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Exposure of brown trout *Salmo trutta* to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation

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Summary

The present study was undertaken to answer two questions relating to the exposure of brown trout *Salmo trutta* to sublethal concentrations of copper and low pH (CLP) for 96 h. (1) What is the effect of these pollutants on the rate of oxygen consumption ($\dot{M}_{O_2}$) at different levels of exercise and (2) why does ammonia accumulate within these fish, when the low external pH should favour the diffusion of NH_3 across the gills? Mean $\dot{M}_{O_2}$ of fish in CLP and control (normal pH and no added copper) conditions were not significantly different from each other at any level of exercise. This suggests that exposure to CLP was not a 'loading' factor at any level of activity. However, both maximum $\dot{M}_{O_2}$ and critical swimming speed (U_{crit}) were significantly lower in the CLP trout (5.5±1.6 mmol O_2 kg^{-1} h^{-1} and 1.12±0.06 *BL* s^{-1}, respectively) than in control fish (18.5±2.3 mmol O_2 kg^{-1} h^{-1} and 2.04±0.11 *BL* s^{-1}, respectively). There was no evidence from cardiovascular variables, such as heart rate and cardiac output, to suggest any changes in the oxygen transport system to compensate for any possible reduction in branchial gas exchange. Thus, it is suggested that oxygen exchange and transport do not limit the swimming performance of CLP trout, but that exposure to CLP reduces the maximum demand for O_2, i.e. it is a limiting factor.

The accumulation of ammonia in the plasma and white muscles during exposure to CLP has already been implicated in reducing the swimming performance of brown trout. Inhibition of cortisol synthesis abolished a large proportion of the increases in both the accumulation and excretion of ammonia that occurred during the second 48 h of the exposure to CLP, but did not inhibit ammonia accumulation completely. It is suggested that CLP not only causes an increase in the rate of production of ammonia, which is enhanced when the level of cortisol starts to increase after 48 h, but that it also inhibits an excretory mechanism (most probably Na^+/NH_4^+ exchange) that is non-obligatory under 'normal' conditions (when passive diffusion is sufficient), but is required in order to respond to unusually high ammonia loads.

Key words: copper, low pH, ammonia, gas exchange, swimming, brown trout, *Salmo trutta.*

Introduction.

Background

The gills of fish are particularly vulnerable to many waterborne pollutants, including heavy metals and low pH. Such pollutants often cause considerable ultrastructural damage, including hyperplasia of epithelial cells, necrosis of chloride cells, swelling and fusion of lamellae and proliferation of mucocytes (Kirk and Lewis, 1993; Wilson and Taylor, 1993; Taylor et al., 1996). In consequence, a major toxic effect of these contaminants is the disruption of the ability of the gill to act effectively in its capacity as an exchange organ either for respiratory gases or ions. At levels of copper that cause mortality within 24 h, rainbow trout *Oncorhynchus mykiss* (Walbaum) experience severe ionoregulatory failure coupled with a progressive systemic hypoxia (Wilson and Taylor, 1993). Perhaps unsurprisingly, these effects have considerable impact upon physiological function. Waiwood and Beamish (1978), for example, showed that levels of copper and low pH that cause 'considerable' mortality to rainbow trout also reduce the swimming performance of the survivors. This, they concluded, was due to the effect of the pollutants upon both oxygen demand and maximum uptake, leading to a greater requirement for oxygen at any given speed ('loading' effect) but a lower maximum rate of uptake ('limiting' effect).

Exposure to sublethal levels of copper and low pH together can also result in gill damage, but to a lesser extent. Brown trout *Salmo trutta* L., exposed for 96 h to 0.08 μmol l^{-1} Cu^{2+} in acidic (pH 5) softwater, were found to have some minor structural deformations of the secondary lamellae, but there was no significant difference in the harmonic mean diffusion distance compared with fish from control conditions (Taylor et al., 1996). While swimming performance of fish exposed to copper and low pH was lower than that of control fish, their rate of oxygen consumption ($\dot{M}_{O_2}$) while routinely active was no different, and there were no differences in the oxygen

content or partial pressure of the arterial blood of exercising fish, which suggests the absence of an underlying diffusional limitation (Beaumont et al., 1995a). A putative role of increased blood viscosity following haematological changes in reducing the supply of oxygen to the tissues is unsupported by the haematological evidence. Plasma sodium, chloride, potassium, haematocrit, haemoglobin and plasma protein concentrations were not affected by CLP exposure, there was a lack of further change in variables such as lactate at the onset of exercise (Beaumont et al., 2000a), and the balance of evidence points away from oxygen delivery being a significant limiting factor during exercise. However, maximum $\dot{M}_{O_2}$ ($\dot{M}_{O_2max}$) was not measured and there were some changes in muscle metabolic status that could be interpreted as evidence of a degree of hypoxic stress (Beaumont et al., 2000a).

A likely alternative is that hyperammonaemia arising from exposure to copper and low pH is the primary cause of the loss of swimming performance (Beaumont et al., 2000a,b; Shingles et al., 2001). Ammonia is a toxic end product of various metabolic processes, in particular the hepatic transdeamination of amino acids (Walton and Cowey, 1982) and, during episodes of stress, its production may be stimulated by an elevation in the circulating levels of catecholamines and cortisol (Wendelaar Bonga, 1997; van Weerd and Komen, 1998). In freshwater fish, most (approximately 90%) excretion of ammonia occurs across the gills, with the remainder being excreted renally or cutaneously (see Wood, 1993). The actual mechanisms of branchial ammonia excretion remain somewhat controversial, however. Possible routes include the passive diffusion of NH_3 or NH_4^+, or an active exchange of NH_4^+ against a counter-ion such as H^+ or Na^+ (Wilson and Taylor, 1992).

It is generally agreed that, in freshwater fishes, transepithelial NH_4^+ diffusion is an unlikely option due to the relative impermeability of the gill epithelium to the ammonium ion, whereas the passive diffusion of NH_3 is normally accepted as an important pathway (e.g. Wood, 1993; Wilkie, 1997). The question of an active excretory mechanism has been a matter of argument, however, not least because of the absence of a method to differentiate between the movement of NH_3 together with a proton and of the NH_4^+ ion alone. Despite numerous attempts to establish the relative importance of the various putative mechanisms for the clearance of ammonia across the gills of fish, the matter is still largely unresolved (e.g. Cameron and Heisler, 1985; Evans and Cameron, 1986; Randall and Wright, 1987). However, recent opinion seems to be that under most conditions, passive diffusion of NH_3 can account for almost all branchial ammonia excretion (e.g. Wilkie, 1997) with a small role perhaps for a non-obligatory Na^+/NH_4^+ exchange (Salama et al., 1999).

The present study

The present study was undertaken to investigate a number of aspects of gas exchange over the gills of brown trout exposed to sublethal copper and low pH. Specifically, the aim was to address two questions. Firstly, what is the effect of these pollutants upon $\dot{M}_{O_2}$ at different levels of exercise? Secondly, why does ammonia accumulate within these fish when the low external pH should favour 'ammonia trapping' (the conversion of the permeable NH_3 to the impermeable NH_4^+ ion), thus maintaining the gradient for the diffusion of this toxic waste product out of the fish as NH_3 (Lin and Randall, 1991)? The rate of oxygen consumption during exercise was measured in a computerised swimming respirometer using fish fitted with Transonic flow-probes in order that cardiovascular changes could also be recorded. Ammonia fluxes and the pattern of ammonia accumulation within trout exposed to copper and low pH were measured, and the role of cortisol investigated by the use of metyrapone, which inhibits cortisol synthesis (Milligan, 1997).

Materials and methods

Animal husbandry

The animal husbandry was identical to that described in our previous studies (Beaumont et al., 1995a,b). Briefly, adult brown trout *Salmo trutta* L. (mass, 300-600 g) were acclimated to the experimental temperature (10±0.2°C) and softwater conditions (approximate composition, in $\mu mol\,l^{-1}$: Ca^{2+}, 50; Na^+, 75; K^+, 5; Mg^{2+}, 40; Cl^-, 100; SO_4^{2-}, 65; NO_3^-, 5) for a minimum of 4 weeks in continuously flowing water.

Rate of oxygen consumption and swimming experiments

Fish were anaesthetised with MS-222 ($100\,mg\,l^{-1}$, buffered to pH 7.5 with $NaHCO_3$). Once ventilation had ceased, the fish were transferred to the operating system where the gills were irrigated continuously with aerated, buffered anaesthetic ($50\,mg\,l^{-1}$). In a total of eight fish, the ventral aorta was exposed by making an incision within the opercular cavity where the vessel could be clearly seen running under the skin. Connective tissue was eased apart and a factory-calibrated flow probe (Transonic Systems Inc, Ithaca, NY, USA) placed around the ventral aorta. The probe was sutured in place and the overlying skin also sutured back into place. To ensure that the wound was sealed and that the probe would not move, a polythene patch was secured with polyacrylamide glue over the wound. The lead from the probe was led from the operculum under the pectoral fin and up to the dorsal fin. It was secured to the fish by further sutures.

The fish was put into clean water and allowed to begin recovery before being placed into the respirometer flume. This is a swimming respirometer developed at the University of Birmingham from an original design provided by Dr John Steffensen (Steffensen et al., 1984). Data acquisition and automatic control of the flume were achieved through a computer with a Lab-PC+ interface card and running software written in the LabWindows/CVI environment (National Instruments). The fish was sealed in a plastic tunnel (maximum cross-sectional area $289\,cm^3$) through which there was a flow of water generated by a propeller and variable speed, d.c. motor. The water flow was adjusted by shaped honeycomb sections both before and after the swim chamber, in order to

minimise turbulent flow at all test velocities. The respirometer had a total volume of 80 litres and was mounted in a larger tank in which the water is cooled to 10±0.2°C and replaced at 3 l min^{-1}. The respirometer can be converted from an open to closed system for measurement of oxygen depletion. Between measurements, water was pumped from the outer tank through the respirometer. Water was sampled by continual gravity feed from the swim chamber and drawn past an oxygen electrode (Strathkelvin Instruments 781 meter and 1302 microcathode electrode) also maintained at 10°C.

After 24 h, automatic oxygen consumption measurements were begun. For each measurement, the water pump was switched off, sealing the respirometer. The pump was restarted before water oxygen saturation in the respirometer fell below 90%. The software calculated the decline by regression analysis and stored the value along with goodness of fit. The software then calculated and stored the rate of oxygen consumption using the appropriate oxygen solubility coefficient (Boutilier et al., 1984) and the fish mass.

After a total of 48 h post-operative recovery, exposure to the test water was begun. For four trout this was 0.08 μmol l^{-1} copper at pH 5 (CLP), and for the remaining four fish, the acclimation water at pH 7 was used, with no added copper (control). At 96 h of exposure, critical swimming speed (U_{crit}; Brett, 1964) was determined using intervals of 0.5 m s^{-1} and time periods of 45 min to ensure that several respirometry measurements could be made at each speed. Data from the blood flow probe were time-stamped and recorded continuously so that they could be analysed in conjunction with those from the respirometer flume.

Ammonia accumulation and flux

A catheter was inserted into the dorsal aorta of 16 fish (Soivio et al., 1972). In six of these, a catheter was also implanted in the ventral aorta. Cannulae were made of PE-50 tubing and, in both cases, the cannulae were inserted through the mouth. The ventral aorta is covered by a layer of cartilage through which a hole was first made using a needle with its tip removed. Cannulae were secured with sutures and polyacrylamide glue. The ventral aorta cannulation had a relatively poor success rate, with some fish losing a significant amount of blood during the operation. Such trout were rejected from the experiment.

Trout were placed in polyacrylamide flux boxes of volume approximately 2.5 litres, constructed following a design provided by Dr Gordon McDonald (McDonald and Rogano, 1986). Pumps circulated water through each flux box and a stream of air bubbles ensured adequate mixing and aeration. The fish were allowed to recover for 48 h prior to the commencement of the 96 h experimental exposure regime. Five trout with only a dorsal aortic catheter, and six with both dorsal and ventral aortic catheters, were exposed to CLP, and five fish, with dorsal aorta catheter only, were left as controls in the acclimation water only. Other than during the flux measurements, there was a continuous flow of water through the flux boxes at a rate of 3 l min^{-1}. Copper was added constantly from a stock solution of $CuCl_2$ to maintain the desired concentration in the experimental water. This was regularly monitored by aniodic stripping voltammetry (Radiometer POL150 polarograph with a hanging-drop mercury electrode and Tracemaster 5 software), which under our experimental conditions, had an experimental detection limit of approximately 0.01 μmol l^{-1}. In the control, artificial softwater, copper concentration was always below this detection limit. The appropriate pH was maintained by titration with either 5% NaOH or 5% H_2SO_4.

Flux measurements were made at 24 h intervals. At the beginning of each measurement period, the pumps were switched off and a 20 ml water sample taken from each box. Three further samples were taken at intervals of 30–60 min. The pH of each sample was measured immediately (Radiometer PHM72 meter with Russell CT757 low-conductivity electrode) and ammonia concentration measured using a micro-modification of the salicylate method (van Verdouw et al., 1978).

Blood samples (0.5–1.0 ml) were taken and haematocrit (Hct) was measured immediately, using a Hawksley micro-haematocrit centrifuge, in order to monitor blood loss. Fish were eliminated from the experiment if Hct fell below 20% of the initial, 'normal' values (see Beaumont et al., 2000a). The remaining sample was centrifuged at 9,000 ***g*** in order to separate plasma and red blood cells, which were subsequently resuspended in saline and reinjected into the fish. Plasma pH was determined using a Cameron BGM200 blood gas system thermostatically controlled to 10°C. Plasma carbon dioxide concentration was determined using a Corning 965 carbon dioxide analyser calibrated with high precision standards (MultiCal, Ciba-Corning). Plasma total ammonia concentration [T_{amm}] ([NH_3]+[NH_4^+]) was measured, within 2 h of sample collection, using Sigma kit no. 171. The remaining plasma was frozen at –70°C for later analysis of cortisol concentration by RIA (ICN immunochem).

Free (NH_3) and ionised ammonia (NH_4^+) concentrations in water and plasma were calculated from the Henderson–Hasselbalch equation:

$$[NH_4^+] = \frac{T_{amm}}{1 + 10^{pH-pK'}}, \quad (1)$$

and

$$[NH_3] = T_{amm} - [NH_4^+], \quad (2)$$

where values of pK′ were estimated from the nomogram of Cameron and Heisler (1983). The NH_3 and CO_2 concentrations and appropriate solubility coefficients (αNH_3, αCO_3, determined from Cameron and Heisler, 1983; Boutilier et al., 1984) were used to calculate the partial pressures of ammonia (Pw_{NH_3}) and carbon dioxide (Pw_{CO_2}).

Cortisol inhibition

Catheters were implanted into the dorsal aorta of six trout, which were subsequently placed into flux chambers as described above (Ammonia accumulation and flux). After 48 h

recovery, metyrapone (Sigma P856525) was injected through the catheter (3 mg 100 g body mass^{-1} in Courtland's saline) prior to the initiation of CLP exposure. Ammonia flux measurements, analysis of a plasma sample (for pH, [T_{amm}] and cortisol levels), and a further metyrapone injection, were made after each 24 h over a 96 h exposure period.

Ammonia infusion

Three fish had catheters inserted into their dorsal aorta and were allowed to recover for 48 h in control water in the flux chambers. Ammonia fluxes were measured and a blood sample taken and analysed for Hct, plasma pH and plasma [T_{amm}]. Ammonia was then infused into each animal *via* the dorsal aorta. The infusion consisted of 0.5 mol l^{-1} NH_4HCO_3 in Courtland's saline, which was infused at a rate of 1 ml h^{-1}. After 24 h, ammonia flux was measured once more and a further blood sample taken for analysis. To avoid contamination with infusate, the first 1 ml of blood sampled was rejected and the second sample analysed.

Results are presented as means ± S.E.M. Unless otherwise stated, significant effects were determined using one- or two-way analysis of variance (ANOVA) and corrected, as appropriate, by *post-hoc* Bonferroni tests, or by univariate repeated-measures analysis using Systat software (Statsoft Inc). Where data did not have a normal distribution, appropriate logarithmic or arcsine transformations were applied prior to the analyses.

Results

Rate of oxygen consumption and swimming experiments

Rate of oxygen consumption

Mean body mass and standard length of trout used in the control group (581±25 g and 386±10 mm) were not significantly different from those of the CLP exposed fish (520±11 g and 370±7 mm). U_{crit} was a significant 45% lower in the CLP fish compared to that in control trout (1.12±0.06 and 2.04±0.11 *BL* s^{-1}, P<0.001, respectively, where *BL*=body length). There was a degree of individual variation in routine mass-specific rate of oxygen consumption ($\dot{M}_{O_2}$) of the control trout, although there was a non-significant trend towards a decline both in the amount of variation and the mean value during the course of the experiment (Fig. 1). $\dot{M}_{O_2}$ of CLP exposed trout was not significantly different from that of the control fish except at 32 h, when it was significantly lower (P<0.05), and at 72 h, when it was significantly higher (P<0.01), than those of the control fish. At the end of the exposure period, $\dot{M}_{O_2}$ in control trout was 2.6±0.3 mmol O_2 kg^{-1} h^{-1} (N=4). Rate of oxygen consumption of control trout increased exponentially with swimming speed ($\dot{M}_{O_2}$=1.68±0.27e$^{1.15\pm0.24U}$, r^2=0.93±0.03, where U is swimming speed in *BL* s^{-1}). Mean $\dot{M}_{O_2}$ of control fish at U_{crit} was 18.5±2.3 mmol O_2 kg^{-1} h^{-1}, whereas $\dot{M}_{O_2}$ of CLP exposed trout at U_{crit} was significantly less at 5.5±1.6 mmol O_2 kg^{-1} h^{-1}. However, CLP exposed trout had a significantly lower U_{crit} than untreated trout and, for any given speed, there was in fact no significant difference in $\dot{M}_{O_2}$ between either group of trout (Fig. 2).

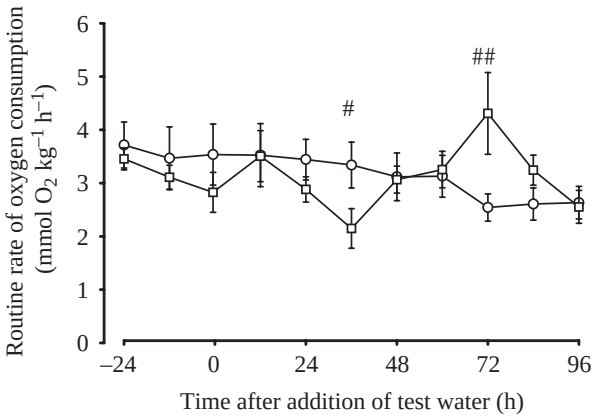


Fig. 1. The routine rate of oxygen consumption of brown trout during 4 days of exposure to either control (pH 7, no added copper) or copper and low pH (CLP; pH 5 and 0.08 µmol l^{-1} Cu) conditions. Values are means ± S.E.M., N=4. Circles, control data; squares, CLP data. In all figures and tables, symbols indicate significant effects as follows: # significant effect of CLP compared to control, † significant effect of CLP compared to pre-exposure value, * significant effect of exercise, ‡ significant effect of CLP and metyrapone. The number of symbols indicates the level of the effect (one, two and three symbols; P<0.05, <0.01 and <0.001, respectively).

Cardiovascular parameters

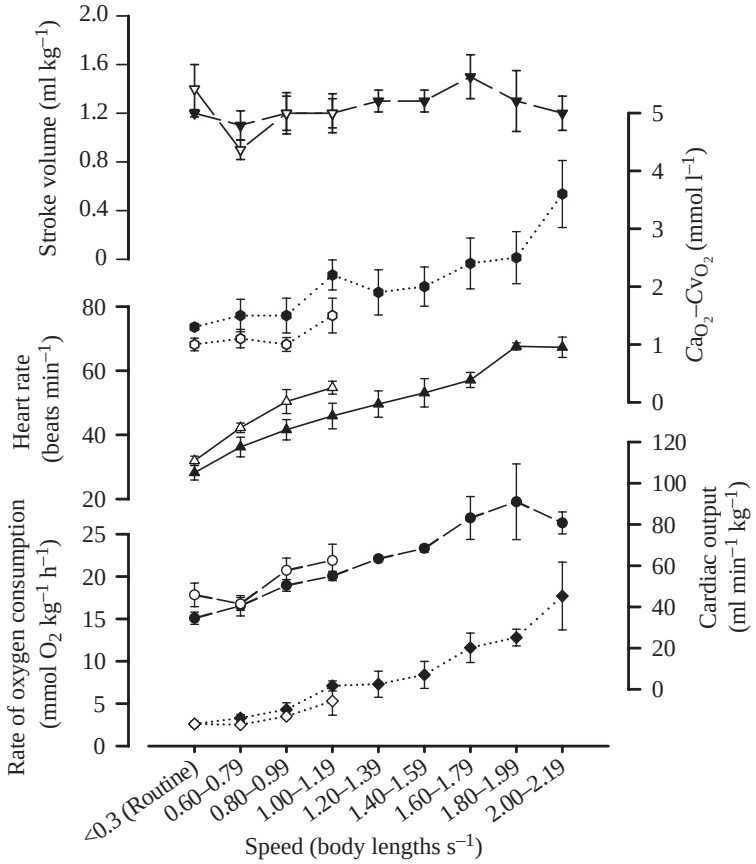
In the control trout, cardiac output at U_{crit} was increased by a significant 2.6 times from the routine rate (Table 1). This was achieved by a significant 2.4 times increase in heart rate while stroke volume remained unchanged (Table 1). Routine cardiac output did not change significantly in trout exposed to CLP (P=0.13; Table 1) in comparison to that in control fish. At U_{crit}, cardiac output of CLP exposed trout had increased significantly but was significantly less than that of control fish at U_{crit} (P=0.05). However, at any given speed, the mean

Table 1. *Cardiovascular data for trout kept in control or copper and low pH (CLP) waters for 96 h and measured at routine levels of activity and at critical swimming speed (*U_{crit}*)*

	Activity level	Control	CLP
Cardiac output	Routine	34.5±3.0	45.8±5.7
(ml min^{-1} kg^{-1})	U_{crit}	90.4±8.1***	63.8±8.5*,#
Heart rate	Routine	28.2±2.3	31.9±1.5
(beats min^{-1})	U_{crit}	68.6±2.8***	54.6±1.7***,##
Stroke volume	Routine	1.2±0.03	1.4±0.20
(ml kg^{-1})	U_{crit}	1.3±0.09	1.1±0.16

Control conditions: pH 7, no copper; N=4; CLP conditions: pH 5, 0.08 µmol l^{-1} copper; N=4.

Superscript symbols denote significant effects; * effect of exercise, # effect of CLP. The number of symbols indicates the level of the effect (one, two and three symbols, P<0.05, <0.01 and <0.001, respectively).



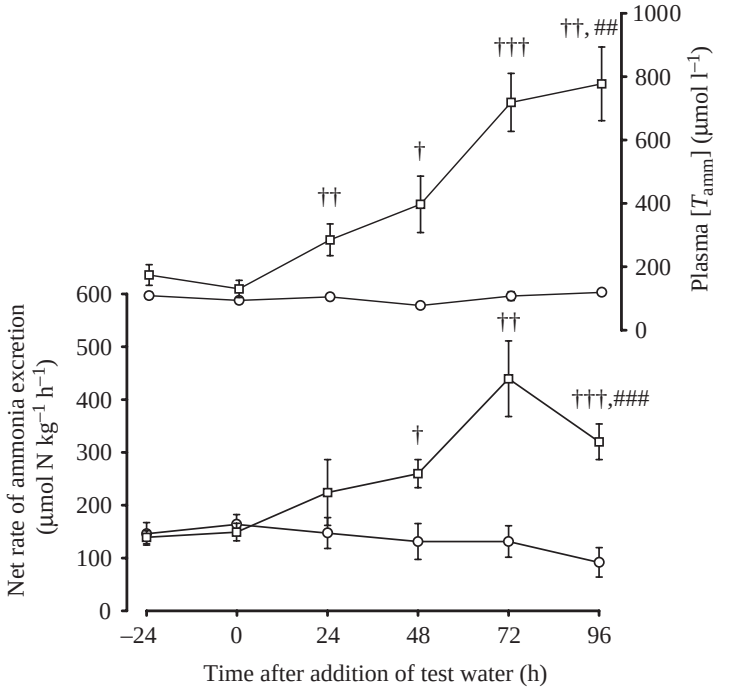


Fig. 3. The net rate of ammonia excretion and total plasma ammonia concentration [T_{amm}] in brown trout during 4 days of exposure to either control (pH 7, no added copper) or copper and low pH (CLP; pH 5 and 0.08 μmol l^{-1} Cu). Symbols as in Fig. 1. Values are means ± S.E.M., N=5. Where vertical lines are not present, S.E.M. is within the limits of the symbol.

Fig. 2. The rate of oxygen consumption and cardiovascular parameters of brown trout while swimming after having been exposed to either control (pH 7, no added copper) or copper and low pH (CLP; pH 5 and 0.08 μmol l^{-1} Cu) conditions for 96 h. Diamonds, rate of oxygen consumption; circles, cardiac output; triangles, heart rate; hexagons, difference in oxygen content between arterial and mixed venous blood Ca_{O_2}–$C\bar{v}_{O_2}$; inverted triangles, cardiac stroke volume. Closed symbols, control data; open symbols, CLP data. Values are means ± S.E.M., N=4. Where vertical lines are not present, S.E.M. is within the limits of the symbol.

cardiac output of CLP trout was not significantly different from that of control fish (Fig. 2). The increase of cardiac output in CLP exposed fish when exercised was achieved by a significant, 1.8-fold increase in heart rate, while stroke volume was unchanged from that at rest (Table 1).

Ammonia accumulation and flux

Mean plasma [T_{amm}] of control fish remained unchanged during the experiment, while in CLP exposed trout, plasma [T_{amm}] increased by almost sixfold from the pre-exposure level of 130.1±27.1 to 776.9±116.5 μmol l^{-1} (N=5, P=0.004; Fig. 3). The rate of increase in plasma [T_{amm}] in CLP exposed trout from 48 h to 96 h (9.6±1.5 μmol l^{-1} h^{-1}) was significantly greater (P<0.05) than that from –0.5 to 48 h (5.8±1.4 μmol l^{-1} h^{-1}).

Net ammonia excretion in trout exposed to control conditions did not change significantly throughout the exposure, ranging from 162.3±15.3 to 105.7±26.7 μmol N kg^{-1} h^{-1} (N=5). In those trout exposed to CLP, net excretion rose to a peak at 72 h (429.4±71.6 μmol N kg^{-1} h^{-1}). After 96 h of exposure to the pollutants, ammonia excretion had declined but was still more than threefold greater than that of control fish (320.0±33.8 μmol N kg^{-1} h^{-1}, P>0.001; Fig. 3).

The increase in plasma [T_{amm}] from the dorsal aortae of the six fish with catheters implanted in both the dorsal and ventral aortae followed a similar pattern during CLP exposure to that previously observed in fish with only a single catheter in the dorsal aorta (Fig. 4). In these fish, ammonia accumulated at a rate of 7.44±0.89 μmol N l^{-1} h^{-1} (r^2=0.96, P=0.004) in the dorsal aorta. [T_{amm}] in plasma from the ventral aorta was some 50–80 μmol l^{-1} greater than that from the dorsal aorta and remained so throughout the exposure. This difference was significant (P<0.001). The accumulation rate of ammonia in the plasma of the ventral aorta was 7.58±0.87 μmol N l^{-1} h^{-1} (r^2=0.96, P=0.003). In the dorsal aorta of control trout, plasma carbon dioxide concentration remained constant throughout the experiment at a mean of 11.1±0.3 mmol l^{-1}. CLP exposure caused no significant difference in plasma carbon dioxide concentration from either aortae (Fig. 4).

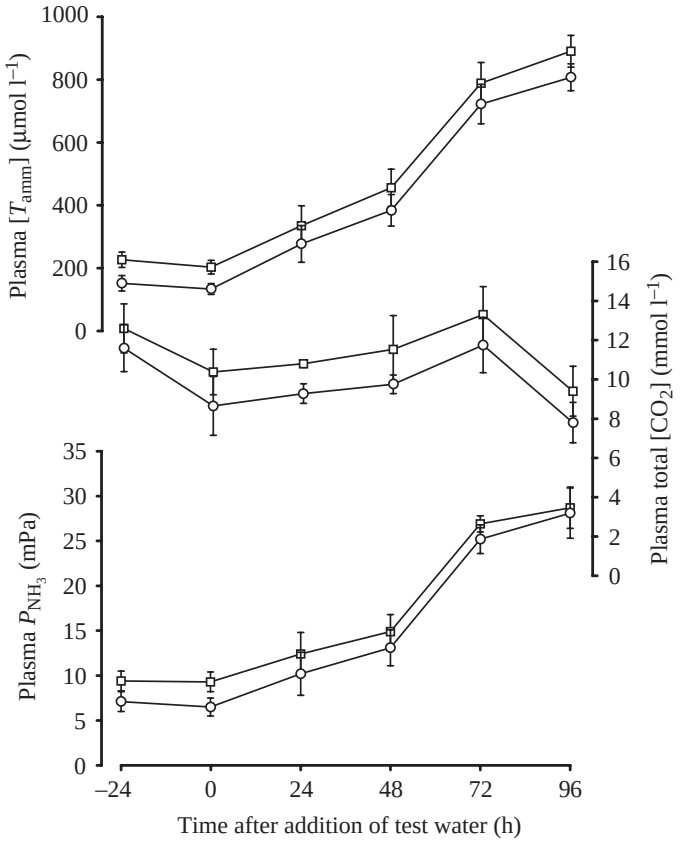


Fig. 4. Plasma total ammonia concentration [T_{amm}], ammonia partial pressure P_{NH_3} and carbon dioxide concentrations [CO_2] in blood taken from the dorsal (circles) and ventral (squares) aortae of brown trout exposed to copper and low pH (CLP; pH 5 and 0.08 μmol l^{-1} Cu). Values are means ± S.E.M., N=6.

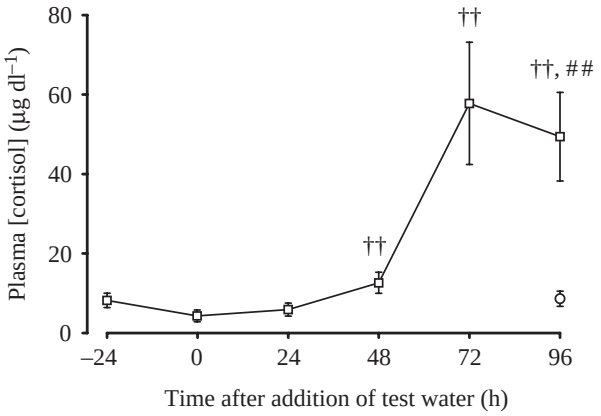


Fig. 5. Plasma cortisol concentration in brown trout during either 4 days of exposure to copper and low pH (CLP; pH 5 and 0.08 μmol l^{-1} Cu) or after 4 days in control (pH 7, no added copper) conditions. Squares, CLP data; circle, control data. Values are means ± S.E.M., N=5.

Plasma cortisol levels were not significantly elevated with respect to pre-exposure values (Fig. 5) until after 48 h of exposure to CLP, when there was a tripling of the mean level (from 4.3±1.5 to 12.6±2.7 μg dl^{-1}, P=0.008, N=5). This peaked at 72 h, with values in some individuals of 20 times the pre-exposure level (mean: 57.8±15.4 μg dl^{-1}, N=5), and remained high at the end of the experiment (49.4±11.1 μg dl^{-1}, N=5).

Cortisol inhibition

Trout exposed to CLP but given daily injections of metyrapone displayed no change in plasma cortisol concentration during the 96 h (Fig. 6). At the end of the experiment, plasma cortisol concentration (7.0±3.3 μg dl^{-1}, N=5) was no different from that of trout kept under control conditions (see Beaumont et al., 2000a). However, both plasma [T_{amm}] and ammonia efflux were elevated in these animals. Net ammonia efflux had increased to a similar level after 96 h as that observed in CLP exposed trout with no metyrapone injections (290.2±50.5 μmol NH_3 kg^{-1} h^{-1} with metyrapone and 320.0±33.8 μmol NH_3 kg^{-1} h^{-1} without metyrapone). However, the peak in ammonia excretion at 72 h was absent from metyrapone treated fish. Plasma [T_{amm}] in the metyrapone treated fish rose during exposure to almost 3.5 times its pre-exposure levels (from 169.3±33.7 to 459.0±40.6; P<0.001, N=6), but this was still less than two-thirds that of the untreated, CLP exposed animals (see *Ammonia accumulation and flux*).

Ammonia infusion

After 24 h of infusion, net ammonia excretion had risen by fivefold to 832.1±40.5 μmol NH_3 kg^{-1} h^{-1} (from 163.2±20.0 μmol NH_3 kg^{-1} h^{-1}; N=3, P=0.003) and plasma ammonia concentration more than doubled from 112.1±9.1 to 298.7±12.1 μmol l^{-1}.

Discussion

Rate of oxygen consumption and cardiovascular variables

Under the conditions of the present experiment, CLP exposure was not a 'loading' factor upon the metabolism of brown trout. At any given level of activity, $\dot{M}_{O_2}$ of CLP and control fish were not significantly different from each other. If it is assumed that $\dot{M}_{O_2max}$ is achieved at U_{crit}, then CLP exposure could be considered as a significant 'limiting' factor of oxygen uptake. The $\dot{M}_{O_2max}$ of CLP exposed trout was less than a third that of the value in control fish and only double that when at rest. However, swimming performance was significantly reduced, and while it might be argued that this was a consequence of an inability of CLP exposed fish to raise oxygen uptake to meet demand, if demand had not increased (i.e. swimming ability was limited by some other factor) then $\dot{M}_{O_2}$ would not rise and this would not necessarily be a measure of maximum capacity. Indeed, the mean $\dot{M}_{O_2}$ of CLP exposed trout at U_{crit} was no different from that of control fish swimming at the same speed. Cardiac output and heart rates were also similar between the two exposure groups when comparisons are made for each speed. In fact, CLP exposed trout may elevate their rate of oxygen consumption above that measured during 'voluntary' swim tests when stressed further

by handling and 'tail-chasing' (M. W. Beaumont, unpublished observations). This suggests that there were no inefficiencies in oxygen transfer across the gills of CLP exposed trout being compensated for by changes elsewhere in the oxygen transport system (cf. Beaumont et al., 1995a, 2000a).

Ammonia accumulation and excretion

Exposure to CLP caused ammonia to accumulate in trout and, since ammonia excretion in these fish is greater than that of trout in control conditions, it is also apparent that this is in some part a consequence of an increase in ammonia production. From the current study, it is clear that there are two phases in the accumulation of ammonia in CLP exposed trout. There is a steady accumulation, which started within the first 24 h of exposure, continued throughout the experiment and did so even in the absence of a change in plasma cortisol concentration (Fig. 6). After 48 h, there is an acceleration in ammonia accumulation associated with an increase in plasma cortisol levels. This latter phase was absent when cortisol production was inhibited. If the bulk of ammonia is excreted across the gills by passive diffusion of NH_3, then its rate of excretion $\dot{M}_{NH_3}$ can be described by Fick's equation in terms of diffusive conductance and the difference in partial pressure:

$$\dot{M}_{NH_3} = G_{NH_3}\Delta P_{NH_3}\,, \qquad (3)$$

where is G_{NH_3} the branchial diffusive conductance for NH_3, and ΔP_{NH_3} the difference in partial pressure of NH_3 between blood and water.

It is worth noting that the delayed increase in plasma cortisol in the CLP fish is probably related to the fact that they were kept in soft water. Brown et al. (1989) reported that exposure of brown trout to acid (pH 4.0–4.6) conditions in water with a high concentration (2.8 mmol l^{-1}) of calcium caused a significant increase in plasma cortisol concentration within 2 days, which returned to the baseline level within 7 days. However, the opposite occurred in fish kept in acid water with a low concentration (0.05 mmol l^{-1}) of calcium; there was no change in plasma cortisol concentration within 2 days, but there was a large, 20-fold, increase within 7 days. Brown et al. (1989) discuss the possible reasons for the difference.

Control conditions

Over the 96 h exposure period, G_{NH_3} of trout in control conditions declined from 37.9±4.9 to 16.4±5.4 μmol N kg^{-1} h^{-1} mPa^{-1}. This occurred due to a fall in $\dot{M}_{NH_3}$ without a corresponding change in ΔP_{NH_3} (from 4.6±0.78 to 6.2±0.86 mPa). Fish were unfed during these experiments and there was probably an associated decline in ammonia production, which would account for the fall in excretion (Brett and Zala, 1975). Conductance of NH_3 depends upon available surface area and epithelial thickness as well as the permeability of the gill to ammonia. At rest, some 40% of the gill lamellae may be redundant (Booth, 1979). In addition to a lower effective surface area, blood flow may be distributed to the basal channels of the lamellae (Farrell et al., 1980), which tend to be buried in filamental tissue. During activity, there is not only an increase in effective surface area of the gills, but their epithelium may also be stretched to accommodate the increased thickness of the vascular sheet, thus causing a narrowing of the diffusion distance between blood and water (Farrell et al., 1980). As activity declines, therefore, a decrease in effective surface area and an increase in epithelial thickness might lead to a decrease in G_{NH_3}, accounting for a lower rate of excretion in the absence of a change in ΔP_{NH_3}.

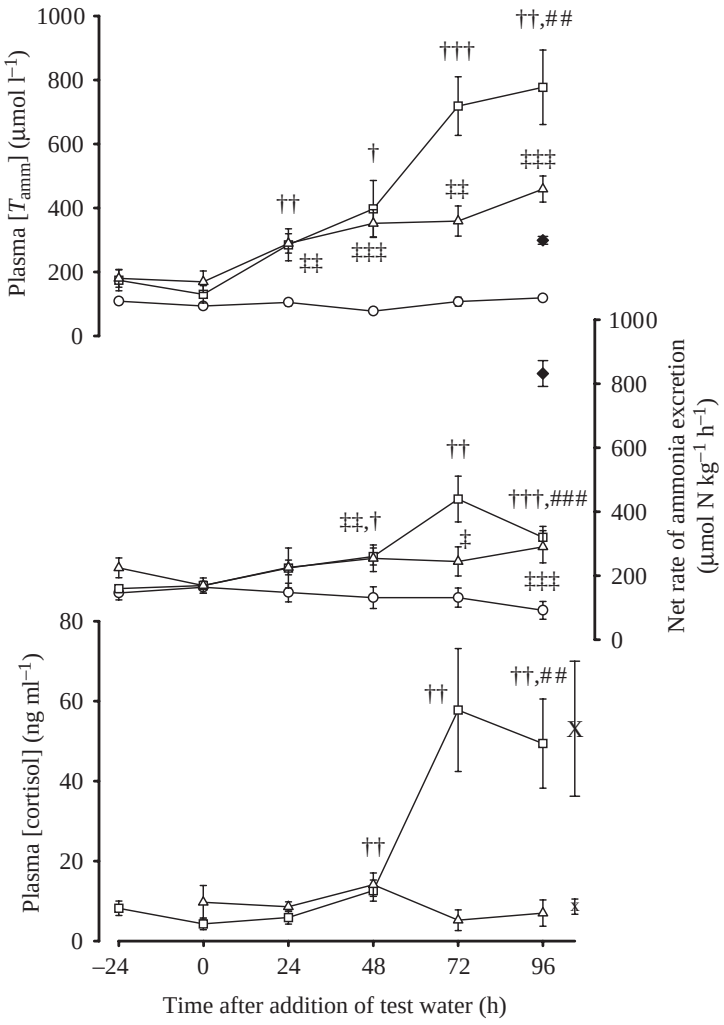


Fig. 6. Combined graphs showing patterns of plasma ammonia concentration [T_{amm}], ammonia flux and cortisol concentration in control (pH 7, no added copper, N=5), CLP-exposed (pH 5 and 0.08 μmol l^{-1} Cu, N=5) and metyrapone-treated CLP-exposed (N=6) trout. Values are means ± S.E.M. Where vertical lines are not present, S.E.M. is within the limits of the symbol. Circles, control data; squares, CLP data; triangles, metyrapone-treated CLP data; filled diamonds, acute ammonia infusion; small cross, control data from Beaumont et al. (2000); large cross, CLP data from Beaumont et al. (2000).

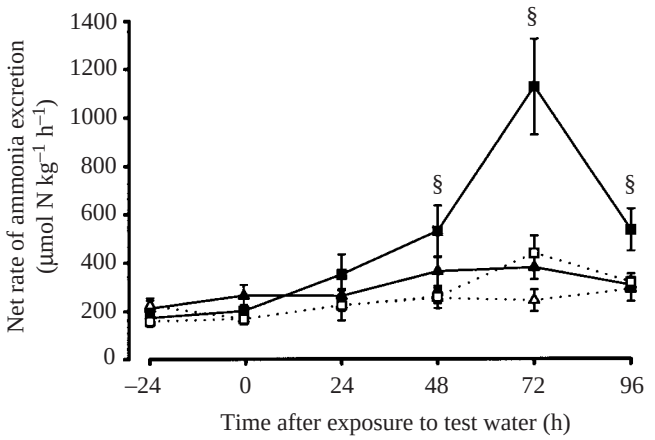


Fig. 7. Observed (open symbols) and predicted (filled symbols) net ammonia excretion rates in brown trout exposed to copper and low pH (CLP; pH 5 and 0.08 μmol l^{-1} Cu) alone (squares) or with metyrapone treatment as well (triangles). Predictions are made using the Fick equation ($\dot{M}_{NH_3}=G_{NH_3}\Delta P_{NH_3}$), measured difference in partial pressure of ammonia (ΔP_{NH_3}) and values of branchial diffusive conductance (G_{NH_3}) calculated from the data from control trout. § indicates a significant difference between observed and expected (χ^2 test, P=0.05, N=5 for all data points, except CLP predicted and observed at 48 h, where N=4).

Table 2. *Comparison of branchial ammonia excretion rate calculated from Equation 4 to the measured net excretion rate from fish prior to (–0.5 h) and after 96 h exposure to CLP conditions*

	Time after addition of test water (h)	
	–0.5	96
Measured $\dot{M}_{NH_3}$ (μmol N kg^{-1} h^{-1})	137.2±16.4	342.1±21.8
$\Delta T_{amm_{(\bar{v}-a)}}$ (μmol l^{-1})	69.8±12.8	82.8±16.7
Cardiac output (ml min^{-1} kg^{-1})	34.5	45.8
Calculated $\dot{M}_{NH_3}$ (μmol N kg^{-1} h^{-1})	144.2±24.7	227.6±51.8§
Calculated $\dot{M}_{NH_3}$/ Measured $\dot{M}_{NH_3}$	0.95±0.1	0.66±0.1

CLP conditions: pH 5, 0.08 μmol l^{-1} copper.
Mean value for cardiac output was the Routine value in Table 1.
§ indicates a significant difference between observed and expected values (χ^2 test, d.f.=4, P=0.05, N=6).

CLP exposure with cortisol inhibition

After 96 h, G_{NH_3} of trout treated with metyrapone and exposed to CLP was 15.4±2.8 μmol N kg^{-1} h^{-1} mPa^{-1}, half that of the pre-exposure value of 31.4±5.2 μmol N kg^{-1} h^{-1} mPa^{-1}. This is similar to the change that occurred in control trout. Unlike the situation in control trout, ΔP_{NH_3} rose in the CLP/metyrapone fish (from 6.2±1.24 to 19.0±0.49 mPa), but was not matched by a proportional increase in $\dot{M}_{NH_3}$. Since oxygen consumption was unchanged by 96 h exposure to CLP/metyrapone, one might speculate that perfusion of the gill follows the same pattern as that of control trout. The observed excretion rates are indeed similar to those predicted by the Fick equation using the appropriate daily mean G_{NH_3} from control trout (Fig. 7).

CLP exposure with cortisol production

In trout exposed to CLP alone, the difference between the magnitude of the rise in ΔP_{NH_3} (from 5.3±0.62 to 32.7±5.29 mPa) and of the increase in ammonia excretion was larger still. G_{NH_3} fell by a third from 34.1±5.6 to 10.9± 1.9 μmol N kg^{-1} h^{-1} mPa^{-1}. Moreover, since data for cardiac output and the difference in arterio – mixed venous [T_{amm}] are available for trout exposed to this treatment, it is possible to use the Fick principle of convection to calculate the rate of branchial ammonia excretion (Table 2):

$$\dot{M}_{NH_3} = \dot{V}_b \times \Delta T_{amm_{(\bar{v}-a)}}, \quad (4)$$

where $\dot{V}_b$ is the cardiac output and $\Delta T_{amm_{(\bar{v}-a)}}$ the difference in total ammonia content between pre- and post branchial blood.

Table 2 shows that approximately 50% of the increase in ammonia excretion in CLP exposed trout is due to an increase in non-branchial ammonia excretion, which increases from approximately 5% to 30% of the total excretion rate. This includes not only urinary and transcutaneous fractions, but also metabolic production of ammonia by the gill, which may account for some 5–8% of net ammonia excretion (Cameron and Heisler, 1983). It is possible that this fraction increased due to CLP-induced gill proteolysis and the subsequent repair of such damage. Otherwise, the increase may have been due to an increase in renal ammonia excretion similar to that shown to occur in the case of prolonged acidosis (McDonald and Wood, 1981; Cameron and Kormanik, 1982).

On the basis of this change in the route of ammonia excretion, G_{NH_3} of CLP exposed trout at 96 h was 7.6± 1.3 μmol N kg^{-1} h^{-1} mPa^{-1}, less than a quarter of the pre-exposure value and considerably lower than that of control fish. In trout exposed to CLP alone, observed excretion rates were considerably lower than those predicted from the Fick equation using the appropriate daily mean G_{NH_3} from control trout (Fig. 7). Both copper and low pH exposures can induce gill damage (e.g. hyperplasia, increased mucus secretion, epithelial thickening and vacuolation), which could result in greater diffusion distances and hence a decreased G_{NH_3}. However, the levels of copper and low pH used in the present study have produced no evidence of such damage in previous investigations (Taylor et al., 1996). It also seems unlikely that NH_3 diffusion would be affected in this manner in the absence of similar effects upon the exchanges of oxygen and carbon dioxide (Figs 2 and 4).

The infusion experiments demonstrate that trout, unexposed to pollutants, have the capacity to elevate the net rate of ammonia excretion by at least fivefold in response to an

infusion of ammonia at a rate that was equivalent to a sixfold increase in ammonia production. It is clear that CLP exposed fish do increase ammonia excretion, but not sufficiently to prevent accumulation. It is interesting to examine the magnitude of the discrepancy. In the present study, ammonia was found to accumulate in the plasma of these fish at a rate of 7.17 $\mu mol\,l^{-1}\,h^{-1}$. This is just 1–3% of the excretion rate. Given that ammonia accumulates in other tissues, data from previous studies (Beaumont et al., 2000a) can be used to estimate the rates of accumulation in red and white muscle as 31.56 and 20.52 $\mu mol\,N\,kg^{-1}\,h^{-1}$. Using measurements of various tissues as percentages of body mass from the same studies and overestimating accumulation in the remainder of the tissues by using the value for red muscle, whole body ammonia accumulation rate is at most 24.2 $\mu mol\,N\,kg^{-1}\,h^{-1}$. Even this overestimate represents only 5–10% of the amount of ammonia being excreted at any given time and demonstrates that, whatever the mechanism of the effect of exposure to CLP, it has a relatively minor effect upon instantaneous excretion rates, but becomes important over time.

In conclusion, previous studies (Beaumont et al., 1995a, 2000a) have inferred an absence of an effect of CLP exposure upon the branchial uptake of oxygen from an absence of effect upon arterial oxygen partial pressure and content. The present study provides evidence of a corresponding lack of effect upon the oxygen transport system. Thus, in answer to the first questioned posed, heart rate, cardiac output and oxygen consumption of CLP exposed trout are no different from those of control fish, at any given level of activity. It is argued that maximum rates are not achieved in CLP exposed fish due to an absence of demand. The answer to the second question is that hyperammonaemia, a possible factor in the loss of exercise performance, arises from two phases of ammonia production. There is a steady increase that occurs in the absence of cortisol production. Once the changes in branchial diffusive conductance observed in control fish during the experiment are taken into account, this ammonia accumulation may simply represent the readjustment of equilibrium, an increase in plasma ammonia to increase the difference in P_{NH3} and, therefore, ammonia excretion by passive diffusion. The second phase of accumulation, associated with a rise in cortisol, does not lead to the level of excretion that would be predicted from simple passive diffusion. Ammonia infusion experiments show that control fish can respond to such an ammonia load. This observation may indicate the presence of an excretory mechanism (most probably Na^+/NH_4^+ exchange) that is non-obligatory in 'normal' conditions but that is required (and inhibited by CLP exposure) in order to respond to unusual ammonia loads.

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