Van:
Verzonden: dinsdag 8 oktober 2013 20:52
Aan:
Onderwerp: RE:

Ik denk dat je inderdaad mijn eerdere reactie op rapporten over de Crusta stun kunt gebruiken.

From:
Sent: dinsdag, oktober 08, 2013 11:21
To:
Subject:

Hoi,
Ik kan denk ik het meeste wat je eerder aan info hebt gestuurd gebruiken voor het beantwoorden van deze brief he?
heb je eventueel nog aanvullende opmerkingen?
alvast bedankt.

Van:
Verzonden: woensdag 24 april 2013 0:17
Aan:
Onderwerp: FW: antwoorden rond vragen bedwelmen krabben
Bijlagen:

Laten we elkaar nog even over de rapporten bellen.

Groet,

From:
Sent: dinsdag, april 23, 2013 15:54

Subject: RE: antwoorden rond vragen bedwelmen krabben

Van
Verzonden: dinsdag 23 april 2013 13:49
Aan:
Onderwerp: RE: antwoorden rond vragen bedwelmen krabben

De samenvatting van de literatuur geeft weer hoe het in diverse landen in Europa toegaat. Er is bij mijn weten geen regelgeving in het VK.
De methoden die bekend zijn, heb ik beschreven.

3

Van:
Verzonden: dinsdag 23 april 2013 10:46
Aan:
Onderwerp: antwoorden rond vragen bedwelmen krabben

Hierbij ontvang je mijn antwoorden.

EFSA rapport
In het EFSA rapport over het doden van dieren in experimenteel onderzoek, wordt het koken van krabben en kreeften ontraden.

The EFSA Journal (2005) 292, 1-46 - Opinion on the "Aspects of the biology and welfare of animals used for experimental and other scientific purposes"

Crusta stun
Over de werking van de Crusta stun is onvoldoende bekend om hierover gefundeerde uitspraken te kunnen doen.

Groet,

10

Van:
Verzonden: maandag 8 april 2013 17:11
Aan:
Onderwerp: aangehouden motie doden van kreeften en krabben

tijdens de behandeling Wet Dieren heeft de PvdD een motie aangehouden waarin verzocht werd om het levend komen van krabben en kreeften te gaan verbieden omsdat er een methode zou bestaan: de crustastun, ontwikkeld door Bristol.

Please consider the environment before printing this e-mail

Senior Policy Advisor Animal Welfare
tel: 00-31-

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Bezuidenhoutseweg 73
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13

The effect of the Crustastun™ on nerve activity in crabs and lobsters

A report by

Professor Douglas Neil

University of Glasgow

July 2010

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Web site of the Langoustine Lab: http://www.gla.ac.uk/langoustinelab/

Introduction

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). A previous report from this laboratory (Albalat *et al.*, 2008) evaluated the flesh quality of langoustines after being killed by the Crustastun.

The present report summarises the results obtained in a number of trials carried out to determine the effect of the Crustastun machine on activity in the nervous system of a typical crab (the shore crab *Carcinus maenas*) and a typical lobster (the Norway lobster or langoustine *Nephrops norvegicus*). On the basis of these results, conclusions have been drawn about the effects of Crustastun usage on the neuronal functioning in these and similar crustaceans.

Aims and objectives

The aims of this study were to use appropriate electrophysiological techniques to record from both the central nervous system and the peripheral nervous system of crabs and lobsters, in order to compare intact animals with those that have been subjected to 'Crustastunning'.

The specific objectives were:

1. To monitor intrinsic and evoked neuronal activity emerging from the 'brain' (supra-oesophageal ganglion) of crabs and lobsters by making extracellular recordings in the circumoesophageal connectives, the main nerves conveying information to and from the brain. This would include making recordings in the head (cephalothorax) of the lobster after isolating it from the tail (abdomen)

2. To monitor intrinsic neuronal activity in the ventral nerve cord of lobsters by making extracellular recordings from neurones in the abdominal ventral nerve cord. This would include making recordings in the tail (abdomen) of the lobster after isolating it from the head (cephalothorax).

3. To record intrinsic activity in the motor nerves leaving the abdominal nerve cord of the lobster to supply the abdominal postural muscles, by making extracellular recordings from the appropriate motor nerves (3rd abdominal roots).

4. To demonstrate evoked motor activity by measuring the muscle forces produced by the activation of the motor neurones supplying a muscle spanning a specific leg segment (the closer muscle of the dactylopodite) in crabs.

5. To determine the sensory activity in the leg nerves of crabs in response to stimulation of specific receptor types: mechanoreceptors in the cuticle (eg. cuticular hairs, campaniform sensillae) and proprioceptors spanning the leg segments internally (chordotonal organs).

These tests were designed to allow the following questions to be addressed, namely, after 'Crustastunning':

- Does any activity continue to be generated spontaneously in the central nervous systems of crabs and lobsters, and if so are its characteristics altered from normal?
- Does any activity, either spontaneous or evoked, remain in the motor and neuromuscular systems of the animals, and if so are their characteristics altered from normal?
- Does any activity remain in the sensory nerves from peripheral mechanosensory organs of the animals, and if so are its characteristics altered from normal?

Anatomy

Decapod crustaceans, the taxonomic group to which crabs and lobsters belong, have nervous systems with the characteristic arthropod plan (Brusca and Brusca, 2002). This involves a ladder-like arrangement of paired nerve cords, with a dorsal brain (supraoeophageal ganglia) separate circumoesophageal connectives and segmental ganglia in the thorax and (if present) in the abdomen, from which nerves arise to supply the segmentally-arranged muscles and sense organs. Lobsters exemplify all these features (Figure 1) whereas in crabs a distinct abdomen has been lost and the thoracic ganglia are condensed into a single thoracic mass, from which all the peripheral nerve roots emerge (Figure 2).

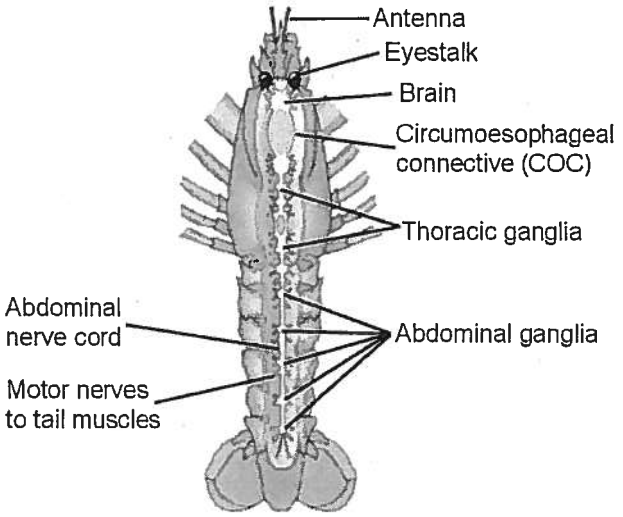

Figure 1. The arrangement of the nervous system in a clawed lobster such as the European lobster *Homarus gammarus* of the Norway lobster *Nephrops norvegicus* .

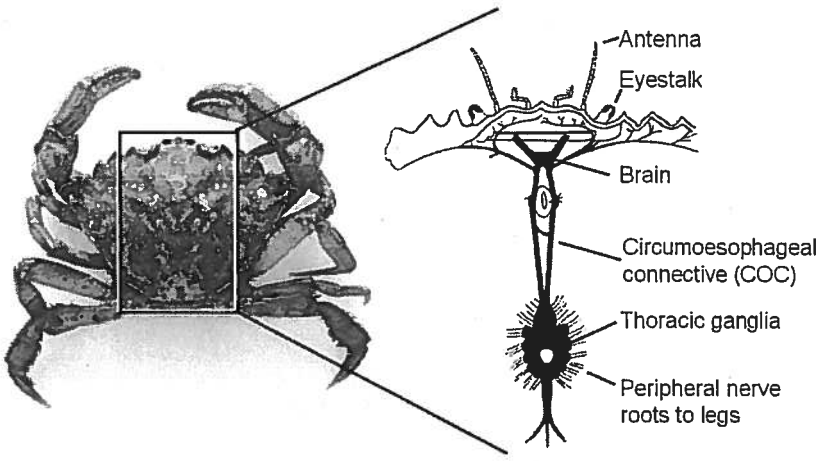

Figure 2. The arrangement of the nervous system in a crab such as the shore crab *Carcinus maenas* .

Each of the four pairs of walking legs (pereiopods) of crabs comprises a series of articulated segments, which are moved by paired muscles (Figure 3). A number of different mechanoreceptors are associated with the leg exoskeleton, including 'funnel canal organs' (a type of campaniform sensilla) which are pressure-sensitive (Libersat, 1987), and innervated cuticular sensory hairs which signal contact and water movement (Garm, 2005). In addition, a series of elastic strands span the various joints, into which are embedded sensory cells which detect joint flexion and extension (Bush, 1965). These so-called chordotonal organs thus act as proprioceptors monitoring the leg movements made by the crab (Hartman *et al.*, 1997). The chordotonal organ spanning the terminal leg segment, between the propopodite and the dactylopodite (the PD chordotonal organ) was selectively activated in this study. The branches (axons) of both the motor and the sensory nerves pass in a mixed leg nerve that travels through the centre of the leg segments.

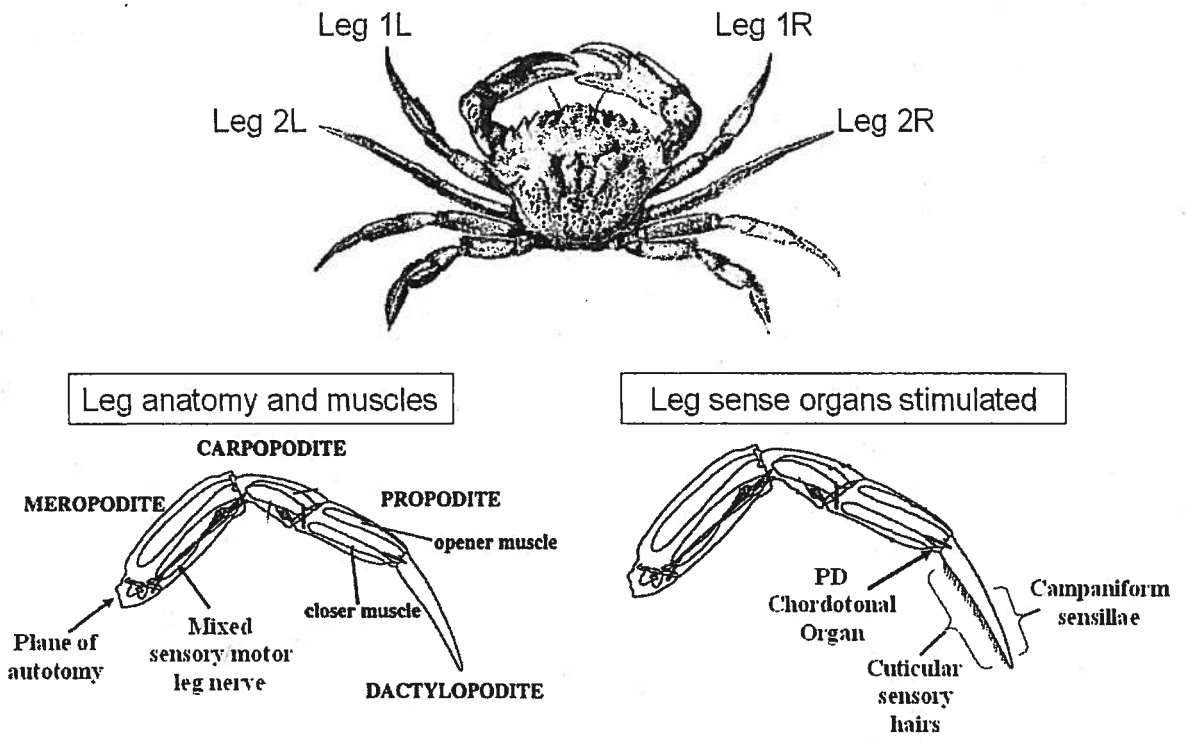

Figure 3. The anatomy of the legs of the crab *Carcinus maenas*, including the arrangement of the muscles and sense organs.

Materials and Methods

Animal supply and holding

All animals used in these tests were obtained live from commercial suppliers (UMBSM Animal Supply Service Millport and Loch Fyne Sea Farms Ltd.) and were retained within a closed seawater circulating system for at least one week before experimentation. Intact and alert animals were held on ice for 30 min immediately prior to the experimental procedures, in order to reduce their metabolic rate.

Crustastunning

The 'Crustastunning' 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 stunned by a 110 volt, 2-5 amp electrical charge for 10 s immediately after removing them from the holding aquaria.

Exposing the nervous systems

In order to expose the central nervous system of the crab for recording, the carapace was removed and the preparation was submerged in a balanced salt solution corresponding in composition and osmolarity to crab haemolymph, at a temperature of 10°C. The internal organs were then removed or displaced in order to expose the circumoesophageal connectives around the base of the stomach. A similar procedure was employed for the lobster, but prior to this the cephalothorax was separated from the abdomen.

To expose the abdominal ventral nerve cord of the lobster for recording, after separating the abdomen from the cephalothorax the dorsal skeletal plates (terga) were detached, and the bulk of the underlying deep flexor musculature was removed. The preparation was then submerged in a balanced salt solution corresponding in composition and osmolarity to lobster haemolymph, at a temperature of 10°C. Selective removal of muscle blocks then revealed the motor roots emerging from the ventral nerve cord.

In order to expose the leg nerve of crabs for recording and stimulating, the leg was first detached from the body of an intact crab by applying pressure to the basipodite segment, which caused the animal to shed its leg naturally by the process of autotomy (McVean, 1976), or by amputation in a stunned crab. The joint between the meropodite and carpopidite (M-C) was then disarticulated, and the muscle tendons spanning this joint were cut with fine scissors. The leg was separated gently at this point, revealing the leg nerve still attached to the distal portion. This isolated leg preparation was submerged in balanced salt solution at a temperature of 10°C until required, and remained viable for many hours.

Electrophysiological recordings

Electrophysiological recordings were made from the exposed nerves using various extracellular techniques. For recording from the circumoesophageal connectives of crabs and lobsters, and from the ventral nerve cord of lobsters, a suction electrode method was used. A fine-tipped polythene electrode containing salt solution was applied to the surface of the nerve, and a gentle suction was applied through attached tubing and a syringe. A silver wire positioned close to the tip of the electrode acted as the indifferent (reference) electrode. Such a recording configuration is termed 'en passant', as it involves attaching the suction electrode to an intact nerve, allowing both directions of nerve transmission to be recorded. However, in some cases the circumoesophageal connective was cut and the electrode was attached to either its anterior or posterior cut end. In this way the presence of active neurones transmitting information in ascending or descending directions could be ascertained.

For recording from the crab leg nerve, the isolated leg was clamped to a Perspex plate and the nerve was passed from an adjacent bath through a wall of petroleum jelly into a second small chamber, both of which contained balanced salt solution (Figure 4, upper panel). A bipolar electrode of two silver wires was used to make contact with the solutions in the inner and outer chambers respectively.

In each case the signals from the extracellular electrodes were passed to a differential pre-amplifier (A101, Isleworth Ltd.) for amplification and filtering. The amplifier output was then passed to an Analog/Digital converter (PowerLab, AD Instruments Ltd) and was both displayed and recorded on a standard PC computer using the associated software (Chart v7, AD Instruments Ltd.)

Stimulating the nerves

To stimulate the motor axons in the crab leg nerve, the bipolar electrodes were connected to an isolated stimulator within the PowerLab (Figure 4, lower panel) and patterns of stimulating pulses at various amplitudes and frequencies were applied using a software 'stimulator control panel' within the Chart v7 software. Typically, stimulus trains of 3 s duration and 10V amplitude were applied at a range of frequencies from 1 – 100 Hz.

Recording muscle force

Although leg nerve stimulation potentially activated motor neurons supplying all of the muscles located more distally in the leg, the forces produced by the closer muscle of the Propopodite/Carpopopodite joint (P-D) were nevertheless recorded selectively. This was achieved by cutting the tendon of the antagonist muscle about that joint (the P-D opener muscle), and then attaching a thread from near the tip of the dactylopodite to the arm of a sensitive force transducer (FT-03, Grass Instruments Ltd.), mounted on a micromanipulator (Figure 4, lower panel). This selectively monitored the forces produced by the dactylopodite closer muscle. The output of the transducer was passed to a custom-

built amplifier (x1000), and then fed to an input of the Powerlab A/D converter. The forces and the stimulus parameters were then both displayed and recorded on a standard PC computer using the Chart v7 software.

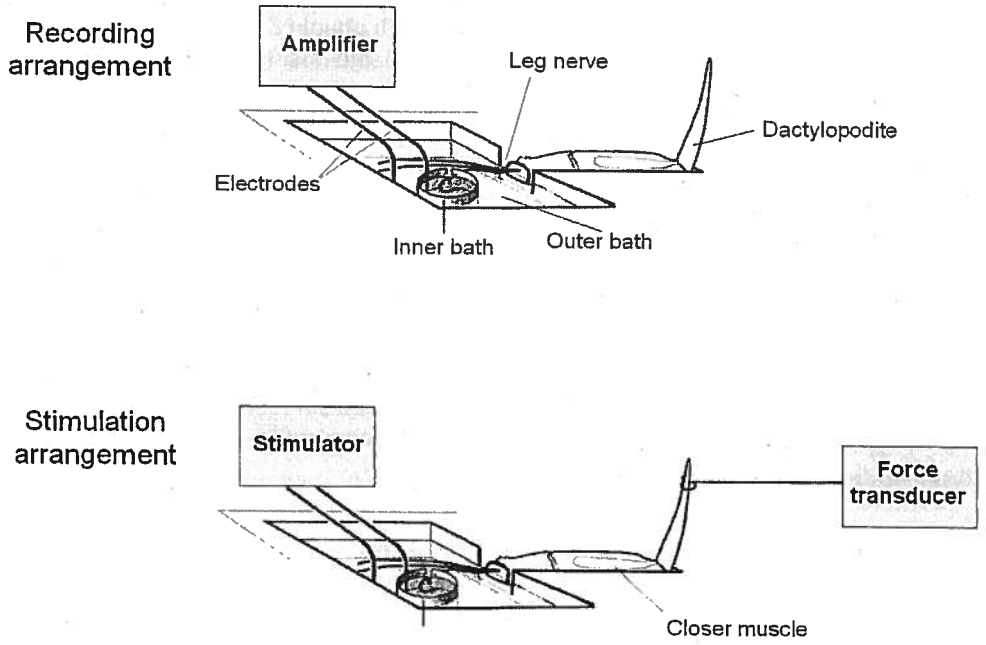

Figure 4. Experimental arrangements for recording from the leg nerve of an autotomised crab leg (upper panel) and for stimulating the crab leg nerve while recording the forces produced by the dactylopodite closer muscle (lower panel).

Results

The Crustastunning procedure was applied to 6 crabs and 6 lobsters, and the same number of intact animals was used as controls. The data are presented as traces of the original electrophysiological recordings and where appropriate also as plots of the muscle forces produced in relation to stimulus parameters. Table 1 (on p. 17) summarises the results obtained.

Activity in the nervous system of intact crabs and lobsters

Recordings made from one or both circumoesophageal connectives in intact crabs indicated that there was a high level of spontaneous neuronal activity passing along the axons of this nerve, even in the absence of any imposed stimulation (Figures 5 and 6). Due to the variety of sizes of the extracellularly-recorded spikes, it can also be concluded

that the signals arose from a large number of different individual nerve axons, of varying diameters.

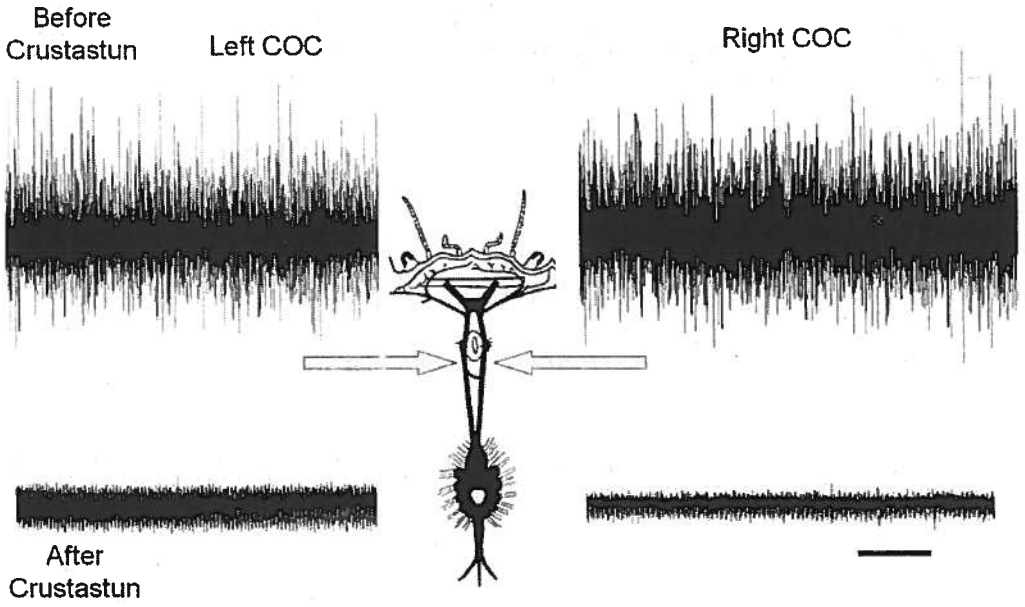

Figure 5. Spontaneous nerve activity recorded extracellularly in the left and right circumoesophageql connectives (COCs) of a shore crab, *Carcinus maenas*. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

When tactile stimuli were applied to the eyestalks or antennae, there were systematic changes in firing frequency in some of these axons, indicating that these were conveying descending activity from the brain. There were also high frequency bursts of activity that corresponded to the animal making struggling movements (fictive locomotion) (Figure 6), although the direction of transmission of this activity was not discernable in en passant recordings. However, recordings made from the cut ends of the circumoesophageal connectives have indicated that spontaneous activity comprises both ascending and descending nerve transmission (data not shown).

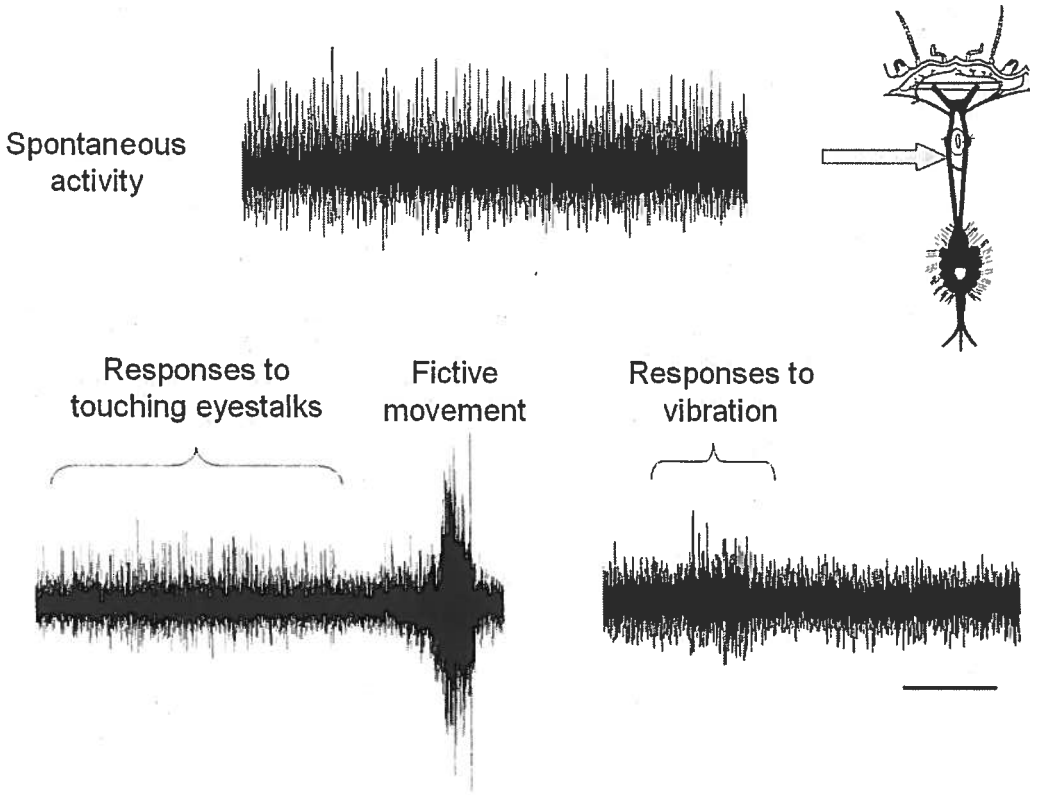

Figure 6. Nerve activity recorded extracellularly in the circumoesophageql connectives of an intact crab. Upper panel, spontaneous activity; left lower panel, responses to touching eyestalks and a burst associated with fictive locomotion; right lower panel, responses to vibration. Scale bar 2.5 s.

Recordings from the circumoesophageal connectives of intact Norway lobsters provided essentially the same results, even when the cephalothorax was detached from the abdomen, with a high level of neuronal activity passing along the axons of this nerve (Figure 7, upper panel).

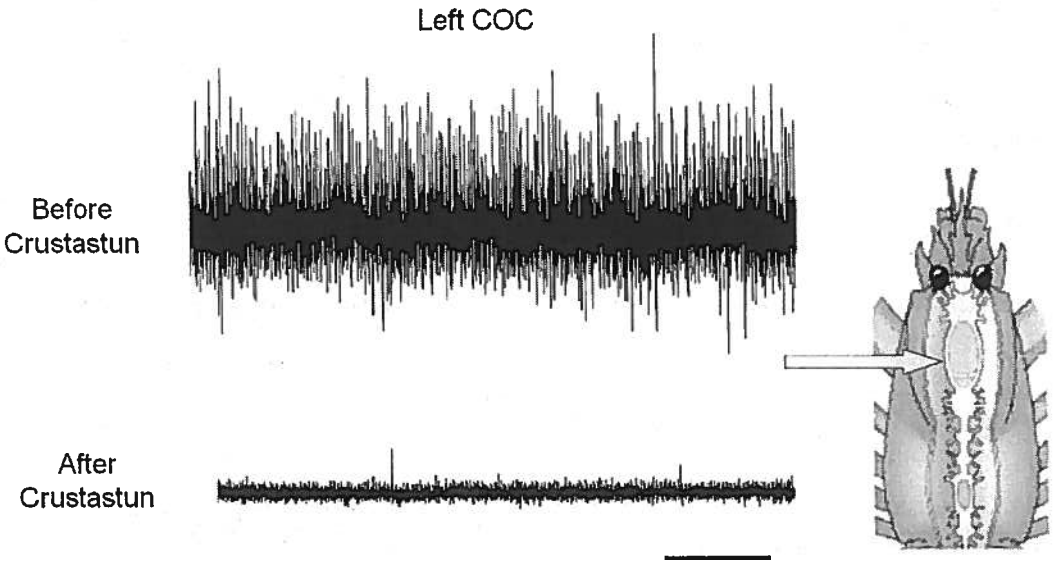

Figure 7. Spontaneous nerve activity recorded extracellularly in the left circumoesophageal connective (COC) of a Norway lobster, *Nephrops norvegicus*. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

Recordings from the abdominal nerve cord of the intact Norway lobsters also encountered spontaneous nerve activity in all cases, even when the abdomen was detached from the cephalothorax (Figure 8).

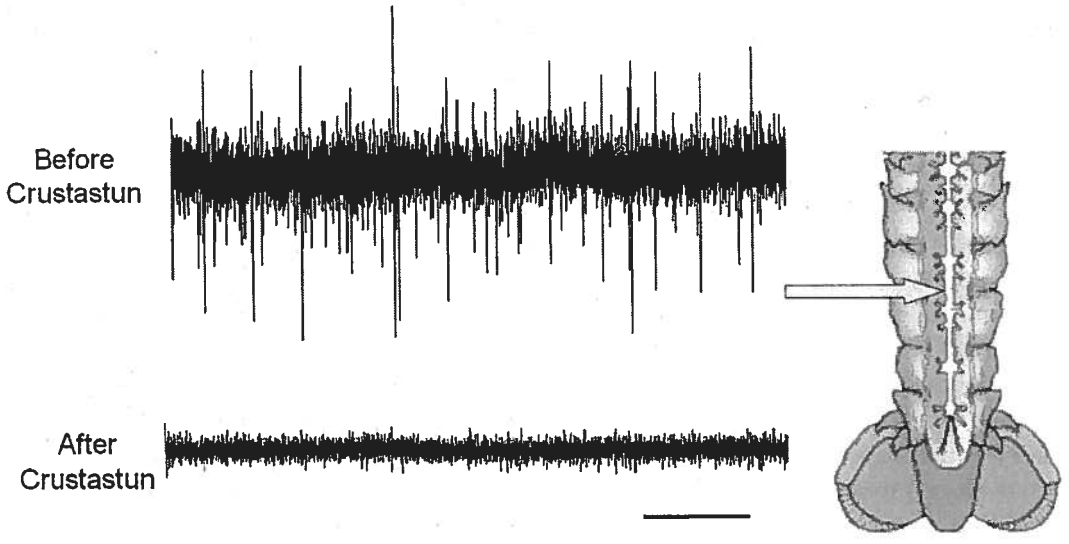

Figure 8. Spontaneous nerve activity recorded extracellularly in the abdominal nerve cord of a Norway lobster. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

Moreover, patterned activity involving a number of motor neurons (represented by different spike sizes) was detectable in all the motor roots emerging from the ventral nerve cord that were surveyed (Figure. 9). This represents evidence for the action of the peripheral nervous system in intact animals, contributing to the generation of muscle tone in the abdominal muscles.

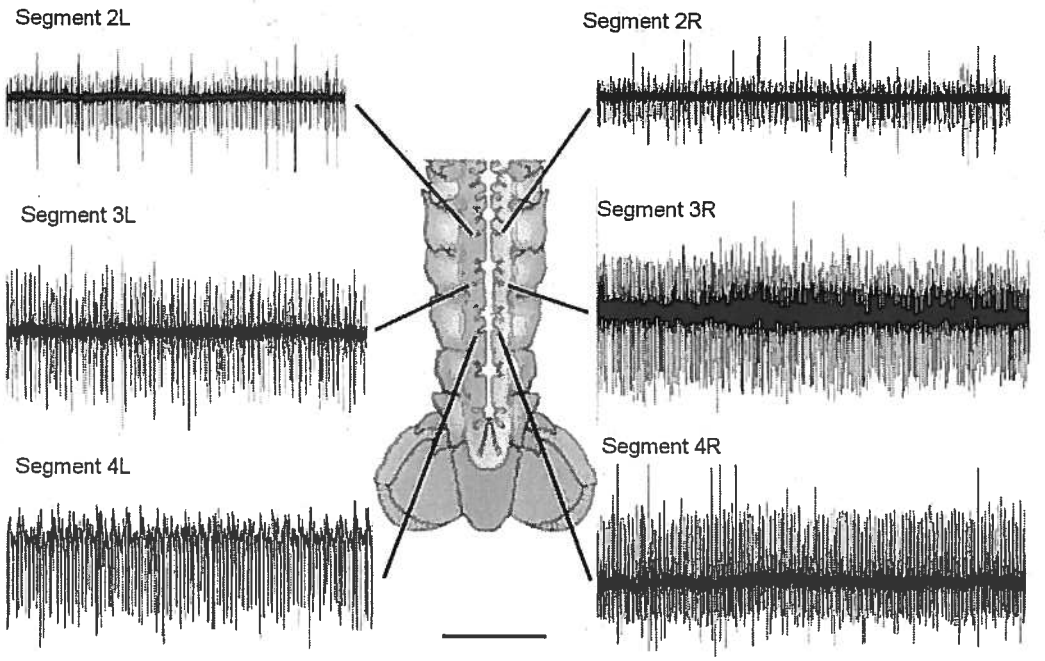

Figure 9. Spontaneous nerve activity recorded extracellularly from motor neurones in the 3rd motor roots of the abdominal nerve cord of an intact Norway lobster. The panels are from the roots indicated. Scale bar 1s.

Further evidence for activity in the peripheral nervous system in intact animals was obtained from the recordings made on the isolated legs of intact crab, following autotomy. Examples from two crabs are presented in Figures 10 and 11. The leg nerve contains a mixture of the axons of sensory and motor neurons, and the application of various stimuli to the distal part of the leg clearly elicited activity in a number of sensory neurons. These patterns of activity were typical for the various sense organs that were stimulated in each case. Thus the responses to the movement and displacement phases of flexions and extensions applied at the P-D joint had characteristic phasic and tonic elements (Figures 10 & 11, left panels). Compression (squeezing) of the cuticle of the dactylopodite elicited persistent tonic responses for the duration of the stimulus (Figures 10 & 11, centre panels). Brushing movements over the cuticle of the dactylopodite produced bursts of activity typical of the responses to displacement of cuticular sensory hairs (Figures10 & 11, right panels).

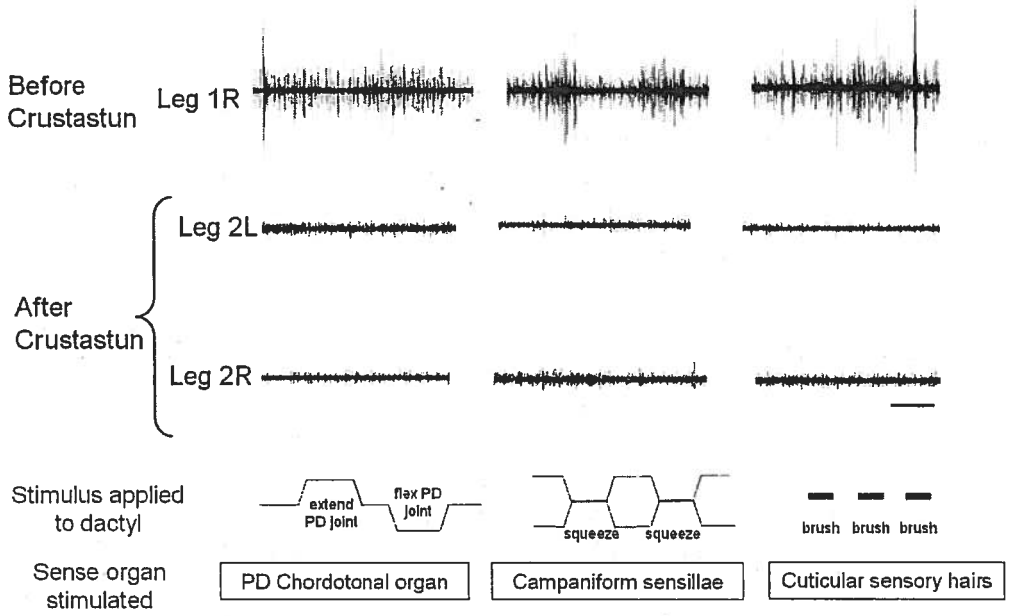

Figure 10. Responses of crab leg nerve to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower two panels, legs autotomised from an animal after Crustastunning. Scale bar 1 s.

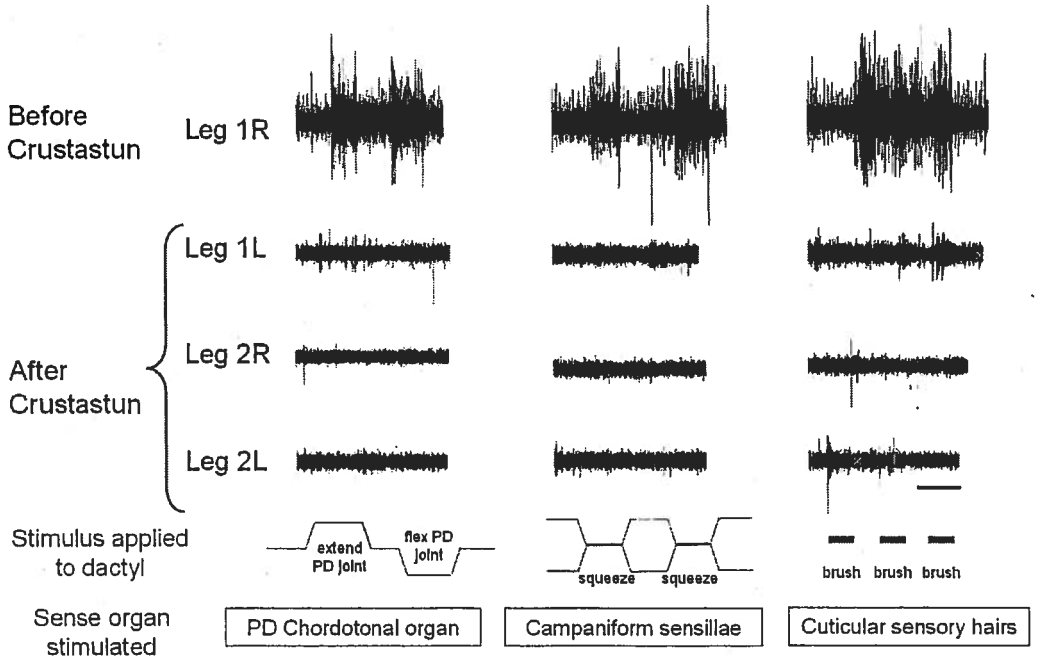

Figure 11. Responses of crab leg nerves in a different preparation to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower three panels, legs autotomised from an animal after Crustastunning. Scale bar 1 s.

Normal operation of the neuromuscular motor pathways in the intact crab was demonstrated by stimulating the leg nerve of an autotomised leg at a range of frequencies while monitoring the force produced by the dactylopodite closer muscle. The force varied in a non-linear frequency-dependent manner that is typical of crustacean neuromuscular systems due to their synaptic properties of summation and facilitation (Figure 12). Mean values obtained form a series of trials on 16 autotomised legs demonstrate this relationship (Figure 13).

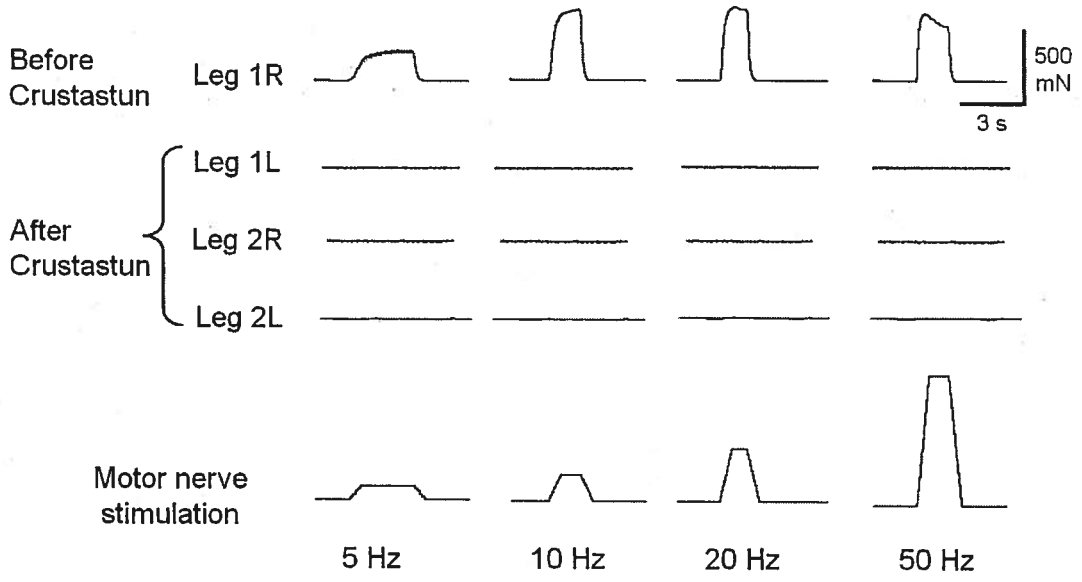

Figure 12. Forces produced by the dactylopodite closer muscle of the crab leg in response to stimulation of the leg nerve at various frequencies. Top panels, leg autotomised from intact crab; lower three panels, legs amputated from the same crab after Crustastunning. Stimulus voltage 10V.

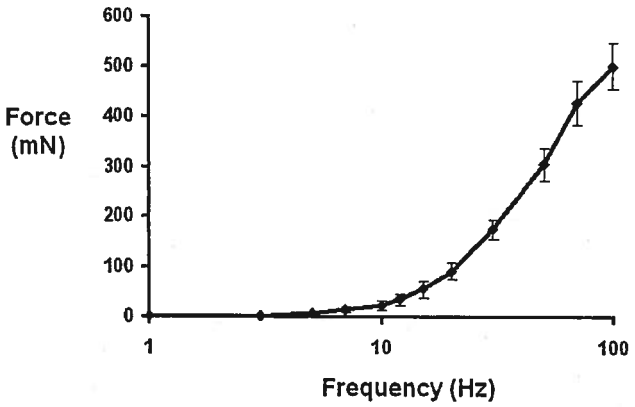

Figure 13. Forces produced by the dactylopodite closer muscle of intact crabs in relation to the frequency of stimulation (at 10V, 3 s duration). Mean ± S.E. values, N = 16 legs.

In order to test the persistence of activity in the nervous systems of intact crabs and lobsters, some preparations were re-tested at intervals of up to several hours. Activity persisted for up to the longest time tested (6 hours) in both their central nervous systems and in the nerves of automised legs (data not shown). A similar persistence was observed when a number of legs that were automised from an intact crab at the same time were held for differing periods of time before being prepared for recording (Figure 14). The sensory responses obtained at 4 h after autotomy were just as strong as those recorded immediately after autotomy.

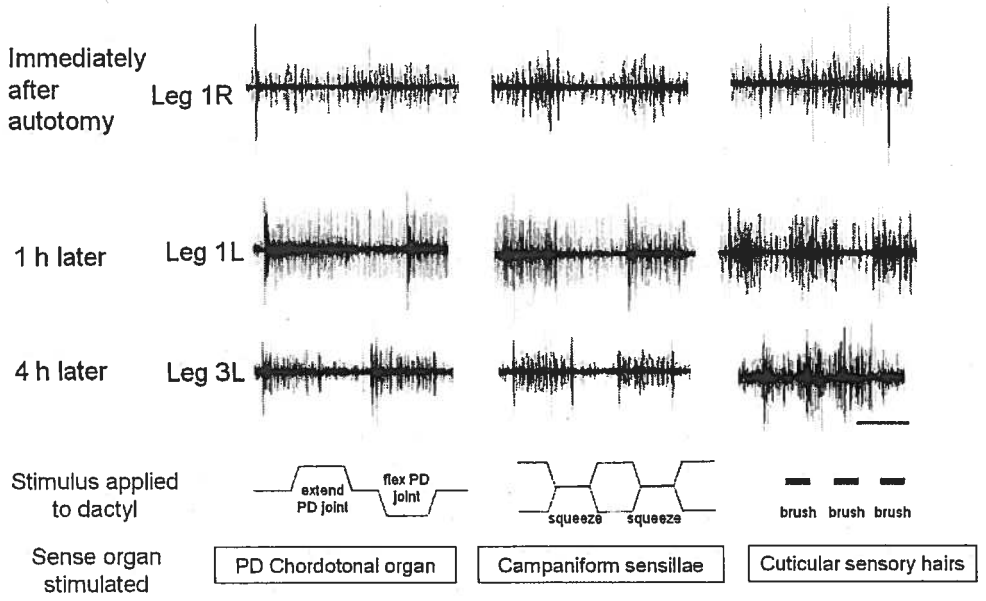

Figure 14. Responses of crab leg nerves from intact crab to three forms of stimulation of the dactylopodite at different times after autotomy. Top panels, immediately after autotomy; middle panels, 1 h after autotomy; bottom panels, 4 h after autotomy. Scale bar 1 s.

Activity in the nervous system of crabs and lobsters following Crustastunning

After Crustastunning there were no visible signs of movement in the body or appendages of the crabs and lobsters. The abdomen of the lobster was strongly flexed and lacked compliancy.

Recordings from the central nervous systems of crabs and lobsters that had been subjected to Crustastunning indicated that no neuronal activity was detectable in the circumoesophageal connectives in any of the individual animals tested of either species (Figures 5 and 7, lower panels). The abdominal nerve cords of the Crustastunned lobsters were also silent, with no indication of spontaneous neuronal activity (Figure 8, lower panel). As expected, due to this lack of central nervous system activity, there was no corresponding motor activity in the abdominal motor nerve roots of these Crustastunned lobsters (Figure 15), which contrasts with the responses obtained in an intact lobster (see Figure 9).

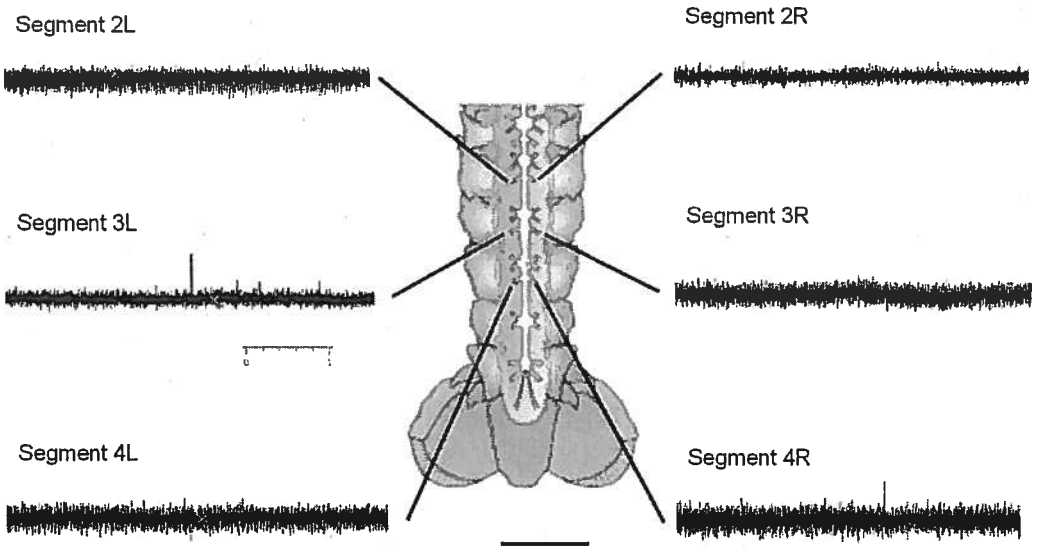

Figure 15. Spontaneous nerve activity recorded extracellularly from motor neurones in the 3rd motor roots of the abdominal nerve cord of a Norway lobster after Crustastunning. The panels are from the roots indicated. Scale bar 1s.

The recordings from the leg nerves of the Crustastunned crabs provided a means of testing whether the peripheral system retained any ability to convey neuronal information, even though the central nervous system might be silent. However, in virtually all the legs tested there were neither sensory responses to the three stimuli applied (Figures 10 and 11, lower panels) nor muscle force development in response to stimulating motor nerves (Figure 12 lower panels).

An exception to this was found in two individual legs from two different Crustastunned crabs. In these cases there was evidence of some recovery of neuronal responsiveness in the leg nerve over a period of minutes after Crustastunning both to sensory stimuli and to evoked motor activity resulting in muscle force development. Figures 16 and 17 show examples of this in the records obtained from one crab. In each case, as here, only one leg of the several tested from individual crabs displayed this recovery of responsiveness, while the other legs from those individuals showed no responsiveness.

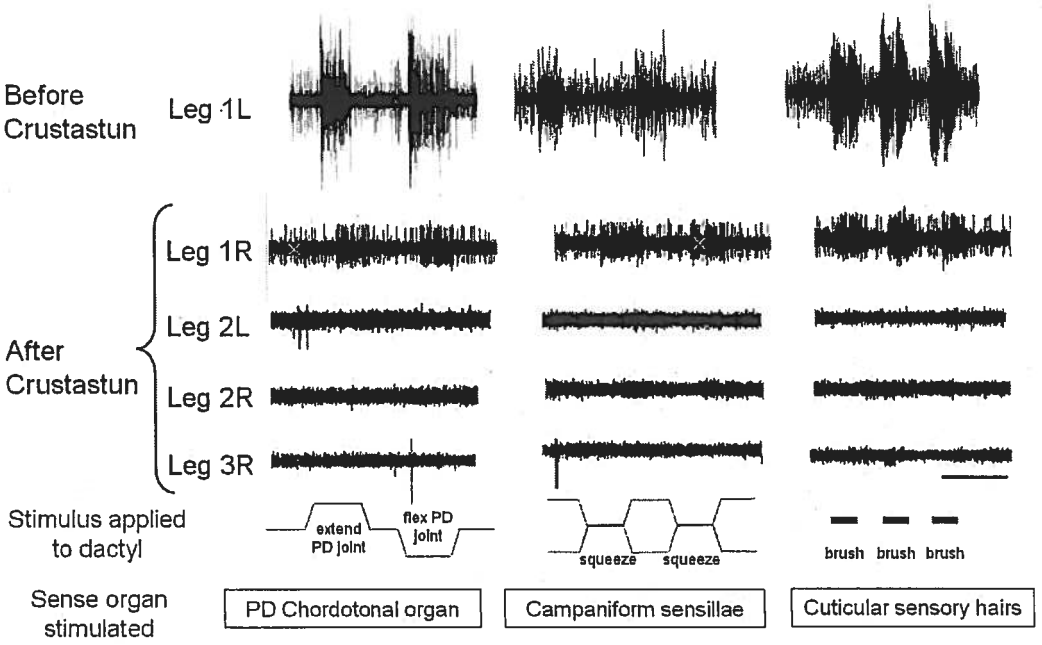

Figure 16. Responses of crab leg nerves to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower three panels, legs autotomised from an animal after Crustastunning. Note the continued responsiveness of the leg nerve from leg 1R, while the other legs show no responses. Scale bar 1 s.

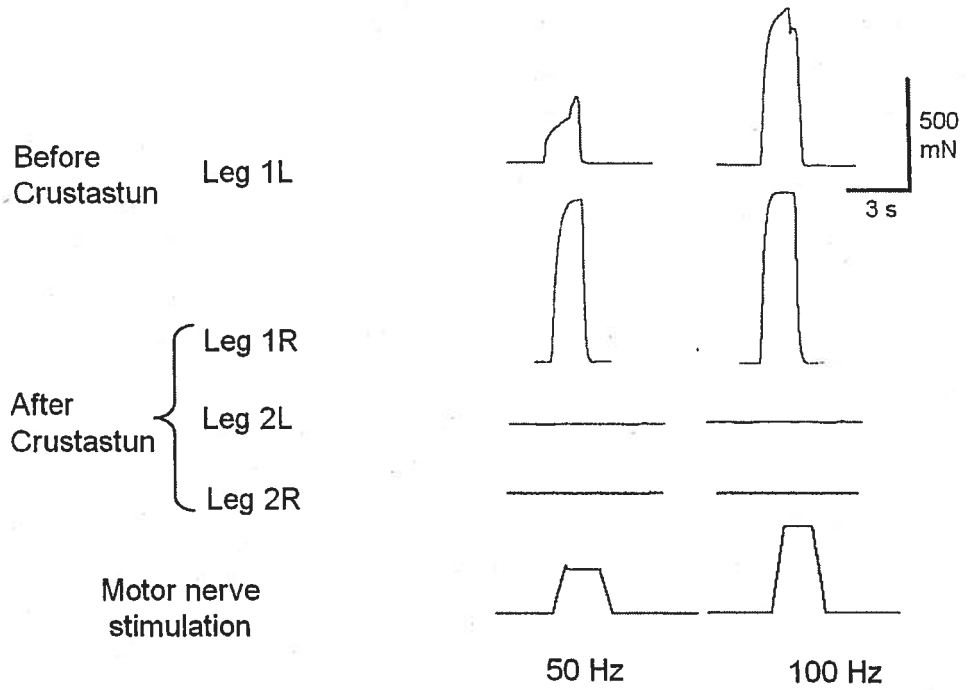

Figure 17. Forces produced by the dactylopodite closer muscle of the crab leg in response to stimulation of the leg nerve at various frequencies. Top panels, leg autotomised from intact crab; lower three panels, legs autotomised from the same crab after Crustastunning. Note the continued development of force by leg 1R, while the other legs produce no force. .Stimulus voltage 10V.

The results of the complete set of trials are summarized in Table 1. These trials were performed on a total of 6 individual animals for each treatment, and in the case of autotomised crab legs three legs per individual were tested.

Table 1. Summary of responses recorded in the nervous systems of intact and Crustastunned crabs and lobsters. Values represent numbers of animals (or autotomised legs) responding as a ratio of the number tested. n/a, not applicable.

	Intact Crab	Crustastunned Crab	Intact Lobster	Crustastunned Lobster
Spontaneous activity in Circumoesophageal Connectives	**6/6**	**0/6**	**6/6**	**0/6**
Spontaneous activity in the Ventral Nerve Cord	n/a	n/a	**6/6**	**0/6**
Spontaneous activity in the Abdominal Motor Roots	n/a	n/a	**6/6**	**0/6**
Sensory responses in leg nerve of autotomised leg	**18/18**	**2/18**	n/a	n/a
Evoked force in P-D closer muscle of autotomised leg	**18/18**	**2/18**	n/a	n/a

Discussion and Conclusions

Activity in the nervous systems

The results obtained here are consistent with the literature on the neurophysiology of crustacean nervous systems (see, for example, the articles in Wiese, 2002) in showing that the central nervous systems of crabs and lobsters display continuous nerve activity, which in turn produces outputs in the motor nerves to the body and limb muscles. A large body of evidence, including studies conducted in this laboratory (Chachri et al., 1994; Holmes et al, 2002), indicates that this activity persists even when parts of the CNS are isolated from each other by severing the nerve cord at one or more levels (Larimer and Moore, 2003). Even isolated single ganglia of the abdominal nerve cord can produce patterned outputs (e.g. Chachri and Neil, 1993), and there is an extensive literature on the most-studied ganglion that can continue to operate in isolation, the stomatogastric ganglion (reviewed by Marder and Bucher, 2007).

It is therefore not surprising to have found in the present study that, as a result of dissection or of detaching the cephalothorax from the abdomen, nerve activity continues to be recorded in the isolated anterior or posterior portions of the body, even though the nerve cord is transected at one or more levels. Also, as expected, this activity includes both descending signals from the brain and ascending signals from more posterior parts of the nervous system.

Although not attempted in these trials, it is without doubt that any procedure that attempted to make a sagittal section through a crab or lobster, in an attempt to destroy the entire nervous system, would inevitably leave small sections untouched and sufficiently intact to be able to continue generating patterned nerve activity, and to respond to sensory stimulation with reflex outputs localized to the muscles in the segments still innervated.

A characteristic feature that is common in these isolated parts of the nervous system is the long-lived nature of continued activity and signal conduction. It is widely reported that, provided the structures are bathed in an appropriate solution, activity can continue for many hours, and indeed this was observed in the present study both with the central nervous preparations and with the isolated crab legs after autotomy. Such robustness makes it easier to interpret any loss of activity following an intervention as due to the intervention itself, rather than to any underlying decline in nervous system responsiveness.

The effects of Crustatunning

The findings obtained on the effect of Crustastunning on nerve activity in crabs and lobsters are relatively conclusive. As far as can be determined from the extracellular recording method used, the various forms of spontaneous activity within the central nervous system are completely arrested. Consistent with this, there are no outputs produced in the motor nerves supplying the abdominal muscles of lobsters, which are known to be synaptically driven from neurones in the CNS.

The recordings made on isolated crab legs allow some further conclusions to be drawn, namely that Crustastunning also has an effect on the functioning of the peripheral parts of the nervous system. There is both a loss of responsiveness to all three types of sensory stimulation, and also a failure in neuromuscular activation. The first of these effects renders the animals insensitive to external stimuli, while the second renders them paralysed and incapable of making movements. Thus it has been found that as a result of Crustastunning the nervous system is incapacitated simultaneously at two levels, i.e. both centrally and peripherally, which completely prevents all normal neuronal functioning.

In terms of identifying the reasons for recording no sensory signals or inducing no motor activity in the peripheral nervous system, the recording method used does not allow definitive conclusions to be made. It is indeed possible that the conduction processes in the axons of both the sensory and motor neurones have been disrupted by the electrical currents generated by the Crustastun. However, it cannot be excluded that the Crustastunning has affected only the sensory transduction processes in the receptor endings of the sense organs, rather than the nerve transmission mechanism in the sensory nerves. Similarly, Crustastunning may have destroyed synaptic transmission at the neuromuscular junctions, and/or excitation-contraction coupling processes within the muscle fibres, rather than the nerve transmission mechanism in the motor nerves. It is of course possible that all of these processes have been affected simultaneously. To distinguish between these possibilities would require an examination of each of the contributing processes by using other, more appropriate, electrophysiological methods in a targeted approach.

The fact that neuronal integrity in the crab leg nerve persisted after Crustastunning in two instances requires an explanation. It involved both continued responsiveness to sensory stimulation and continued ability to produce motor activation of muscle contraction in response to artificial stimulation. In each case it involved only one of the legs, while the others from the same crab showed complete unresponsiveness. It is possible that in these cases one appendage was positioned within the Crustastun machine in such a way that the electrical current did not pass through it as completely as it did through the body of the animal or through the other legs. It is therefore suggested that the pathways of current flow through the animals when subjected to Crustastunning are examined in detail, and recommendations made for positioning the animal and its limbs to eliminate the possibility of such a failure to inactivate the limb nerves.

However, in terms of the consequences for neuronal action, it should be noted that the central nervous systems of animals involved in these two cases were silenced by the Crustastunning, and so despite the fact that sensory information may have reached the thoracic ganglion, this would have produced no neuronal reaction in the CNS. Likewise, although the motor pathways to the muscles were still competent, no motor commands would have been generated by the CNS to initiate movement.

Scope of conclusions

The interpretations of the results obtained in this study of the effects of Crustastunning apply most directly to the two species used (the crab *Carcinus maenas* and the Norway lobster *Nephrops norvegicus*). However, due to their virtually identical anatomies and physiologies, there is no reason to believe that similar results would not be obtained in other commercially-important UK species such as the edible brown crab, *Cancer pagurus* and the European lobster *Homarus gammarus,* or indeed in other species commonly supplied live to the restaurant trade, such as the American lobster *Homarus americanus* or rock lobster species such as *Palinurus elephas, Panulirus argus* or *Jasus lalandii.* Confirmation of this could only be obtained, however, by directly testing them in a similar manner to that described here.

Acknowledgements

This work was carried out by Professor Douglas Neil as a Consultancy Project for Studham Technologies Ltd. The technical assistance of Mr Graham Adam is acknowledged.

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14

Evaluation of the quality of Langoustines after being killed by the Crustastun

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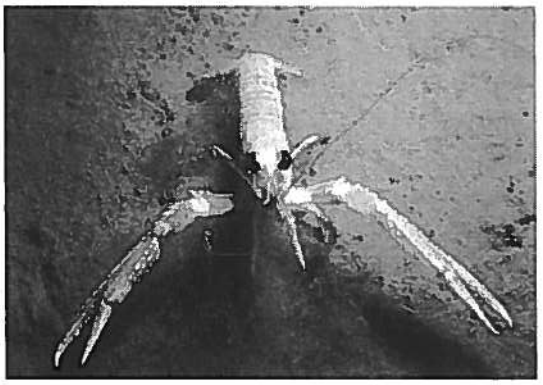
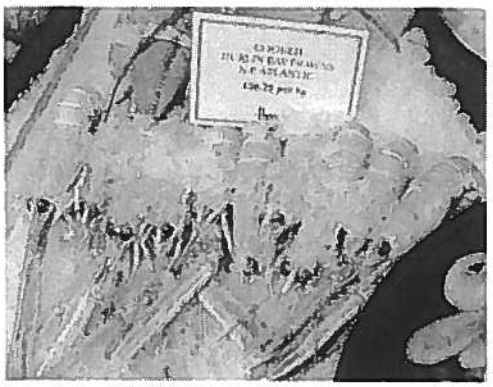
May 2008





University of Glasgow | Faculty of Biomedical & Life Sciences

Evaluation of the quality of Langoustines after being killed by the Crustastun

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This report is provided in confidence to Mr Simon Buckhaven, Crustastun, Greystones, 53 Church Road, Studham, Bedfordshire, LU6 2QD.

May 2008

Introduction

The Crustastun is a device designed to administer a lethal electric shock to shellfish (such as lobsters, crabs, and crayfish) before cooking, to avoid boiling a live shellfish (**www.crustastun.com**). It works by applying a 110 volt, 2-5 amp electrical charge to a shellfish.

It is stated by Simon Buckhaven, the inventor of the CrustaStun, that it is not only a fast and efficient way of killing a lobster, but that as it reduces the stress that the animals goes through, and also the quality of the meat in terms of texture and flavour are improved.

An evaluation of the effects of using the Crustastun to kill langoustines (*Nephrops norvegicus* (L.)) on established quality measures has been performed using a set of assays developed by the Langoustine Lab at the University of Glasgow. For comparison, langoustines killed by holding them continuously on ice have also been evaluated.

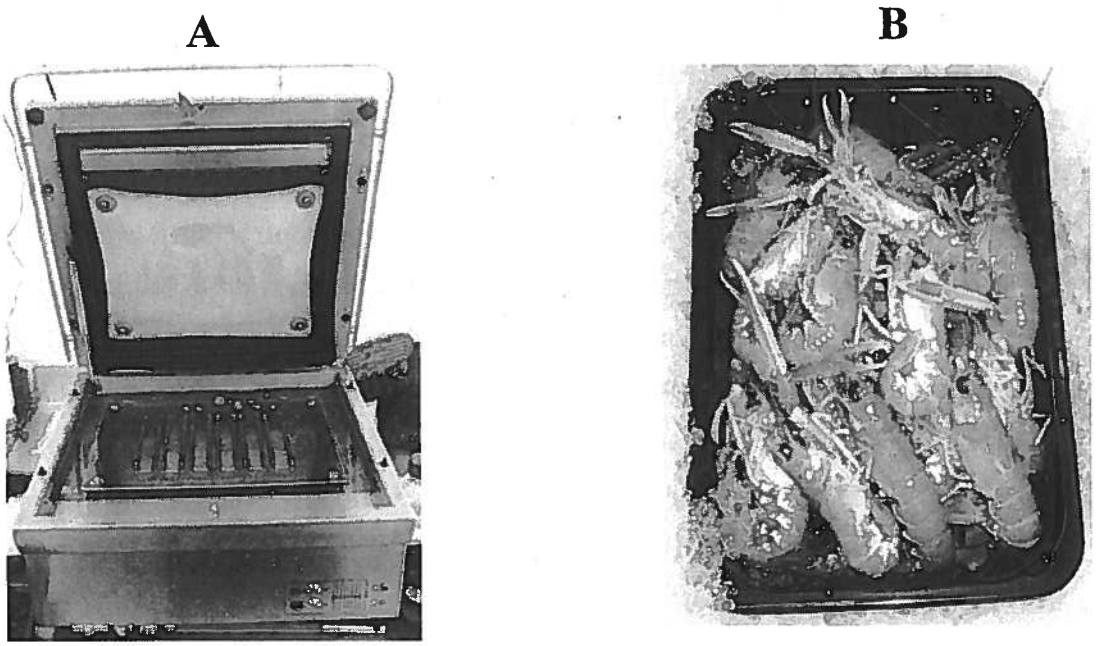

Figure 1. **A.** The Crustastun Prototype I. **B.** Langoustines on ice after being electrocuted in the Crustastun.

Methods

Capture, holding and transportation

Langoustines (*Nephrops norvegicus*) were caught by otter trawl in the Clyde Sea area on two occasions, (the 17/08/2007 and 23/10/2007) by the vessel RV Aplysia. Once on board, animals were held in running seawater and arrived to the University Marine Biological Station Millport (UMBSM) within 4 hours, at which time they were still alive and vigorous.

The Crustastun process

From the trawl catch obtained on 23/10/07, once in the UMBSM, a group of 30 animals was killed using the Crustastun Prototype I machine with factory settings (110 volt, 2-5 amp delivered for 5s – lobster symbol). Seven cycles of operation were performed with 4-5 animals per cycle. Before and after this procedure, both photographs and thermal images (Fluke Ti20 thermal imager) of the animals were taken, and the temperature of the brine in the Crustastun chamber was recorded. In addition, samples of the brine solution were taken from the chamber before use, and also after 7 cycles of operation, for analysis of bacterial content.

Quality assessments

From the trawl catch obtained on 17/08/07, once in the UMBSM, animals were separated into 2 groups (each of 50-60 animals). One group of animals was killed using the Crustastun Prototype I machine with factory settings (110 volt, 2-5 amp delivered for 5s – lobster symbol). Twelve cycles of operation were performed with 4-5 animals per cycle. The other (Control) group of animals was placed directly on ice. Ten samples of tail meat were taken from animals immediately after being Crustastun-killed, and the same number of samples was taken simultaneously from animals of the ice-killed group ("fresh"). After that, all the remaining animals from the two groups were transported on ice to the University of Glasgow where they were kept at a temperature of 0-2°C for up to 7 days. Further assessments were made and samples of tail meat were taken on days 1, 3, 5 and 7. At each time point some or all of the following parameters were measured:

- Visual assessment of the animals
- Melanosis score
- Measurement of the nucleotide breakdown products (ATP, ADP, AMP, IMP, INO, HX)
- The K-value (a freshness indicator) calculated the ratio of nucleotide breakdown products
- The pH of the meat
- The bacterial load in the meat (measured as total bacteria counts, H_2S producers, luminescent bacteria and *Pseudomonas* sp. bacteria)
- The nitrogenous breakdown product trimethylamine oxide (TMA)
- From the trawl catch of 23/10/07, samples from Day 1 from both the Crustastun-killed and the Ice-killed groups were frozen, and at a later date were assessed by an independent sensory panel trained previously to evaluate the sensory properties of langoustine meat.

The principles and methods of the quality assays

Visual Assessment

A visual assessment was used that is based on five different parameters (appearance of head; appearance of claws; appearance of upper-side of tail; appearance of under-side of tail; odour), each of which is allocated four demerit scores. The Tables used for the visual assessment of the animals are presented in the Appendix 1.

Melanosis score

Melanosis (also known as enzymatic browning or blackspot syndrome) causes a blackening of food produce surfaces, shells and membranes and affects fruits, vegetables and seafood. In seafood, melanosis occurs primarily in crustaceans and is a major problem during post-harvest storage. The blackening is caused by the action of the enzyme polyphenol oxidase (PPO). Several stress-related factors can activate the PPO enzyme, such as the capture process, rough handling and in general 'traumatic' events (Bartolo and Birk, 1998). Moreover, once melanosis is triggered and becomes established several factors affect the rate of blackening, such as pH and

temperature. The Tables used for the scoring of melanosis in langoustines are presented in the Appendix 2.

Measurement of the nucleotide breakdown products

The amounts of the nucleotide breakdown products ATP, ADP, AMP, IMP, INO and HX in langoustine tail meat samples were determined using high performance liquid chromatography (HPLC). A supernatant of muscle homogenate was extracted, and analysed by HPLC following a protocol modified by Ryder (1985) using a reverse-phase column.

Freshness Index (K-value)

The K-value is one of the most useful indicators to evaluate fish and shellfish freshness (Connell, 1980). Strong correlations between sensory loss of freshness and increase in K-value have been found in many fish species. The K-value is calculated from the concentrations of ATP, the main metabolic energy source in muscle, and its breakdown products. The degradation of ATP follows a general pathway:

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{INO} \rightarrow \text{Hx}$$

The first steps of this degradation are catalyzed by endogenous tissue enzymes (autolytic phase) while further stages are slower and besides tissue enzymes they also involve bacteria (bacterial phase). Taken together, the concentrations of all these compounds make it possible to calculate the K-index or K-value, as described first by Saito (1959) and later by Ryder (1985):

$$\text{K- index} = \frac{[\text{Ino+Hx}]}{[\text{ATP+ADP+AMP+IMP+Ino+Hx}]}$$

The higher the value of the K-index, the greater the loss of freshness. In several fish species a K-value of 20% has been set as a freshness limit, although this index is species-dependent and therefore does not apply to all fish species. The K-index is widely used today; for example it is a standard fish freshness index in Japan. Furthermore, some of these compounds are related to different tastes. In this sense,

IMP has been described to give a nice, meaty taste to food and fish in particular while Hx has been attributed to give a bitter-off taste to fish products. In many fish species these changes correlate well with sensory assessment.

The pH of the meat

Low values of muscle pH in live or early post-mortem stages indicate ante-mortem exhaustion of reserves and gradual formation of anoxic conditions in the muscle. Under these conditions, an accumulation of lactic acid and other acidic products as a result of glycolysis (the breakdown of glycogen) produces low pH values. In this study, the pH of langoustine tail meat samples was determined from muscle homogenates using a standard semi-micro pH electrode (Jenway).

The bacterial load in the meat

To count the number of marine (psychtrophic) bacteria in langoustine tail meat, samples of the tail meat with added sterile sea water containing bacterial peptone were homogenized, serially diluted and plated onto marine agar plates with added marine broth (for total marine bacteria) or with added iron (for H_2S-producing bacteria). Plates were incubated at 20°C for 48h, and the colonies were counted and converted to values of colony forming units per gram (cfu g^{-1}). For H_2S-producing bacteria, the total of black colonies on the iron agar plates was determined. The number of luminous bacteria strains was also determined by counting the number of luminous colonies on the MIA plates in a dark room after 48 h. For the determination of *Pseudomonas* sp., an agar with an added selective 'CFC' supplement was used.

The nitrogenous breakdown product trimethylamine oxide (TMA)

Decomposition of some non-protein nitrogenous compounds during post-mortem metabolism causes undesirable properties such as the loss of freshness and the development of putrefaction. Among these compounds is TMA, a volatile odorous

compound that gives a fishy or ammonia-like smell to raw fish products. Many bacteria are capable of producing TMA that comes from the reduction of TMAO. In this study, the TMA in samples of langoustine tail meat was determined using the method of Dyer (1959) as modified by Stroud *et al.* (1982). Seven different biogenic amines (putrescine, cadaverine, histamine, tyramine, agmatine, tryptamine and spermidine) were also analysed, although the results are not presented in this report.

Sensory Evaluation

In order to assess if the Crustastun had any impact on cooked langoustine tail meat, an independent sensory assessment was performed in the Food Innovation Institute at the Midlothian Innovation Centre, Edinburgh. A trained Panel performed the Quantitative Descriptive Analysis (QDA) method to profile each langoustine sample in terms of its sensory attributes related to aroma, appearance, texture and flavour. For each sensory attribute of this QDA test, a two-anchored linear scale (0-10) was used, in which the score of 5 is the mid-point. This scale is objective and has nothing to do with the like or dislike of a given panelist. The trained Panel was also asked to provide a subjective score for their 'degree of like or dislike' to obtain the 'overall liking' on a linear scale (0-10). On this scale, 0 equals extremely disliked, 10 equals extremely liked and 5 equals the mid-point.

Samples from animals killed with the Crustastun or killed on ice were frozen on Day 1 and then stored at -22°C until they were sent on dry ice to the Sensory Testing Laboratory. Sample preparation involved thawing the animals, and boiling the tails for 3 minutes to ensure core temperature of 75°C (in compliance with EU regulations) and then peeling the tails to obtain the meat. Sensory evaluation sessions were carried out by a trained professional panel of ten members, and all tests were conducted and all the data were gathered and analysed using a specialised computer software package (FIZZ) and Microsoft Excel. For information on the FIZZ specialised software for sensory evaluation see http://www.biosystemes.com.

Results

The Crustastun process

The Crustastun process (110 volt, 2-5 amp delivered for 5s – lobster symbol) was found to reliably kill all the animals (up to 5) placed in the chamber, as judged by the fact that they showed no further body or limb movements after treatment. The stunning process was found in most cases to induce a flexure of the tail (Figure 2), presumably due the occurrence of tetanic contraction of the tail flexor muscles.

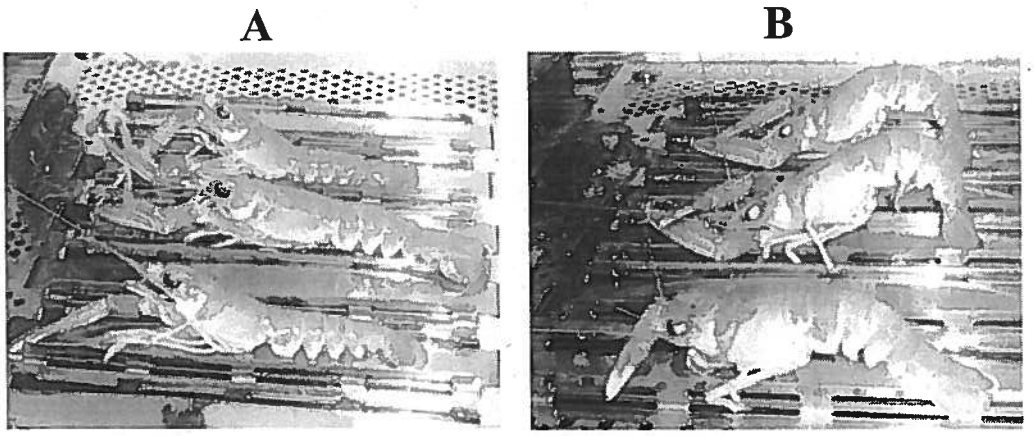

Figure 2. **A.** Three live langoustines mounted in the Crustastun chamber. **B.** The same three langoustines immediately after the Crustastun process

Before being Crustastun-killed, the animals landed on 23/10/2007 had body temperatures of 12.5-13.0°C, and the brine in the Crustastun chamber had a temperature of 15-16°C. After being electrocuted, the temperature of the animals increased significantly, especially in the region of dorsal cephalothorax or "head" (the part of the body that would have been in contact with the upper electrode in the lid) (Figure 3). The temperature of the dorsal surface of the cepahalothorax reached around 24-27°C on average (with maximum temperatures measured of around 30°C). In some cases (Figure 4) the Crustastun process also resulted in an increase in the temperature of the dorsal side of the abdomen ("tail"), but such increases were never as pronounced (the highest recorded being around 22°C) and in many cases tails were not affected at all. Thermal images of the transverse section of the cephalothorax (Figure 5) reveal the thermal gradient from the dorsal surface to the ventral surface. This indicates that it is predominantly the upper side of the cephalothorax that is

affected by the increase in temperature, while the ventral side suffered practically no change.

After 7 cycles of operation, in which a total of 30 animals were killed, the temperature of the brine in the Crustastun chamber increased by only 1°C (from 16°C to 17°C), However, it is probable that this temperature increase would have been larger if more animals had been Crustastun-killed over a larger number of cycles, and/or over a shorter period of time or if ambient air temperature had been higher.

A) Before

B) After

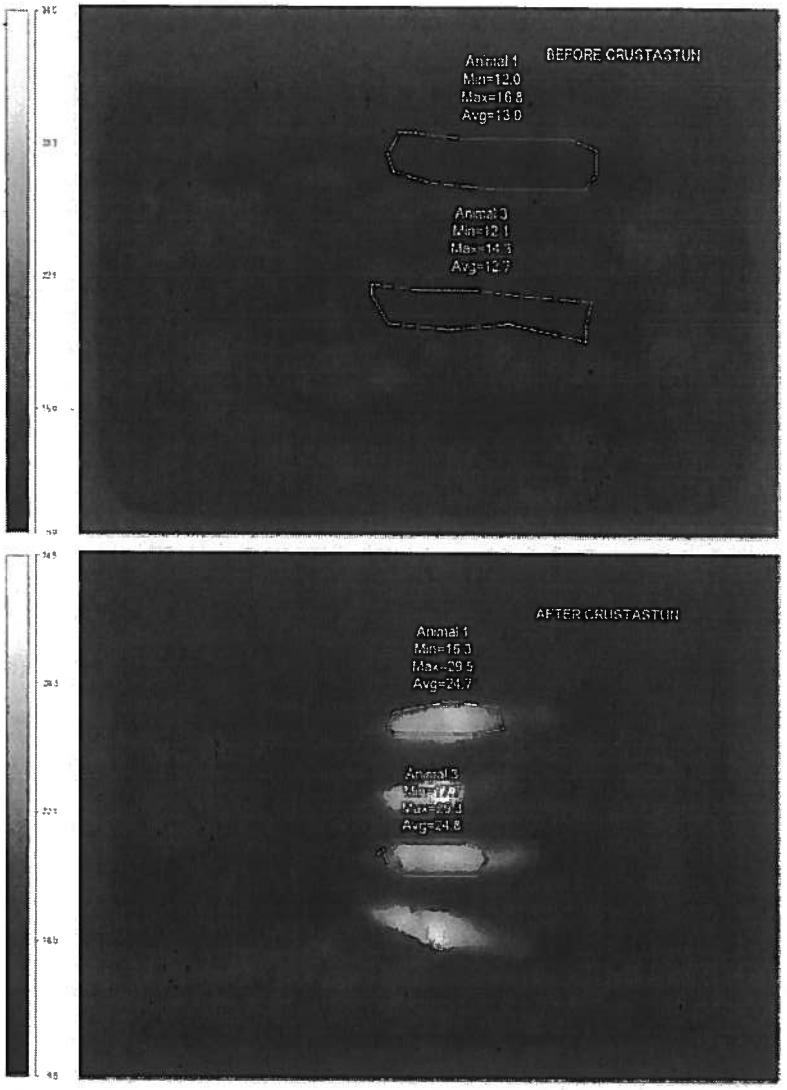

Figure 3. Thermal images of a group of four langoustines A) before and B) after being killed using the standard Crustastun procedure. Values refer to the outlined areas.

Figure 4. Thermal images of a second group of four langoustines after being killed using the Crustastun machine. In this case there was some change in the temperature of at least two of the tails. Values refer to the outlined areas.

A) Before **B) After**

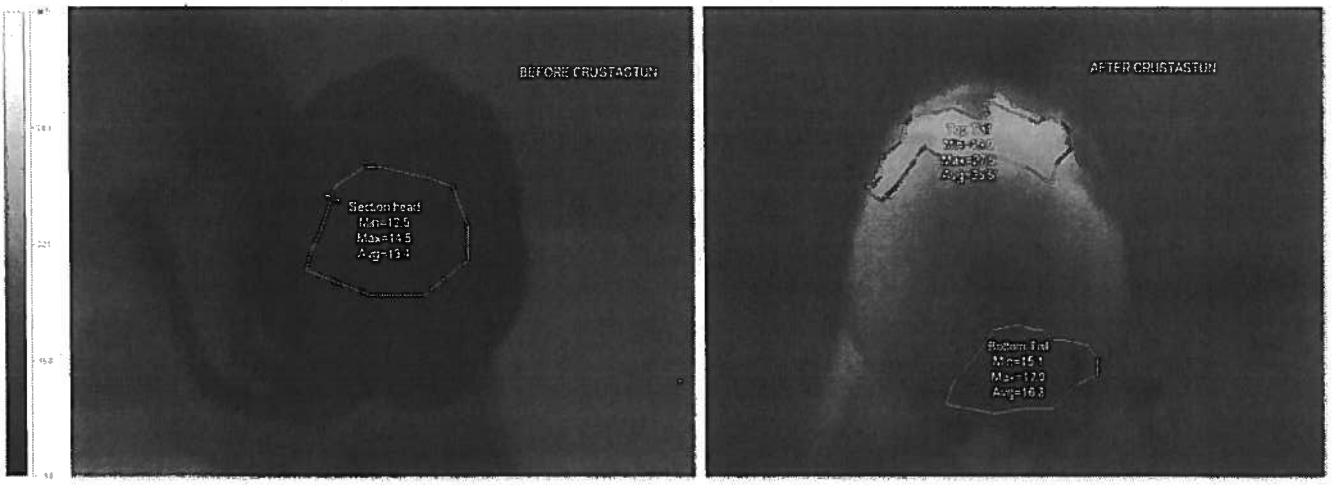

Figure 5. Thermal images taken through a transverse section of the cephalothorax ("head") A) before and B) after being subjected to a standard Crustastun. procedure. Values refer to the outlined areas.

Quality measures

Visual assessment

Animals killed with the Crustastun had visual assessment (QIM) scores (as per the scheme in Appendix 1) very similar to those of animals killed on ice, both on Day 1 (first assessment) and also up to 7 days (Figure 6).

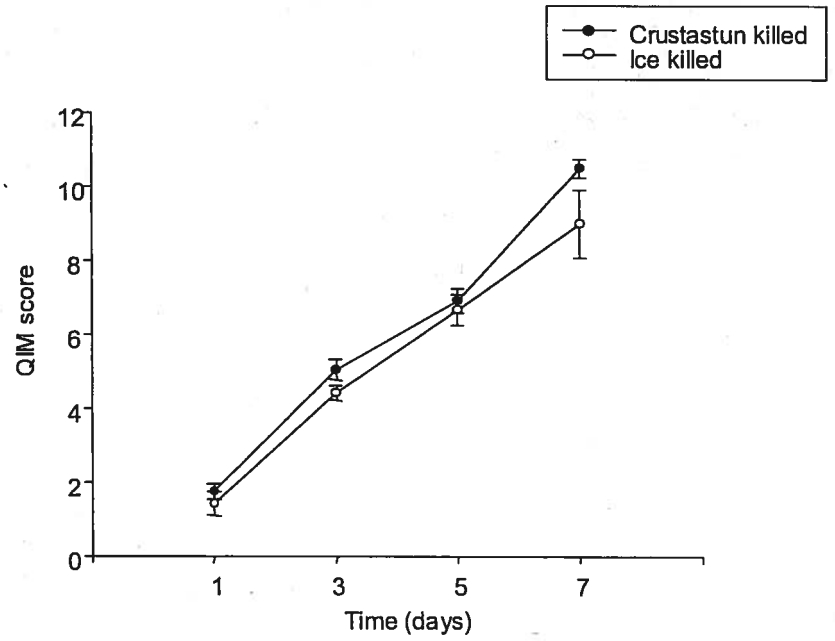

Figure 6. Scores obtained from visual assessment of langoustines killed with Crustastun or ice and stored at 0-2°C for up to 7 days. Values are the mean ± S.E.M. of ten different animals.

Melanosis Score

In terms of melanosis development (using the scoring scheme in Appendix 2) it was found that animals killed with the Crustastun developed black discoloration (melanosis) in the dorsal cephalothorax region ("head") more rapidly, compared to the animals killed on ice (Figure 7). This effect was initially indicated on Day 1 (first assessment) and became clear after 3 days of storage (Figure 8). However this effect of the Crustastun on the development of melanosis was only observed in this one region of the body, while values similar to those of the ice-killed Control group were recorded for the other parts of the body, i.e. the first (clawed) legs, the ventral cephalothorax & pereiopods, the pleopod appendages, tail and tail fan (Figure 7).

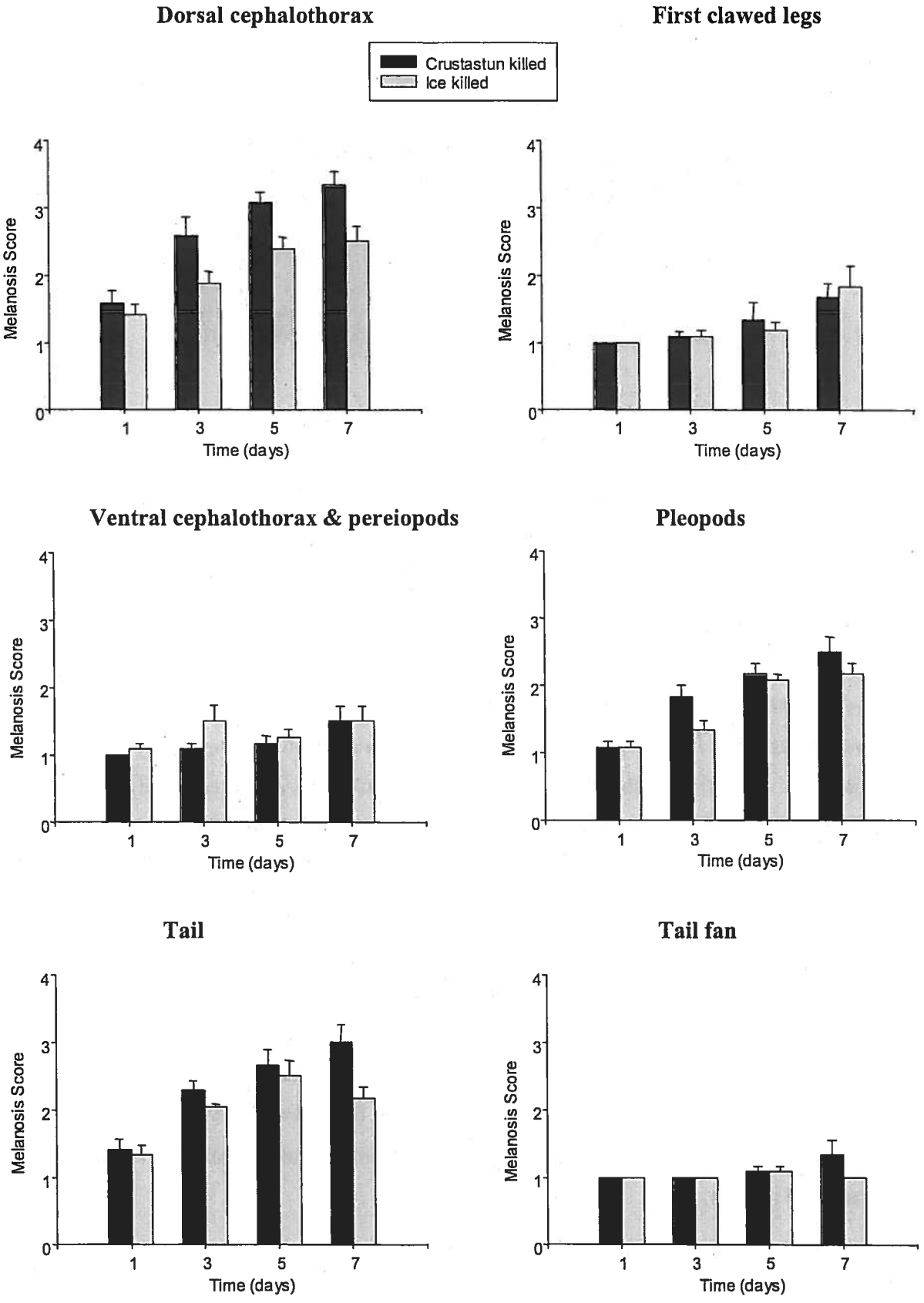

Figure 7. Melanosis score in langoustines killed with Crustastun or ice and stored at 0-2°C for up to 7 days in different parts of the body. Values are the Mean ± S.E.M. of ten different tails.

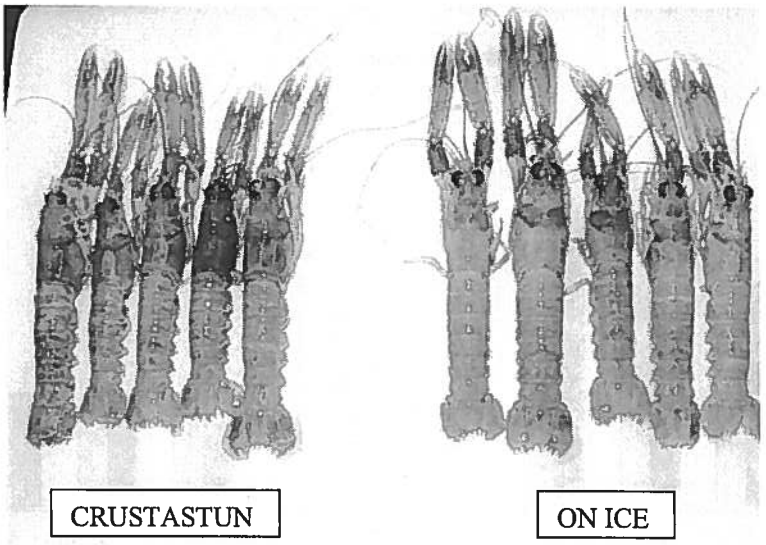

Figure 8. Melanosis development in langoustines on Day 3 after being killed using Crustastun (left group) or ice (right group).

Breakdown of nucleotides

The degradation of ATP and its breakdown products was analysed and the results are shown in Figure 9. In both groups AMP was the main nucleotide immediately after the animals were killed, which is consistent with results previously obtained from animals captured by trawling. On Day 1, IMP was the main nucleotide in Crustastun-killed animals, while AMP was still high in ice-killed animals. These results indicate that on Day 1 the processes of autolysis were more advanced in Crustastun-killed animals compared to ice-killed animals.

The fact that IMP increased sooner in Crustastun-killed animals could be advantageous in terms of the taste of meat of these animals over the early stages of storage (first 24 hours), if, as reported for several fish species, IMP gives the enhanced taste to langoustine meat. This possibility was separately evaluated by the independent sensory panel.

The differences between the nucleotide profiles of the two groups were only observed in the early storage stages, but from Day 5 onwards there was no difference between them. For instance, Hx concentrations (related to a bitter-off taste) were very similar

between Crustastun-killed (0.33 ± 0.09 µmol/g) and iced-killed animals (0.34 ± 0.01 µmol/g) at the end of the 7 day storage period.

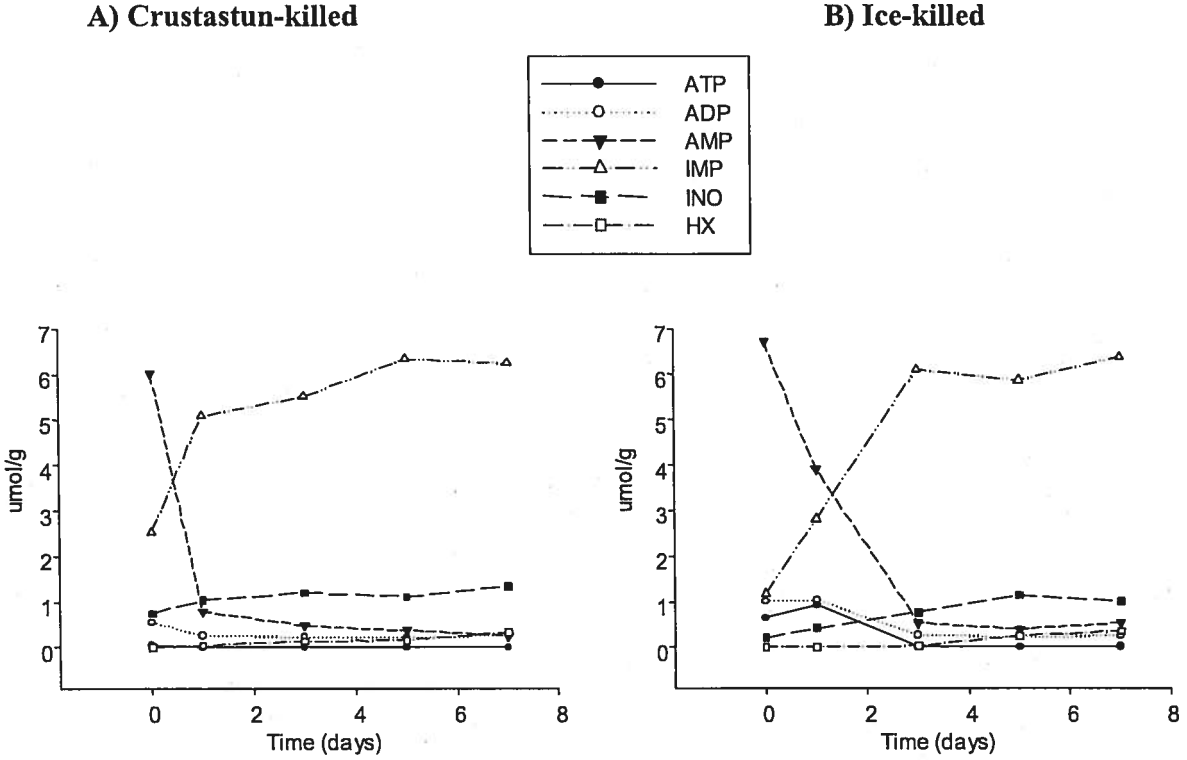

Figure 9. Nucleotide profiles in langoustine meat during ice storage after being killed using A) Crustastun or B) left on ice. Values are the mean of three different determinations.

The Freshness Index (K value)

The K-values calculated from the concentrations presented in Figure 9 were initially higher in Crustastun-killed animals than in ice-killed animals (Figure 10), and this difference persisted up to Day 5.

Our interpretation of this difference is that there was a more rapid autolytic phase after Crustastun killing. Moreover, the eventual similarity of the curves can be ascribed to there being a similarity in the bacterial phase of spoilage in the two groups.

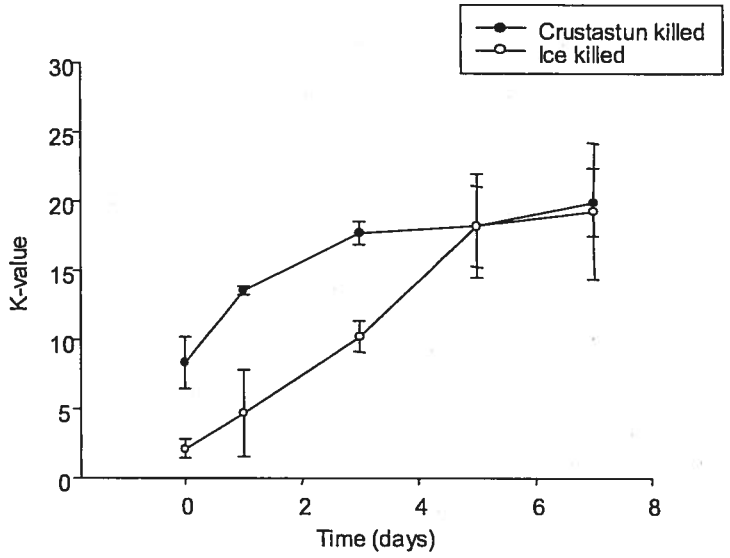

Figure 10. K-values in Langoustine meat during ice storage after being killed using different methods. Values are the mean of three different determinations.

The pH of the meat

When sampled fresh, immediately after landing, the pH values in the tail meat of both groups was lower than obtained from aquarium held animals (the meat of which typically has a pH of around 7.4). This difference is explicable by the fact that the animals used in the present trials had been obtained by trawling, which is known to cause elevated lactic acid production. Moreover, the Crustastun-killed group had a mean value of 6.71, while the pH in the tail meat of the ice-killed group had a mean pH value 6.82 (Figure 11). These values are significantly different, suggesting that the processes of glycolysis were reduced in Crustastun-killed langoustines.

On Day 1, the pH values in the tail meat of both groups had increased, but the Crustastun-killed animals continued to have a higher pH than those of ice-killed animals. This again indicates that the post-mortem autolytic processes were more advanced in this group, with the consequent breakdown of nitrogenous compounds leading to increases in the pH. This result is consistent with the measurements of the nucleotide breakdown rates. By comparison, the pH of the tail meat of animals killed on ice remained lower, indicating that they were in earlier stages of post-mortem change. Thereafter, in both groups the pH increased steadily with storage time, and from Day 3 onwards there was no difference between them in this measure.

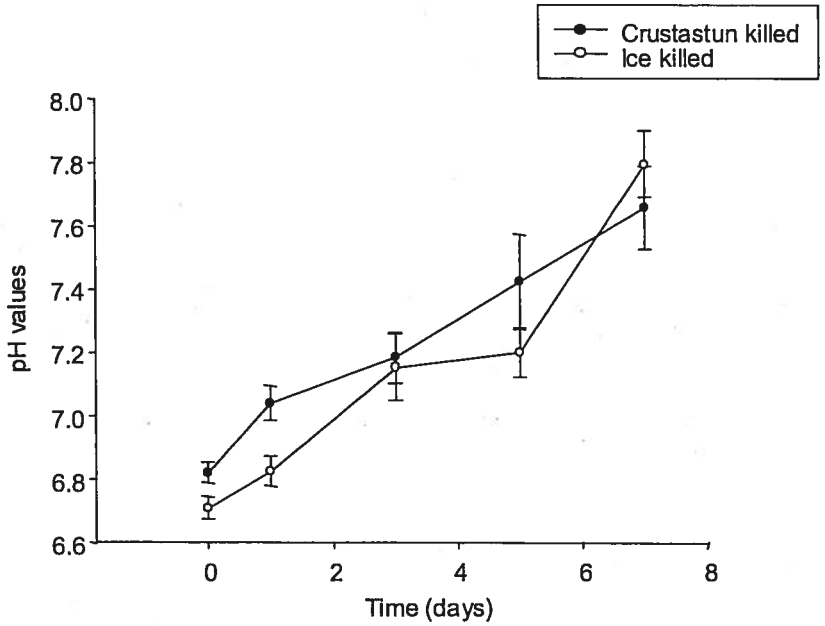

Figure 11. Changes in muscle pH in langoustines during ice storage after being killed using Crustastun on left on ice. Values are the mean ± S.E.M. of ten different animals.

We interpret the results of the nucleotide breakdown rates and the changes in muscle pH, to mean that the action of the Crustastun kills the animals almost instantaneously, and this electrocution suppresses glycolytic activity leading to an earlier onset of the post-mortem autolytic processes. As a consequence, the condition of the meat in Crustastun-killed animals is similar in these terms to that of the meat of ice-killed animals at a time point around 2 days later.

Bacterial load in the meat

The load of psychotrphic marine bacteria in the meat was obtained by plating muscle homogenates onto marine iron agar plates (Figure 12). Crustastun-killed animals had significantly lower total bacterial numbers than ice-killed animals on both Day 1 and Day 3 of storage. These results indicate that up to Day 3 the Crustastun process delayed the growth of internal psychotrophic bacteria. However, this effect was not very strong, and bacteria numbers from Crustastun-killed animals later increased to be the same as in ice-killed animals by Day 7. This suggests that there might be no actual shelf-life extension induced by killing the animals using Crustastun.

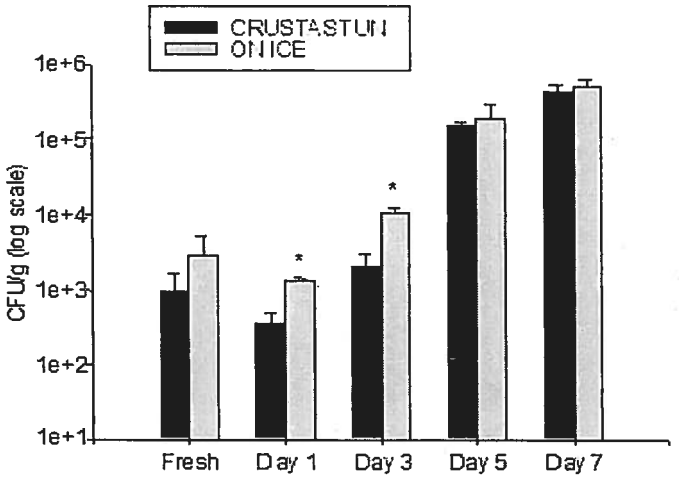

Figure 12. Changes in total bacteria counts measured on marine agar for langoustine meat during ice storage after being killed using different methods. Values are the mean ± S.E.M. of three different determinations (3 tails were pooled for each determination). Asterisks indicate statistically significant differences.

Separate determinations of the numbers of H_2S producing bacteria and of luminescent bacteria produced no differences between Crustastun-killed and ice-killed groups (data not shown). However, Interestingly, *Pseudomonas* sp. bacteria were found in higher numbers in Crustastun-killed animals, measured directly after the stunning process (Figure 13).

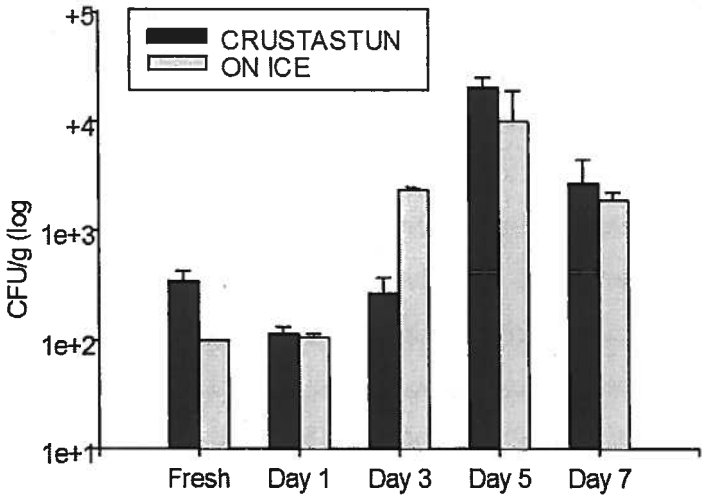

Figure 13. *Pseudomonas* Sp. bacteria in langoustine meat during ice storage after being killed using different methods. Values are the mean ± S.E.M. of three different determinations (3 tails were pooled for each determination).

When transferred to ice storage, this difference in *Pseudomonas* numbers between the Crustastun-killed and ice-killed animals disappeared or even reversed, although both then showed a pattern of increasing numbers (Figure 13). The finding of higher *Pseudomonas* sp. numbers initially in Crustastun-killed animals may have been due to the accumulation of these bacteria in the brine in the Crustastun chamber over repeated cycles of operation. This possibility was therefore tested in a separate trial (below).

Trimethylamine (TMA) concentration

Trimethylamine (TMA) is a product of bacterial spoilage, and was measured to assess the possibility that the lower bacterial numbers found on Days 1-3 in Crustastun-killed animals reduced their later spoilage activity. The results show that TMA concentrations were closely similar in the Crustastun-killed and ice-killed groups over the whole period of storage, and moreover that the amount produced did not begin to increase until Day 5 in each group (Figure 15).

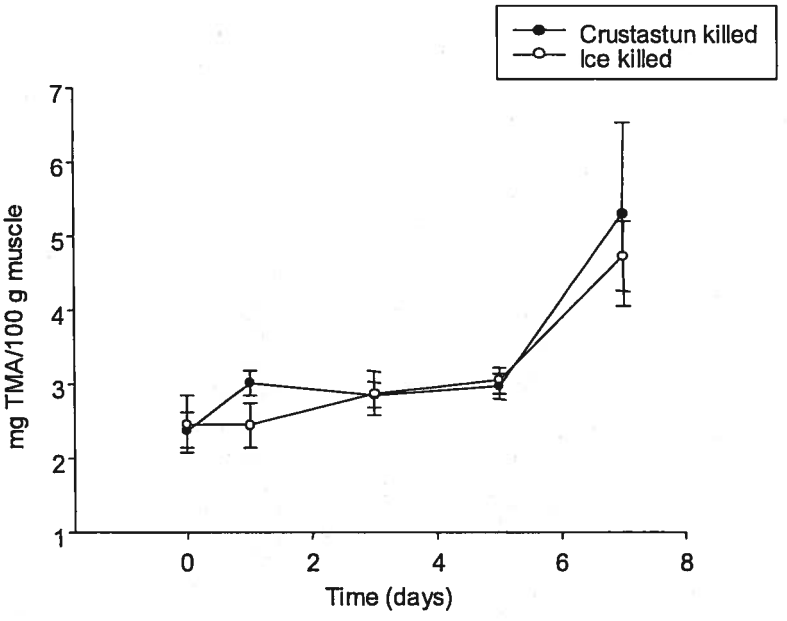

Figure 15. TMA concentration in langoustine meat during storage after being killed using different methods. Values are the mean ± S.E.M. of four different determinations.

We interpret these results to indicate that the method of killing did not affect the production of TMA during subsequent ice storage. Therefore, the lower levels of

bacteria that were found on Days 1 and 3 in Crustastun-killed animals did not affect production of TMA significantly. These results suggest that either the reduction in bacteria was not large enough to change the TMA levels or that the bacteria species affected by Crustastun were not TMA producers. Measures of other non-protein nitrogenous compounds (biogenic amines) that are known to be produced by bacterial action provided essentially the same result (data not shown). Our conclusion is therefore that the bacterial phase of spoilage is little affected by Crustastun killing, and that differences found in total bacteria counts immediately after this treatment may not be relevant in terms of increasing the shelf-life of the product.

Accumulation of bacteria in the brine within the Crustastun chamber

In conjunction with the trial on 23/10/2007, in which 30 langoustines were Crustastun-killed in 7 cycles of operation, samples of the brine in the Crustastun chamber were collected at the start and at the end of the trial. The bacterial load in each water sample was analysed in terms of the total counts, and also specifically for *Pseudomonas* sp.. The results (Figure 16) indicate that the chamber contained more bacteria in total and *Pseudomonas* sp. in particular after the trial compared to before it.

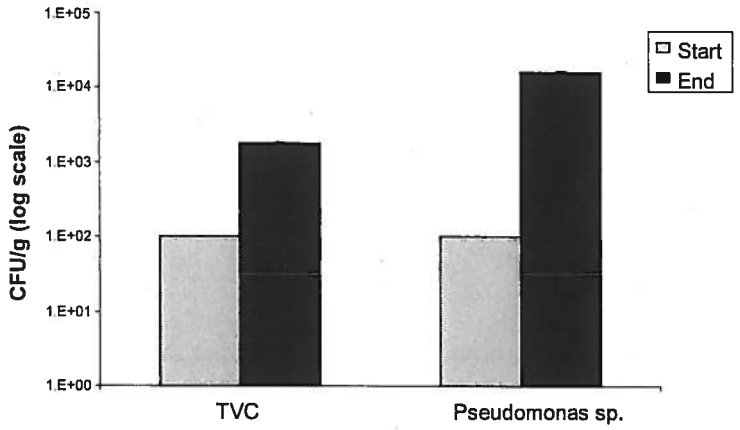

Figure 16. Different types of bacteria (TVC: total bacterial load and *Pseudomonas* sp.) in water collected from the Crustastun chamber before and after 7 cycles of operation (30 animals killed). Values are the mean ± S.E.M. of three different determinations.

Sensory Evaluation of langoustines killed with Crustastun

Sensory evaluations of langoustine tail muscle samples were carried out by a trained professional panel. The scores obtained are shown in Table 1. Samples from Crustastun-killed langoustines scored higher in smell strength and lower in firmness and chewiness. For the other parameters the results were very similar, and no significant differences observed in smell characteristics, springiness, moistness, flavour and aftertaste.

Table 1. Scores obtained on sensory attributes of langoustine cooked meat after being killed on ice or with Crustastun.

		Smell Character.	Smell Strength	Springiness	Firmness	Chewiness	Moistness	Flavour	Aftertaste
Crustastun	Mean	**5.63**	**5.07**	**6.09**	**6.09**	**5.80**	**5.73**	**6.51**	**6.11**
	St. D.	0.39	1.22	2.87	1.09	1.12	0.86	1.28	1.27
On ice	Mean	**5.01**	**3.89**	**5.91**	**7.01**	**7.46**	**5.81**	**6.57**	**6.20**
	St. D.	1.71	2.20	1.44	2.13	2.43	1.09	1.45	1.65

From the comments of the panellists, both samples were perceived as sweet with a sweet aftertaste. However, differences were found in texture attributes. Tail muscle samples from Crustastun-killed langoustines were less firm and chewy (tendencies evaluated as positive). Therefore, the texture of the meat from Crustastun-killed langoustines was described as "nice" or "good" while samples from langoustines killed on ice were described by some panellists as "too chewy" or "rubbery". Possibly because of these textural features, the overall liking was slightly (but not significantly) higher for the samples from Crustastun-killed langoustines compared to those from ice-killed animals (Figure 17).

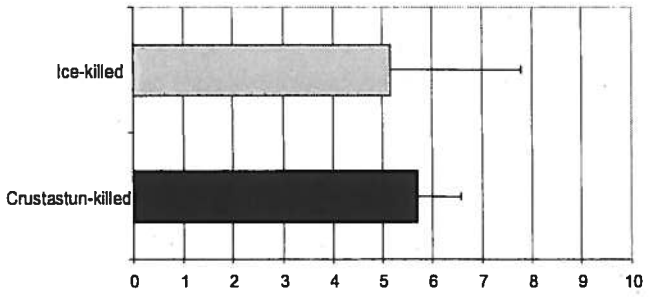

Figure 17. Overall liking of cooked langoustine meat after being Crustastun-killed or ice-killed

Conclusions

- The Crustastun machine was very effective in killing langoustines
- The animals adopted a characteristic tail curvature during the stunning process
- Visual assessment of the langoustines killed using the Crustastun or killed on ice were very similar throughout the storage period studied (7 days).
- The Crustastun process produced an increase in the temperature of the langoustines. This increase was particularly noticeable in the dorsal cephalothorax region, but less so or not at all in the tail.
- The Crustastun-killed langoustines developed black discoloration (melanosis) in the dorsal region of the cephalothorax more rapidly (within 3 days) compared to animals killed on ice. This is ascribed to the temperature increase in these parts, which must either trigger or potentiate the melanosis reactions.
- Post-mortem autolysis progressed more rapidly in the Crustastun-killed langoustines, possibly due to a suppression of gylcolyis by the electrocution process, leading to an acceleration of the breakdown of nucleotides (ATP).
- As a consequence, IMP was the predominant nucleotide from Day 1 in Crustastun-killed langoustines, whereas in ice-killed animals AMP remained high for longer.
- The earlier appearance of IMP in Crustastun-killed langoustines may contribute to a flavour enhancement, if they are consumed within a short period (say 24h) of electrocution.
- The total load of marine (psychotrophic) bacteria was reduced by the Crustastun process, with numbers being lower on Days 1 - 3 of storage.
- However, this lower level of bacteria did not either delay or reduce the appearance of typical products of bacterial spoilage such as hypoxanthine, TMA and biogenic amines, that can affect the quality of the meat
- *Pseudomonas* sp. bacteria numbers were initially higher in the meat of Crustastun-killed langoustines.
- *Pseudomonas* sp. bacteria numbers increased in the brine within the Crustastun chamber after it had been operated for several cycles of electrocution of langoustines.

- Taken together, these results suggest that the brine in the Crustastun chamber should be changed regularly, and that high levels of hygiene should be maintained.
- When meat from Crustastun-killed and ice-killed langoustines was assessed by an independent Sensory Panel, several differences in the objective assessments were reported between the two groups, particularly in some texture parameters. Thus at the time point of 1 day after electrocution, compared to ice-killing, the meat texture of Crustastun-killed langoustines was judged to be less firm and less chewy, features that were evaluated as positive.
- The subjective assessment of the panel in terms of overall liking was also slightly more positive for the Crustasun-killed langoustines, compared to those killed on ice.

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Appendices

Appendix 1. Visual assessment scoring table used for langoustines

Attributes	criteria	index points
appearance claws:	carrot orange, white, sharp contrast	0
	orange fading, bleached, dull	1
	light grey, slightly more algae green	2
	bleached , more algae green, creamy/yellowish	3
appearance head:	sharp contrast, carrot orange, pinkish, black eyes	0
	less shiny, bleached, slightly grey, creamy ends	1
	dull, dead, more grey, brownish, bleached eyes	2
	Black, dark grey, dull, grey eyes	3
Uper site tail	Fresh orange, pinkish, white ends, some tail ends already brown	0
	bleached orange, tail ends more grayish and darker brown	1
	light brown over orange, creamy ends, bleached, brown tail ends.	2
	Distinct brown, green lines, black tail ends.	3
Under site tail	Transparent feet, pinkish, translucent meat	0
	discolored feet, milky meat	1
	Brown feet, yellowish meat	2
Odor:	Fresh, hey, marine	0
	Les fresh, neutral	1
	Old seaweed, musty, slightly ammonia	2
	Sour, musty, ammonia	3
Total score		0 - 14

Appendix 2. Melanosis scoring table used for langoustines

1	Total absence of black spots or blackening
2	Few black spots or blackening less than 30 %
3	Considerable blackening (between 30-70 %)
4	Substantial blackening (more than 70 %)

15

The effect of the Crustastun™ on nerve activity in two commercially important decapod crustaceans: the edible brown *Cancer pagurus* and the European lobster *Homarus gammarus*

Scientific Report to Studham Technologies Ltd.

by

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May 2012

University of Glasgow

INTRODUCTION

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). Crustastunning kills the animals instantaneously, and imposes no additional physiological stress as judged by indicative biochemical measures (Neil and Thompson, 2012).

A previous investigation (Neil, 2010) evaluated the effect of the Crustastun™ on nerve activity in a typical crab (the shore crab *Carcinus maenas*) and a typical clawed lobster (the Norway lobster or langoustine *Nephrops norvegicus*). The present report summarises the results obtained in a number of trials carried out to determine the effect of the Crustastun machine on activity in the nervous system of two other decapod crustaceans: the edible brown *Cancer pagurus* and the European lobster *Homarus gammarus*. These are important species that are commonly supplied live to processors and to the restaurant trade in the UK and other European countries. Moreover, the closely related species of crab, the Dungeness crab *Metacarcinus* (formerly *Cancer*) *magister,* and species of lobster, the American lobster *Homarus americanus*, are widely consumed seafood in North America. On the basis of the results obtained in this study, conclusions have been drawn about the effects of Crustastun usage on the neuronal functioning in these commercially important crustaceans.

Aims and objectives

The aims of this study were, as in the previous study (Neil, 2010), to use appropriate electrophysiological techniques to record from both the central nervous system and the peripheral nervous system of, in this case, the brown crab *Cancer pagurus* and the European lobster *Homarus gammarus*, in order to compare intact animals with those that have been subjected to 'Crustastunning'.

The specific objectives were:

1. To monitor intrinsic and evoked neuronal activity emerging from the anterior of the central nervous system, the 'brain' (supra-oesophageal ganglion) of crabs and lobsters, by making extracellular recordings in the circumoesophageal connectives, the main nerves conveying information to and from the brain. This would include making recordings in the "head" (cephalothorax) of the lobster after isolating it from the tail (abdomen)

2. To monitor intrinsic neuronal activity from the posterior of the central nervous system of lobsters by making extracellular recordings from neurones in the abdominal ventral nerve cord. This would include making recordings in the tail (abdomen) of the lobster after isolating it from the head (cephalothorax).

3. To record intrinsic activity from the peripheral nervous system, the motor nerves leaving the abdominal nerve cord of the lobster to supply the abdominal postural muscles, by making extracellular recordings from the appropriate motor nerves (3rd abdominal roots).

4. To demonstrate evoked motor activity in the peripheral nervous system of the crab by measuring the muscle forces produced by the activation of the motor neurones in the leg nerve supplying a muscle spanning a specific leg segment (the closer muscle of the dactylopodite) in crabs.

5. To demonstrate evoked sensory activity in the peripheral nervous system of crabs and lobsters by recordings from the sensory neurones in the leg nerve in response to stimulation of specific receptor types: mechanoreceptors in the cuticle (eg. cuticular hairs, campaniform sensillae) and proprioceptors spanning the leg segments internally (chordotonal organs).

These tests were designed to allow the following questions to be addressed, namely, after 'Crustastunning':

- Does any activity continue to be generated spontaneously in the central nervous systems of the brown crab and the European lobster, and if so are its characteristics altered from normal?

- Does any activity, either spontaneous or evoked, remain in the motor and neuromuscular systems of the animals, and if so are their characteristics altered from normal?

- Does any activity remain in the sensory nerves from peripheral mechanosensory organs of the animals, and if so are its characteristics altered from normal?

Anatomy

Decapod crustaceans, the taxonomic group to which crabs and lobsters belong, have nervous systems with the characteristic arthropod plan (Brusca and Brusca, 2002). This involves a ladder-like arrangement of paired nerve cords, with a dorsal brain (supraoeophageal ganglia) separate circumoesophageal connectives and segmental ganglia in the thorax and (if present) in the abdomen, from which nerves arise to supply the segmentally-arranged muscles and sense organs. Lobsters exemplify all these features (Figure 1) whereas in crabs a distinct abdomen has been lost and the thoracic ganglia are condensed into a single thoracic mass, from which all the peripheral nerve roots emerge (Figure 2).

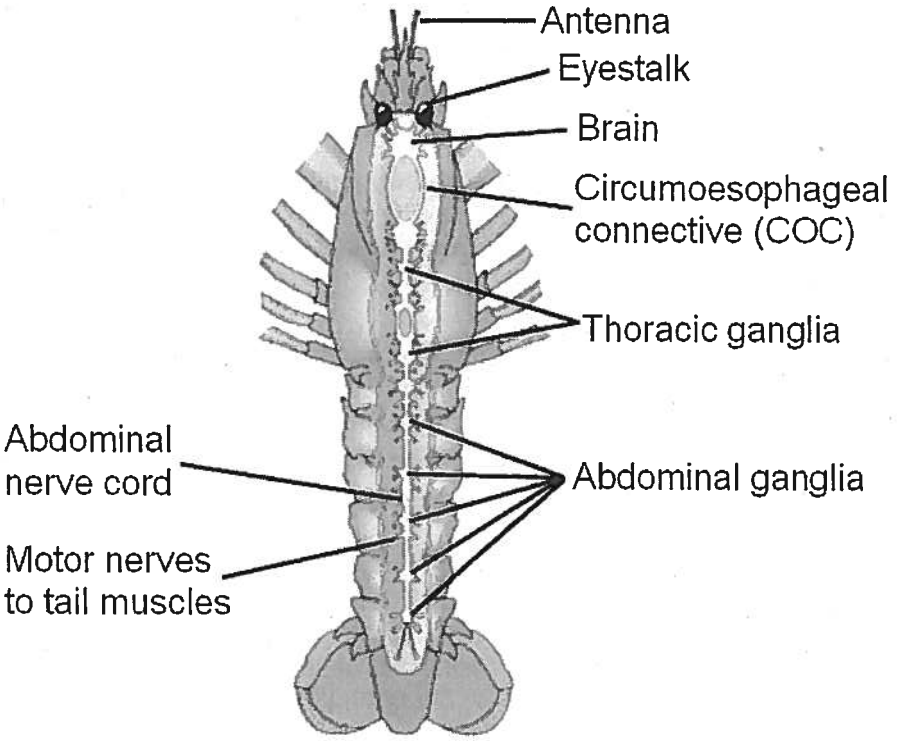

Figure 1. The arrangement of the nervous system in a clawed lobster such as the European lobster *Homarus gammarus*

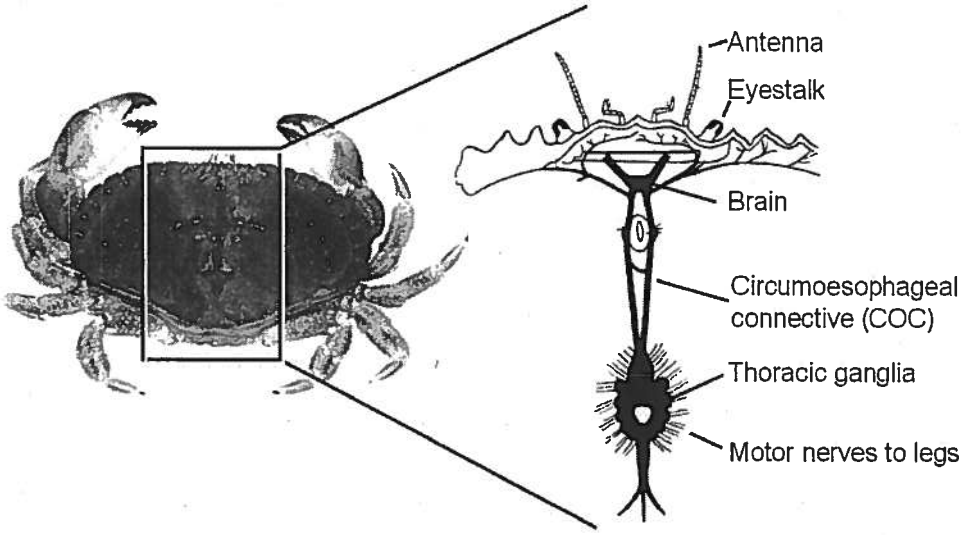

Figure 2. The arrangement of the nervous system in a crab such as the brown crab *Cancer pagurus*

Each of the four pairs of walking legs (pereiopods) of crabs and lobsters comprises a series of articulated segments, which are moved by paired muscles (Figure 3). A number of different mechanoreceptors are associated with the leg exoskeleton, including innervated cuticular sensory hairs which signal contact and water movement (Garm, 2005), and 'funnel canal organs' (a type of campaniform sensilla) which are pressure-sensitive (Libersat, 1987). In addition, a series of elastic strands span the various joints, into which are embedded sensory cells which detect joint flexion and extension (Bush, 1965). These so-called chordotonal organs thus act as proprioceptors monitoring the leg movements made by the crab (Hartman *et al.*, 1997). The chordotonal organ spanning the terminal leg segment, between the propopodite and the dactylopodite (the PD chordotonal organ) was selectively activated in this study. The branches (axons) of both the motor and the sensory nerves pass in a mixed leg nerve that travels through the centre of the leg segments.

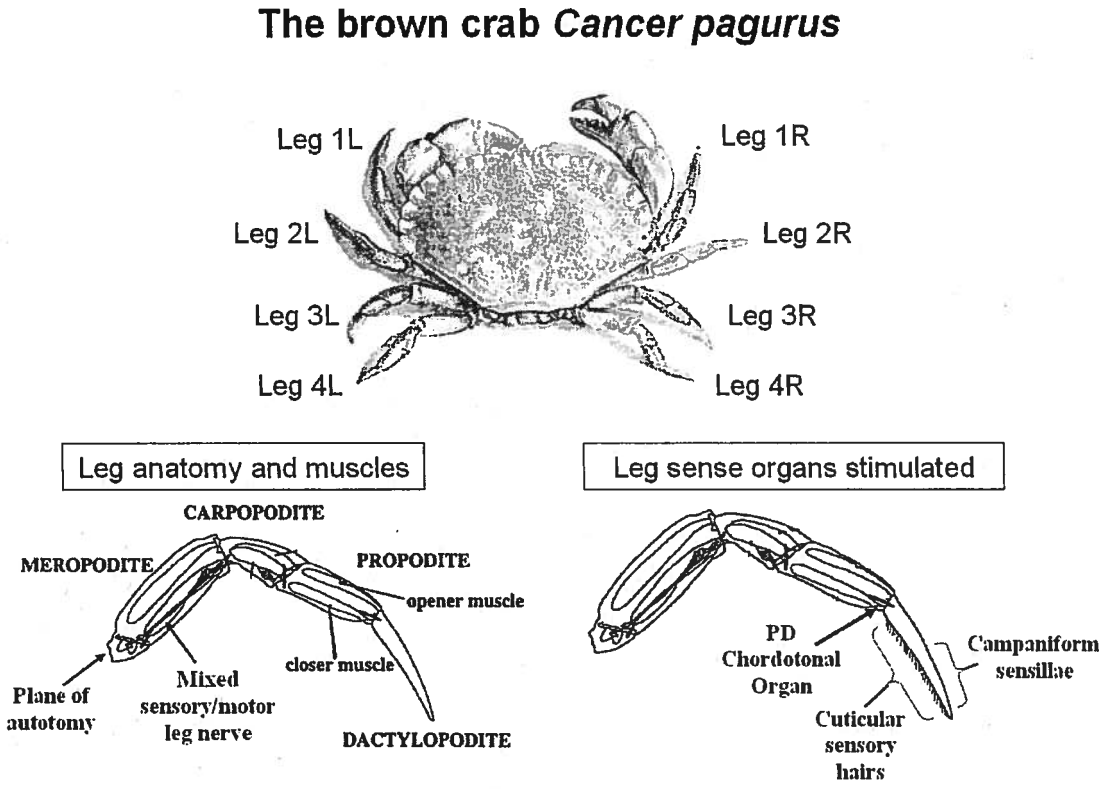

Figure 3. Schematic diagram of the anatomy of the legs of the crab *Cancer pagurus*, including the arrangement of the muscles and sense organs.

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, and were 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°C for at least two weeks before experimentation.

Crustastunning

The Crustastunning' 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 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°C - 12°C).

Exposing the nervous systems

In order to expose the central nervous system of the crab for recording, the carapace was removed and the preparation was submerged in a balanced salt solution corresponding in composition and osmolarity to crab haemolymph, at a temperature of 10°C. The internal organs were then removed or displaced in order to expose the circumoesophageal connectives around the base of the stomach. A similar procedure was employed for the lobster, but prior to this the cephalothorax was separated from the abdomen.

To expose the abdominal ventral nerve cord of the lobster for recording, after separating the abdomen from the cephalothorax the dorsal skeletal plates (terga) were detached, and the bulk of the underlying deep flexor musculature was removed. The preparation was then submerged in a balanced salt solution corresponding in composition and osmolarity to lobster haemolymph, at a temperature of 10°C. Selective removal of muscle blocks then revealed the motor roots emerging from the ventral nerve cord.

The leg nerves of crabs and lobsters were exposed for recording and stimulating using the following procedures. The intact animal was induced to shed a leg spontaneously (autotomy) by applying pressure to the basipodite segment (McVean, 1976, Smith and Hines, 1991). For the Crustastunned crabs and lobsters, which were effectively killed and did not express the autotomy reflex, a leg was detached by amputation. In either case the joint between the meropodite and carpopidite (M-C) was then disarticulated, and the muscle tendons spanning this joint were cut with fine scissors. The leg was separated gently at this point, revealing the leg nerve still attached to the distal portion. This isolated leg preparation was submerged in a balanced salt solution at a temperature of 10°C until required, and remained viable for many hours.

Electrophysiological recordings

Electrophysiological recordings were made from the exposed nerves using various extracellular techniques. For recording from the circumoesophageal connectives of crabs and lobsters, and from the ventral nerve cord of lobsters, a suction electrode method was used. A fine-tipped polythene electrode containing salt solution was applied to the surface of the nerve, and a gentle suction was applied through attached tubing and a syringe. A silver wire positioned close to the tip of the electrode acted as the indifferent (reference) electrode. Such a recording configuration is termed 'en passant', as it involves attaching the suction electrode to an intact nerve, allowing both directions of nerve transmission to be recorded. However, in some cases the circumoesophageal connective was cut and the electrode was attached to either its anterior or posterior cut end. In this way the presence of active neurones transmitting information in ascending or descending directions could be ascertained.

For recording from the crab and lobster leg nerves, the isolated leg was clamped to a Perspex plate and the nerve was passed from an adjacent bath through a wall of petroleum jelly into a second small chamber, both of which contained balanced salt solution (Figure 4, upper panel). A bipolar electrode of two silver wires was used to make contact with the solutions in the inner and outer chambers respectively.

In each case the signals from the extracellular electrodes were passed to a differential pre-amplifier (A101, Isleworth Ltd.) for amplification and filtering. The amplifier output was then passed to an Analog/Digital converter (PowerLab, AD Instruments Ltd) and was both displayed and recorded on a standard PC computer using the associated software (Chart v7, AD Instruments Ltd.)

Stimulating the nerves

Motor responses in the legs were measured only in crabs, since their leg anatomy was more compatible with the force measuring procedure (see Figure 4, lower panel). To stimulate the motor axons in the crab leg nerve, the bipolar electrodes were connected to an isolated stimulator within the PowerLab (Figure 4, lower panel) and patterns of stimulating pulses at various amplitudes and frequencies were applied using a software

'stimulator control panel' within the Chart v7 software. Typically, stimulus trains of 3 s duration and 4 V amplitude were applied at a range of frequencies from 10 – 100 Hz.

Recording muscle force

Although stimulation of the crab leg nerve potentially activated motor neurons supplying all of the muscles located more distally in the leg, the forces produced by the closer muscle of the propopodite/dactylopopodite joint (P-D) were nevertheless recorded selectively. This was achieved by cutting the tendon of the antagonist muscle about that joint (the P-D opener muscle), and then attaching a thread from near the tip of the dactylopodite to the arm of a sensitive force transducer (FT-03, Grass Instruments Ltd.), mounted on a micromanipulator (Figure 4, lower panel). This selectively monitored the forces produced by the dactylopodite closer muscle. The output of the transducer was passed to a custom-built amplifier (x1000), and then fed to an input of the Powerlab A/D converter. The forces and the stimulus parameters were then both displayed and recorded on a standard PC computer using the Chart v7 software.

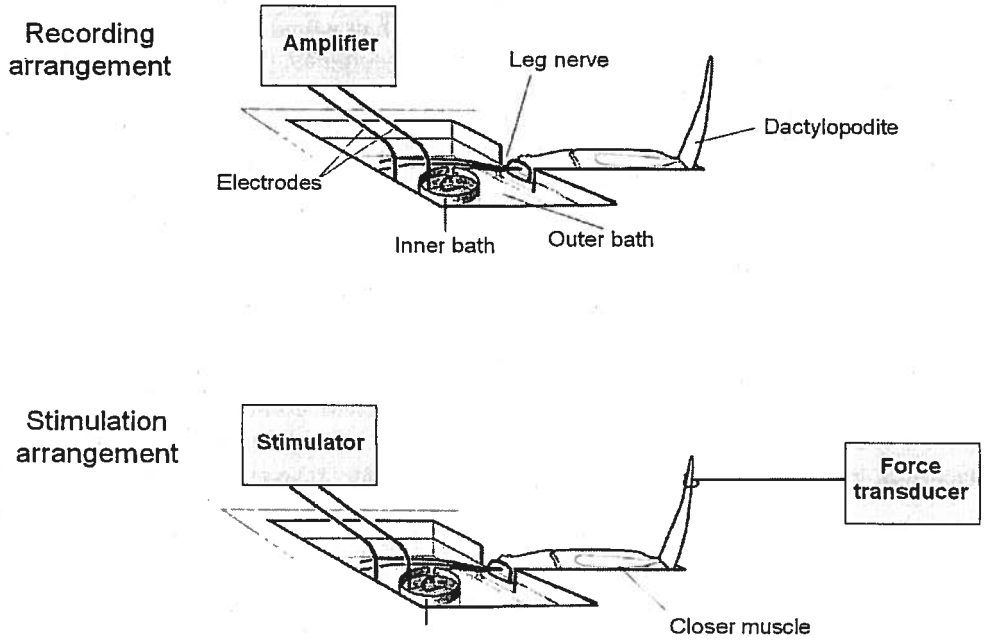

Figure 4. Experimental arrangements for recording from the nerve of an isolated crab or lobster leg (upper panel) and for stimulating the crab leg nerve while recording the forces produced by the dactylopodite closer muscle (lower panel).

RESULTS

For each species, *C. pagurus* and *H. gammarus*, the complete set of trials involved a total of 6 individual animals that were Crustastunned and the same number of intact animals as a control group. In the case of isolated legs, three legs per individual were tested. The neuronal data are presented as traces of the original electrophysiological recordings and where appropriate also as plots of the muscle forces produced in relation to stimulus parameters.

Activity in the central nervous system (CNS) of intact crabs and lobsters

Recordings made from one or both circumoesophageal connectives in intact *C. pagurus* crabs indicated that there was a high level of spontaneous neuronal activity passing along the axons of this nerve, even in the absence of any imposed stimulation (Figure 5 upper panel). Due to the variety of sizes of the extracellularly-recorded spikes, it can also be concluded that the signals arose from a large number of different individual nerve axons, of varying diameters.

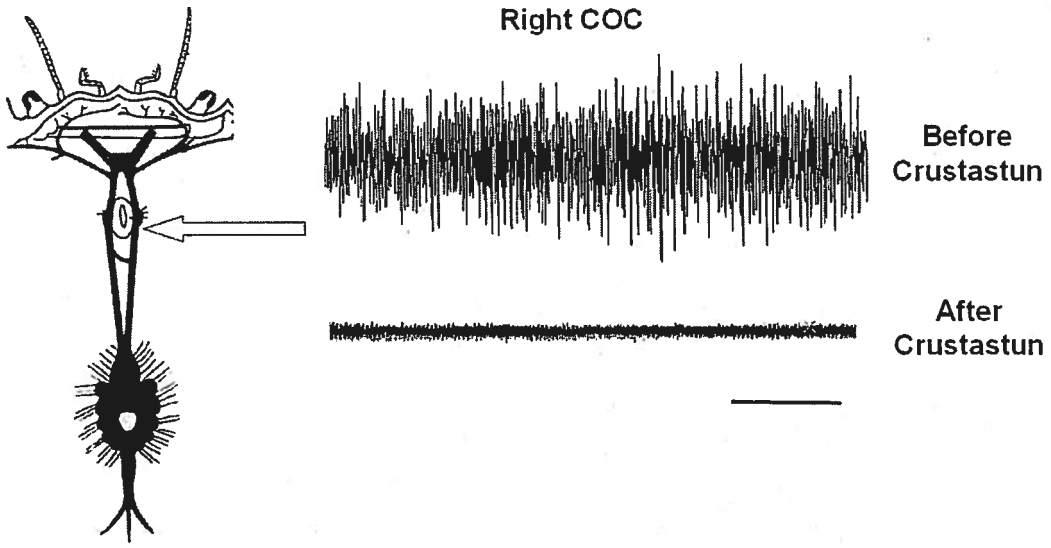

Figure 5. Spontaneous nerve activity recorded extracellularly in the right circumoesophageal connective (COC) of a brown crab, *Cancer pagurus*. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

When tactile stimuli were applied to the eyestalks or antennae, there were systematic changes in firing frequency in some of these axons, indicating that these were conveying descending activity from the brain. There were also high frequency bursts of activity that corresponded to the animal making struggling movements (fictive locomotion) (data not shown).

Recordings from the circumoesophageal connectives of intact *H. gammarus* lobsters provided essentially the same results, even when the cephalothorax was detached from the abdomen, with a high level of neuronal activity passing along the axons of this nerve (Figure 6, upper panel).

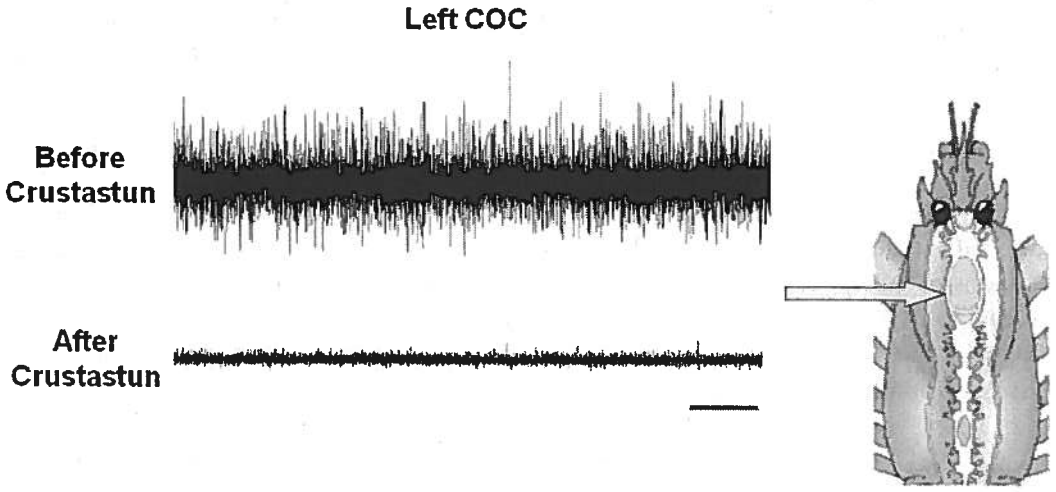

Figure 6. Spontaneous nerve activity recorded extracellularly in the left circumoesophageal connective (COC) of a *H. gammarus* lobster. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

Recordings from the abdominal nerve cord of the intact lobsters also encountered spontaneous nerve activity in all cases, even when the abdomen was detached from the cephalothorax (Figure 7 upper panel).

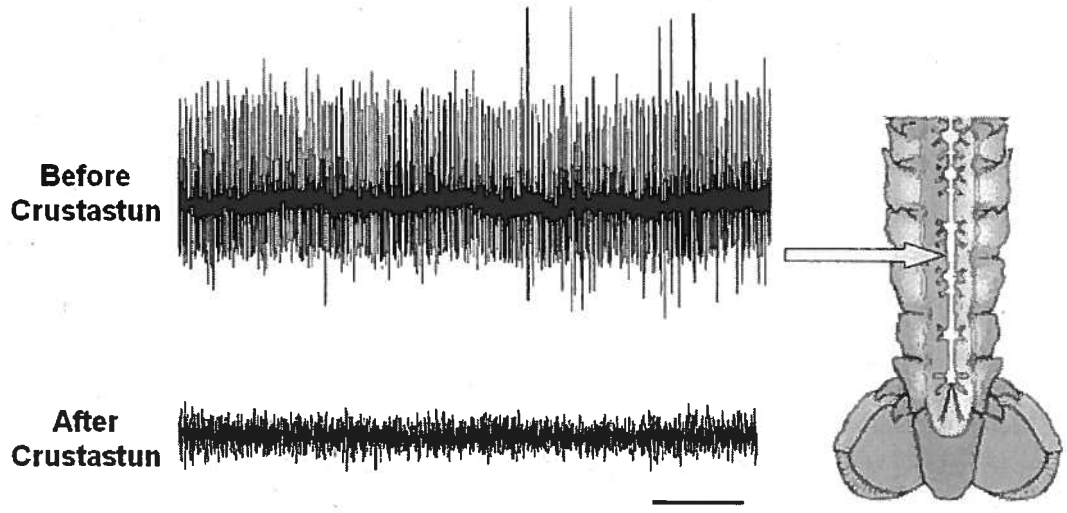

Figure 7. Spontaneous nerve activity recorded extracellularly between the 3rd and 4th ganglia in the abdominal nerve cord of a *H. gammarus* lobster. Upper panel, intact animal; lower panel, animal after Crustastunning. Scale bar 1s.

Activity in the peripheral nervous system of intact crabs and lobsters – motor responses

Patterned activity involving a number of motor neurons (represented by different spike sizes) was detectable in motor roots emerging from the ventral nerve cord of the lobster *H. gammarus* (Figure 8). This represents evidence for the action of the peripheral nervous system in intact animals, contributing to the generation of muscle tone in the abdominal muscles.

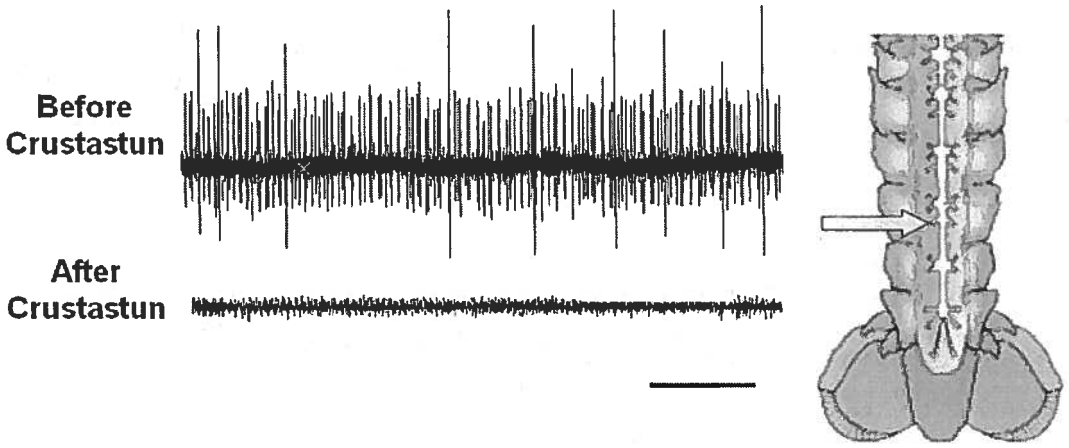

Figure 8. Spontaneous nerve activity recorded extracellularly from motor neurones in the 3rd motor root of the 4th abdominal ganglion of an intact *H. gammarus* lobster. Scale bar 1s.

Due to the relative inaccessibility of the motor roots emerging from the thoracic ganglia of crabs, equivalent recordings were not obtained from *C. pagurus*. However the normal operation of the motor pathways of the peripheral nervous system of this crab was demonstrated by stimulating the leg nerve of an autotomised leg at various frequencies while monitoring the force produced by the dactylopodite closer muscle. The force varied in a frequency-dependent manner typical of crustacean neuromuscular systems due to their synaptic properties of summation and facilitation (Figure 9).

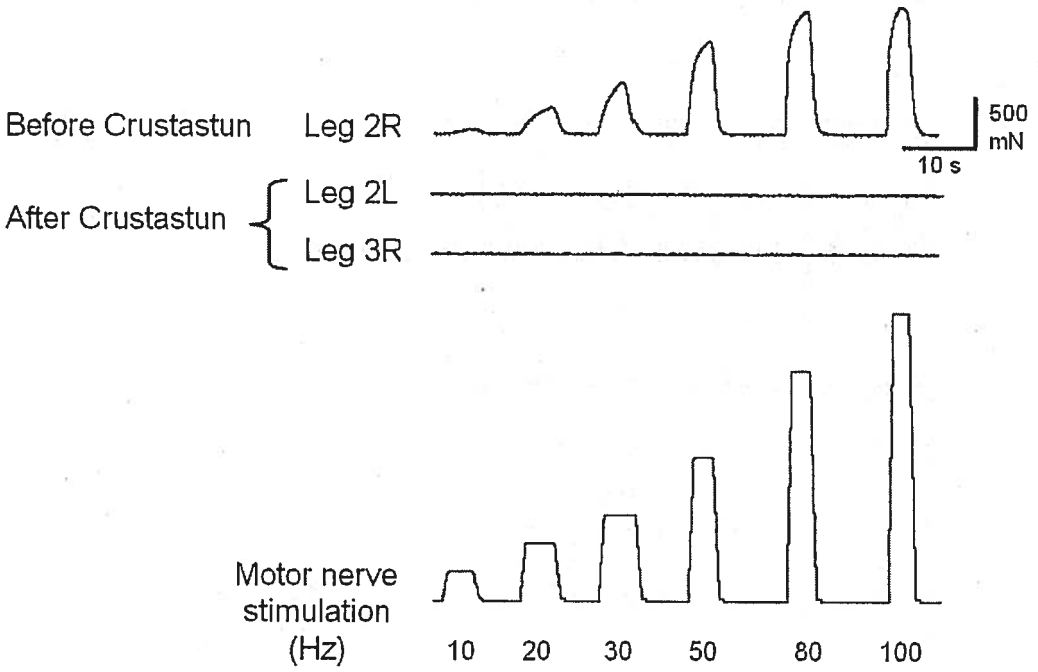

Figure 9. Forces produced by the dactylopodite closer muscle of the leg of *Cancer pagurus* in response to stimulation of the leg nerve at various frequencies. Top panels, leg autotomised from intact crab; lower two panels, legs amputated from the same crab after Crustastunning. Stimulus voltage 4V.

Activity in the peripheral nervous system of intact crabs and lobsters – sensory responses

Further evidence for activity in the peripheral nervous system in intact crabs and lobsters was obtained from the recordings of sensory activity made in their isolated legs, following autotomy. Examples from two lobsters are presented in Figures 10 and 11, and from two crabs in Figures 12 and 13.

The leg nerve of a crab or lobster contains a mixture of the axons of sensory and motor neurons, and the application of various stimuli to the distal part of the leg clearly elicited activity in a number of sensory neurons. These patterns of activity were typical for the various sense organs that were stimulated in each case. Thus brushing movements over the cuticle of the dactylopodite produced bursts of activity typical of the responses to displacement of cuticular sensory hairs (Figures 10-13, left panels). Compression (squeezing) of the cuticle of the dactylopodite elicited persistent tonic responses for the duration of the stimulus (Figures 10-13, centre panels). The responses to the movement and displacement phases of flexions and extensions applied at the P-D joint had characteristic phasic and tonic elements (Figures 10-13, right panels).

In order to test the persistence of activity in the nervous systems of intact crabs and lobsters, some preparations were re-tested at intervals of up to several hours. Activity persisted for up to the longest time tested (6 hours) in both their central nervous systems and in the nerves of autotomised legs (data not shown). A similar persistence was observed when a number of legs that were autotomised from an intact crab at the same time were held for differing periods of time before being prepared for recording. The sensory responses obtained at 4 h after autotomy were just as strong as those recorded immediately after autotomy.

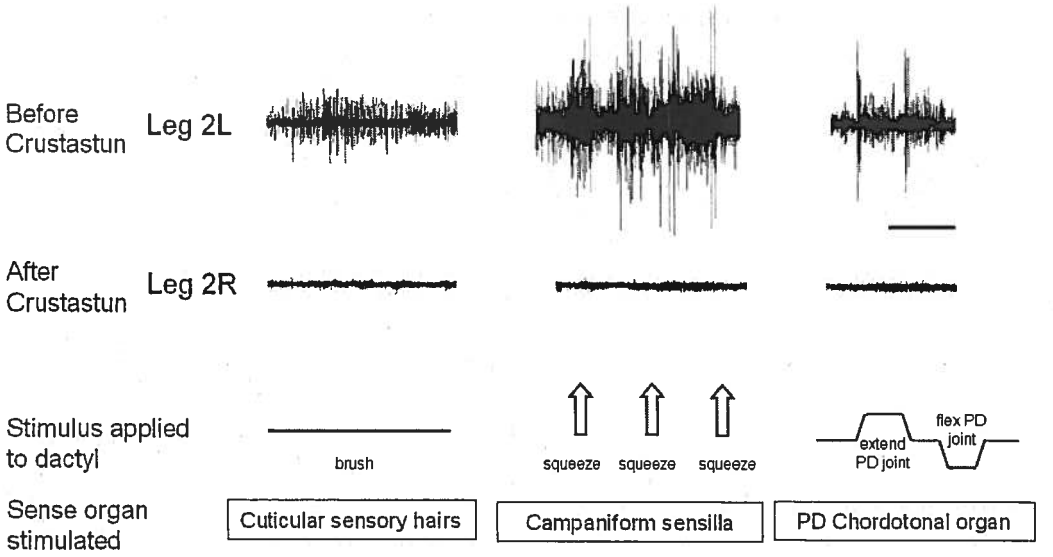

Figure 10. Responses of the leg nerve of the lobster *H. gammarus* to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower panels, leg amputated from an animal after Crustastunning. Scale bar 5 s.

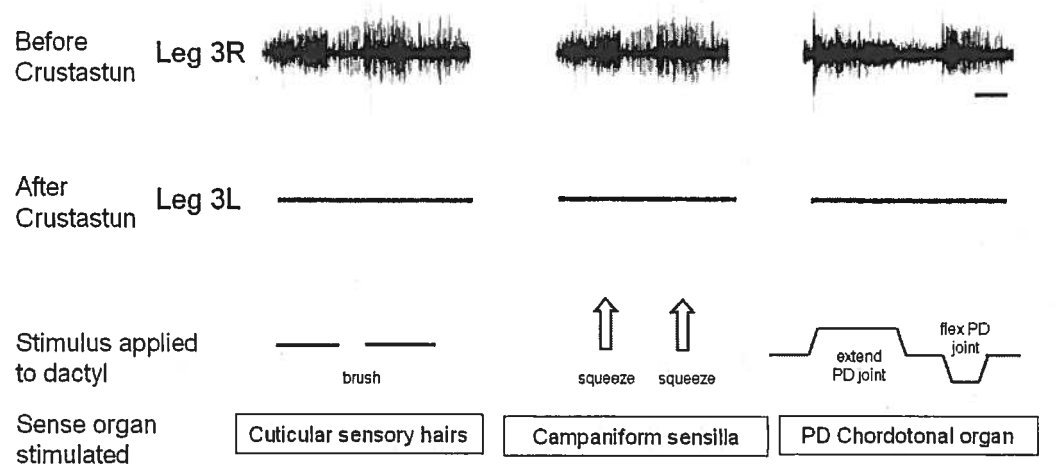

Figure 11. Responses of the leg nerve of a different lobster *H. gammarus* preparation to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower three panels, legs amputated from an animal after Crustastunning. Scale bar 5 s.

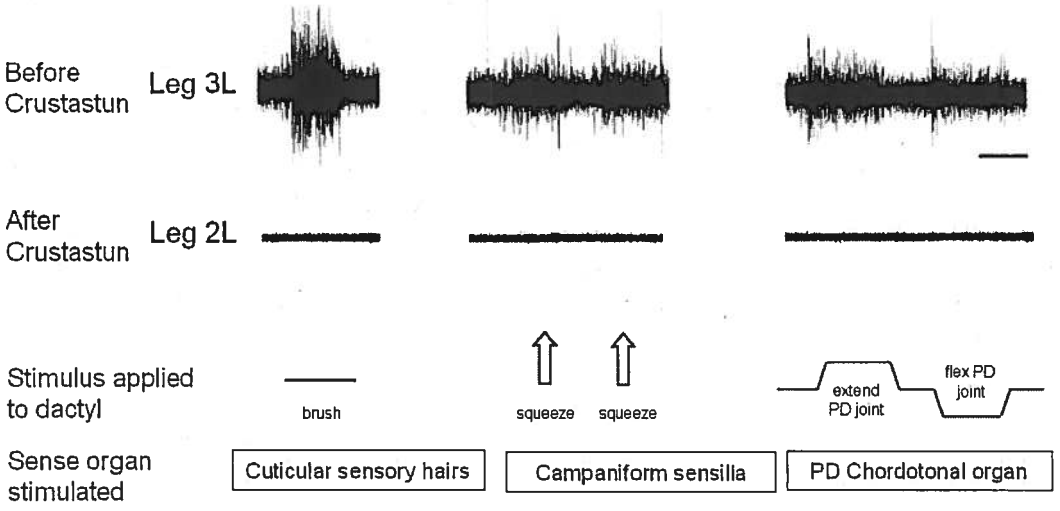

Figure 12. Responses of the leg nerve of the crab *C. pagurus* to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower panels, leg amputated from an animal after Crustastunning. Scale bar 5 s.

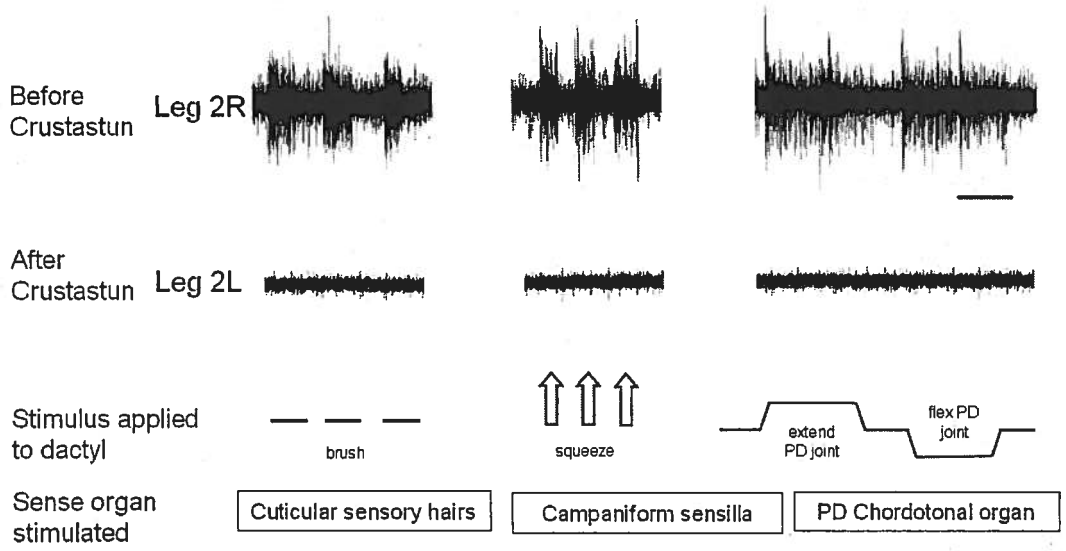

Figure 13. Responses of the leg nerve of a different crab *C. pagurus* preparation to three forms of stimulation of the dactylopodite. Top panels, leg autotomised from intact animal; lower three panels, legs amputated from an animal after Crustastunning. Scale bar 5 s.

Activity in the nervous system of crabs and lobsters following Crustastunning

After Crustastunning the stunned crabs showed no further visible movements (limb movement, antennule flicking, a ventilation current and eye retraction reflexes), and never recovered, i.e they were effectively killed. After Crustastunning the stunned lobsters showed either no further visible movements (limb movement, antennule flicking, a ventilation current, pleopod beating and eye retraction reflexes), or in a few cases showed some transient movements of the mouthpart exopodites and abdominal pleopods, lasting for a few seconds, and thereafter became immobile and never recovered, and were then effectively killed. One feature that was never observed in either the crabs or the lobsters as a result of Crustastunning was an evoked autotomy of either the claws or the walking legs (pereiopods).

Recordings from the central nervous systems of crabs and lobsters that had been subjected to Crustastunning indicated that no neuronal activity was detectable in the circumoesophageal connectives in any of the individual animals tested of either species (examples in Figures 5 and 6, lower panels). The abdominal nerve cords of the Crustastunned lobsters were also silent, with no indication of spontaneous neuronal activity (Figure 7, lower panel). As expected, due to this lack of central nervous system activity, there was no corresponding motor activity in the abdominal motor nerve roots of these Crustastunned lobsters (Figure 8, lower panel), which contrasts with the responses obtained in an intact lobster (see Figure 8, upper panel).

The recordings from the leg nerves of the Crustastunned crabs and lobsters provided a means of testing whether the peripheral system retained any ability to convey neuronal information, even though the central nervous system might be silent. However, in all the legs tested of either species there were no sensory responses to the three stimuli applied (Figures 10 and 11, lower panels for *H. gammarus*; Figures 12 and 13, lower panels for *C. pagurus*) and, in the tests performed on crabs, there was no muscle force development in response to stimulating motor nerves (Figure 9, lower panels).

DISCUSSION AND CONCLUSIONS

Activity in the nervous systems

The results obtained here from intact *Homarus gammarus* lobsters and *Cancer pagurus* crabs are very similar to those obtained previously on *Nephrops norvegicus* lobsters and *Carcinus maenas* crabs (Neil, 2010). They are also consistent with the literature on the neurophysiology of crustacean nervous systems (see, for example, the articles in Wiese, 2002) in showing that the central nervous systems of lobsters and crabs display continuous nerve activity, which in turn produces outputs in the motor nerves to the body and limb muscles. A large body of evidence, including studies conducted in this laboratory (Chachri et al., 1994; Holmes et al, 2002), indicates that this activity persists even when parts of the CNS are isolated from each other by severing the nerve cord at one or more levels (Larimer and Moore, 2003). Even isolated single ganglia of the abdominal nerve cord can produce patterned outputs (e.g. Chachri and Neil, 1993), and there is an extensive literature on the most-studied ganglion that can continue to operate in isolation, the stomatogastric ganglion (reviewed by Marder and Bucher, 2007).

It is therefore not surprising to have found in the present study that, as a result of dissection or of detaching the cephalothorax from the abdomen of the lobster, nerve activity continues to be recorded in the isolated anterior or posterior portions of the body, even though the nerve cord is transected at one or more levels. Also, as expected, this activity includes both descending signals from the brain and ascending signals from more posterior parts of the nervous system.

Although not attempted in these trials, it is without doubt that any procedure that attempted to make a sagittal section through a lobster or crab, in an attempt to destroy the entire nervous system, would inevitably leave small sections untouched and sufficiently intact to be able to continue generating patterned nerve activity, and to respond to sensory stimulation with reflex outputs localized to the muscles in the segments still innervated.

A characteristic feature that is common in these isolated parts of the nervous system is the long-lived nature of continued activity and signal conduction. It is widely reported that, provided the structures are bathed in an appropriate solution, activity can continue for many hours, and indeed, as in other lobsters and crabs (Neil, 2010), this was observed in the present study both with the central nervous preparations and with the isolated *H. gammarus* and *C. maenas* legs after autotomy. Such robustness makes it easier to interpret any loss of activity following a procedure such as Crustastunning as due to the intervention itself, rather than to any underlying decline in nervous system responsiveness.

The use of autotomised legs

Autotomy is a natural process for defence (Juanes and Smith, 1995), invoked by particular stimuli (Smith and Hines, 1991). In decapod crustaceans it involves a specific neuromuscular reflex acting across a fixed plane (McVean, 1976), although there may be a degree of voluntary control (Fleming *et al.*, 2007). In addition to specific mechanosensory

stimuli, crabs show autotomy when an appendage has been subjected to various other stimuli such as heating, shock, wounding, acetic acid injection or minor electric shocks to the appendages (Elwood et al. (2009), or when cooled by placing the animals on ice.

Induced autotomy was used in the present study to obtain an isolated leg from an intact lobster or crab, since it has been found that this process has virtually no measurable effect on the stress levels in the animal, as indicated by the low levels of L-lactate circulating in the haemolymph (Patterson *et al.*, 2007). Forced removal of a limb in the living animals was not employed, since it is known to induce a significantly greater stress, due to the greater extent of tissue damage imposed (Patterson et al., 2007). Limb amputation was only used on animals which had been killed by the Crustastunning process.

The effects of Crustastunning

The findings obtained on the effect of Crustastunning on nerve activity in lobsters and crabs are relatively conclusive. As far as can be determined from the extracellular recording method used, the various forms of spontaneous activity within the central nervous system were completely and almost instantaneously arrested. Consistent with this, there were no outputs produced in the motor nerves supplying the abdominal muscles of lobster, which are known to be synaptically driven from neurones within the CNS.

The recordings made on isolated lobster and crab legs allow some further conclusions to be drawn, namely that Crustastunning also has a specific effect on the functioning of the peripheral parts of the nervous system. There was both a loss of responsiveness to all three types of sensory stimulation, and also, as tested specifically in the crab, a failure in neuromuscular activation. The first of these effects would have rendered the animals insensitive to external stimuli, while the second would have rendered them paralysed and incapable of making movements.

Crustastunning did not induce autotomy in either of the species used in the present study, nor in the two other species studied previously (Neil, 2010). This finding, which is consistent with those made during routine commercial use of this instrument that autotomy of claws or legs never occurs, suggests that the observed inactivation of the sensory and motor divisions of the nervous system must have included the neuromuscular reflex pathways underlying autotomy. This result is especially striking since other attempts to electrically stun crabs using a low electrical field strength to the whole animal (Roth and Øines, 2010) failed to inactivate the animals, but actually caused extensive autotomy. Moreover, Elwood *et al.* (2009) found that weak electrical stimuli applied to the legs induced them to autotomise. A plausible interpretation of these different findings is that weak electrical stimuli artificially activate the sensory and/or motor pathways involved in the autotomy reflex, resulting in the shedding of the limb, whereas the Crustastunning inactivates these pathways more extensively and completely, so that no limb losses occur. Moreover, this would imply that neuronal inactivation by Crustastunning occurs very rapidly, before the reflex neuromuscular action underlying autotomy can be elicited. It is therefore possible that the inactivation of the central and peripheral nervous system found more widely in the present study following Crustastunning would also have occurred

almost instantaneously, although the methods used here were not appropriate to detect this directly.

Taken together these results indicate that as a result of Crustastunning the nervous system is incapacitated simultaneously at two levels, i.e. both centrally and peripherally, which completely prevents all normal neuronal functioning.

In terms of identifying the reasons for recording no sensory signals or inducing no motor activity in the peripheral nervous system, the recording method used does not allow definitive conclusions to be made. It is indeed possible that the conduction processes in the axons of both the sensory and motor neurones have been disrupted by the electrical currents generated by the Crustastun. However, it cannot be excluded that the Crustastunning has affected only the sensory transduction processes in the receptor endings of the sense organs, rather than the nerve transmission mechanism in the sensory nerves. Similarly, Crustastunning may have destroyed synaptic transmission at the neuromuscular junctions, and/or excitation-contraction coupling processes within the muscle fibres, rather than the nerve transmission mechanism in the motor nerves. It is of course possible that all of these processes have been affected simultaneously. To distinguish between these possibilities would require an examination of each of the contributing processes by using other, more appropriate, electrophysiological methods in a targeted approach.

Scope of conclusions

The results obtained in this study of the effects of Crustastunning on the brown crab, *Cancer pagurus* and the European lobster *Homarus gammarus* are consistent with those found previously in two other decapod crustacean species (the crab *Carcinus maenas* and the Norway lobster *Nephrops norvegicus*) (see Neil, 2010). This is to be expected considering the virtually identical anatomies and physiologies of the two species of each group. As such, the findings will also be applicable to species that are closely related to *C. pagurus* and to *H. gammarus*, namely the Dungeness crab *Metacarcinus* (formerly *Cancer*) *magister,* and the American lobster *Homarus americanus*, which are widely consumed seafood species in North America. In all these commercially important crustaceans, Crustastunning almost instantaneously arrests spontaneous activity within the central nervous system, with an accompanying loss of sensory responsiveness and a failure in neuromuscular activation.

Acknowledgements

This research has been conducted independently by DMN, using a Crustastun machine kindly loaned by Studham Technologies Ltd.

Thanks to Dr Keith Todd of the St Abbs Marine Station and the fishermen of St Abbs for the supply of animals. The technical assistance of Mr Graham Adam is gratefully acknowledged.

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16

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 to Studham Technologies Ltd.

by

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May 2012

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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°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°C - 12°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°C - 12°C). 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).

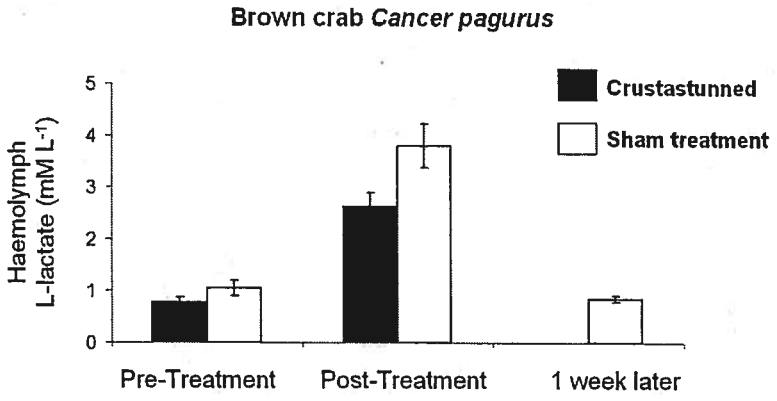

Figure 1. Haemolymph L-lactate concentrations in male brown crabs, *Cancer pagurus*, before and after Crustastunning or sham-treatment. Mean values ± SEM. N=6 for each treatment group.

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).

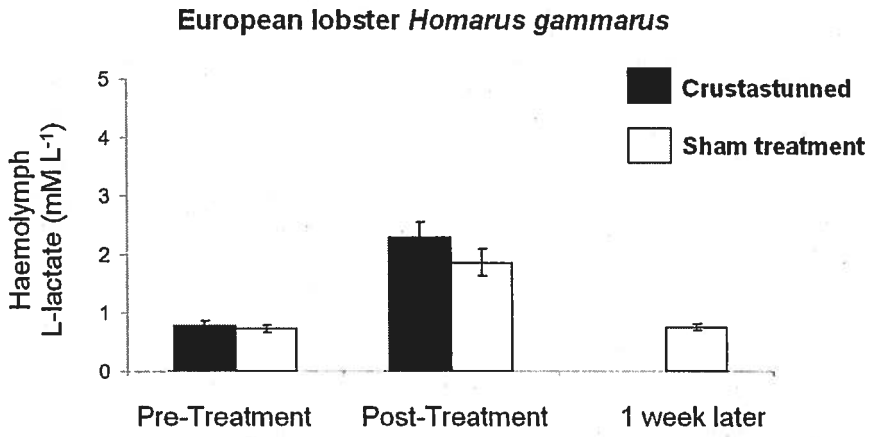

Figure 2. Haemolymph L-lactate concentrations in male European lobsters, *Homarus gammarus*, before and after Crustastunning or sham-treatment. Mean values ± SEM. N=6 for each treatment group.

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).

Acknowledgements

This research has been conducted independently by DMN and JT, using a Crustastun machine kindly loaned by Studham Technologies Ltd.

Thanks to Dr Keith Todd of the St Abbs Marine Station and the fishermen of St Abbs for the supply of animals. The technical assistance of Mr Graham Adam, the advice of Dr Andrew Watts and Ms Rosanna Milligan on statistical analysis, and the comments of Dr Amaya Albalat on the report are all gratefully acknowledged.

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