The stress induced by the Crustastun™ process in two commercially important decapod crustaceans: the edible brown *Cancer pagurus* and the European lobster *Homarus gammarus*

Scientific Report

by

Professor Douglas Neil &
Dr John Thompson

Institute of Biodiversity, Animal Health and Comparative Medicine
School of Medical Veterinary and Life Sciences
University of Glasgow
Scotland
UK

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Introduction

The impacts of various stunning methods on the welfare of decapods have recently been compared by Roth and Øines (2010) using mainly behavioural measures. However, they did not include any biochemical measures of stress, and they did not evaluate the effect of stunning using the Crustastun™ device. The present report summarises the results of a systematic study of the stress imposed by Crustastunning in two commercially important edible crustaceans, namely the brown crab *Cancer pagurus* and the European lobster *Homarus gammarus*, as indicated by a biochemical measure of stress.
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Using the Crustastun on crabs and lobsters

The Crustastun™ is a device designed to administer a lethal electric shock to shellfish such as crabs and lobsters before cooking, to avoid boiling a live shellfish (www.crustastun.com). It works by applying a 110 volt, 2-5 amp electrical charge to the shellfish. These parameters were determined by Robb (1999) and the effectiveness of the Crustastun in achieving the required stun currents was evaluated by Sparrey (2005).

Measuring stress responses in Crustacea

The most studied stress responses of crustaceans are the alterations in variables related to fuel metabolism (e.g. hyperglycemia) that occur in order to satisfy the energy demands imposed by stress (see Neil, 2012a). Such responses are analogous to the secondary stress responses of fish, although the neuroendocrine mechanisms (primary stress responses) are completely different and less well understood than for vertebrates. Crustacean hyperglycemic hormone (CHH) probably represents the most recognized neuroendocrine mechanism mediating such stress responses in crustaceans (see Chang, 2005, Lorenzon, 2005, Fanjul-Moles, 2006 and Webster *et al*., 2012 for recent reviews of CHH).

CHH release is modulated by several neuromodulators, including catecholamines (CA). The role of CA as components of the primary stress response in crustaceans has been addressed by analogy to the well known involvement of the sympathoadrenal system as a stress response mediator in vertebrates (Wendelaar-Bonga, 1997), although the specific pathways and the particular CA involved could be different. However a few studies do indicate that CA, particularly dopamine (Zou *et al.*, 2003) and noradrenaline (Aparicio-Simón *et al.*, 2010), may exert control of CHH secretion.

Hormones from the superfamily of hormones to which CHH belongs also control other physiological processes (Webster *et al.*, 2012). These include ionic and osmotic regulation (Spanings-Pierrot *et al.*, 2000), water uptake during ecdysis (Chung *et al.*, 1999), gonad maturation, inhibition of moulting, and secretion of enzymes by the hepatopancreas. Moreover, the activity and effects of CHH have been reported in a wide variety of crustaceans subjected to various environmental stresses: hypoxia (Albert and Ellington, 1985), temperature and salinity changes (Keller *et al.*, 1994), capture by trawling (Paterson and Spanoghe, 1997), emersion (Ridgway *et al.*, 2006), changes in light intensity (Fanjul-Moles *et al.*, 1998) and exposure to bacterial endotoxins (Lorenzon *et al.*, 1997), parasitism by dinoflagellates (Stentiford *et al.*, 2001) and heavy metal pollutants (Lorenzon *et al.*, 2004). Also, exercise seems to be a potent elicitor of CHH release (Morris *et al.*, 2010).

Crustaceans subject to this range of stressors release CHH, which elevates the haemolymph glucose concentrations (Webster, 1996; Bergmann *et al.*, 2001; Toullec *et al.*, 2002). This occurs by mobilisation of intracellular glycogen, brought about because CHH stimulates the breakdown of glycogen (glycogenolysis) in muscle and in the hepatopancreas. It does this by inhibiting glycogen synthase and activating glycogen phosphorylase (Sedlmeier, 1982, 1988; Keller and Orth, 1990). The glucose thus formed either moves to haemolymph, thus causing hyperglycemia, or is converted intracellularly to L-lactate via glycolysis, which also then transfers to the haemolymph causing hyperlactemia (Stentiford *et al.*, 2001; Verri *et al.*, 2001). This is analogous to the responses of vertebrates.

CHH participates in these adaptive mechanisms to stressful conditions by means of a dual feedback control system (see Figure 2 in Fanjul-Moles, 2006). Hormone synthesis and secretion is homeostatically controlled, being under negative feedback control of the haemolymph glucose (Santos and Keller, 1993a,b; Glowik *et al.*, 1997; Santos *et al.*, 2001). In a second positive feedback loop, circulating L-lactate in the haemolymph, resulting from the increasing glycogenolysis in the muscle tissues and hepatopancreas, stimulates a release of CHH, which in turn stimulates further glycogenolysis (Santos and Keller, 1993a,b). This leads to parallel changes in haemolymph CHH and L-lactate occurring under applied stresses, for example high levels of exercise in a crab (see Figure 2 in Morris *et al.*, 2010) and emersion in air in a lobster (see Table 2 in Ridgway *et al.*, 2006).

Lactate is a good indicator of the stress response in crustaceans simply because it is the major end product of anaerobic metabolism, with higher concentrations indicating an attempt by the animal to mitigate the effect of a stressor (Albert and Ellington, 1985). However, several lines of evidence suggest that L-lactate is not only a metabolic end product, but may itself perform specific signaling functions related to stress. Thus L-lactate may act as a metabolic alarm signal, by helping the animal to sense unfavourable conditions and initiate behavioural and metabolic changes, e.g. behavioural hypothermia as reported by De Wachter *et al.* (1997). Catecholamines may also play a role in mediating such an emergency response, since low but significant positive correlations were found in that study between levels of L-lactate and levels of adrenaline, octopamine and tryptophan (a precursor of serotonin).

A further indication of the specific role of L-lactate in stress signaling is provided by the rapidity of its increase in the haemolymph (hyperlactemia), which often precedes hyperglycemia. Webster (1996) demonstrated this immediate hyperlactemia preceding hyperglycemia 30 min post air exposure in *C. pagurus*. Patterson *et al.* (2007) showed that forced de-clawing of a single claw from the edible crab, *C. pagurus*, caused marked short-term physiological changes consistent with a stress response in which the lactate response was particularly rapid, and significant within a few minutes. Similar short-term responses of L-lactate to a range of other stressors have been reported for other crustaceans. e.g in the crab *Liocarcinus depurator* and the squat lobster *Munida rugosa* immediately after trawling (Bergmann *et al.*, 2001) and in the lobster, *Panulirus cygnus* after post-capture handling (Paterson and Spanoghe, 1997).

For all these reasons, the haemolymph L-lactate concentration provides an appropriate measure of stress in crustaceans, and is particularly useful since a large number of

published studies using a wide range of environmental stresses on numerous decapod crustaceans provide extensive data for comparison. The evaluation of the stresses induced in crabs and lobsters by the Crustastun machine has therefore been performed using measures of haemolymph L-lactate.

Materials and Methods

Ethical statement
The number of animals used in these trials was kept to the minimum necessary to obtain scientific results, considering that the gain in knowledge and long term benefit to the subject will be significant. All the live animals used were treated with proper care in order to minimize their discomfort and distress.

Animal supply and holding
Male brown crabs, *Cancer pagurus* of carapace width 120-140 mm, and male European lobsters, *Homarus gammarus* lobsters of carapace length 80-95 mm were used in these trials. All animals were in the intermoult stage with a hard exoskeleton. They were captured by commercial fishermen using baited traps (creels) laid offshore from St Abbs on the east coast of Scotland. After banding the claws of the lobsters, and nicking the tendons of the crab claws (both standard commercial practices) they were held initially in seawater tanks at the St Abbs Marine Station, then transferred in chilled containers to the University of Glasgow. Here they were retained individually in tanks within a closed seawater circulating system at 10^{o}C for at least two weeks before experimentation.

Experimental design
From the stock of 12 crabs and 12 lobsters, groups of 6 animals of each species, chosen randomly from the holding tanks, were subjected to one of two treatments: either the Crustastunning procedure or a sham treatment in which the animals were handled in exactly the way, but not stunned. This sham treatment was used to provide a control for the effects of the handling itself which inevitably occur during the Crustastunning procedure.

Specifically, for 'Crustastunning' the procedure was applied without prior anaesthesia using a machine supplied by Studham Technologies Ltd., according to the manufacturer's operating instructions. The chamber was filled with a salt solution (~3g L^{-1}). Individual crabs or lobsters were removed from their holding tanks and an initial haemolymph sample was taken for L-lactate determination ("pre-stun" value). The animal was then placed into the Crustastun machine, the lid was closed and the animal was stunned by a 110 volt, 2-5 amp electrical charge for 10 s. The animal was then returned to its seawater container (water temperature 10^{o}C - 12^{o}C). These procedures entailed the animal being emersed into the air for no more than 2 minutes. A second haemolymph sample (from the contralateral side) was taken at a time point of 10 minutes after the Crustastunning procedure, for L-lactate determination ("post-stun" value). No further samples were taken at later time points as the animals were effectively killed by the Crustastunning procedure, and were then in a post-mortem state.

For the "Sham" treatment, individual crabs or lobsters were removed from their holding tanks and an initial haemolymph sample was taken for L-lactate determination ("pre-sham" value). The animal was then placed into the Crustastun machine and the lid was closed for 10 s, but without activation of the electrical charge. The animal was then returned to its

seawater container (water temperature 10^oC - 12^oC). Again, for these procedures the animal was emersed into the air for no more than 2 minutes. A second haemolymph sample (from the contralateral side) was taken at a time point of 10 minutes after the treatment, for L-lactate determination ("post-sham" value). Also, in order to gauge recovery, a further haemolymph sample was taken from animals of the sham-treated group one week later.

Haemolymph sampling and measuring L-lactate

Haemolymph samples were taken from the sinus at the base of a 5th pereiopod of both the crabs and lobsters using a 25-gauge needle and a disposable syringe. The -lactate concentration was measured in the haemolymph samples with a portable lactate analyser (Accutrend ®, Roche Diagnostics, Basel, Switzerland) using freshly extracted samples. The accuracy of the portable lactate analyser for the determination of L-lactate in decapod crustacean haemolymph samples had previously been determined by analysing a set of haemolymph samples from the Norway lobster using an enzymatic method (see Albalat *et al.*, 2010 for details) and comparing these values with those obtained using the lactate analyser. It was found that there was a highly significant correlation (r^2=0.960) between the values for haemolymph L-lactate obtained using the two methods.

Statistical analysis

Statistical analyses were carried out for each measure by a General Linear Model (GLM), treating stunned and sham-treated animals as separate experiments. The response variable was the haemolymph L-lactate concentration measure and the explanatory variable was the treatment (as a categorical factor). The residuals were assessed visually for normality. Data are reported as mean values ± standard error of mean (SEM). The differences between Crustastunned animals and sham-treated animals at the two common sampling times were analysed by independent samples t-tests, and P-values lower than 0.05 were considered statistically significant.

Results

The brown crab *Cancer pagurus*

The results obtained are shown in Figure 1. The haemolmph L-lactate values in the two groups of 6 rested crabs taken randomly from the holding tanks for either Crustastunning or the sham treatment had mean values of 0.78 ± 0.09 mM L^{-1} (pre-stun) and 1.05 ± 0.15 mM L^{-1} (pre-sham) respectively. These values did not differ significantly from each other ($F_{1,11}$=2.47, P=0.147).

Following Crustastunning the haemolymph L-lactate in the crabs increased to a mean value of 2.63 ± 0.26 mM L^{-1}, which was significantly greater than the pre-stun value for this group ($F_{1,11}$=45.00, P=0.000).

After the sham treatment the haemolymph L-lactate in the crabs also increased, with a mean value of 3.80 ± 0.42 mM L^{-1} being obtained. This was also significantly greater than the pre-sham value for this group ($F_{1,11}$=38.49, P=0.000).

The increases of haemolymph L-lactate following the two treatments were compared by considering the changes in the values for individual crabs, and it was found that the mean increase for the Crustastunned crabs (1.85 ± 0.30 mM L^{-1}) was not significantly different

from the mean increase for the sham-treated crabs (2.75 ± 0.36 mM L^{-1}) ($F_{1,11}$ = 3.68, P=0.084).

In terms of their subsequent fates, when returned to their holding tanks the stunned crabs showed no further visible movements, and never recovered. However the sham-treated crabs showed normal behaviour when returned to their holding tanks (limb movement, antennule flicking, a ventilation current and eye retraction reflexes) which continued thereafter. Samples taken from these sham-treated crabs one week later showed that they had a mean haemolymph L-lactate concentration of 0.85 ± 0.06 mM L^{-1}, which was not significantly different from the pre-sham value of 1.05 ± 0.15 mM L^{-1} for this group ($F_{1,11}$=1.64, P=0.229).

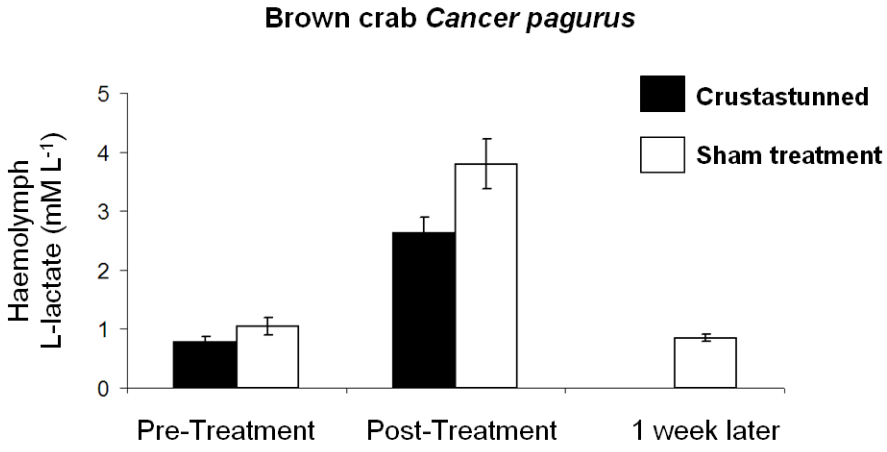

Figure 1. Haemolymph L-lactate concentrations in male brown crabs, *Cancer pagurus*, before and after Crustastunning or sham-treatment. Mean values ± SEM. N=6 for each treatment group.

The European lobster *Homarus gammarus*

The results obtained are shown in Figure 2. The haemolymph L-lactate values in the 2 groups of 6 rested lobsters taken randomly from their holding tanks for either Crustastunning or the sham treatment had mean values of 0.77 ± 0.10 mM L^{-1} (pre-stun) and 0.72 ± 0.06 mM L^{-1} (pre-sham) respectively. These values did not differ significantly from each other ($F_{1,11}$=0.19, P=0.675).

Following Crustastunning the haemolymph L-lactate in the lobsters increased to a mean value of 2.28 ± 0.19 mM L^{-1}, which was significantly greater than the pre-stun value for this group ($F_{1,11}$=50.68, P=0.000).

After the sham treatment the haemolymph L-lactate in the lobsters also increased, with a mean value of 1.85 ± 0.23 mM L^{-1} being obtained. This was also significantly greater than the pre-sham value for this group ($F_{1,11}$=22.36, P=0.001).

The increases of haemolymph L-lactate following the two treatments were compared by considering the changes in the values for individual lobsters, and it was found that the mean increase for the Crustastunned lobsters (1.52 ± 0.18 mM L^{-1}) was not significantly

different from the mean increase for the sham treated lobsters (1.13 ± 0.19 mM L^{-1}) ($F_{1,11}$ = 2.14, P=0.174).

In terms of their subsequent fates, when returned to their holding tanks the stunned lobsters showed either no further visible movements, or in a few cases some transient movements of the mouthpart exopodites and abdominal pleopods, lasting for a few seconds. Thereafter they became immobile and never recovered. However the sham treated lobsters showed normal behaviour when returned to their holding tanks (limb movement, antennule flicking, a ventilation current, pleopod beating and eye retraction reflexes) which continued thereafter. Samples taken from these sham-treated lobsters one week later showed that they had a mean haemolymph L-lactate concentration of 0.75 ± 0.06 mM L^{-1}, which was not significantly different from the pre-sham value of 0.72 ± 0.06 mM L^{-1} for this group ($F_{1,11}$=0.16, P=0.694).

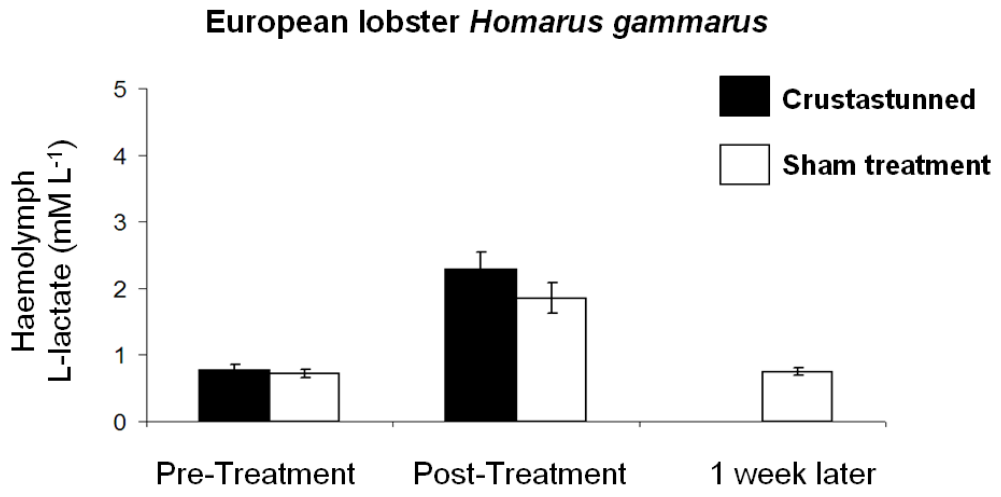

Figure 2. Haemolymph L-lactate concentrations in male European lobsters, *Homarus gammarus*, before and after Crustastunning or sham-treatment. Mean values ± SEM. N=6 for each treatment group.

Discussion

The results obtained for the crab *Cancer pagurus* and for the lobster *Homarus gammarus* were generally similar, with only differences of detail. The main findings were that there was a measurable effect of both Crustastunning and the sham treatment on the haemolymph L-lactate concentrations of both the crab and lobster, but that there was no statistically significant difference between the effects of these two treatments in either species.

Accepting, as outlined in the Introduction, that the haemolymph L-lactate concentration is an appropriate indicator of both acute and chronic stresses in decapod crustaceans, it can be concluded that the animals were stressed to a level above the resting value by both of the imposed treatments. However the fact that the increase in haemolymph L-lactate concentration was not statistically different after Crustastunning than after the sham treatment indicates that the stress imposed during the stunning procedure was in fact no

greater than that induced by the brief emersion (aerial exposure), handling and haemolymph sampling that were common to both treatments. This implies that there was in fact no measurable additional stress due to the electrical stunning process itself.

Having established that the stresses measured in *C. pagurus* and *H. gammarus* undergoing the two procedures can be attributed predominantly to the periods of emersion in combination with handling and blood sampling involved, it becomes relevant to consider what these levels of acute stress represent in absolute terms. This can be judged by considering the values of haemolymph L-lactate obtained here in relation to those obtained in other studies on these and other decapod crustacean species in response to a range of other stresses. Table 1 summarises the results from a number of such studies.

Table 1. Haemolymph L-lactate concentrations measured in decapod crustaceans under various applied stresses.

Species	Stress	Haemolymph L-lactate initially (mM L^{-1})	Haemolymph L-lactate after stress (mM L^{-1})	Reference
Cancer pagurus	Crustastun	0.8	2.6	Present study
Cancer pagurus	Sham	1.1	3.8	Present study
Cancer pagurus	Transport	0.3	10.0	Lorenzon *et al*. (2008)
Cancer pagurus	Simulated transport	3.5	>20.0	Barrento *et al*. (2011)
Homarus gammarus	Crustastun	0.8	2.3	Present study
Homarus gammarus	Sham	0.7	1.9	Present study
Homarus gammarus	Transport	0.4	12.5	Lorenzon *et al.* (2007)
Jasus lalandii	Emersion	1.0	18.5	Haupt *et al.* (2006)
Nephrops norvegicus	Emersion	0.6	19.6	Ridgway *et al.* (2006)
Nephrops norvegicus	Trawling	-	12.0	Albalat *et al.* (2010)
Liocarcinus depurator	Trawling + emersion	-	14.7	Giomi *et al.* (2008)
Orconectes limosus	Emersion	-	19.7	Gäde (1984)
Gecarcoidea natalis	Exercise	0.46	>20.0	Morris *et al.* (2010)

It can be seen that in the species studied here, *C. pagurus* and *H. americanus*, haemolymph L-lactate concentrations can reach much higher values when the animals are exposed to more extreme stresses, such as the emersion and handling associated with transportation, having been at similar initial resting values. Thus values of 10.0 mM L^{-1} have been reported for *C. pagurus* after transportation (Lorenzon *et al.*, 2008), and indeed can reach more than double that value when combined with emersion at elevated temperatures (Barrento *et al.*, 2011, but note higher initial value). Similarly, for *H. gammarus* a mean value of 12.5 mM L^{-1} was obtained by Lorenzon *et al.* (2007) following transportation. From a survey of other stress experiments on a range of decapod crustacean species it can be seen that values of haemolymph L-lactate well in excess of 10.0 mM L^{-1}, and often around 20.0 mM L^{-1}, have been recorded (Table 1). These indicate the possible L-lactate concentrations that can occur in the haemolymph, and so define the range within which the values obtained in the present study lie.

The relative increases in the measures are also relevant. Thus the increases in haemolymph L-lactate concentrations from before to after Crustastunning or sham treatment in the present study represent around a 3.4 fold increase for *C. pagurus* and around a 2.8 fold

increase for *H. gammarus*. These increases are of the order of ten times smaller than those induced by the most extreme stresses.

The return of haemolymph L-lactate concentrations in sham-treated crabs and lobsters to pre-treatment resting levels after one week is as expected, and although the detailed time course of this was not documented, other studies suggest that it would to have taken several hours to subside following the imposed stress (see for example Albalat *et al.*, 2010). In contrast, since Crustastunning killed the animals, it was not relevant to continue measuring haemolymph L-lactate concentrations at later time points. This is because the animals were then in a post-mortem state, and it is known that during this period there is an extensive anaerobic fermentation in the tissues, leading to a rapid production of large amounts of L-lactate (see Figure 3 in Gornik *et al.*, 2008). This highlights the fact that the interpretation of L-lactate data as an indication of stress has to be made with caution, since they can reflect *in vivo* stress or exhaustive exercise, or *post-mortem* processes, depending on the situation.

Conclusions

Consideration of both the absolute values of haemolymph L-lactate that can occur, and the relative increases in them that can be induced by these various stressors, allows the conclusion to be drawn that the handling stresses imposed in the present study by the sham treatment are at the mild end of a spectrum of possible intensities. This is not surprising considering the short duration of the emersion and the careful handling involved, relative to the more prolonged and severe stresses applied in the other cited studies.

What is more unexpected is the finding that the results obtained provide no evidence that the Crustastunning process itself induces any additional measurable stress, beyond that which can be attributed to the emersion and handling involved (as demonstrated by the sham treatment). The reasons for this can only be speculated, but may relate to the almost instantaneous cessation of neuronal (and hence presumably also neuroendocrine) activity in these animals that has been found to occur (see Neil, 2012b), results that are consistent with previous data from other decapod crustaceans (Neil, 2010).

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References

Albalat, A., Sinclair, S., Laurie, J., Taylor, A.C. and Neil, D.M. (2010). Targeting the live market: Recovery of Norway lobsters *Nephrops norvegicus* (L.) from trawl-capture as assessed by stress-related parameters and nucleotide breakdown. Journal of Experimental Marine Biology and Ecology 395, 206–214.

Albert, J.L. and Ellington, W.R. (1985) Patterns of energy-metabolism in the stone crab, *Menippe mercenaria*, during severe hypoxia and subsequent recovery. Journal of Experimental Zoology 234, 175–183.

Aparicio-Simón B., Piñón M., Racotta R. and Racotta, I.S. (2010). Neuroendocrine and metabolic responses of Pacific whiteleg shrimp *Litopenaeus vannamei* exposed to acute handling stress. Aquaculture 298, 308–314.

Barrento, S., Marques, A. Vaz-Pires, P. and Nunes, M.L. (2011). *Cancer pagurus* (Linnaeus, 1758) physiological responses to simulated live transport: Influence of temperature, air exposure and AQUI-S. Journal of Thermal Biology 36, 128–137.

Bergmann M., Taylor, A.C. and Moore, P.G. (2001) Physiological stress in decapod crustaceans (*Munida rugosa* and *Liocarcinus depurator*) discarded in the Clyde *Nephrops* fishery. Journal of Experimental Marine Biology and Ecology 259,215–229.

Chang, E.S. (2005). Stressed-Out Lobsters: Crustacean Hyperglycemic Hormone and Stress Proteins. Integrative and Comparative Biology 45, 43–50.

Chung, J.S., Dircksen, H. and Webster, S.G. (1999). A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*, Proceedings of the National. Academy of Science USA 96, 13103–13107.

De Wachter, B., Sartoris, F-J and Pörtner, H-O. (1997). The anaerobic endproduct lactate has a behavioural and metabolic signalling function in the shore crab C*arcinus maenas.* The Journal of Experimental Biology 200, 1015–1024.

Fanjul-Moles, M.L. (2006). Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: Review and update. Comparative Biochemistry and Physiology, Part C 142, 390–400.

Gäde G. (1984). Effects of oxygen deprivation during anoxia and muscular work on the energy metabolism of the crayfish *Orconectes limosus*. Comparative Biochemistry and Physiology A 77, 495–502.

Giomi, F., Raicevich, S., Giovanardi, O., Pranovi, F., Di Muro P. and Beltramini, M. (2008). Catch me in winter! Seasonal variation in air temperature severely enhances physiological stress and mortality of species subjected to sorting operations and discarded during annual fishing activities. Hydrobiologia 606, 195–202.

Glowik, R.M., Golowasch, J., Keller, R. and Marder, E. (1997). D-glucose-sensitive neurosecretory cells of the crab *Cancer borealis* and negative feedback regulation of blood glucose level. The Journal of Experimental Biology. 200, 1421–1431.

Gornik, S.G., Albalat, A., Atkinson, R.J.A., Coombs, G.H. and Neil, D.M. (2008). Early post-mortem processes in the abdominal muscle of the Norway lobster (*Nephrops norvegicus*). Marine and Freshwater Behaviour and Physiology 41, 241–256.

Haupt, P., Brouwer, S. L., Banch, G. M. and Gäde, G. (2006). Effects of exposure to air on the escape behaviour and haemolymph chemistry of the South African Cape Lobster *Jasus lalandii*. Fisheries Research 81: 210–218.

Keller, R. and Orth, H.P., 1990. Hyperglycemic neuropeptides in crustaceans. Progress in Clinical and Biological Research 342, 265–271.

Keller R., Haylett B. and Cooke, I. (1994). Neurosecretion of crustacean hyperglycemic hormone evoked by axonal stimulation or elevation of saline K+ concentration quantified by a sensitive immunoassay method. The Journal of Experimental Biology 188, 293–316.

Lorenzon, S. (2005). Hyperglycemic stress response in Crustacea. Invertebrate Survival Journal 2, 132-141.

Lorenzon, S., Giulianini, P.G and Ferrero, E.A. (1997). Lipopolysaccharide-induced hyperglycemia is mediated by CHH release in crustaceans, General and Comparative Endocrinology 108 395–405.

Lorenzon, S., Edomi, P., Giulianini, P.G., Mettulio, R. and Ferrero, E.A. (2004). Variation of crustacean hyperglycemic hormone (CHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress, The Journal of Experimental Biology.207, 4205–4213.

Lorenzon, S., Giulianini, P.G., Martinis, M. and Ferrero, E.A. (2007). Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology147, 94–102.

Lorenzon, S., Giulianini, P.G., Libralato, S., Martinis, M. and Ferrero, E.A. (2008). Stress effect of two different transport systems on the physiological profiles of the crab *Cancer pagurus*. Aquaculture 278,156–163.

Morris, S., U. Postel, U., Mrinalini, Turner, L.M., Palmer, J. and Webster, S.G. (2010). The adaptive significance of crustacean hyperglycaemic hormone (CHH) in daily and seasonal migratory activities of the Christmas Island red crab *Gecarcoidea natalis*, The Journal of Experimental Biology 213, 3062–3073.

Neil, D.M. (2010). The effect of the Crustastun™ on nerve activity in crabs and lobsters. Scientific Report to Studham Technologies.

Neil, D.M. (2012a). Ensuring crustacean product quality in the post-harvest phase. Journal of Invertebrate Pathology 110, 267-275.

Neil, D.M. (2012b). The effect of the Crustastun™ on nerve activity in the brown crab *Cancer pagurus* and the European lobster *Homarus gammarus*. Scientific Report to Studham Technologies.

Paterson, B.D. and Spanoghe, P.T. (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.

Patterson, L., Dick, J.T.A. and Elwood, R.W. (2007). Physiological stress responses in the edible crab, *Cancer pagurus*, to the fishery practice of de-clawing. Marine Biology 152, 265–272.

Ridgway, I.D., Taylor, A.C., Atkinson, R.J.A., Stentiford, G.D., Chang, E.S., Chang, S.A. and Neil, D.M. (2006). Morbidity and mortality in Norway lobsters, *Nephrops norvegicus*: physiological, immunological and pathological effects of aerial exposure. Journal of Experimental Marine Biology and Ecology 328, 251–264.

Robb, D. (1999). The humane slaughter of Crustacea: Electrical stunning. Department of Food Animal Science, University of Bristol: Langford, UK. Unpublished research report.

Roth, B. and Øines, S. (2010). Stunning and killing of edible crabs (*Cancer pagurus*). Animal Welfare 19, 287-294.

Santos, E.A. and Keller, R. (1993a). Regulation of circulating levels of the crustacean hyperglycemic hormone-evidence of a dual feedback control system. Journal of Comparative Physiology 163A, 374–379.

Santos, E.A. and Keller, R. (1993b). Effect of exposure to atmospheric air on blood glucose and lactate concentration in two crustacean species: a role of the crustacean hyperglycemic hormone (CHH). Comparative Biochemistry and Physiology A 106, 343–347.

Santos, E.A., Keller, R., Rodríguez, E. and López, L. (2001). Effects of serotonin and fluoxetine on blood glucose regulation in two decapod species. Brazilian. Journal of . Medical and Biological Research 34, 75–80.

Sedlmeier, D., 1982. The mode of action of the crustacean neurosecretory hyperglycemic hormone (CHH): II. Involvement of glycogen synthase. General and Comparative Endocrinology 47, 426–432.

Sedlmeier, D. (1988). The crustacean hyperglycemic hormone (CHH) releases amylase from the crayfish midgut gland. Regulatory. Peptides 20, 91–98.

Spanings-Pierrot, C., Soyez, D., Van Herp, F., Gompel, M., Skaret, G., Grousset, E. and Charmantier, G. (2000). Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab Pachygrapsus marmoratus. General and Comparative Endocrinology 119, 340–350.

Sparrey J. (2005). Testing of Crustastun single crab and lobster stunner. Unpublished research report.

Stentiford, G.D., Chang, E.S., Chang, S.A. and Neil, D.M. (2001). Carbohydrate dynamics and the crustacean hyperglycemic hormone (CHH): Effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*), General and Comparative Endocrinology 121, 13–22.

Toullec J.Y., Vinh J., Le Caer J.P., Shillito B. and Soyez D. (2002). Structure and phylogeny of the crustacean hyperglycemic hormone and its precursor from a hydrothermal vent crustacean: the crab *Bythograea thermydron*. Peptides 23, 31–42.

Verri, T., Mandal, A., Zilli, L., Bossa, D., Mandal, P.K., Ingrosso, L., Zonno, V., Vilella, S., Ahearn, G.A. and Storelli, C. (2001) D-Glucose transport in decapod crustacean hepatopancreas. Comparative Biochemistry and Physiology 130, 585–606

Webster, S.G. (1996). Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. The Journal of Experimental Biology 199, 1579–1585.

Webster, S.G., Keller, R. and Dircksen, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. General and Comparative Endocrinology 175, 217–233.

Wendelaar-Bonga, S.E. (1997). The stress response in fish. Physiological Reviews 77, 591–625.

Zou, H.S., Juan, C.C., Chen, S.C., Wang, H.Y. and Lee, C.Y. (2003). Dopaminergic regulation of crustacean hyperglycemic hormone and glucose levels in the hemolymph of the crayfish *Procambarus clarkii*. Journal of Experimental Zoology A 298, 44–52.