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The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure

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Abstract. Acutely lethal (24 h) exposure of adult rainbow trout *(Oncorhynchus mykiss)* to 4.9 μmol copper · l^{-1} in fresh water (pH 7.9, $[Ca^{2+}] \approx 0.8$ mEq · l^{-1}) caused a rapid decline of plasma Na^+ and Cl^- and arterial O_2 tension, and initially a pronounced tachycardia. The internal hypoxia probably resulted from histopathologies observed in the gills of fish exposed to copper, such as cell swelling, thickening and curling of the lamellae, and haematomas. Copper cannot therefore be considered purely as an ionoregulatory toxicant during acutely lethal conditions. Mortality during exposure to copper could not simply be explained by the plasma ionic dilution, nor by the internal hypoxia, since arterial O_2 content remained relatively unchanged. Secondary to the ionoregulatory and respiratory disturbances were a number of deleterious physiological responses which included a massive haemoconcentration (haematocrit values as high as 60%) and a doubling of the mean arterial blood pressure. The time-course of these changes suggest that cardiac failure was the final cause of death. In this respect copper exposure resembles low pH exposure in freshwater trout (Milligan and Wood 1982). Copper and H^+ appear to be similar in both the primary site of their toxic action (the gills) and the secondary physiological consequences which result from acutely lethal exposures. Furthermore, the acute toxicity syndrome observed may be common to many metals which cause ionoregulatory and/or respiratory problems in freshwater fish.

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Abbreviations: C_aO_2, arterial oxygen content; FR, water flow rate; Hb, haemoglobin; Hct, haematocrit; ΔH_m^+, net metabolic acid load; IU, international unit; MABP, mean arterial blood pressure; MCHC, mean corpuscular haemoglobin content; $\dot{M}O_2$, rate of oxygen consumption; P_aCO_2, arterial carbon dioxide tension; P_aO_2, arterial oxygen partial pressure; T_{amm}, total ammonia ($=NH_3+NH_4^+$); TCO_2, total carbon dioxide; TOC, total organic carbon; %Hb-O_2, percentage of haemoglobin saturated with oxygen

Key words: Copper toxicity – Ionoregulation – Gas exchange – Cardiovascular function – Trout, *Oncorhynchus mykiss*

Introduction

The assessment of copper toxicity has been the subject of numerous studies and reviews (e.g. Spear and Pierce 1979; Alabaster and Lloyd 1980; Campbell and Stokes 1985; Flemming and Trevors 1989). Such studies of toxicity and the modulating effects of varying water quality are useful in predicting the toxicity of pollutants in real environmental situations. However, accurate predictions of the toxicity of pollutants, especially when in combination, require a better understanding of the actual mechanisms of toxic action, the physiological changes produced by lethal and sublethal levels of pollutants (Sprague 1971) and how these mechanisms might vary under different environmental conditions.

The present study is one part of a project relating to the potential problems caused by copper and ammonia discharges into the Tees estuary in the North-East of England. Here, euryhaline fish will be exposed to high levels of copper and/or ammonia at varying environmental salinities. This paper deals with the physiological changes elicited by acute exposure to copper alone in freshwater-adapted rainbow trout. The responses found in trout adapted to higher salinities (33% and 100% sea water) are dealt with in a separate paper (Wilson and Taylor 1993).

Previous studies have shown that acute exposure to copper (from 0.23 to 31.3 μmol · l^{-1}) causes a reduction in plasma $[Na^+]$ and $[Cl^-]$ in freshwater fish (McKim et al. 1970; Lewis and Lewis 1971; Christensen et al. 1972; Schreck and Lorz 1978; Stagg and Shuttleworth 1982). Laurén and McDonald (1985, 1986) demonstrated that this was due to the disruption of gill ionoregulatory function. More specifically, they found that copper caused (i) an inhibition of active branchial ion uptake [at

[Cu] > 12.5 μg · l^{-1} (0.2 μmol · l^{-1})], together with (ii) a stimulation of passive branchial ionic effluxes [at [Cu] > 100 μg · l^{-1} (1.57 μmol · l^{-1})]. However, relatively little is known about the effect copper has on other important physiological functions of the gill such as respiratory gas exchange and acid-base balance.

In terms of branchial ionoregulation, the impact of increasing ambient copper levels resembles the response to low pH (increased [H$^+$]) in freshwater fish (e.g. McDonald and Wood 1981; McDonald et al. 1983). Indeed, Laurén and McDonald (1985) proposed that copper and H$^+$ might share a common toxic mechanism at the gills. Research on the mechanisms of acid toxicity have shown that although gill ionoregulation is the primary target for H$^+$, secondary physiological disturbances such as fluid volume changes and circulatory collapse may be the key to mortality in acid-exposed fish (Milligan and Wood 1982; Wood and McDonald 1982; Wood 1989). Although the ionoregulatory effects of copper have been well documented, the secondary physiological consequences of exposure to copper have received relatively little attention. In the present study we have investigated the ionoregulatory, respiratory, acid-base, haematological, and cardiovascular responses, together with a qualitative examination of gill histopathology in rainbow trout exposed to an acutely lethal level of copper (4.9 μmol · l^{-1}, pH = 7.9, moderately soft water). This is 25% lower than the peak levels encountered in the Tees estuary, but would be expected to induce symptoms of acutely lethal copper toxicity in freshwater trout within a 24-h period (Howarth and Sprague 1978) and to cause a severe impairment of branchial ionoregulation (Laurén and McDonald 1985, 1986).

Materials and methods

Animals. Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 300–800 g were obtained from Zeals Fish Farm, Wiltshire, UK. Following transportation to the ICI Environmental Laboratory, Brixham, Devon, fish were maintained in continuously flowing, dechlorinated Brixham tapwater ([Na$^+$] ≈ 0.6, [Cl$^-$] ≈ 0.5, [Ca^{2+}] ≈ 0.8; titratable alkalinity ≈ 0.62 meq · l^{-1}; TOC < 1.66 mg · l^{-1}; pH = 7.0–7.8; T = 10–18 °C). At least 2 weeks prior to experimentation the water temperature was adjusted to 15 ± 0.5 °C. Fish were kept on a maintenance diet (1% body weight per day) of commercial trout pellets but were starved for 4 days prior to surgery to avoid post-prandial changes in metabolic rate and excretion of nitrogenous waste during experiments (Jobling 1981).

Experimental protocol. To enable repeated blood sampling, fish were first anaesthetised with a solution of MS222 (100 mg · l^{-1}, Sigma; buffered to ≈ pH 7.5 with $NaHCO_3$) and then fitted with a chronic indwelling dorsal aortic catheter (Soivio et al. 1972) whilst the gills were irrigated with a lower concentration of oxygenated anaesthetic (60 mg · l^{-1} MS222). Following surgery catheters were filled with Na-heparinised trout saline [Perry et al. (1984); heparin = 50 IU · ml^{-1}] and fish were transferred to individual, darkened Perspex respirometer tubes (40 cm × 10 cm) supplied with aerated water ($P_{O_2} \geq 19.3$ kPa) at 500–1000 ml · min^{-1} and allowed to recover for a minimum of 36 h. When required, a stock solution of $Cu(NO_3)_2 \cdot 7H_2O$ was dosed into mixing cells situated immediately upstream from the respirometers using peristaltic pumps (Watson-Marlow) to produce the desired water concentration of 4.9 μmol copper · l^{-1}. Water pH was maintained at 7.9 by controlled addition of 2 N NaOH using a programmable peristaltic pump (Watson-Marlow, 202U/AA) in conjunction with pH and reference electrodes (Corning) and a pH meter (Kent Industrial).

Thirteen catheterised trout (445 ± 34 g) were exposed to 4.88 ± 0.88 μmol copper · l^{-1} (n = 45) at pH = 7.89 ± 0.01 (n = 33). To act as controls, a further five fish (541 ± 33 g) were subjected to the same blood sampling regime but exposed to zero nominal copper (< 0.05 μmol · l^{-1}). A single pre-exposure blood sample was taken from each fish about 1 h before the exposure began followed by subsequent samples after 1, 5, 16 and 19 h.

Analytical techniques. Arterial blood samples (1.0 ml) were anaerobically drawn into chilled Hamilton gas-tight syringes. A 400-μl subsample was immediately centrifuged for 3 min at 13500 × g (MSE Microcentaur) for later plasma T_{amm} and ion analysis. Another 50-μl subsample was deproteinised in 100 μl ice-cold perchloric acid (0.8 mol · l^{-1}) for 5 min, centrifuged for 5 min at 13000 × g (MSE Microcentaur or Wifug) and the clear supernatant withdrawn and frozen in liquid N_2 until subsequent analysis for lactate. The remainder of the sample was used for the determination of whole-blood P_aO_2, Hct, [Hb] and the pH and TCO_2 of both whole blood and plasma. The blood used for P_aO_2 measurement (≈ 250 μl), was then returned to the animal followed by infusion of enough saline to replace the net blood volume removed (≈ 750 μl). Whole-blood and true plasma pH were measured using a Radiometer G279/G2 glass capillary electrode coupled with a K497 calomel reference electrode. Both electrodes were thermostatted to the experimental temperature (15 °C) and used in conjunction with a pH/blood gas monitor (Radiometer PHM73). TCO_2 and C_aO_2 were measured on 50-μl subsamples by the methods of Cameron (1971) and Tucker (1967) using PCO_2 and PO_2 electrodes (Radiometer E5037 and E5046) connected to the same pH/blood gas monitor as above. For plasma pH and TCO_2 measurements, plasma was taken from blood centrifuged anaerobically in microhaematocrit tubes used for Hct determination (see below). P_aO_2 and water PO_2 were measured using O_2 electrodes (Radiometer E5046) thermostatted to experimental temperature and in conjunction with oxygen meters (Strathkelvin). For each blood sample Hct was determined in duplicate, using 80-μl Na-heparinised microhaematocrit tubes centrifuged at 12000 × g in a haematocrit centrifuge (Hawksley) for 2 min. Tubes were centrifuged anaerobically by covering the upper surface of blood with a drop of liquid paraffin.

Hb concentration was determined in duplicate on 20-μl aliquots of whole blood using the cyanomethaemoglobin method (Sigma kit no 525). Whole-blood lactate concentration was measured enzymatically (L-lactate dehydrogenase/NADH at 340 nm) on 67-μl deproteinised samples using Sigma reagents. T_{amm} was measured in 200-μl subsamples using a specific enzymatic assay (GlDH/NADH; Sigma 170–UV at 340 nm). Hb, lactate, and ammonia assays were performed using a dual-beam spectrophotometer (Uvikon 860 Kontron). Another 40-μl subsample of plasma was immediately diluted 100 times in deionised water and frozen (−20 °C) for later measurement of the plasma cations Na^+, K^+, Ca^{2+} and Mg^{2+} by atomic absorption spectrophotometry (Pye Unicam SP9) and plasma protein (Sigma kit no. P5656). Plasma Cl^- concentration was measured by amperometric titration on 50-μl aliquots of undiluted plasma (Aminco-Cotlove automatic chloride titrator). Water total dissolved copper levels were analysed by atomic absorption spectrophotometry (Perkin-Elmer 2380 AAS) following acidification of samples with concentrated nitric acid.

$\dot{M}O_2$ was monitored by measuring the difference between the O_2 tension of the water entering and exiting the respirometer. $\dot{M}O_2$ could then be calculated from this difference (ΔPO_2), FR, fish mass and O_2 solubility coefficient [$\alpha_W O_2$ = 2.0101 μmol · l^{-1} · torr^{-1} for fresh water at 15 °C; Boutilier et al. (1984)] using the Fick principle:

$$\dot{M}O_2 = \frac{(\Delta PO_2 \cdot \alpha_W O_2 \cdot FR)}{\text{Body mass}}$$

For each measurement input water was gravity fed past a thermostatted O_2 electrode at a constant and low rate (≈ 1 ml $\cdot$ min^{-1}) so as not to significantly alter the flow rate to the respirometer. The average PO_2 value was noted over a 4-min period. This was then repeated for the output water, and a value for ΔPO_2 obtained. O_2 consumption was monitored over a 30-min period at each sampling time (three separate readings of input and output water) to limit confusion by instantaneous changes in $\dot{M}O_2$, and an average value calculated for that sampling time.

Connecting dorsal aortic catheters to a blood pressure transducer (Druck PCDR 75/2) and recording the output on a curvilinear pen recorder (Washington) allowed measurement of arterial blood pressure and heart rate. Heart rates were counted manually from the pressure pulse recordings, and MABP was calculated according to Burton (1972):

$$\text{MABP} = \frac{(1\ \text{systolic} + 2\ \text{diastolic})}{3}$$

To assess the effects of copper on gill morphology, ten uncatheterised trout were divided into two groups of five and transferred to 600-l static tanks containing either 4.9 μmol $\cdot$ l^{-1} or zero nominal copper (controls) at pH 7.9 (pH manually adjusted using NaOH). Mortality was lower in this uncatheterised group exposed to copper (one out of five). After 19 h of exposure fish were killed by a blow to the head. The second and third gills arches (from the left hand side only) were rapidly excised and immediately placed in ice-cold fixative for 2 h (2.5% glutaraldehyde, 2% formaldehyde in 0.1 mol $\cdot$ l^{-1} sodium cacodylate buffer at pH 7.4). Gills were then post-fixed for 1 h in 1% OsO_4 in cacodylate buffer. For light microscopy gill filaments were dehydrated in graded ethanol solutions (70–90–100% ethanol) and embedded in "LR White" resin (London Resin Company). Saggital sections (1 μm) of filaments were cut using an ultramicrotome (Reichert OM4), stained with 1% toluidine blue and examined and photographed using a Leitz Ortholux II microscope with an Orthomat-W automatic camera system. For scanning electron microscopy analysis some of the post-fixed gill filaments were dehydrated in graded acetone solutions (50–70–90–100% dried acetone) and then critical point dried (Polaron E3000). Individual filaments were sputter-coated with platinum (Emscope SC500) and examined and photographed using a Jeol 1200 EX or Hitachi S2300 electron microscope.

Calculation of derived variables. Blood PCO_2 and plasma bicarbonate values were calculated from measurements of plasma TCO_2 and whole blood pH, using a rearrangement of the Henderson-Hasselbalch equation and values for CO_2 solubility and pK′ derived from Boutilier et al. (1984). The net metabolic acid load (ΔH_m^+) was calculated according to the formula of McDonald et al. (1980) using non-bicarbonate buffer values estimated from the blood [Hb] and the regression relationship of Wood et al. (1982). The MCHC (mmol $\cdot$ l red cells^{-1}) was calculated as the ratio of [Hb] (mmol $\cdot$ l whole blood^{-1}) to Hct as a decimal $\left(\text{MCHC} = \frac{[\text{Hb}]}{\text{Hct}}\right)$. %Hb-$O_2$ was calculated from the C_aO_2 and [Hb] (i.e. C_aO_2 max) after correcting for dissolved O_2:

$$\%\text{Hb-O}_2 = \frac{\{C_aO_2 - (P_aO_2 \cdot \alpha O_2)\}}{[\text{Hb}]}$$

where αO_2 represents the tabulated value for the O_2 solubility of human plasma at 15 °C (Boutilier et al. 1984).

Statistics. Values are expressed as mean ± one standard error (n) throughout the text. Significant differences ($P < 0.05$) within each group were tested with a paired Student's two-tailed t-test. Comparisons between the control and copper-exposed groups at each sample time were tested using an unpaired Student's t-test ($P < 0.05$).

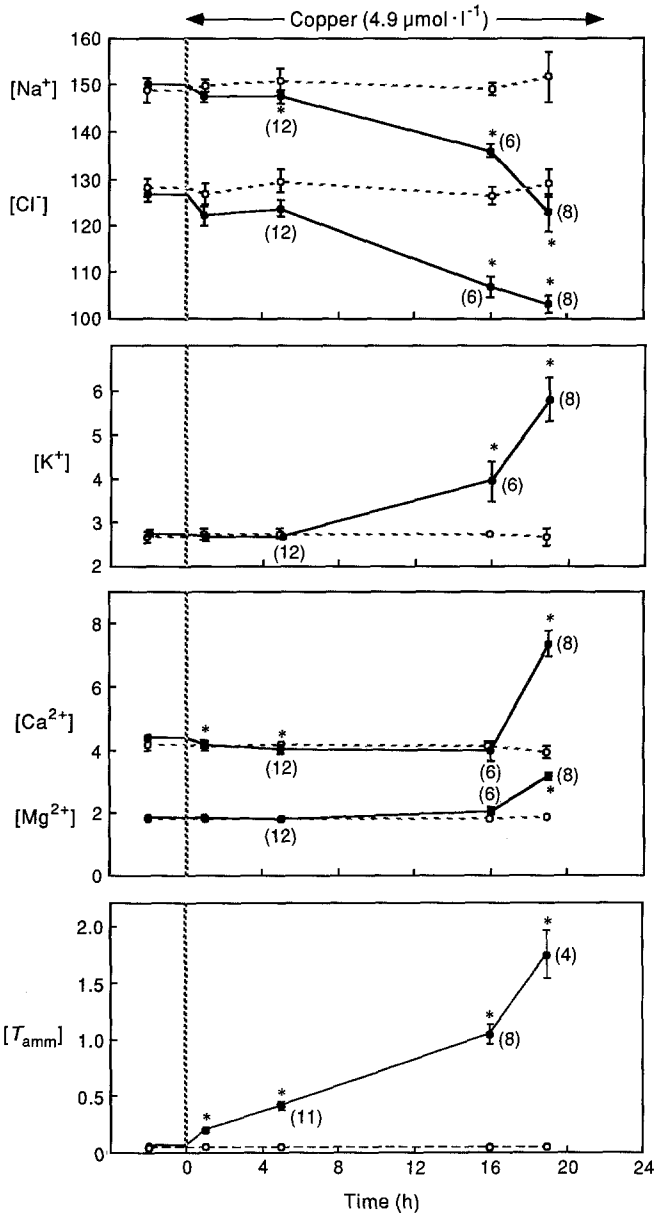

Fig. 1. Changes in plasma ion and total ammonia concentrations (meq $\cdot$ l^{-1}) in the arterial blood of control rainbow trout (*open symbols* –○–), and in trout exposed to 4.9 μmol copper $\cdot$ l^{-1} (*solid symbols*, –●–). Points represent mean values (±1 SEM) during the pre-exposure period and following 1, 5, 16, and 19 h of exposure at pH 7.9. For copper-exposed trout $n = 13$ except where indicated in parentheses. For control fish $n = 5$ at all points.* denotes mean values significantly different from their respective pre-exposure means ($P < 0.05$; Student's paired t-test)

Results

Mortality

Mortality occurred within 24 h in all 13 catheterised rainbow trout exposed to copper (mean time to death = 18.3 ± 0.9 h). No mortalities occurred in the 5 control trout. In the control group Hct, [Hb] and C_aO_2 all declined with time as expected due to the progressive removal of red cells by blood sampling. There were

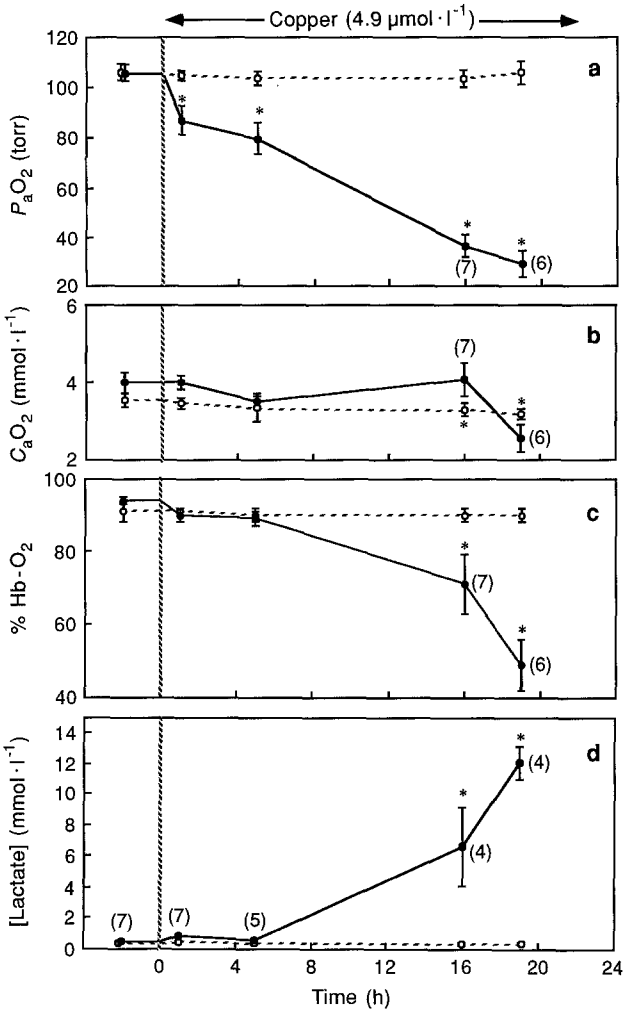

Fig. 2. Changes in (**a**) oxygen tension (P_aO_2), (**b**) oxygen content (C_aO_2), (**c**) percentage saturation of haemoglobin and (**d**) lactate concentration in the arterial blood of control rainbow trout (*open symbols*, $n=5$) and trout exposed to 4.9 μmol copper · l^{-1} (*solid symbols*) at pH 7.9. For copper-exposed trout $n=13$ except where indicated in parentheses. For control fish $n=5$ at all points. * denotes mean values significantly different from their respective pre-exposure means ($P<0.05$; Student's paired *t*-test)

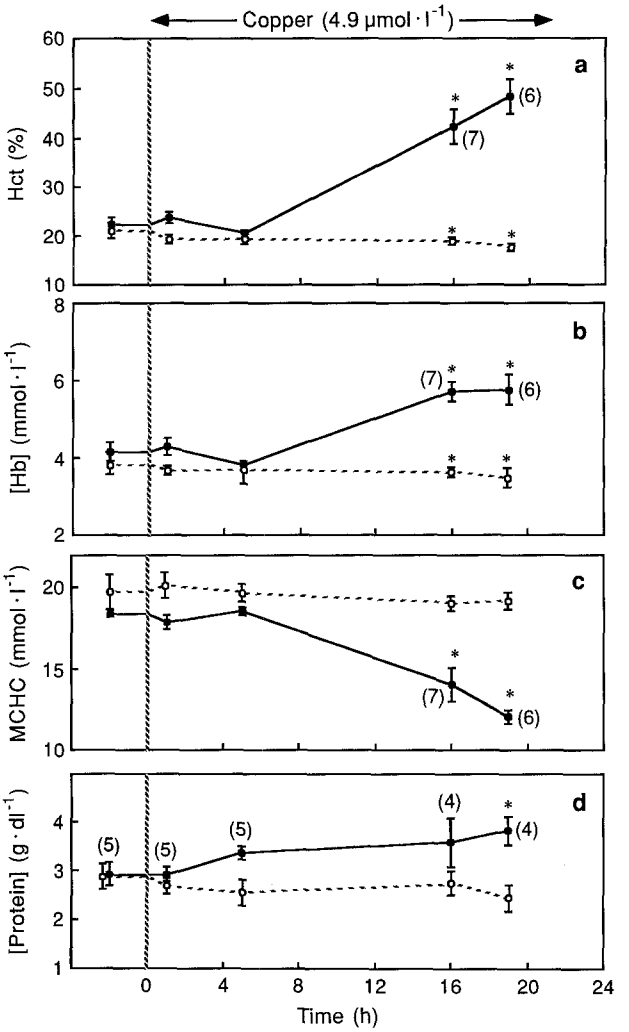

Fig. 3. Changes in (**a**) arterial blood haematocrit (**b**), haemoglobin concentration, (**c**) mean corpuscular haemoglobin content, and (**d**) plasma protein concentration in control rainbow trout (*open symbols*, $n=5$) and trout exposed to 4.9 μmol copper · l^{-1} at pH 7.9 (*solid symbols*). For copper-exposed trout $n=13$ except where indicated in parentheses. For control fish $n=5$ at all points. * denotes mean values significantly different from their respective pre-exposure means ($P<0.05$; Student's paired *t*-test)

otherwise no significant changes in any of the blood variables measured in the control group.

Ionoregulatory responses

Plasma [Na^+] and [Cl^-] declined in an approximately linear fashion throughout exposure to copper (Fig. 1). By 19 h [Na^+] and [Cl^-] had fallen by 16 and 24 meq · l^{-1}, respectively. The reduction in plasma [Cl^-] was therefore 50% greater than the corresponding fall in plasma [Na^+]. Plasma [K^+] was initially unaffected but increased exponentially during the last few hours before death reaching 110% of the initial level after 19 h (Fig. 1). Similar patterns were seen in plasma [Ca^{2+}] and [Mg^{2+}] (Fig. 1) with only minor changes observed initially but both increasing dramatically after 19 h (by 67% and 72%, respectively). Plasma T_{amm} increased linearly with time during copper exposure, to a value almost 28-fold greater than the pre-exposure value after 16 h (Fig. 1).

Respiratory responses

Copper exposure caused large and rapid decreases in arterial PO_2 (Fig. 2). The reduction in P_aO_2 was most rapid during the first hour but subsequently fell linearly with time, reaching 29±6 torr after 19 h. The amount of Hb-bound O_2 (expressed as %Hb-O_2 saturation) also declined throughout the copper exposure (Fig. 2c). However, a comparable decrease in C_aO_2 was not observed (Fig. 2b). C_aO_2 never fell significantly below the pre-exposure value ($P=0.124$ after 19 h) and was never statistically different from the corresponding value in the control group (Student's unpaired *t*-test).

$\dot{M}O_2$ was elevated by 29–53% throughout most of the copper exposure (Fig. 6) but decreased below the pre-

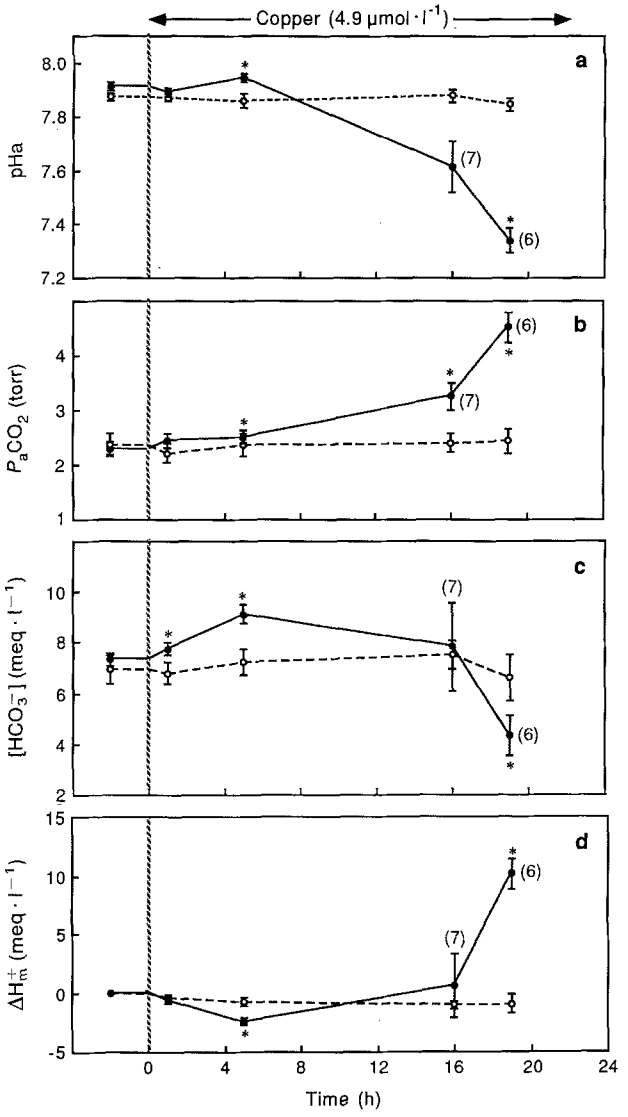

Fig. 4a–d. Arterial blood acid-base variables in control rainbow trout (*open symbols, n=5*) and trout exposed to 4.9 μmol copper · l^{-1} (*solid symbols*) at pH 7.9: **a** extracellular pH; **b** P_aCO_2; **c** $[HCO_3^-]$; **d** metabolic acid load (ΔH_m^+). For copper-exposed trout $n=13$ except where indicated in parentheses. * denotes mean values significantly different from their respective pre-exposure means ($P<0.05$; Student's paired *t*-test)

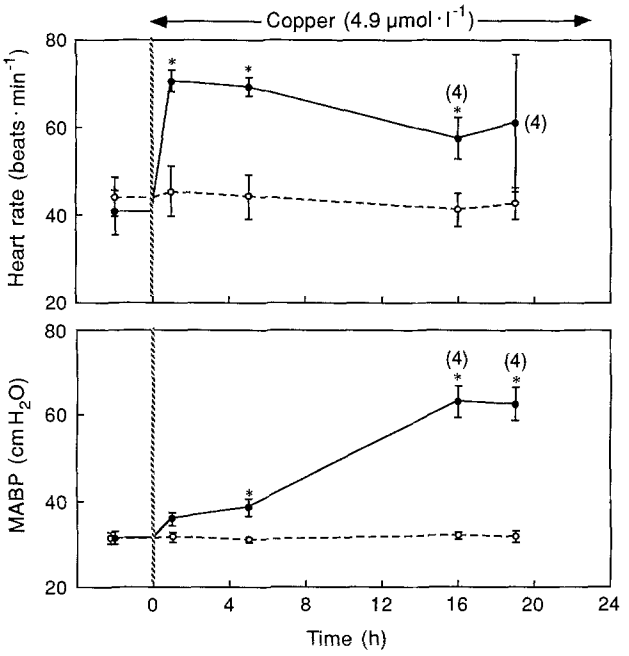

Fig. 5. Heart rate and mean arterial blood pressure in control rainbow trout (*open symbols, n=5*) and trout exposed to 4.9 μmol copper · l^{-1} (*solid symbols; n=6* except where indicated in parentheses). * denotes mean values significantly different from their respective pre-exposure means ($P<0.05$; Student's paired *t*-test)

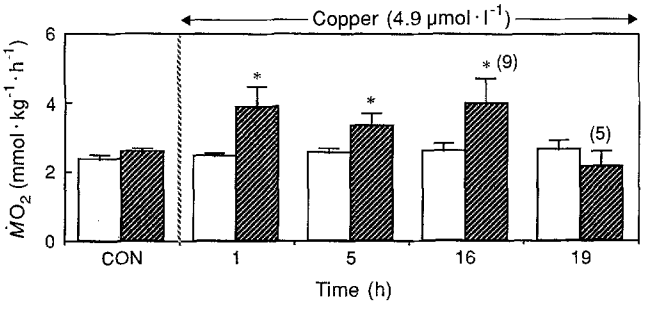

Fig. 6. Oxygen consumption in catheterised rainbow trout exposed to 4.9 μmol copper · l^{-1} (*hatched bars*) or zero nominal copper (*open bars;* control group, $n=5$). For copper-exposed trout $n=13$ except where indicated in parentheses. * denotes mean values significantly different from their respective pre-exposure means ($P<0.05$; Student's paired *t*-test)

exposure $\dot{M}O_2$ during the very last stages before death (not significant). $\dot{M}O_2$ was not affected by either time or sampling in the control group (Fig. 6).

Haematological and plasma protein changes

A pronounced haemoconcentration was observed in the copper-exposed trout (Fig. 3). Hct increased from 22.4±1.4% ($n=13$) to 42.1±3.5% ($n=7$) after 16 h ($P=0.005$), and 48.2±3.5% ($n=6$) after 19 h ($P=0.001$). This doubling of the packed cell volume of blood was associated with a 38% increase in whole blood [Hb], a 35% decrease in MCHC, and a 30% increase in plasma protein concentration after 19 h (see Fig. 3).

Acid-base disturbances

A slight but significant increase in extracellular pH was observed after 5 h exposure to copper ($P=0.030$; Fig. 4). Arterial pH subsequently declined and a severe extracellular acidosis had developed by 19 h [$pH_a=7.341\pm0.044$ ($n=6$) versus 7.913±0.015 ($n=13$) pre-exposure value; $P=0.001$]. The small initial alkalosis was non-respiratory (P_aCO_2 had actually increased slightly after 5 h; $P=0.012$) and was associated with an increase of 2 meq · l^{-1} in plasma $[HCO_3^-]$ over the initial 5 h of exposure ($P<0.001$. The fall in pH_a and $[HCO_3^-]$ after 19 h was concurrent with a massive build up of lactate and metabolic acid (ΔH_m^+) in the blood (Figs. 2d, 4d). The terminal acidosis was therefore primarily of metabolic

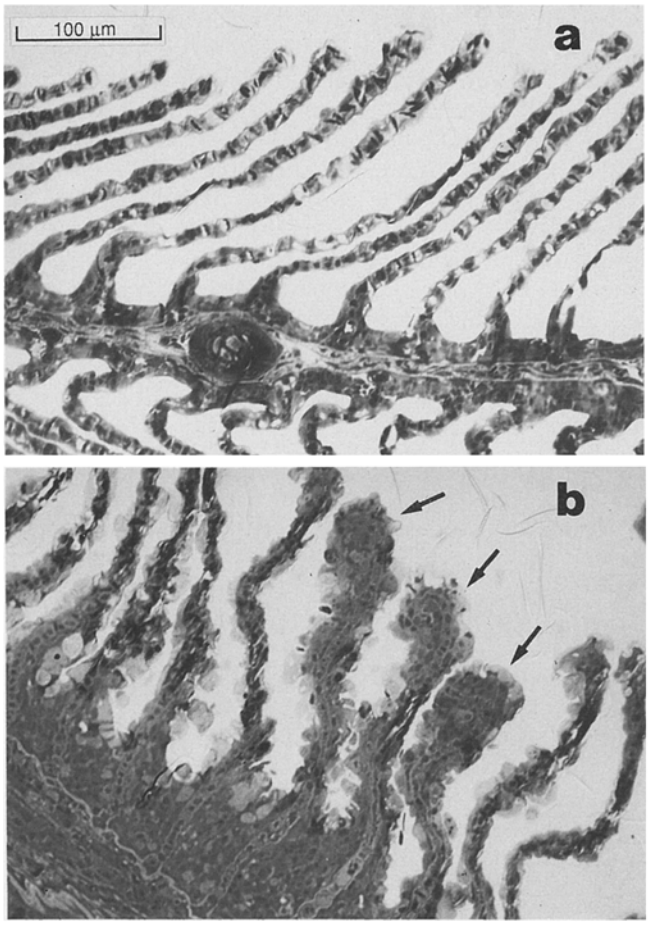

Fig. 7. Representative light micrographs of gills from (**a**) control fish and (**b**) trout exposed to 4.9 µmol copper · l^{-1}. *Bar* represents 100 µm. In the control fish lamellae are thin and uniform with a minimal blood-water diffusion distance. Cells on the surface of lamellae appear swollen in the copper-exposed trout (**b**) causing a thickening of the respiratory epithelium and hence an increase in the blood-water diffusion distance. *Arrows* indicate lamellae where curling has occured leading to a further reduction in the effective surface area for gas exchange

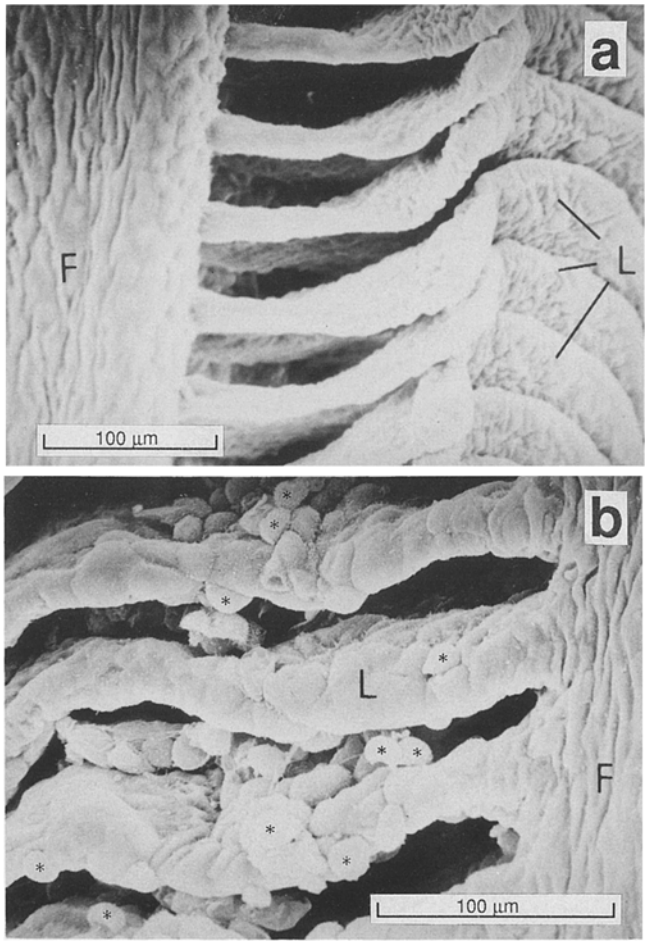

Fig. 8. Representative scanning electron micrographs of gills from (**a**) control fish and (**b**) trout exposed to 4.9 µmol copper · l^{-1}. *Bars* indicate 100 µm. *Asterisks* on micrograph (**b**) mark respiratory pavement cells (as indicated by their microridged surface under higher magnification) which have become almost completely detached from the surrounding lamellar epithelium in copper-exposed trout. *L* = lamellae, *F* = filament

origin, despite the fact that P_aCO_2 steadily increased throughout the exposure in a reciprocal fashion to the fall in P_aO_2. The large increase in P_aCO_2 between 16 and 19 h was associated with the lactacidosis causing a leftward shift in the equilibrium $CO_2 \leftrightarrow HCO_3^- + H^+$

Cardiovascular responses

Copper exposure resulted in a rapid 74% elevation in heart rate within the first hour (Fig. 5). Mean heart rate remained highly elevated but declined slightly as the exposure continued. After 19 h the mean heart rate (61.0 ± 15.8 beats · min^{-1}) was still 50% faster than the control level but no longer significant due to the large range of values (30–82 beats · min^{-1}). In contrast, mean arterial blood pressure increased gradually, reaching a maximum value of 63.2 ± 3.8 cm H_2O after 16 h (a 100% increase over the pre-exposure mean). This doubling of blood pressure was maintained over the last few hours of copper exposure even when, in some fish, heart rate fell below the control level. No significant changes in either heart rate or mean arterial blood pressure were observed in the control group (Fig. 5).

Gill histopathology

When compared with gills of control trout, a number of distinct histopathologies were observed in trout exposed to copper when examined using light and scanning electron microscopy (Figs. 7, 8). Lamellae were thickened, lamellar epithelial cells were swollen and curling of individual lamallae was common (Fig. 7b). Under scanning electron microscopy swollen epithelial cells were frequently observed protruding from the normally smooth surface of the lamellar epithelium (Fig. 8b). In some cases these cells appeared almost completely detached (see

asterisks on Fig. 8b). Haematomas (not shown) were also a common feature in gills of fish exposed to copper (rounded, blood-filled lamellae where the pillar cell structure had disintegrated).

Discussion

Recent studies on the physiological effects of copper in fish have concentrated on its interference with branchial ionoregulation (Laurén and McDonald 1985, 1986, 1987; Reid and McDonald 1988; Reader et al. 1989; Sayer et al. 1991b). However, it is clear from the present study that during acute toxicity mortality cannot be solely attributed to ionoregulatory failure. We have demonstrated that exposure to 4.9 μmol copper $\cdot l^{-1}$ in moderately soft water at pH 7.9 causes both ionoregulatory *and* respiratory toxicity in rainbow trout, with death probably resulting from the severe haematological and cardiovascular disturbances which develop secondary to the deleterious action of copper at the gills.

Ionoregulatory toxicity

The depression of plasma $[Na^+]$ and $[Cl^-]$ (11 and 19%, resprectively) would almost certainly be insufficient to cause death in itself as freshwater salmonids are known to tolerate up to 30% reductions in plasma electrolytes during longer exposures to lower copper levels (Laurén and McDonald 1985), acid pH (Wood 1989) or during migrations from sea water to fresh water (Miles 1971). However, it has been pointed out that in acid-exposed trout mortality is not simply a function of the magnitude of the ion losses incurred, but is more dependent on the *rapidity* of these ion losses (Wood 1989).

The elevation of plasma $[K^+]$ during copper-exposure was presumably the result of an efflux from the intracellular compartment. K^+ is the dominant intracellular cation and the plasma ionic dilution would favour efflux of K^+ into the extracellular fluid (Lee et al. 1983; Stuart and Morris 1985). Increased $[K^+]$ is therefore only secondary to changes in plasma $[Na^+]$ and $[Cl^-]$. In addition, some of the observed hyperkalaemia may be related to the terminal acidosis, as low blood pH induces K^+ release from muscle [in exchange for the entry of H^+; Ladé and Brown (1963)].

The marked increases in $[Ca^{2+}]$ and $[Mg^{2+}]$ can perhaps be explained as a release of skeletal calcium and magnesium salts which would result from the terminal blood acidosis as observed following severe exercise in trout (Ruben and Bennett 1981). Haemoconcentration through fluid volume shifts would add to the concentration of these divalent ions (and K^+) in plasma. However, the haemoconcentration developed gradually during exposure, whereas the increase in plasma $[Ca^{2+}]$ and $[Mg^{2+}]$ only occurred over the last few hours and coincided with the acidic blood pH at this time. From the timing of these events it seems more likely that the blood acidosis was the principal cause of elevated plasma $[Ca^{2+}]$ and $[Mg^{2+}]$. Release of divalent ions from bone may help buffer the blood acidosis by simultaneously liberating phosphate (phosphate is the major calcium salt in vertebrate bone tissue). This would resemble the mobilisation of $CaCO_3$ from the exoskeleton of various crustaceans which is observed during periods of acidosis and acts as a source of HCO_3^- buffer (Taylor and Wheatly 1981; Taylor et al. 1987). However, the potential benefits of an increase in extracellular phosphate buffer may well be outweighed by the disadvantages produced by a concomitant hypercalcaemia. In mammals hypercalcaemic disorders are known to induce many deleterious physiological responses such as bradycardia, cardiac arrhythmia and hypertension (Parfitt and Kleerekoper 1980).

Under normal conditions ammonia excretion across the gills is probably the result of a flexible combination of gaseous NH_3 diffusion and ionic exchange of NH_4^+ for Na^+ (Wright and Wood 1985). Both these avenues for ammonia excretion would be impaired during exposure to copper due to the detrimental effects on branchial gas exchange and active ion uptake. The linear increase in plasma T_{amm} would result from a gradual build up of endogenously produced ammonia as branchial excretion became more impaired with time. Although accumulation of endogenous ammonia could contribute to toxicity, similarly elevated levels of plasma T_{amm} have been observed in seawater trout exposed to high ambient copper and ammonia levels simultaneously, but without significant mortality (R.W. Wilson and E.W. Taylor, unpublished results). The hyperammoniaemia probably contributed little to the overall toxic syndrome in our freshwater trout exposed to copper.

Respiratory and acid-base disturbances

The gill histopathologies provoked by acute exposure to copper (cell swelling, thickening and curling of lamellae, haemattommas etc.) would all tend to increase the blood-water diffusion distance and thus impair branchial gas transfer (Hughes and Perry 1976; Satchell 1984; Mallatt 1985). This was presumably the cause of the internal hypoxia-hypercapnia in copper-exposed fish. Copper is often considered purely as an ionoregulatory toxicant, and the possibility of copper-induced respiratory changes has largely been ignored in studies relating to freshwater fish. However, it seems logical that any disruption of *trans*epithelial ion transport at the gill will simultaneously inhibit mechanisms involved in the iono- and osmoregulatory homeostasis of the branchial epithelial cells themselves. At sufficiently high concentrations any stressor affecting ion fluxes at the gill might also cause a breakdown in cellular volume control and hence an impairment of the respiratory function of the gills. The pattern of physiological changes observed in this study may therefore be typical of a general acute toxicity syndrome common to many toxic metals rather than one specific to copper.

P_aO_2 was reduced to values lower than normally reported for venous blood in trout [30–40 torr; Holeton and Randall (1967); Kiceniuk and Jones (1977)]. Despite this severe internal hypoxia, the copper-exposed trout

never became truly hypoxaemic. C_aO_2 was maintained within the normal range even during the terminal acidosis which would tend to reduce the Hb-O_2 binding affinity via a Bohr shift. The major contributor to the maintenance of C_aO_2 was a compensatory 38% increase in blood [Hb] and hence O_2 carrying capacity. However, it would appear that the blood O_2 affinity remained relatively unchanged despite the acidosis. The %Hb-O_2 saturation and P_aO_2 values all corresponded well with published in vitro blood O_2 dissociation curves for rainbow trout blood at normal pH [7.8; Tetens and Lykkeboe (1981)]. This probably reflects the adrenergically-mediated regulation of red cell pH which "protects" the intracellular environment from factors which would normally lower the red cell O_2 content (such as hypoxia and extracellular acidosis).

Even though blood O_2 content was not substantially reduced there was a massive accumulation of blood lactate towards the latter stages of exposure indicating that O_2 delivery was insufficient to meet the tissue O_2 demand even though C_aO_2 was maintained. It seems reasonable to assume that insufficient O_2 delivery was therefore due to limitations of blood perfusion rather than simply a reduction in P_aO_2. The net effect was an accumulation of lactate from anaerobic glycolysis and a severe metabolic acidosis. The rapidity of the resultant fall in pH_a makes it unlikely that acidosis was the actual cause of death, but rather a consequence of the other physiological changes leading to an impairment of O_2 delivery to the tissues.

Haematological and fluid volume disturbances

One of the more immediately obvious changes occuring in the blood of fish exposed to copper was a massive increase in Hct (values as high as 60% were observed up to 2 h prior to death). There are three probable causes of this haemoconcentration: (i) red cell swelling, (ii) reduced plasma volume, and (iii) release of erythrocytes from storage organs such as the spleen. All three were probably involved in the present scenario. Firstly, red cell swelling was evident from a 35% decrease in the MCHC. Secondly, plasma protein concentration increased, which is a good indicator of a contracting plasma fluid volume (McDonald et al. 1980). This in turn reflects the osmotic imbalance produced when ion losses from the extracellular compartment occur more rapidly than those from the intracellular compartment (Lee et al. 1983; Stuart and Morris 1985; Wood 1989). Thirdly, an increase in red cell numbers would occur if splenic release were activated by high catecholamine levels (Nilsson and Grove 1974). This also seems likely in view of the internal hypoxia and acidosis observed in the copper-exposed fish, both being associated with elevated catecholamines in trout (Tetens and Christensen 1987; Boutilier et al. 1986; Witters et al. 1991).

Milligan and Wood (1982) showed that haemoconcentration coincides with an increase in blood viscosity. They demonstrated that blood viscosity doubled in acid-exposed rainbow trout when Hct increased from 21.3 to 47.3% and plasma protein concentration increased from 2.27 to 3.46 $g \cdot dl^{-1}$. Assuming a similar relationship between viscosity and these two variables in trout from the present study, a similar increase in blood viscosity would be expected as the corresponding Hct and protein concentration changes were almost identical (Hct from 22.4 to 48.2%, protein concentration from 2.93 to 3.82 $g \cdot dl^{-1}$) during exposure to copper.

Cardiovascular disturbances

The rapid 50% elevation in heart rate after just 1 h of copper exposure, without a simultaneous increase in blood pressure, indicates a reduction of vagal tone rather than an adrenergic response, as the latter would simultaneously stimulate systemic vasoconstriction in rainbow trout (Wood 1976). The pronounced tachycardia may be a reflex response to the increase in ambient copper since it occurred prior to any severe changes in the other physiological variables measured. In contrast, the much slower and more progressive increase in MABP follows the decline in P_aO_2, the increase in Hct and presumably an increase in circulating catecholamines. Elevated blood pressure may be the result of a gradual increase in adrenergically-mediated peripheral vasoconstriction coupled with an increase in blood viscosity as demonstrated for acid-exposed trout (Milligan and Wood 1982). However, in the present study the increase in MABP is considerably greater (60% higher) than that found by Milligan and Wood (1982) in trout following 3 days of acid exposure, although Hct and plasma protein concentration values are almost identical in both studies. Blood viscosity would therefore appear to be a less important contributory factor towards the increase in blood pressure during exposure to copper. An excessive increase in circulating catecholamine levels could well be the key to the extremely high blood pressures in these fish.

The massive and sustained increase in blood pressure (double the control value) must impose an extraordinary load on the heart. Comparable levels of hypertension are normally only encountered transiently during events such as exhaustive exercises (e.g. Kiceniuk and Jones 1977). The fact that heart rate gradually declined throughout the remainder of the exposure to high copper levels, even though increased catecholamine levels may have exerted a cardioacceleratory effect (Wood 1976), implies that the demand on the heart could not be maintained. Indeed, in the cases where cardiovascular variables were being monitored very close to the actual point of death, heart rate and pulse pressure dropped considerably before blood pressure started to fall. We conclude that systemic vasoconstriction was the predominant factor responsible for the sustained high blood pressure and the ensuing circulatory failure.

There are many similarities between the suite of physiological events occurring in rainbow trout during the present acutely lethal copper exposures and the 3-day acid exposures described by Milligan and Wood (1982), the main difference between the present and the latter studies being that respiratory impairment was not in-

volved in the overall toxic syndrome during exposure to pH 4.0–4.5. However, under more extreme acid conditions (pH 3.0–3.5) impaired respiratory function has been observed (Packer 1979; Ultsch and Gros 1979), indicating that the difference mentioned above is probably due to the relative concentrations of copper and H^+ used rather than any specific differences in their mode of action. This further supports the idea that the pattern of physiological events observed with copper-exposed trout is typical of a general acute toxicity syndrome which may be common to many toxic metals. Indeed, similar disturbances have been observed (Witters et al. 1990) or implicated (Sayer et al. 1991a) in salmonids exposed to acid and aluminium where both ionic and respiratory impairments are also prevalent.

Laurén and McDonald (1985) proposed that copper and H^+ ions might share a common toxic mechanism at the gills. Our results further emphasize the similarity between these two stressors by demonstrating that the secondary physiological disturbances are also remarkably similar. What is surprising is the fact that the overall toxic syndrome leading to circulatory collapse appears to be the same whether or not respiratory toxicity is involved. The key to this may well be the ubiquity of the adrenergic response in fish suffering ionoregulatory and/or respiratory stresses. The role of the adrenergic response in the acute toxicity syndrome caused by metals and acid is currently under investigation.

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