

Acid-base status, haemolymph composition and tissue copper accumulation in the shore crab *Carcinus maenas* exposed to combined copper and salinity stress

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ABSTRACT: The effects of copper exposure on the shore crab *Carcinus maenas* (L.) were investigated at constant and changing salinities. Crabs were acclimated to 20 ppt artificial seawater (SW) for 7 d at 15 °C, and were subsequently assigned to 1 of 6 groups: (1) maintained in 20 ppt SW; (2) transferred to 10 ppt SW; (3) transferred to 30 ppt SW; (4) exposed to copper (0.75 mg Cu l⁻¹) in 20 ppt SW; (5) exposed to copper with concurrent salinity change to 10 ppt SW; or (6) exposed to copper with concurrent salinity change to 30 ppt SW. Salinity change was associated with altered total ionic concentration of the haemolymph but only small changes in acid-base status. Copper exposure induced metabolic acidosis, with a progressive decrease in haemolymph pH and HCO₃⁻, both at constant and changing salinities. Thus, the effects of copper on acid-base status overrode the effects of salinity change. The acidosis in copper-exposed crabs was paralleled by a rise in haemolymph Ca⁺⁺. Crabs in poor condition and close to death exhibited low haemolymph pH values, suggesting that haemolymph pH may be a useful biomarker of physiological status. With metal exposure, copper accumulated in the gills and midgut gland but not in muscle. The accumulation of copper in gill tissue was positively correlated with salinity. During copper exposure the haemolymph copper concentration decreased. Haemolymph total protein and haemocyanin remained constant in crabs which were not exposed to copper, whereas these parameters decreased in copper-exposed crabs.

INTRODUCTION

Environmental changes often disturb the acid-base balance in aquatic animals. Both salinity change and copper exposure affect acid-base balance in crustaceans. In the shore crab *Carcinus maenas* (L.), transfer from full-strength seawater to dilute seawater induces metabolic alkalosis, while the reverse transfer induces an acidosis (Truchot 1981). Exposure to copper typically induces metabolic acidosis (Boitel & Truchot 1989, 1990). Responses to copper exposure with concurrent salinity changes are unknown. In Danish coastal waters, however, shore crabs inhabit waters of fluctuating salinity. Therefore the effects of exposure to another environ-

mental stressor (e.g. copper) should be considered in the light of effects due to concurrent changes in salinity.

A decrease in salinity is often associated with an increased uptake rate of trace metals (especially zinc and cadmium) in marine crustaceans (Nugegoda & Rainbow 1989a, b). Thus metal toxicity is potentially enhanced at low salinities due to the increased bio-availability of free metal ions (Zirone & Yamamoto 1972). Such metal species have been identified as the principal chemical form bioavailable to organisms (Depledge & Rainbow 1990). *Carcinus maenas*, however, is a resilient euryhaline crustacean, and there is the possibility that physiological adaptation to dilute media may not only help this crab to minimise ionic and osmotic fluxes, but also impair the uptake of dissolved trace metals (Depledge 1990).

Carcinus maenas is one of the most studied crustacean species (at least in northern climes). With regard

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to the responses of *C. maenas* to pollutants, the effects of copper have been studied most intensively. Copper uptake occurs both from food and seawater (see Depledge & Rainbow 1990). Copper that is not bound to metallothionein in tissues at the site of entry (i.e. the gill when the seawater route is involved or in the midgut gland when uptake is via the food route) or to other cellular constituents, enters the haemolymph where non-specific binding to haemolymph proteins occurs (principally haemocyanin). The copper is then distributed among the tissues with accumulation occurring in the order: gill > midgut gland > muscle > carapace (Depledge 1989). Depending on exposure concentration, copper has been shown to alter tissue respiration rates (Kerkut & Munday 1962), disturb circulatory and ventilatory activity (Depledge 1984a), disrupt feeding behaviour and endogenous rhythms (Depledge 1984b), and impair osmoregulatory ability (Bjerregaard & Vislie 1986). Recent studies by Hansen et al. (1992a, b) indicate that the biochemical mechanism underlying loss of osmoregulatory ability involved the inhibition of gill Na^+, K^+ -ATPase activity by copper ions. Other important effects following copper exposure included marked depression of the activities of phosphofructokinase in midgut gland and citrate synthetase in posterior gills. Also, lactate levels in gills, haemolymph and midgut gland were raised, as was glucose concentration in the haemolymph, indicating altered utilisation of aerobic vs anaerobic metabolic pathways. Energy charge potential was unaffected (Hansen et al. 1992b). Such changes are associated with the development of pathological processes leading to death.

In the present study the effects of copper on *Carcinus maenas* at both constant and changing salinity has been examined. In particular, changes in haemolymph composition and acid-base balance were investigated.

MATERIALS AND METHODS

Experimental crabs. Adult male *Carcinus maenas* were caught in seine nets from the coastal waters around Fyn, Denmark. The salinity of the water in which the crabs were caught varied approximately between 17 and 20 ppt (depending on prevailing wind and current). Crabs were held subsequently in running seawater (SW) (with a salinity of ca 20 ppt) at the Odense University Marine Station at Bøgebjerggaard prior to use in experiments. Intermoult crabs (40 to 50 g wet wt) were assigned to one of twenty-four 10 l acid-washed aquaria (with 4 crabs per aquarium). Crabs were acclimated to 20 ppt artificial seawater (hw marinemix + Bioelements, Germany; background copper concentra-

tion $5 \mu\text{g Cu l}^{-1}$) at 15°C , under constant aeration and a 12 h light:12 h dark regime for 7 d prior to experimentation. The water was changed daily. Crabs were not fed during the acclimation and experimental periods. Subsequently, they were assigned to 1 of 6 groups: (1) maintained in 20 ppt SW; (2) transferred to 10 ppt SW; (3) transferred to 30 ppt SW; (4) exposed to copper ($0.75 \text{ mg Cu l}^{-1}$) in 20 ppt SW; (5) exposed to copper with a concurrent salinity change to 10 ppt SW; or (6) exposed to copper with concurrent salinity change to 30 ppt SW. Copper was added from a stock solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (analytical grade, Merck, Germany).

Haemolymph samples were taken from crabs on Day 0 (i.e. control crabs in 20 ppt SW, the normal salinity for the population from the southern Kattegat used in these experiments) and thereafter on Days 1, 2, 4 and 7. Sampling involved quickly withdrawing ca 800 μl of haemolymph from each crab using a hypodermic needle inserted through the arthroal membrane at the base of the 4th or 5th walking leg. These samples were used for the determination of ionic haemolymph composition. A separate group of crabs were used for investigating acid-base effects. These were sampled (200 μl) at Times 0, 2 h, 1 d, 3 d and 7 d. All crabs were individually marked and sampled repeatedly throughout the experiment.

At the end of the experiment, the crabs were sacrificed and samples of midgut gland, gill and muscle tissue were removed, weighed and freeze-dried prior to metal analysis.

Analytical procedures. Haemolymph pH was measured immediately after sampling using the capillary pH-electrode of a Radiometer BMS 3 electrode assembly (Copenhagen, Denmark). Haemolymph total carbon dioxide content (C_{CO_2}) was determined by the method of Cameron (1971). Partial pressure of carbon dioxide P_{CO_2} was calculated from:

$$P_{\text{CO}_2} = \frac{C_{\text{CO}_2}}{\alpha_{\text{CO}_2}(10^{(\text{pH} - \text{pK}') + 1})} \quad (1)$$

and $[\text{HCO}_3^-]$ from:

$$[\text{HCO}_3^-] = C_{\text{CO}_2} - (\alpha_{\text{CO}_2} \cdot P_{\text{CO}_2}) \quad (2)$$

using α_{CO_2} (the CO_2 solubility) and pK' values predicted by the formulae of Heisler (1989).

Haemolymph chloride concentrations were determined colourimetrically (Radiometer CMT 10). After acid digestion of 0.05 ml haemolymph samples, sodium, potassium, magnesium, calcium and copper total contents were measured by flame atomic absorption spectrophotometry (Perkin-Elmer 2380). To eliminate interactions among the various ions during analysis, mixed standards with ion concentrations similar to those in crab haemolymph were used. La_2O_3 was added to prevent phosphate interactions. Concentrations of haemocyanin ($[\text{Hc}]$ in mmol O_2 binding sites

l^{-1}) were determined from the absorption peak near 335 nm of 1:9 dilutions of haemolymph samples, using a millimolar extinction coefficient of 17.3. The remainder of each haemolymph sample was stored at -18°C for later use. Protein concentrations were measured by the Lowry technique (Lowry et al. 1951).

Dried tissue samples were digested at 100°C with 65% nitric acid (analytical grade, Merck, Germany) and evaporated to dryness. The residue was then re-dissolved in 2 ml 0.2% HNO_3 . Digests (and/or dilutions thereof as appropriate) were analysed for total copper using atomic absorption spectrophotometry (flame atomisation, Perkin-Elmer 2380). All tissue metal concentrations are expressed in $\mu\text{g g}^{-1}$ dry wt.

Data are presented as means \pm standard error and the significance of differences between means ($p < 0.05$) was tested using 2-tailed Student's *t*-tests or by analysis of variance (ANOVA), both *a priori* to test for differences amongst all means, and *a posteriori* for differences between selected groups according to Sokal & Rohlf (1981).

RESULTS

Acid-base disturbances

Salinity changes alone were associated with only minor acid-base disturbances. When salinity was lowered from 20 to 10 ppt, haemolymph pH remained at ca 7.84 during the 7 d of exposure (Fig. 1). An increase in salinity from 20 to 30 ppt caused a minor metabolic acidosis after 7 d. Exposure to copper was associated with significantly larger acid-base disturbances, both at constant and changing salinities. Crabs exposed to copper at constant salinity (20 ppt) gradually developed a metabolic acidosis with haemolymph pH and $[\text{HCO}_3^-]$ declining whilst carbon dioxide tension was almost unchanged (Fig. 1, middle panel). Copper-exposure together with simultaneous salinity changes from either 20 to 10 ppt or from 20 to 30 ppt also led to typical metabolic acidoses (Fig. 1 upper and lower panels). In crabs kept at 20 ppt in the absence of copper, haemolymph pH was 7.84. The degree of depression of haemolymph pH after 7 d of copper exposure was dependent on salinity. The most severe acidosis occurred in copper-exposed crabs at 10 ppt with haemolymph pH falling to 7.58. At a salinity of 20 ppt, haemolymph pH fell to 7.64 while at 30 ppt, pH was reduced to 7.70.

Effects on haemolymph ion composition

A change in salinity in the absence of copper caused only limited changes in the major haemolymph ions, characterised by a minor increase in the total haemo-

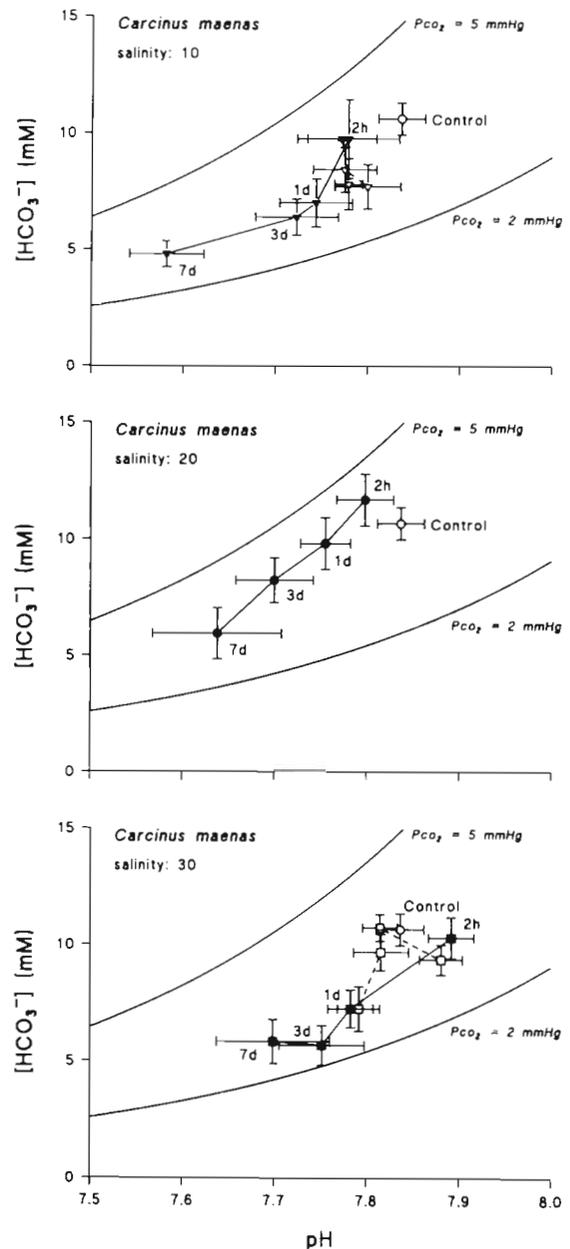


Fig. 1. *Carcinus maenas*. Haemolymph $[\text{HCO}_3^-]$ vs pH diagram with PCO_2 isopleths depicting the changes in extracellular acid-base status (means \pm SEM) of shore crabs exposed to $0.75 \text{ mg Cu l}^{-1}$ at constant (20 ppt) salinity (middle panel) or with a concurrent salinity change to 10 ppt (upper panel) or 30 ppt (lower panel). (○) Control acid-base status of crabs at salinity 20 ppt; (●, ●, ■) copper-exposed crabs; (▽, □) crabs exposed to salinity change alone. Numbers at points indicate time of copper exposure in hours (h) and days (d)

lymph ionic concentrations following an increase in salinity. Conversely, a small reduction in total haemolymph ions occurred after salinity was lowered. The data reflect the well-known ability of *Carcinus maenas* to regulate haemolymph osmolality at an almost con-

stant level in the face of changing external osmolalities. Analysis of the data by ANOVA has shown that in the presence of copper, there was a significant decrease in haemolymph Na^+ (21%, $p < 0.01$) and Cl^- (11%, $p < 0.05$) concentrations when the crabs were exposed to a salinity of 10 ppt (Fig. 2a, b). Similarly, concomitant exposure to copper and a salinity change to 30 ppt resulted in a significant (30%, $p < 0.01$) increase in haemolymph Cl^- to values above those measured in crabs not exposed to copper (Fig. 2b). Thus, copper appeared to impair the ability of *C. maenas* to osmoregulate.

A major effect of copper exposure was that it caused a significant rise in haemolymph calcium, irrespective of

the salinity regime to which the crabs had been subjected (Fig. 2c). In the absence of copper, haemolymph Ca^{++} remained relatively constant. Haemolymph Mg^{++} ion concentrations showed similar changes following both salinity transfer and copper-exposure. A significant ($p < 0.01$) increase in magnesium concentration was induced in crabs transferred to a salinity of 30 ppt SW, whereas crabs transferred to salinities of 10 and 20 ppt exhibited a significant ($p < 0.05$) decrease in haemolymph magnesium (Fig. 2d).

ANOVA showed that haemolymph protein, haemocyanin and copper concentrations did not change in response to salinity transfer alone, whereas there was a significant ($p < 0.001$) decrease in all these parameters in all copper-exposed crabs (Fig. 3).

Tissue copper concentrations

There were no significant changes in tissue water contents during the 7 d of either salinity or copper exposure (i.e. no changes in the dry wt/wet wt ratio of the individual tissues were detected). Copper exposure brought about a several fold increase in gill tissue copper concentration, the degree of copper accumulation increasing with increasing salinity (Fig. 4). The copper concentration in the midgut gland of copper-exposed crabs increased significantly at salinities of 10 and 20 ppt but not at 30 ppt (Fig. 4). Copper exposure did not result in a significant increase in the copper concentrations in muscle tissues (Fig. 4).

DISCUSSION

Shore crabs acclimated to full strength seawater (33 ppt) and exposed to dissolved copper gradually developed a non-lactic haemolymph metabolic acidosis (Boitel & Truchot 1989). The present study further elucidates this finding by demonstrating a metabolic acidosis in shore crabs from brackish water exposed to copper at both constant and changing (increasing or decreasing) salinity. This shows that the effects of copper on acid-base status override the effects of concurrent salinity changes.

Salinity changes *per se* caused only minor acid-base disturbances in crabs in

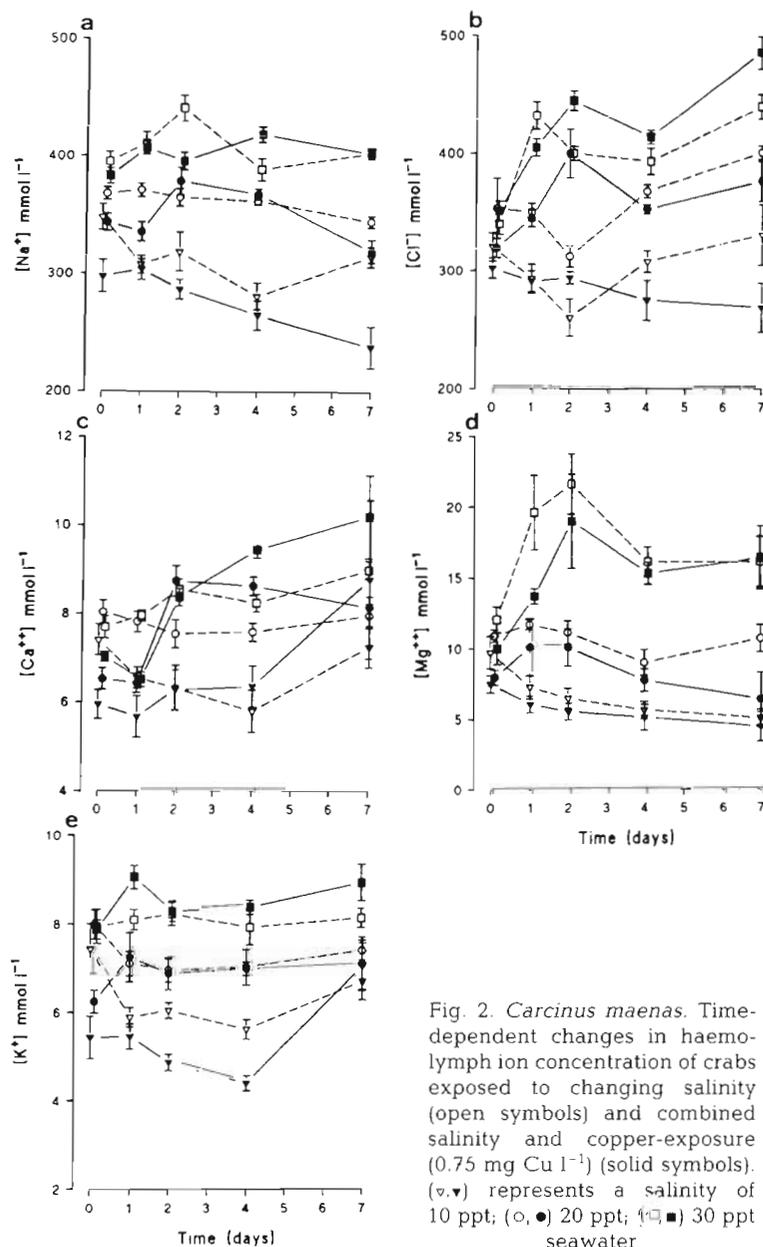


Fig. 2. *Carcinus maenas*. Time-dependent changes in haemolymph ion concentration of crabs exposed to changing salinity (open symbols) and combined salinity and copper-exposure ($0.75 \text{ mg Cu l}^{-1}$) (solid symbols). (\circ, \bullet) represents a salinity of 10 ppt; (\square, \blacksquare) 30 ppt seawater

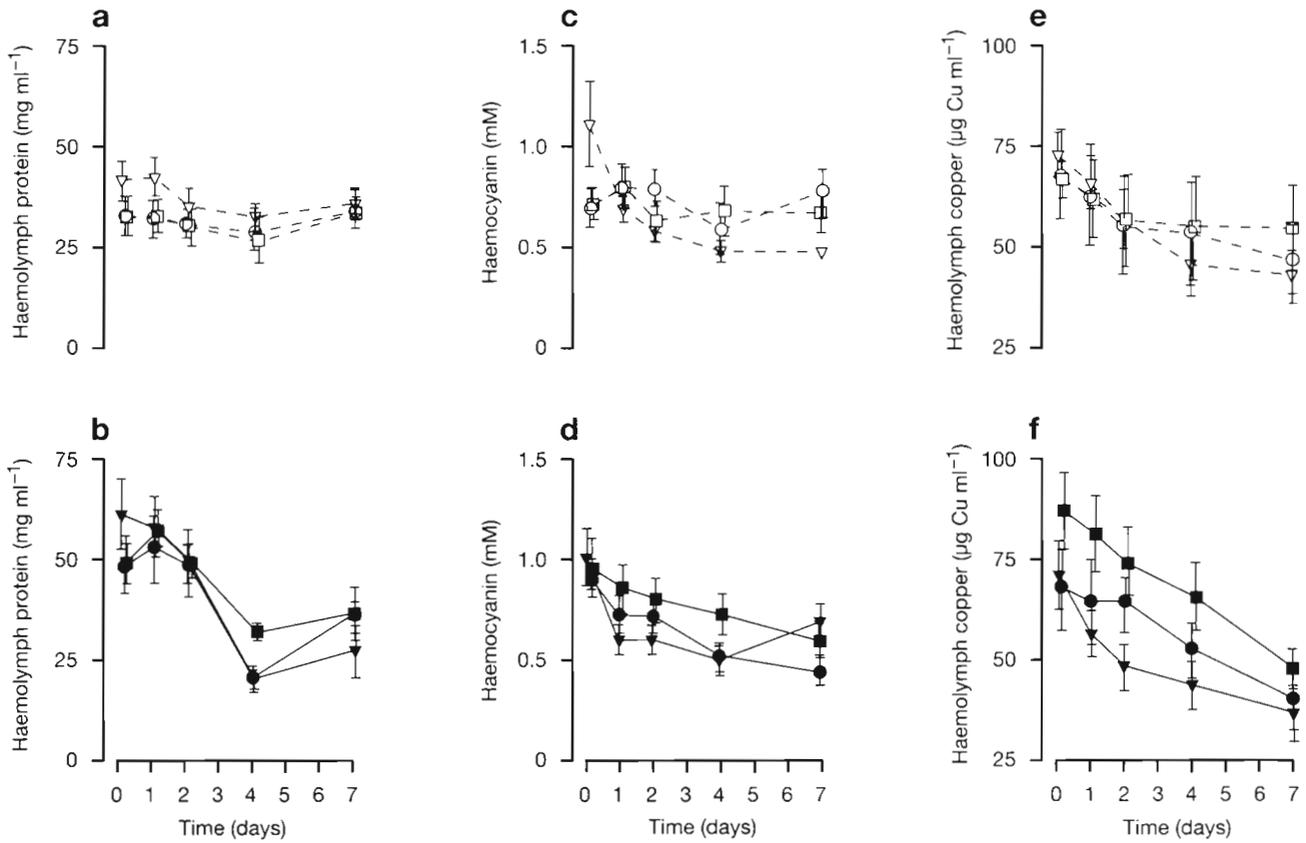


Fig. 3. *Carcinus maenas*. Time-dependent changes in (a, b) haemolymph protein, (c, d) haemocyanin and (e, f) haemolymph copper of crabs exposed to changing salinity (open symbols) and combined salinity and copper exposure (0.75 mg Cu l⁻¹) (closed symbols). (∇, ▼) represents a salinity of 10 ppt; (○, ●) 20 ppt; (■, □) 30 ppt seawater

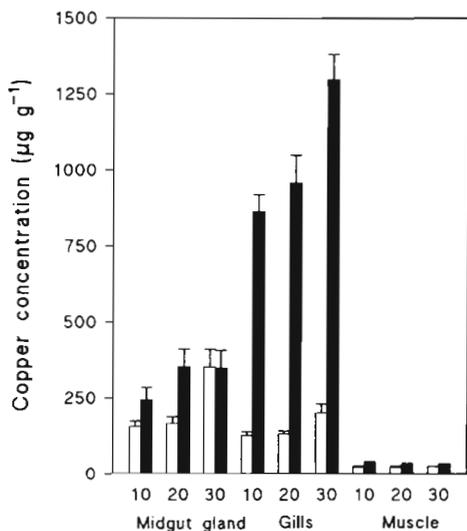


Fig. 4. *Carcinus maenas*. Mean (\pm SEM) copper concentrations ($\mu\text{g g}^{-1}$ dry wt) in the midgut gland, gills and muscle tissues of crabs following 14 d exposure to either salinity change (unshaded bars) or combined salinity change with concomitant copper exposure (0.75 mg Cu l⁻¹) (shaded bars)

the present study compared to those reported elsewhere (e.g. Truchot 1981, Boitel & Truchot 1990). This may indicate that Danish crabs, which are exposed to very marked salinity variation in their natural habitat, regulate salinity-induced acid-base disturbances more rapidly and efficiently than Atlantic coast crabs (as used by Truchot 1981) that normally encounter relatively stable, higher salinities. Animals living under conditions of rapidly fluctuating salinity would clearly benefit from an ability to alter their surface permeability to minimize ionic and osmotic fluxes. Thus, Danish *Carcinus maenas* may be more readily able to alter their physiology to suit ambient conditions than crabs from more constant environments (Chan et al. 1992).

Although metabolic acidosis in copper-exposed crabs was quite marked, it was probably insufficient to result in death of the crabs. Crabs in poor condition, and close to death, invariably had low haemolymph pH values. Therefore, the likelihood of survival during the 24 h period after the final pH measurement could be predicted with a high degree of accuracy. Such a biomarker of condition might prove useful in future

assessments of the physiological well-being of crabs exposed to a wide range of environmental perturbations (Depledge et al. 1992).

This present study showed relatively few and only minor changes in haemolymph ion concentrations upon exposure to salinity or copper stress (cf. Bjerregaard & Vislie 1986). In full strength seawater, marine decapods are more or less iso-osmotic and iso-ionic with the ambient water, whereas in dilute seawater the haemolymph ionic concentrations is regulated at a hyperosmotic level by means of increased branchial ion uptake (Zanders 1980a). In the present study, crabs exposed to copper at a 10 ppt salinity experienced a marked decrease in haemolymph sodium and chloride concentrations, which may be indicative of a net ion efflux at the branchial epithelium. This effect may be dependent on both copper concentration and season. Bjerregaard & Vislie (1986) reported no effect upon haemolymph Na^+ , K^+ and Cl^- concentrations following exposure to $0.25 \text{ mg Cu l}^{-1}$. Na^+ and Cl^- concentrations decreased upon exposure to 0.5 mg Cu l^{-1} , the reduction being most significant during May and June. Copper-induced ion regulatory disturbances may be related to a direct inhibitory effect of copper on gill Na^+ , K^+ -ATPase in *Carcinus maenas* (Hansen et al. 1992a, b), but might also be caused by increased passive ion fluxes due to increased branchial permeability.

At a salinity of 10 and 20 ppt the crabs in this study maintained their haemolymph magnesium concentrations at ca 8 to 11 mmol l^{-1} both in the presence and absence of copper (Fig. 2). At a salinity of 30 ppt, however, a marked increase in haemolymph magnesium concentration was induced. This finding contradicts the suggestion by Zanders (1980b) that *Carcinus maenas* maintains a low magnesium concentration by excretion via the antennal glands/bladder system (Zanders 1980b). It is possible that Danish crabs from low salinity environments take up magnesium to establish concentrations that are normal for Atlantic coast crabs (ca 18 mM ; Chan 1990).

Haemolymph calcium concentrations increased in all copper-exposed crabs irrespective of salinity. This rise may reflect buffering of extracellular acidosis by means of dissolution of exoskeletal calcium carbonate (cf. Truchot 1987). Acidosis is, however, not always associated with a rise in haemolymph Ca^{++} in crustaceans. Thus, the freshwater crayfish *Astacus astacus* developed the same degree of extracellular acidosis when exposed to acid water in the presence or absence of aluminium, but haemolymph calcium was only elevated in the aluminium-exposed specimens (Jensen & Malte 1990). This finding suggests that the presence and accumulation of metal in the gill tissue may somehow stimulate the carapace dissolution process. This could be via an inhibition of the branchial ion ex-

change mechanisms that mediates transfer of acid-base equivalents between the animal and the environment and guarantees that normally (i.e. in the absence of metal) the external water rather than the carapace carbonate predominates as the proton sink (Cameron 1985).

Copper concentrations in the gills, muscle and midgut gland from control *Carcinus maenas* in the present study were consistent with the values reported by Bjerregaard & Vislie (1986). The haemolymph present in the extracellular space in muscle may have led to an overestimation of muscle copper concentration in this study. Exposure to copper augmented concentrations of copper in the gill and midgut gland but not in the muscle. Earlier studies have indicated that the main target of metal action is the gill. Acute exposure to copper resulted in profound changes in gill ultrastructure and the production of excessive quantities of 'mucus' on the outer surface of the gills of the isopod *Jaera nordmanni* (Bubel 1976). Passive adsorption of copper onto the surfaces of the gill probably plays a role in copper uptake. Further, gill ion exchange components are probably affected by metal pollutants (Hansen et al. 1992a, b).

Total haemolymph protein concentrations in decapods are known to vary widely both inter- and intraspecifically (Depledge & Bjerregaard 1989). The values measured in this study were in agreement with Bjerregaard & Vislie (1986) (i.e. ca 57 mg ml^{-1}). Boone & Schoffeniels (1979) reported for *C. maenas* exposed to hypo-osmotic seawater (17 ppt) a doubling of haemocyanin concentration within 48 h, increasing from 15 – 20 to 35 mg ml^{-1} . This was associated with an increase in haemolymph copper from 27 to $62 \text{ } \mu\text{g ml}^{-1}$, but with no apparent increase in body copper. Calculations by Depledge & Bjerregaard (1989) have cast considerable doubt on this finding, since mass balance determinations clearly demonstrate that the rise in haemocyanin copper concentration could not have been fulfilled without a major increase in whole body copper load. In the present study, salinity change alone did not affect haemolymph protein, haemocyanin or copper levels significantly. Weeks & Rainbow (1992) also failed to show any increase in copper concentrations in the haemolymph of 2 species of talitrid amphipods following short-term hypo-osmotic stress. Combined salinity and copper stress, however, resulted in a lowering of all 3 crab haemolymph parameters. It is well known that many metabolic adjustments occur at the tissue level following changes of ambient salinity; these processes may well be transitory, however, the time course of re-adjustments (if any) is not known. These re-adjustments may be further complicated in the presence of dissolved copper. One interpretation of the observed decline in haemolymph copper in

copper-exposed crabs may be that of 'overcompensation'. Copper taken up via the haemolymph at ambient concentrations is removed to the midgut gland, maintaining the copper level in the haemolymph and other internal organs relatively constant (Bryan 1976). At lowered salinities, and combined with an additional stress such as copper, then the rate of copper removal from the haemolymph to the midgut gland (with the involvement of metallothionein detoxification and a storage system) may be enhanced, to such an extent as to cause the observed lowering of haemolymph copper with a concomitant increase in midgut gland copper.

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LITERATURE CITED

- Bjerregaard, P., Vislie, T. (1986). Effect of copper on ion- and osmoregulation in the shore crab *Carcinus maenas*. *Mar Biol.* 91: 69–76
- Boitel, F., Truchot, J.-P., (1989). Effects of sublethal and lethal copper levels on haemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted seawater. *Mar. Biol.* 103: 495–501
- Boitel, F., Truchot, J.-P. (1990). Comparative study of the effects of copper on haemolymph ion concentrations and acid-base balance in shore crabs *Carcinus maenas* acclimated to full-strength or dilute seawater. *Comp. Biochem. Physiol.* 95C: 307–312
- Boone, W. R., Schoffeniels, E. (1979). Hemocyanin synthesis during hypo-osmotic stress in the shore crab *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* 63B: 207–214
- Bryan, G. W. (1976). Heavy metal pollution in the sea. In: Johnston, R. (ed.) *Marine pollution*. Academic Press, London, p. 185–302
- Bubel, A. (1976). Histological and electron microscopical observations on the effects of different salinities and heavy metal ions, on the gills of *Jaera nordmanni* (Rathke) (Crustacea: Isopoda). *Cell. Tissue Res.* 167: 65–95
- Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. appl. Physiol.* 31: 632–634
- Cameron, J. N. (1985). Compensation of hypercapnic acidosis in the aquatic blue crab, *Callinectes sapidus*: the predominance of external seawater over carapace carbonate as the proton sink. *J. exp. Biol.* 114: 197–206
- Chan, H. M. (1990). Aspects of the biology of zinc in crabs with particular emphasis on the shore crab *Carcinus maenas* (L.). Ph.D. thesis, University of London
- Chan, H. M., Bjerregaard, P., Rainbow, P. S., Depledge, M. H. (1992). Uptake of zinc and cadmium by two populations of shore crabs *Carcinus maenas* at different salinities. *Mar. Ecol. Prog. Ser.* 86: 91–97
- Depledge, M. H. (1984a). Disruption of circulatory and respiratory activity in shore crabs (*Carcinus maenas* L.) exposed to heavy metal pollution. *Comp. Biochem. Physiol.* 78C: 445–459
- Depledge, M. H. (1984b). Disruption of endogenous rhythms in *Carcinus maenas* (L.) following exposure to mercury pollution. *Comp. Biochem. Physiol.* 78A: 375–379
- Depledge, M. H. (1989). Re-evaluation of metabolic requirements for copper and zinc in decapod crustaceans. *Mar. environ. Res.* 27: 115–126
- Depledge, M. H. (1990). Interactions between heavy metals and physiological processes in estuarine invertebrates. In: Chambers, P. L., Chambers, C. M. (eds.) *Estuarine ecotoxicology*. JAPAGA, Ashford, Ireland, p. 89–100
- Depledge, M. H., Bjerregaard, P. (1989). Haemolymph protein composition and copper levels in decapod crustaceans. *Helgoländer Meeresunters.* 43: 207–223
- Depledge, M. H., Amaral-Mendes, J. J., Daniel, B., Halbrook, R., Kloepper-Sams, P., Moore, M., Peakall, D. B. (1992). The conceptual basis of the biomarker approach. In: Peakall, D. B., Shugart, L. R. (eds.) *Strategy for biomarker research and application in the assessment of environmental health*. Lewis Publishers, Boca Raton (in press)
- Depledge, M. H., Rainbow, P. S. (1990). Models of regulation and accumulation of trace metals in marine invertebrates. *Comp. Biochem. Physiol.* 97C: 1–7
- Hansen, J. I., Mustatta, T., Depledge, M. (1992a). Mechanisms of copper toxicity in the shore crab, *Carcinus maenas* I. Effects on Na,K-ATPase activity, haemolymph electrolyte concentrations and tissue water contents. *Mar. Biol.* 114: 253–257
- Hansen, J. I., Mustafa, T., Depledge, M. (1992b). Mechanisms of copper toxicity in the shore crab, *Carcinus maenas* II. Effects on key metabolic enzymes, metabolites and energy charge potential. *Mar. Biol.* 114: 259–264
- Heisler, N. (1989). Parameters and methods in acid-base physiology. In: Bridges, C. R., Butler, P. J. (eds.) *Techniques in comparative respiratory physiology: an environmental approach*. Cambridge University Press, Cambridge, p. 305–332
- Jensen, F. B., Malte, H. (1990). Acid-base and electrolyte regulation, and haemolymph gas transport in crayfish, *Astacus astacus*, exposed to soft, acid water with and without aluminium. *J. comp. Physiol. (Sect. B)* 160: 483–490
- Kerkut, G. A., Munday, K. A. (1962). The effect of copper on the tissue respiration of the crab, *Carcinus maenas*. *Cah. Biol. mar.* 3: 27–35
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* 193: 265–275
- Nugegoda, D., Rainbow, P. S. (1989a). Effects of salinity changes on zinc uptake and regulation by the decapod crustaceans *Palaemon elegans* and *Palaemonetes varians*. *Mar. Ecol. Prog. Ser.* 51: 57–75
- Nugegoda, D., Rainbow, P. S. (1989b). Salinity, osmolality and zinc uptake in *Palaemon elegans* (Crustacea: Decapoda). *Mar. Ecol. Prog. Ser.* 55: 149–157
- Sokal, R. R., Rohlf, F. J. (1981). *Biometry. The principles and practice of statistics in biological research*. W. H. Freeman & Co., San Francisco, p. 776
- Truchot, J.-P. (1981). The effect of water salinity and acid-base state on the blood acid-base balance in the euryhaline crab, *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* 68A: 555–561
- Truchot, J.-P. (1987). *Comparative aspects of extracellular acid-base balance*. Springer-Verlag, Berlin, p. 248
- Weeks, J. M., Rainbow, P. S. (1992). The effect of salinity on copper and zinc concentrations in three species of talitrid amphipods (Crustacea). *Comp. Biochem. Physiol.* 101C: 399–405

- Zanders, I. P. (1980a). Regulation of blood ions in *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* 65A: 97–108
- Zanders, I. P. (1980b). The control of magnesium and sulphate excretion in *Carcinus maenas* (L.). *Comp. Biochem.*

Physiol. 66A: 69–76

- Zirino, A., Yamamoto, S. (1972). A pH-dependent model for the chemical speciation of copper, zinc, cadmium and lead in seawater. *Limnol. Oceanogr.* 17: 661–671

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