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Extra- and intracellular acid-base balance and ionic regulation in cod (Gadus morhua) during combined and isolated exposures to hypercapnia and copper

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Abstract Cod (Gadus morhua) were exposed to hypercapnia (water Pco₂ = 7.5 mmHg), elevated copper level (0.4 ppm) or a combination of both in order to study extra- and intracellular acid-base regulation and the influence hereupon of copper. During pure hypercapnia, the extracellular respiratory acidosis was completely compensated within 12 to 24 h via a chloride-mediated increase in extracellular [HCO₃⁻]. Exposure to copper in normocapnic seawater caused a large and progressive increase in plasma [Na⁺] and [Cl⁻] and a metabolic acidosis. Exposure to copper in hypercapnic seawater was associated with smaller elevations of plasma [Na⁺] and [Cl⁻] than in normocapnic seawater, showing that hypercapnia had a protective effect on the copper-induced osmoregulatory disturbances. The compensation of the hypercapnic acidosis was, however, slow and incomplete in fish exposed to both copper and hypercapnia. Extracellular pH remained depressed by 0.3 pH units after 72 h. The data reveal that acid-base regulation was immediately and persistently inhibited by copper. The limited acid-base regulation during combined copper and hypercapnia exposure was chloride-mediated as during hypercapnia alone. Intracellular pH recovery was complete and very rapid in ventricular and skeletal muscle tissues during environmental hypercapnia, whereas acid-base compensation in liver tissue was slower, the kinetics being similar to that in the extracellular compartment. Intracellular pH compensation was significantly slowed down by copper. Copper concentration increased drastically in gill tissue already at 3 h, while copper concentrations in liver, muscle and plasma were significantly elevated only after 48 h, with liver showing the largest elevation.

Introduction

Metals occur naturally in marine environments due to land drainage and weathering, deep-sea volcanism and emission, but due to widespread use of copper in industries, agriculture etc., anthropogenic inputs are the most important sources of copper contamination (Bryan 1976). The background concentration of copper in open seawater is about 0.5 μg l⁻¹, while concentrations of up to 0.6 mg l⁻¹ have been reported for coastal areas. Copper is essential to all living organisms, by playing an important role in a large number of biological processes. However, in polluted areas copper concentration may reach levels which are harmful for several aquatic animals (Bryan 1976).

The acute physiological effects of elevated water copper concentration on fish have been intensively studied, especially in freshwater fishes (reviewed by McDonald et al. 1989; McDonald and Wood 1993). Relatively few experiments have been carried out in seawater (Cardeilhac et al. 1979; Stagg and Shuttleworth 1982b; Wilson and Taylor 1993b). The primary target for acute copper toxicity is gill ionic transport, and mainly the transport of monovalent ions (McDonald and Wood 1993). Effects of copper on extracellular acid-base status have been studied in some freshwater fishes, but hardly in marine fishes. Information on intracellular acid-base status during Cu exposure is absent. In addition to inducing direct acid-base disturbance, copper may potentially affect the ability to regulate an acid-base disturbance caused by other factors such as e.g. environmental hypercapnia. Regulation of acid-base balance in fish is primarily via gill ionic exchangers (Heisler 1986b). It appears likely that interaction of copper with these or with related transport proteins may influence

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the capacity for acid-base regulation. This hypothesis has not yet been investigated.

Materials and methods

Specimens and holding conditions

Atlantic cod (Gadus morhua), 230 to 525 g in weight (N = 83), were obtained from the Biological Station on Helgoland, Germany, and transported to the Institute for Polar and Marine Research, Bremerhaven, Germany. Cod were kept in 900 litres of recirculated, well-aerated seawater (34%, 12 °C) for at least 1 week before they were used in experiments. The maximum number of fish in the tank at any time was 25. Individuals were subjected to a 12 h light:12 h dark rhythm and fed with shrimp twice a week until 2 d before use in experiments.

Series I: experiments with cannulated fish

In order to enable repetitive blood sampling without disturbing the fish, cod (415 ± 64 g, mean ± SD, N = 26) were cannulated with PE50 tubing in the afferent branchial artery of the third gill arch under MS222 (3-aminobenzoic acid ethyl ester, Sigma) anaesthesia (1:15000). During surgery, the gills were continuously irrigated with aerated seawater containing the anaesthetic. After surgery the cod were placed in individual darkened aquaria containing 21 litres of well-aerated (PO₂ > 150 mmHg) artificial seawater (sea salt dissolved in deionized water, 34%, 12 °C) and allowed to recover from cannulation and anaesthesia for 48 h. In each aquarium, 15 litres of the water was renewed daily, also during succeeding experiments.

Individuals were subjected to one of the following four experiments: (i) control, fish kept under control conditions (normocapnic-normoxic water) for 24 h; (ii) hypercapnia (Hyp), fish exposed to 0.99% CO₂ for 24 h; (iii) copper (Cu), fish exposed to 0.4 mg l⁻¹ total copper for 48 h in normocapnic seawater; or (iv) hypercapnia plus copper (HCu), fish exposed to 0.99% CO₂ and 0.4 mg l⁻¹ total copper for 72 h. Copper was added to the aquarium as CuSO₄ and constancy of copper concentration was checked daily before water renewal. Normocapnic-normoxic conditions were obtained by equilibration with air, and hypercapnic conditions by equilibration with a CO₂/air gas mixture (0.99% CO₂/99.01% air) delivered by Wösthoff gas mixing pumps. The water surface in the aquarium was covered by a sheet of poly styrene to minimize escape of CO₂. Within 1.5 h after the onset of equilibration, water PO₂ had increased to a constant level of 7.5 mmHg. This caused water pH to decrease from about 8.0 to 7.0. Water PO₂ remained high, and did not fall below 145 mmHg at any time.

Blood samples (0.8 ml) were drawn at 0, 3, 5, 12 and 24 h in all groups. Additional blood samples were taken after 48 h in the Cu group and after 48 and 72 h in the HCu group. After withdrawal of the final blood sample, samples of white muscle, liver, ventricle and gill tissues were excised for intracellular measurements (see following section).

Series II: kinetics of changes in intracellular parameters

Since only terminal tissue samples could be obtained from the four experimental groups in Series I, a further series was conducted to investigate intracellular parameters at earlier times during the exposures. Unanesthetized cod (307 ± 54 g, mean ± SD, N = 57) were transferred to the individual aquaria and left overnight to recover from handling stress. The fish were exposed to (i) hypercapnia for 3, 5 or 12 h; (ii) copper for 12 or 24 h; or (iii) hypercapnia + copper for 3, 5, 12, 24 or 48 h, whereupon tissue samples were taken (see following section). The intracellular values from the control group in Series I were taken as "true" controls. Tissue sampling in the Cu group was started 12 h after copper addition, as only minor changes were found in the extracellular acid-base status prior to 12 h. During the experiment it became clear that copper caused large mortality in the Cu group, and that it would be difficult to obtain enough 72-h samples in this group. It was therefore decided to end the experiment in this group after 48 h.

Sampling of tissue

Individuals were anaesthetized by adding a concentrated solution of MS222 to the aquaria to a final concentration of 0.15 to 0.2 g l⁻¹. Fish quickly lost balance and were fully anaesthetized after about 3 min. A sample of caudal skeletal muscle was excised from the side of the fish, whereafter, a liver sample was taken and the ventricle was dissected out. All samples were freeze-clamped immediately, wrapped in aluminium foil and stored under liquid nitrogen. These samples were used for intracellular acid-base measurements. The individual was subsequently decapitated, whereupon further samples of caudal muscle, liver and gill were taken for analysis of ions and copper accumulation. Gill samples were briefly rinsed in saline (0.9% NaCl) to remove mucus. Samples used for ion analyses were stored at -80 °C until use.

Measurements on blood and plasma

Extracellular pH (pHe), PO₂ and PO₄ were determined in blood immediately after sampling using a gas analyzer equipped with Eschweiler electrodes (Kid, FRG). Total CO₂ was determined on 50 μl true plasma by acidification (converting HCO₃⁻ and CO₃⁻ to CO₂) and subsequent measurement of the free CO₂ with a gas chromatograph (Hach Carle 100 AGC). Bicarbonate concentration was calculated from the Henderson–Hasselbach equation. The apparent pKₐ and the CO₂ solubility coefficient a were calculated according to Heisler (1986a). The extensive change in ionic concentrations in some of the experimental groups was taken into account by calculating pKₐ and a from the time-dependent mean concentrations of plasma ions in each group.

Total concentration of haemoglobin (Hb), haematocrit (Hct), number of red blood cells (RBCs) per unit volume of blood (N₉RBC), mean cellular volume (MCV), mean cellular Hb content (MCH), and mean cellular Hb concentration (MCHC), mmol l⁻¹ RBC were measured/calculated as described by Jensen (1990).

Plasma osmolality was measured by vapour pressure osmometry (Wescor 5500). Plasma chloride concentration was measured by coulometric titration (Radiometer CMT 10) and sodium by a flame photometer (Instrumentation Laboratory 243). Potassium, calcium, magnesium and copper were measured by atomic absorption spectrophotometry (Perkin Elmer 2380). Plasma lactate was assayed with the Boehringer-Mannheim lactate dehydrogenase method.

The concentration of K⁺ in whole blood was measured in supernatants from blood samples after deproteinization with equal volumes of 12% trichloroacetic acid. The red cell concentration of potassium (mmol l⁻¹ RBC) and the mean cellular potassium content (MCK, in mol cell⁻¹) were calculated from blood [K⁺], plasma [K⁺], Hct and N₉RBC (Jensen 1990).

Intracellular acid-base measurements

Intracellular pH (pHi) and total CO₂ (CICO₂) in skeletal muscle tissue, and pH in liver and ventricle tissues were measured by the homogenization technique developed by Pörtner et al. (1990), using nitrioltriacetic acid (NTA) and potassium fluoride for metabolic inhibition. The NTA and KF concentrations were 6 and 150 mM, respectively, and similar to those used for trout muscle (Pörtner et al. 1990). pHi was measured by a radiometer capillary pH electrode (PHM93, G297162) and total CO₂ (CICO₂) by gas chromatography (Hach Carle 100 AGC). CICO₂ was measured in 50 μl aliquots from 2 to 4 homogenates prepared from the same muscle powder used for pHi measurements. CICO₂ in cell water was calcu-
lated according to Pörtner et al. (1990), taking the high extracellular concentrations of bicarbonate into account and assuming interstitial [HCO₃⁻] to be slightly higher than plasma [HCO₃⁻] due to a Donnan effect (Donnan factor of 1.05). A value of 0.11 for fractional extracellular water was adopted from Heisler (1986a). Intracellular pK′ and α values were calculated according to Heisler (1986a), assuming [Na⁺] = 0.02 M, [M] = 0.21 mol l⁻¹, 1 = 0.12 mol l⁻¹ and [Protein] = 220 g l⁻¹ (Pörtner et al. 1990). Po₂ and [HCO₃⁻] in cell water could then be calculated from intracellular pH and CO₂ according to the Henderson–Hasselbalch equation.

Intracellular ion concentrations

The frozen tissue samples were freeze-dried to constant weight and digested in concentrated HNO₃; the remaining salts were bleached in H₂O₂, and finally redissolved in 0.2% HNO₃. Only the gill filaments were used from the gill samples, and the skin was removed from muscle samples before freeze drying. Cu, K, Ca and Mg contents were measured in muscle tissue, whereas only Cu was measured in liver and gill tissues.

The data are presented as means ± SEM. Statistical significance of within-group differences in extracellular values were evaluated by analysis of variance (ANOVA) for repeated measures followed by the Tukey test. Differences between groups during the first 24 h were evaluated using two-way ANOVA followed by the Tukey test. Values obtained at 48 and 72 h in the HCU and Cu groups were compared only to control values of each group at time zero. Differences in intracellular values of exposure groups and the control group were evaluated using one-way ANOVA followed by the Tukey test.

Results

Hypercapnia decreased copper toxicity

Copper was found to be significantly more toxic under normocapnic than under hypercapnic conditions. No mortality was observed in cod exposed to copper under hypercapnic conditions for 72 h, whereas 10 out of 17 fish died within 48 h of normocapnic Cu exposure.

Extracellular acid-base status and oxygen pressure

Blood sampling per se did not influence acid-base status within 24 h, as neither Pco₂, pH or plasma [HCO₃⁻] changed significantly in the control group (Fig. 1). Exposure of cod to 0.4 mg Cu l⁻¹ under normocapnic conditions gradually induced an extracellular acidosis, which was of metabolic origin as plasma [HCO₃⁻] decreased, while Pco₂ remained constant (Fig. 1B, C). Elevation of ambient Pco₂ to 7.5 mmHg increased blood Pco₂ from 3.5 to 9–10 mmHg in the pure hypercapnia (Hyp) group. In the group exposed to hypercapnia plus copper (HCU), blood Pco₂ rose to about 11 mmHg, which was significantly higher than in the Hyp group (Fig. 1A). This elevation of Pco₂ was established within 3 h and caused a large decrease in pH (Fig. 1B). The pH deflection at 3 h was, however, more than twice as large in the HCU group (ΔpH = 0.42) than in the Hyp group (ΔpH = 0.19). The respiratory acidosis was quickly compensated in fish exposed to pure hypercapnia. Complete pH recovery was achieved within 12 to 24 h. Fish exposed to hypercapnia plus copper showed a very slow and incomplete compensation. After 72 h, pH remained depressed by almost 0.3 units compared to the time zero value (Fig. 1B). These differences in pH regulation were reflected in a more pronounced and faster extracellular accumulation of bicarbonate in the Hyp group than in the HCU group. Plasma [HCO₃⁻] in the Hyp group was elevated significantly above that in the HCU group already at 3 h (explaining the different pH deflection at 3 h), and the total increase in plasma [HCO₃⁻] was twice as large.

The degree of pH compensation can be calculated as: 100 × (pH₅ – pH₅₀)/(pH₅ – pH₅₀), where pH₅ is pH measured at hour x, pH₅₀ is the time zero control.
value and $pH_{std}$ is the "theoretical" plasma pH that corresponds to the $P_{CO_2}$ at hour x if [HCO$_3^-$] had remained at the control value (standardized pH, Claiborne and Heisler 1986). After 24 h, fish exposed to pure hypercapnia exhibited 100% compensation, whereas pH compensation in the HCu group was about 35%. HCu fish reached a final compensation of 45% after 72 h.

The mixed venous oxygen pressure ($P_{O_2}$) was relatively unaffected except in fish exposed to hypercapnia plus copper, where it decreased from about 35 mmHg to a significantly lower value of 23 mmHg at 72 h (not illustrated).

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**Fig. 2 Gadus morhua.** Time-dependent changes in plasma A osmolality, B sodium, C chloride, D potassium, E calcium, F magnesium and G lactate in cod exposed to four different experimental conditions. Values are means ± SEM; N values and probability as in Fig. 1.

Ionic status

Plasma osmolality remained constant in the control group and in the pure hypercapnia group (Fig. 2A). Copper exposure, in contrast, caused a progressive increase in osmolality from 325 to 417 mOsm within 48 h (Fig. 2A). This drastic change was due to elevation of both [Na$^+$] (Fig. 2B) and [Cl$^-$] (Fig. 2C) by more than 40 mM during the experiment. Osmoregulation was also critically perturbed during combined hypercapnia and copper exposure (HCu group), but the elevations of osmolality and ion concentrations were smaller than
during normocapnic copper exposure (Cu group). Osmolality in the HCu group increased by 45 mOsm after 48 h, which was only half the change observed in the Cu group (Fig. 2A). The difference was due to less pronounced elevations of [Na\(^+\)] and [Cl\(^-\)] in the HCu group (Fig. 2B, C). During the 72 h HCu exposure, the mean values of [Na\(^+\)] and [Cl\(^-\)] increased by 29 and 18 mM, respectively. Hypercapnia by itself caused a pronounced and fast decrease in plasma [Cl\(^-\)], which was significant already after 3 h. The total decrease after 24 h was about 20 mM (Fig. 2C).

Acid-base status and ionic status are interrelated through the concept of "strong ion difference"; [SID] = ([Na\(^+\)] + [Ca\(^{2+}\)] + [K\(^+\)] + [Mg\(^{2+}\))] - ([Cl\(^-\)] + [lactate\(^-\)]). At constant Pco\(_2\) and nonbicarbonate buffer concentration an increase in [SID] results in an equimolar increase in plasma [HCO\(_3^-\)], and an elevation of pH, while the opposite occurs at decreasing [SID] (Stewart 1983). During pure hypercapnia, Cl\(^-\) was the only strong ion which changed significantly, and the 20 mM decrease in [Cl\(^-\)] (Fig. 2C) was paralleled by a 20 mM increase in [HCO\(_3^-\)] (Fig. 1C). The situation was more complicated during Cu and HCu exposures. During Cu exposure, the rises in [Na\(^+\)] and [Cl\(^-\)] were almost equimolar. Together with minor changes in other strong ions (Fig. 2) this added up to a metabolic acidosis with a minor decrease in [SID] and in the [HCO\(_3^-\)] concentration of 3.31 mM within 48 h. During HCu exposure, Cl\(^-\) increased less than Na\(^+\). On the basis of only small changes in other strong ions, [SID] was determined by [Na\(^+\)] - [Cl\(^-\)], which rose by 11 mM during 72 h. This compared well with the 11 mM rise in [HCO\(_3^-\)] (Fig. 1C), which partly compensated the respiratory acidosis.

Plasma [K\(^+\)] (Fig. 2D) increased in the Cu group during the experiment. This may have been due to an increased muscular activity level, as it was observed that cod exposed to Cu alone were rather active, already after 12 h throughout the experiment. Fish exposed to hypercapnia plus Cu remained calm as did control fish and fish exposed to hypercapnia, where plasma [K\(^+\)] was not elevated. Plasma [Ca\(^{2+}\)] (Fig. 2E) decreased and plasma [Mg\(^{2+}\)] (Fig. 2F) increased significantly in the HCu group, while it remained constant in the three other groups. Cu fish showed a small significant increase in plasma [lactate] to about 2 mM at 48 h (Fig. 2G), whereas no significant changes were found in the other groups.

Haematological parameters

Mean cellular volume (MCV) of red blood cells (RBCs) was unchanged in controls and transiently increased at the onset of hypercapnia (Hyp and HCu groups) (Fig. 3). In copper-exposed fish (Cu and HCu groups), the gradual and profound elevation of plasma osmolality induced a progressive shrinkage of the RBCs. MCV decreased by 28% in the Cu group, and by 13% in the HCu group (Fig. 3A). The larger decrease in MCV in the Cu group correlated with the larger increase in osmolality in this group. The cellular content of haemoglobin (MCH) (Fig. 3C) did not change, so RBC shrinkage significantly increased MCHC (Fig. 3B). Similarly, the red cell K\(^+\) content (MCK) was relatively constant (Fig. 3D), and RBC shrinkage therefore induced pronounced elevation of [K\(^+\)]\(_{RBC}\) in copper-exposed fish.

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**Fig. 3 Gadus morhua.**
A Mean cellular volume (MCV). B mean cellular haemoglobin concentration (MCHC). C mean cellular haemoglobin content (MCH) and D mean cellular potassium content (MCK) in red cells from cod exposed to four different experimental conditions. Values are means ± SEM; N values and probability as in Fig. 1.
the HCu group $[K^+]_{RBC}$ increased from 108 to 143 mmol l$^{-1}$ RBC after 72 h, and in the Cu group $[K^+]_{RBC}$ increased from 105 to 186 mmol l$^{-1}$ RBC.

Intracellular pH status

pHi in caudal skeletal muscle from control fish was 7.344 ± 0.008 (mean ± SD) (Fig. 4B). During Cu exposure, muscle pHi was significantly lowered compared to the control value, but the difference of 0.056 pH units in 48 h (Fig. 4B) was smaller than the 0.2 pH unit decrease seen extracellularly (Fig. 1). Hypercapnia caused an increase in intracellular Pco$_2$ from about 5.5 mmHg (control group) to 14–15 mmHg (Fig. 4A). This caused a small and only transient intracellular respiratory acido-

sis. In Hyp fish, the deviation from control pHi was only 0.068 pH units, and complete compensation was achieved after 12 h. The fast intracellular pH compensation was achieved by a rapid increase in intracellular $[HCO_3^-]$ from about 4 to 8 mM after 3 h and further to 11 mM at 12 h (Fig. 4C). Regulation of muscle pHi was also relatively fast in HCu fish, but it was slower than in Hyp fish. Hyp fish reached 100% intracellular pH compensation after 12 h, whereas HCu fish at the same time showed only 80% compensation. HCu fish obtained 100% pH compensation after 48 h, but pHi decreased again at 72 h.

pHi in heart and liver tissues (control pHi = 7.057 ± 0.026 and 7.067 ± 0.085, respectively, means ± SD) (Fig. 5) were lower than in white muscle. Intracellular pH compensation was very fast in cardiac muscle as reflected by transient and nonsignificant decrease in pHi in groups Hyp and HCu (Fig. 5A). No significant differences from control pHi were observed in Cu fish. In liver, the initial deviations from control pHi were larger than in skeletal muscle, and compensation was slower (Fig. 5B). It took 24 h for liver pHi to return to the control level during hypercapnia. In the HCu group, a significant drop in pHi was observed at 72 h. A significant lowering of liver pHi was also observed in Cu fish, at 24 and 48 h.

Fig. 4 Gadus morhua. Time-dependent changes in intracellular A Pco$_2$, B pHi and C cell water bicarbonate concentration in caudal skeletal muscle of cod exposed to four different experimental conditions. All exposure values are statistically compared to control values (means ± SD). (*p < 0.05), N = 6 to 7 for all points.

Fig. 5 Gadus morhua. Time-dependent changes in intracellular pH of A heart (ventricle) tissue and B liver tissue. All exposure values are statistically compared to control values (means ± SD). N = 6, 7, 7, and 6 in groups control, Hyp, HCu and Cu, respectively (*p < 0.05)
Tissue water and Cu ion content

The large increase in extracellular osmolality in copper-exposed fish significantly reduced the water content of skeletal muscle from 80.85 ± 0.22% in controls to 79.39 ± 0.52 (means ± SD) in HCU fish after 72 h, and to 78.49 ± 0.21% in Cu fish after 48 h. The muscle water content in Cu fish was significantly lower (p < 0.05) than in HCU fish. In gill tissue, water content was close to 81% but no significant treatment effects were detected. Water content in liver tissue was lower (i.e. approximately 65%) and varied more than in muscle and gill tissue (due to variation in fat content), and significant changes were not detected during the experiments.

Copper concentration of the mucous-free gill tissue was significantly elevated in the HCU group already after 3 h of exposure to copper (Fig. 6A). After 72 h the concentration was increased by 320% to 19 µg g⁻¹ dry wt. In the Cu group, gill [Cu] rose somewhat slower but [Cu] reached 17 µg g⁻¹ dry wt after 48 h, which was similar to values in HCU fish. The liver Cu concentration was about four times higher than in gill tissue and 15 times higher than in muscle tissue (Fig. 6B). During copper exposure, mean liver [Cu] rose by about 60 and 90% in the HCU and Cu groups, respectively. The copper concentration of muscle tissue was low but slightly elevated upon copper exposure (Fig. 6C). The increase was by about 40% in both copper-exposed groups, but only the final values were significantly higher than controls. Plasma [Cu] rose significantly from 0.6 to 0.7 µg ml⁻¹ during copper exposure (Fig. 6D).

The muscle content of K was about 725 mmol g⁻¹ dry wt, and the contents of Ca and Mg were both about 12 mmol g⁻¹ dry wt. No significant changes in the levels of these tissue cations were found during the different exposures.

Discussion

This study provides new information on copper-induced effects and on acid-base regulation in marine fish. Notably, copper-induced ion regulatory disturbances were shown to be strongly reduced by environmental hypercapnia, and the rates and capacity for extra- and intracellular acid-base regulation were shown to be lowered by copper exposure.

Extracellular acid-base status

Atlantic cod exposed to environmental hypercapnia (Hyp) achieved full compensation of the extracellular acidosis, within 12 to 24 h (Fig. 1). This rapid acid-base regulation correlates well with other studies on marine fish (Oncorhynchus kisutch, Perry 1982; Conger conger, Toews et al. 1983; Squalus acanthias, Caliborne and Evans 1992). The often faster regulation of a hypercapnic acidosis in marine fishes than in freshwater fishes has been attributed to the high buffer capacity of seawater and the high concentrations of ions available for acid-base regulatory ionic exchange across the gills (Perry 1982; Heisler 1986b). High environmental [HCO₃⁻] seems to be particularly important for acid-base compensation during hypercapnia in both freshwater and seawater (Heisler 1986b; Larsen and Jensen 1997),
but the high [Na⁺] and [Cl⁻] characterizing seawater are also beneficial (Iwama and Heisler 1991). Under pure hypercapnia, the compensation of the respiratory acidoses was chloride mediated, as reflected by a progressive decrease in plasma [Cl⁻] and constancy in plasma [Na⁺] (Fig. 2B, C). This apparent dependency on chloride rather than sodium-mediated mechanisms seems to be general during hypercapnia in both freshwater and marine fishes (i.e. Toews et al. 1983; Jensen and Weber 1985; Perry et al. 1987; Larsen and Jensen 1997). Measurements of Na⁺ and Cl⁻ fluxes between animal and environment support this conclusion, as changes in Na⁺ fluxes are relatively small and short, whereas changes in Cl⁻ fluxes are larger and longer lasting (Wood et al. 1984; Goss and Wood 1990; Goss et al. 1992). Thus, it appears that modulation of gill Cl⁻/HCO₃⁻ exchange plays a central role in acid-base regulation during hypercapnia and that Na⁺/H⁺ exchange is of minor importance.

Pure copper exposure resulted in a metabolic acidosis, which was significant after 5 h (Fig. 1). The elevations of plasma [Na⁺] and [Cl⁻] were of similar magnitude, but as [Cl⁻] increased slightly more than [Na⁺], a metabolic acidosis was induced. The elevation of plasma [Na⁺] and [Cl⁻] (Fig. 2B, C) can be attributed to an inhibition of the basolateral Na⁺/K⁺-ATPase (see below), which is a well-known effect of copper in both freshwater (Lorz and McPherson 1976; Laurén and McDonald 1987) and marine teleost fish (Stagg and Shuttleworth 1982a). Acid-base effects associated with copper-induced disturbances of branchial ion transport are, however, variable. Whereas a significant metabolic acidosis was induced in cod, arterial pH was unchanged in seawater-acclimated rainbow trout exposed to the same concentration of copper for 24 h (Wilson and Taylor 1993b). Variable effects on acid-base balance during copper exposure have also been reported in freshwater fish (e.g. Wilson and Taylor 1993a; Pilgaard et al. 1994). Marine invertebrates, such as shore crabs, respond with a metabolic acidosis (i.e. Boitel and Truchot 1989, 1990; Weeks et al. 1993), as observed in cod. However, these animals do not hypoosmoregulate as marine fishes, complicating direct comparison.

The present study is the first to show an immediate and persistent inhibitory effect of copper on the capacity for acid-base regulation during hypercapnia. During combined copper and hypercapnia exposure the acute pH deflection was larger than during pure hypercapnia, and the compensatory extracellular HCO₃⁻ accumulation was retarded for at least 72 h (Fig. 1). The basis for the copper-induced inhibition of acid-base regulation cannot be evaluated from the present experiments. It may be the integrative result of several actions of copper on the branchial epithelium. In addition to its inhibitory effect on the Na⁺/K⁺-ATPase in chloride cells, copper may influence the function of the branchial HCO₃⁻/Cl⁻ exchanger. Copper may also inhibit gill carbonic anhydrase, and thereby limit the conversion of CO₂ to HCO₃⁻ inside the cells. Finally, copper may affect gill ion permeability and thus passive fluxes (see following section).

Even though copper clearly inhibited acid-base regulation, the HCu-exposed fish achieved a limited degree of pH compensation. Interestingly, this pH compensation appeared to be Cl⁻ mediated, as during pure hypercapnia. Thus, whereas the increases in plasma [Na⁺] and [Cl⁻] were approximately equimolar during copper exposure, plasma [Cl⁻] rose less than [Na⁺] during combined copper and hypercapnia exposure. This suggests that acid-base regulatory Cl⁻/HCO₃⁻ exchange across the gills (tending to decrease plasma [Cl⁻]) and increase plasma [HCO₃⁻]) occurred in addition to the copper-induced rises in plasma [Na⁺] and [Cl⁻].

Osmo/Ion regulation

In general, copper strongly affects the regulation of monovalent ions, inducing a net ion loss in hypoosmoregulating freshwater fish (Laurén and McDonald 1985; Wilson and Taylor 1993a) and a net ion uptake in hypoosmoregulating marine fish (Cardeilhac et al. 1979; Stagg and Shuttleworth 1982b). The increases in extracellular [Na⁺], [Cl⁻] and osmolality were very large in cod during Cu exposure. During combined hypercapnia and copper exposure these parameters increased significantly (Fig. 2), showing that hypercapnia protected the cod against the copper-induced osmoregulatory disturbances. Copper directly inhibits Cl⁻ secretion in seawater fish, via its inhibition of the baso-lateral Na⁺/K⁺-ATPase in chloride cells, and it reduces Na⁺ secretion indirectly by lowering the transepithelial potential (Stagg and Shuttleworth 1982a). Copper may additionally have caused an internal ion load by increasing the ion and water permeabilities of the gills. Laurén and McDonald (1985) observed a large stimulation of the passive efflux of NaCl (reflecting an increase in permeability) in freshwater rainbow trout, an effect which presumably was due to displacement of Ca²⁺ by Cu²⁺ in tight junctions of the gill epithelium. An increased passive ion influx in seawater cod would elevate plasma [Na⁺] and [Cl⁻], and an increased water loss would lead to haemoconcentration. The data suggests a greater water loss from both intra- and extracellular spaces in Cu fish than in HCu fish. Water content of skeletal muscle was significantly lower in Cu fish than in control and HCu fish. Also, blood sampling caused a significant decrease in [Hb] and N_rbc by 25% in HCu fish, whereas in Cu fish these parameters decreased nonsignificantly and only by 12%. Since the Cu and HCu fish were of the same size and the same amount of blood was taken, the data supports the idea that haemoconcentration was largest in Cu fish. The protective effect of hypercapnia on the copper-induced osmoregulatory disturbances could have been caused by a reduced gill permeability, limiting water loss and passive ion influx. It is, however, also possible that effects on active branchial ion trans-
port, drinking rate and ion/water uptake in the gut are involved.

Red blood cell volume

The steep increase in osmolality caused a reduction in RBC volume in the two copper-exposed groups (Fig. 3). The red cell shrinkage was directly related to the degree by which plasma osmolality increased. The extreme red cell shrinkage in the Cu group may have caused a large decrease in blood O$_2$-affinity (by increasing the cellular concentration and complexing of haemoglobin and nu-
cleotide triphosphates) as demonstrated in shrunken carp red cells (Jensen 1990). Possible disturbances of blood O$_2$ transport may, however, have been limited by a high arterial Po$_2$. Plasma [lactate] only increased to 2 mM in Cu fish.

Intracellular acid-base status

The pH i control value for skeletal muscle in cod was close to that reported in fish in other studies (e.g. Höbe et al. 1984; Pörtner et al. 1990; Tang and Boutillier 1991; Wang et al. 1994). The ventricle pH i was similar to that found by Pörtner et al. (1990) for toad ventricle using the same technique.

During hypercapnia, the intracellular acidoses in skeletal and ventricle muscles were smaller and more rapidly compensated than the extracellular acidosis. This was not the case for liver, suggesting that this tissue deviates from the general trend of a more efficient intracellular than extracellular acid-base regulation. Tissue-specific differences in the velocity of acid-base regulation may predominantly be related to different capacities for transfer of acid-base equivalents across cell membranes.

During combined hypercapnia and copper exposures, the slow and incomplete extracellular compensation also slowed down the intracellular compensation. It is, however, remarkable that intracellular pH compensation in skeletal muscle was 80% complete after 5 h. It is possible that a more efficient transfer of HCO$_3^-$ equivalents from the extracellular to the intracellular space than from the water to the extracellular space caused an intracellular compensation at the expense of the extracellular compartment, as reported in Synbranchus marmoratus during air breathing (Heisler 1982).

Copper accumulation

The gills, owing to their large surface area and intimate contact with the water, are the primary target of wa-
terborne copper. As in earlier studies (e.g. Stagg and Shuttleworth 1982b; Pilgaard et al. 1994; Pelgrom et al. 1995), copper was accumulated in gill tissue. This accumulation was significant already few hours after copper exposure (Fig. 6). [Cu] increased only slightly in muscle and plasma but was also accumulated in liver. Liver [Cu] increased by about 60 and 90% in HCu and Cu fish, respectively. This is much less than reported by Stagg and Shuttleworth (1982b) and by Pilgaard et al. (1994) during copper exposures of longer duration. Copper is known to induce metallothionein production in liver and gill tissues (McCarter and Roch 1984; Roesijadi 1992). This leads to accumulation but also detoxification of the metal in these tissues, since binding of Cu to metallothionein lowers the free concentration of the metal. Copper accumulation was slightly different during hypercapnia and normocapnia. Apparently, copper accumulated faster in the gills during hyper-
capnia (Fig. 6A), while under normocapnia more Cu entered the body and ended up in the liver (Fig. 6B).

Final remarks

Mortality in copper-exposed cod seemed to be caused by osmoregulatory collapse. Hypercapnia significantly decreased the toxicity of copper, by limiting the copper-induced osmoregulatory disturbances. The protective effect of hypercapnia on osmoregulation has not previously been reported. Cod were far more sensitive to copper than seawater-adapted rainbow trout (Wilson and Taylor 1993b) and flounder (Stagg and Shuttle-
worth 1982b). It is difficult to evaluate these differences, but differences in ionregulatory mechanisms and in gill area could play a role. Furthermore, physicochemical parameters of seawater may differ between studies. Natural seawater contains organic compounds which complex with copper, and decrease the toxicity of copper (Pagenkopf 1986). The water used in the present study was made from sea salt, and the amount of organic compounds may have been less than in natural seawater, increasing the concentration of toxic copper species. Copper toxicity is also pH dependent (Pagenkopf 1986). A decrease in pH, as occurring during hypercapnia, will change the composition of copper species, and possibly toxicity.

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References

Bryan GW (1976) Heavy metal contamination in the sea. In: Jon-


Iwama GK, Heisler N (1991) Effect of environmental water salinity on acid-base regulation during environmental hypercapnia in the rainbow trout (Oncorhynchus mykiss). J exp Biol 158: 1–18


Larsen BK, Jensen FB (1997) Influence of ambient ionic composition on acid-base regulation in rainbow trout (Oncorhynchus mykiss) exposed to environmental hypercapnia. Fish Physiol Biochem (in press)

Lauren DJ, McDonald DG (1985) Effects of copper on branchial ionoregulation in the rainbow trout, Salmo gairdneri, Richardson. J comp Physiol (B) 155: 635–644


Perry SF (1982) The regulation of hypercapnic acidosis in two salmonids, the freshwater trout (Salmo gairdneri) and the seawater salmon (Oncorhynchus kisutch). Mar Behav Physiol 9: 73–79


Pigaard L, Malte H, Jensen FB (1994) Physiological effects and tissue accumulation of copper in freshwater rainbow trout (Oncorhynchus mykiss) under normoxic and hypoxic conditions. Aquat Toxic 29: 197–212


Stagg RM, Shuttleworth TJ (1982a) The effects of copper on ionic regulation by the gills of the seawater-adapted flounder (Platichthys flesus L.). J comp Physiol (B) 149: 83–90


Wilson RW, Taylor EW (1993b) Differential responses to copper in rainbow trout (Oncorhynchus mykiss) acclimated to sea water and brackish water. J comp Physiol (B) 163: 239–246