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Variation of crustacean hyperglycemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress

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Summary

This study investigates (by means of bioassays and ELISA using an antibody against recombinant cHH) the variation of cHH levels in the eyestalks and haemolymph of *Palaemon elegans* (Decapoda, Caridea) following exposure to various stresses (heavy metals and lipopolysaccharide), and correlates them with the variation in amount and time course of blood glucose. The dose-relationship between exposure to copper and quick release of cHH from the eyestalk into haemolymph was confirmed by variation of blood glucose with a dose-related hyperglycaemia, that peaked 2 h after immersion in contaminated seawater. Animals exposed to a sublethal concentration of mercury showed the same dose relation between toxicant, release of cHH from the eyestalk, increment of circulating hormone level and subsequent hyperglycaemia as observed for copper contamination. It is of note that although the highest lethal mercury concentration induced the release of cHH from the eyestalk into the haemolymph, it was not followed by a significant variation of blood glucose. Step doses of a bacterial contaminant [such as lipopolysaccharide (LPS) from *E. coli* injected into shrimps] confirmed the dose-relationship and convergent chain of events that bring about hyperglycaemia. These are the first data that relate the release of cHH from the eyestalk, the circulating hormone level and the consequent glycaemic response to stress. Moreover, they confirm the dose-related pathway that leads to variation of blood glucose as a quantitative biomarker of environmental quality, even at sublethal toxicant concentrations.

Key words: Crustacea, cHH, glucose, heavy metals, lipopolysaccharide, water quality.

Introduction

Physiological processes are mostly coordinated by hormones; and in crustaceans, changes in hormone levels are expected to occur soon after exposure to environmental stresses, such as pollutants, eventually acting as endocrine disruptors (Fingerman et al., 1996, 1997). Hyperglycaemia is a common stress response of many aquatic animals. In vertebrates, hyperglycaemia is mediated by elevated plasma cortisol, whereas in crustaceans it occurs following the involvement of the crustacean hyperglycemic hormone (cHH) produced in the eyestalk. cHH mainly regulates glucose homeostasis; it belongs to a neuropeptide family of about 8.5 KDa, synthesized in the eyestalk by the medulla terminalis X-organs (MTOX), and is accumulated by, and released from, the sinus gland (SG; Fingerman, 1987, 1992; Garcìa and Aréchiga, 1998; Bocking et al., 2001; Wilcockson et al., 2002). Exposure to atmospheric air induces a large, but transitory, increase in blood glucose levels in the intertidal crab *Chasmagnathus granulata* (Santos and Colares, 1986). Hyperglycaemia was reported in the giant prawn *Macrobrachium rosenbergii* as a response to cold shock (Kuo and Yang, 1999). Cadmium (Cd), mercury (Hg) and copper (Cu) induce hyperglycaemia in the freshwater prawn *Macrobrachium kistenensis* and the crab *Barytelphusa canicularis* (Nagabhushanam and Kulkarni, 1981; Machele et al., 1989) and *Scylla serrata* (Reddy and Bhagyalakshmi, 1994). Moreover, $CdCl_2$ induces hyperglycaemia in intact crayfish *Procambarus clarkii*, but not in the absence of the eyestalks, suggesting a cHH-mediated response (Reddy et al., 1996). An increase in blood glucose level was found in *P. elegans* and other crustacean species after injection of bacterial lipopolysaccharide (LPS). The hyperglycemic effect is probably mediated by cHH because it does not occur in eyestalk-ablated animals, and it is dose related (although by what extent depends on the source of LPS; Lorenzon et al., 1997, 2002).

Our studies (Lorenzon et al., 2000) on the effect of heavy metals on blood glucose levels in *P. elegans* showed that the intermediate sublethal concentrations of Hg, Cd and lead (Pb) produced significant hyperglycaemic responses, while the highest concentrations elicited no hyperglycaemia in the 24 h following treatment. By contrast, animals exposed to Cu and zinc (Zn) showed hyperglycaemia, even at high concentrations. This difference in response can probably be explained by the physiological roles of the essential elements Cu and Zn in

crustaceans, and consequent tolerance adaptations, as opposed to the toxic xenobiotic heavy metals Cd, Hg and Pb. Hyperglycaemic responses to both groups of heavy metals are not elicited in eyestalk-ablated animals, therefore, they involve MTXO-SG hormones, probably cHH.

However, in spite of the richness of information about blood glucose variation following stress, much less is known about the stress-induced variation of cHH content in the sinus gland and circulating in the haemolymph. In the crayfish *Orconectes limosus* undergoing hypoxia, cHH titres of around 120 pmol l^{-1} are reached within 15 min (Keller and Orth, 1990). In *Cancer pagurus*, emersion induced an increase of the haemolymph cHH after 4 h (Webster, 1996). Chang et al. (1998) used ELISA to monitor the blood cHH variation in *Homarus americanus* following various environmental stresses. Emersion proved to be a potent stimulator for elevation of cHH, while temperature and salinity variations were less effective.

Moreover, in *C. maenas* it has been shown that the concentration of cHH in the haemolymph increases dramatically during moulting: from 1–5 fmol 100 μl^{-1} in the intermoult, up to 150–200 fmol 100 μl^{-1} during ecdysis (Chung et al., 1999). More recently, variation of cHH titre was reported in the haemolymph of *Nephrops norvegicus* infected by the parasitic dinoflagellate *Hematodinium* sp. (Stentiford et al., 2001). Finally, increased water temperature in *Cancer pagurus* and *Procambarus clarckii* induced an increment in blood cHH (Wilcockson et al., 2002; Zou et al., 2003). The aim of this paper was to monitor the variation of cHH in the eyestalks and haemolymph of *P. elegans* (Decapoda, Caridea) following various stresses (heavy metals and LPS), and to relate cHH levels to the variation of amount and time course of blood glucose.

Materials and methods

Animal supply and maintenance

Specimens of *Palaemon elegans* (Rathke) (Decapoda, Caridea, 4–6 cm in length), an eurythermal and euryhaline species distributed widely along the coastal areas of Europe, were supplied by commercial fishermen and caught by cages in the Gulf of Trieste (Upper Adriatic Sea). They were stocked in 120 l glass tanks with closed-circuit (filtered and thoroughly aerated) 36% salinity artificial sea water (Prodac® Padova, Italy), at 16–18°C, and a natural L:D photoperiod, 300 lux intensity (type 49 fluorescent tubes by Philips, Monza, Italy). They were fed *ad libitum* with bits of shrimp, cuttlefish or fish every second day; dead animals were removed daily. Apparently healthy animals of both sexes, non-ovigerous and intermoult, having a body weight 1–1.5 g were used. 48 h before use, animals were housed individually in 500 ml plastic net cages immersed in larger tanks, for individual recognition. From this point on, and during the experiments, the animals were not fed.

Haemolymph sampling and determination of glycaemia

The animals were blotted dry and haemolymph (50 µl) was withdrawn from the pericardial sinus into a sterile 1 ml syringe fitted with 25 g needles. Animals (N=10 for each treatment) were bled at 0 h, usually between 9–10 a.m., to reduce possible interference due to circadian change in blood-glucose level (Kallen et al., 1990).

Haemolymph glucose content was quantified by using One touch® II Meter (Lifescan, Miltipas, CA, USA) and commercial kit test strips (precision of strips±3% coefficient of variation in the tested range). Owing to the speed of processing, no anticoagulant was needed. In the results, variations of glycaemia defined as increments are given as the mean of: [(experimental value)/(value displayed by the same animal at 0 h)]–1.

Effect of $HgCl_2$, $CuCl_2$ and lipopolysaccharide (LPS) on blood glucose level

Variation of glycaemia was tested on groups of intact *P. elegans* (N=10 for each treatment) following exposure to Hg^{2+} (0.1, 0.5 and 5 mg l^{-1}, administered as $HgCl_2$), Cu^{2+} (0.1 and 5 mg l^{-1}, administered as $CuCl_2$) in seawater, and the controls maintained in uncontaminated water. At 0, 0.5, 1, 2, 3 and 24 h after exposure to heavy metals, animals were bled as described above.

For LPS, groups of 10 animals were injected with 0.1 and 2 mg g^{-1} live weight of LPS from *Escherichia coli* 0111:B4; variation of glycaemia was determined as described above at 0, 0.5, 1, 2, 3, 5 and 24 h. Control groups injected with saline were tested at the same time; sterile saline for marine crustaceans was prepared with pyrogen-free distilled water and analytical grade chemicals, according to Smith and Ratcliffe (1978) and autoclaved for 25 min. All reagents were supplied by Sigma-Aldrich (St Louis, Missouri, USA).

Eyestalk homogenate and haemolymph treatment for ELISA measurement of cHH variation

Groups of 10 *P. elegans* were exposed to Hg^{2+} (0.1, 0.5 and 5 mg l^{-1}), Cu^{2+} (0.1 and 5 mg l^{-1}) or injected with LPS (0.1 and 2 mg g^{-1} live weight); untreated animals and saline-injected animals were used as controls. Eyestalks were removed at time 0 and then at 0.5, 1, 2 and 3 h of exposure (and at 5 h only for LPS) from the 10 animals of each different experimental group. Animals were anaesthetized for 1 min on ice before ablation. The eyestalk was quickly frozen and the pigmented eyecup dissected. Eyestalk homogenate was prepared from 20 eyestalks homogenized in 2 ml cold phosphate-buffered saline (PBS Sigma) pH 8.0, and then centrifuged for 1 h at 930 ***g*** and 4°C, and the pellet discarded. Homogenates were quickly deep frozen at –20°C and stored until required for study.

Haemolymph was withdrawn from different groups of 10 *P. elegans* for each treatment and time, as described above at time 0 and then at 0.5, 1, 2 and 3 h (and at 5 h only for LPS), immediately centrifuged for 1 min at 10,300 ***g*** and 4°C, and the supernatants then stored at –20°C.

Direct enzyme-linked immunosorbent assay (ELISA) of cHH

The samples of eyestalk homogenate were always tested at

the concentration of 1 sinus gland equivalent (SGe) in 100 µl and at dilutions of 0.5, 0.1, 0.05, 0.01 and 0.001 SGe in PBS (pH 8.0). Haemolymph was tested undiluted and at dilutions of 0.5, 0.1, 0.05 in PBS. The standards were known amount (from 1 to 0.001 µg in 100 µl of PBS) of 6×His-*Nen*cHHwt (M_r=11 KDa) recombinant protein (Mettulio, 2002).

100 µl of the samples (eyestalk homogenate, or haemolymph, from the different treatments and standards) were loaded onto a 96-microwell plate (Costar, Bethesda, MD, USA) and incubated in duplicate overnight at 4°C. The content of the wells was discarded and the wells were washed four times with 250 µl of PBS-T (PBS+0.1% Tween20, pH 7.4), then filled with 100 µl of 3% bovine serum albumine (BSA, Sigma) solution in PBS pH 7.4 plus 5% foetal calf serum (FCS, Sigma) and left for 2 h at room temperature (RT). The content was discarded and the plates washed four times as described above. 100 µl of the biotinylated anti-*Nen*cHH (anti *N. norvegicus* cHH; Giulianini et al., 2002) antibody (1 µg µl^{-1}) diluted 1:1000 was then added to each well and the plate was incubated for 3 h at 36°C.

After removal of the biotinylated antibody, plates were washed extensively with PBS-T, followed by the addition of 100 µl of streptavidin-peroxidase solution (Sigma) diluted 1:5000 and incubated for 1 h at RT. The plates were once again washed four times with PBS-T and developed with 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid solution (Sigma; liquid substrate ready for use) in darkness for 1 h at RT (100 µl per well). The absorbance was measured in a multiwell plate reader (Anthos 2020 version 1.1; Krefeld, Germany) at 405 nm.

Fig. 1 presents an example of the standard curve obtained from dilutions of a known amount of a 6×His-*Nen*cHHwt. The graph shows the mean and standard deviations (after subtraction of the background absorption) of the quadruplicate determinations of recombinant cHH plotted against the optical density (OD) with the calculated linear regression (Fig. 1). Sample values were then inserted into the equation and the amount of unknown cHH thereby determined.

Statistical analysis

All statistics were performed by using a SPSS 9® (SPSS Inc., Chicago, IL, USA) for Windows package, and data are given as arithmetic means ± S.D. Effects of experimental treatments on blood glucose levels were analysed. Analysis of variance (ANOVA) and Student's *t*-test were used to test the null hypotheses that all treatment means were equal, and then all the data were tested by the LSD and Dunnett *post hoc* test. The levels of significance were then calculated by Student's *t*-test for paired or independent data. A probability value of <0.05 of the statistical tests between the control and experimental values (mean ± S.D.) was considered significant. To test the statistical significance of ELISA values compared between experiments, Student's *t*-test was used at $P<0.05$. Time scale of graphs is not proportional, to provide a better visual inspection of data and size of the illustration.

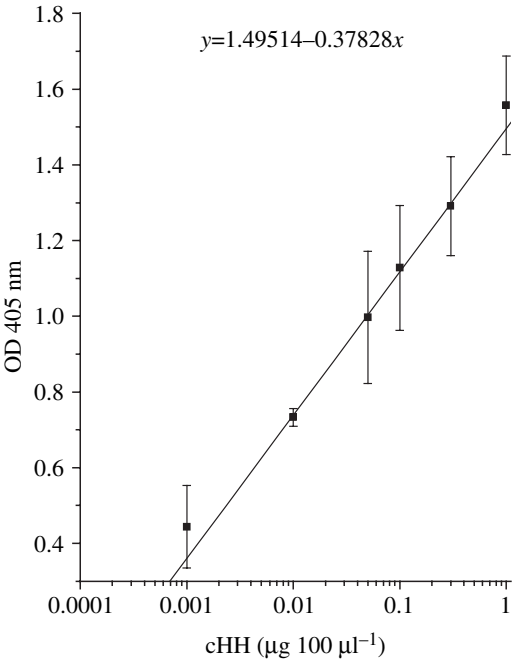

Fig. 1. Standard curve of known amounts of 6xHis-*Nen*cHHwt ELISA. The *x* axis represents the amount of peptide added per well (100 µl). Absorbance was read in a multiwell reader at 405 nm. Values of OD are means ± S.D. (N=4); correlation coefficients of the fitted curves are >0.99 for all the data (R=0.9937, S.D.=0.44, $P<0.001$).

Results

The mean concentration of glucose in the haemolymph of untreated *P. elegans* was 10.06±2.22 mg dl^{-1} (N=90). In untreated animals bled at times 0, 0.5, 1, 2, 3, 5 and 24 h, no significant ($P>0.05$) variation of blood glucose was revealed compared with time 0 value of 10.70±3.06 mg dl^{-1} (N=10; data included in Fig. 10). Control animals injected with saline (N=10) reached a peak of glycaemia (see Materials and methods) of 0.37±0.22 (12.90±2.32 mg dl^{-1}) 2 h after injection, which is significantly (P=0.014) different from the initial value of 9.70±2.91 mg dl^{-1}. Thereafter, the glucose level returned to the initial level until the end of the experiment. No significant difference in variation of glycaemia ($P>0.05$) was noticed between the control animals that were bled only and those that were treated with saline (data included in Fig.10). The latter more-conservative control was used throughout for statistical comparison. In untreated animals, the cHH resting level in the eyestalk was 5.60±2.6 pmol SGe^{-1} (N=14) and in the haemolymph it was 1.13±0.28 pmol ml^{-1} (N=11), and these baseline values were used as the control for experimental animals exposed to heavy metals.

In animals injected with saline, the level of cHH in the eyestalk reached a minimum of 5.3±1.7 pmol SGe^{-1} (N=3) 1 h after injection, a value that was not significantly different from the resting value of the untreated animals (P=0.855); in the haemolymph, a maximum of circulating cHH of 1.23±0.603 pmol ml^{-1} was detected 30 min after injection of saline, which was not significantly different (P=0.930) from

the resting value of untreated animals. Sterile saline was also used as control for animals injected with LPS.

Time course of cHH levels in the eyestalk and haemolymph, and of blood glucose, following exposure to Cu^{2+}, Hg^{2+} and LPS

The effects of exposure to Cu^{2+} on cHH level in the eyestalk of *P. elegans* are shown in Fig. 2, where 0.1 mg l^{-1} is a sublethal concentration and 5 mg l^{-1} is a lethal concentration (as determined by Lorenzon et al., 2000).

The highest Cu^{2+} concentration induced a massive release of cHH from the sinus gland. After 30 min the cHH content is 0.71±0.21 pmol SGe^{-1}, significantly lower (P=0.006) than in the untreated controls (used as time 0) and the hormone level reached a minimum of 0.45±0.27 pmol SGe^{-1} (P=0.004 *vs* control) after 2 h. Thereafter, the cHH started to recover with a content of 2.70±3.94 pmol SGe^{-1} detected 3 h after exposure and not significantly different (P=0.126) from the control.

The Cu^{2+} concentration of 0.1 mg l^{-1} (Fig. 2) caused a gradual release of cHH. The value of cHH decreased to 3.37±2.99 pmol SGe^{-1} 1 h after exposure, which is not significantly different from the control (P=0.207). At 2 h, the significant minimum content of cHH of 2.29±0.81 pmol SGe^{-1} (P=0.04) was reached. Thereafter, from 3 h onwards the cHH titre returned to the pre-treatment level of 5.04±4.90 pmol SGe^{-1} (P=0.774 *vs* untreated control).

Exposure to Cu^{2+} 5 mg l^{-1} caused a significant elevation of haemolymph cHH (Fig. 3) that rose up to 8.67±2.99 pmol ml^{-1} after 2 h, a value that was significantly different (P=0.001) from that of the untreated animals (1.13±0.28 pmol ml^{-1}). The circulating cHH remained significantly (P<0.05) high throughout the experimental period. In animals exposed to the lowest Cu^{2+} concentration, the blood cHH remained on resting levels and only after 2 h increased slightly, but significantly (P=0.013 *vs* control), up to 1.68±0.34 pmol ml^{-1}. Exposure of *P. elegans* to Cu^{2+} induced a dose-related release of cHH from eyestalk to haemolymph.

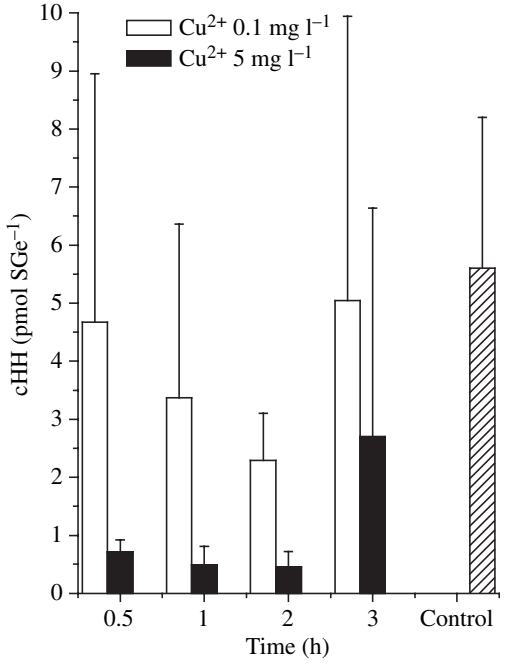

Fig. 2. Time course of cHH in the eyestalk homogenates pooled from 10 *P. elegans* after exposure to different concentrations of Cu^{2+} and in the untreated control. Values are expressed as means ± S.D.

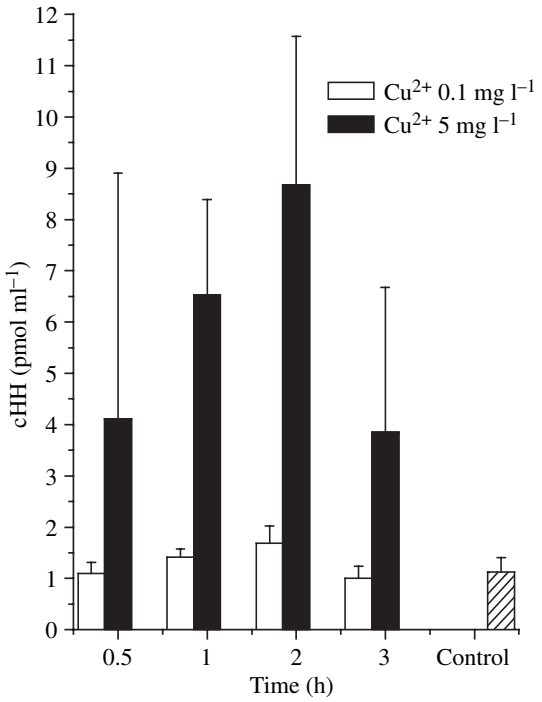

Fig. 3. Time course of cHH in the hemolymph pooled from 10 *P. elegans* after exposure to different concentrations of Cu^{2+} and in the untreated control. Values are expressed as means ± S.D.

Fig. 4 shows the consequent dose-related variation of glycaemia. At the highest concentration, in the first 3 h after exposure, a significant increase in blood glucose became evident, with a peak of increment of 2.60±0.49 (33.80±4.89 mg dl^{-1}) at 2 h that was significantly different from the initial value (9.50±1.65 mg dl^{-1}; P=0.001) and also from the control at the same time (13.30±2.75 mg dl^{-1}; P=0.001). Animals exposed to 0.1 mg l^{-1} Cu^{2+} showed a similar time course of hyperglycaemia but with a lower increment in blood glucose. A maximum increment of 0.77±0.25 (15.00±1.49 mg dl^{-1}) was revealed at 2 h, which is significantly different from the control (13.30±2.75 mg dl^{-1}; P=0.046) and from the value at the higher concentration (P=0.001) at the same time.

Fig. 5 shows the time course of cHH eyestalk content after exposure of *P. elegans* to Hg^{2+} at the sublethal concentrations (as defined by Lorenzon et al., 2000) of 0.1 and 0.5 mg l^{-1}, and at a lethal concentration of 5 mg l^{-1}, compared with the untreated control. At the lethal concentration, a rapid and massive release of cHH from the sinus gland became evident; in fact after 30 min from exposure, the hormone content decreased to 1.22±0.39 pmol SGe^{-1}, significantly (P=0.012)

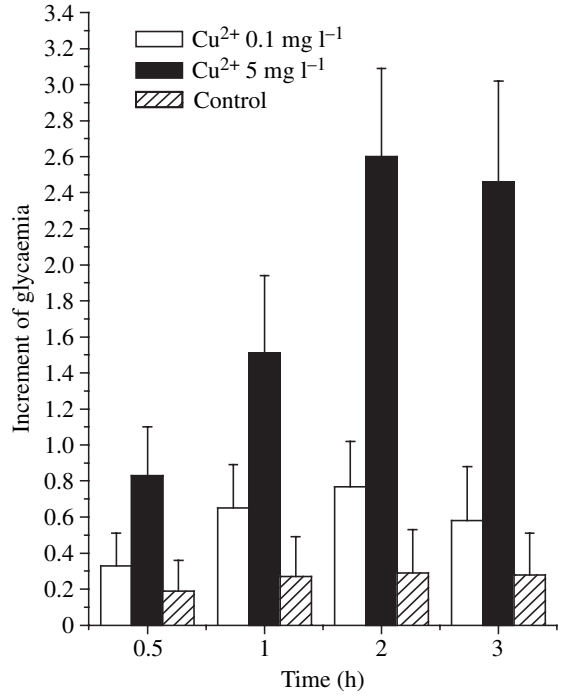

Fig. 4. Time course of glycaemia in the haemolymph of *P. elegans* after exposure to different concentrations of Cu^{2+} and in the untreated control group. Values of increment (see Materials and methods) are expressed as means ± S.D.

lower than the control (5.60±2.6 pmol SGe^{-1}). Values remained significantly (P<0.05) below the resting level for all the experimental period with a minimum of 0.72±0.25 pmol SGe^{-1} detected at 2 h.

The intermediate Hg^{2+} concentration (0.5 mg l^{-1}; Fig. 5) was even more effective at inducing release of cHH: after 30 min the hormone content in the eyestalk was 0.19±0.07 pmol SGe^{-1} (P=0.003 *vs* control) and the eyestalk remained massively and significantly (P<0.05) depleted of the hormone content throughout the experimental time considered. Finally, the lowest concentration of 0.1 mg l^{-1} induced a limited release of cHH that was significantly different (P=0.033) from the control at 1 h after exposure with a cHH level of 1.97±0.62 pmol SGe^{-1}.

Exposure to Hg^{2+} (Fig. 6), at the concentration of 5 mg l^{-1}, induced a rapid increase of cHH concentration in the haemolymph with a peak of 5.90±0.68 pmol ml^{-1} after 1 h that was significantly different (P=0.001) from the unexposed control (1.13±0.28 pmol ml^{-1}). Thereafter, the circulating cHH remained significantly (P<0.05) higher than in the control. The intermediate concentration was most effective in elevating blood cHH: the hormone concentration rapidly increased and remained significantly (P<0.05) higher than in control group, with a maximal concentration of circulating hormone (6.92±1.27 pmol ml^{-1}) at 2 h (Fig. 6). At the lowest Hg^{2+} concentration (0.1 mg l^{-1}), a significant increase (P<0.05) of circulating cHH was recorded 1 and 2 h after exposure with a maximum of 2.14±0.74 pmol ml^{-1}. Variation

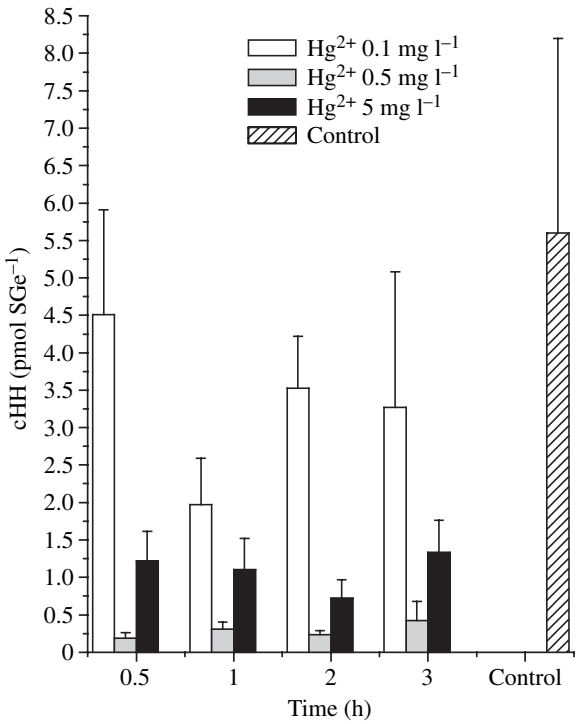

Fig. 5. Time course of cHH in the eyestalk homogenates pooled from 10 *P. elegans* after exposure to three different concentrations of Hg^{2+} and in the untreated control. Values are expressed as means ± S.D.

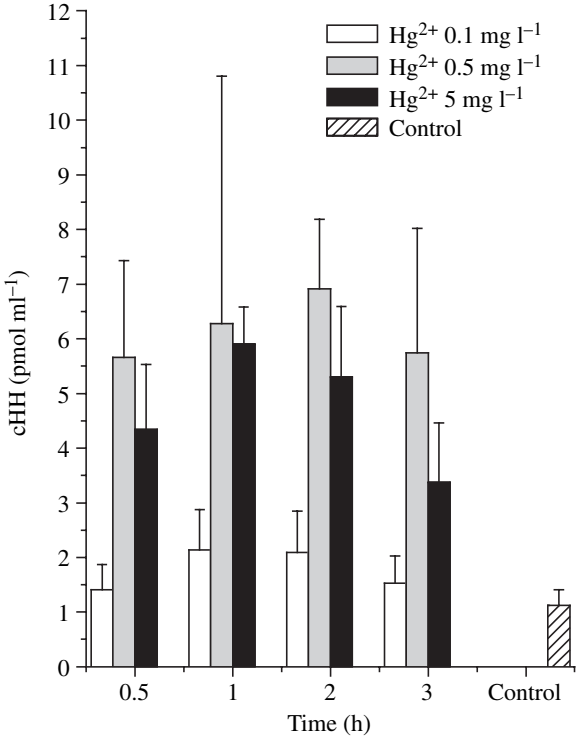

Fig. 6. Time course of cHH in the haemolymph pooled from 10 *P. elegans* after exposure to three different concentrations of Hg^{2+} and in the untreated control. Values are expressed as means ± S.D.

of eyestalk and blood cHH in the case of exposure to Hg^{2+} is not dose related; in fact, the intermediate concentration proved to be the most effective in altering physiological resting values, with a higher significant (P<0.05) release of cHH from the eyestalk.

This is paralleled by the variation of blood glucose. Fig. 7 shows time course of glycaemia after exposure of *P. elegans* to different concentrations of Hg. In animals exposed to 0.5 mg l^{-1} Hg, marked hyperglycaemia became evident after 1 h, which peaked at 3 h with an increment of 1.35±0.42 (22.20±5.45 mg dl^{-1}), and a concentration that was significantly different from the initial value of 9.40±1.35 mg dl^{-1} (P=0.001) and from the control at the same time 0.28±0.23 (13.10±2.75 mg dl^{-1}, P=0.001). By contrast, at the highest concentration (Fig. 7) no significant variation of blood glucose was revealed for all the experimental times: the maximum increment of 0.28±0.22 (13.88±2.38 mg dl^{-1}) recorded at 2 h was not significantly different from the initial value (P>0.05) and from the control (P>0.05). Animals exposed to 0.1 mg l^{-1} (Fig. 7) showed slight hyperglycaemia at 2 h with an increment of 0.58±0.20 (16.30±1.83 mg dl^{-1}), significantly different from the time 0 value (10.40±1.51 mg dl^{-1}; P=0.001) and from the control, which, at the same time, revealed an increment of 0.29±0.24 (13.30±2.75 mg dl^{-1}; P=0.009).

P. elegans injected with 2 mg g^{-1} living weight of LPS from *E. coli* 0111:B4 showed a massive release of cHH from the eyestalk (Fig. 8). After 30 min, the hormone level was 0.26±0.03 pmol SG^{-1}, which is significantly different from the initial value of 5.60±2.6 pmol SG^{-1} (P=0.003) and from the saline control at the same time point (5.47±1.86 pmol SGe^{-1}; P=0.008). After 3 h, the cHH level rose, slightly, to 0.75±0.14 pmol SGe^{-1} and, eventually, 5 h after injection the hormone content was 1.42±0.39 pmol SGe^{-1}, which was still significantly lower (P=0.007 and P=0.016, respectively) than the saline control. The lower dose of 0.1 mg g^{-1} (Fig. 8) induced the cHH release in the first hour after injection, with a minimum eyestalk content, revealed at 30 min, of 2.39±0.93 pmol SGe^{-1}, which is also significantly different from the saline control (P<0.05). Thereafter, we observed a gradual recovery of the hormone level. From 2 h onwards the cHH eyestalk content (4.11±2.59 pmol SGe^{-1}) was not significantly different from control (5.41±0.3 pmol SGe^{-1}; P=0.435) and after 5 h the value returned back to a normal resting value of 5.21±1.23 pmol SGe^{-1} (P=0.804 *vs* control).

Following the massive cHH release from the eyestalk in animals that had been treated with 2 mg g^{-1} of LPS (Fig. 9), high levels of circulating hormone were detected in the haemolymph. After 30 min, the cHH level rose to 7.12±1.81 pmol ml^{-1}, which was significantly different from the initial value in untreated (P=0.001) animals (1.13±0.28 pmol ml^{-1}) and from the saline-injected control

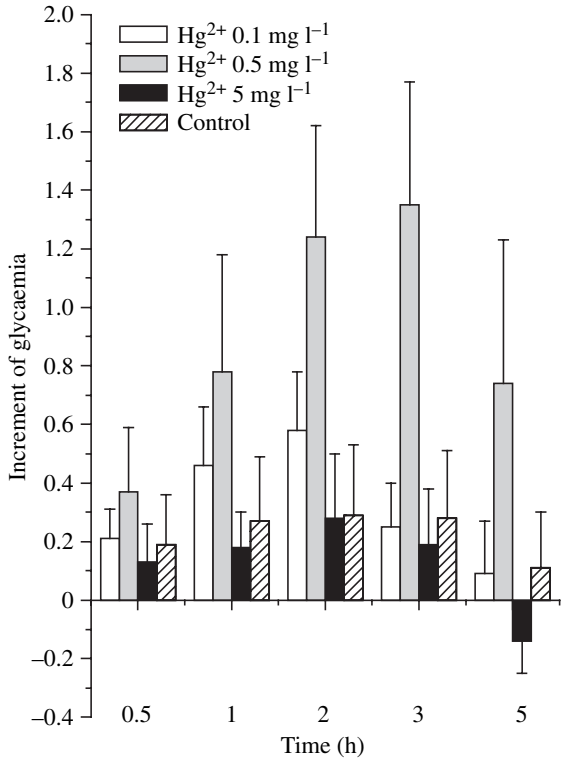

Fig. 7. Time course of glycaemia in the haemolymph of *P. elegans* after exposure to three different concentrations of Hg^{2+} and in the untreated control group. Values of increment (see Materials and methods) are expressed as means ± S.D.

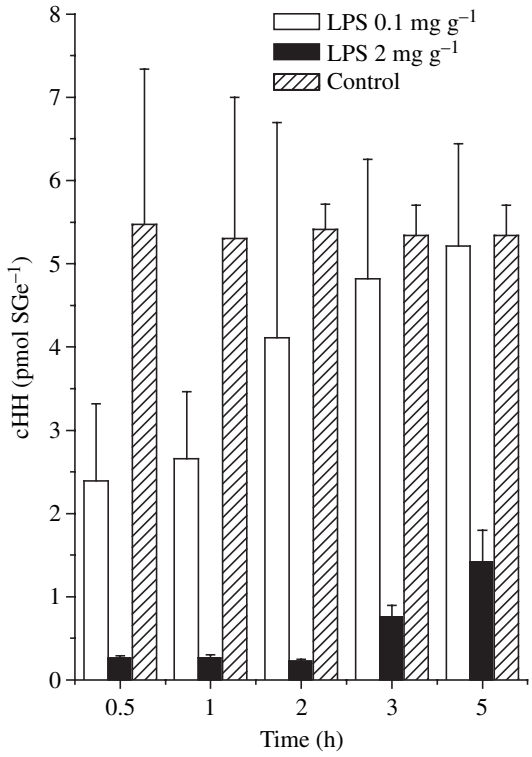

Fig. 8. Time course of cHH in the eyestalk homogenates pooled from 10 *P. elegans* after injection of two different concentrations of lipopolysaccharide and in the control. Values are expressed as means ± S.D.

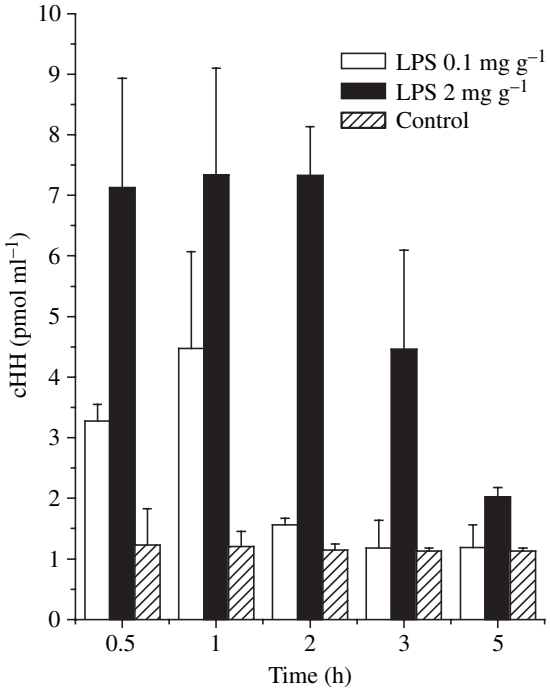

Fig. 9. Time course of cHH in the haemolymph pooled from 10 *P. elegans* after injection of two different concentrations of lipopolysaccharide and in the control. Values are expressed as means ± S.D.

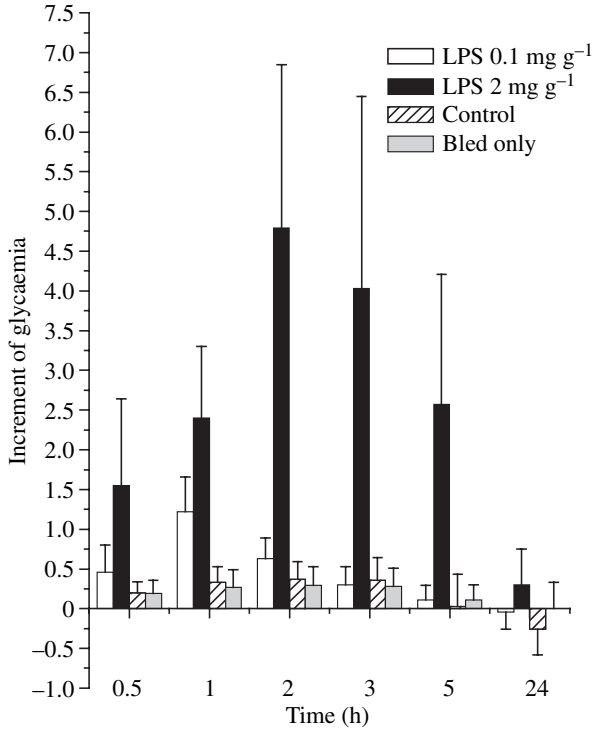

Fig. 10. Time course of glycaemia in the haemolymph of *P. elegans* after injection of two different concentrations of lipopolysaccharide and in the saline control and bled only control groups. Values of increment (see Materials and methods) are expressed as means ± S.D.

analysed at the same time point (1.23±0.6 pmol ml^{-1}; P=0.006). Circulating hormone concentration remained high until 2 h; afterwards the level gradually decreased to 4.45±1.65 pmol ml^{-1} (P=0.001 *vs* saline control) at 3 h and to 2.02±0.16 pmol ml^{-1} after 5 h, which is still significantly higher than control (P=0.001). In animals injected with 0.1 mg g^{-1} of LPS (Fig. 9), an increase in circulating cHH was detected from 30 min with a significant (P=0.001 *vs* control) peak of 4.47±1.26 pmol ml^{-1} at 1 h, then the level decreased and after 3 h there was no significant difference from the control (P>0.05; Fig. 9).

Blood glucose variation after injection of LPS (Fig. 10) follows the time course of haemolymph cHH. At the highest dose, from 30 min after injection, an increment of glycaemia of 1.55±1.09 (26.90±12.40 mg dl^{-1}) is revealed – a concentration that was significantly different from initial value (10.70±2.71 mg dl^{-1}; P=0.001) and from the control at the same time (11.40±2.37 mg dl^{-1}, P=0.001). At 2 h, injected animals showed a peak of increment of 4.79±2.06 (58.90±16.93 mg dl^{-1}), and in the next 24 h blood glucose level gradually recovered to resting values. The lower dose (0.1 mg g^{-1}) induced a slight hyperglycaemia at 1 h with a maximal increment of 1.22±0.44 (19.10±4.01 mg dl^{-1}) significantly different from time 0 value (8.70±1.42 mg dl^{-1} P=0.001) and from control (P=0.001). Thereafter, no significant variation (P>0.05) of blood glucose was recorded.

Discussion

This paper describes (using ELISA and bioassays) the relationship between environmental stress, the release of cHH from the eyestalk into the haemolymph and the hyperglycemic response in the shrimp *Palaemon elegans*. Moreover, this work validates the use of a cross-reactive antibody anti-*Nen*cHH (Giulianini et al., 2002) to assess cHH content in the eyestalk and haemolymph of *P. elegans*. Finally, this present study is the first, to our knowledge, to quantify the variation of cHH after a challenge with different stressors and to follow it through to its effects in the blood compartment.

The resting value of cHH in *P. elegans* eyestalk was found to be 5.6±2.6 pmol SGe^{-1} (62±29 ng SGe^{-1}), which is in the range of those already detected in other crustacean species. Huberman et al. (1995) identified in *Procambarus bouvieri* two isoforms of the cHH (cHH-I e cHH-II) in approximate concentrations of 60 and 20 ng SG^{-1}, respectively. Chang et al. (1990) found in *Homarus americanus* a cHH content in the sinus gland of 3 pmol SG^{-1}. More recently, Marco et al. (2000) quantified the content of cHH-I in the spiny lobster *Jasus lalandii* as 20 pmol SG^{-1} and cHH-II as 3 pmol SG^{-1}. In *C. maenas* and *C. pagurus*, a larger cHH content of 180 pmol SG^{-1} and 125 pmol SG^{-1}, respectively, was detected (Keller et al., 1985; Webster, 1996). All former data were obtained by calibration on HPLC purified SG cHH and

antibody raised against it. We are presently unable to relate immunoreactivity of our recombinant cHH and antibody with the natural purified neuropeptide. Because of cross immunoreactivity, western blotting, and similar distribution (Giulianini et al., 2002) and quantitative profiles, we are confident that immuno-localization and quantification is specific for the native cHH as well.

In *P. elegans*, exposure to Cu induced a dose-related rapid and massive release of cHH from the eyestalk into haemolymph at the higher, lethal concentration, whereas a gradual and reduced discharge was revealed at the lower concentration. The relationship between exposure to toxicant and release of cHH is confirmed by variation of blood glucose with a dose-related hyperglycaemia that peaked 2 h after exposure to Cu.

Animals exposed to previously defined (Lorenzon et al., 2000) sublethal concentrations of Hg showed the same quantitative and time-course relationships between toxicant and release of cHH from the eyestalk, increment of hormone level in the haemolymph and subsequent hyperglycaemia (as already described for Cu contamination). Interestingly, however, the highest, lethal concentration of 5 mg l^{-1} (Lorenzon et al., 2000) induced the release of cHH from the eyestalk to the haemolymph but was not followed by a significant variation of blood glucose. This situation could be related to the high toxicity of Hg, which may interfere with the finer mechanisms that regulate the hyperglycaemic response. It is not caused by a synaptic blockage of the superimposed neuronal release network (Lorenzon et al., 1999) or to a limited release of circulating cHH because high levels of cHH are discharged from the SG into the blood compartment. Likewise, it is not due to inhibition of peripheral receptors on glucogenolytic target organs; indeed, native SG homogenate injected into eyestalk-less shrimps exposed to lethal Hg^{2+} for 3 h can still cause hyperglycaemia (Lorenzon et al., 2000). High concentrations of Hg, instead, may change the functionality of the prepro-cHH processed during secretory steps, due to Hg ability to bind cysteines – six of which represent a highly conserved feature of the peptide structure (Lacombe et al., 1999) – thereby altering the active conformation of the peptide, as seen in another system (Rodgers et al., 2001), but not its immunoreactivity. Moreover, Hg is known to impair osmoregulatory capability in the crab *Eriocheir sinensis* (Péqueux et al., 1996); and in *P. clarkii* Hg induces an inhibition of the acetylcholinesterase activity (Devi and Fingerman, 1995). Therefore, the altered response in *P. elegans* exposed to high concentrations of Hg^{2+} may be related to physiological modifications induced by Hg^{2+} at a different systemic level.

Contamination with different doses of a bacterial thermostable endotoxin, such as LPS from *E. coli*, confirms the dose-related and convergent chain of events that bring about hyperglycaemia. It suggests that blood glucose elevation is a general purpose response to stressors and is likely to perform a protective role. Variation in the level of circulating cHH has been reported following exposure to various stresses. In *Cancer pagurus*, variation of haemolymph cHH is reported after emersion; hormone titre rapidly increases from undetectable levels to 30 pmol l^{-1} after 4 h with a simultaneous increase of blood glucose (Webster, 1996).

Chang et al. (1998) used ELISA to measure the levels of circulating cHH in *Homarus americanus* following three different kinds of stress: emersion, temperature elevation and salinity change. Emersion was the most potent stimulator for the elevation of haemolymph cHH increasing the baseline values of 4 fmol ml^{-1} to 168.1 fmol ml^{-1} after 4 h; thermal and salinity stress caused only a slight increase in circulating hormone. In *H. americanus*, the maximum increment of glycaemia is reached at 2 h after emersion while the maximum cHH level in the haemolymph is revealed at 4 h. By contrast, in our experiment, the peak of circulating cHH precedes, or at least coincides with, the maximum level of blood glucose. Recently, Zou et al. (2003) demonstrated that variation of water temperature from 24 to 34°C increases cHH levels from 32.4±4.9 fmol ml^{-1} up to 123.3±21.1 fmol ml^{-1} after 2 h in *P. clarkii*.

Variation of blood cHH has been reported during the moult of *C. maenas* (Chung et al., 1999) and, in particular, a dramatic increase of the cHH level became evident during ecdysis (from 1–5 fmol 100 μl^{-1} of intermoult to 200 fmol 100 μl^{-1}). In this case, the release of cHH is not dependent on the eyestalk, as an increase in circulating cHH is revealed also in eyestalk-less animals and release from the gut-associated neuroendocrine tissue. However, additional eyestalk release could be related with the cells containing cHH, as identified by Chang et al. (1999) in the second roots of the thoracic ganglia and in the suboesophageal ganglion.

Infection of *Nephrops norvegicus* by the parasitic dinoflagellate *Hematodinium sp*. induces an elevation of the cHH titre in the haemolymph (Stentiford et al., 2001). Concentration of blood hormone increases with the severity of the infection from 32.2 fmol ml^{-1} in uninfected animals to 77.2 and 107.65 fmol ml^{-1}. In this case, the concentration of glucose in the haemolymph is significantly reduced in infected animals, probably due to the use of glucose as a substrate for the growth of the parasite in the haemolymph (Stentiford et al., 2001).

The basal level of cHH in the haemolymph of *P. elegans* was 1.13±0.28 pmol ml^{-1} (12±0.3 ng ml^{-1}), which is higher than in the species discussed above. This could depend on the species tested or, in the presence of a similar cHH eyestalk content and release, on a smaller volume of the body-fluid compartment and possibly slower turnover of the circulating hormone. Therefore, further information is needed about the time course and fine mechanisms that regulate the release of cHH, its binding to receptors, and about the half-life and the catabolism of this hormone in the haemolymph in species belonging to different taxa and/or of different inner-fluid-compartment volumes.

In conclusion, the results presented in the present study are the first data that: (1) relate the release of cHH from the eyestalk, the circulating hormone level and the consequent glycaemic response to stress; (2) provide evidence of the

interference by Hg^{2+} on the regulation of this mechanism; (3) confirm the dose-related pathway that leads to variation of blood glucose as a quantitative biomarker of environmental quality, even at sublethal toxicant concentration.

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