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The adaptive significance of crustacean hyperglycaemic hormone (CHH) in daily and seasonal migratory activities of the Christmas Island red crab *Gecarcoidea natalis*

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*Professor Morris died on 11th August 2009 before this work was completed. This paper is dedicated to his memory.

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SUMMARY

The Christmas Island red crab *Gecarcoidea natalis* undergoes extreme changes in metabolic status, ranging from inactivity during the dry season, to a spectacular annual breeding migration at the start of the wet season. The dramatic change in metabolic physiology that this polarisation entails should be reflected in changes in endocrine physiology, particularly that of the crustacean hyperglycaemic hormone (CHH), of which we know relatively little. CHH levels were measured using a novel ultrasensitive time-resolved fluoroimmunoassay (TR-FIA), together with metabolites (glucose, lactate), in the field at several scales of temporal resolution, during migratory activities (wet season) and during the inactive fossorial phase (dry season). Release patterns of CHH were measured during extreme (forced) exercise, showing for the first time an unexpectedly rapid pulsatile release of this hormone. A seasonally dependent glucose-sensitive negative-feedback loop was identified that might be important in energy mobilisation during migration. Haemolymph lactate levels were strongly correlated with CHH levels in both field and experimental animals. During migration, CHH levels were lower than during the dry season and, during migration, daytime CHH levels (when most locomotor activity occurred) increased. However, the intense dawn activity in both dry and wet seasons was not always associated with repeatable hyperglycaemia or CHH release. The results obtained are discussed in relation to the life history and behaviour of *G. natalis*.

Key words: *Gecarcoidea natalis*, Christmas Island, crustacean hyperglycaemic hormone (CHH), exercise, migration, activity patterns.

INTRODUCTION

It is well established that energy metabolism in malacostracan crustaceans is controlled by crustacean hyperglycaemic hormones (CHHs). The first described role of CHH was in the regulation of carbohydrate metabolism (for reviews, see Keller et al., 1985; Böcking et al., 2002; Chan et al., 2003; Fanjul-Moles, 2006). CHH release from the sinus gland results in hyperglycaemia due to the inhibition of glycogen synthase in the midgut gland and muscle (Sedlmeier, 1985). However, it is now widely appreciated that these hormones are pleiotropic; related biological functions include secretagogue activity (Sedlmeier, 1988) and lipid mobilisation (Santos et al., 1997), and a plethora of functions unrelated to regulation of energy mobilisation have now been discovered. These include the repression of ecdysteroid synthesis by the Y-organ, and thus a possible involvement in moult control (Webster and Keller, 1986; Chang et al., 1990), the inhibition of reproductive processes via the inhibition of methyl farnesoate synthesis (Liu et al., 1997), and the inhibition of *vitellogenin* (*Vg*) mRNA, protein synthesis (Khayat et al., 1998) or *Vg* gene expression (Tsutsui et al., 2005). An involvement in iono-osmoregulatory processes is also documented (Charmantier-Daures et al., 1994; Chung et al., 1999; Serrano et al., 2003), and it appears that CHH is involved in stimulation of ion transport across respiratory epithelia (Spanings-Pierrot et al., 2000) and in dipsogenesis during ecdysis (Chung et al., 1999).

Although CHH has long been considered to be secreted and released by the X-organ sinus glands in malacostracan crustaceans, it is now known that a variety of nervous and non-nervous tissues synthesize this hormone, or a similar one derived from alternative splicing. For example, intrinsic cells of the pericardial organs produce a CHH splice variant in several decapod crustaceans (Dircksen et al., 2001; Toullec et al., 2006; Ohira et al., 2006; Chung and Zmora, 2008), cells in the suboesophageal and second thoracic ganglion produce CHH in the lobster *Homarus americanus* (Chang et al., 1999), as do paraneurones in the gut of *Carcinus maenas* during the premoult (Chung et al., 1999), and peripheral abdominal neurones of zoea larvae (Chung and Webster, 2004).

Despite these fundamental advances, which relate mainly to the structures, functions and distributions of CHH, we still know remarkably little about the adaptive significance of these hormones. Functions related to energy metabolism might be readily defined by experimental manipulation and careful measurement of hormone titres, together with the measurement of changes in glucose levels, yet in relatively few instances has this been done with sufficient precision and temporal resolution to enable unequivocal conclusions to be drawn. CHH release occurs after stressful episodes (Keller and Orth, 1990; Webster, 1996; Chang et al., 1998; Wilcockson et al., 2002; Zou et al., 2003; Chung and Webster, 2005; Chung and Zmora, 2008), exposure to heavy metal pollutants (Lorenzon et al., 2004) or parasite load (Stentiford et al., 2001). Nevertheless, we still know relatively little about the adaptive roles of CHH in the life history of crustaceans.

To address this issue, we reasoned that a suitable model crustacean would be one that experiences both profound and extreme changes in seasonal variables, and thus in physiology, particularly for those processes relating to energy mobilisation and ionic homeostasis. Thus, a suitable model could be the Christmas Island red crab, *Gecarcoidea natalis*. This crab undergoes extensive annual

migrations of several km in order to mate and spawn; these migrations are triggered by monsoonal rains, and this activity is the most energetically demanding and extreme rapid migration known in crustaceans. Furthermore, during the dry season, this crab must also undergo extended periods of inactivity in a xeric environment (Hicks, 1985; Green, 1997; Adamczewska and Morris, 2001a; Adamczewska and Morris, 2001b). So clearly, physiological demands related not only to energy mobilisation, but also to ionic homeostasis are germane, particularly when the proposed ionoregulatory functions of CHH are considered. Thus, the unique annual polarisation in life history of this crab might allow us to address pertinent questions regarding the roles of CHH. In this study, we have measured hyperglycaemic hormone levels in the field, together with relevant metabolites, over seasonal cycles, during specific behavioural patterns and during exercise, to further define the role of CHH in energy mobilisation and seasonal adaptation.

MATERIALS AND METHODS

Animals and experiments

Field sampling

Specimens of intermoult male 240–400 g *Gecarcoidea natalis* (Pocock) were collected under permit from Parks Australia, from sampling sites within the Christmas Island National Park that have been described previously (Adamczewska and Morris, 2001a). During the dry season (June–November/December) the 'Pre-migration' sample site was used. During the monsoon season (November/December–May) the 'Migration' sample site was used. Haemolymph samples (1.25 ml) for CHH estimations were taken from the base of a walking leg with a hypodermic syringe and needle and immediately mixed with an equal volume of anticoagulant (0.3 mol l^{-1} NaCl, 20 mmol l^{-1} trisodium citrate, 26 mmol l^{-1} citric acid, 1 mmol l^{-1} EDTA) in a Minisorp tube (NUNC Thermo Fisher Scientific, Roskilde, Denmark), then snap-frozen on dry ice. Small haemolymph samples (0.5 ml) were also taken at this time for lactate and glucose estimations. Care was taken to take samples as rapidly as possible, within 1 min of capture, and with minimum trauma. Following sampling, crabs were marked with a non-toxic acrylic aerosol spray to avoid subsequent re-sampling and were returned to the rainforest. Dry season sampling was carried out during July and August 2006–2008. Sampling at the beginning of the wet (monsoon) season, when crabs were migrating, was carried out during November and December 2006–2007. In an attempt to maximise the amount of correlative information obtained from field collections, particularly in view of probable short-term changes involving episodic release of CHH, balanced against longer term changes in metabolites, a variety of sampling regimes for non-migrating (dry season) and migrating (wet season) crabs were followed, from short (minutes) to long (seasonal) timescales. These were as follows. (1) Dawn sampling at 20–30 min intervals (N=10–12) from 04:00 to 09:00 h, to encompass the *ca.* 2 h activity peak that occurs just after sunrise (Green, 1997). (2) Twenty-one hour sampling at 3 hourly intervals (N=8–16) from 05:00 to 02:00 h during the dry and wet seasons. (3) Eighteen hour sampling at 6 hourly intervals (N=15–16) from 05:00 to 23:00 h for migrating and non-migrating crabs. (4) Seasonal sampling (N=15–20) during the dry season, seaward migration, burrowing, fighting and upon the landward return of crabs to the forest. Times of sunrise/sunset, twilight (which occurred about 25 min later) and astronomical twilight (50–70 min before or after sunrise/sunset) were recorded. Additionally, at some of these times, samples of muscle from a walking leg were taken, by causing autotomy of the second walking leg by pinching the merus with pliers. Muscle tissue (0.3–0.6 g) was rapidly removed and stored on dry ice. Tissues were subsequently processed for glycogen estimation (see below).

Field experiments

During both dry and wet seasons (June and December 2007), crabs (*ca.* 240 g) were collected and held in a large 3×3 m enclosure in the rainforest, in an attempt to minimise capture stress. Crabs were then separated in groups of 12 into polypropylene boxes of approximately 0.5 m^2 area, with free access to water, and were injected at 8 h intervals for 3 days with 150 µl red crab saline (Morris and Ahern, 2003) containing one of the following: (1) no addition; (2) glucose, to give an estimated haemolymph concentration of 3 mmol l^{-1}; (3) sodium lactate, to give an estimated haemolymph concentration of 10 mmol l^{-1}; (4) amino acids (proline, alanine, glycine, asparagine) to give estimated concentrations of 0.3 mmol l^{-1} of each amino acid; (5) phospholipid/lipids, a mixture of lecithin and corn oil, dissolved in glycerol, which was mixed with saline to give final estimated concentrations around 10 mg of each lipid per crab. Estimates of final circulating metabolite concentrations (and hence dilution factors needed) were made assuming a haemolymph volume of 72 ml for a 240 g crab. Crabs were then individually removed, initial 1.25 and 0.2–0.5 ml haemolymph samples taken as described earlier, and a final single injection of one of the appropriate treatments shown above given. Each crab was exercised by persuading it to walk continuously for 10 min by tapping gently on the carapace and providing visual stimulation. Further haemolymph samples (1.25 and 0.2–0.5 ml) were then taken.

Laboratory experiments

Specimens of male *G. natalis* were air freighted to the UK. Appropriate export licences for live animals and tissue samples were obtained from The Department of the Environment and Water Resources, Australia, and likewise, for licences for import to the UK, from the Department for Environment, Food and Rural Affairs (DEFRA). Crabs were held in a large 1 m^2 earth-filled terrarium (25°C, light:dark 12 h:12 h, 80–100% relative humidity). They were fed a diet of leaves, carrot, apple and dried cat food, and had free access to water. Under these conditions, healthy animals could be kept for over a year. Treated crabs (N=5) were exercised as detailed earlier, controls (N=5) were kept quiescent. Haemolymph samples (500 µl) were taken at 0, 5, 10, 20, 30, 60 and 120 min intervals after the start of the 10 min exercise period.

Purification of CHH and antiserum production

G. natalis were collected from Christmas Island during the dry season (June 2006, June 2007). Freshly collected batches of crabs were anaesthetised on ice until they were unresponsive, eyestalks removed, and sinus glands (SGs) microscopically dissected in the field and immediately snap-frozen in microcentrifuge tubes packed in dry ice. Batches of SGs (1500 total) were air freighted on dry ice to the UK. SGs were extracted in ice-cold 2 M acetic acid in small batches (*ca*. 30), centrifuged (14,000 ***g***, 5 min, 4°C) and immediately separated by HPLC [4.6×300 mm Jupiter C_{18} 300 Å (Phenomenex, Macclesfield, UK), 40–80% solvent B over 40 min, 1 ml min^{-1}, detection at 210 nm; solvent A, 0.11% trifluoroacetic acid (TFA); solvent B, 60% acetonitrile, 0.1% TFA]. The peak corresponding to CHH was tentatively identified by dot-blot (Dircksen et al., 1987) on nitrocellulose strips, using existing CHH antisera raised against *Carcinus maenas* and *Cancer pagurus* CHH. Complete identification of *G. natalis* CHH (S.M., U.P., M., L.M.T., J.P. and S.G.W., unpublished observations) was verified by cDNA cloning from eyestalk RNA, via degenerate primer PCR, 3′,

5′RACE, and mass determination by MALDI-TOF MS (GenBank Accession No. EF095546). CHH was quantified by amino acid analysis as detailed previously (Webster, 1991). Antiserum against *G. natalis* CHH was raised commercially (Davids Biotechnologie, Regensberg, Germany). A single New Zealand White rabbit was immunised by multiple subcutaneous injection of 10 nmol CHH at thirty-day intervals. The first immunisation used Freund's complete adjuvant, the subsequent two and a final booster (5 nmol) used incomplete adjuvant. The rabbit was terminally exsanguinated after 90 days under deep anaesthesia. All national animal welfare legislation was followed. Antiserum specificity was confirmed by dot-blotting HPLC fractions of SG and immunohistochemistry of paraffin-embedded eyestalks.

CHH time resolved-fluoroimmunoassay (TR-FIA)

Anti-CHH IgG was purified on a Sepharose CL-4B protein A column, as previously described (Webster, 1993), quantified on a NanoDrop ND-1000 (NanoDrop products, Wilmington, DE, USA), and stored at 10 mg ml^{-1} at –20°C in PBS. The IgG (1.2 mg) was buffer exchanged into 100 µl 0.1 mol l^{-1} borate buffer, pH 8.2, and was biotinylated, using 10 mmol l^{-1} Sulfo-NHS-LC-biotin (Thermo Pierce, Warrington, UK) at 20-fold molar excess, at 0°C overnight, with constant stirring. Excess reagent was firstly removed using a Zeba spin column (Thermo Pierce), and then by extensive ultrafiltration on an Amicon Ultracel YM-30 cartridge (Millipore, Billerica, MA, USA). Biotinylated IgG was quantified as described, and stored at 5 mg ml^{-1} at –20°C. Under these conditions the conjugated IgG was stable for at least one year. Non-competitive 'sandwich type' immunoassays were performed in 96-well high-binding microtitre plates (Costar 3590, Corning, VWR International, East Grinstead, UK). Plates were coated (overnight, 4°C) with 100 µl/well 10 µg ml^{-1} anti-CHH IgG in 0.1 mol l^{-1} sodium bicarbonate buffer, pH 9.3. Plates were then washed with the same buffer (3×), incubated for 1 h with 250 µl/well blocking buffer (0.1% BSA in 50 mmol l^{-1} Tris pH 8.0 containing 0.85% NaCl) and blotted thoroughly. Standard CHH dilution series (50 fmol–50 amol per 100 µl) or purified haemolymph samples, dissolved in assay buffer (0.05 mol l^{-1} sodium phosphate pH 7.5, 0.15 mol l^{-1} NaCl, 0.01 mol l^{-1} $MgCl_2$, 0.05% casein), were added in duplicate to the wells and incubated overnight at 4°C. After washing (6×) in Delfia buffer (PerkinElmer, Waltham, MA, USA), plates were incubated in 100 µl/well biotinylated anti-CHH IgG (5 µg ml^{-1}) for 6 h at room temperature (RT). Plates were then washed (6×) and incubated (2 h, room temperature) with 100 µl/well Europium-labelled streptavidin (PerkinElmer) diluted to 100 ng ml^{-1} in proprietary assay buffer (PerkinElmer). Plates were then washed extensively (8×) and 50 µl/well enhancement solution (PerkinElmer) added followed by vigorous orbital shaking for 2 min (Heidolph Vibramax 100). Time-resolved fluorescence of Europium was measured on a PerkinElmer Victor^2 1420 instrument (Wallac, Turku, Finland) equipped with proprietary software (Workout) for data analysis. To detect possible assay drift, every plate assayed included a duplicated internal standard sample derived from a large sample of purified haemolymph, which had been spiked with CHH prior to purification, to give a nominal concentration (per well) of 2.5 fmol/50 µl haemolymph. It was essential that samples were purified before assay. After gentle thawing, 1 ml of haemolymph (which contained an equal volume of anticoagulant) was diluted 1:1 with water, and slowly passed over 200 mg Strata-X polymeric reverse-phase cartridges (Phenomenex) previously conditioned with 3 ml isopropanol and 10 ml water, using a 20 port vacuum manifold (International Sorbent Technology, Glamorgan, Wales). Cartridges were then washed with 10 ml water before peptides were eluted with 3 ml 60% isopropanol and dried on a vacuum centrifuge. Samples were then reconstituted with assay buffer (see above), sonicated thoroughly, and volumes adjusted to give a final dilution equivalent to 50 µl haemolymph per well (100 µl). Spike/recovery experiments showed that CHH recovery was greater than 95% under these conditions (data not shown). A standard curve (fitted, 4 parameter logistic), obtained from two independent experiments, with duplicated samples is shown in Fig. 1. The assay range covered three orders of magnitude, and the detection limit of the assay was always less than 50 amol/well.

Biochemical assays: glucose, lactate, glycogen

Glucose concentrations in haemolymph samples were measured using the glucose oxidase/peroxidase method essentially as described by Webster (Webster, 1996), except that 50 µl haemolymph samples were deproteinised with an equal volume of 6% perchloric acid before being neutralised with 20 µl 2.5 mol l^{-1} K_2CO_3. The enzyme reagent consisted of 125 µg horseradish peroxidase, Type VI-A, 5 mg glucose oxidase, Type VII, 100 mg ABTS dissolved in 100 ml 0.2 mol l^{-1} sodium phosphate, pH 7.5 (reagents from Sigma, Poole, UK). For haemolymph lactate determination, deproteinised haemolymph samples were assayed using a commercially available kit (BioSenTec, Toulouse, France) that measures the formation of NADH from the action of lactate dehydrogenase in the presence of glutamate pyruvate transaminase. For muscle glycogen determination, the modified method of Bergmeyer (Bergmeyer, 1985), as detailed by Adameczeska and Morris (Adameczeska and Morris, 2001b) was used. All of the above enzyme assays were performed as end-point determinations on a Fluostar Optima ABS microplate reader (BMG Labtech, Aylesbury, UK).

Statistical analysis

Calculation of descriptive statistics and analyses were performed using SPSS 15.0. On the basis of calculation of distributions (normality, or otherwise), appropriate parametric (Tukey–Kramer multiple comparisons) or non-parametric (Dunn's multiple comparisons, Scheirer-Ray-Hare multiple ANOVA, Spearman's test) statistical tests were performed.

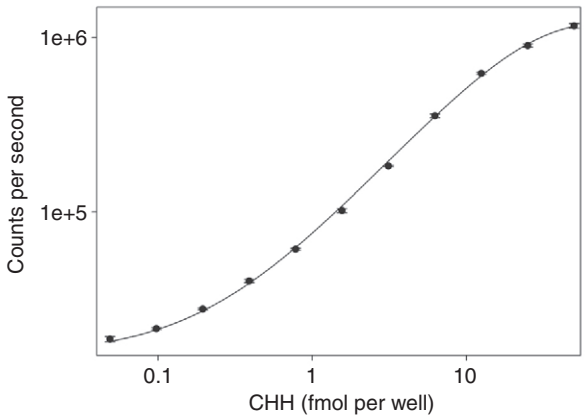

Fig. 1. Standard curve for *Gecarcoidea natalis* crustacean hyperglycaemic hormone (CHH) time-resolved fluoroimmunoassay (TR-FIA). Results (means ± s.e.m.) are from two independent experiments with duplicated standards (50 fmol–50 amol per well).

RESULTS

Laboratory experiments: exercise/recovery

Experiments designed to determine the effect of short, intense exercise regimes were performed on laboratory acclimated crabs, repeatedly measuring CHH, glucose and lactate levels in the haemolymph of individuals for 110 min following 10 min exercise. The results are shown in Fig. 2. Within 5 min of the start of exercise, lactate increased dramatically (all results are shown as mean ± s.e.m., 0.24±0.07 to 11.0±1.28 mmol l^{-1}), a highly significant increase compared with that of controls (0.18±0.07 to 0.54±0.09 mmol l^{-1}). Highly significant differences (P<0.001) between exercised and control crabs remained at 110 min post-exercise. For glucose, significant (P<0.05) differences between control and exercised crabs only became apparent at 50 min post-exercise (control, 0.46±0.08 mmol l^{-1}; exercised, 2.01±0.22 mmol l^{-1}), and at 110 min post-exercise highly significant differences (P<0.001) in haemolymph glucose between exercised and control crabs were observed (control, 2.87±0.655 mmol l^{-1}; exercised, 7.29±0.99 mmol l^{-1}).

CHH levels increased very rapidly (fivefold) during the exercise period (t=0, 10.1±1.5 pmol l^{-1}; t=10, 50.8±9.6 pmol l^{-1}) compared with controls, which exhibited a modest twofold increase (t=0, 8.4±0.78 pmol l^{-1}; t=10, 16.0±2.28 pmol l^{-1}). The difference compared with controls was highly significant (P<0.01) at this time. Much greater variability was seen in the exercised crabs, but all animals showed rapid increases in CHH during exercise, and a decline during the post-exercise recovery period; indeed, within 50 min of recovery, CHH levels were essentially the same (but still higher than at the beginning of the experiment) in both control and exercised crabs.

Field experiments: feedback loops

To determine possible feedback loops involved in CHH release, crabs were injected with a variety of substrates/metabolites that could be considered to be potentially important in energy metabolism. Glucose, lactate, phosholipids, amino acids and a saline control were injected at 8 h intervals for 3 days, followed by a final injection and an intensive 10 min exercise period. Changes in circulating CHH levels are shown in Fig. 3. For experiments carried out during the dry season (June/July 2007), 10 min of exercise induced a massive release of CHH for crabs injected with lactate, phospholipids, amino acids or control saline: Levels of CHH increased significantly (P<0.001) from *ca.* 13–30 to 62–72 pmol l^{-1}. For crabs injected with glucose, this increase was much less marked but still significant (P<0.05; 26±3.8 to 46±4.3 pmol l^{-1}). When these experiments were performed during the wet (monsoon) season (December 2007), when crabs were migrating, exercise after injection of lactate, phospholipids, amino acids or control saline again resulted in highly significant (P<0.001) increases in CHH from *ca*. 17–40 to 75–111 pmol l^{-1}. However, when crabs were injected with glucose at this time, exercise resulted in a very small, insignificant rise (17±4.3 to 29±4.4 pmol l^{-1}) in circulating CHH levels. By pooling the data for all crabs, but excluding those injected with glucose, the highly significant (P<0.001) increases in CHH levels (from 24.7±2.0 to 82.7±3.6 pmol l^{-1}) following exercise were associated with highly significant (P<0.001) and dramatic increases in haemolymph lactate (from 0.47±0.08 to 13.2±0.89 mmol l^{-1}), as shown in Fig. 4. Before exercise there was a highly significant correlation (P=0.0003, R=0.38) between lactate and CHH levels; this correlation still existed after exercise, when both CHH and lactate levels had increased, but it was much less marked (P=0.05, R=0.21).

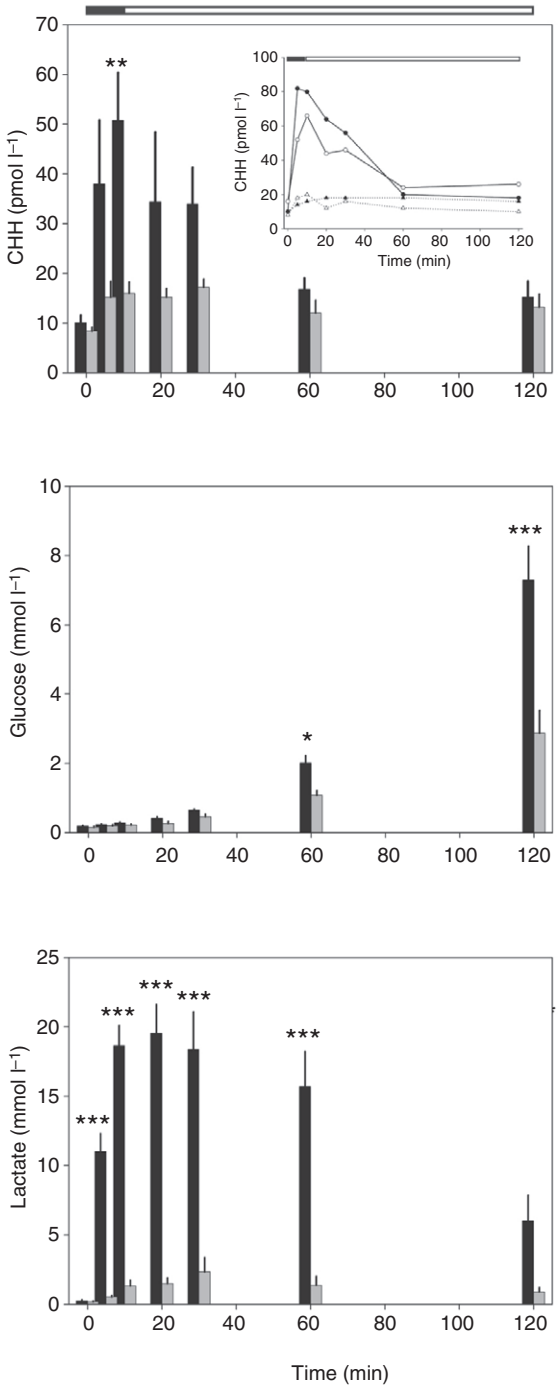

Fig. 2. The effect of exercise on circulating CHH, glucose and lactate levels. *Gecarcoidea natalis* (N=5) were exercised for 10 min (black bar), followed by a 110 min recovery period (white bar). Black columns show exercised crabs, grey columns, unexercised controls (means + s.e.m.). Inset shows individual CHH profiles from two exercised (solid lines, circles) and two control (dotted lines, triangles) crabs. For CHH, **P<0.01 (Dunn's multiple comparisons); for glucose and lactate, *P<0.05, ***P<0.001 (Tukey–Kramer multiple comparisons). Statistical tests were performed by comparing controls with exercised crabs at each timepoint.

Field sampling

Seasonal changes

Haemolymph samples were analysed from field-collected animals during the dry season (when crabs were inactive) and at the

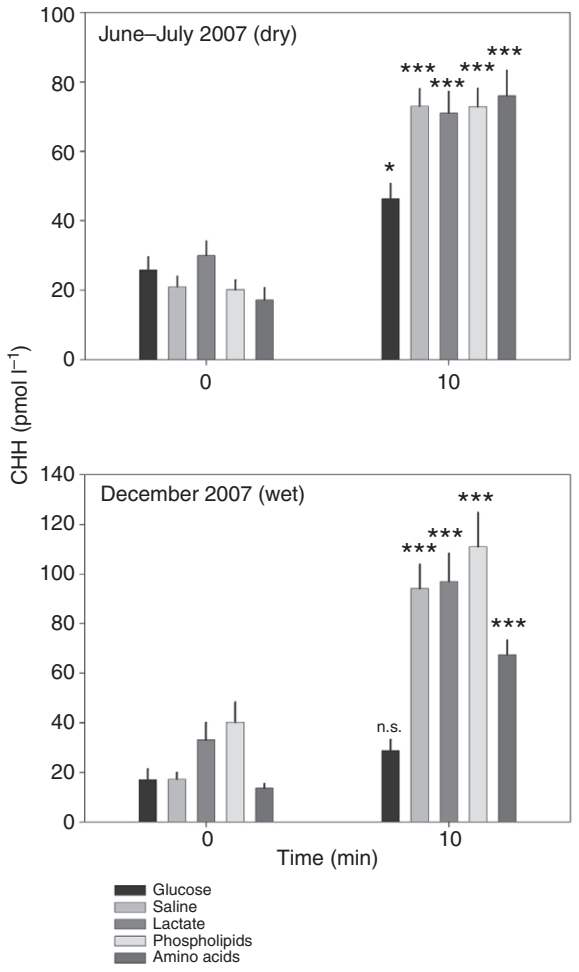

Fig. 3. The effect of exercise on haemolymph CHH levels in *G. natalis* previously injected with metabolites (glucose, lactate, phospholipids, amino acids) or control saline as detailed in the text. Haemolymph samples were taken before and after 10 min exercise periods, for dry season (June and July 2007, *N*=10) and wet season (December 2007, *N*=12) crabs. Bars show means + s.e.m. **P*<0.05, ****P*<0.001, n.s.=not significant (Tukey–Kramer multiple comparisons).

beginning of the wet season (when crabs were migrating) from 2006–2008. Summarised data for haemolymph glucose, lactate, CHH and muscle glycogen are shown in Table 1. For glucose, dry season haemolymph concentrations were generally about 0.1 mmol l^{-1}. By contrast, during the wet season (migration and digging phases), glucose levels were significantly elevated (0.2±0.04 to 0.67±0.01 mmol l^{-1}). For crabs returning to the rainforest after spawning, glucose concentrations were within the range seen during the dry season. Haemolymph lactate levels were generally rather invariant, between 1 and 2 mmol l^{-1}; however, for crabs engaged in digging and fighting behaviours, levels were often elevated (for the samples taken during digging and fighting in 2007, mean lactate levels were 5.6±0.8 mmol l^{-1} and 3.65±0.98 mmol l^{-1}, respectively). Circulating CHH levels were, in general, between 20 and 40 pmol l^{-1}. However, on occasion, very low mean CHH titres (<10 pmol l^{-1}) were observed, in particular in the wet season during migrating and digging phases; these values were significantly lower than all of the others (*P*<0.05–0.01). For muscle glycogen levels, the somewhat limited sampling showed that levels were generally between 17 and 36 mmol kg^{-1}. However, for crabs returning to the rainforest after the 2006 spawning migration, muscle glycogen was dramatically depleted (4.6 mmol kg^{-1}).

Daily changes

A summary of data for samples taken at 6 h intervals during the wet season of 2007 and the dry season of 2008 is shown in Table 2. Seasonal differences in circulating glucose levels were apparent: during the wet season, levels were between 0.14 and 0.25 mmol l^{-1}; and in the dry season, between 0.06 and 0.15 mmol l^{-1}. The highest circulating glucose levels were seen in the morning (11:00 h). These differences were highly significant, with respect to season (*P*<0.0005) and time (*P*=0.02; Scheirer-Ray-Hare multiple ANOVA). Mean CHH levels were once again significantly lower (*P*<0.05–0.001) in the wet season of 2007 (7.3–9.4 pmol l^{-1}) than in the dry season of 2008 (25.5–43.1 pmol l^{-1}). Circulating lactate levels were rather invariant (1–2 mmol l^{-1}), but were generally lower during darkness, when the crabs were unresponsive and quiescent. This was particularly marked during the wet season, when daytime levels (*ca.* 2 mmol l^{-1}) were significantly depressed at night (*ca.* 0.8 mmol l^{-1}). Muscle glycogen levels were fairly constant (15–35 mmol kg^{-1}). However, levels in crabs sampled during the morning in the wet season (35–37 mmol kg^{-1}) were significantly higher (*P*<0.05–0.01, Tukey–Kramer multiple comparisons) than in those sampled at other times of the day, and during the dry season (15–24 mmol kg^{-1}).

For crabs sampled at 3 h intervals over 24 h during the wet (2006) and dry (2007) seasons (Fig. 5), levels of CHH (30–40 pmol l^{-1}) lactate (2.5–3.5 mmol l^{-1}) and glucose (0.01–0.1 mmol l^{-1}) were quite invariant during the dry season. However, during the wet season, daytime elevations of both CHH and lactate were noted. CHH levels increased significantly (*P*<0.05–0.001) from *ca.* 10 pmol l^{-1} at dawn to *ca.* 30 pmol l^{-1} during the day, declining again to low levels (10 pmol l^{-1}) by the end of the night. Lactate levels also increased significantly (*P*<0.05–0.001) during the day from *ca.* 1 mmol l^{-1} at dawn to *ca.* 3 mmol l^{-1} during the day, declining to dawn values by the end of the night. When CHH and lactate levels in individuals were compared for 3 h sampling periods during wet and dry seasons, highly significant (*P*<0.001) correlations were observed (Fig. 5). Glucose levels in wet season crabs were variable (0.2–0.6 mmol l^{-1}), but again, much higher than in dry season crabs (0.02–0.09 mmol l^{-1}). Comparison of samples taken during the 3 h sampling experiments in the wet season of 2006 (Fig. 5) with those taken during 6 hourly sampling in the corresponding wet season of 2007 (Table 2) showed that levels of both CHH and glucose were both lower during the wet season of 2007 than during that of 2006, which presumably reflects yearly variation.

Samples of haemolymph taken from crabs at 20–30 min intervals during the night/day transition during the dry season, the wet season migration, and burrow digging (2007–2008) were assayed for CHH and glucose (Fig. 6). For crabs sampled during burrow digging, glucose levels were somewhat invariant (0.05–0.15 mmol l^{-1}). For CHH, a clear peak (*ca.* 25 pmol l^{-1}) was seen about 40 min before sunrise. However, this pattern was not seen in migrating crabs. In these, glucose levels were somewhat higher than in burrow-digging crabs, between 0.1 and 0.3 mmol l^{-1}, and there was no obvious correlation of CHH level with time of day. For dry season crabs, CHH and glucose levels were variable, and showed no obvious pattern, except for a post-dawn hyperglycaemia and an increase in CHH levels at dawn, followed by an increase in hormone levels about 1 h later (September 2008).

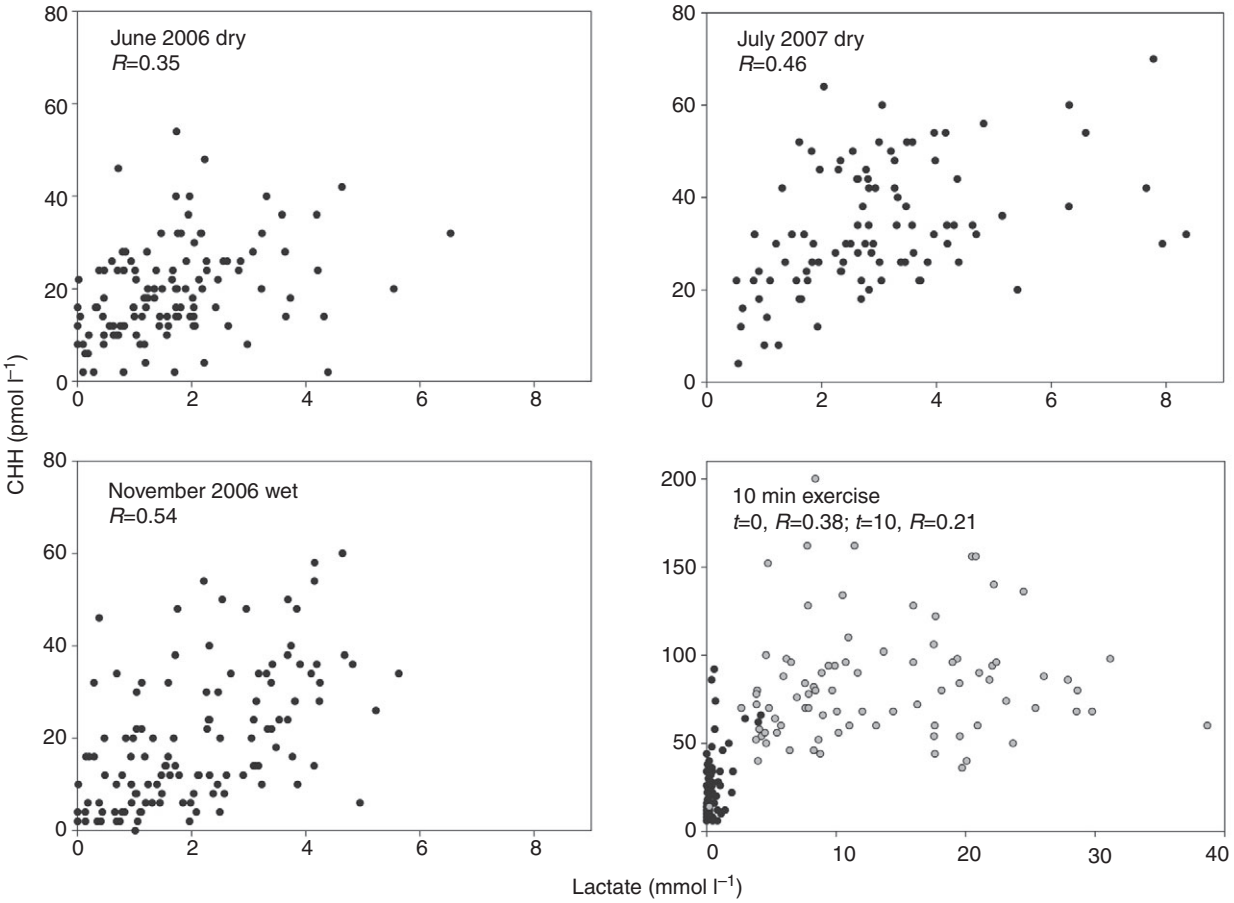

Fig. 4. Correlation of circulating lactate levels with CHH. Haemolymph samples were taken at 3 h intervals over 24 h cycles during the dry season (June 2006, *N*=122; July 2007, *N*=96) and the wet season (November 2006, *N*=122). Highly significant correlations (*P*<0.001; the Spearman's correlation coefficient, *R*, is shown in each panel) between CHH and lactate levels were observed. Lower right panel shows correlation between CHH and lactate levels, before (black circles) and after (grey circles) 10 min exercise, in crabs (*N*=86) previously injected with metabolites as detailed in Fig. 3, but excluding those injected with glucose. At *t*=0, lactate levels were highly significantly correlated with CHH (*P*=0.0003, Spearman's *R*=0.38). After exercise, levels of both CHH and lactate were raised significantly (*P*<0.001, Dunn's multiple comparisons), and correlation between CHH and lactate was still significant (*P*=0.05, Spearman's *R*=0.21).

DISCUSSION

In the present study, the adaptive significance of CHH in metabolic adaptation of the Christmas Island red crab *Gecarcoidea natalis* was investigated using primarily a field-based experimental approach in which haemolymph CHH and relevant metabolite levels were measured. We reasoned that this crab would be an ideal model for such studies in view of (1) the extreme annual breeding migration that involves extensive (4–5 km) and occasionally rapid (5–6 day) migration with maximum walking speeds of up to 6 m min^{-1} (Adamczewska and Morris, 2001a); and (2) the fact that, in extreme contrast, during the dry season they are relatively inactive, becoming fossorial, only venturing out of their burrows to feed at dawn and late afternoon (Hicks, 1985; Green, 1997). Dry season activity is probably restricted to avoid dessication, and thus the physiology of energy mobilisation and osmo/ionoregulation of dry season crabs could be entirely different to those of wet season crabs, as previously suggested (Adamczewska and Morris, 2001a). To perform accurate measurements of circulating CHH levels, development of an ultrasensitive and specific immunoassay was mandatory. Previously we have used ^{125}I-based radioimmunoassays (RIA) to measure CHH in the haemolymph of various crabs (Webster, 1996; Chung and Webster, 2005), but these, whilst being reasonably sensitive, require relatively large (1–2 ml) haemolymph samples to be taken, and possibly preclude repeated sampling from individuals, as this might involve undue stress. Furthermore, we previously knew nothing regarding the basal circulating levels of CHH in *G. natalis*, which might have been very small. However, by using the strategy first reported for the measurement of vanishingly low levels of moult-inhibiting hormone (MIH) in the crayfish *Procambarus clarkii* (Nakatsuji and Sonobe, 2003), an ultrasensitive time-resolved fluoroimmunoassay (TR-FIA) was developed using a polyclonal antibody raised against native, HPLC-purified *G. natalis* CHH. The exceptional sensitivity and precision of this assay (detection limit $<50\times10^{-18}$ moles per well) allowed small (<100 μl) haemolymph samples to be readily assayed, with the possibility of measuring multiple samples from the same animal in the laboratory or field, and thus permitting rapid (within minutes) 'real-time' changes in hormone levels to be measured, the first time this has been done, to our knowledge, for any crustacean.

Seasonal breeding migrations require extensive locomotor activity, and most crustaceans have quite a limited aerobic scope and rapidly become anaerobic, with obvious lactacidosis, when

forced to undergo sustained walking at maximum speed (for reviews, see McMahon, 1981; Booth and McMahon, 1985; Head and Baldwin, 1986; Herreid and Full, 1988; Greenaway et al., 1988; Forster et al., 1989; Van Aardt, 1990; Henry et al., 1994; Adamczewska and Morris, 1994; Adamczewska and Morris, 1998). Thus, we were interested in determining CHH release profiles, in conjunction with the measurement of pertinent metabolites (glucose, lactate), during brief periods of forced exercise (near or at maximum capacity) and also during the recovery period. Within the first 5 min of forced exercise, circulating lactate levels rose dramatically to *ca.* 10 mmol l^{-1}, and increased further to *ca.* 20 mmol l^{-1}, and these levels were sustained long after (1 h) the termination of exercise. These patterns of lactate accumulation and recovery were extremely similar to those recorded from identical exercise regimes in other land crabs (Henry et al., 1994). During the first 5 min of exercise, CHH was released, reaching maximum levels after 10 min, when the crabs' walking speed declined dramatically. Thereafter, CHH levels decreased quite rapidly, and within an hour, levels were similar to those of controls. Although the half-time of injected CHH was not measured in this study, it is likely to be brief, in the order of *ca.* 10 min, as we have observed for other crabs (Webster, 1996; Chung and Webster, 2005), thus the rapid decline in CHH levels observed following exercise is likely to reflect a dramatic reduction or cessation of CHH release within 10 min of the end of this period. Although a significant increase in haemolymph glucose (*ca.* 2 mmol l^{-1}) was first observed at 50 min post-exercise in accordance with earlier work (Adamczewska and Morris, 1994), it should be noted that, in that study, significant increases in muscle glucose were seen between 10 and 20 min into the exercise period, and that muscle glucose concentrations at this time were much higher (*ca.* 10 mmol l^{-1}) than circulating levels (*ca.* 1 mmol l^{-1}). Thus it seems likely that CHH release causes a significant tissue hyperglycaemia within 10 min, which is in keeping with the view that signal transduction cascades following receptor binding by peptide hormones have timescales of just a few minutes (Ruf et al., 2007).

In view of the coincident patterns of hormone release and raised lactate levels, it could reasonably be proposed that raised lactate levels cause CHH release, as suggested by Santos and Keller (Santos and Keller, 1993). In exercise experiments (Fig. 2), it was clear that increases in lactate and CHH were correlated, and in the experiments concerned with exercise and metabolite-feedback loops, increases in CHH were accompanied by dramatic increases in circulating lactate, as summarised in Fig. 4. Furthermore, there were highly significant correlations between CHH levels and lactate in crabs sampled over 24 h cycles at 3 h intervals (Fig. 5). Thus, raised CHH levels are associated with correspondent increased circulating lactate levels, as would be expected given that locomotory activities beyond aerobic scope could well be an effective stressor, and clearly CHH release would be of adaptive significance in this situation.

With regard to other possible candidate compounds involved in regulation of CHH release, it has previously been suggested that dopamine or Leu-enkephalin might be involved (Jaros, 1990; Lüschen et al., 1993; Sarojini et al., 1995). However, these neurotransmitters do not evoke changes in the depolarisation patterns of CHH perikarya in *Cancer borealis* (Glowik et al., 1997). Because it has been shown that dopamine stimulates release of CHH from isolated eyestalk ganglia of *Procambarus clarkii*, it seems possible that this neurotransmitter could be involved in CHH release (Zou et al., 2003). However, there are a number of contradictory studies that implicate a variety of neurotransmitters and neuromodulators in CHH release [see Fanjul-Moles (Fanjul-Moles, 2006) for examples], and an unequivocal demonstration of their roles

Table 1. Seasonal changes (2006–2008) in circulating glucose, lactate, CHH and muscle glycogen in migrating (wet season) and inactive (dry season) *Gecarcoidea natalis*

Sampling date	21.07.06	12.11.06	16.11.06	26.11.06	30.11.06	3.12.06	8.12.06	30.06.07	7.11.07	18.11.07	21.11.07	24.11.07	28.08.06	30.8.08
Season and behaviour	Dry, inactive	Late dry, inactive	Late dry Inactive	Wet, migrating	Wet, digging	Wet, fighting	Wet, returning	Dry, inactive	Wet, migrating	Wet, digging	Wet, fighting	Wet, returning	Mid dry, inactive	Mid dry, inactive
Glucose mmol l^{-1}	0.1±0.01 (19)	0.09±0.02 (15)	0.11±0.02 (15)	0.4±0.03* (13)	0.19±0.04 (15)	0.27±0.03 (16)	0.07±0.01 (16)	0.09±0.02 (16)	0.36±0.04* (16)	0.67±0.01* (16)	0.2±0.23 (15)	0.21±0.03 (14)	0.3±0.04 (16)	0.15±0.03 (16)
Lactate mmol l^{-1}	1.41±0.26 (20)	2.12±0.34 (15)	2.03±0.34 (15)	0.94±0.17 (16)	2.57±0.41 (15)	2.79±0.73 (16)	0.85±0.21 (16)	1.36±0.23 (16)	1.77±0.33 (16)	5.59±0.83 (15)	3.65±0.98 (15)	2.41±0.53 (16)	1.41±0.21 (16)	1.91±0.32 (16)
CHH pmol l^{-1}	27.1±2.96 (20)	24.88±2.51 (16)	n.d.	**9.73±1.52** (15)	37.2±5.24 (15)	41.33±6.16 (18)	44.4±5.8 (16)	21.75±1.8 (16)	**11.0±1.72** (16)	**9.38±1.11** (16)	36.8±2.92 (15)	n.d.	35.6±4.71 (15)	43.13±3.95 (16)
Glycogen mmol kg^{-1}	n.d.	n.d.	n.d.	n.d.	n.d.	9.80±2.05 (18)	4.66±0.92 (8)	n.d.	35.74±2.20 (16)	28.84±3.34 (16)	7.25±1.07 (15)	n.d.	16.65±1.61 (16)	16.95±1.83 (16)

Values are means ± s.e.m. Numbers sampled are given in parentheses. All samples were taken between 09:00 and 11:00 h. Bold font indicates significant differences in haemolymph CHH from other means (P<0.05–0.01, Dunn's multiple comparisons).
*Corresponding mean glucose levels.
n.d., not determined.

Table 2. Seasonal changes in circulating glucose, lactate, CHH and muscle glycogen in migrating (wet season) and inactive (dry season) *Gecarcoidea natalis*, during 18 h cycles, samples taken at 6 h intervals

Sample time/date	11:00 h 10.12.07	17:00 h 10.12.07	23:00 h 10.12.07*	05:00 h 11.12.07*	11:00 h 7.08.08	17:00 h 7.08.08	23:00 h 7.08.08*	05:00 h 8.08.08*
Season and behaviour	Wet, active migrating	Wet, active migrating	Wet, unresponsive	Wet, inactive/alert	Dry, inactive foraging	Dry, inactive foraging	Dry, inactive unresponsive	Dry, inactive/alert
Glucose mmol l^{-1}	0.25±0.04 (16)	0.17±0.03 (16)	0.18±0.03 (16)	0.14±0.02 (16)	0.15±0.03 (16)	0.15±0.03 (16)	0.12±0.03 (15)	0.06±0.007 (16)
Lactate mmol l^{-1}	2.23±0.27 (15)	1.89±0.29 (16)	**0.81±0.17 (15)†**	**0.75±0.19 (16)‡**	1.91±0.32 (15)	1.96±0.32 (15)	1.57±0.29 (16)	0.91±0.18 (16)
CHH pmol l^{-1}	**9.43±1.37 (14)§**	**9.75±1.26 (16)¶**	**5.25±1.17 (16)§**	**7.25±1.06 (16)§**	43.13±3.95 (16)	29.37±3.65 (16)	25.47±2.45 (15)	26.13±2.28 (15)
Muscle glycogen mmol kg^{-1}	**37.26±4.80 (15)‡**	24.35±3.10 (16)	23.0±3.63 (14)	**35.32±5.21 (15)†**	16.95±1.83 (16)	15.84±2.09 (16)	18.08±2.14 (16)	20.01±1.58 (15)

Values are means ± s.e.m. Numbers sampled are given in parentheses. Bold font indicates significant differences from means (compared by season).
*During darkness.
†$P<0.05$; ‡$P<0.01$ (Tukey-Kramer multiple comparisons).
§$P<0.001$; ¶$P<0.05$ (Dunn's multiple comparisons).

requires further, crucial study. This is exemplified by Basu and Kravitz (Basu and Kravitz, 2003) who have shown, using a combination of immunohistochemistry and electrophysiological techniques, that the neurotransmitters octopamine and serotonin inhibit spontaneous bursting of CHH immunoreactive neurones in the second thoracic nerve roots of *Homarus americanus*.

In view of previous work either supporting the idea that CHH levels might be controlled via feedback mechanisms involving metabolites such as glucose (Santos and Keller, 1993; Glowik et al., 1997), or proposing the involvement of CHH in lipid metabolism following the observation that CHH injection elevates haemolymph phospholipids and triglycerides in *Carcinus maenas* (Santos et al., 1997), experiments were designed in which various compounds (glucose, lactate, phospholipids, amino acids) were repeatedly injected over several days to raise their concentration in the haemolymph. Subsequently, crabs were exercised and their CHH-release response measured. Only glucose showed a clear effect and, surprisingly, this was dependent upon the season. During the wet season, when crabs were migrating, glucose injection completely abrogated exercise-dependent CHH release. By contrast, during the dry season, when crabs were inactive, a small but significant release of CHH was seen following exercise, but this was much less than that observed following other treatments or in controls. With the other treatments, there was no evidence for the involvement of lactate, amino acids or phospholipids in feedback control of CHH release. The results from these experiments support those obtained *in vitro*, in which glucose causes hyperpolarisation of CHH neurones in a dose-dependent and, crucially, physiologically relevant manner (Glowik et al., 1997).

Although lactate injection did not further promote the release of CHH during exercise, there was a clear positive correlation of raised lactate levels with CHH in field experiments, which might suggest a positive-feedback mechanism. Together with the results for glucose, which clearly show a negative-feedback loop, these results strengthen the proposal that these are relevant control mechanisms for CHH release in crustaceans, as suggested by Santos and Keller (Santos and Keller, 1993).

The seasonal dependence of the proposed negative-feedback loop is intriguing. In terms of adaptation to migration and attendant energy demands, negative feedback would make sense, particularly as the crabs are feeding during migration (Adamczewska and Morris, 2001b). By contrast, because dry season crabs are highly fossorial, and have quite proscribed periods of limited activity, at dawn and in the late afternoon, it is possible that the uncoupling of a negative-feedback loop might be advantageous, or that CHH release during the dry season might be primarily important in other physiologies, such as those related to ionic homeostasis. In this context, it is interesting to note that the highest levels of CHH were generally (but not invariably) seen in dry season crabs, and the lowest in wet season, migrating crabs.

To investigate the adaptive significance of CHH in the life history of *G. natalis* in the field, a large number of haemolymph samples were taken from crabs from 2006 to 2008, in both dry and wet seasons. Because the probable episodic release of CHH and its short half-life would inevitably increase the signal to noise ratio (in contrast to measurements of longer lived metabolites, such as glucose and lactate, for which correlations might be made), we sampled over seasonal, daily, hour and minute timescales. In this way, we hoped to see differences between true patterning and stochastic events.

For seasonal differences, glucose levels were quite variable but, in general, rather higher during the wet rather than during the dry season, and these differences were statistically highly significant despite this variability, which has also been commented upon in an earlier study (Adamczewska and Morris, 2001b). It seems likely that the elevated wet season glucose levels overall reflect much higher activity levels, and feeding activities, at this time. Lactate levels were raised in crabs that were digging or fighting, but not in migrating crabs, but in these, glucose levels were raised. It has previously been noted (Adamczewska and Morris, 2001b) that migration, but not burrow digging or fighting, occurs without recourse to anaerobiosis, despite the fact that on occasion walking speeds of crabs exceed those that promote this process, which occurred during the forced exercise experiments shown in this study. Moreover, it has previously been established that red crabs avoid exceptional lactate build up during migration by walking slowly or intermittently during large parts of their migration (Adamczewska and Morris, 1994). Although migration did not seem to involve any significant reduction in energy reserves (muscle glycogen), probably because during migration crabs continue feeding (Adamczewska and Morris, 2001b), for crabs undergoing burrowing and fighting behaviours, and particularly upon return migration, muscle glycogen reserves were depleted. This phenomenon has been noted previously by these authors.

With regard to seasonal differences in CHH levels, during migration in both 2006 and 2007, and during burrow digging in

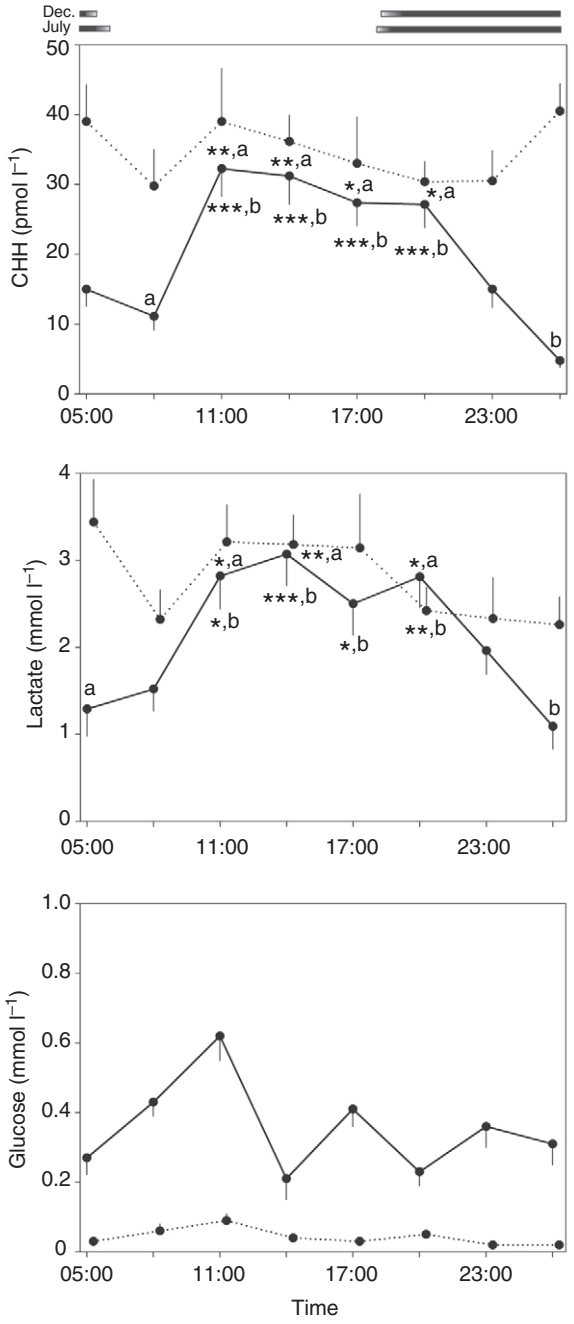

Fig. 5. Profiles of CHH, glucose and lactate levels in *G. natalis* haemolymph samples taken at 3 hourly intervals over 24 h from dry season (July 2007, *N*=8; dotted lines) and wet season, migrating (December 2006, *N*=15–16; solid lines) crab populations. Sampling sites were as detailed in the text. Means ± s.e.m. are shown. Day-to-night transitions are schematically indicated by shaded bars, sunrise and sunset are unshaded, and times following astronomical twilight are black. **P*<0.05, ***P*<0.01, ****P*<0.001. a and b indicate the timepoints for which statistical tests (CHH, Dunn's multiple comparisons; lactate, Tukey–Kramer multiple comparisons) were made for wet season crabs.

2007, it was notable that CHH levels were significantly lower than in all of the dry season samples. This is somewhat paradoxical given the depletion of glycogen reserves after migration and considering the fundamental role of CHH in mobilisation of glycogen reserves (Sedlmeier, 1985). It is also clear that these low CHH levels do not positively correlate with glucose levels in migrating crabs, which were amongst the highest observed. One explanation for this might be that the negative-glucose-feedback loop is functioning at this time. Because the crabs are feeding during their migration, this would ensure the conservation of glycogen stores for future strenuous activities (fighting, burrowing) after which glycogen is depleted. However, an alternative explanation might be that, during the dry season, CHH levels are elevated compared with those in the wet season. This might be relevant if a role of CHH in osmoregulation is invoked. CHH release during ecdysis by gut endocrine cells causes water uptake via drinking in *Carcinus* (Chung et al., 1999), and the corresponding hormone in insects, ion transport peptide (ITP), has well-established antidiuretic functions in promoting water uptake across the hindgut, by the stimulation of ileal chloride transport (Audsley et al., 1992; Phillips et al., 1998a; Phillips et al., 1998b; King et al., 1999). Thus, it is possible that the raised CHH levels seen in dry season crabs (compared with in wet season animals) might be associated primarily with iono/osmoregulatory demands involved with water retention. Clearly further laboratory-based studies on the role of CHH in osmoregulation in red crabs are now timely.

When seasonal changes in CHH and metabolite levels were studied at a finer temporal scale (6 hourly samples) during a consecutive wet and dry season (2007–2008), the same features were seen regarding CHH levels and season as in the single-sampling regime, namely that CHH levels were lower in the wet season (migrating) than in the dry season (inactive) crabs. Thus, this repeatable phenomenon could be a real rather than a stochastic variation, reflecting a seasonal change in endocrine physiology. An interesting feature of these measurements concerned circulating lactate levels. Although no clear seasonal pattern was evident (Table 1), the 6 h sampling experiments (Table 2) showed that, for migrating crabs, lactate levels declined during night-time. This observation is entirely in keeping with their behaviour.

For the 3 h sampling experiments, the night-time nadir in lactate concentration was once again notable in migrating crabs. For migrating crabs, CHH levels during most of the period when they are active (11:00–18:00 h) through to early night-time (20:00 h) were significantly higher than during late night-time (02:00–05:00 h), when crabs are in an inactive, almost 'sleep-like', unresponsive state. Although the general patterns of CHH levels were strikingly similar to those of circulating lactate, it is as yet premature to say that they are causally related. However, they might be in that they reflect the overall activity levels of the crabs. Nevertheless, the diel changes in CHH observed in these experiments did not correlate with any pattern of hyperglycaemia. As migrating animals are known to continue feeding during the daytime as alluded to earlier, and considering the observation that in the shrimp *Penaeus monodon* feeding is associated with rapid hyperglycaemia (Hall and Van Ham, 1998), this variability might well reflect this behaviour rather than any causal relationship with CHH release.

A notable feature of the measurements of CHH made in free-ranging animals in the field, whether taken from inactive (dry season) or migrating (wet season) crabs, was that, although the levels of CHH were very similar to those taken prior to experimentation in the forced exercise or metabolite/forced exercise experiments, (10–30 pmol l^{-1}), the peak levels of CHH seen in these experiments (70–110 pmol l^{-1}) were very rarely observed in the field. Clearly these can only be obtained following extreme stress, and it seems likely that CHH is released tonically, or more likely as short-lived pulses, as we have suggested previously from our studies on CHH and MIH release in *Carcinus* (Chung and Webster, 2005).

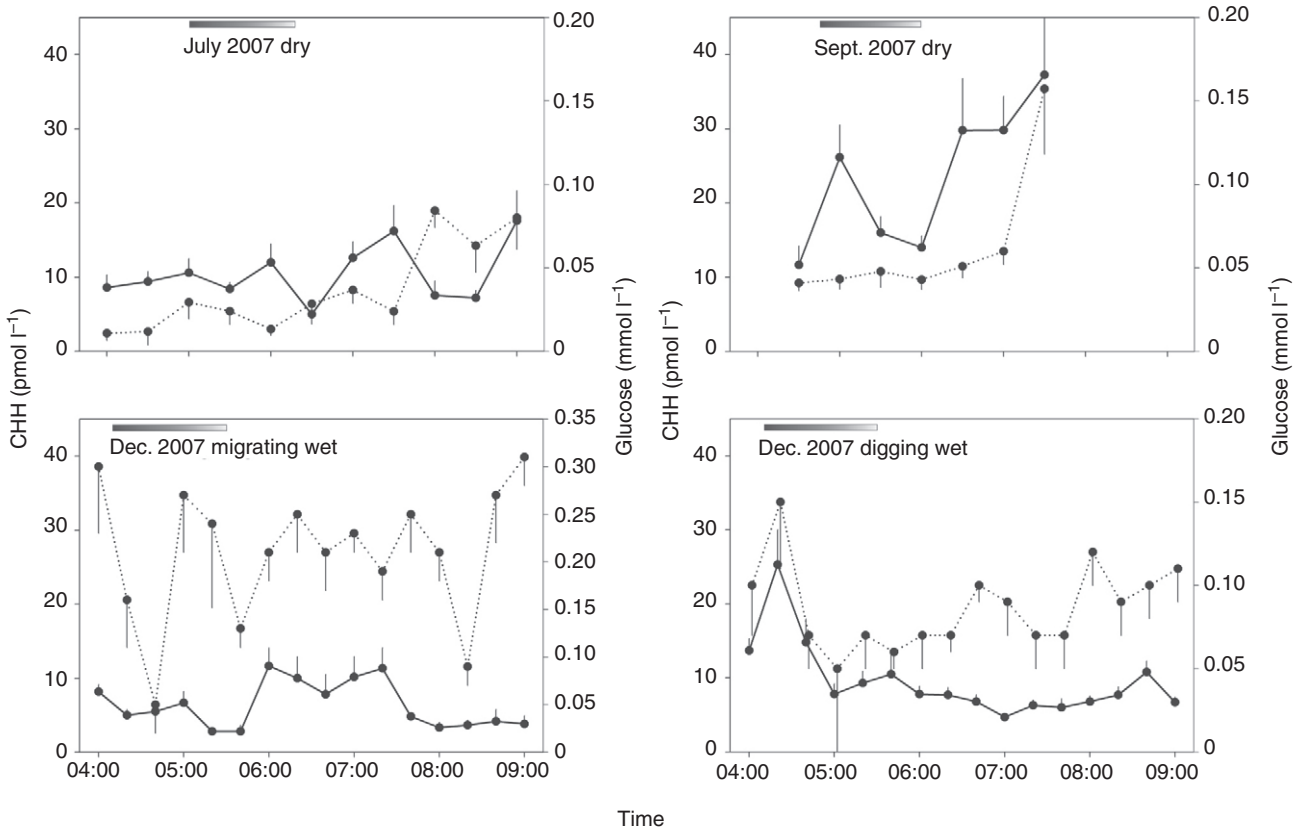

Fig. 6. Profiles of haemolymph CHH (solid lines) and glucose (dotted lines) in *G. natalis*. Samples taken at 20–30 min intervals, over the dawn period (shaded bars show schematically the period from the start of astronomical twilight until sunrise), during the dry season (July 2007 and September 2007; *N*=10), during the wet season for migrating and for digging crabs (December 2007, *N*=12). Means ± s.e.m. are shown. Sampling sites were as detailed in the text.

To determine still finer temporal resolution of CHH levels, we decided to investigate changes in CHH levels during the night-to-day transition. Just before dawn, for about 2 h, red crabs become very active. We reasoned that this should place significant energy demands upon the crabs, and previous observations had suggested that the night-to-dawn transition was in *G. natalis* often associated with hyperglycaemia (S.M., unpublished observations). Dawn hyperglycaemia has been observed in the prawn *Macrobrachium rosenbergii* (Kuo and Yang, 1999), conversely both hyperglycaemia and CHH release associated with the day-to-night transition has been seen in a nocturnally active crustacean, *Orconectes limosus* (Kallen et al., 1990). However, for the shrimp *Penaeus monodon*, diel variations in haemolymph glucose were not observed (Hall and Van Ham, 1998). Because dawn activity is associated with foraging in *G. natalis*, it seems possible that feeding-related hyperglycaemia could mask any endogenous diurnal rhythmicity in glucose levels, and consequently could inhibit CHH release. Further laboratory-based experiments are now needed to resolve this issue.

Several comparisons in both dry and wet seasons showed that dawn hyperglycaemia was a rather unpredictable phenomenon, and was not associated with CHH release in any obvious way (Fig. 6). Because these field experiments were, by necessity, not performed by repeated measurements on the same animals, little more can be said at present; however, these experiments clearly point to a need to obtain real-time repeated measures of circulating CHH in single crabs, to eliminate the confounding effects of individual variability. Clearly, in view of its sensitivity, the assay described here is eminently suitable for repeatedly measuring CHH levels in many sequentially sampled small volume (*ca*. 50 µl) haemolymph extracts, and a promising approach would be to employ continuous perfusion and microdialysis. This technique has been used to obtain hourly profiles of amino acids and GABA from free-moving crayfish (Cebada et al., 2006) and, in particular, it has been used to qualitatively profile circulating neuropeptides using contemporary mass spectrometric techniques (Behrens et al., 2008). Thus, if issues relating to the rapid clotting of the haemolymph of some crabs (in particular *G. natalis*) can be addressed, microdialysis coupled with ultrasensitive TR-FIA might be an ideal way to measure relatively rapid pulsatile fluctuations in CHH levels in free-ranging animals, without repeated, possibly stressful, episodes of haemolymph sampling that in themselves will cause a small release of CHH (as we observed in the control crabs that were sampled repeatedly over 2 h in the exercise experiments (Fig. 2). Nevertheless, obtaining sufficient volumes of haemolymph (50 µl) using this technique would limit sampling to, at best, hourly intervals; thus, for measurement of near instantaneous changes in CHH levels, careful repeated sampling still seems to be the most useful procedure.

This study was pioneering as it was the first attempt to measure CHH on a large scale in field populations of a terrestrial crab, and to correlate these with relevant metabolites, during significant stages of life history, and obtain ‘real-time’ measurements of changing CHH levels following experimental manipulation. The study has revealed an unexpected dynamism in CHH levels and novel feedback loops, which have been correlated with significant events in the life history of *G. natalis*. Future work, which this study has highlighted, will be to continuously record rapid changes in CHH

levels in crabs during experimental manipulation, and to compare and contrast specific behaviours for individuals in the field with changes in CHH levels and correspondent metabolic physiology. Indeed, it could be concluded that research into land crab endocrinology, particularly for those species that offer dramatic polarisations of metabolic physiology during their life history, such as in the present example, is potentially insightful, not least in underscoring Krogh's principle (Krogh, 1929).

LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
BSA	bovine serum albumin
cDNA	complementary DNA
CHH	crustacean hyperglycaemic hormone
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
MALDI-TOF MS	matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
RACE	rapid amplification of complementary ends
SG	sinus gland
TFA	trifluoroacetic acid
TR-FIA	time-resolved fluoroimmunoassay

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REFERENCES

Adamczewska, A. M. and Morris, S. (1994). Exercise in the terrestrial Christmas Island Red Crab *Gecarcoidea natalis*. II. Energetics of locomotion. *J. Exp. Biol.* **188**, 257-274.

Adamczewska, A. M. and Morris, S. (1998). Strategies for migration in the terrestrial Christmas Island red crab *Gecarcoidea natalis*: intermittent *versus* continuous locomotion. *J. Exp. Biol.* **201**, 3221-3231.

Adamczewska, A. M. and Morris, S. (2001a). Ecology and behaviour of *Gecarcoidea natalis*, the Christmas Island red crab, during the annual breeding migration. *Biol. Bull.* **200**, 305-320.

Adamczewska, A. M. and Morris, S. (2001b). Metabolic status and respiratory physiology of *Gecarcoidea natalis*, the Christmas Island red crab, during the annual breeding migration. *Biol. Bull.* **200**, 321-335.

Audsley, N., McIntosh, C. and Phillips, J. E. (1992). Actions of ion transport peptide from locust corpus cardiacum on several hindgut processes. *J. Exp. Biol.* **173**, 275-288.

Basu, A. C. and Kravitz, E. A. (2003). Morphology and monoaminergic modulation of crustacean hyperglycaemic hormone-like immunoreactive neurons in the lobster nervous system. *J. Neurocytol.* **32**, 253-263.

Behrens, H. L., Chen, R. and Li, L. (2008). Combining microdialysis, nanoLC-MS, and MALDI-TOF/TOF to detect neuropeptides secreted in the crab, *Cancer borealis*. *Anal. Chem.* **80**, 6949-6958.

Bergmeyer, H. U. (1985). *Methods of Enzymatic Analysis*, Vol 6, 2nd edn. Weinheim: VCH Verlagsgesellschaft.

Böcking, D., Dircksen, H. and Keller, R. (2002). The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In *The Crustacean Nervous System* (ed. K. Wiese), pp. 84-97. Heidelberg: Springer.

Booth, C. E. and McMahon, B. R. (1985). Short communications. Lactate dynamics during locomotor activity in the blue crab, *Callinectes sapidus*. *J. Exp. Biol.* **118**, 461-465.

Cebada, J., Alvardo-Álvarez, R., Becerra, E., Neri-Bazán, L., Rocha, E. and García, U. (2006). An improved method for long-term measuring of haemolymph fluctuations of non-essential amino acids, GABA and histamine from freely moving crayfish. *J. Neurosci. Meth.* **153**, 1-7.

Chan, S., Gu, P. L., Chu, K. H. and Tobe, S. S. (2003). Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies. *Gen. Comp. Endocrinol.* **134**, 214-219.

Chang, E. S., Prestwich, G. D. and Bruce, M. J. (1990). Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. *Biochem. Biophys. Res. Commun.* **171**, 818-826.

Chang, E. S., Keller, R. and Chang, S. A. (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen. Comp. Endocrinol.* **111**, 359-366.

Chang, E. S., Chang, S. A., Beltz, B. S. and Kravitz, E. A. (1999). Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the suboesophageal ganglion and thoracic second roots. *J. Comp. Neurol.* **414**, 50-56.

Charmantier-Daures, M., Charmantier, G., Janssen, K. P. C., Aiken, D. E. and Van Herp, F. (1994). Involvement of eyestalk factors in the neuroendocrine control of osmoregulation in adult American lobster, *Homarus americanus*. *Gen. Comp. Endocrinol.* **94**, 281-293.

Chung, J. S. and Webster, S. G. (2004). Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. *Development* **131**, 4751-4761.

Chung, J. S. and Webster, S. G. (2005). Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycaemic hormone in the shore crab, *Carcinus maenas*. *Endocrinology* **46**, 5545-5551.

Chung, J. S. and Zmora, N. (2008). Functional studies of crustacean hyperglycemic hormone (CHHs) of the blue crab, *Callinectes sapidus*- the expression and release of CHH in the eyestalk and pericardial organ in response to environmental stress. *FEBS J.* **275**, 693-704.

Chung, J. S., Dircksen, H. and Webster, S. G. (1999). A remarkable, precisely timed release of hyperglycaemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl. Acad. Sci. USA* **96**, 13103-13107.

Dircksen, H., Zahnow, C. A., Gaus, G., Keller, R., Rao, K. R. and Riehm, J. P. (1987). The ultrastructure of nerve endings containing pigment dispersing hormone (PDH) in crustacean sinus glands: Identification by an antiserum against synthetic PDH. *Cell Tissue Res.* **250**, 377-387.

Dircksen, H., Böcking, D., Heyn, U., Mandel, C., Chung, J. S., Plosch, T., Jaros, P. P., Waelkens, E., Keller, R. and Webster, S. G. (2001). Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. *Biochem. J.* **356**, 159-170.

Fanjul-Moles, M. L. (2006). Biochemical and functional aspects of hyperglycemic hormone in decapod crustaceans: Review and update. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **142**, 390-400.

Forster, M. E., Waldron, F. M. and Taylor, H. H. (1989). Recovery from exhausting exercise in a bimodally breathing crab, *Leptograpsus variegatus* (Decapoda, Grapsidae). *J. Exp. Mar. Biol. Ecol.* **127**, 165-173.

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

Green, P. T. (1997). Red crabs in rain forest on Christmas Island, Indian Ocean: activity patterns, density and biomass. *J. Trop. Ecol.* **13**, 17-38.

Greenaway, P., Morris, S. and McMahon, B. R. (1988). Adaptations to a terrestrial existence by the robber crab *Birgus latro*. II. *In vivo* respiratory gas exchange and transport. *J. Exp. Biol.* **140**, 493-509.

Hall, M. R. and Van Ham, E. H. (1998). Diurnal variation and effects of feeding on blood glucose in the giant tiger prawn, *Penaeus monodon*. *Physiol. Zool.* **71**, 574-583.

Head, G. and Baldwin, J. (1986). Energy metabolism and the fate of lactate during recovery from exercise in the Australian freshwater crayfish *Cherax destructor*. *Aust. J. Mar. Freshwater Res.* **37**, 641-646.

Henry, R. P., Booth, C. E., Lallier, F. H. and Walsh, P. J. (1994). Post-exercise lactate production and metabolism in three species of aqautic and terrestrial crustaceans. *J. Exp. Biol.* **186**, 215-234.

Herreid, C. F. and Full, R. J. (1988). Energetics and locomotion. In *Biology of the Land Crabs* (ed. W. W. Burggren and B. R. McMahon), pp. 333-337. New York: Cambridge University Press.

Hicks, J. W. (1985). The breeding behaviour and migrations of the terrestrial crab *Gecarcoidea natalis* (Decapoda: Brachyura). *Aust. J. Zool.* **33**, 127-142.

Jaros, P. (1990). Enkephalins, biologically active peptides in invertebrates, with special reference to crustaceans. In *Frontiers in Crustacean Neurobiology* (ed. K. Wiese, W. D. Krenz, J. Tautz, H. Reichert and B. Mulloney), pp. 471-482. Basel: Birkäuser.

Kallen, J. L., Abrahamse, S. L. and Van Herp, F. (1990). Circadian rhythmicity of the crustacean hyperglycaemic hormone (CHH) in the hemolymph of the crayfish. *Biol. Bull.* **179**, 351-357.

Keller, R. and Orth, H. P. (1990). Hyperglycemic neuropeptides in crustaceans. In *Progress in Comparative Endocrinology* (ed. A. Epple, C. Scanes and M. Stetson), pp. 265-271. New York: Wiley-Liss.

Keller, R., Jaros, P. P. and Kegel, G. (1985). Crustacean hyperglycemic neuropeptides. *Am. Zool.* **25**, 207-221.

Khayat, M., Yang, W., Aida, K., Nagasawa, H., Tietz, A., Funkenstein, B. and Lubzens, E. (1998). Hyperglycemic hormones inhibit protein and mRNA synthesis in *in vitro* incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*. *Gen. Comp. Endocrinol.* **110**, 307-318.

King, D. S., Meredith, J., Wang, Y. J. and Phillips, J. E. (1999). Biological actions of synthetic locust ion transport peptide (ITP). *Insect Biochem. Mol. Biol.* **29**, 11-18.

Krogh, A. (1929). The progress of physiology. *Am. J. Physiol.* **90**, 243-251.

Kuo, C. M. and Yang, Y. H. (1999). Hyperglycemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **169**, 49-54.

Liu, L., Laufer, H., Wang, Y. J. and Hayes, T. K. (1997). A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. *Biochem. Biophys. Res. Commun.* **237**, 694-701.

Lorenzon, S., Edomi, P., Giulianini, P. G., Mettulio, R. and Ferrero, E. A. (2004). Variation of crustacean hyperglycaemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress. *J. Exp. Biol.* **207**, 4205-4213.

Lüschen, W., Willig, A. and Jaros, P. P. (1993). The role of biogenic amines in control of blood glucose levels in the decapod crustacean, *Carcinus maenas*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **105**, 291-296.

McMahon, B. R. (1981). Oxygen uptake and acid-base balance during activity in decapod crustaceans. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid and C. R. Fourtner), pp. 299-390. New York: Plenum Press.

Morris, S. and Ahern, M. D. (2003). Regulation of urine reprocessing in the maintenance of sodium and water balance in the terrestrial Christmas Island red

crab *Gecarcoidea natalis*, investigated under field conditions. *J. Exp. Biol.* **206**, 2869-2881.
Nakatsuji, T. and Sonobe, H. (2003). Measurement of molt-inhibiting hormone titer in hemolymph of the Americaln crayfish, *Procambarus clarkii*, by time-resolved fluoroimmunoassay. *Zool. Sci.* **20**, 999-1001.
Ohira, T., Tsutsui, N., Nagasawa, H. and Wilder, M. N. (2006). Preparation of two recombinant crustacean hyperglycaemic hormones from the giant freshwater prawn, *Macrobrachium rosenbergii*, and their hyperglycaemic activities. *Zool. Sci.* **23**, 383-391.
Phillips, J. E., Meredith, J., Audsley, N., Richardson, N., Macins, A. and Ring, M. (1998a). Locust ion transport peptide (ITP): A putative hormone controlling water and ionic balance in terrestrial insects. *Am. Zool.* **38**, 461-470.
Phillips, J. E., Meredith, J., Audsley, N., Ring, M., Macins, A., Brock, H., Theilmann, D. and Litteford, D. (1998b). Locust ion transport peptide (ITP): function, structure, cDNA and expression. In *Recent Advances in Arthropod Endocrinology* (ed. G. M. Coast and S. G. Webster), pp. 210-226. Cambridge: Cambridge University Press.
Ruf, F., Hayot, F., Park, M.-J., Ge, Y., Lin, G., Roysam, B. and Sealfon, S. C. (2007). Noise propagation and scaling in regulation of gonadotrope biosynthesis. *Biophys. J.* **93**, 4474-4480.
Santos, E. A. and Keller, R. (1993). Regulation of circulating levels of the crustacean hyperglycaemic hormone: evidence for a dual feedback control system. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **163**, 374-379.
Santos, E. A., Nery, L. E. M., Keller, R. and Gonçalves, A. A. (1997). Evidence for the involvement of the crustacean hyperglycaemic hormone in the regulation of lipid metabolism. *Physiol. Zool.* **70**, 415-420.
Sarojini, R., Nagabhushanam, R. and Fingerman, M. (1995). Dopaminergic and enkephalinergic involvement in the regulation of blood glucose in the red swamp crayfish, *Procambarus clarkii*. *Gen. Comp. Endocrinol.* **97**, 160-170.
Sedlmeier, D. (1985). Mode of action of the crustacean hyperglycemic hormone. *Am. Zool.* **25**, 223-232.
Sedlmeier, D. (1988). The crustacean hyperglycaemic hormone releases amylase from the crayfish midgut gland. *Regul. Pept.* **20**, 91-98.
Serrano, L., Blanvillain, G., Soyez, D., Charmantier, G., Grousset, E., Aujoulat, F. and Spanings-Pierrot, C. (2003). Putative involvement of crustacean hyperglycaemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus*. *J. Exp. Biol.* **206**, 979-988.
Spanings-Pierrot, C., Soyez, D., Van Herp, F., Gompel, M., Skaret, G., Grousset, E. and Charmantier, G. (2000). Involvement of crustacean hyperglycaemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol.* **119**, 340-350.
Stentiford, G. D., Chang, E. S., Chang, S. A. and Neil, D. M. (2001). Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). *Gen. Comp. Endocrinol.* **121**, 13-22.
Toullec, J.-Y., Serrano, L., Lopez, P. and Spanings-Pierrot, C. (2006). The crustacean hyperglycemic hormones from an euryhaline crab *Pachygrapsus marmoratus* and a freshwater crab *Potamon ibericum*: eyestalk and pericardial isoforms. *Peptides* **27**, 1269-1280.
Tsutsui, N., Katayama, H., Ohira, T., Nagasawa, H., Wilder, M. N. and Aida, K. (2005). The effects of crustacean hyperglycaemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus*. *Gen. Comp. Endocrinol.* **144**, 232-239.
Van Aardt, W. J. (1990). Oxygen uptake and haemocyanin oxygen affinity of *Potamonautes warreni* Calman after exercise. *S. Afr. J. Zool.* **25**, 11-17.
Webster, S. G. (1991). Amino acid sequence of putative moult-inhibiting hormone (MIH) from the crab *Carcinus maenas*. *Proc. R. Soc. Lond. B. Biol. Sci.* **244**, 247-252.
Webster, S. G. (1993). High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane bound receptors on the Y-organ of the shore crab *Carcinus maenas*. *Proc. R. Soc. Lond. B. Biol. Sci.* **251**, 53-59.
Webster, S. G. (1996). Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *J. Exp. Biol.* **199**, 1579-1585.
Webster, S. G. and Keller, R. (1986). Purification, characterization and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **156**, 617-624.
Wilcockson, D. C., Chung, J. S. and Webster, S. G. (2002). Is crustacean hyperglycaemic hormone precursor-related peptide a circulating neurohormone in crabs? *Cell Tissue Res.* **307**, 129-138.
Zou, H. S., Juan, C. C., Chen, S. C., Wang, H. Y. and Lee, C. Y. (2003). Dopaminergic regulation of crustacean hyperglycaemic hormone and glucose levels in the hemolymph of the crayfish *Procambarus clarkii*. *J. Exp. Zool.* **298**, 344-352.