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Review

Hyperglycemic stress response in Crustacea

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Abstract

Blood glucose level in crustaceans is controlled by the crustacean Hyperglycemic Hormone (cHH), released from the eyestalk neuroendocrine centres both under physiological and environmental stress conditions. Hyperglycemia is a typical response of many aquatic animals to pollutants and stress and, in crustaceans, increased circulating cHH and hyperglycemia are reported to result from exposure to several environmental stressors. Biogenic amines and enkephalin have been found to mediate the release of several neurohormones from crustacean neuroendocrine tissue and a model of the controlling network is proposed.

Key words: Crustacea; glucose; crustacean Hyperglycemic Hormone (cHH); stress response; neuroendocrine control

Introduction

Hyperglycemia is a typical response of many aquatic animals to harmful physical and chemical environmental changes. In crustaceans increased circulating crustacean Hyperglycemic Hormone (cHH) titres and hyperglycemia are reported to occur following exposure to several environmental stressors (Durand *et al.*, 2000; Lorenzon *et al.*, 1997; 2002; Santos *et al.*, 2001) in intact but not in eyestalkless animals (Fig.1), suggesting a cHH mediated response (Fingerman *et al.*, 1981; Reddy and Bhagyalakshimi, 1994; Reddy *et al.*, 1996; Lorenzon *et al.*, 2000, 2004a).

Toxicity induced by a pollutant is the result of interaction of the compound or one of its metabolites, with the biochemical events involved in the homeostatic control of a physiological process (Brouwer *et al.*, 1990). Physiological processes are mostly coordinated by hormones.

Anthropogenic chemicals can alter the hormonal (endocrine) systems of wildlife and the effects of organic and inorganic contaminants on functions regulated by hormones in crustaceans are being investigated with increased frequency because several of these phenomena could be used as biomarkers of environmental contamination. Heavy metals and organic compounds have been found to negatively affect hormonally-regulated functions, such as reproduction, molting, blood glucose level and pigmentary effectors in crustaceans (Fingerman *et al.*, 1998; Depledge and Billinghurst, 1999). Therefore, biosentinel parameters and "early warning" of toxicity can be identified by looking for alterations in endocrine patterns (Fingerman *et al.*, 1996).

Neurosecretory structures in the eyestalk are the most important components of the neuroendocrine system of the stalk-eyed crustaceans. The hemolymph glucose concentration is mainly controlled by the cHH synthesized within the X-organ (XO) and released from the sinus gland (SG) complex in the eyestalk (Abramowitz *et al.*, 1944; Fingerman, 1987).

Biogenic amines and enkephalin (L/M-Enk) control the release of neurohormones from the crustacean neuroendocrine tissue.

Serotonin (5-HT) is involved in regulating important aspects of behaviour and a variety of systemic physiological functions. 5-HT has long been known (Bauchau and Mengeot, 1966) to have a potent hyperglycemic effect in several crustacean species (Lorenzon *et al.*, 1999, 2004b; Lee *et al.*,

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2000; Komali *et al.*, 2005;), while dopamine (DA) and enkephalin showed conflicting results in different species (Sarojini *et al.*, 1995; Lorenzon *et al.*, 1999, 2004b; Zou *et al.*, 2003; Komali *et al.*, 2005).

The crustacean Hyperglycemic Hormone (cHH)

Multiple forms of the cHH represent one member of an eyestalk neuropeptide family (Bocking *et al*, 2001), that includes the moult inhibiting hormone (MIH) and the gonad inhibiting hormone (GIH): the cHH/MIH/GIH family. These neuropeptides, synthesized in the XO, a cluster of neuron perikarya located in the medulla terminalis of the eyestalk, are transported to and stored in the axon terminals forming a neurohemal organ named SG and released by exocytosis into the hemolymph (Fig. 2).

The main function of cHH is the regulation of hemolymph sugar level: cHHs are also involved in other functions such as reproduction (De Kleijn *et al.*, 1998; De Kleijn and van Herp, 1998), molting (Chung *et al.*, 1999; Webster *et al.*, 2000), lipid metabolism (Santos *et al.*, 1997), stress response (Lorenzon *et al.*, 1997; 2002;Chang *et al.*, 1999; Durand *et al.*, 2000; Santos *et al.*, 2001) and hydromineral regulation (Spanings-Pierrot *et al.*, 2000; Serrano *et al.*, 2003).

On the basis of the primary structure, the cHH/MIH/GIH family can be divided into two sub-families (De Kleijn *et al.*, 1995; Lacombe *et al.*, 1999): the cHH sub-family characterized by the cHH precursor-related peptide (CPRP) and the MIH/GIH sub-family without CPRP. The prepropeptide cHH consists of a signal peptide, CPRP and a peptide with 72–74 amino acids. Usually, the mature peptide has an amidated carboxyl terminus (De Kleijn and van Herp, 1998; Lacombe *et al.*, 1999), which is important in conferring hyperglycemic activity in *Penaeus japonicus* as evidenced by bioassay of recombinant peptide (Katayama *et al.*, 2003). In several crustacean species, different isoforms of cHH exist. In the American lobster *Homarus americanus*, cHH-A (8.583 Da) and cHH-B (8.638 Da) have been found, with different actions during the female biannual reproductive cycle (De Kleijn *et al.*, 1995).

Role of biogenic amines and enkephalin in blood glucose regulation

Neurotransmitters such as 5-HT, DA and L/M-enk play a fundamental role in hormone modulation (Fingerman *et al.,* 1994) and at the same time their level and functions can be altered by pollutants (Amiard-Triquet *et al.*, 1986, Reddy *et al.*, 1997).

5-HT is well known as a neurotransmitter in crustaceans on several grounds, and its levels have been measured in the nervous system and hemolymph of various crustacean species (Elofsson *et al.*, 1982; Laxmyr, 1984; Kulkarni and Fingerman 1992), thus suggesting a possible role as a neurohormone (Rodriguez-Soza *et al.*, 1997).

In crustaceans 5HT is linked with discrete circuits that control movements of the foregut, escape behaviour, locomotion and posture as well as with higher-order behaviours such as aggression (Sosa *et al.*, 2004). In addition 5-HT levels are sensitive to environmental stress.

5-HT has long been known to have a potent hyperglycemic effect in several crustacean species (Bauchau and Mengeot, 1966; Keller and Beyer 1968; Lüschen *et al.*, 1993; Kuo *et al.*, 1995; Santos *et al.*, 2001). In our laboratory (Lorenzon *et al.*, 1999, 2004b) we have demonstrated that 5-HT elevates blood glucose in *Palaemon elegans*, *Astacus leptodactylus* and *Squilla mantis*. However no such effects were found in eyestalkless individuals of these species, suggesting the involvement of the eyestalk hormone cHH in the hyperglycemic response. In all the species injection of the antagonist, ketanserin and CPH (cyproheptadine, 5-HT_1 receptor inhibitor) were able to inhibit the hyperglycemic effect of 5-HT. 5-HT_1 like receptors seemed to be more likely involved in mediating 5-HT action, as CPH was a more effective antagonist than ketanserin (5-HT_2 receptor inhibitor and also putative DA antagonist). These data agree with those by Lee *et al.* (2000) in *Procambarus clarkii* suggesting that 5-HT induced hyperglycemia is mediated by 5-HT_1 and 5-HT_2 like receptors.

Using ELISA very recently we have demonstrated in *P. elegans* that injection of 5-HT induced a rapid and massive release of cHH from the eyestalk into the hemolymph followed by hyperglycemia. On the contrary DA did not significantly affect cHH release and hyperglycemia (Lorenzon *et al.* 2005).

DA and enkephalins showed conflicting results in different species (Table 1). Injection of DA induced marked decrease in blood glucose levels in *P. elegans* and *S. mantis* (Lorenzon *et al.*, 1999, 2004b). On the other hand injection of the DA receptor blocker inhibits the effects on blood glucose, apparently allowing the release of cHH. These findings are in contrast with those by Lüschen *et al.*, (1993) for C*arcinus maenas*, Kuo *et al.* (1995) for *Penaeus monodon* and Komali *et al.* (2005) for *Macrobrachium malcolmsonii* where DA induced hyperglycemia in intact animals.

As for enkephalins, L/M-Enk elicited hypoglycemic response in intact *S. mantis* but not in eyestalkless individuals (Lorenzon *et al.* 2004a). These results confirm those of Jaros (1990), Lüschen *et al.* (1991), Rothe *et al.* (1991) and Sarojini *et al.* (1995) who reported that L/M-Enk induced hypoglycemia in *Uca pugilator, C. maenas* and *P. clarkii* respectively. On the other hand L-enk induced hyperglycemic response in intact but not in eyestalkless *A. leptodactylus* (Lorenzon *et al.* 2004). These observations are consistent with our previous findings in *P. elegans* (Lorenzon *et al.*, 1999) and also with recent reports on *Oziotelphusa senex senex* (Reddy and Basha, 2001), on the mud crab *Scylla serrata* (Reddy and Kishori, 2001) and in the two prawns, *Penaeus indicus* and *Metapenaeus monocerus* (Kishori *et al.*, 2001). In *S. mantis* injection of the opioid antagonist naloxone reversed the inhibitory effect on blood glucose of L-enk while in *A. leptodactylus* an additive effect on hyperglycemia was recorded (Lorenzon *et al.*, 2004b).

All these results corroborate the commonly held view that 5-HT, is a potent hyperglycemic effector and exerts its effect through cHH release from the

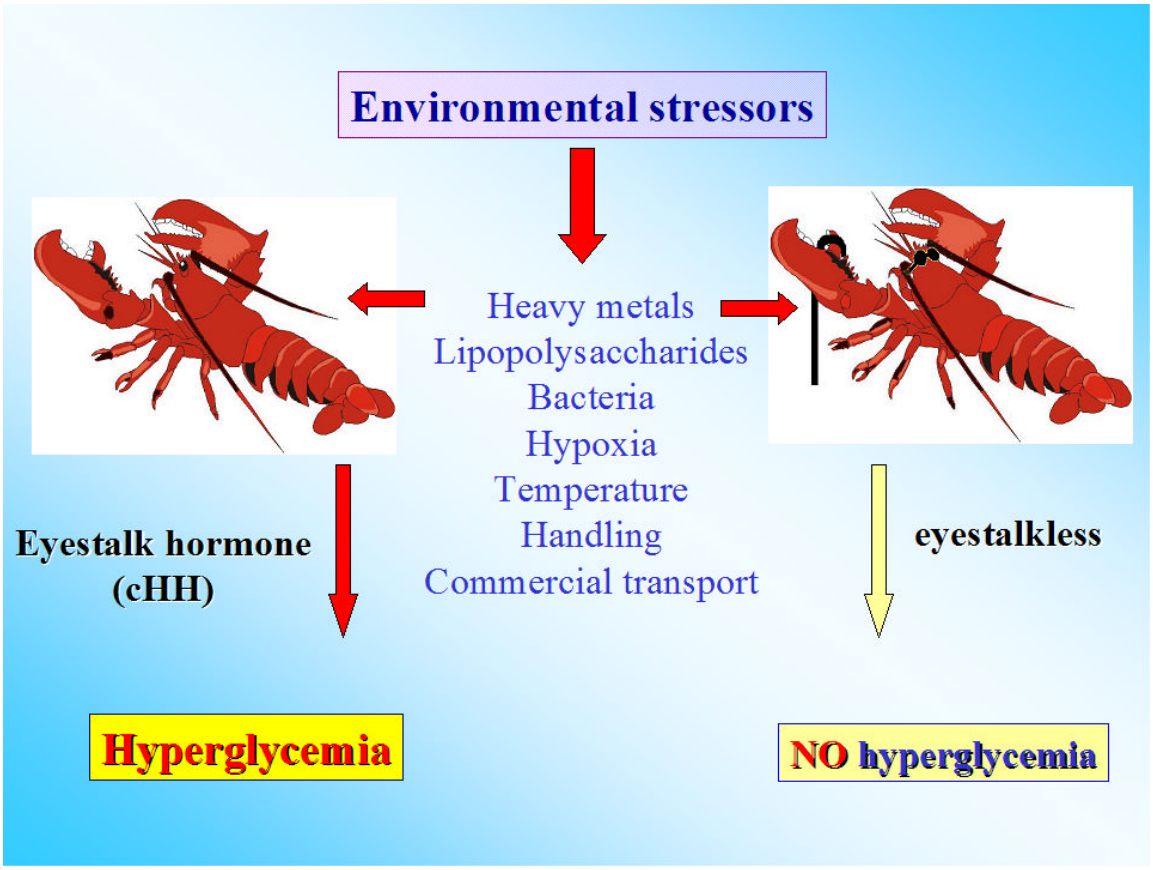

Fig.1 Stress response in Crustacea.

medulla terminalis XO-sinus gland complex (MTXO-SG), mediated by modulation of electrical activity of XO cells (Saenz *et al.*, 1997). A detailed reconstruction of the underlying neural circuitry suffers from lack of precise identification of neurosecretory cell types, contrasting results of electrophysiological evidence and discrepancies due to interspecific differences (Glowik *et al.*, 1997; Saenz *et al.*, 1997).

Finally 5-HT appears to provide a major control mechanism for glucose mobilization whereas DA and L/M-enk act as modulators whose plasticity in use or actions varied among even closely related species.

Stress response

Stress induced by changes in environmental parameters, emersion, handling and transport during commercial processes requires homeostatic regulation that brings about behavioural and physiological alterations in aquatic animals.

Hemolymph glucose concentration can change significantly with altered physiological and environmental conditions. Exposure to air during commercial transport and hypoxia are reported to induce hyperglycemia in many crustacean species like the spiny lobster, *Jasus edwardsii* (Morris and Oliver 1999; Speed *et al.*, 2001), the crab, *Eriocheir sinensis* (Zou *et al.*, 1996), the spider crab, *Maia squinado* (Durand *et al.*, 2000) and the Norway lobster, *Nephrops norvegicus* (Spicer *et al.,* 1990). Moreover hyperglycemia is reported in the giant prawn, *Macrobrachium rosenbergii* as a response to cold shock (Kuo and Yang, 1999).

Blood glucose level increased in *P. elegans* and other crustacean species after injection of lipopolysaccharide (LPS) and the hyperglycemic effect, is likely mediated by the cHH since it does not occur in eyestalkless animals. It is dose-related and dependent on the different Gram negative bacterial LPS (Lorenzon *et al.*, 1997, 2002).

Heavy metals like Cd, Hg, and Cu cause hyperglycemia in the freshwater prawn, *Macrobrachium kistenensis*, the crab, *Barytelphusa canicularis* (Nagabhushanam and Kulkarni, 1981, Machele *et al.*, 1989) and *S. serrata* (Reddy and Bhagyalakshmi, 1994). Moreover, $CdCl_2$ induces hyperglycemia in intact crayfish *P. clarkii*, but not in the absence of the eyestalks, suggesting a cHH mediated response (Reddy *et al.*, 1996).

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 Pb produced significant hyperglycemic responses 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 could be explained on the basis of the physiological roles these two essential

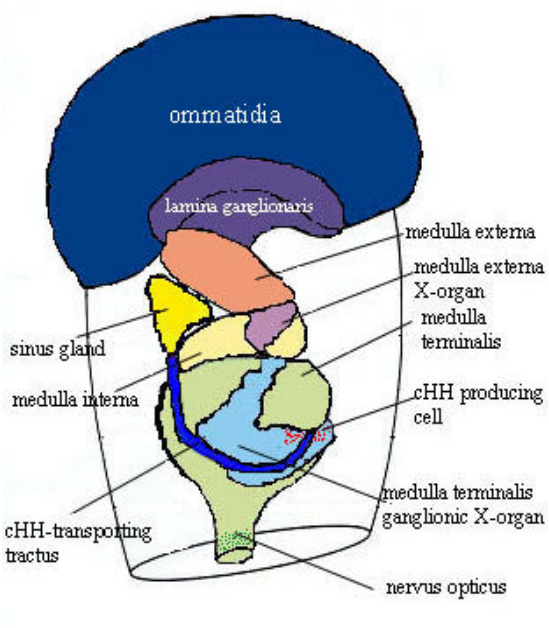

Fig. 2 General organization of neuroendocrine tissues in the eyestalk of crustaceans.

elements play in crustaceans, and consequent tolerance adaptations, as opposed to the toxic, xenobiotic heavy metals Cd, Hg and Pb. On the other hand both groups of heavy metals failed to elicit a hyperglycemic responses in eyestalk ablated animals suggesting the involvement of MTXO-SG hormones, most likely cHH. However, in spite of the richness of information regarding variations in blood glucose levels following stress, much less is known about the stress-induced variation in cHH levels in the sinus gland and in the hemolymph.

In the crayfish *Orconectes limosus* subjected to hypoxia, blood cHH titers raise within 15 min (Keller and Orth, 1990). In *Cancer pagurus* emersion induced an increase in the hemolymph cHH after 4 h (Webster, 1996). Using ELISA Chang *et al.* (1998) observed variation in the blood cHH in *Homarus americanus* following exposure to various environmental stresses. Emersion was found to be a potent stimulator of blood cHH while temperature and salinity variations were less effective.

Moreover an increase in water temperature increased blood cHH in *C. pagurus* and *P. clarckii* (Wilcockson *et al.* 2002; Zou *et al.*, 2003). In *C. maenas* it has been shown that the concentration of the cHH in the hemolymph increases dramatically during molting from 1-5 fmol $100\mu L^{-1}$ in the intermolt up to 150-200 fmol $100\mu L^{-1}$ during ecdysis (Chung *et al.*, 1999). Variation in the hemolymph cHH titer were also observed in *N. norvegicus* infected by the parasitic dinoflagellate *Hematodinium* sp. (Stentiford *et al.*, 2001).

Using ELISA and bioassay tests we have recently demonstrated the relationship between an environmental stressor and the release of cHH from the eyestalk into the hemolymph and the hyperglycemic response in the shrimp, *P. elegans* (Lorenzon *et al.*, 2004a). Moreover with this work we validated the use of a cross reactive antibody, anti-NencHH, to assess cHH level in the eyestalk and hemolymph of *P. elegans*. With the help of standard immunocytochemistry the antibody had previously been tested for recognition of cHH in the eyestalks of different species belonging to systematic groups increasingly remote in the phylogenetic tree: the decapods *A. leptodactylus, N. norvegicus*, *P. elegans, Munida rugosa* and the stomatopod *S. mantis* (Giulianini *et al.*, 2002). Finally we have quantified the variations in the hemolymph cHH after a challenge with different stressors. In *P. elegans* exposure to copper induced a dose-related rapid and massive release of cHH from the eyestalk into the hemolymph at the higher, lethal concentration while a gradual and reduced discharge was observed at the lower concentration (Fig. 3).

The relationship between exposure to a toxicant and release of the cHH was confirmed by variation in blood glucose with a dose related hyperglycemia that peaked 2 h after exposure to copper (Fig. 4).

Animals exposed to sublethal concentrations of Hg showed similar quantitative and time course relations between toxicant, release of cHH from the eyestalk, increment of hormone level in the hemolymph and subsequent hyperglycemia as already described for copper contamination. Interestingly, however, the highest, lethal concentration induced the release of cHH from the eyestalk into the hemolymph but was not followed by a significant variation in blood glucose (Figs 5, 6).

This situation could be related to the high toxicity of Hg which may interfere with the finer mechanisms that regulate hyperglycemic response. It is neither due to synaptic blockage of the superimposed neuronal release network (Lorenzon *et al.*, 1999) nor limited release of circulating cHH as high levels of cHH are discharged from the SG into the hemolymph. It is not due to inhibition of peripheral receptors on glycogenolytic target organs: indeed native SG homogenate injected into eyestalkless shrimps exposed to lethal concentration of Hg for 3 h is still able to cause hyperglycemia (Lorenzon *et al.*, 2000). High concentrations of Hg, instead, may change the functionality of the prepro-cHH processed during secretory steps and due to its ability to bind cysteines - six of which represent a highly conserved feature of the peptide structure (Lacombe *et al.*, 1999) - Hg might alter the active configuration of the peptide, as seen in other systems (Rodgers *et al.*, 2001), but not its immunoreactivity. Moreover Hg is known to impair osmoregulatory mechanisms in the crab, *Eriocheir sinensis* (Péqueux *et al.*, 1996); and inhibit acetylcholinesterase activity in *P. clarkii* (Devi and Fingerman, 1995). The altered response in *P. elegans* exposed to high concentrations of Hg may also be related to physiological modifications induced by Hg at a different systemic level (Lorenzon *et al.*, 2004a). Cu contamination induced variations of 5-HT of the eyestalk and hemolymph of *P. elegans* (Lorenzon *et al.*, 2005). The release of 5-HT from the eyestalk appears to be very rapid and dose dependent. In the hemolymph 5-HT peak occurs after 30 min and again the concentration of circulating 5-HT is dose dependent. After 1 h the level of 5-HT slowly decreases to the basal level (Fig. 7).

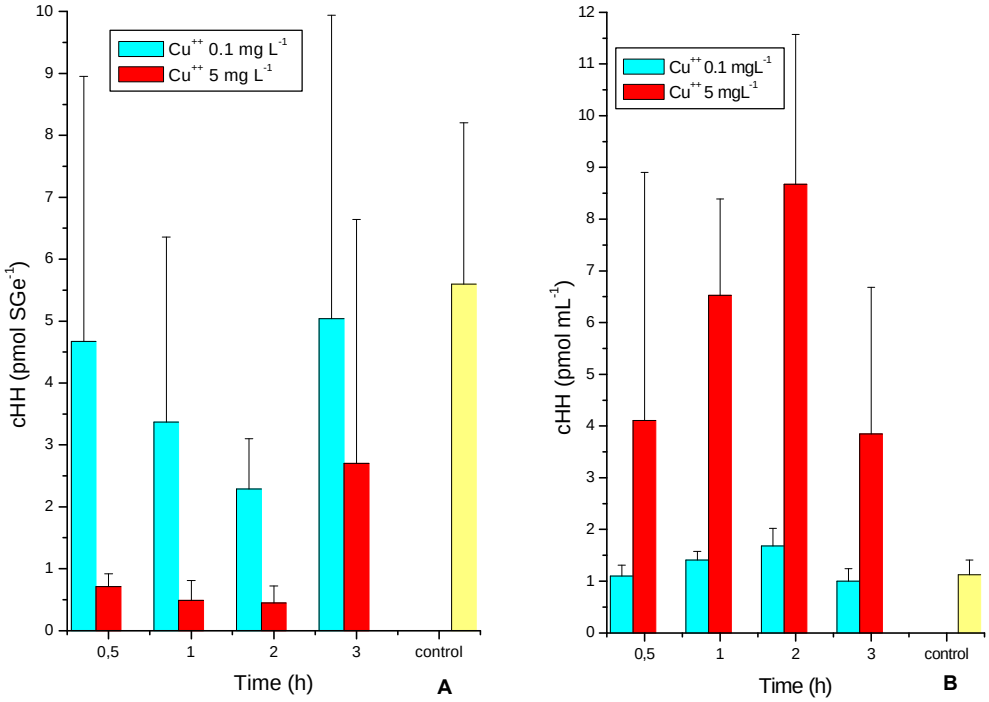

Fig. 3 Time course of cHH in the eyestalk homogenates (A) and in the hemolymph (B) of *P. elegans* after exposure to different concentrations of Cu^{++} and in relation to untreated controls. Values are expressed as means ± SD (n=4 repeated measures).

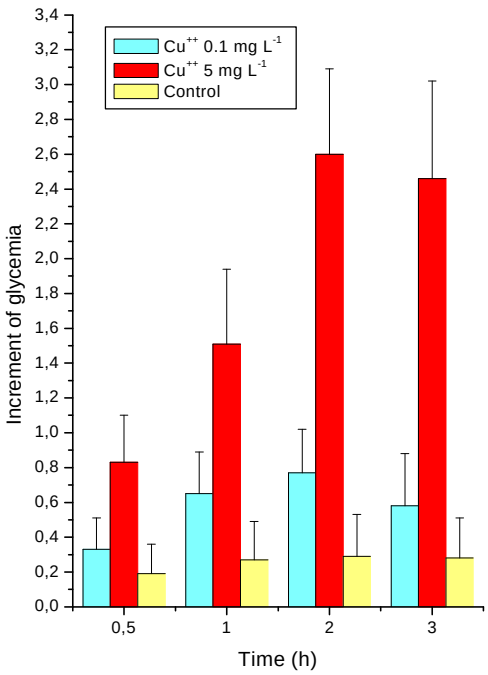

Fig. 4 Time course of glycemia in the hemolymph of *P. elegans* after exposure to different concentrations of Cu^{++} and in relation to untreated controls. Values of increment given as: [(experimental value)/ (value displayed by the same animal at 0 h)]$^{-1}$, are expressed means ± SD (N=10 repeated measures).

The release of 5-HT from the eyestalk into the hemolymph after Cu exposure precedes in its time course the release of cHH, confirming its role as neurotransmitter acting on cHH neuroendocrine cells. The rapid and massive release of 5-HT from the eyestalk of individual species following exposure to Cu might have induced release of the cHH resulting in hyperglycemia in intact but not in eyestalkless animals.

Lastly contamination with different doses of LPS, a bacterial thermostable endotoxin from *E. coli,* confirms the dose-related and convergent chain of events that leads to hyperglycemia. This suggests that blood glucose elevation is a general-purpose response to stressors and is likely to perform a protective role (Lorenzon *et al.*, 2004a).

Conclusion

In spite of the vastness of information on hyperglcemic stress response in Crustacea, there still exist many questions. In the scheme presented in figure 8 a possible model of the controlling network is proposed.

Stressors have been demonstrated to release the cHH and 5-HT from the eyestalk leading to an increase in their hemolymph concentrations. 5-HT exerts a positive influence inducing the release of cHH from the SG into the blood. The cHH then acts upon the target organs to release more than normal level glucose resulting in hyperglycemia. The DA

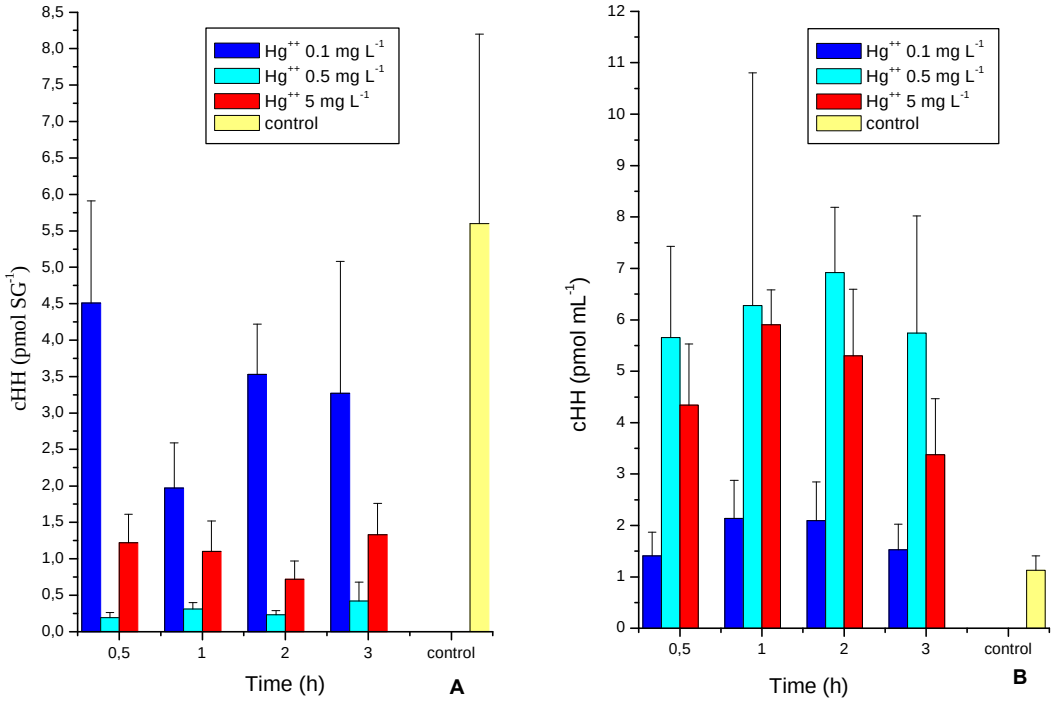

Fig. 5 Time course of cHH in the eyestalk homogenates (A) and in the hemolymph (B) of *P. elegans* after exposure to three different concentrations of Hg^{++} and in relation to untreated control. Values are expressed means ± SD (N=4 repeated measures).

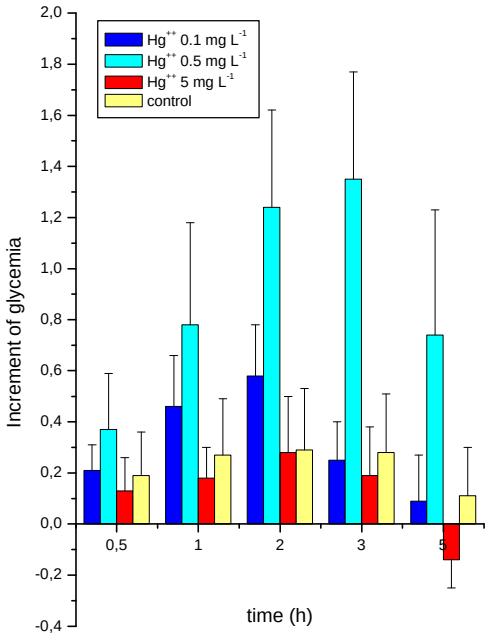

Fig. 6 Time course of glycemia in the hemolymph of *P. elegans* after exposure to three different concentrations of Hg^{++} and in relation to untreated controls. Values of increment given as: [(experimental value)/ (value displayed by the same animal at 0 h)]$^{-1}$, are expressed means ± SD (N=10 repeated measures).

receptor blocker, spiperone, inhibited the hypoglycemic action of DA and was found not to affect the ability of L/M-Enk to produce hypoglycemia. On the other hand, naloxone blocked the action of both L/M-Enk and DA, thereby allowing the release of cHH (Sarojini *et al.*, 1995, Lorenzon *et al.*, 1999). Apparently DA and L-enk produced hypoglycemia by inhibiting cHH release. These results suggest that in the chain of neurons terminating at the neuroendocrine cells that secrete cHH, dopaminergic neurons precede enkephalinergic neurons.

We also suggest a role for the hemocytes in the hyperglycemic stress response as stressors affect both the total (THC) and the differential haemocyte count and that exocytosis of cHH granules from the eyestalk neuroendocrine cells can be elicited either by an early release from hemocytes of cytokines and/or other circulating messengers like 5-HT.

Moreover LPS treated eyestalkless animals undergo less haemocytopenia than intact individuals. This suggests that previous cHH release and hyperglycemia can cause a decrease in THC, which eventually exerts a protective function (Lorenzon *et al.*, 2002).

In summary it may be said that indicators of stress responses are useful in assessing the short-term well-being or long-term health status of an animal (Fossi *et al.*, 1997; Paterson and Spanoghe, 1997) and, such indicators have received considerable attention in commercially important species of decapod crustaceans (Paterson

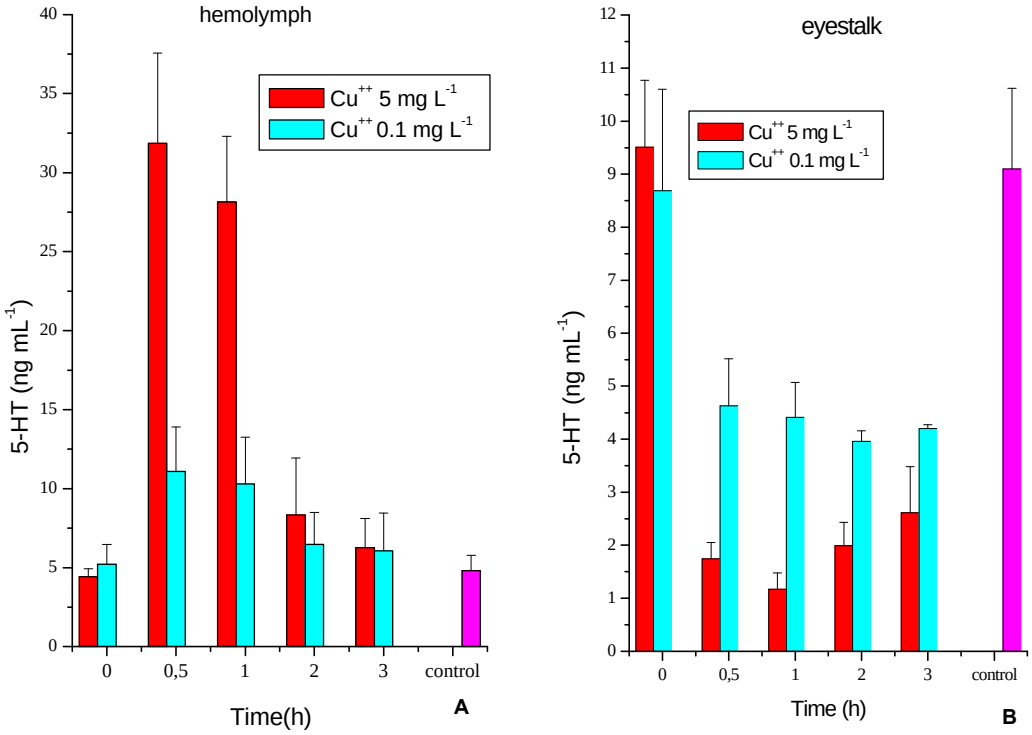

Fig. 7 Time course (0.5-3 h) of 5-HT in the hemolymph (A) and in the eyestalk (B) of *P. elegans* after exposure to different concentrations of Cu^{++} and in relation to untreated controls. Values are expressed means ± SD (n=4 repeated measures).

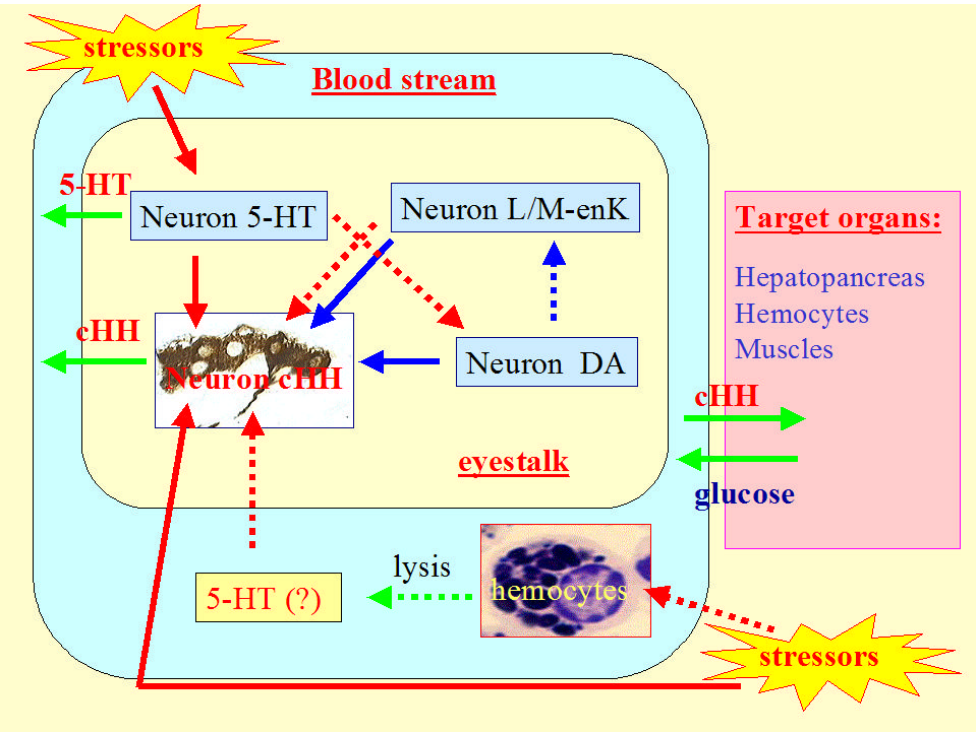

Fig. 8 Possible model of hyperglycemic stress response controlling network. In the scheme: continuous arrow=demonstrated effect, dotted arrow= hypothesized effect, red arrow= stimulation, blue arrow= inhibition, green arrow=release.

and Spanoghe, 1997; Chang *et al.*, 1999). A number of researchers have suggested different methods for quantifying the stress responses in crustaceans; which include the measurement of different hemocyte types in the hemolymph (Jussila *et al.*, 1997 Lorenzon *et al.*, 1999, 2001), and the physiological, biochemical (Paterson and Spanoghe, 1997; Stentiford *et al*.. 1999), and molecular changes in the tissues and the hemolymph (Fossi *et al.*, 1997). Thus variations in the hemolymph glucose concentration in the hemolymph and of the cHH level in relation to stressors could be used as a tool to monitor a variety of stress responses.

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