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Pre-exposure to Cadmium or Zinc Alters the Heart Rate Response of the Crayfish *Procambarus clarkii* Towards Copper

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Abstract Exposure to sub-lethal concentrations of a pollutant induces, in some organisms, an acclimation process which increases their resistance to other substances (cross-acclimation). Understanding this phenomenon is important as a basis for a better comprehension of the effects of pollutants in ecosystems. In this paper we investigated whether the exposure to Cd or Zn is able to modify the heart rate response of the crayfish *Procambarus clarkii* to acute Cu stress. A first set of experiments provided the basis to understand heart rate changes induced by varying Cd or Zn concentrations. In a second set of experiments crayfish were acclimated for 96 h to control water, Cd or Zn enriched water, and then exposed to a 10 mg L^{-1} Cu solution, known to induce bradycardia in this species. Bradycardia was suppressed in specimens previously exposed to Cd or Zn but not in those exposed to clean water, providing a clear evidence of a cross-acclimation in the heart rate response of *P. clarkii*.

Keywords Heart rate · Crayfish · Heavy metals · Pre-exposure · Cross-acclimation

Metals represent one of the major classes of contaminants in aquatic environments and are today a worldwide threat to aquatic species (Gautam et al. 2014). Cd is a widespread environmental contaminant, potentially hazardous to wildlife even at very low concentrations (Wright and Welbourn 1994; Qu et al. 2013) and freshwater crustaceans are amongst the most sensitive of macroinvertebrate species to this non-essential element (Williams et al. 1985). On the contrary, Zn and Cu are essential elements, needed for biological activities in trace amounts, but may have toxic effects at higher concentrations (Peña et al. 1999; Brinkman and Johnston 2012). Both these metals are widespread contaminants in aquatic ecosystem and their ecological relevance is well understood (e.g. Hebel et al. 1997; Naito et al. 2010).

The monitoring of physiological, biochemical and functional responses of aquatic organisms to metal exposure is receiving growing interest, due to their potential use as exposure or stress biomarkers (Luoma and Rainbow 2008). One of the possible markers used to detect the effect of metallic ions on the metabolism of aquatic invertebrates is cardiac activity, which involves changes in both heart rate and stroke volume. Heart rate, in particular, is widely used as a biomarker in water quality assessments (e.g. Bamber and Depledge 1997).

The effects of a few metals on heart rate modulation of crustaceans are known and among those utilized in the present study (i.e., Cd, Zn and Cu), Cu is certainly the best studied. A range of effects from an irregular heartbeat, temporary acardia, and even an increase in heart rate have been observed in crustaceans exposed to this metal (e.g. Lundebye and Depledge 1998; Brown et al. 2004; Camus et al. 2004; Ketpadung and Tangkrock-olan 2006). Less information is available about the effect of Cd or Zn, and available reports show either a reduction (bradycardia) or an increase of heart rate frequency in different species (Mali and Afsar 2011; More 2011).

It is known that in many species exposure to sub-lethal concentrations of some pollutants, which include both metals and organic compounds, can induce an acclimation process, increasing the resistance of organisms to a subsequent exposure to the same substance (e.g. Muyssen and

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Janssen 2002; Mouneyrac et al. 2003; Silvestre et al. 2006; Wang and Xing 2009; Farwell et al. 2012). Interestingly, pre-exposure to sub-lethal amounts of a pollutant may in some cases elicit an unspecific response leading to increased resistance to other substances. This cross resistance, which has been described for both metals (e.g. McGeer et al. 2007) and organic substances such as PCBs or pesticides (e.g. Cosper et al. 1987), can basically be acquired through individual physiological acclimation or genetic adaptation (Sun et al. 2014). Individual acclimation is a rapid, plastic change in phenotype, driven by an external environmental factor, and is largely based on the plasticity of a particular trait (West-Eberhard 1989). On the contrary, genetic adaptation describes genetically based changes acquired at a population level, through the survival of tolerant genotypes and the demise of those that are sensitive (e.g. Klerks and Weis 1987). Individual acclimation can therefore induce a rapid but reversible phenotypic adaptation, which increases individual resistance and may quickly disappear when the stressor is removed. On the contrary, the effects of genetic adaptation may be maintained for several generations, and can persist for some time even after the removal of stress factor.

The red swamp crayfish *Procambarus clarkii* is native to north Mexico and south USA, but has become a widely distributed invasive species in many other parts of the world (Gherardi 2006). Owing also to its worldwide distribution, population abundance, ease of identification and collection and the ability to colonize both polluted and unpolluted sites, this crayfish is becoming an important model species to investigate the effect of exposure to trace metals (Alcorlo et al. 2006; Henriques et al. 2014). Furthermore, many details of the physiological responses of this species to trace metals are fairly well known. As for example, several studies have demonstrated that exposure to high concentrations of such pollutants produces toxic effects and causes the induction of metallothioneins-like and vitellogenin/vitellin-like proteins (Martin-Diaz et al. 2005, 2006). *P. clarkii* is also an efficient bioaccumulator of heavy metals, as demonstrated by several field and laboratory studies (Kouba et al. 2010; Suárez-Serrano et al. 2010). Finally, the effect of Cu on heart rate of this species was described by Bini and Chelazzi (2006), who showed that a sub-lethal Cu induces bradycardia. This study also demonstrated that the effect of Cu on heart rate can be reduced by a 96 h pre-exposure to lower concentrations of the same metal, suggesting an acclimation effect.

The present paper reports the results of experiments to assess the effect of metals on the heart rate of *P. clarkii*. In particular, we wished to test if previous short-term exposure to a sub-lethal concentration of Cd or Zn was able to trigger an unspecific individual acclimation, leading to increased resistance to Cu. We firstly investigated the heart rate response of this species to a short term (3–6 h) exposure to sub-lethal Cd and Zn concentrations, providing the baseline information for the following experiment. Second, we investigated if a longer (96 h) exposure to these metals was able to suppress the bradycardia induced by a following acute Cu exposure.

Materials and Methods

Specimens of *P. clarkii* (body length 6.5–8.5 cm) were collected from Fucecchio Marshes, central Italy (43°45′N; 10°48′E) in May–July 2006. Immediately after collection, groups of crayfish were transported to the laboratory and acclimated for 7 days in 20 L of aerated tap water (control water) at 20 ± 1°C. Control water was characterized as follows: pH 7.4; hardness 19.2°F; Dissolved Organic Carbon 0.3 mg L^{-1}. During the acclimation period, the crayfish were maintained under a natural day–night cycle and fed once with pieces of beef liver, which is sufficient not to induce starvation-dependent changes in heart rate (Bini and Chelazzi 2006). Before the start of any test, each animal was individually placed into an 800 mL test tank.

Metal solutions used in the tests were obtained by adding the appropriate quantity of Cu (II) sulphate 5-hydrate (purity ≥ 98 %, Sigma), Cd sulphate hydrate or Zn sulphate 7-hydrate (purity ≥ 99 %, Fluka) to control water.

Heart rate was monitored using a non-invasive technique established for invertebrates (De Pirro et al. 1999). Therefore, each crayfish was equipped with an optoelectronic sensor glued to the carapace in a dorsal position over the heart and left undisturbed in clean water for 1 h before any treatment to permit complete stress recovery (Bini and Chelazzi 2006). During all experimental sessions the test medium was renewed daily.

Cd and Zn concentrations were chosen on the basis of the results of a preliminary study (Bini 2006), showing the ability of crayfish of the Fucecchio population to resist high metal concentrations. The 96 h LC50 values reported by Bini (2006) for these metals (lower–upper 95 % CI) are 156 (121–231) and 812 (623–1210) mg L^{-1} for Cd and Zn, respectively. The used concentrations were control, 15, and 30 mg L^{-1} for Cd and control, 100 and 200 mg L^{-1} for Zn. The concentrations used in this study were rather high, although still not able to cause any mortality after a 96 h exposure. These concentrations were not chosen as representative of those that crayfish could experience in the field, even at severely polluted sites. Instead, they were chosen since these concentrations trigger detectable responses, even after a short exposure time, in this highly resistant population of the studied species (Bini 2006). In the first experiment, 42 animals were randomly divided into six groups (n = 7), each exposed to a different Cd or

Zn concentration for up to 6 h. The heart rate (HR) of each specimen was measured in the acclimation water, just before metal administration (HR_0), and 3 (HR_3) and 6 (HR_6) h after metal exposure. The individual HR measured after 3 and 6 h were expressed relative to the values measured before metal administration as $RHR_{3h} = HR_3/HR_0$ and $RHR_{6h} = HR_6/HR_0$, respectively. In the second experiment, 60 crayfish were randomly divided into six groups (n = 10) and each group was exposed to water with a different Cd or Zn concentration as in previous experiment (control, 15 and 30 mg L^{-1} Cd; control, 100 and 200 mg L^{-1} Zn) for 96 h. After 96 h exposure, all specimens were washed in clean water for 3 h to remove any metal, and then exposed to Cu (10 mg L^{-1}) for three consecutive hours. The HR of each specimen was measured in the washing water just before Cu administration (HR_0), and then 3 h after exposure to Cu-bearing water (HR_3). The individual variations of heart rate were expressed as $RHR = HR_3/HR_0$.

Data were analysed using ANOVA, following Underwood (1996). Cochran's C test was used to assess the homogeneity of variances. Data were log transformed to cope with homogeneity of variance and non-normality. When significant differences were observed, the Tukey multiple comparison test was applied.

Two water samples for each treatment level used in the experiments were collected and analysed (using ICP-OES) to provide actual amounts of metals in exposure water. Average values were as follows. Zn: 0.25 (controls), 101.34 (expected = 100) and 202.17 (expected = 200) mg L^{-1}, respectively. Cd: <0.005 (controls), 14.98 (expected = 15) and 30.44 (expected = 30) mg L^{-1}. Cu: <0.005 (controls), 10.25 (expected = 10) mg L^{-1}. The instrument detection limits were (in µg L^{-1}): 2.3 (Cd), 1.2 (Zn) and 3.6 (Cu). Certified reference materials (grade BCR, Fluka Analytical, Sigma-Aldrich) were used to verify the accuracy and the precision of the methods.

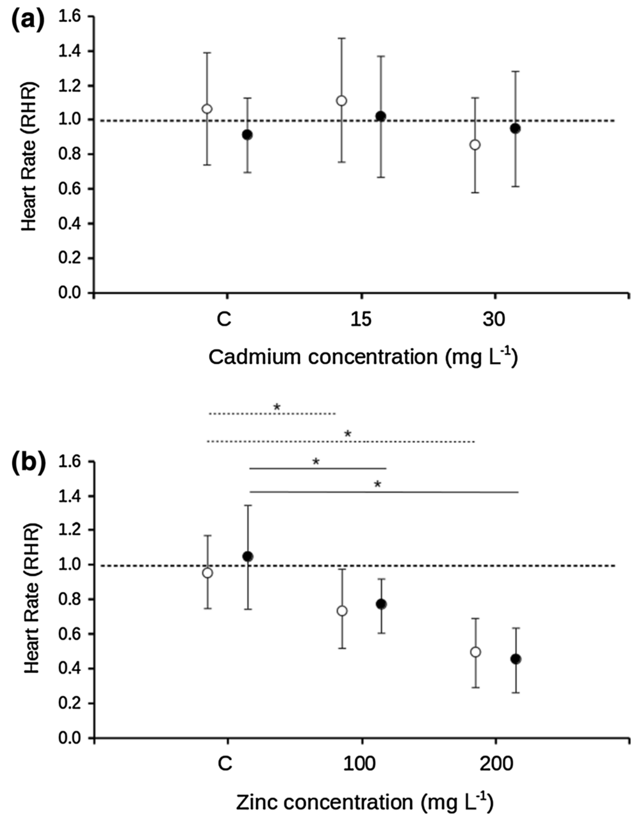

Fig. 1 Implications of **a** Cd and **b** Zn exposure on the heart rate of *P. clarkii*. *Circles* represent the mean (±SD) RHR measured after 3 (*open circles*) and 6 h (*closed circles*) of exposure to different metal concentration (*C* controls). *Horizontal lines* show significant differences between metal-exposed animals and controls (Tukey test, $*p < 0.05$) after 3 (*dotted lines*) and 6 h (*continuous lines*) of exposure to the metal. Value = 1 (*dashed line*) means no variation in the HR caused by the exposure; values >1 and <1 represent increased or decreased HRs, respectively. Sample size was n = 7 for each group

Results and Discussion

Results of the first experiment showed that 6 h of exposure to control water and both Cd concentrations (15 and 30 mg L^{-1}) did not caused death nor modify crayfish heart rate (Fig. 1a). RHR values were very close to one (indicating that no change in heart rate frequency had occurred) and no significant difference was detected independent of the exposure duration ($F_{2,19} = 1.11$, $p = 0.350$; $F_{2,19} = 0.27$, $p = 0.766$, respectively). On the contrary, exposure to Zn induced a significant concentration-dependent reduction in heart rate (Fig. 1b), after both 3 and 6 h ($F_{2,19} = 6.10$, $p = 0.009$; $F_{2,19} = 10.54$, $p = 0.0008$).

In the second experiment, no significant difference was observed in the heart rate of the three Cd exposed groups (controls, 15 and 30 mg L^{-1}), at the end of the wash period ($F_{2,28} = 0.12$, $p = 0.887$). A subsequent wash period was also effective at cancelling the bradycardia induced by exposure to Zn and no difference in heart rate of the three Zn exposed groups ($F_{2,28} = 0.58$, $p = 0.566$) was detected. After 3 h of exposure to 10 mg Cu L^{-1} an 57 % reduction in heart rate was evident in the group acclimated to control water, but not in the two Cd-exposed groups (Fig. 2a). This difference was significant ($F_{2,28} = 15.76$, $p = 0.00003$) and Tukey test showed that values observed for both the 15 and 30 mg Cd L^{-1} exposure groups were significantly different from those measured in animals acclimated to control water, but not from each other. A similar trend was observed in Zn-exposed groups. The 3 h exposure to 10 mg Cu L^{-1} caused a significant reduction

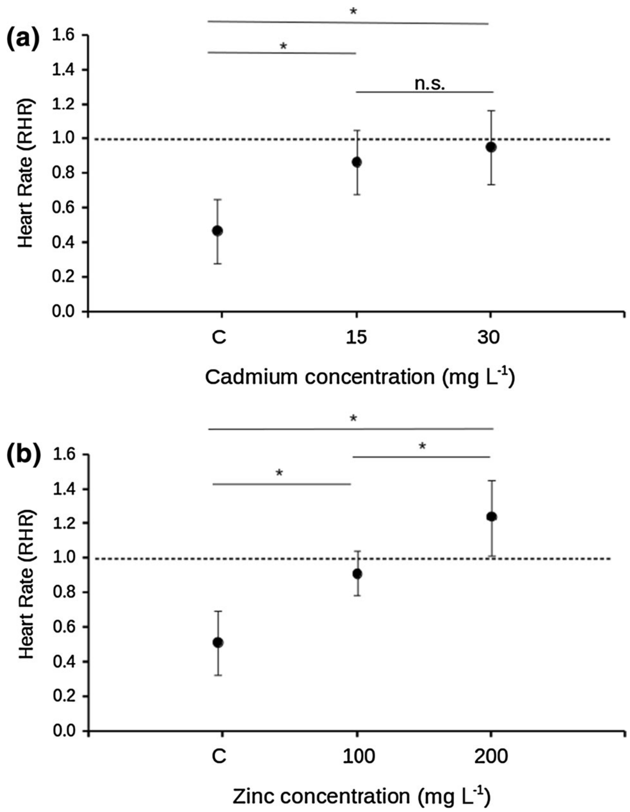

Fig. 2 Implications of previous exposure to **a** Cd and **b** Zn on the heart rate response of *P. clarkii* exposed to 10 mg L^{-1} Cu solution. *Circles* represent the mean (±SD) RHR of specimens preliminarily exposed to different metal concentrations (C = animals acclimated to control water before the exposure to Cu). *Horizontal continuous lines*: Tukey test: $*p < 0.05$, n.s. = not significant. Value = 1 (*dashed line*) means no variation in the HR caused by the exposure; values >1 and <1 represent increased or decreased HRs, respectively. Sample size was n = 10 for each group

of heart rate in crayfish exposed to control water, but not in those exposed to 100 mg Zn L^{-1} (Fig. 2b). A slight increase in heart rate was observed in crayfish exposed to 200 mg Zn L^{-1}. All these differences were significant ($F_{2,28} = 29.17$, $p < 0.00001$) and Tukey test showed that both 100 and 200 mg Zn L^{-1} exposed groups were significantly different from the control-acclimated group and not from each other.

Previous exposure to Cd and Zn concentrations prevented the bradycardia expected to be induced by the subsequent exposure to Cu, and this effect was consistent for all the concentrations of the two metals used. This finding is in line with previous observations regarding the effect of previous exposure to metals on heart rate of this species (Bini and Chelazzi 2006), but also extends our knowledge to cross-acclimation effects. Cross acclimation is a known phenomenon, described to occur both for metals and organic substances, but to our knowledge, this is the first study where it was investigated using heart rate changes as a biomarker.

The physiological and molecular mechanisms underpinning this acclimation effect are unknown, and are deserving further investigation. The effect of previous exposure was not simply due to an alteration of metabolic rate induced by the Cd or Zn concentrations. In fact, exposure to Cd did not cause any detectable change in heart rate, and the bradycardic effect induced by Zn was completely cancelled by a 3 h wash in clean water. Among the possible mechanisms leading to acquired resistance, two seem most likely for this specific case. In many aquatic organisms, an increase in environmental metal concentration induces metallothionein-like proteins – MTLPs – (Amiard et al. 2006). MTLPs play a primary role in the regulation and homeostasis of several essential metals, including Cu and Zn, but are also involved in the detoxification of non-essential ones, such as Cd (Coile et al. 2002). Several studies demonstrated that acquired tolerance to metals may relate to the body content of these proteins (reviewed in Amiard et al. 2006). Induction of these proteins can occur in a rather short time and, as for example, a 2–24 h exposure to Cd proved to be sufficiently long to induce MTLPs in several aquatic crustaceans, including *P. clarckii* (e.g. Martinez et al. 1993; Del Ramo et al. 1995). Such a short time span is fully compatible with the acclimation times used in the present study, making the induction of MTPLs a likely candidate to explain the observed acclimation in *P. clarkii.* On the other side, it is also known that in some invertebrates pre-exposure to a metal may induce a reduction of that metal's uptake from the ambient medium (reviewed by Wang and Rainbow 2005). However, despite this effect seems to be common in molluscs (e.g. Blackmore and Wang 2002; Wang and Rainbow 2005) contradictory evidences were produced for crustaceans. As for example, Truchot and Rtal (1998) demonstrated an important reduction of Cu uptake from the ambient medium, after long term exposure to sub-lethal doses of this metal in the crab *Carcinus maenas*, whereas metal pre-exposure did not alter the uptake rate in barnacles (Rainbow et al. 2004). No information on the effect of pre-exposure on metal uptake is available for *P. clarkii*. Furthermore, given that, to our knowledge, there are no clear demonstrations that pre-exposure to one element may influence the uptake of others, this mechanism seems less likely to explain the changes in heart rate observed in *P. clarkii.*

Finally, it is important to discuss whether our results support the idea that heart rate changes following an acute metal stress could be used to infer a previous exposure to metal contamination. Previous investigations on heart rate activity of marine invertebrates (gastropod molluscs) invoked an a-specific cross-acclimation effect to explain why specimens collected from polluted and unpolluted sites responded differently to an acute metal (Cu) stress

(Chelazzi et al. 2004; Bini et al. 2008). In these species, the heart rate of animals from metal-polluted shores was, in fact, not affected by a subsequent acute exposure to Cu, contrarily to what happened in limpets from unpolluted sites. This finding was interpreted as due to an increase in tolerance determined by a cross-acclimation triggered by previous exposure. However, despite a superficial agreement between this explanation and our results, there are some caveats that need to be acknowledged, before our findings could be used in support of this explanation. In the examples provided by Chelazzi et al. (2004) and Bini et al. (2008), in fact, it is not clear to what extent increased tolerance originates from individual acclimation (as in our study), genetic selection or a mixture of both. Until rigorous tests to separate all these effects have been carried out, generalisations may be useless, when not dangerous (Morgan et al. 2007; Gall et al. 2013).

In conclusion, the results of this study provided a clear evidence of a cross-acclimation effect in the heart rate response of *P. clarkii* after exposure to metal pollutants. In particular, the results showed that even a relatively short exposure to metals is able to induce a cross-acclimation on heart rate activity of this species. To our knowledge, this is the first time that such an effect is described on the heart rate of a crustacean, and this finding may contribute to a better understanding of the action of metals on the cardiac physiology this group. Our results, however, prompted for further investigations to clarify the role of the specific physiological mechanisms leading to acquired resistance, and to ascertain how short term acclimation effects interact with longer-term selective processes.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All applicable national guidelines for the care and use of animals were followed.

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