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Heavy Metal Toxicity and Differential Effects on the Hyperglycemic Stress Response in the Shrimp *Palaemon elegans*

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Abstract. Agricultural and industrial activities cause heavy metal pollution of the aquatic environment. The sensitivity of crustaceans to heavy metals is well documented. However, the hormonal and metabolic target of physiological functions affected by sublethal toxicity and stress responses have been scarcely investigated. Exposure of *Palaemon elegans* to increasing concentrations of heavy metals dissolved in artificial sea water resulted in an order of toxicity tested by LC_{50} for 96 h in intact and eyestalkless animals in which Hg is the most toxic, followed by Cd, Cu, Zn, and Pb. Eyestalkless animals were found to be more sensitive than intact individuals. Heavy metals affect the blood glucose levels, yet manipulative stress does not. The intermediate sublethal concentrations of Hg, Cd, and Pb produced significant hyperglycemic responses within 3 h, while the highest concentrations elicited no hyperglycemia in 24 h. In contrast, animals exposed to Cu and Zn showed hyperglycemia even at high concentrations. This difference in response between Cu or Zn and the nonessential heavy metals Cd, Hg, or Pb can probably be explained by the physiological roles of the former in crustaceans and by tolerance adaptations. Involvement of the crustacean hyperglycemic hormone (cHH) was tested by routine bioassay on eyestalkless individuals; each group was injected with a two-eyestalk-equivalent extract from control animals or from shrimp exposed to high concentrations of Cd, Hg, Pb, or low concentrations of Cu or Zn. All showed a hyperglycemic response within 2 h. In contrast, extracts of eyestalk removed from animals that had developed a full hyperglycemic reaction after exposure to low concentrations of Hg, Cd, Pb, or high concentrations of Cu and Zn were depleted of cHH as shown by the attenuation of the response after injection of the extracts into eyestalkless animals. This generalized and predictable sublethal response can be used as a quantitative physiological biomarker for water quality monitoring assessment.

Agricultural and industrial activities cause environmental contamination of the aquatic environment due to heavy metals. Metals can enter the food chain and, as a result of bioaccumulation, cause serious health problems to humans.

The sensitivity of crustaceans to heavy metals is well documented (Ahsanullah *et al*. 1981; Migliore and de Nicola Giudici 1990); biotic factors, which seem to modify the sensitivity of crustaceans to heavy metals, include life stage, size, reproductive status, molting stage, and nutritional condition (Vernberg *et al*. 1974; Madsen 1992; McGee *et al*. 1998). Two heavy metals biologically essential for crustaceans are copper, which is important for the metabolic functioning of hemocyanin, and zinc, a component of many enzymes (Bryan 1984; Rainbow 1988). In excess, however, these metals become toxic. Other nonessential metals may exert a noxious influence as well. Small amounts of absorbed trace metals may be stored in a metabolically available form for essential biochemical purposes. Alternatively, when absorbed they may be detoxified into metabolically inert chemical forms and stored in the body either temporarily or permanently. The accumulation strategies of crustaceans varies depending on metals and species (Rainbow 1988, 1997).

Initial studies of the effects of metals on marine organisms dealt mainly with LC_{50}. The earliest response of a marine organism to a physiological challenge by sublethal concentrations of metals might occur at molecular level. Therefore, it seems more appropriate to carry out studies at sublethal rather than at lethal concentrations, given that the disturbance of the inmost biological mechanisms allows for early detection of biological deterioration of the environment (Amiard-Triquet *et al*. 1986). The toxicity induced by a pollutant could be the result of interference by the compound or one of its metabolites with biochemical events involved in the homeostatic control of a physiological process (Brouwer *et al*. 1990).

In crustaceans, physiological processes are often coordinated by hormones. Changes in hormone levels are expected to occur soon after exposure to a pollutant. Therefore, it follows that biosentinel parameters of toxicity can be identified by looking for alterations and modifications in endocrine patterns (Fingerman *et al*. 1996, 1998).

Heavy metals induce variations in the physiology of crustaceans. Mercury and cadmium in *Procambarus clarkii* cause the inhibition of ovarian maturation (Reddy *et al*. 1997); moreover,

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exposure to cadmium induces a reduction of fecundity and hatching success (Naqvi and Howell 1993). Inhibition of growth by dissolved copper was assessed in *Penaeus merguiensis* and *P. monodon* (Ahsanullah and Ying 1995). Hg, Cd, and Zn were found to inhibit limb regeneration and molting in *Uca pugilator* and other fiddler crabs (Weis 1978; 1980). Hyperglycemia is a frequent response of many aquatic animals to environmental stressors, and in crustaceans both hyperglycemia and the involvement of the eyestalk crustacean hyperglycemic hormone (cHH) were reported. Exposure to atmospheric air induces a large but transitory increase in blood glucose levels in the intertidal crab *Chasmagnathus granulata* (Santos and Colares 1986). Hyperglycemia was reported in the giant prawn *Macrobrachium rosenbergii* as a response to cold shock (Kuo and Yang 1999). Moreover, in *Crangon crangon* and *Carcinus maenas* a combination of Cu and Zn and hypoxia increases blood glucose (Johnson 1987). Similar effects are elicited by lipopolysaccharide (LPS) endotoxin injection in several crustacean species (Lorenzon *et al*. 1997). Cd, Hg, and Cu induce hyperglycemia in the freshwater prawn *Macrobrachium kistenensis*, and the crab *Barytelphusa canicularis* (Nagabhushanam and Kulkarni 1981; Machele *et al*. 1989). Hemolymph sugar levels are increased in the edible crab *Scylla serrata* exposed to a sublethal concentration (2.5 ppm) of cadmium chloride (Reddy and Bhagyalakshmi 1994). Organic pesticides can also induce hyperglycemia in several crustaceans (Fingerman *et al*. 1981; Reddy *et al*. 1983). Moreover, $CdCl_2$ induces hyperglycemia in intact crayfish *Procambarus clarkii*, but not in the absence of the eyestalks, suggesting a cHH-mediated response (Reddy *et al*. 1993). The aim of this paper is the *in vivo* investigation of the heavy metal–mediated stress response affecting glucose homeostasis and its eyestalk hormone control in *Palaemon elegans* an eurythermal and euryhaline crustacean species widespread along the coastal areas of Europe.

Materials and Methods

Animal Supply and Maintenance

The shrimp *P. elegans* Rathke 1837 (Decapoda, Caridea, 4–6 cm in length) was used as a standard test species. The animals were supplied by commercial fishermen in four batches of 600 in November and February 1997 and 1998. Animals was caught by small fish traps at the mouth of the River Tagliamento (Upper Adriatic Sea), a relatively uncontaminated area on the basis of the report of Donazzolo *et al*. (1981, 1984) regarding the characterization of the marine sediment in the northern Adriatic Sea area for heavy metal contents (Hg, Pb, Cd, Cr, Zn, and Fe).

Groups of 200 animals were stocked at controlled conditions throughout, *i.e*., in 120-L glass tanks with closed-circuit filtered and thoroughly aerated 36‰ salinity artificial (Prodac) sea water and seasonal L:D photoperiod, 300 lux intensity. They were fed *ad libitum* with bits of shrimp, cuttlefish, or fish every second day; dead animals were removed daily. The animals were allowed to acclimate for at least 2 weeks prior to experimentation. Apparently healthy animals of both sexes and intermolt having a body weight of 1–1.5 g were used. Forty-eight hours before use in an experiment, animals were housed individually in 500-ml plastic net cages immersed in 40-L glass tanks to allow for individual recognition and were kept unfed.

Identification of LC_{50} at Increasing Postexposure Times

For the identification of LC_{50}, groups of intact *P. elegans* or groups of ovigerous females or eyestalkless animals ($n = 10$ for each treatment) in 12 L were used for each concentration of heavy metals and for the controls in a static acute assay. Dead animals were removed daily for up to 96 h. Independent experiments were carried out in duplicate for each concentration of metal.

The concentration of heavy metals used for the LC_{50} experiment was related to the mean value of each heavy metal found in the sediment as reported by Donazzolo *et al*. (1981, 1984). Moreover, the lowest concentration tested for each metal was above the legal threshold content (91/271/CEE as per Italian dlgs152/99) for waters to be discharged into superficial water bodies.

Stock solutions of reagent-grade (Sigma) $CdCl_2$ (10, 5, 2, 1, 0.5, 0.2, and 0.1 mg L^{-1}), $HgCl_2$ (5, 2, 1, 0.5, 0.2, and 0.1 mg L^{-1}), $CuCl_2$ $2H_2O$ (20, 10, 5, 2, 1, 0.5, 0.2, and 0.1 mg L^{-1}), $ZnCl_2$ (50, 20, 10, 5, 2, and 1 mg L^{-1}), and $Pb(NO_3)_2$ (500, 200, 100, 50, 20, and 10 mg L^{-1}) were dissolved in sea water. $Pb(NO_3)_2$ was preferred to other lead salts for its higher solubility in water; the complete solubility in each solution was controlled using an ICP (inductively coupled plasma) mass spectrometer.

The 24-, 48-, or 96-h mortality data were processed by calculating the respective LC_{50} time by probit analysis.

Hemolymph Sampling and Determination of Glycemia

For the determination of glycemia groups of intact *P. elegans* or of eyestalkless animals ($n = 10$ for each treatment) in 12-L, individually partitioned tanks were used for each concentration of heavy metals: $CdCl_2$ (0.1, 0.2, 0.5, 1, 2, 5, and 10 mg L^{-1}), $HgCl_2$ (0.05, 0.1, 0.2, 0.5, 1, 2, and 5 mg L^{-1}), $CuCl_2$ $2H_2O$ (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 mg L^{-1}), $ZnCl_2$ (1, 2, 5, 10, 20, and 50 mg L^{-1}), $Pb(NO_3)_2$ (10, 20, 50, 100, 200, and 500 mg L^{-1}), and for the controls. The range of concentrations tested was chosen to span two $\log_{10}$ units around the LC_{50} 96-h value detected.

The hard-shelled animals were blotted dry, and hemolymph was withdrawn from the pericardial sinus with a sterile 1-ml syringe fitted with a 25-g needle. Animals ($n = 10$ for each treatment) were bled, 50 μl hemolymph each time, at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h, and 24 h after exposure to heavy metals. Glucose content was quantified by using One touch® II Meter (Lifescan) and commercial kit test strips (precision strips ±3% CV in the tested range). Given the short processing time, no anticoagulant was needed. Controls, maintained in contaminant-free water, assessed the effects of repetitive handling stress. In the results, variations of glycemia are given as the mean of (actual experimental value/value displayed by the same animal at 0 h) − 1.

Eyestalk Ablation and Homogenate Treatment

Bilateral eyestalk ablation of *P. elegans* was performed 48 h prior to the start of an experiment; animals were anesthetized for 1 min on ice, eyestalks pulled out at their basal articular membrane by fine tweezers, and then immediately returned to the tank and left to recover; mortality was minimal, *i.e*., less than 10%. Batches of 40 eyestalks were quickly deep frozen at −20°C and stored until required for study. Eyestalks from Hg^{++} (0.5 or 5 mg L^{-1}), Cd^{++} (0.5 or 5 mg L^{-1}), Cu^{++} (0.5 or 5 mg L^{-1}), Zn^{++} (5 or 50 mg L^{-1}), Pb^{++} (50 or 500 mg L^{-1}), or from untreated animals were removed 3 h after the exposure when the effect on glycemia was maximal. The eyestalk sinus gland represents the major store of cHH, accounting for 35% of the *P. elegans* neurohormones accumulated there (Lorenzon *et al*. 1997).

The eyecups of a batch of 40 frozen eyestalks were cut off, and their

basal stumps were homogenized in 1 ml cold sterile saline and then centrifuged for 30 min at 930 *g* and 4°C. For the standard bioassay of cHH eyestalk content (Smullen and Bentley 1994) a volume of 50 µl corresponding to two eyestalk equivalents was then injected into eyestalkless animals. The subsequent determination of glycemia was performed as stated above.

Statistical Methods

LC_{50} was calculated using the probit method (Wardlaw 1992). All statistics were performed by using a SPSS 9® for Windows package, and data are given as arithmetic means and ± values thereafter reported are standard deviations. Analysis of variance (ANOVA) and ANOVA–RM was 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 or less between the control and experimental values was considered significant.

Results

Toxicity of Heavy Metals

The toxicity of the five metals for *P. elegans* (both sexes) increased with time (Table 1). Hg was the most toxic metal in the 96-h assay, followed by Cd, Cu, Zn, and Pb. This order of toxicity did not change during the entire testing period.

Table 2 shows the LC_{50} values of ovigerous females exposed to Hg, Cd, and Cu. The order of toxicity at 96 h remained the same, but ovigerous females were nearly twice as sensitive to these metals as intact shrimps of both sexes.

An increase in the toxicity of metals in time was observed in eyestalkless animals as well (Table 3). Again, Hg proved to be the most toxic metal and, comparing Tables 1 and 3, the eyestalkless animals were from about 3 to 50 times more sensitive to the heavy metals than the intact ones, depending on the metal tested and the time elapsed.

Table 1. LC_{50} values in intact *P. elegans* of both sexes

	24-h LC_{50} (mg/L)	48-h LC_{50} (mg/L)	96-h LC_{50} (mg/L)	n
$HgCl_2$	9.54	3.54	0.67	20
$CdCl_2$	49.77	8.91	1.46	20
$CuCl_2$	249.46	12.79	3.27	20
$ZnCl_2$	not assessable	166.1	26.3	20
$Pb(NO_3)_2$	5,623.4	2,398.8	167	20

Table 2. LC_{50} values in ovigerous female *P. elegans*

	24-h LC_{50} (mg/L)	48-h LC_{50} (mg/L)	96-h LC_{50} (mg/L)	n
$HgCl_2$	6.65	2.40	0.47	20
$CdCl_2$	23.70	10.90	1.17	20
$CuCl_2$	13.41	6.24	2.47	20

Table 3. LC_{50} values in eyestalkless *P. elegans* of both sexes

	24-h LC_{50} (mg/L)	48-h LC_{50} (mg/L)	96-h LC_{50} (mg/L)	n
$HgCl_2$	0.73	0.26	0.15	20
$CuCl_2$	4.07	0.64	0.61	20
$CdCl_2$	18.19	3.16	0.78	20
$ZnCl_2$	263.0	66.1	8.71	20
$Pb(NO_3)_2$	3,630.7	338.8	66.1	20

Heavy Metal–Induced Hyperglycemic Response in Intact P. elegans

The mean initial value of glycemia of intact animals was of 10.25 ± 3.52 SD mg dL^{-1} ($n = 260$) that is, significantly different ($F = 43.958$, $p < 0.001$; $t = 6.630$, $p < 0.001$) from the initial value obtained in eyestalkless animals 8.23 ± 3.10 SD mg dL^{-1} ($n = 220$).

Exposure of intact shrimps to increasing concentrations of Hg^{++} (0.05, 0.1, 0.2, 0.5, 1, 2, and 5 mg L^{-1}) caused variations in the blood glucose level (Table 4). A significant (Dunnet's test, $p < 0.05$) peak increment of glycemia ranging between 0.97 ± 0.56 and 1.15 ± 0.88 (Student's *t* test, $p < 0.05$ versus control) was reached at the four intermediate concentrations of 0.1, 0.2, 0.5, and 1 mg L^{-1} from 1 to 3 h while at the highest concentrations of 2 and 5 mg L^{-1} a significant (Student's *t* test, $p < 0.05$ versus control) decrease of blood glucose level was revealed. No significant increase of blood glucose level (Dunnet's test, $p > 0.05$) was detected at the lowest concentrations of 0.05 mg L^{-1} (Student's *t* test, $p > 0.05$ versus control).

After exposure of intact *P. elegans* to Cd^{++} (0.1, 0.2, 0.5, 1, 2, 5, and 10 mg L^{-1}; Table 5) glycemia was found to follow a similar course. In fact at the highest concentrations of 2, 5, and 10 mg L^{-1}, the blood glucose levels significantly decreased at 5 h as compared with the control (Student's *t* test, $p < 0.05$) and at the highest concentration this effect was prolonged for up to 8 h. At the intermediate concentrations of 0.2, 0.5, and 1 mg L^{-1}, a significant hyperglycemia (Dunnet's test, $p < 0.05$), with an increment ranging from 1.48 ± 0.6 to 1.99 ± 1.08, occurred at 3 h (Student's *t* test, $p < 0.05$ versus control). No significant variation of blood glucose level was revealed at the lowest concentration tested of 0.1 mg L^{-1} (Student's *t* test, $p > 0.05$ versus control).

Exposure of intact *P. elegans* to Pb^{++} (10, 20, 50, 100, 200, and 500 mg L^{-1}; Table 6) caused a significant increase (Dunnet's test, $p < 0.05$) of blood glucose from 1.26 ± 0.69 to 1.83 ± 0.92 (Student's *t* test, $p < 0.05$ versus control) at the three intermediate concentrations of 20, 50, and 100 mg L^{-1} from 1 to 5 h, while at the highest concentrations of 200 and 500 mg L^{-1} no significant (Dunnet's test, $p > 0.05$) variation of blood glucose was revealed during the 24 h of the test (Student's *t* test, $p > 0.05$ versus control). The lowest concentration of 10 mg L^{-1} caused no significant (Dunnet's test, $p > 0.05$) variation in glycemia during the entire experiment (Student's *t* test, $p > 0.05$ versus control).

Figure 1 shows the blood glucose level at 3 h (identified as time of maximum effect) in *P. elegans* exposed at dif-

Table 4. Variations in the blood glucose (expressed in mg dL^{-1}) level in intact *P. elegans* exposed to Hg

Concentration	0 h	n	1 h	n	3 h	n	5 h	n	8 h	n	24 h	n	F	p
5 mg L^{-1}	11.60 ± 3.60	10	11.50 ± 3.95	10	13.40 ± 12.80	5							0.418	
2 mg L^{-1}	11.30 ± 4.00	10	10.30 ± 4.06	10	9.33 ± 4.77	9	10.67 ± 2.73	6	12.60 ± 4.16	5			0.469	
1 mg L^{-1}	13.90 ± 5.49	10	24.30 ± 11.76	10	28.00 ± 15.16	10	21.33 ± 15.73	9	18.11 ± 10.83	9	12.44 ± 4.42	9	2.691	*
0.5 mg L^{-1}	11.40 ± 2.50	10	20.70 ± 8.92	10	23.60 ± 8.85	10	18.40 ± 4.48	10	13.90 ± 4.43	10	13.25 ± 2.81	8	6.133	***
0.2 mg L^{-1}	10.50 ± 2.80	10	23.80 ± 5.57	10	21.70 ± 5.19	10	18.20 ± 3.58	10	15.40 ± 2.72	10	12.44 ± 2.07	9	17.467	***
0.1 mg L^{-1}	13.30 ± 2.31	10	18.30 ± 2.67	10	16.20 ± 2.62	10	14.60 ± 2.46	10	13.50 ± 1.96	10	13.50 ± 2.55	10	6.664	***
0.05 mg L^{-1}	12.00 ± 4.14	10	15.40 ± 3.86	10	13.70 ± 3.30	10	11.90 ± 2.92	10	11.80 ± 2.30	10	12.20 ± 3.05	10	1.884	
Control	12.20 ± 4.07	10	14.50 ± 3.95	10	15.90 ± 3.73	10	15.70 ± 2.79	10	13.30 ± 3.37	10	12.10 ± 3.07	10	2.958	*

* = $0.05 < p < 0.01$.
** = $0.01 < p < 0.001$.
*** = $p < 0.001$.

Table 5. Variations in the blood glucose (expressed in mg dL^{-1}) level in intact *P. elegans* exposed to Cd

Concentration	0 h	n	1 h	n	3 h	n	5 h	n	8 h	n	24 h	n	F	p
10 mg L^{-1}	10.00 ± 3.37	10	12.30 ± 4.95	10	13.00 ± 15.62	10	11.30 ± 4.81	10	8.40 ± 2.41	10	7.50 ± 1.51	9	2.591	*
5 mg L^{-1}	9.80 ± 4.24	10	12.10 ± 4.23	10	11.10 ± 3.54	10	10.40 ± 3.84	9	9.67 ± 4.23	6	10.17 ± 2.99	6	0.495	
2 mg L^{-1}	10.40 ± 3.03	10	10.80 ± 3.65	10	11.60 ± 3.44	10	10.63 ± 3.46	8	9.63 ± 3.34	8	9.75 ± 2.49	8	0.445	
1 mg L^{-1}	9.10 ± 2.77	10	17.70 ± 6.40	10	21.90 ± 6.38	10	18.67 ± 5.61	9	15.38 ± 4.98	8	10.38 ± 2.88	8	10.305	***
0.5 mg L^{-1}	10.30 ± 2.41	10	21.60 ± 9.31	10	30.50 ± 12.47	10	26.56 ± 11.42	9	23.13 ± 10.92	8	11.63 ± 5.24	8	6.907	***
0.2 mg L^{-1}	12.60 ± 4.65	10	23.30 ± 6.25	10	32.20 ± 9.39	10	26.5 ± 9.14	10	13.33 ± 9.45	9	12.88 ± 3.00	8	10.154	***
0.1 mg L^{-1}	7.00 ± 3.53	10	9.20 ± 3.01	10	11.30 ± 2.88	10	10.30 ± 2.87	10	7.10 ± 2.60	10	6.90 ± 2.60	10	3.732	**
Control	12.20 ± 4.07	10	14.50 ± 3.95	10	15.90 ± 3.73	10	15.70 ± 2.79	10	13.30 ± 3.37	10	12.10 ± 3.07	10	2.958	*

* = $0.05 < p < 0.01$.
** = $0.01 < p < 0.001$.
*** = $p < 0.001$.

Table 6. Variations in the blood glucose (expressed in mg dL^{-1}) level in intact *P. elegans* exposed to Pb

Concentration	0 h	n	1 h	n	3 h	n	5 h	n	8 h	n	24 h	n	F	p
500 mg L^{-1}	8.00 ± 1.33	10	9.60 ± 3.66	10	11.30 ± 6.07	10	10.50 ± 4.58	10	8.22 ± 1.99	9	9.00 ± 0.63	8	1.152	
200 mg L^{-1}	9.20 ± 2.70	10	10.90 ± 3.75	10	14.10 ± 4.79	10	12.33 ± 4.58	9	10.75 ± 5.04	8	9.63 ± 2.97	6	1.920	***
100 mg L^{-1}	8.00 ± 2.83	10	17.00 ± 6.60	10	21.10 ± 5.13	10	18.30 ± 5.48	10	11.80 ± 2.31	10	10.56 ± 1.51	9	14.103	***
50 mg L^{-1}	8.90 ± 1.37	10	15.90 ± 4.75	10	20.70 ± 2.75	10	16.90 ± 2.28	10	11.30 ± 2.31	10	10.56 ± 1.51	9	26.644	***
20 mg L^{-1}	8.70 ± 1.89	10	14.90 ± 4.36	10	19.20 ± 5.31	10	15.30 ± 4.37	10	12.67 ± 3.91	9	9.00 ± 2.06	9	10.616	***
10 mg L^{-1}	11.40 ± 3.69	10	15.60 ± 5.72	10	18.30 ± 6.99	10	17.70 ± 5.91	10	15.22 ± 4.99	9	12.44 ± 2.83	9	2.702	**
Control	9.50 ± 2.88	10	11.50 ± 5.50	10	12.10 ± 4.43	10	12.60 ± 4.95	10	12.00 ± 4.57	10	9.70 ± 2.54	10	9.390	**

* = $0.05 < p < 0.01$.
** = $0.01 < p < 0.001$.
*** = $p < 0.001$.

ferent concentrations of the nonessential metals Pb, Hg, and Cd. Looking at the two physiological metals, the time course of blood glucose levels in intact *P. elegans* exposed to copper and zinc showed a time- and dose-related response curve.

Intact shrimps exposed to Cu^{++} (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 mg L^{-1}; Table 7), show significant hyperglycemia (Dunnet's test, $p < 0.05$) with an increment ranging between 1.11 ± 0.57 and 2.62 ± 0.66 (Student's *t* test, $p < 0.05$ versus control) depending on the concentration from 1 to 5 h; blood glucose returned to initial levels at 24 h. Variation observed for the lowest concentrations of 0.1 and 0.2 mg L^{-1} (Dunnet's test, $p < 0.05$) was not significant (Student's *t* test, $p > 0.05$) compared to the contaminant-free control.

Similarly *P. elegans* exposed to concentrations of Zn^{++} (1, 2, 5, 10, 20, and 50 mg L^{-1}; Table 8) showed time- and dose-related variations of glycemia and a significant (Dunnet's test, $p < 0.05$) increase of blood glucose level was noted between 3 to 5 h with an increment from 1.55 ± 0.76 to 2.82 ± 1.37 (Student's *t* test, $p < 0.05$ versus control); the glucose level then slowly returned to the initial values. Variation observed for the lowest concentrations of 1 and 2 mg L^{-1} (Dunnet's test, $p < 0.05$) was not significant (Student's *t* test, $p > 0.05$) compared to the control.

Figure 2 shows the blood glucose level at 3 h in *P. elegans* exposed to different concentrations of the essential metals Cu and Zn. The plotted curves identify a sigmoid course that is drastically different from the peaking course of the nonessential metals (Figure 1).

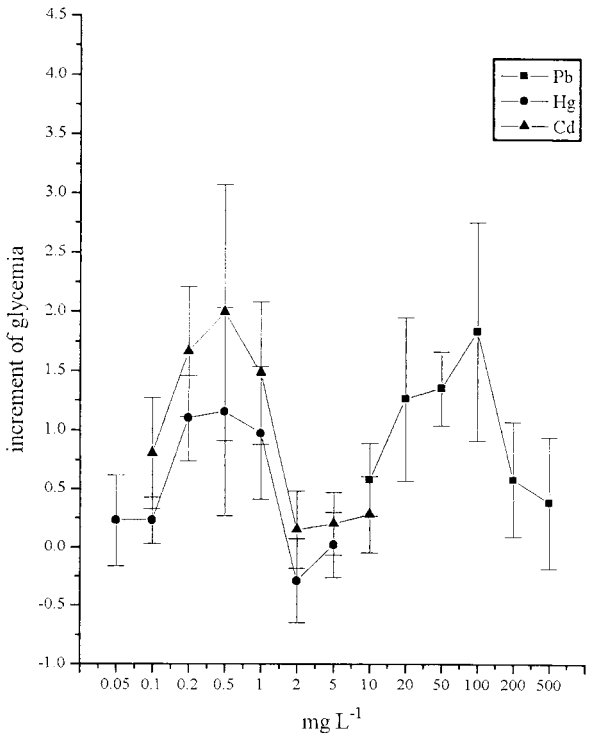

Fig. 1. Mean values of glycemia (as increments versus initial values ± SD bars) occurring 3 h after exposure of intact *P. elegans* to dissolved Pb, Hg, and Cd at the concentrations reported in the x axis on a $\log_{10}$ scale

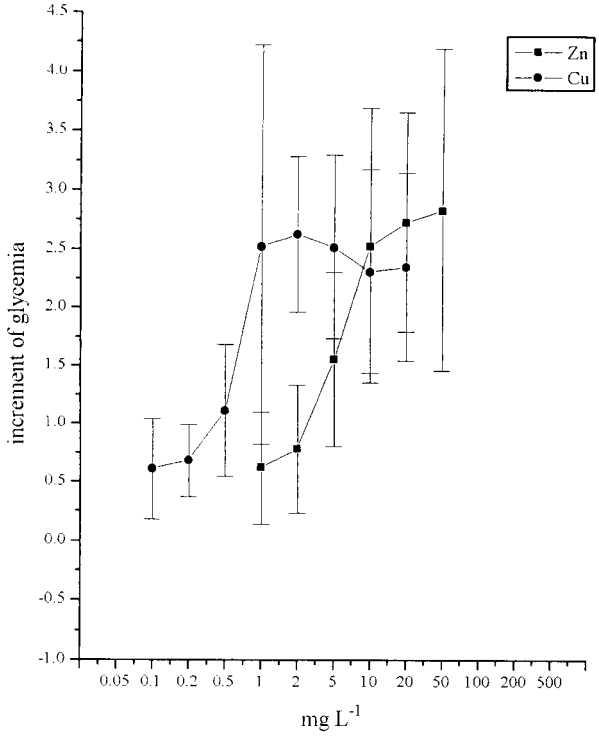

Fig. 2. Mean values of glycemia (as increments versus initial values ± SD bars) occurring 3 h after exposure of intact *P. elegans* to dissolved Zn and Cu at the concentrations reported in the x axis on a $\log_{10}$ scale

Table 7. Variations in the blood glucose (expressed in mg dL^{-1}) level in intact *P. elegans* exposed to Cu

Concentration	0 h	n	1 h	n	3 h	n	5 h	n	8 h	n	24 h	n	F	p
20 mg L^{-1}	8.02 ± 3.39	10	15.20 ± 5.70	10	23.78 ± 9.34	10	16.91 ± 6.08	10	12.98 ± 5.62	9	9.51 ± 4.27	8	9.029	***
10 mg L^{-1}	8.50 ± 2.64	10	16.30 ± 3.50	10	27.20 ± 7.80	10	19.00 ± 5.27	9	15.11 ± 4.70	9	10.50 ± 3.07	8	18.091	***
5 mg L^{-1}	9.50 ± 3.10	10	22.20 ± 8.95	10	32.10 ± 8.97	10	23.77 ± 5.09	9	15.38 ± 3.25	8	12.00 ± 2.45	5	24.327	***
2 mg L^{-1}	9.80 ± 2.44	10	24.50 ± 7.74	10	34.30 ± 5.70	10	23.70 ± 6.50	10	17.67 ± 5.82	6	11.83 ± 3.54	6	23.29	***
1 mg L^{-1}	12.30 ± 2.98	10	30.50 ± 11.45	10	40.80 ± 16.94	10	26.78 ± 9.97	9	21.33 ± 19.31	9	9.86 ± 4.02	7	11.083	***
0.5 mg L^{-1}	13.00 ± 21.71	10	21.00 ± 5.89	10	26.40 ± 4.79	10	21.40 ± 3.78	10	16.44 ± 3.36	9	11.75 ± 2.12	8	10.051	***
0.2 mg L^{-1}	11.40 ± 2.91	10	15.30 ± 3.80	10	18.70 ± 3.62	10	16.30 ± 2.71	10	13.78 ± 2.11	9	10.78 ± 11.92	9	6.404	***
0.1 mg L^{-1}	10.90 ± 2.60	10	17.70 ± 6.17	10	17.30 ± 14.30	10	14.30 ± 3.56	10	11.10 ± 1.91	10	10.56 ± 2.24	9	2.958	***
Control	12.20 ± 4.07	10	14.50 ± 3.95	10	15.90 ± 3.73	10	15.70 ± 2.79	10	13.30 ± 3.37	10	12.10 ± 3.07	10	2.958	*

* = $0.05 < p < 0.01$.
** = $0.01 < p < 0.001$.
*** = $p < 0.001$.

Table 8. Variations in the blood glucose (expressed in mg dL^{-1}) level in intact *P. elegans* exposed to Zn

Concentration	0 h	n	1 h	n	3 h	n	5 h	n	8 h	n	24 h	n	F	p
50 mg L^{-1}	7.60 ± 2.32	10	17.50 ± 4.60	10	27.50 ± 9.19	10	26.20 ± 9.61	10	16.80 ± 8.20	10	9.90 ± 4.01	10	13.886	
20 mg L^{-1}	8.00 ± 2.21	10	16.80 ± 4.59	10	18.80 ± 7.98	10	24.60 ± 4.90	10	18.50 ± 4.45	10	10.80 ± 2.70	10	26.724	
10 mg L^{-1}	9.30 ± 3.20	10	20.40 ± 9.05	10	31.40 ± 10.39	10	26.00 ± 8.29	10	17.60 ± 5.40	10	8.70 ± 2.16	10	16.132	
5 mg L^{-1}	11.40 ± 3.10	10	18.20 ± 7.35	10	28.30 ± 10.09	10	21.30 ± 11.38	10	15.20 ± 5.98	10	10.80 ± 1.69	10	7.879	
2 mg L^{-1}	12.30 ± 2.00	10	16.20 ± 4.69	10	21.30 ± 6.00	10	19.40 ± 6.87	10	16.80 ± 4.52	10	11.30 ± 2.36	10	6.769	
1 mg L^{-1}	10.60 ± 3.60	10	13.20 ± 3.19	10	16.60 ± 5.17	10	14.70 ± 4.60	10	12.50 ± 3.27	10	11.00 ± 1.63	10	3.677	
Control	9.50 ± 2.88	10	11.50 ± 5.50	10	12.10 ± 4.43	10	12.60 ± 4.95	10	12.00 ± 4.57	10	9.70 ± 2.54	10	9.390	**

* = $0.05 < p < 0.01$.
** = $0.01 < p < 0.001$.
*** = $p < 0.001$.

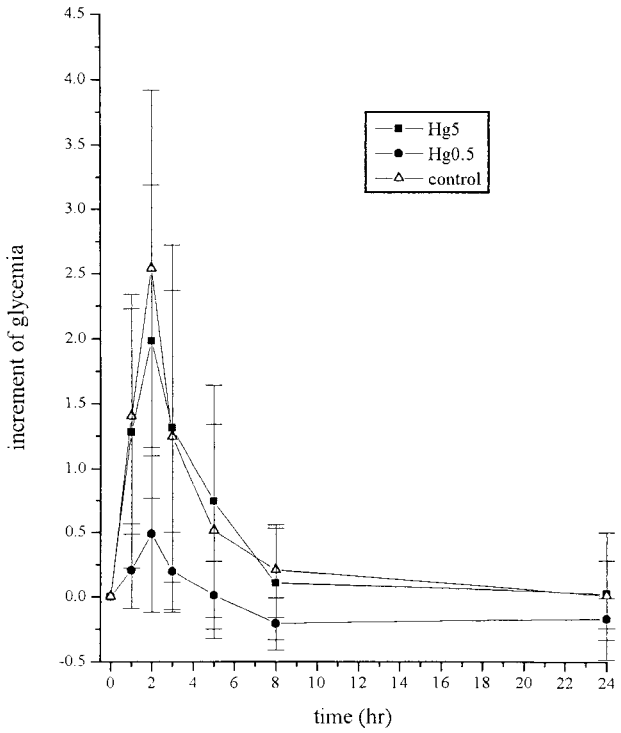

Fig. 3. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extracts from Hg^{++} (5 and 0.5 mg L^{-1})–treated animals and from contaminant-free animals as control (n = 10). In the legend Hg5 = 5 mg L^{-1} Hg^{++} and Hg0.5 = 0.5 mg L^{-1} Hg^{++}

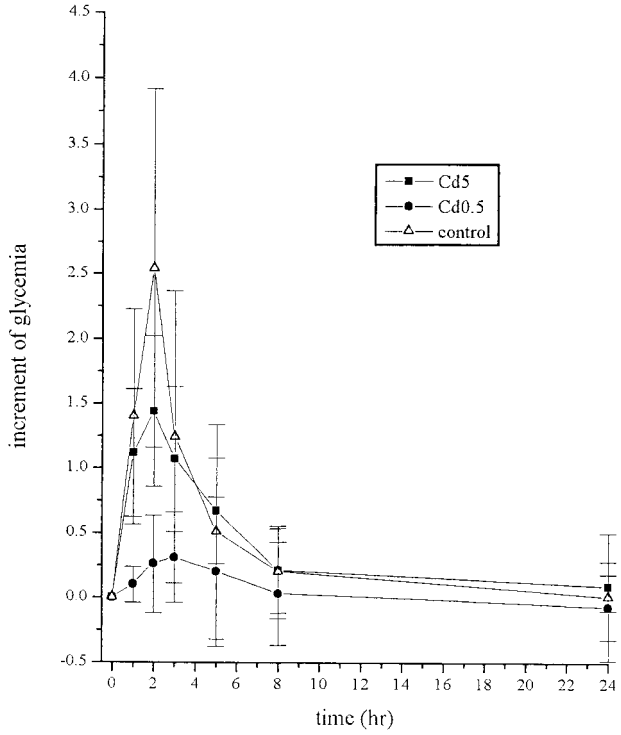

Fig. 4. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extracts from Cd^{++} (5 and 0.5 mg L^{-1})–treated animals and from contaminant-free animals as control (n = 10). In the legend, Cd5 = 5 mg L^{-1} Cd^{++} and Cd0.5 = 0.5 mg L^{-1} Cd^{++}

Involvement of Eyestalk Hormones

Exposure of eyestalkless animals (n = 10 for each treatment) to 5 or 0.5 mg L^{-1} Hg^{++}, Cd^{++}, and Cu^{++}, did not cause a significant variation (Dunnet's test, $p > 0.05$) in the blood glucose levels during the experimental period compared with untreated animals (Student's *t* test, $p > 0.05$). In eyestalkless *P. elegans* exposed to Zn^{++} (5 and 50 mg L^{-1}) a slight but significant (Dunnet's test, $p < 0.05$) hyperglycemia was present at the lowest concentration until 3 h (Student's *t* test, $p < 0.05$ versus control), then the blood glucose subsided to the control level. In contrast, animals exposed to Pb^{++} at the concentration of 50 mg L^{-1} showed a significant hypoglycemia (Dunnet's test, $p < 0.05$) from 3 h until the end of the experiment (Student's *t* test, $p < 0.05$ versus control); at the concentration of 500 mg L^{-1} the blood glucose level decreased slightly (Dunnet's test, $p > 0.05$) and the hypoglycemia became significant (Student's *t* test, $p < 0.05$ versus control) at 24 h (data not presented).

A standard quantification bioassay of eyestalk hormones involved in the general glycemic response was then performed. Figures 3, 4, and 5, respectively, show the blood glucose curve in eyestalkless animals (n = 10) injected with two eyestalk equivalents from Hg^{++} (5 or 0.5 mg L^{-1}), Cd^{++} (5 or 0.5 mg L^{-1}), Pb^{++} (500 or 50 mg L^{-1})–exposed animals and untreated shrimps.

Injection of the eyestalk extract from animals treated with high concentrations of Hg^{++}, Cd^{++} (5 mg L^{-1}) or Pb^{++} (500 mg L^{-1}) induced hyperglycemia (Dunnet's test, $p < 0.05$) within 2 h, but this effect was not significantly different from that of the control injected with eyestalk extract from untreated animals (Student's *t* test, $p > 0.05$); no significant hyperglycemic effect (Dunnet's test, $p > 0.05$) was seen in eyestalkless *P. elegans* after injection of extract from animals exposed to low concentrations of Hg^{++}, Cd^{++} (0.5 mg L^{-1}), or Pb^{++} (50 mg L^{-1}), and this was significantly different compared with the control (Student's *t* test, $p < 0.05$).

By contrast, injection of eyestalk extract from animals treated with high concentrations of Cu^{++} 5 mg L^{-1} (Figure 6) or Zn^{++} 50 mg L^{-1} (Figure 7) caused no hyperglycemic effect (Dunnet's test, $p > 0.05$) in eyestalkless *P. elegans* and resulted significantly different (Student's *t* test, $p < 0.05$) from those injected with control animal extract. Eyestalkless animals treated with eyestalk extract from animals exposed to low concentration of Cu^{++} 0.5 mg L^{-1} (Figure 6) or Zn^{++} 5 mg L^{-1} (Figure 7) developed a hyperglycemic response (Dunnet's test, $p < 0.05$) within 3 h of injection, which is not significantly different (Student's *t* test, $p > 0.05$) from control.

Alternately, the responsiveness to cHH of contaminant-exposed animals was tested. Eyestalkless animals (n = 10) exposed to Hg^{++} 0.5 or 2 mg L^{-1} and injected with 50 μL of eyestalk extract from untreated animals showed (Figure 8) a significant increment (Dunnet's test, $p < 0.05$) of blood glucose levels from 1 to 8 h with a maximum peak of about 2.5 at 2 h that is not significantly different (Student's *t* test, $p > 0.05$)

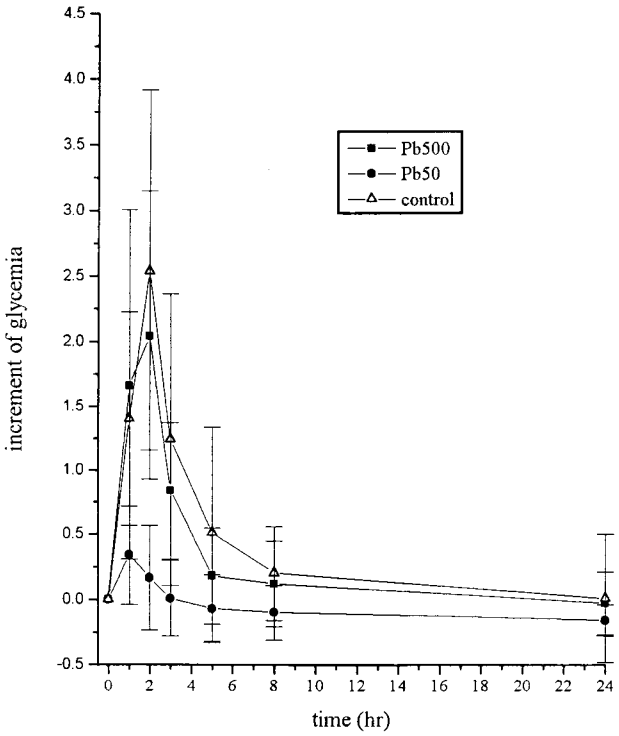

Fig. 5. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extracts Pb^{++} (500 and 50 mg L^{-1})–treated animals and from contaminant-free animals as control (n = 10). In the legend Pb500 = 500 mg L^{-1} Pb^{++} and Pb50 = 50 mg L^{-1} of Pb^{++}

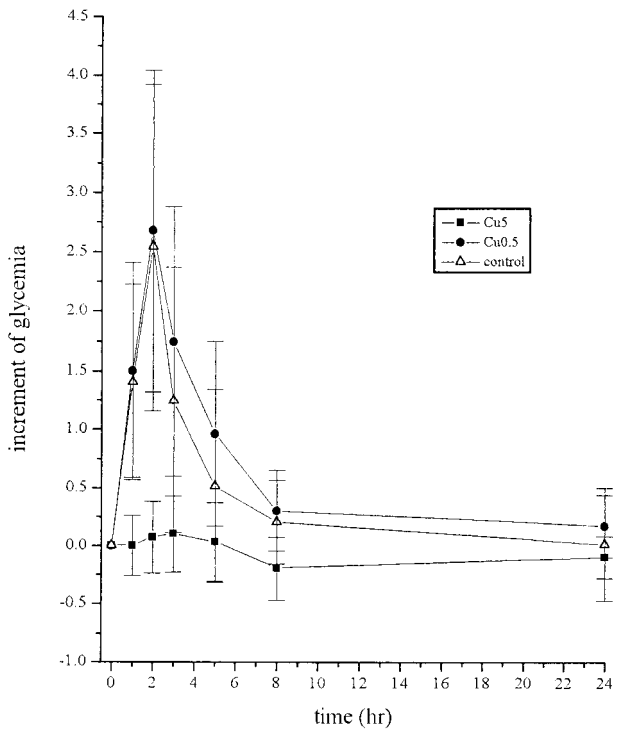

Fig. 6. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extract from Cu^{++} (5 and 0.5 mg L^{-1})–treated animals and from contaminant-free animals as control (n = 10). In the legend Cu5 = 5 mg L^{-1} Cu^{++} and Cu0.5 = 0.5 mg L^{-1} of Cu^{++}

from the control animals injected with the eyestalk extract but maintained in contaminant-free water. The level of blood glucose returned to the initial value at 24 h (Dunnet's test, $p > 0.05$). The same course of hyperglycemia (Figure 9) was revealed in eyestalkless shrimps exposed to Cd^{++} 0.5 or 5 mg L^{-1} and injected with eyestalk extract from untreated animals.

Eyestalkless shrimp exposed to Pb^{++} 50 or 500 mg L^{-1} and injected with 50 μL of eyestalk extract from contaminant-free animals showed (Figure 10) a significant (Dunnet's test, $p < 0.05$) increment of glycemia from the 1 h that is not significantly different (Student's *t* test, $p > 0.05$) from the control animals injected with extract but maintained in uncontaminated water. Blood glucose level returned to initial values in 24 h (Dunnet's test, $p > 0.05$). Therefore, the target organs were responsive to cHH at both high and low pollutant concentrations.

Discussion

Eyestalkless *P. elegans* are the most sensitive to heavy metals, followed by intact ovigerous females and both sexes of intact shrimps. From the LC_{50} results for the latter group compared to other decapods, *P. elegans* appears to be intermediate in its sensitivity to acute toxicity (see Ahsanullah *et al*. 1981; Denton and Burdon-Jones 1982; Ferrero *et al*. 1994 for references).

For instance, in shrimps *P. elegans* is more resistant to cadmium than *Crangon septemspinosa* (LC_{50} at 96 h 0.32 mg L^{-1}; Portmann 1968) but less than *Palaemon serratus* (LC_{50} at 96 h 4 mg L^{-1}; Thebault *et al*. 1996) and other *Palaemon* sp. (LC_{50} at 96 h 6.6 mg L^{-1}; Ahsanullah 1976). *P. elegans* presents the same order of resistance to mercury as does *Palaemonetes pugio* (Barthalmus 1977), but appears to be more resistant than *Penaeus merguensis* (LC_{50} 96 h 0.03 mg L^{-1}; Ashanullah and Ying 1995). In general, *P. elegans* presents an intermediate tolerance to zinc and copper when compared with other crustacean species (Ahsanullah 1976; Ahsanullah *et al*. 1981). The differences of toxicity between species may also be due to differences in duration of exposure and in salinity, temperature, and age of the animals (Vernberg *et al*. 1974; Denton and Burdon-Jones 1982; Madsen 1992; McGee *et al*. 1998).

The increased toxicity of the heavy metals to the ovigerous females compared to the entire population of *P. elegans* gives an important warning signal of the toxic impact of heavy metals on stock recruitment at low levels of exposure. Similar data for Ni and Cd have been obtained with egg carrying cladocerans (McCahon and Pascoe 1988; Ravera and Gatti 1988). The lower toxicity reported in maturing females instead (Ananthalashmikumari *et al*. 1990) is probably due to the sequestration of pollutants in the developing oocyte stores depending on the lipophilic or polar nature of the toxicant.

Eyestalkless animals are the most sensitive to heavy metals, and this result suggests a resistance homeostatic reaction mediated by neurohormones in the intact shrimp.

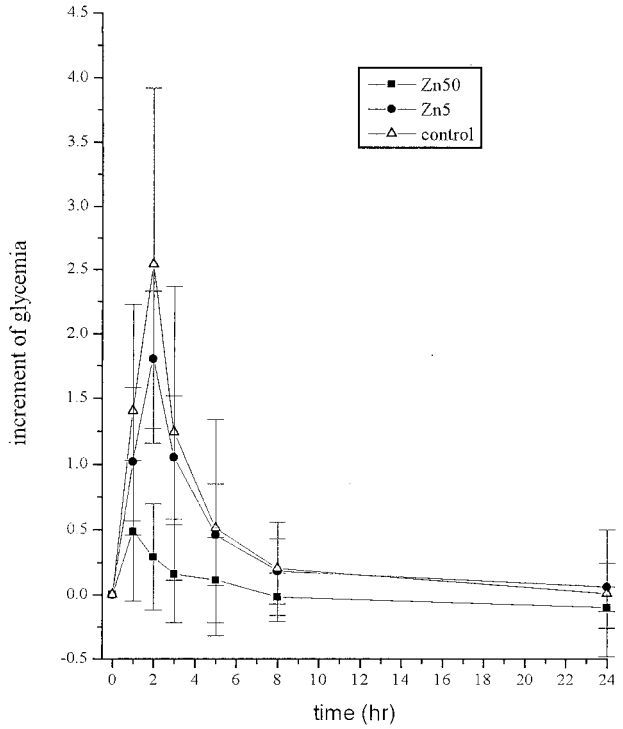

Fig. 7. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extract from Zn^{++} (50 and 5 mg L^{-1})–treated animals and from contaminant-free animals as control (n = 10). In the legend Zn50 = 50 mg L^{-1} Zn^{++} and Zn5 = 5 mg L^{-1} of Zn^{++}

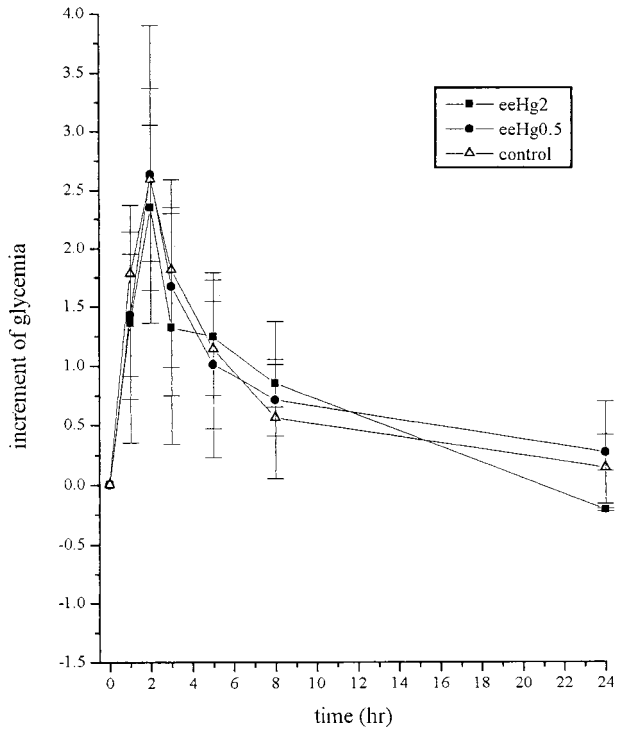

Fig. 8. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extract from contaminant-free animals and exposed to dissolved Hg^{++} 0.5 or 2 mg L^{-1} or to uncontaminated water as control (n = 10). In the legend eeHg2 = animal exposed to Hg^{++} 2 mg L^{-1} and eeHg0.5 = animal exposed to Hg^{++} 0.5 mg L^{-1}

From the results reported herein for *P. elegans*, as a standard test species, heavy metals induce variations in the blood glucose level, and this response is independent of manipulative stress, which causes no hyperglycemic effect.

Variation in blood glucose is observed in crustaceans when they are submitted to stressful conditions, such as exposure to hypoxia in *P. clarkii*, to organic pollutants or naphthalene in *U. pugilator*, to DDT in *B. guerini*, to starvation or anoxia in *O. limosus*, to heavy metals in *M. kistenensis* and *B. canicularis* (Fingerman *et al*. 1998), and to thermal shock in *M. rosenbergii* (Kuo and Yang 1999).

The highest concentrations of Hg, Cd, and Pb elicited no hyperglycemia in 24 h, while the intermediate sublethal concentrations of these metals produced a significant hyperglycemic response. In contrast, animals exposed to Cu and Zn showed hyperglycemia even at high concentrations. The lack of a hyperglycemic response to a high concentration of Hg, Cd, and Pb is not due to a block of peripheral effectors; in fact, eyestalkless animals exposed to either high or low heavy metal concentrations both exhibited a fully developed hyperglycemic response when challenged with injected eyestalk extract. This difference in response between the physiologically required Cu and Zn and the nonessential heavy metals Cd, Hg, and Pb is probably related to the physiological roles of Cu and Zn in crustaceans and their tolerance adaptations.

The two physiological heavy metals Cu and Zn are the most effective in causing hyperglycemia; Pb is the most effective of the nonphysiological metals in elevating the blood glucose level. Apparently there is an inverse relationship between effectiveness in eliciting hyperglycemia and toxicity, which further supports the hypothesis for a protective role exerted by the eyestalk neuroendocrine organs through a stress reaction.

Eyestalkless animals exposed to high and low concentrations of heavy metals do not show a relevant hyperglycemic effect except for a slight increase after Zn exposure. This further proves the significant involvement of the eyestalk neuroendocrine centers while the residual response can be interpreted as a peripheral mechanism. It is elicited either by noneyestalk-based neuroendocrine control or directly affected by the action of heavy metals or of messengers released by their action on the target tissues. The difference between intact and unresponsive eyestalkless animals has already been reported by Reddy *et al*. (1994) for *P. clarkii* exposed to Cd and in *U. pugilator* exposed to Cd and naphthalene (Reddy *et al*. 1996) and also for *P. elegans* injected with LPS as well (Lorenzon *et al*. 1997).

To assess whether the lack of a hyperglycemic response was due to a central blockage of cHH release rather than a peripheral block of the effector's response, the suggested involvement of cHH was tested by a routine bioassay (Smullen and Bentley 1994; Lorenzon *et al*. 1997) on eyestalkless individuals. Each of 10 animals was injected with a two-eyestalk-equivalent extract from control unexposed animals or from high concentrations of Cd, Hg, Pb, or low concentrations of Cu- or Zn-exposed animals, and they showed a hyperglycemic response. In contrast, extracts of eyestalks removed from ani-

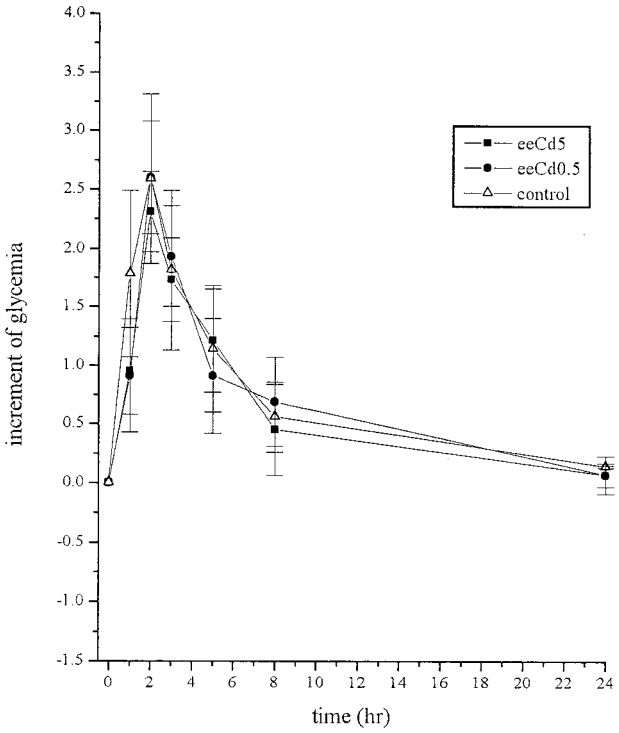

Fig. 9. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extract from contaminant-free animals and exposed to dissolved Cd^{++} 0.5 or 5 mg L^{-1} or to uncontaminated water as control (n = 10). In the legend eeCd5 = animal exposed to Cd^{++} 5 mg L^{-1} and eeCd0.5 = animal exposed to Cd^{++} 0.5 mg L^{-1}

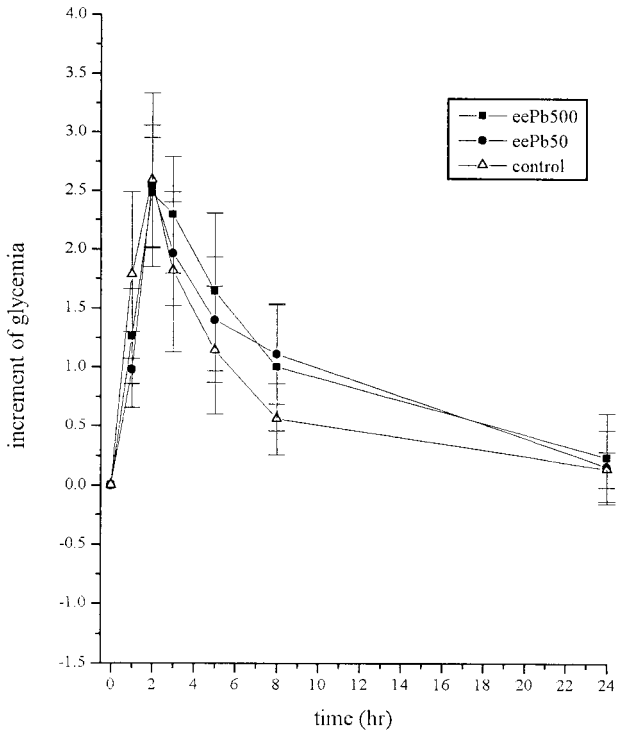

Fig. 10. Time course of glycemia (mean values of increments versus initial values ± SD bars) in eyestalkless *P. elegans* injected with eyestalk extract from contaminant-free animals and exposed to dissolved Pb^{++} 50 or 500 mg L^{-1} or to uncontaminated water as control (n = 10). In the legend eePb500 = animal exposed to Pb^{++} 500 mg L^{-1} and eePb50 = animal exposed to Pb^{++} 50 mg L^{-1}

mals that had developed a full hyperglycemic reaction after exposure to a low concentration of Hg, Cd, Pb, or a high concentration of Cu or Zn were depleted of cHH as shown by the significant attenuation of the response after extract injection into eyestalkless animals.

Another blood parameter, *i.e.*, variation in the total hemocyte counts (THC), is also affected by exposure of intact *P. elegans* to heavy metals Hg, Cd, Cu, Zn, or Pb for 96 h. The results showed that heavy metals induced a decrease of THC in the first 8 h after exposure and in the following hours the hemocyte count returned to the initial level. The percentage decrease of circulating hemocytes induced by all metals was significantly different from the control. The time required to reach the minimum level of THC and the degree of hemocytopenia depended on the doses and metal tested. Pb was the most effective metal in inducing hemocytopenia, followed by Zn, Hg, Cr, Cu, and Cd (Lorenzon *et al.* in preparation).

In conclusion, sublethal heavy metal concentrations cause a variation of blood glucose levels mediated by eyestalk hormone in *P. elegans* within a 24-h exposure period. Therefore the differential effect on the glycemia stress response proves to be a generalized and predictable sublethal reaction that can be used as a quantitative physiological biomarker for water quality monitoring assessment. The combined use of several physiological biomarkers might then enable to discriminate between the effects of different heavy metals and their concentrations.

The results presented above call for further studies on the finer mechanisms that regulate the inhibition of hyperglycemic responses in *P. elegans* at high concentrations of nonessential metals and how they may interfere with the normal functioning of the endocrine system. Heavy metals acting on neuroendocrine secretion—such as the recently defined broader category of "endocrine disruptors"—may cause a host of problems with development, behavior, and reproduction, which are modulated by the endocrine system.

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