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Effects of sublethal and lethal copper levels on hemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted sea water

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Abstract

Hemolymph acid-base balance and ion concentrations were measured in shore crabs, *Carcinus maenas* (L.), kept in full-strength sea water (~33‰ S) and exposed to sublethal (0.5 mg l^{-1}) and lethal (1 and 2 mg l^{-1}) levels of copper in water. The study was conducted throughout the year 1987 on crabs collected near Arcachon (France). Whatever the dose, waterborne copper induced metabolic acidosis without marked changes of hemolymph ion concentrations. At the sublethal copper level, the acidosis was non-lactic and partly compensated by transitory hypocapnia. Complete recovery was observed within 20 d. At intermediate and lethal copper levels, this primary acidosis was later reinforced by hypercapnia and accumulation of lactic acid, indicating that restriction of respiratory gas exchange is the probable cause of death. A steep increase in the hemolymph calcium concentration before death suggests buffering of the acidosis by calcium-carbonate stores from the exoskeleton.

Introduction

Of natural origin or not, many metal ions are presently found in aquatic environments. Copper levels, for example, amount to 0.5 to 20 $\mu g\, l^{-1}$ in unpolluted marine waters (Baker 1969, Bjerregaard and Vislie 1986), but anthropogenic inputs can result in much higher values, around 1 mg l^{-1} (Bjerregaard and Vislie 1986).

Many studies of the effects of waterborne heavy-metal pollutants have been performed on aquatic organisms. Most are concerned with toxicity tests, but often information about the kind of physiopathological disorders which may account for the effects of the pollutant in lethal and especially sublethal conditions is lacking. This presumably results from the fact that the most important target(s) of the toxic action is not yet known with certainty. Heavy-metal ions presumably bind to various protein structures (-SH groups for example) and thereby may inhibit or activate multiple processes leading to the disorganization of various functions. Among these, those concerned with exchanges between the organism and its environment are without doubt of primary importance. Hence, the gills, the main site of respiratory and ionic exchanges, are often considered as the first target of waterborne pollutants. As a result, it is commonly thought that mortality of aquatic animals at lethal doses of heavy metals is mainly linked to a disruption of the gill function. Indeed, previous studies in fish have shown that the gill structure is profoundly affected by high concentrations of copper or other metal ions in water (Baker 1969, Skidmore and Towell 1972, Olson et al. 1973). Such branchial damage obviously impedes respiratory gas-exchange, leading to respiratory disturbances, with internal hypoxia. Many investigations have also documented marked disturbances of ionic regulation in fishes exposed to heavy metals in water. While hypoionic marine teleost fish tend to gain ions, freshwater teleost demineralize upon exposure to copper (Stagg and Shuttleworth 1982a, Lauren and McDonald 1985). In addition, it has been recently reported that sublethal levels of heavy metals induce extracellular acid-base perturbations. Fish exposed to zinc or aluminium displayed mainly lactic extracellular acidosis (Spry and Wood 1985, Jensen and Weber 1987).

Relatively few studies have been devoted so far to the physiological effects of sublethal doses of heavy-metal pollutants on crustaceans. Euryhaline crabs, such as the shore crab *Carcinus maenas*, hyperosmoregulate in diluted media but are isosmotic and nearly isoionic in full-strength sea water. When exposed to sublethal and lethal copper concentrations in diluted sea water, shore crabs lose chloride and sodium, as do freshwater teleosts (Bjerregaard and Vislie 1986). Such ionic disturbances would be expected to be of lesser magnitude in undiluted sea water. On the other hand, nothing is presently known about the possible effects of heavy metals on acid-base balance in crustaceans.

Therefore, the present study investigated the effects of sublethal and lethal copper concentrations on hemolymph

acid-base and ionic status in shore crabs maintained in full-strength sea water.

Materials and methods

Male *Carcinus maenas* (L.), of 40 to 70 g wet wt, were collected near Arcachon (France) and kept in tanks with aerated running sea water (~33‰ S) for several weeks throughout the year 1987. They were fed weekly with frozen mussels until the beginning of the experiments.

Experimental protocols

For each experiment, a group of six or ten crabs at the intermoult stage was held in a glass aquarium containing 130 litres undiluted sea water (~33‰ S) thermostatted at 15 °C. Steady bubbling of CO_2-free air prior to the introduction of the crabs and during the entire exposure period ensured normoxic ambient conditions. Using a pH-CO_2-stat (Dejours and Armand 1980) and maintaining a constant water-titration alkalinity (TA) by appropriate corrections, the partial pressure of carbon dioxide was regulated at a constant value of 0.75 torr. Copper was added as a $CuCl_2$ solution in sea water to simulate a pulse input of contaminant with subsequent ageing. The toxic material was not replenished during the course of an experiment, but serial measurements by atomic absorption spectrophotometry (GBC, Model 901) of the total copper in the water showed that initial levels were maintained.

Four series of experiments were conducted. One control series (C) without metal added (copper levels undetectable) tested for the effects of maintenance conditions and sampling. In three experimental series, different total copper concentrations were used: a sublethal level (S) of 0.5 mg l^{-1}, at which no mortality was observed up to 20 d; an intermediate level (I) of 1 mg l^{-1}, at which limited mortality occurred after 4 d (median lethal time = ~12 d); and a lethal level (L) of 2 mg l^{-1}, at which all crabs died within 3 to 10 d (median lethal time = 5 d). For these three series, the crabs were acclimated for 24 h in normal sea water before addition of copper. Water temperature, oxygen partial pressure (P_{O_2}), pH, TA and ionic concentrations were checked daily, and the range of values is given in Table 1. Within each series, these parameters remained almost constant, but water composition differed slightly between series. Venous hemolymph samples, of ~0.7 ml, drawn by syringe and needle puncture at the base of a walking leg, were repetitively obtained from each crab before copper addition and then at various time intervals during metal exposure, which lasted from 10 d (lethal level) to up to 20 d (sublethal level).

Measurement techniques

Water pH and P_{O_2} were measured with Radiometer electrodes (GK 2401 C and E 5046/0, respectively) at 15 °C. Water TA was determined by a Gran titration procedure (Anderson and Robinson 1946, Culberson et al. 1970). The chloride concentration in hemolymph and water was determined coulometrically with a Radiometer CMT 10 chloride titrator; these measurements allowed the calculation of water salinity. Vapour pressure osmometry (Wescor 5500) was used for osmotic pressure determinations in blood and water. Hemolymph and water concentrations of sodium, potassium and total calcium were obtained by flame photometry (Eppendorf FCM 6341). Blood pH (pH_b) was assayed with a thermostatted capillary pH electrode (Radiometer G 299 A), while partial pressure of CO_2 in blood (P_{bCO_2}) was determined by interpolation using the Astrup equilibration method (Astrup 1956). Bicarbonate concentration ($[HCO_3^- + CO_3^{2-}]$) was calculated with a solubility coefficient and dissociation constants from Truchot (1976). Total blood L-lactate concentration was measured by an enzymatic method (Boehringer Kit No. 139084) on a deproteinized aliquot of the sample with 8% perchloric acid.

Table 1. Water composition. Range of values for some parameters measured each day in sea water sampled from the experimental system throughout experimental series. P_{O_2}: partial pressure of oxygen; P_{CO_2}: partial pressure of carbon dioxide; TA: water-titration alkalinity

Parameter	Range	
	min.	max.
T (°C)	14.8	15.1
P_{O_2}	air	saturation
pH	7.83	7.97
TA (meq l^{-1})	2.26	2.85
P_{CO_2} (torr)	0.74	0.76
$[Cl^-]$ (mmol l^{-1})	513	544
$[Na^+]$ (mmol l^{-1})	445	472
$[K^+]$ (mmol l^{-1})	9.3	10.2
$[Ca^{2+}]$ (mmol l^{-1})	8.9	10.8

Data presentation

Mean values for all parameters are reported ±1 SEM, except at the lethal copper level a few days before death, since at this stage data were no longer representative of the group as a whole; two examples of surviving individuals at this stage are shown in Figs. 1 and 4. For all series, the number n of crabs still alive at Time t, is indicated in legend to Fig. 1. Within each series, the statistical significance of differences between the reference mean value (N), i.e., before copper addition, and means obtained during copper exposure were evaluated using Student's paired t-tests in series where no mortality occurred (each crab serving as its own control). Student's unpaired t-tests were applied in series where mortality occurred during copper exposure.

Results

At the three tested concentrations of the toxicant, *Carcinus maenas* became clearly agitated within a few minutes after

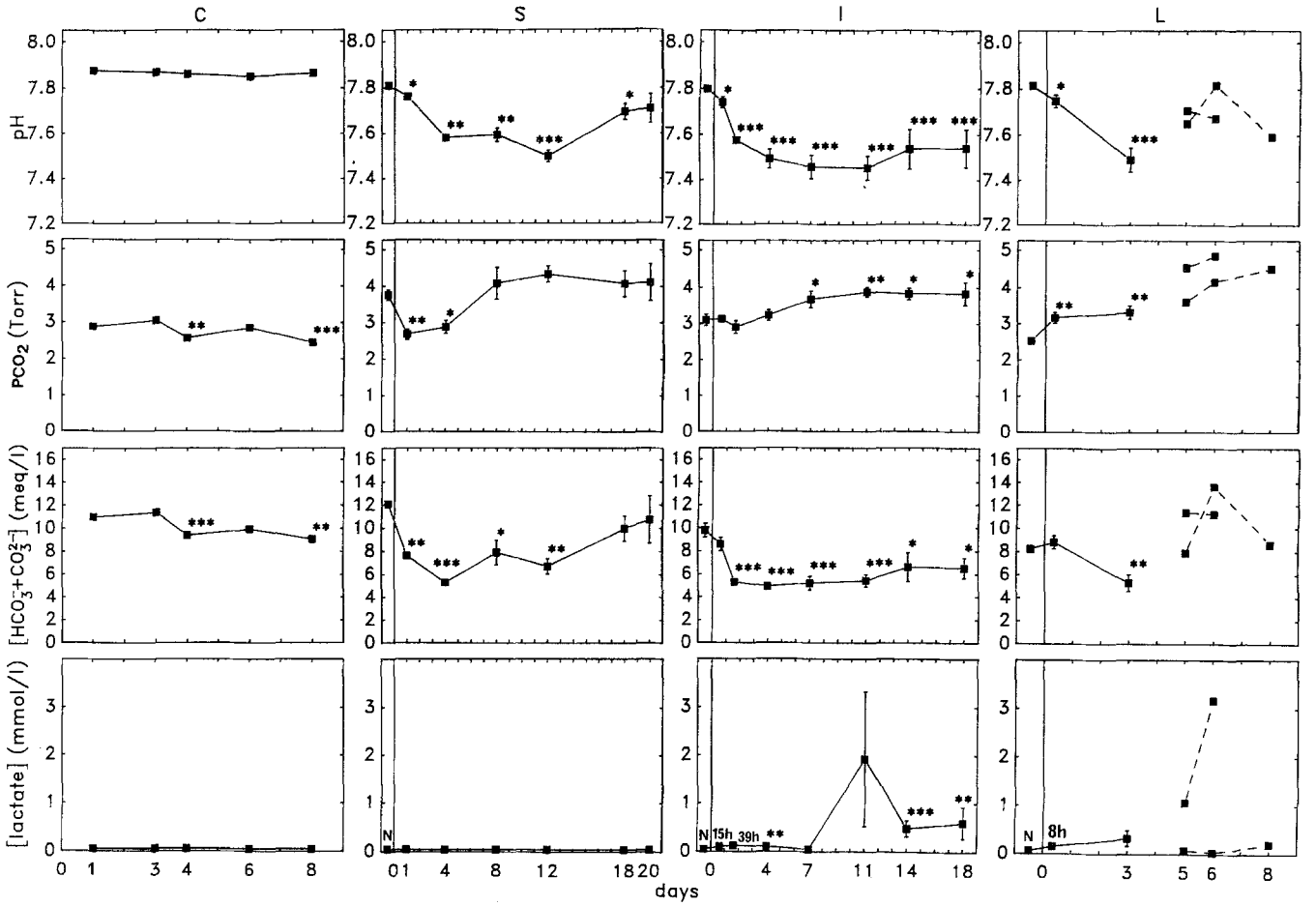

Fig. 1. *Carcinus maenas*. Time-dependent changes in hemolymph acid-base parameters pH, CO_2 partial pressure (P_{CO_2}), bicarbonate plus carbonate concentration ($[HCO_3^- + CO_3^{2-}]$) and blood-lactate concentration in control (C) crabs and in crabs exposed to 0.5, 1.0 and 2.0 mg Cu l^{-1} (S, I and L, respectively). Copper exposure began at Time 0, after measuring a reference value (N) plotted on left of Graphs S, I and L. $n=10$ and 6 individuals for all data points in C and S, respectively. For I, n declined with time, being, from left to right, 10, 10, 10, 9, 7, 6, 3, 3; for L, n was 10, 10, 9 for the first three time points, dashed lines show two examples among surviving individuals for the remainder of the exposure (6, 4 and 2 individuals remained alive at the last three time points, respectively). All values except those plotted on dashed lines are means ± SEM; asterisks denote means significantly different from N (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), using paired Student's t-test for C and S and unpaired Student's t-test for I and L

addition of copper. This increase in locomotory activity, probably corresponding to an escape behaviour, lasted several tens of minutes. Then, the crabs settled and became as quiet as before contamination.

Blood acid-base status

Fig. 1 shows the time course of blood pH, P_{CO_2}, bicarbonate and lactate concentrations for control individuals and for crabs at the three copper levels tested.

There were no major changes in acid-base balance in crabs kept in uncontaminated water (Fig. 1 C). pH_b remained steady, P_{bCO_2} and $[HCO_3^- + CO_3^{2-}]$ showed only a slight trend to decrease, and there was no sign of lactate in hemolymph. In contrast, important acid-base disturbances took place in all crabs exposed to copper. The pattern of these changes characteristically depended on the dose.

At the sublethal level of 0.5 mg l^{-1} (Fig. 1 S), blood pH decreased slowly but significantly from 7.80 to a minimum of 7.45 by 12 d, and then recovered to values not significantly different from control by 20 d. Blood P_{CO_2} transiently decreased during the first 4 d, retained control values by 8 d, and remained at this level for the rest of the exposure period. Bicarbonate concentration decreased rapidly from 11.13 to 4.79 meq l^{-1} at 4 d, and then recovered slowly, reaching a value not significantly different from control by 20 d. $[HCO_3^- + CO_3^{2-}]$ plotted as a function of pH (Fig. 2) revealed the main component of the acidosis to be metabolic, the pH decrease being limited by blood hypocapnia during the first 4 d of copper exposure. However, the lactate concentration remained very low (Fig. 1 S). Thus, sublethal copper exposure elicited non-lactic, reversible metabolic acidosis.

At the intermediate copper level of 1 mg l^{-1} (Fig. 1 I), there was also initially non-lactic acidosis, but without significant changes in blood P_{CO_2}. From 7 d onwards, P_{bCO_2} increased significantly, and lactate appeared in the hemolymph, especially in those individuals which became moribund. At the end of 18 d, pH_b and bicarbonate concentra-

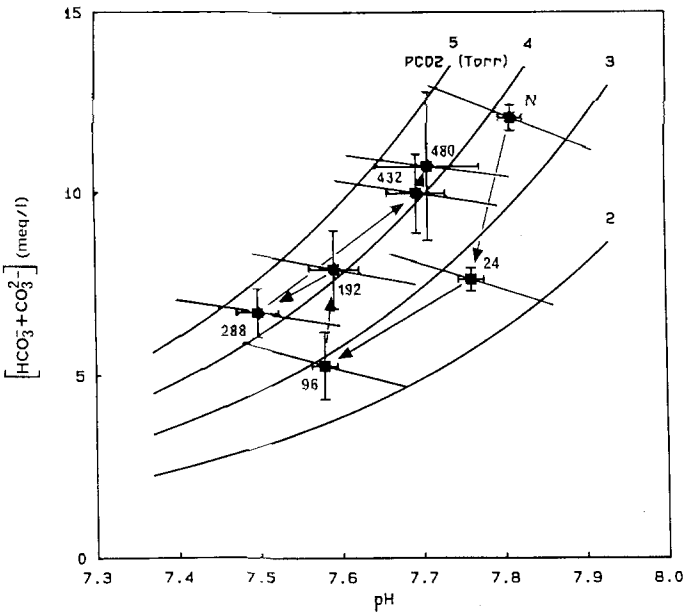

Fig. 2. *Carcinus maenas*. Changes in prebranchial hemolymph acid-base status of crabs exposed to 0.5 mg Cu l^{-1}. Diagram shows $[HCO_3^- + CO_3^{2-}]$ vs pH and includes carbon-dioxide-tension isopleths for 5, 4, 3 and 2 torr (15 °C; 32.5‰ S); *N*: reference value before copper addition. Values near each data point indicate time of exposure to copper, in hours; time-course is indicated by arrowed lines. Values are means ± SEM ($n = 5$). Straight oblique lines through each data point are mean *in vitro* buffer data

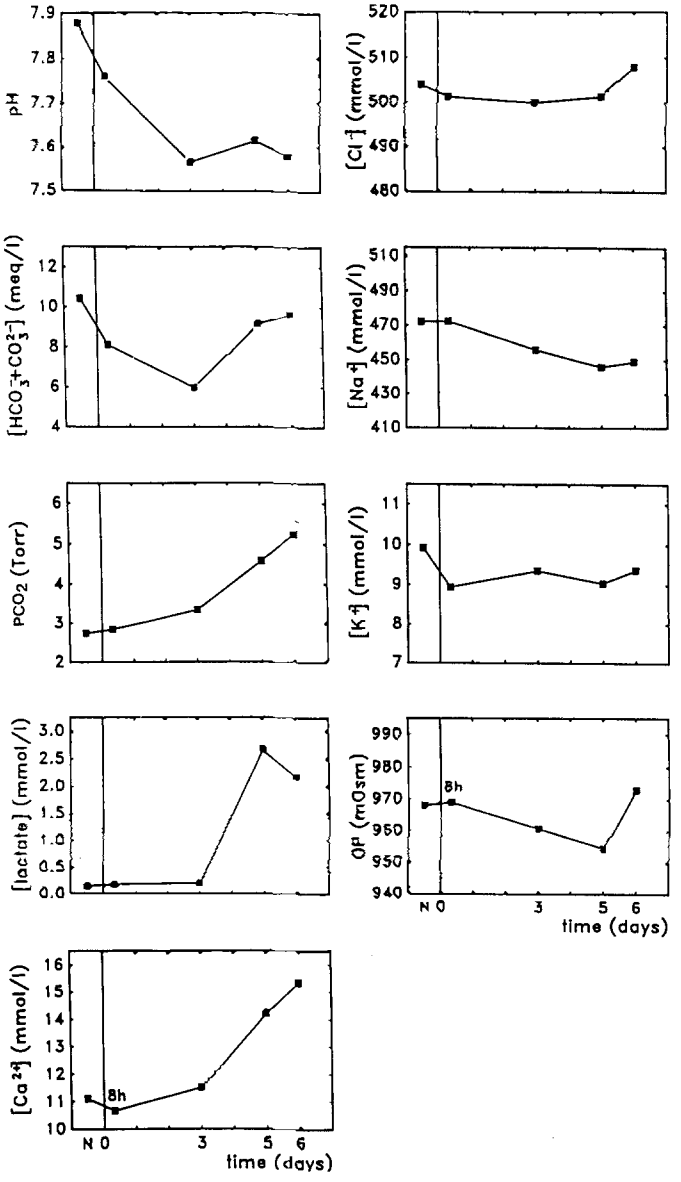

Fig. 3. *Carcinus maenas*. Time-dependent changes in blood acid-base parameters (pH, bicarbonate concentration ($[HCO_3^- + CO_3^{2-}]$) and CO_2 partial pressure (P_{CO_2})), lactate, ion concentrations and osmotic pressure (OP) in one representative crab from experimental series at 2 mg Cu l^{-1} prior to (reference value *N*) and during environmental copper exposure

tion were still low and there was no evidence of acid-base recovery in individuals surviving copper exposure.

At the lethal copper level of 2 mg l^{-1} (Fig. 1 L), the pattern of acid-base variations was the same for all crabs, but differed considerably in time-course between individuals, probably reflecting differential resistances to the toxicant. The most important changes apparently took place rapidly during the few hours before death, and were not fully observed in all crabs since the time of death could not be predicted. Therefore, Fig. 1 L gives mean values only up to 3 d, during which period all but one crab remained alive, and two individual examples for the remainder of the exposure period. A representative pattern of acid-base changes at this copper level is also illustrated for an individual in Fig. 3. There was initially both metabolic and respiratory acidosis, since blood P_{CO_2} increased significantly compared to control, with a moderate rise in the lactate level (Fig. 1 L). Immediately preceding death, blood pH and bicarbonate levels either remained constant or increased, whereas strong hypercapnia developed and lactate often increased abruptly, as illustrated in Fig. 3.

Hemolymph ions

Fig. 4 shows the time-course of hemolymph chloride, sodium, potassium and calcium concentrations for crabs maintained in uncontaminated sea water or exposed to copper. Since the patterns of changes were practically the same for copper levels of 0.5 and 1 mg l^{-1}, only the data obtained at 1 mg l^{-1} are shown.

In control crabs (Fig. 4 C), relatively few changes were observed in blood-ion levels, except for chloride concentration which increased slightly but significantly.

Exposure to 1 mg l^{-1} copper (Fig. 4 I) produced characteristic initial disturbances during the first 1 to 2 d, with increased chloride, sodium and calcium concentrations and a steep decrease in potassium concentration. From the fourth day onwards, $[Cl^-]$ and $[Na^+]$ gradually increased, $[K^+]$ reached levels slightly higher than control, and $[Ca^{2+}]$ remained nearly constant at moderately elevated values. Although significant, none of these changes was important;

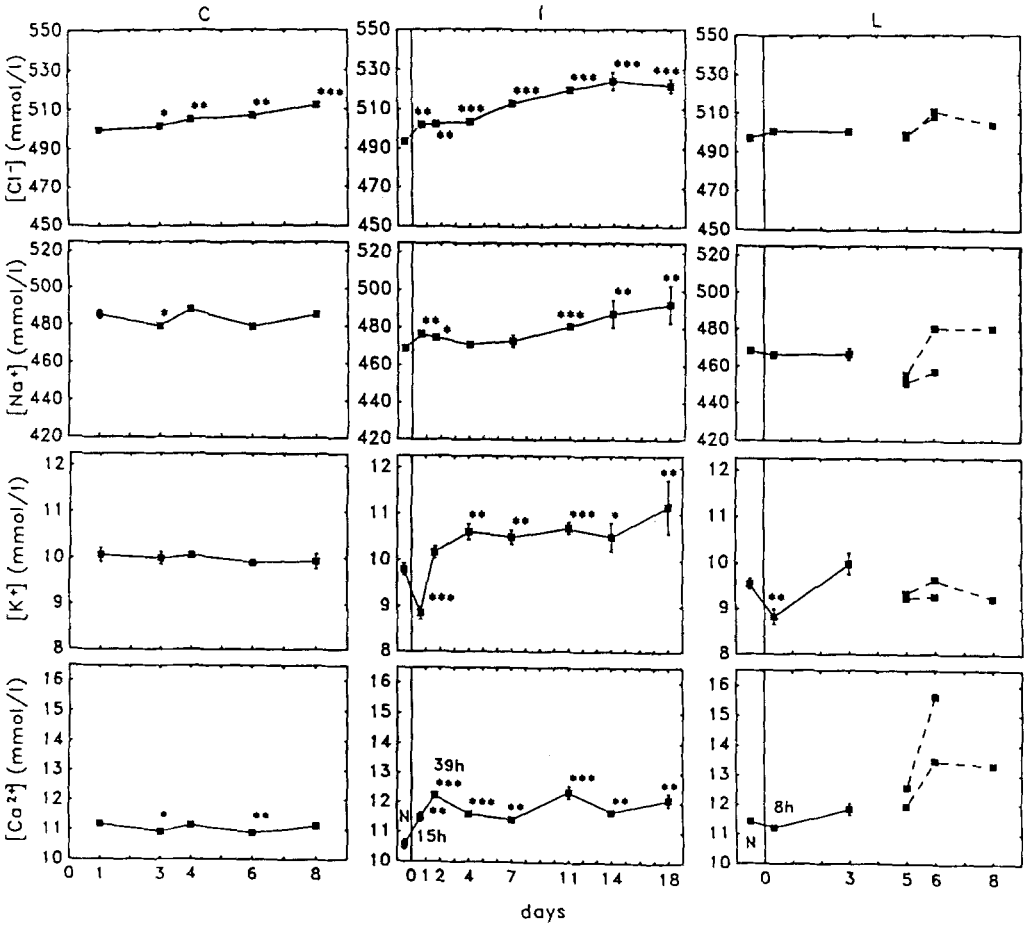

Fig. 4. *Carcinus maenas*. Time-dependent changes in hemolymph ion concentrations in controls (C) crabs and in crabs exposed to 1 (I) and 2 (L) mg Cu l^{-1}. Further details as in legend to Fig. 1

e.g. [Cl^-], [Na^+] and osmolality (data not shown) never increased by more than 4 to 5% above control values.

Fig. 4 L shows mean values of hemolymph ion concentrations during the first 3 d exposure to a lethal copper level of 2 mg l^{-1}. Except for a transient decrease in potassium concentration, no significant changes were observed. Fig. 4 L also illustrates two examples of individual patterns of hemolymph-ion variations before death; a third example is shown in Fig. 3 together with acid-base and lactate data. No major changes in chloride, sodium, or potassium concentrations were observed before death. In contrast, the hemolymph calcium concentration increased steeply, coinciding with parallel elevations of blood P_{CO_2} and lactate concentration (Fig. 3).

Discussion

Our results indicate that copper exposure elicits profound extracellular acid-base disturbances without major changes in hemolymph ion concentrations in shore crabs, *Carcinus maenas*, kept in undiluted sea water. Whatever the dose, waterborne copper initially induced metabolic, non-lactic acidosis. At a sublethal copper level of 0.5 mg l^{-1}, complete recovery was observed after 20 d. At higher lethal levels, lactacidosis and hypercapnia additionally developed before death.

The present study has concentrated on long-term effects of metal exposure. As already noted by Depledge (1984), we observed that shore crabs exposed to copper exhibited increased locomotory activity for a few tens of minutes. The significance of this active response remains to be established, but Depledge has shown it to be accompanied by marked changes in cardiac and respiratory activities.

Most studies on the toxicity of waterborne heavy metals in aquatic organisms have stressed that the first and most important effects are at the gill level. Ion regulation as well as respiratory function may be affected. In teleost fish, copper for example is thought to increase the passive ion permeability and to inhibit active ion transport at the gill (Lorz and McPherson 1976, Stagg and Shuttleworth 1982b, Lauren and McDonald 1985). This results in salt loss in hyperionic freshwater fish (McKim et al. 1970, Lewis and Lewis 1971, Stagg and Shuttleworth 1982a, Lauren and McDonald 1985) and on increase of plasma electrolytes in hypoionic sea water fish (Cardeilhac et al. 1979, Stagg and Shuttleworth 1982a). In full-strength sea water, marine decapods such as the shore crab *Carcinus maenas* are nearly isosmotic and isoionic and chloride and sodium seem to be

distributed passively between blood and medium (Zanders 1980). Accordingly, our data show relatively few changes in hemolymph ion concentrations upon exposure to copper, in contrast to low-salinity-acclimated hyperionic crabs, in which a marked decrease in hemolymph osmolality, chloride and sodium concentrations have been found (Thurberg et al. 1973, Bjerregaard and Vislie 1986). Nevertheless, at least at a copper level of 1 mg l^{-1}, we did observe a slight, progressive increase in hemolymph chloride and sodium concentrations. These changes could not be related to a parallel rise in water ion concentrations, but may possibly be linked to a shift of water from the extracellular fluid to the tissues. Also, a consistent but transient decrease in hemolymph potassium concentration took place after copper addition, which agrees with the observation of a significant potassium loss in trout by Lauren and McDonald (1985).

In fish, exposure to heavy metals not only disrupts ionic balance but also induces extracellular acid-base changes (see "Introduction"). Large disturbances of the hemolymph acid-base balance were also found in shore crabs exposed to copper. Their pattern and time course differed according to the dose applied.

In sublethal conditions (0.5 mg l^{-1}), the slowly developing metabolic acidosis was initially limited by concomitant hypocapnia, which may have been linked to increased gill ventilation. Hyperventilatory response was observed in fish upon zinc exposure by Hughes and Adeney (1977). Furthermore, this acid-base disturbance was reversible in the long term, blood pH and bicarbonate having regained control levels after 20 d. Since hemolymph lactate levels remained low throughout the exposure period, the metabolic acidosis probably originated from perturbations of the inorganic anion and cation balance and subsequent changes in the strong ion difference (SID); i.e., even if copper exposure apparently induced only minor disturbances in ionic exchanges, these disturbances were large enough to profoundly affect the acid-base balance. Unfortunately, the limited number of ions measured as well as the high background levels of hemolymph chloride and sodium in sea-water crabs precludes any accurate estimation of concomitant SID changes.

At an intermediate copper level of 1 mg l^{-1}, metabolic acidosis developed more rapidly, without compensatory hypocapnia, and no evidence of recovery was found. Rather, partial mortality was accompanied by a significant increase in hemolymph P_{CO_2} and lactacidosis. These features were much accentuated at the rapidly lethal dose of 2 mg l^{-1} copper, where increases in P_{bCO_2} and lactate were evident on the first day of exposure, becoming very marked a few hours before death. This indicates that the toxic dose probably greatly restricted respiratory gas-exchange, ultimately leading to tissue hypoxia and death. Interestingly, hemolymph pH and bicarbonate concentration did not decrease much at this stage, but tended to stay constant or increase. The marked concomitant rise in hemolymph calcium levels, which was also noted by Bjerregaard and Vislie (1986), suggests that a large acid load may have been buffered by calcium-carbonate stores, probably from the exoskeleton.

Comparison of the results obtained in the three experimental series throws some light upon the causes of death of crabs exposed to waterborne copper. In no case could the acidosis be considered lethal. At least in the initial phase of intoxication its extent was not dose-dependent, being similar in magnitude in both lethal and sublethal conditions. Further, acidosis was reversed slowly but completely at the sublethal copper level. Finally, despite a marked increase in hemolymph lactate, dying crabs were hypercapnic but not particularly acidotic. Most probably, death was linked neither to ionic disturbances nor to acid-base changes, but rather to a reduced efficiency of respiratory gas-exchange at the gills.

In summary, our data allow us to delineate the succession of events which characterize copper intoxication in seawater-acclimated shore crabs. Ionic exchanges appear to be first affected, leading to slight changes in blood ion concentrations but to a marked non-lactic acidosis. At a sublethal level, this metabolic acidosis is reversible, with no sign of respiratory dysfunction. At a toxic level, respiratory gas-exchanges are additionally affected, most probably because of anatomical gill damage (own unpublished observations). The rate of disruption of the respiratory function increases with increasing copper level, inducing internal hypoxia which is probably the primary lethal mechanism.

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