

Crustacean Hyperglycemic Hormone and Hemolymph Metabolites: Stress Responses in Two Lobster Species

Ernest S. Chang

Bodega Marine Laboratory
University of California-Davis
P.O. Box 247, Bodega Bay
California 94923, USA,
eschang@ucdavis.edu

Grant D. Stentiford

Center for Environment
Fisheries and Aquaculture Science
Weymouth Laboratory, Weymouth DT4
8UB
Dorset, UK,
g.d.stentiford@cefas.co.uk

Douglas M. Neil

Division of Environmental and
Evolutionary Biology
University of Glasgow
Glasgow G12 8QQ
Scotland, UK,
d.m.neil@bio.gla.ac.uk.

Sharon A. Chang

Bodega Marine Laboratory
University of California-Davis
P.O. Box 247, Bodega Bay
California 94923, USA,
sachang@ucdavis.edu.

Abstract

Our laboratory has developed several methods for the quantification of stress in crustaceans. An ELISA was developed for the crustacean hyperglycemic hormone (CHH) from the American lobster (*Homarus americanus*). It is sensitive to as little as 0.2 fmol of peptide. Increases in hemolymph CHH were observed under conditions of hypoxia, elevated temperature, and altered salinity. In addition, elevated CHH concentrations were observed in Norway lobsters (*Nephrops norvegicus*) that were parasitized with the dinoflagellate *Hematodinium* and in other lobsters following the stress of capture by trawling.

Introduction

Stress responses occur in all animals when regulated physiological systems are extended beyond their normal range by external stressors. Failure of all or part of the integrated homeostatic response may lead to increasing physiological disturbance and ultimately death. Indicators of such stress responses are therefore useful in assessing the short-term well-being or long-term health status of an animal and such indicators have received considerable attention in commercially important decapod crustacean species (Paterson and Spanoghe, 1997).

Hyperglycemia as a response to various kinds of stress is well documented in decapod crustaceans. Regulation of hemolymph glucose is mediated by the release of the crustacean hyperglycemic hormone (CHH) that is synthesized in the eyestalk x-organ and stored prior to release from the sinus gland (for review see Böcking *et al.*, 2002). We have developed an enzyme-linked immunosorbent assay (ELISA) for CHH as a tool for the quantification of various environmental stresses in lobsters (hypoxia, thermal stress, salinity

stress). We also examined the effects of the stresses imposed by parasitism and capture upon CHH and hemolymph metabolites.

Materials and Methods

Animal sampling

Sampling of hemolymph and methods for inducing stress for CHH and metabolite measurements in American lobsters (*Homarus americanus*) have been described (Chang *et al.*, 1998). Norway lobsters (*Nephrops norvegicus*) were captured from two sites on the west coast of Sweden. One subset of lobsters (n=20) was captured using a standard otter trawl, towed for 1 h in the Skagerrak Sea area at the mouth of the Gullmarsfjord. Another subset (n=20) was captured using baited creels set within the same area (57°39.927N, 11°29.658E). Trawled and creeled lobsters were sampled immediately upon landing. One ml of hemolymph was drawn from the base of the 5th pereopod of each lobster into a 2-ml syringe fitted with a 23-gauge needle and frozen immediately by immersing the syringe into liquid nitrogen. Samples of hepatopancreas were removed, placed into vials and frozen as above. Upon reaching the laboratory, all samples were lyophilized overnight in a freeze dryer prior to storage at 4°C. Characterization of the parasitization of *N. norvegicus* by the dinoflagellate *Hematodinium* sp. has been published (see Field and Appleton, 1995).

CHH analysis of hemolymph

Production of an antibody against purified CHH-A from *H. americanus* and development of the ELISA have previously been described (Chang *et al.*, 1998). Prior studies demonstrated that *N. norvegicus* CHH could be detected using this antibody (Stentiford *et al.*, 2001). In the current study, freeze-dried plasma samples were resuspended to their original volume with water and assayed for CHH with ELISA. Due to the lack of available purified *N. norvegicus* CHH, it was not possible to quantify absolute concentrations of CHH in *N. norvegicus*. The data are therefore presented as *Orconectes limosus* equivalents, which were used as the standards and which allow relative quantification in *N. norvegicus*.

Glycogen analysis of hepatopancreas

For determination of glycogen, 400 µl of 30% potassium hydroxide were added to 20 mg of the freeze-dried samples of *N. norvegicus* hepatopancreas. The samples were heated for 20 min in a water bath maintained at 95-100°C. Samples were cooled and added to 700 µl absolute ethanol before being placed on ice for 2 h. Following precipitation, samples were spun at 17,000g for 10 min and the supernatant discarded. One ml of water was added to each sample before sonication. Fifty µl of each sonicated sample were incubated at 95-100°C in 1 ml of anthrone reagent before measurement of total glycogen (see Carroll *et al.*, 1956).

Glucose analysis of hemolymph

Glucose concentration in the lobster hemolymph was measured using the glucose oxidase method (Boehringer-Mannheim) in a multi-well plate method as described by Webster (1996). Briefly, 50 μl samples of deproteinized plasma were added to 450 μl of 0.2 M phosphate buffer (pH 7.4) and 100 μl samples of this solution were used in the assay with 200 μl of the enzyme chromogen reagent. Concentrations of plasma glucose were read from a standard curve constructed for glucose.

Lactate analysis of hemolymph

Lactate was measured using a kit (Sigma #826). The reagents were prepared according to its protocol. Anticlotting buffer (glycine ethyl ester; Chang *et al.*, 1998) was added to hemolymph (1:1, v:v) and centrifuged. Sample supernatants (20 μl) and standards (10 μl and 10 μl of anticlotting buffer) were added to individual wells of a 96-well plate. Reagent (lactate dehydrogenase and nicotinamide adenine dinucleotide) (200 μl) was added to each well. After mixing and a 10-min incubation, the plate was read at 540 nm. Concentrations of lactate were determined from the standard curve.

Results

CHH in *H. americanus*

We observed that emersion is a potent stimulator for the elevation of hemolymph CHH (Chang *et al.*, 1998). **Figure 1** shows that it increases from resting values of 4.0 fmol/ml to 168.1 fmol/ml after 4 h emersion. Although handling stress slightly increases CHH, the additional stress of emersion is significantly above the handling stress observed in immersed controls (from 11.3 to 20.6 fmol/ml) held at the same temperature (13°C) as the emersed lobsters.

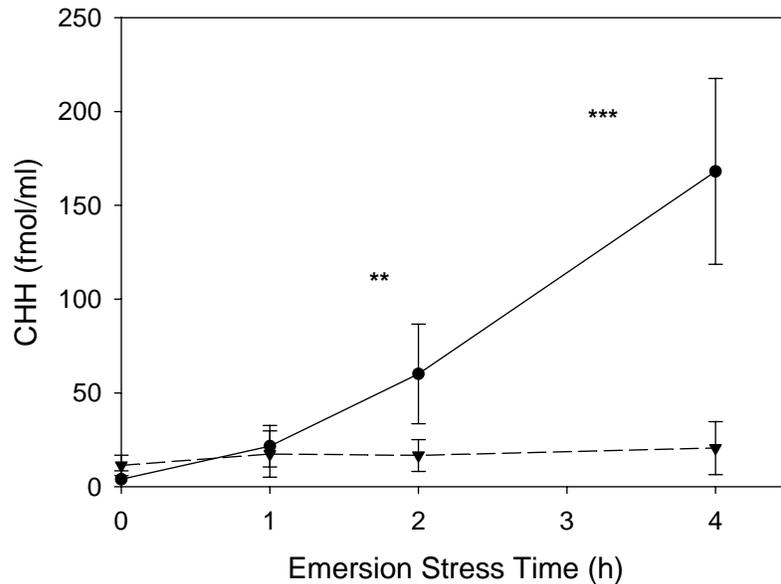


Figure 1. Effects of emersion on *H. americanus* hemolymph CHH (circles, solid line). Lobsters (n=7) were sampled as previously described (Chang *et al.*, 1998). Means±SD are shown. Control data are represented by the triangles with the broken line (n=8). Asterisks indicate significant differences from immersed controls at $P<0.01$ (**) and at $P<0.001$ (***). Modified from Chang *et al.* (1998).

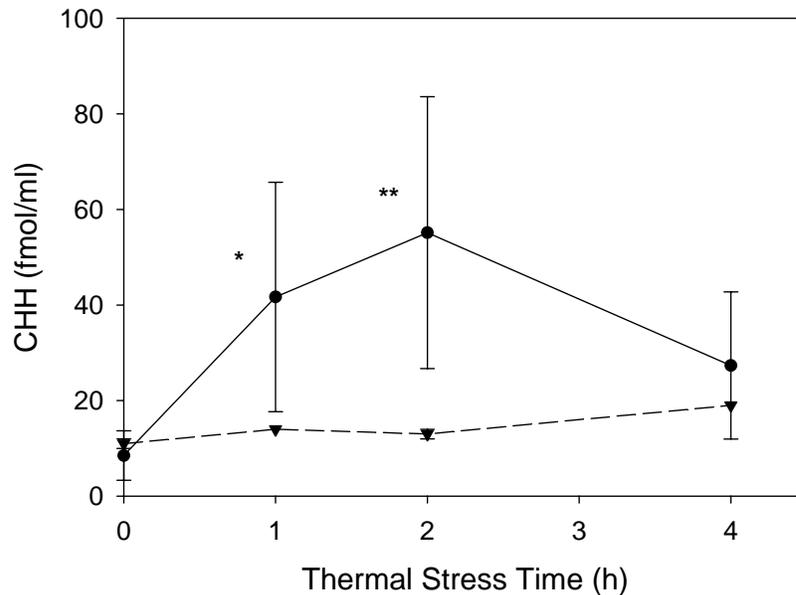


Figure 2. Hemolymph CHH concentrations (means±SD; circles, solid lines) in immersed *H. americanus* held at 23°C (n=8). Hemolymph was sampled and assayed by ELISA. Controls (triangles with broken line) are the same animals used in Fig. 1 (error bars have been

omitted). Asterisks indicate significant differences from 13°C controls at $P<0.05$ (*) and at $P<0.01$ (**). Modified from Chang *et al.* (1998).

Thermal stress caused an increase in hemolymph CHH. **Figure 2** shows that a 10°C change in temperature to 23°C caused an increase in CHH relative to ambient (13°C) controls. No significant changes in hemolymph CHH were observed following a 5° temperature elevation nor were changes seen during cold stress (data not shown). Both hyposalinity (50%) and hypersalinity (150% seawater) resulted in significant alterations in hemolymph CHH after 2 h. This elevation in CHH relative to the controls was not significant at later time points (**Fig. 3**).

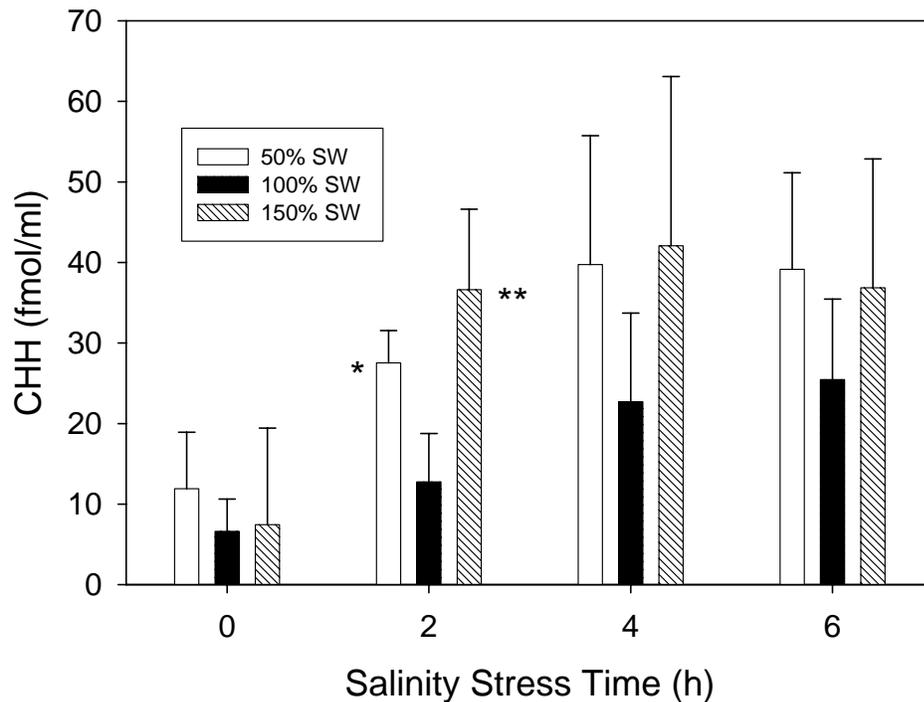


Figure 3. Hemolymph CHH concentrations (means±SD) in immersed *H. americanus* held in 50% (white bars), 100% (black bars), or 150% (hatched bars) seawater (n=5 for each salinity). Hemolymph was sampled and assayed by ELISA. Asterisks indicate significant differences from the 100% seawater controls at $P<0.05$ (*) and at $P<0.01$ (**). Modified from Chang *et al.* (1998).

CHH and hemolymph metabolites in N. norvegicus

The mean concentration of CHH in the plasma of uninfected *N. norvegicus* was 32.2 fmol/ml. The mean concentration of CHH in the plasma of lobsters subpatently infected with the dinoflagellate *Hematodinium* sp. (107.65 fmol/ml) was significantly higher than that of Stage 0 (uninfected) animals ($P<0.05$). At Stage 1 (light patent infection), the mean concentration was higher (though not significantly, $P=0.057$) than that of Stage 0 and lower

(though not significantly, $P=0.070$) than that of subpatently infected lobsters. In later stages of patent *Hematodinium* infection, the plasma CHH concentration was significantly higher than that of uninfected animals [Stage 2 (77.2 fmol/ml, $P<0.001$) and Stage 3–4 (106.6 fmol/ml, $P<0.001$)]. The significant increase in plasma CHH concentration between Stage 1 and Stage 3–4 animals ($P<0.05$) and the almost significant increase between Stage 1 and Stage 2 animals ($P=0.080$) suggest that the titer of CHH increases with the severity of patent infection, but also that initiation of subpatent infection may involve a temporary rise in plasma CHH titer. It is interesting to note, however, that the mean plasma CHH titer of subpatently infected lobsters is not significantly different from that of Stage 3–4 lobsters ($P=0.997$) (Fig. 4).

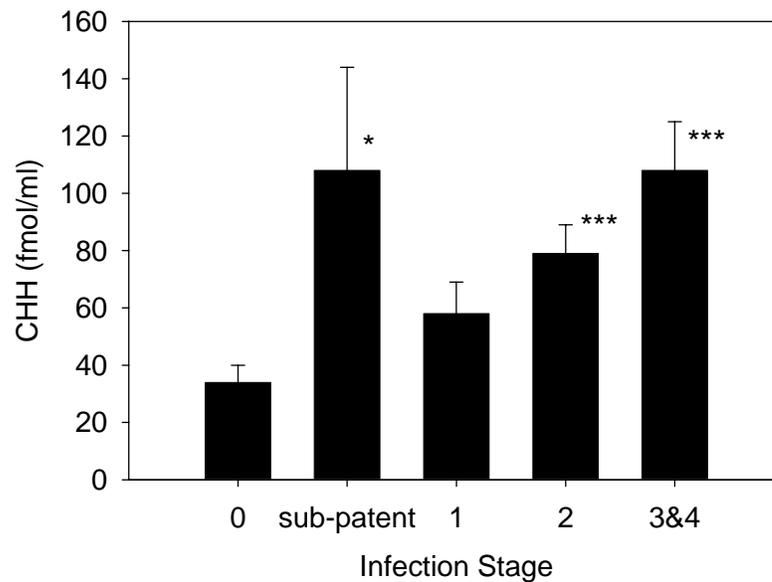


Figure 4. Concentration of CHH (fmol/ml) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (subpatent and Stages 1, 2, 3–4) *N. norvegicus*. Statistically significant difference from Stage 0 value given as * ($P<0.05$), and *** ($P<0.001$). Stage 0, $n=22$; subpatent, $n=8$; Stage 1, $n=22$; Stage 2, $n=33$; Stage 3–4, $n=15$. Modified from Stentiford *et al.* (2001).

The mean concentration of CHH in the hemolymph of lobsters captured by trawling was 97.7 fmol/ml and was significantly higher than in those lobsters captured by passive trapping (2.34 fmol/ml) (Mann Whitney test, $W=55.0$, $P<0.001$). The mean concentration of glycogen in the hepatopancreas of lobsters captured by creeling (8.59 ± 1.4 $\mu\text{g/g}$ dry weight) was lower than those captured by trawling (12.26 ± 2.41 $\mu\text{g/g}$ dry weight) but the difference was not significant (Mann-Whitney test, $W=220.0$, $P>0.05$).

Glucose concentration in the hemolymph of lobsters captured by trawling was 197.5 ± 28.6 $\mu\text{g/ml}$. The mean concentration of glucose in the hemolymph of lobsters captured by creeling (138.3 ± 12.8 $\mu\text{g/ml}$) was lower, but the difference was not significant (ANOVA test, $F=5.06$, $P>0.05$). The concentration of lactate in the hemolymph of lobsters captured by trawling was 607.1 ± 14.8 mg/ml. The concentration of lactate in the hemolymph of lobsters

captured by creeling (12.9 ± 5.0 mg/ml) was significantly lower than in those captured by trawling (ANOVA test, $F=55.0$, $P<0.001$).

Discussion

We have demonstrated that various environmental stresses and physiological states resulted in elevations in the hemolymph CHH concentration in American lobsters. These stresses included emersion (producing hypoxia), temperature elevation, and salinity changes. Our results are in agreement with those of Webster (1996) on *Cancer pagurus*. He found that emersion causes a significant increase of CHH in the hemolymph 15 min after emersion. Similarly, in our lobsters, a significant increase was measurable after 20 min. Webster (1996) discussed the physiological significance of this mechanism of endocrine metabolic adaptation for *C. pagurus*, which may be subjected to emersion and hypoxia in the intertidal zone. Lobsters may also occasionally experience hypoxia in warm, intertidal waters (Lawton and Lavalli, 1995). The increase of CHH in response to thermal stress may be related either to the hypoxic conditions existing in warm seawater or to increased general metabolism at higher temperatures. Our data are consistent with observations made on thermal stress on crabs by Chung and Webster (1996).

Although *H. americanus* occasionally experiences hyposaline environments (Lawton and Lavalli, 1995), they are considered to be stenohaline. Apparently, the limited ability to osmoregulate would appear to be consistent with the limited metabolic adaptation to salinity changes. The observation that CHH increases only slightly upon salinity stress may reflect this situation. There are indications that other stresses, such as heavy metals (Reddy *et al.*, 1996) and pesticides (Chang and De Guise, unpublished) may cause hyperglycemia via increased secretion of CHH.

In Norway lobsters patently infected with *Hemtodinium*, the plasma CHH concentration shows a steady and significant increase in relation to infection severity (Fig. 4). As the parasite burden increases, a steadily increasing demand is placed upon the hosts' hemolymph glucose (Stentiford *et al.*, 2001). A feedback loop likely results in the release of additional CHH from the sinus gland. The parasites could also diminish the partial pressure of oxygen in the hemolymph via a reduction in hemocyanin. Thus, the elevated hemolymph CHH concentration in patent infection may be due primarily to a "functional hypoxia" in the infected lobster, which elicits a cascade response similar to that seen during the "environmental hypoxia" caused by emersion.

Our results suggest that a one-hour trawling duration causes a small but not statistically significant increase in the hemolymph glucose of Norway lobsters. The hepatopancreas of lobsters captured by trawling had a slightly increased (though not significant) concentration of glycogen. The most significant differences between trawled and creeled lobsters were observed in the concentrations of lactate and CHH in the hemolymph. Lactate was elevated almost 40-fold and CHH was elevated almost 50-fold in the hemolymph of trawled lobsters. Reduced glucose normally results in increased CHH release from the sinus gland via a negative feedback loop. Since glucose was not reduced in the hemolymph of trawled animals, it is unlikely that the elevated CHH is caused by this mechanism. Instead, the elevated hemolymph lactate, a feature known to drive the positive feedback of CHH release from the sinus gland, is more likely to lead to the elevated CHH concentration observed in the trawled lobsters.

Since trawled lobsters undergo exhaustive exercise in an attempt to escape capture by a typical trawl net and may further undergo additional exercise within the cod-end of the net, it is likely that the elevated hemolymph lactate was derived from the large muscles responsible for flexion of the abdomen. Excess lactate is transferred from the muscle to the hemolymph in an attempt to reduce tissue acidosis. With continued exercise it accumulates in the hemolymph.

We believe that the results presented in this paper demonstrate that measurements of hemolymph CHH (and selected circulating metabolites) will be useful for monitoring a variety of stress responses in lobsters and to study the role of CHH in the metabolic regulation of crustaceans. Our CHH research will complement other stress indicators, such as the induction of stress proteins (Spees *et al.*, 2002a,b, 2003). These studies should have utility in the monitoring of ecosystem health and in improving fisheries and aquaculture practices.

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Literature Cited

- Böcking, D., H. Dirksen and R. Keller.** 2002. The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. *The Crustacean Nervous System* (ed. K. Wiese). Springer, pp. 84-97.
- Carroll, N. V., R. W. Longley and J. D. Roe.** 1956. The determination of glycogen in liver and muscle by use of the anthrone reagent. *Journal of Biological Chemistry* 220:583-593.
- Chang, E. S., R. Keller and S. A. Chang.** 1998. Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *General and Comparative Endocrinology* 111:359-366.
- Chung, J. S. and S. G. Webster.** 1996. Does the N-terminal pyroglutamate residue have any physiological significance for crab hyperglycemic neuropeptides? *European Journal of Biochemistry* 240:358-364.

- Field, R. H. and P. L. Appleton.** 1995. A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. *Diseases of Aquatic Organisms* 22:115-128.
- Lawton, P. and K. Lavalli.** 1995. Postlarval, juvenile, adolescent, and adult ecology. *Biology of the Lobster Homarus americanus* (ed. J. R. Factor). Academic Press, pp. 47-88.
- Paterson, B. D. and P. T. Spanoghe.** 1997. Stress indicators in marine decapod crustaceans, with particular reference to the grading of western rock lobsters (*Panulirus cygnus*) during commercial handling. *Marine and Freshwater Research* 48:829-834.
- Reddy, P. S., R. V. Katyayani and M. Fingerman.** 1996. Cadmium and naphthalene-induced hyperglycemia in the fiddler crab, *Uca pugilator*: differential modes of action on the neuroendocrine system. *Bulletin of Environmental Contamination and Toxicology* 56:425-431.
- Spees, J. L., S. A. Chang, M. J. Snyder and E. S. Chang.** 2002a. Osmotic induction of stress-responsive gene expression in the lobster *Homarus americanus*. *Biological Bulletin* 203:331-337.
- Spees, J. L., S. A. Chang, M. J. Snyder and E. S. Chang.** 2002b. Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin. *Cell Stress and Chaperones* 7:97-106.
- Spees, J. L., S. A. Chang, D. L. Mykles, M. J. Snyder and E. S. Chang.** 2003. Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. *Cell Stress and Chaperones* 8:258-264.
- Stentiford, G. D., E. S. Chang, S. A. Chang and D. M. Neil.** 2001. Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). *General and Comparative Endocrinology* 121:13-22.
- Webster, S. G.** 1996. Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *Journal of Experimental Biology* 199:1579-1585.