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# Effect of copper exposure on histamine concentrations in the marbled crayfish (*Procambarus fallax* forma *virginalis*)

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**Abstract**
Crustaceans can store excess copper in the hepatopancreas, an organ playing a role in digestive activity as well as in neurosecretory control. Here, we studied the effect of copper exposure on the level of histamine, an indicator of food spoilage in edible crustaceans. Histamine is also a neuromodulator in the intestinal nervous system of crustaceans, and a human allergen. Marbled crayfish (*Procambarus fallax* forma *virginalis*) were exposed to average measured values of 0.031 mg Cu/l and 0.38 mg Cu/l, respectively, for 14 days and then transferred to copper-free water for another 14 days. Concentrations of copper and histamine in the hepatopancreas and muscle were evaluated at different time points. Histamine levels were significantly higher in hepatopancreas and muscle tissues at the highest exposure level, but only after transfer of the animals to copper-free water. The increased histamine concentration following copper exposure may be explained by a (delayed) stress response, and by up-regulated histidine synthesis induced by copper, followed by decarboxylation to histamine.

**Keywords**
Copper; hepatopancreas; histamine; intestinal nervous system; neuromodulator

## Introduction

Metal bioaccumulation in aquatic animals is an important phenomenon in ecotoxicology and well documented for many years (Spehar et al., 1978; Calabrese et al., 1984; Khan et al., 1989; Baden et al., 1999; Canivet et al., 2001; Luoma and Rain-

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bow, 2005; Veltman et al., 2008; Martins et al., 2011). Among the metal pollutants in water bodies and sediments, copper is interesting, because it is both essential and toxic depending on its available concentration. Especially in crustaceans, copper binds oxygen in hemocyanin, the respiratory protein in the crustacean blood (hemolymph) (Anderson et al., 1997). In aquatic crustaceans exposure to excess copper causes physiological disturbances, due to bioaccumulation. For instance, Bini and Chelazzi (2006) reported that upon exposure to 0.5 mg Cu/l, *Procambarus clarkii* showed an alteration in the cardiac and ventilatory rates. Several researchers reported variability in the physiological status of crustaceans caused by copper, including, among others, reduced osmoregulatory capacity (Harris and Santos, 2000) and changed moulting cycle (Engel and Brouwer, 1993). Furthermore, physiological effects are preceded by, or coincide with, changes in biochemistry of the animals. Li et al. (2007) reported copper effects on the structure of the gills and hepatopancreas in juvenile giant freshwater prawns coinciding with an impact on the content of metallothionein.

In a previous study (Soedarini et al., 2012), marbled crayfish showed accumulation of copper in different tissues at increased exposure concentrations in the water with excess copper especially being stored in the hepatopancreas. The pattern of copper bioaccumulation in crayfish is similar to that in other aquatic crustaceans, such as the penaeid shrimp *Metapenaeus dobsoni* (Manisseri and Menon, 1995), the blue crab *Callinectes* spp. (Sastre et al., 1999) and the freshwater prawn *Macrobrachium rosenbergii* (Reddy et al., 2006). In crustaceans, the hepatopancreas plays an important role in digestive activity, synthesis of hemocyanin and biotransformation of xenobiotics (Loizzi, 1971). Several studies have shown that histamine, a biogenic amine, plays a role as an inhibitory neurotransmitter or neuromodulator in the stomatogastric nervous system of crustaceans and so might regulate digestive activity of the hepatopancreas (Pulver et al., 2003; Cebada and Garcia, 2007). Histamine is also an indicator of food spoilage in shelf-life studies with edible crustaceans (Taylor et al., 1989).

In this study we ask the question whether copper accumulation interacts with the histamine content of crayfish. Currently there are no studies on effects of pollution on the histamine level in aquatic crustaceans. We assess histamine concentrations in marbled crayfish tissues under the influence of different copper exposure levels.

## Materials and methods

### *Test animals*

Marbled crayfish (*Procambarus fallax* (Hagen, 1870) forma *virginalis*; Malacostraca, Decapoda, Cambaridae) were obtained from Alterra, part of Wageningen University and Research Centre, The Netherlands. This freshwater crayfish is known only from aquaria and populations established from released individuals. Using molecular markers, Martin et al. (2010) have shown recently that marbled crayfish is a parthenogenetic form of *Procambarus fallax* from Florida. The mar-

bled crayfish itself is not a common edible species, but being a freshwater and parthenogenetic species it is more easily cultured and used in experiments than salt water species. The animals used measured 3.5 to 7.2 cm body length, weighed 1.3 to 7.4 g, and were acclimated in an aquarium filled with aerated, filtered copper-free water (pH 7.3 ± 0.1; $CaCO_3$ hardness 2-4 mmol/l) for one week prior to exposure. The crayfish were incubated in a climate-controlled room at 20 ± 1°C; day/night cycle of 12 hours light and 12 hours dark and fed with commercial crayfish food pellets (Tetra Wafer Mix) three times a week.

*Copper exposure*

Aqueous copper concentration of 0.05 mg Cu/l and 0.5 mg Cu/l were prepared by dissolving $CuSO_4 \cdot 5H_2O$ (Merck, p.a.) in copper-free tap water (pH 7.3 ± 0.1; hardness 2-4 mmol $CaCO_3$/l). Copper-free tap water was obtained from a special piping system without copper linings available at VU University. The test animals were randomly divided in three groups and exposed individually in 800 ml glass jars at 20 ± 1°C. The first two groups, each composed of 31 animals, were exposed to the two different levels of copper (0.05 and 0.5 mg Cu/l). The third group, containing seven animals, was the control and kept in copper-free water. The animals were incubated in a climate-controlled room under the same conditions as during acclimation. They were fed three times a week with commercial crayfish food pellets, shortly (three hours) before renewing the exposure solution. The exposure took 14 days after which all remaining animals were transferred to copper-free water. At different time points during the copper uptake (1, 2, 4, 8 and 14 days) and elimination phases (15, 16, 18, 22 and 28 days) three animals from each treatment, in the inter-moult stage, were sampled and killed by decapitation using a lancet. Control animals were sampled only at 0 and 28 days (three animals each on both sampling days). The animals were dissected to collect the gills, hepatopancreas, ovaries, muscle and exoskeleton. The organs were placed in plastic tubes (with lids), frozen in liquid nitrogen, weighed and kept in a freezer (−20°C) until analysis.

Exposure solutions and animal organs were analysed for copper concentrations as described by Soedarini et al. (2012).

*Histamine analysis*

Histamine was extracted from the wet tissue samples (hepatopancreas and muscle). Samples at a precise weight between 0.1 and 0.2 g F.W. were placed in 15 ml plastic tubes and 10 ml 0.1 M ethylene diamine tetra acetic acid (EDTA) – 2Na solution (pH 8.0) was added. The samples were boiled for 20 minutes and then cooled on ice. The samples were subsequently filtered through a membrane filter (0.2 $\mu$m cellulose acetate syringe filter WHAT10462701, Whatman, UK) (Sato et al., 2005). To determine the amount of histamine in the filtrate, the histamine EIA Kit was used (Oxford Biomedical Research), which is a competitive direct enzyme-linked immunosorbent assay (ELISA) in a micro-well format that allows users to obtain

histamine concentrations in ng/ml range. A series of standard solutions containing pre-defined amounts of histamine (0-50 ng/ml) was used. Absorbance was read at 650 nm using a Versamax 340-750 nm plate reader (MTX Lab systems, Inc.)

*Data analysis*

The effect of the copper concentration in the water and time of exposure on the histamine concentrations in the crayfish organs were analyzed using two-way analysis of variance (two-way ANOVA). One-way ANOVA with Tukey's post-hoc test was applied to determine the significance of copper effects on histamine concentrations in the hepatopancreas and the muscle. Pearson rank correlation was applied to determine significance of the relationships between histamine and copper levels in the crayfish muscle and hepatopancreas tissues. Statistical analyses were run in the statistics software SPSS, version 17.0 (SPSS Inc. Chicago, Illinois, USA).

## Results

In total 92.8% of the animals survived untill the moment of sampling and mortality was not related to copper exposure. Data on measured copper concentrations in the water, crayfish growth and copper uptake and elimination kinetics have been reported by Soedarini et al. (2012) and are summarized here. Average measured copper concentrations in the water during the first 14-day test period were 0.031 mg Cu/l and 0.38 mg Cu/l, respectively. The animal mass steadily increased during the experiment and did not show any differences between the copper treatment levels and the controls, suggesting the absence of any toxic effects. The animals showed no changes in the copper levels in any of the organs analyzed upon exposure to the lowest copper level of 0.031 mg Cu/l. At 0.38 mg Cu/l, copper level in the muscle tissue showed a slight increase during the 14-day exposure period after which it decreased again; copper concentrations in the hepatopancreas steadily increased with time and showed only a slight decline during the second 14-day incubation period in clean water (Soedarini et al., 2012).

Development with time of the histamine concentrations in the hepatopancreas and the muscle of marbled crayfish is shown in fig. 1. Results of the corresponding statistical analysis are presented in table 1. Overall, histamine levels in the animals were significantly higher at the highest than at the lowest copper exposure level (ANOVA: $F_{1,20} = 12.495$, $P = 0.001$ for muscle tissue; $F_{1,20} = 10.453$, $P = 0.003$ for hepatopancreas), but this seemed mainly due to the levels in the second part of the test when the animals were no longer exposed to copper (fig. 1). Time did not affect histamine levels in the animals, except for histamine in the hepatopancreas during the first 14-day test period, which was significantly affected by time (ANOVA: $F_{4,10} = 3.173$; $P = 0.040$). A closer look at the data shows that this is mainly caused by the relatively low histamine levels at the start and after 1 day (fig. 1); this suggests a rapid build-up of histamine in the hepatopancreas in the beginning of the experiment, probably unrelated to copper exposure. The latter

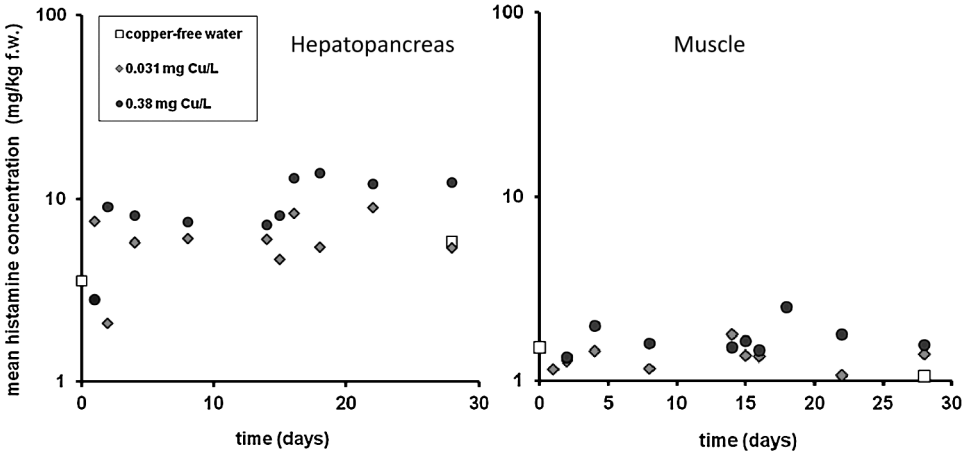


**Figure 1.** Histamine concentrations in the hepatopancreas and the muscle of marbled crayfish (*Procambarus fallax* f. *virginalis*) measured at different exposure times to two different Cu concentrations for 14 days and after transfer to copper-free water for another 14 days. Each data point represents the averaged measured concentration in three test animals.

assumption, however, cannot be confirmed due to the absence of control measurements during these initial days of exposure (except for t = 0).

To better visualize effects of copper exposure concentration, histamine concentrations averaged over all exposure times are shown in fig. 2. Copper exposure concentrations did not affect histamine concentrations in the muscle but showed a clear effect on the histamine levels in the hepatopancreas. Averaged histamine concentrations in the muscle of animals exposed to copper-free water, 0.031 mg Cu/l and 0.38 mg Cu/l were 1.28 ± 0.31, 1.28 ± 0.38 and 1.62 ± 0.51 mg histamine/kg

**Table 1.**
Probability (p) values resulting from the two-way analysis of variance (ANOVA) of the effect of copper exposure level and time on histamine concentrations in the muscle and the hepatopancreas of the marbled crayfish (*Procambarus fallax* f. *virginalis*).

| Source of variation | Histamine in hepatopancreas | | | Histamine in muscle | | |
|---|---|---|---|---|---|---|
| | Total | Day 1-14 | Day 15-28 | Total | Day 1-14 | Day 15-28 |
| Copper | 0.003* | 0.774 | <0.001* | 0.001* | 0.182 | 0.003* |
| Time | 0.056 | 0.040* | 0.528 | 0.073 | 0.541 | 0.411 |
| Copper × time | 0.001* | 0.148 | 0.003* | 0.099 | 0.014* | 0.765 |

Values are given for the total 28-day incubation period and separated for the uptake phase (day 1-14) and the elimination phase (day 15-28). In the uptake phase, the animals were exposed to measured total copper concentrations of 0.031 and 0.38 Cu mg/l. After 14 days of exposure, all animals were transferred to and incubated in copper-free water for another 14 days. Copper refers to copper exposure level, time refers to different exposure times, and copper × time indicates the interaction of both factors. See text for further explanation.

* Asterisk indicates significance.

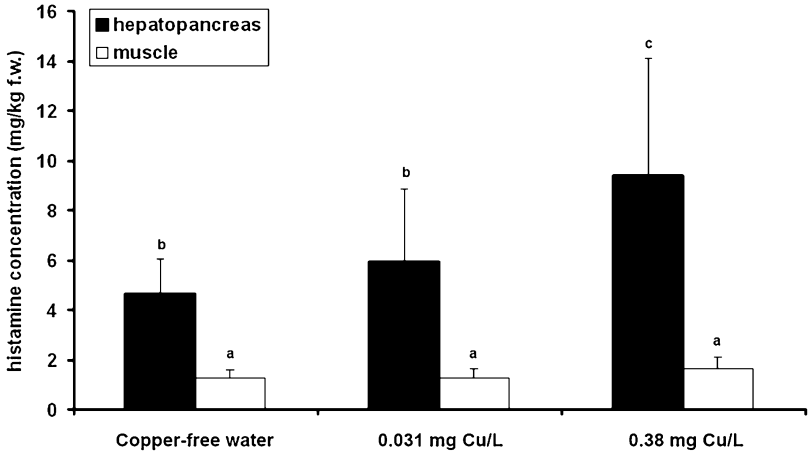


**Figure 2.** Average histamine concentrations in the hepatopancreas and the muscle tissue of marbled crayfish (*Procambarus fallax* f. *virginalis*) exposed for 14 days to different copper concentration levels and transferred to copper-free water for another 14 days. Error bars represent the standard deviations ($N = 6$, 27 and 29 for copper-free water, 0.031 mg Cu/l and 0.38 mg Cu/l, respectively). In case of copper-free water, all animals were included that were sampled at the start of the exposures and after 28 days of incubation. Different letters indicate significantly different histamine levels between treatments (one-way ANOVA followed by Tukey's post-hoc test).

fresh weight (mean ± SD), respectively. Average histamine concentrations in the hepatopancreas of crayfish exposed to 0.031 mg Cu/l did not differ from the control, with values of 4.67 ± 1.37 and 5.95 ± 2.89 mg histamine/kg fresh weight (mean ± SD), respectively. In contrast, histamine concentrations in the hepatopancreas of crayfish exposed to 0.38 mg Cu/l were significantly higher, reaching an average value of 9.41 ± 4.72 mg/kg (mean ± SD).

Figure 3 relates histamine concentrations to the copper levels in the analyzed tissues. Histamine levels were significantly correlated with copper concentrations in the hepatopancreas (Pearson rank correlation: $R = 0.454$, $N = 62$, $P < 0.01$), but not in the muscle of marbled crayfish (*Procambarus fallax* f. *virginalis*).

## Discussion

Histamine is a well-known neuromodulator of the intestinal nervous system, with a local (paracrine) action on motor neurons. In the lobster *Homarus americanus*, histamine reduces the firing of neurons associated of the stomatogastric system (Pulver et al., 2003). The increase of histamine levels under the influence of copper, observed in our experiments, may be part of the stress response of the animal by which it attempts to prevent further uptake of copper. Alternatively, histamine could arise because of an increase of the concentration of histidine, followed by decarboxylation to histamine. Histidine has an important role in the copper-containing, oxygen carrying molecule of crustaceans. Each hemocyanin molecule contains two copper ions and each copper ion is coordinated by three histidine residues. A highly

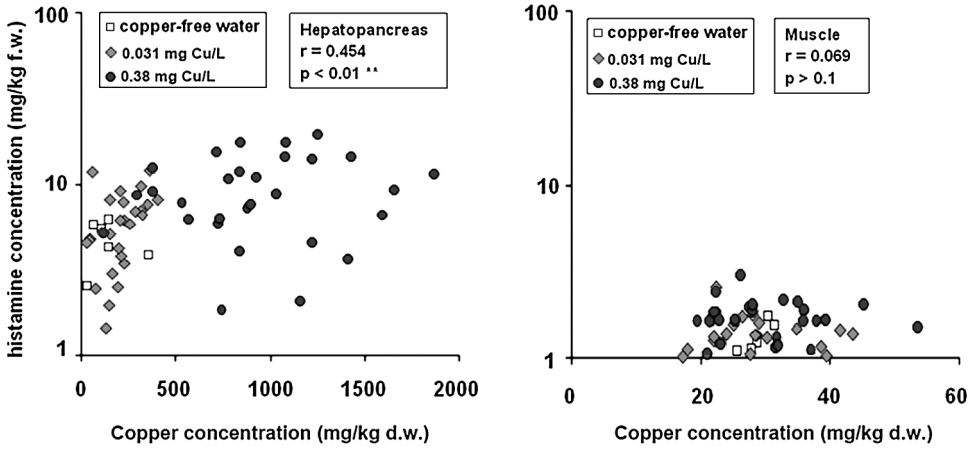


**Figure 3.** Histamine concentrations in the hepatopancreas and the muscle of marbled crayfish (*Procambarus fallax* f. *virginalis*) expressed as a function of copper concentrations in these tissues. Animals were exposed for 14 days to different copper exposure levels and then transferred to copper-free water for another 14 days. Each data point represents an individual measurement. Abbreviations and symbols: r, Pearson correlation coefficient; p, degree of linear relationship between the two variables; **, significant correlation between copper concentration and histamine concentration in the hepatopancreas of marbled crayfish (SPSS, Pearson correlation test).

up-regulated synthesis of histidine could be part of a mechanism by which the animal attempts to scavenge and store copper in the hepatopancreas. Any excess of histidine produced could be metabolized to histamine.

Which of these possibilities on the functional significance role of increased histamine is true (upregulated neurosecretion or upregulated copper scavenging) is difficult to say, as both are speculative. However, since we observed an increase of histamine concentrations only in the hepatopacreas, and since this was also the only organ in which copper was selectively retained, a causal link between histamine increase and copper accumulation is very likely. Both explanations may also be valid when trying to explain the reason why histamine levels showed a kind of delayed response, with higher levels arising especially in the second part of the test when the animals were transferred to copper-free water.

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