diets recovered from the acid stress, while fish fed the low-salt diets had significantly lower [Na^+] than at day −1.

There were no significant differences among treatments for hct, though it did increase by day 28 from the pre-acid-challenge values measured on day −1 (Table 2). Plasma protein concentration (initially 3.6 g [100 mL]$^{-1}$) changed during the exposure (Table 2), ending with lower levels in starved fish (2.8 g [100 mL]$^{-1}$ at day 28) compared with fish fed the regular-energy/low-salt diets (5.8 g [100 mL]$^{-1}$ at day 28). The other three groups exhibited intermediate levels of plasma protein at this time.



Whole-Body Ions and Proximate Composition

Whole-body [Na^+] and [Cl^-] did not change significantly immediately after the acid challenge, in contrast to whole-body [K^+], which increased (Fig. 6), and plasma [Na^+], which fell (Fig. 5). By day 11 of the exposure, fish fed low-salt diets exhibited significantly reduced levels of whole-body [Na^+] relative to the preexposure value (Fig. 6). For all three electrolytes, levels in the trout on low-salt diets were generally lower than for fish on regular-salt diets (Fig. 6). These differences were not evident for whole-body [Na^+] on day 20, but whole-body [Na^+] was again lower in the fish on low-salt diets at day 28, especially in the fish fed the regular-energy/low-salt diets, while the starved fish exhibited the highest whole-body [Na^+] at this time. Whole-body [K^+] also showed the same trend in these treatments on days 11 and 20 but not on day 28. Whole-body [Cl^-] exhibited generally similar trends to whole-body [Na^+], with lower levels in the low-salt diets. Whole-body [Ca^{2+}] showed no treatment effects during the exposure (data not shown). The overall mean whole-body [Ca^{2+}] was 152.7 ± 2.3 mmol kg^{-1} ($N = 236$).

Whole-body protein concentration did not change in any of the treatments over the 28 d, with levels staying between approximately 8.6% and 10% (Fig. 7). However, lipids and carbohydrates both showed variations among the treatments. On day 28, lipid levels were lower in the two low-energy treatments and the starvation treatment (6.4%–7.0%) compared with fish fed the regular-energy diets (8.7%–8.9%), and these differences were significant relative to the preexposure lipid content. Water content was inversely proportional to lipid levels; thus, starved fish had the highest percentage of water, and the regular-energy/regular-salt-fed fish had the lowest, but none of the differences were significant. Stored carbohydrates (glucose, glycogen, and lactate), which made up a much smaller percentage of the proximate composition (0.2%–0.6%) than either protein or lipid, showed no definite pattern. Highest levels were seen in fish fed the low-energy/low-salt diet and the regular-energy/regular-salt diets.

Discussion

Time-Dependent Effects of Low-pH Exposure

Most of the acute effects of the acid challenge had attenuated or disappeared by day 20 of chronic low-pH exposure in all groups. However, effects clearly became more severe again between days 21 and 28, as indicated by the increased mortality rate, the generally higher plasma cortisol concentrations, and the lower levels of plasma Na^+ and whole-body ions in some groups. There were no changes in water pH or ionic composition at this time, so two explanations, which are by no means mutually exclusive, seem possible. The first is that the physiological resistance of the fish generally deteriorated as the duration of chronic acid exposure became greater. The second is that the gradual decline in water temperature (18°–13.5°C over 28 d, with the final 1.2°C occurring in the last 7 d) exacerbated the deleterious effects of low pH from day 21 onward. Neither of these explanations finds direct support in the literature. In previous chronic low-pH studies that were severe enough to cause ionoregulatory disturbance in rainbow trout, most parameters had stabilized but had not returned to normal by this time (e.g., Audet et al. 1988; Wilson and Wood 1992; Audet and Wood 1993; Wilson et al. 1994). Furthermore, lower temperature in this range is generally considered to be modestly protective, at least against acute acid stress (Reid et al. 1996). However, no previous studies have examined the possible interactive effects of slowly declining temperature and prolonged low-pH exposure.

Whatever the explanation for these time-dependent alterations, the results at day 28 suggest that fish fed regular-salt diets were able to restore and maintain normal electrolyte status during chronic acid exposure. In contrast, those fed low-salt diets suffered deleterious effects, regardless of the energy content of the food. These results indicate that it is the salt rather than the energy in food that is responsible for preventing and/or correcting ionoregulatory disturbances. This result also indirectly supports the hypothesis put forth in previous studies by Dockray et al. (1996) and by D'Cruz et al. (1998) that increased voluntary food consumption seen in trout chronically exposed to sublethal low pH is associated with the requirement for increased dietary salt intake. Fish can apparently use dietary salt to replace branchial ion losses during chronic acid exposure, a prime example of how the quality and quantity of a diet can modify the toxicity of a pollutant (Lanno et al. 1989; Cho and Kaushik 1990). In addition, this study shows that on a fixed ration, a balanced ratio of energy and salt are needed to protect against the deleterious effects of acid exposure.

Dietary Salts and Acid Exposure

Calculations based on food ion concentrations (Table 1), feeding ration (0.6% body weight d^{-1}), and whole-body ion pools (Fig. 6) found that fish on low-salt diets consumed about 0.6%–1.1% (258–468 μmol Na^+ kg^{-1}) of their Na^+ pool per day, an amount that was insufficient to maintain internal Na^+ levels by day 28. Fish on the regular-salt diets consumed about 3.4%–3.9% (1,462–1,694 μmol Na^+ kg^{-1}) of their Na^+ pool

Figure 6. Whole-body ion concentrations (mmol kg^{-1}; means ± SEM, $N = 10–20$). *A*, [Na^+]; *B*, [Cl^-]; and *C*, [K^+]. An asterisk indicates significant differences ($P < 0.05$) between concentrations before and after the acid challenge. Significant differences ($P < 0.05$) among treatments at one sampling period are indicated by treatment groups that do not share a common letter. If no letters are present for the sampling period, there were no significant differences between means.

per day, which was sufficient to correct for any ionoregulatory imbalances. This is substantially lower than the 10% of the Na^+ pool per day that trout voluntarily consume when they are allowed to feed to satiation under chronic acid stress (Dockray et al. 1996).

Na^+ net flux measurements have been made on trout maintained on a limited dietary regime (similar to that of the present study) of commercial fish food (Zeigler Diet; Table 1) and chronically exposed to pH 5.2; such fish lose Na^+ at a net rate of about 50 μmol kg^{-1} h^{-1} (L. M. D'Cruz, I. J. Morgan, and C. M. Wood, unpublished data). Thus, the present fish were probably losing approximately 1,200 μmol kg^{-1} day^{-1}, which was substantially higher than dietary Na^+ intake from the low-salt diets, explaining the decrease in whole-body [Na^+] in fish on the latter regime. In comparison, fish fed the regular-salt diets were taking in a small excess of Na^+.

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

Dietary Energy Sources and Acid Exposure

In the present study, when fish were fed regular-energy diets, there was a marked increasing trend (32%–47% elevations) in M_{O_2} following feeding, while fish maintained on low-energy diets generally showed no postprandial elevation in M_{O_2}. This increase due to feeding is attributed to specific dynamic action (SDA). SDA is the term used to describe the energy expended by fish during digestion, absorption, interconversion, and resynthesis of substrates for retention in tissues, and formation and excretion of metabolic wastes (Beamish 1974; Cho and Kaushik 1990). The main biochemical basis for SDA appears to be the energy expended for interconversion and synthesis (Brown and Cameron 1991) and not the mechanical work. For example, the same increase in M_{O_2} occurs if amino acids are delivered intravenously rather than ingested (reviewed in Cho et al. 1982; Brown and Cameron 1991), and sham feeding does not increase resting metabolic rate (Smith et al. 1978*a*, 1978*b*). In the present study, feeding with low-energy diets high in cellulose similarly caused no SDA effect.

Oxygen uptake and ion losses are thought to be linked through permeability and surface area changes at the gills. Indeed, the ability to balance the two processes, respiration and ionoregulation, has been coined the "osmorespiratory compromise" (Randall et al. 1972). For example, Wood and Randall (1973) and Gonzalez and McDonald (1992) showed that increases in M_{O_2} in exercising freshwater fish result in increased ion loss across the gills, with some ability of the fish to limit ion losses during extended exercise. We hypothesize therefore that an increase in oxygen transfer for SDA will likely be associated with an increase in net ion losses, though the quantitative relationships between these two factors may not be the same as during exercise. Furthermore, we suggest that fish fed the regular-energy diets may have suffered greater branchial ion losses due to the SDA response evoked by the regular-energy diets. This, of course, would be detrimental in fish fed the regular-energy/low-salt diets, where the loss of ions through the gills associated with the SDA effect would not be replaced from the diet.

Starvation and Acid Exposure

In the present study, starved fish exhibited the lowest M_{O_2} (and, of course, no SDA effect) at day 15. Beamish (1964) and Dickson and Kramer (1971) reported a decrease in the standard metabolism of fish during the first 2 or 3 d of starvation. Similarly, Lauff and Wood (1996) showed a steady decline in M_{O_2} over time during starvation. Food deprivation leads to a reduction in ribosomal number and a fall in protein synthetic capacity. This decrease in protein synthesis capacity is thought to give rise to an overall reduction in protein turnover, and the net result may be a reduction in metabolic activity and conservation of energy reserves (Cowey and Sargent 1979). Protein was also conserved in our starved fish (similar to Stirling [1976]; Lauff and Wood [1996]), and the decreased catabolism of this stored energy reserve may have also aided in maintaining a low M_{O_2}. In turn, this low M_{O_2} probably minimized diffusive loss of ions at the gills and maintained ionoregulatory needs at a minimal level.

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

Indicators of Stress

Several studies have reported prolonged elevation of cortisol during low-pH exposure (Brown et al. 1989; Audet and Wood 1993; D'Cruz et al. 1998). In contrast, other studies have found that the pituitary-interrenal axis in acid-exposed fish was not substantially activated (Brown et al. 1984; Goss and Wood 1988; Balm and Pottinger 1993; D'Cruz et al. 1998). The present study, as well as the differing results reported in two separate experiments by D'Cruz et al. (1998), suggests that dietary factors may explain the apparent dichotomy.

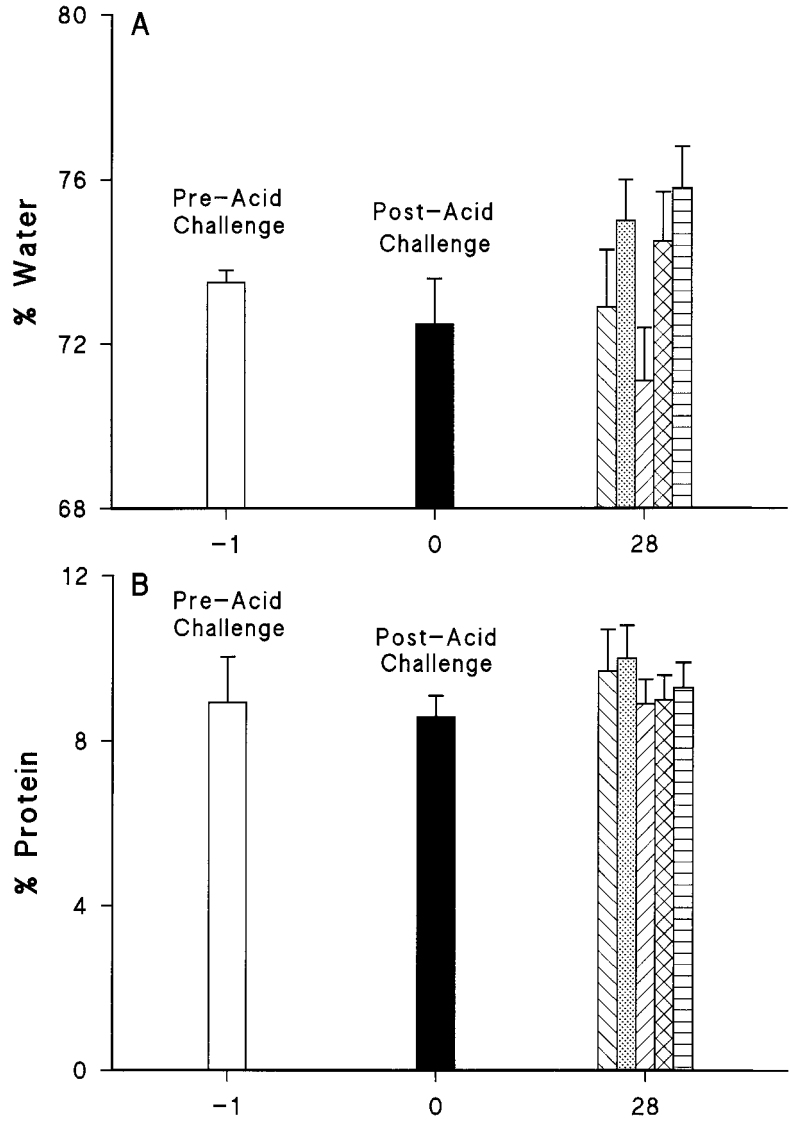


Figure 7. Whole-body composition (% of total wet weight; means ± SEM, $N = 10–20$) for fish before and after the acid challenge and after the 28 d exposure to pH 5.2. *A*, Water; *B*, protein; *C*, lipid; and *D*, carbohydrate concentrations. Significant differences ($P < 0.05$) are indicated among treatment means that do not share a common letter. If no letters are present, there are no significant differences between means.

The mobilization of cortisol appears to coincide with two interrelated factors regarding feeding. First, when fish are fed daily either to satiation (D'Cruz et al. 1998) or a relatively large ration (2% of their body weight daily; Balm and Pottinger 1993), no cortisol elevation occurs, and there are no ionoregulatory disturbances during acid exposure. Additionally, when fish are starved, no cortisol response is evoked (Brown et al. 1984; Goss and Wood 1988); this result is similar to the results reported by Anderson et al. (1991) in non-acid-exposed fish. However, when ration is limited or feeding is periodic (Audet and Wood 1993; D'Cruz et al. 1998), cortisol levels are elevated, and ionoregulatory disturbances occur during sublethal low-pH exposure. Hence, elevated cortisol levels during acid exposure can apparently result from restricted ration, ionoregulatory disturbances, or their interaction. This conclusion concurs with the fact that in the present study cortisol levels were highest in acid-exposed fish on a regular-energy/low-salt diet, that is, a situation where ration was restricted and ionoregulatory disturbances were greatest. One benefit of increased cortisol levels is chloride cell proliferation (Foskett et al. 1983; Laurent and Perry 1990). Moreover, when fish were in a catabolic state, the cortisol response was not as pronounced, with low levels not only in the starved fish but also in the fish fed low-energy diets. This may be due to atrophy of the adrenotrophic tissue (Love 1970) or because food depriva-

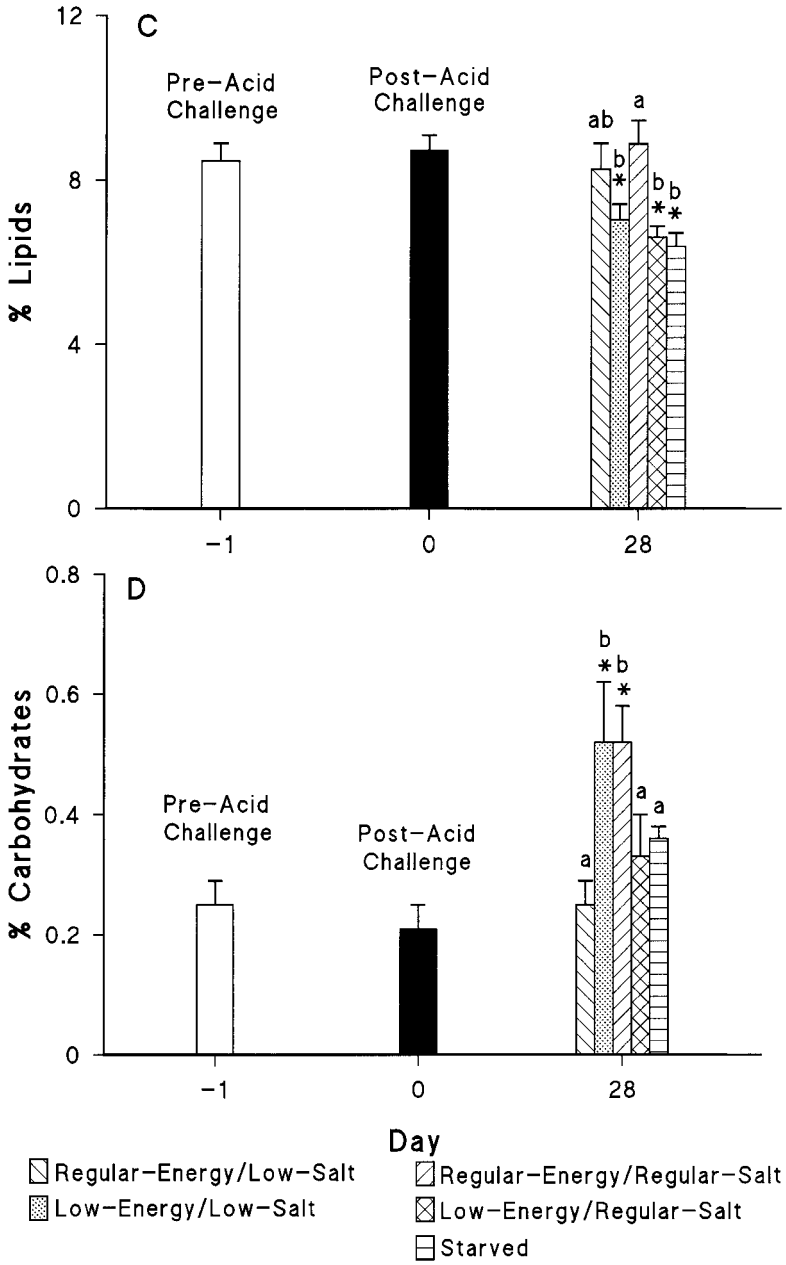


Figure 7 (*Continued*)

tion reduces the normal stress response of fish (Anderson et al. 1991). The stress response may not be elicited during satiation feeding (Balm and Pottinger 1993; D'Cruz et al. 1998) because dietary salts are compensating for branchial loss.

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