

## RESEARCH ARTICLE

# Roles of crustacean hyperglycaemic hormone in ionic and metabolic homeostasis in the Christmas Island blue crab, *Discoplax celeste*

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### SUMMARY

There is a growing body of evidence implicating the involvement of crustacean hyperglycaemic hormone (CHH) in ionic homeostasis in decapod crustaceans. However, little is known regarding hormonally influenced osmoregulatory processes in terrestrial decapods. As many terrestrial decapods experience opposing seasonal demands upon ionoregulatory physiologies, we reasoned that these would make interesting models in which to study the effect of CHH upon these phenomena. In particular, those (tropical) species that also undergo seasonal migrations might be especially informative, as we know relatively little regarding the nature of CHHs in terrestrial decapods, and hormonally mediated responses to seasonal changes in metabolic demands might also be superimposed or otherwise integrated with those associated with ionic homeostasis. Using *Discoplax celeste* as a model crab that experiences seasonal extremes in water availability, and exhibits diurnal and migratory activity patterns, we identified two CHHs in the sinus gland. We biochemically characterised (cDNA cloning) one CHH and functionally characterised (in terms of dose-dependent hyperglycaemic responses and glucose-dependent negative feedback loops) both CHHs. Whole-animal *in situ* branchial chamber <sup>22</sup>NaCl perfusion experiments showed that injection of both CHHs increased gill Na<sup>+</sup> uptake in a seasonally dependent manner, and <sup>51</sup>Cr-EDTA clearance experiments demonstrated that CHH increased urine production by the antennal gland. Seasonal and salinity-dependent differences in haemolymph CHH titre further implicated CHH in osmoregulatory processes. Intriguingly, CHH appeared to have no effect on gill Na<sup>+</sup>/K<sup>+</sup>-ATPase or V-ATPase activity, suggesting unknown mechanisms of this hormone's action on Na<sup>+</sup> transport across gill epithelia.

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### INTRODUCTION

The most widely studied crustacean neurohormone, crustacean hyperglycaemic hormone (CHH) has long been known to be centrally involved in regulation of energy metabolism in many malacostracan crustaceans (reviewed by Böcking et al., 2002; Fanjul-Moles, 2006; Chung et al., 2010; Webster et al., 2012). In recent years, a variety of other functions for these hormones have been established, involving (for example) inhibition of ecdysteroid (Webster and Keller, 1986; Chang et al., 1990; Chung and Webster, 2003; Chung and Webster, 2005), methyl farnesoate (Liu et al., 1997) and ovarian protein synthesis (Khayat et al., 1998; Avarre et al., 2001). A subject of topical interest is the involvement of CHH in ionoregulatory processes (Charmantier-Daures et al., 1994; Charmantier et al., 1999; Chung et al., 1999; Townsend et al., 2001; Serrano et al., 2003; Chung and Webster, 2006). Whilst it is known that sinus gland extracts cause rapid and reversible changes in transepithelial potentials, and Na<sup>+</sup> transport in isolated perfused gills of *Pachygrapsus marmoratus* (Spanings-Pierrot et al., 2000), the precise mechanisms regulated by CHH in crustacean gill tissues remain poorly investigated, despite our extensive knowledge of branchial membrane exchangers and pumps (Lucu, 1990; Onken et al., 1991; Onken and Putzenlechner, 1995; Lucu and Towle, 2003;

Weihrauch et al., 2004; Tsai and Lin, 2007; Masui et al., 2008). Additionally, because the insect equivalent of CHH-ion transport peptide (ITP) has long been known to stimulate water uptake in the locust hindgut by opening Cl<sup>-</sup> channels (Phillips and Audsley, 1995; Phillips et al., 1998), it is surprising that the ionoregulatory processes controlled by CHH have not attracted further interest.

Whilst significant progress in understanding crustacean branchial membrane ionic transport processes have been made, by utilising euryhaline crab models such as *Eriocheir sinensis* (Onken and Graszynski, 1989; Trausch et al., 1989; Bianchini and Gilles, 1990; Detaille et al., 1992; Riestenpatt et al., 1994; Onken and Putzenlechner, 1995; Riestenpatt et al., 1995; Mo et al., 1998; Onken, 1999; Onken et al., 2000; Mo et al., 2003), our approach in investigating possible roles for CHH involved a rather less well known terrestrial/amphibious gecarcinid, the Christmas Island blue crab, *Discoplax celeste* (Ng and Davie, 2012). We reasoned that this might be a useful model, because as with all terrestrial crabs, conservation of water and salts occurs *via* reprocessing of primary urine from the antennal glands over the gills, to produce a hypoionic final urine 'P' (Wolcott and Wolcott, 1985), with less than 30% of the ions measured in the primary urine and 5% of ions found in the haemolymph (Morris, 2001). Thus it seemed feasible that CHH may

have a role in the production of primary urine as well as in its branchial reprocessing. Moreover, as this crab experiences seasonal extremes in habitat (dry and wet seasons), resulting in contrasting diel activity patterns, and also undergoes seasonal migration to the sea to breed, the associated metabolic demands that are mediated *via* CHH might be superimposed upon, or otherwise integrated with, contrasting seasonal changes in ionoregulatory demands, namely conservation of water during the dry season *versus* conservation of ions during the monsoon (Morris and Ahern, 2003).

The seasonal status and osmoregulatory role of CHH in *D. celeste* were investigated *in situ* on Christmas Island using a variety of approaches. CHHs were firstly isolated and characterised. Changes in CHH titre were correlated with season and exposure to different salinity regimes. The effect of CHH on the ability of the branchial chamber system to absorb salts was studied by *in vivo* perfusion of branchial chambers, measurement of  $^{22}\text{Na}$  flux and gill  $\text{Na}^+/\text{K}^+$ -ATPase and V-ATPase activities. Possible roles of CHH in antennal gland haemolymph/urine filtration were examined by measuring  $^{51}\text{Cr}$ -EDTA clearance. Furthermore, the perfusion and infusion experiments were also conducted on the Christmas Island red crab *Gecarcoidea natalis* Pocock 1888 to compare and contrast any differences in the response of a closely related land crab species, albeit one with a more truly terrestrial (independent of freshwater) ecology. By using this integrated ecophysiological approach, we hoped to obtain a better understanding of the roles of CHH in osmoregulation of land crabs.

## MATERIALS AND METHODS

### Animal collection and husbandry

Adult intermoult male *D. celeste* were collected, under permit from Parks Australia, near permanent freshwater seeps at Ross Hill Gardens, Christmas Island, Indian Ocean ( $10^{\circ}29'11''\text{S}$ ,  $105^{\circ}40'41''\text{E}$ ), and shipped *via* air-freight to the UK within 3 days of collection. Export licences for live animals and tissue samples were obtained from The Department of the Environment and Water Resources, Australia, and import licences for the UK were obtained from the Department of Environment, Food and Rural Affairs.

Crabs were maintained at  $25^{\circ}\text{C}$  under a 12h:12h light:dark cycle at 80–100% relative humidity in communal plastic terraria, and were fed once a week (chopped apples and dried cat biscuits). Continuous access to water (2% seawater, changed daily) was provided.

### Purification and identification of CHH

Sinus glands (SGs) were microscopically dissected from ice-anaesthetised *D. celeste* on Christmas Island in batches of ~50–100 and immediately snap-frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . HPLC purification of  $2\text{mol l}^{-1}$  acetic acid extracts of SGs was performed as previously described (Morris et al., 2010). CHHs were identified by bioassay and cDNA cloning. For bioassay, peptides corresponding to HPLC peaks 1–7 (see Fig. 1A) were quantified by amino acid analysis (Webster, 1991) [with conditions as described in Chung et al. (Chung et al., 1998)]. One hundred picomoles of peptide dissolved in  $100\ \mu\text{l}$  *D. celeste* saline (in  $\text{mmol l}^{-1}$ : 288.7 NaCl, 4.6 KCl, 3.6  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.8  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 1  $\text{NaHCO}_3$ ) or saline only (controls) was injected into crabs ( $N=8$ ) *via* the arthroal membrane at the base of the fourth walking leg. After 90 min a  $200\ \mu\text{l}$  haemolymph sample was taken and processed for glucose determination as described below.

### CHH time-resolved fluoroimmunoassay

#### Antiserum production and purification

Antisera directed against CHHa and CHHb were raised in rabbits (Davids Biotechnologie, Regensburg, Germany) against HPLC-purified *D. celeste* CHHa and *D. celeste* CHHb. For production of each antibody, a New Zealand white rabbit was immunised intramuscularly as well as subcutaneously with an initial injection of 5 nmol peptide emulsified in complete Freund's adjuvant, and at four-weekly intervals with 5, 5 and 3 nmol peptide in incomplete Freund's adjuvant. Four weeks after final immunization, blood was collected by terminal exsanguination under anaesthesia. Principles of laboratory animal care and specific national laws were followed. Immunoglobulin G purification and biotinylation was performed as detailed in Morris et al. (Morris et al., 2010). Antiserum specificity was determined by direct and 'sandwich type' enzyme-linked

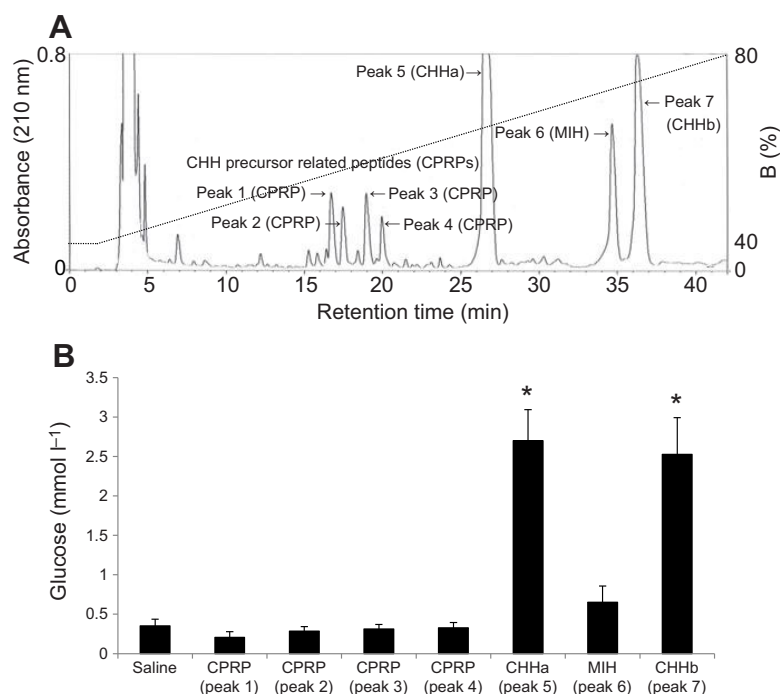


Fig. 1. (A) HPLC fractionation of an extract from 57 sinus glands from *Discoplax celeste*. The acetonitrile gradient is indicated by a dotted line [(A) 0.11% TFA; (B) 0.1% TFA in 60% acetonitrile]. (B) [Glucose] in the haemolymph of *D. celeste* ( $\text{mmol l}^{-1}$ ) 90 min post-injection with ~100 pmol of peptide (crab body mass = ~300 g). Asterisks indicate a significant increase compared with saline-injected crabs. Kruskal–Wallis ANOVA and Mann–Whitney tests for multiple comparisons were used for statistical analysis. Bars represent means  $\pm$  s.e.m. ( $N=8$ ).

immunosorbent assays of HPLC-purified SG fractions and by immunohistochemistry of paraffin-embedded eyestalks.

#### Time-resolved fluoroimmunoassay

Haemolymph samples collected in the field were immediately mixed with an equal volume of anticoagulant (Morris et al., 2010) and snap-frozen on dry ice. The time-resolved fluoroimmunoassay (TR-FIA) was carried out according to the methods detailed in Morris et al. (Morris et al., 2010). Before use in the assay, haemolymph samples were purified on 300 mg Strata-X solid phase extraction cartridges (Phenomenex, Torrance, CA, USA), as previously described. Standards were CHHa (50–0.05 fmol well<sup>-1</sup>). All assays were performed in duplicate wells. Time-resolved fluorescence of europium was measured on a PerkinElmer Victor<sup>2</sup> 1420 instrument (Wallac, Turku, Finland) equipped with proprietary software (Workout) for data analysis. Using these methods, spike-recovery experiments demonstrated that recovery of CHHa was greater than 95%.

#### Glucose and lactate bioassays

For use in glucose and lactate bioassays, haemolymph (normally 200 µl) was taken with a chilled 1 ml syringe using a 26 G needle *via* the arthrodial membrane of a walking leg. The sample was immediately mixed (1:1) with 0.6 mol l<sup>-1</sup> HClO<sub>4</sub> to denature proteins and neutralised with 2.5 mol l<sup>-1</sup> K<sub>2</sub>CO<sub>3</sub> (usually 80 µl) followed by storage at -20°C. Deproteinised haemolymph samples were used to measure glucose and lactate concentrations as detailed in Morris et al. (Morris et al., 2010).

#### Laboratory experiments

##### Dose-responses for CHHa (peak 5) and CHHb (peak 7)

Groups of eight crabs were injected with 100 µl of 0.1, 1, 10 or 100 pmol of CHHa (peak 5) or CHHb (peak 7). Two additional groups of animals (*N*=8) were also used as controls. One of these was injected with moult-inhibiting hormone (MIH; peak 6) (0.1, 1, 10 and 100 pmol) and the other with saline. After 90 min, haemolymph was sampled, stored at -20°C and subsequently assayed for glucose.

#### Field experiments

##### Exercise experiments

On Christmas Island, field experiments were undertaken to examine the effects of exercise on haemolymph CHH, glucose and lactate concentrations. *Discoplax celeste* (*N*=5) were exercised for 10 min by inducing continuous walking; controls (*N*=5) were not disturbed. Haemolymph samples (~1.25 ml) were taken at 0, 5, 10, 20, 30, 60 and 120 min intervals after the start of the 10 min exercise period. Samples were subsequently assayed for CHH, glucose and lactate.

Further experiments were conducted on Christmas Island during both the dry (July–August 2009) and wet (February 2012) seasons. Groups of *D. celeste* (*N*=7) were collected from the rainforest and housed in communal terraria. Every 8 h for 3 days crabs were injected with 100 µl lactate at 10<sup>-6</sup> mol g<sup>-1</sup>, glucose at 10<sup>-6</sup> mol g<sup>-1</sup> or a saline control. Immediately after the final injection, a haemolymph sample was taken, following which the crabs were exercised for 10 min by inducing continuous walking. After 10 min of exercise and a 5 min recovery, a further haemolymph sample was taken. CHH, glucose and lactate concentrations of samples were determined.

##### Branchial infusion and perfusion experiments

The branchial infusions and perfusions were carried out using methods essentially the same as those detailed in Morris et al.

(Morris et al., 1991; Morris et al., 2000) and Morris and Ahern (Morris and Ahern, 2003).

On Christmas Island, infusion experiments were conducted to determine the effects of CHH (CHHa and b) compared with saline (control) on the antennal gland filtration rate using <sup>51</sup>Cr-EDTA (Perkin-Elmer, Melbourne, VIC, Australia) as a tracer. These experiments were conducted during both the dry (July–August 2009) and wet (February 2012) seasons on *D. celeste* and *G. natalis* (using *G. natalis* CHH). The radioactivities of the <sup>51</sup>Cr-EDTA samples were measured using a gamma counter (LKB Wallac Cline Gamma Counter, Wallac). Rates of <sup>51</sup>Cr-EDTA clearance, urine production and fluid resorption within the urinary system were calculated according to Greenaway et al. (Greenaway et al., 1990) and Morris and Van Aardt (1998).

Branchial perfusion experiments were conducted on *D. celeste* and *G. natalis* on Christmas Island to determine the effects of CHH (for *D. celeste* CHHa or CHHb was used, for red crabs, *G. natalis* CHH was used) compared with saline (control) on the unidirectional flux (*J*<sub>in</sub>) of Na<sup>+</sup> using <sup>22</sup>NaCl (Perkin-Elmer) as a tracer. For *D. celeste*, experiments were conducted in five field seasons on Christmas Island that were characterised by varying amounts of rainfall (Fig. 2). For *G. natalis*, experiments were conducted in three successive dry and wet seasons. The radioactivities of the <sup>22</sup>Na samples were measured using a beta scintillation counter (Tri-Carb 2900TR Liquid Scintillation Analyzer, Perkin-Elmer). Samples were also analysed for Na<sup>+</sup> concentration using flame atomic absorption spectrophotometry (Avanta Σ AAS, GBC, Dandenong, VIC, Australia). The net rate of Na<sup>+</sup> uptake was calculated based on the methods described by Morris and Van Aardt (Morris and Van Aardt, 1998), Morris et al. (Morris et al., 1991) and Morris and Ahern (Morris and Ahern, 2003).

#### Seasonal metabolic and osmotic status of *D. celeste*

Samples were collected on Christmas Island to measure haemolymph CHH and glucose concentration during different seasons from animals sampled either directly from the rainforest or after acclimation for 14 days (which involved both drinking and immersion behaviours) to freshwater collected from the nearby freshwater seepages, 30% seawater or, in some cases, 50% seawater. Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase and V-ATPase activities were determined in crabs collected directly from the rainforest or after acclimation to either 10% or 50% seawater.

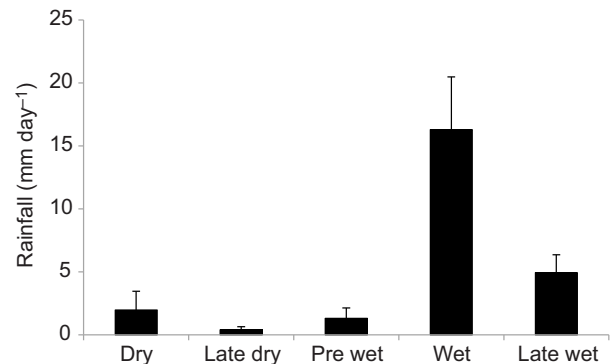


Fig. 2. Daily rainfall (mm) recorded in the five field seasons in which sampling was conducted on Christmas Island: dry (July 2007), late dry (August–September 2008), pre wet (November–December 2007), wet (February–March 2008) and late wet (July–August 2009). Values are means ± s.e.m. (*N*=22–27). Rainfall data were obtained from the Australian Government Bureau of Meteorology ([www.bom.gov.au](http://www.bom.gov.au)).

### Gill enzyme activities

Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was determined in gill tissue using the methods described in Morris and Ahern (Morris and Ahern, 2003). V-ATPase activity was determined using the methods of Onken et al. (Onken et al., 2000).

### Statistical analysis

Statistical analyses were carried out using Minitab 15 (Minitab, State College, PA, USA). The normal distribution of data was verified using the Anderson–Darling test prior to all statistical tests. Where the data were not normally distributed, the data were transformed (stated in figure legends). *T*-tests or one- or two-way ANOVA were carried out on data (or transformed data) that exhibited normal distributions. *Post hoc* testing was carried out using Tukey's tests when significant differences were indicated by one- or two-way ANOVA. If transformation of the data did not produce normally distributed data sets, non-parametric Kruskal–Wallis or Scheirer–Ray–Hare tests followed by Mann–Whitney tests were carried out. Where appropriate, a Bonferroni correction for multiple testing was used.

## RESULTS

### Identification of CHH

HPLC of *D. celestes* SG extracts gave typical separation profiles, as shown in Fig. 1A. On the basis of matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) of the prominent peaks [masses (all MALDI-TOF, M+H<sup>+</sup>): peak 1, 3697.71 Da; peak 2, 3698.3 Da; peak 3, 3713.0 Da; peak 4, 3712.56 Da; peak 5, 8512.74 Da; peak 6, 8976.14 Da; peak 7, 8503.6 Da] and amino acid analysis (data not shown), four peptides (peaks 1–4) were tentatively identified as CHH precursor related peptides (CPRPs). Two prominent peaks were markedly hyperglycaemic in the *D. celestes* bioassay (Fig. 1B), and were referred to as CHHa (peak 5) and CHHb (peak 7). Peak 6 did not induce hyperglycaemia. In view of its mass and amino acid composition, this probably represented an MIH, although its biological activity (repression of ecdysteroid biosynthesis) was not determined. A strategy involving mRNA isolation from the medulla terminalis, cDNA cloning, degenerate PCR, and 3' and 5' rapid amplification of cDNA ends (RACE) from medulla terminalis mRNA gave a full-length product encoding an open reading frame corresponding exactly to CHHb and a CPRP (GenBank accession no. JF894384) (see the Appendix for materials and methods for cloning and sequencing of *D. celestes* CHH). The mass of the conceptually translated CHHb agreed exactly with that measured by MALDI-TOF (M+H<sup>+</sup>; 8503.6 Da). Despite extensive sequencing, we could not identify any further clones corresponding to CHHa. Dose–response experiments showed that CHHa was somewhat more potent in eliciting hyperglycaemia than CHHb (Fig. 3). The antisera raised against CHHa and CHHb were used in direct enzyme immunoassay of HPLC-fractionated material to establish specificity. Only the antiserum raised against CHHb was of suitable titre and affinity, and this cross-reacted equally with both CHHs (results not shown). This was used to develop a sensitive TR-FIA, with a detection limit (for 'total' CHHa and b) of less than 0.1 fmol per well (Fig. 4).

### Exercise/recovery and feedback loops

Experiments were conducted in the field to examine the effect of exercise on CHH, glucose and lactate concentrations in the haemolymph of *D. celestes* (Fig. 5). Glucose haemolymph levels significantly increased ( $P=0.0147$ ) in the exercised crabs 20 min post-exercise ( $1.65\pm 0.42$  mmol l<sup>-1</sup> compared with  $0.49\pm 0.11$  mmol l<sup>-1</sup> in the controls). There was much variability seen in the remainder (after

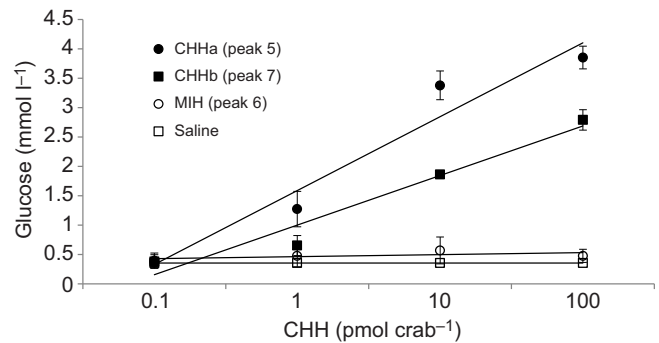


Fig. 3. [Glucose] (mmol l<sup>-1</sup>) in the haemolymph of *D. celestes* 90 min post-injection with ~0.1, 1, 10 and 100 pmol of crustacean hyperglycaemic hormone (CHH) (crab body mass = ~300 g). Non-parametric ANCOVA was used for statistical analysis. Data points represent means  $\pm$  s.e.m. ( $N=8$ ).

20 min post-exercise) of the haemolymph glucose data, but all exercised animals continued to show higher haemolymph glucose concentrations than those of the unexercised controls. However, in the case of lactate haemolymph concentration, this significantly increased ( $P=0.037$ ) after 5 min of exercise from  $1.91\pm 0.45$  to  $21.8\pm 4.72$  mmol l<sup>-1</sup> in exercised animals compared with  $0.50\pm 0.30$  to  $2.02\pm 1.05$  mmol l<sup>-1</sup> in the control crabs. Significant differences remained between exercised and control animals until 50 min post-exercise ( $P=0.012$ ). CHH levels in the haemolymph significantly increased ( $P=0.035$ ) also within 5 min of exercise ( $335.44\pm 50.86$  mmol l<sup>-1</sup> compared with  $83.2\pm 32.66$  mmol l<sup>-1</sup> in the controls). This difference persisted during the remainder of the exercise period ( $P=0.044$ ), but post-exercise the levels of CHH in the haemolymph rapidly returned to those of unexercised animals (Fig. 5).

To examine possible feedback loops (and their seasonality) involved in CHH release, *D. celestes* were injected with compounds that are important in energy metabolism (i.e. glucose, lactate). Changes in circulating CHH levels are shown in Fig. 6. In the dry season, CHH levels increased following exercise after injection with glucose ( $P=0.045$ ), lactate ( $P=0.011$ ) or a saline control ( $P=0.011$ ). In the wet season there was no significant difference in haemolymph CHH concentration after exercise in those animals previously injected with glucose ( $144.57\pm 27.91$  pmol l<sup>-1</sup> compared with

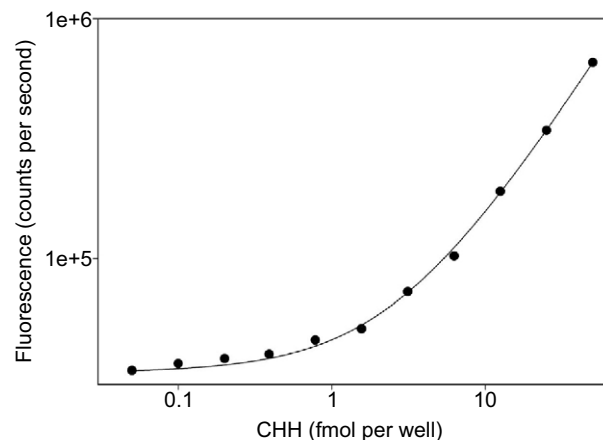


Fig. 4. Typical standard curve for the *D. celestes* CHHa time-resolved fluoroimmunoassay. The working range (limits of the assay) is between 0.5 and 0.5 fmol 100  $\mu$ l<sup>-1</sup>. Standards (duplicates) were diluted from quantified (HPLC purified) sinus gland extract.

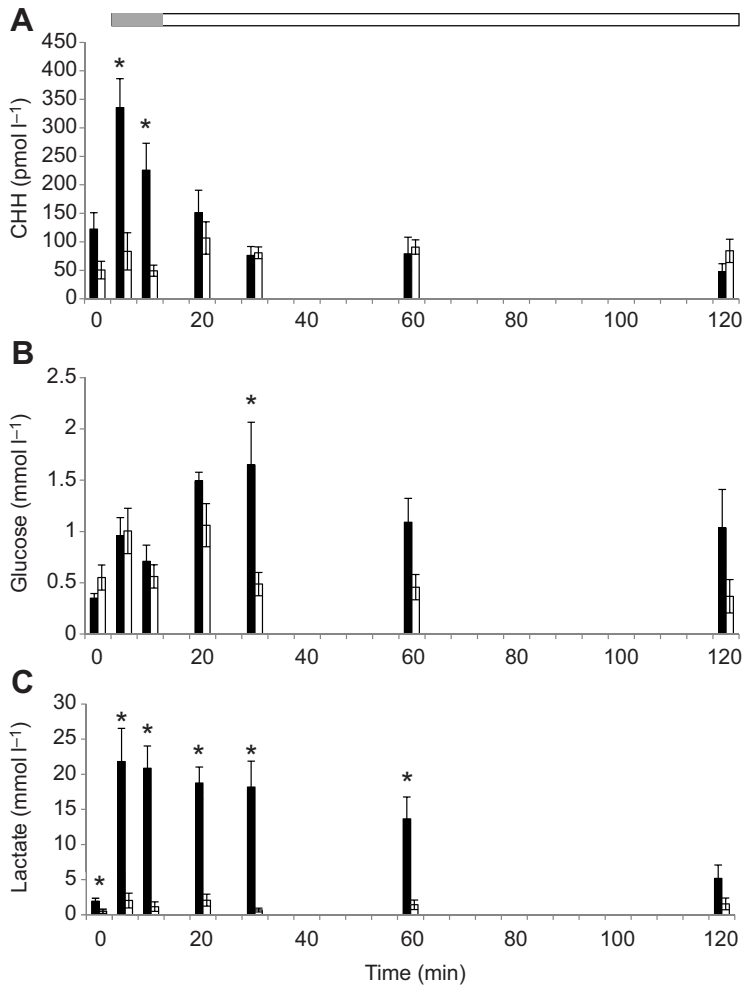


Fig. 5. The effect of exercise on circulating (A) CHH, (B) glucose and (C) lactate levels. *Discoplax celestes* were exercised for 10 min (grey bar) followed by a 110 min recovery period (white bar). Black columns show exercised crabs, white columns unexercised controls. Asterisks indicate a significant increase compared with unexercised crabs. Bars represent means  $\pm$  s.e.m. ( $N=5$ ). Statistical tests were performed by comparing controls with exercised crabs at each time point (one-way ANOVA and Tukey's tests).

92.43 $\pm$ 4.58 pmol l<sup>-1</sup>), but there was a significant difference in CHH haemolymph concentration in those crabs injected with either lactate ( $P=0.048$ ) or the saline control ( $P=0.023$ ; Fig. 6). However, it should be noted that differences in resting CHH levels between seasons (Fig. 6) are not directly comparable because crabs used in this experiment were held in captivity for 3 days prior to the start of this experiment. Haemolymph glucose concentration did not significantly increase post-exercise in those crabs previously injected with glucose or lactate (Fig. 6C,D). However, lactate levels increased after exercise in all three groups of crabs (Fig. 6E,F).

#### Branchial infusion and perfusion

In the dry season (July–August 2009) there was a significant increase in urine production in *D. celestes* infused with CHH compared with those infused with the saline control ( $P=0.047$ ; Fig. 7). In the wet season (February 2012) there was no effect of CHH on urine production ( $P=0.140$ ). When the same experiment was conducted on *G. natalis* in both the dry ( $P=0.804$ ) and the wet ( $P=0.506$ ) seasons there was no significant effect of CHH on urine production.

Determination of branchial Na<sup>+</sup> uptake ( $J_{in}$ ) in *D. celestes* demonstrated that there were no significant differences in the effects of CHHa or CHHb when compared with the saline-injected crabs ( $P=0.863$ ), but the season the crabs were sampled in did have a significant effect on unidirectional Na<sup>+</sup> uptake ( $P<0.001$ ). However, this seasonal effect was dependent on which CHH was injected ( $P<0.001$ ; Fig. 8A). The injection of CHHa significantly increased Na<sup>+</sup>  $J_{in}$  in the very dry (pre-wet) season and the injection

of CHHb significantly increased Na<sup>+</sup>  $J_{in}$  in the wet season, when the crabs were experiencing the driest and wettest conditions, respectively (Fig. 8A). Determination of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the gills of *D. celestes* following perfusion with CHH demonstrated that there were no significant differences in the effects of CHHa or CHHb when compared with the saline-injected crabs ( $P=0.864$ ), but the sampling season had a significant effect ( $P<0.001$ ). However, this seasonal effect was again dependent on which CHH was injected ( $P=0.034$ ; Fig. 8B). CHHb significantly elevated Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the gills of *D. celestes*, but this was only the case in the dry season. In *G. natalis*, CHH significantly decreased branchial Na<sup>+</sup>  $J_{in}$  in the dry season ( $P=0.038$ ) and increased Na<sup>+</sup>  $J_{in}$  in the wet season ( $P=0.015$ ) when compared with those crabs injected with saline (Fig. 9). Determination of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the gills of *G. natalis* demonstrated that there was no significant effect of CHH on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity at the gills ( $P=0.849$ ; supplementary material Fig. S1).

#### Seasonal metabolic and osmotic status of *D. celestes*

There was a significant effect of sampling season on the seasonal haemolymph CHH concentration ( $P<0.001$ ), with the highest levels of CHH recorded from animals in the dry season (315.63 $\pm$ 45.07 pmol l<sup>-1</sup>, compared with 80.07 $\pm$ 11.46 pmol l<sup>-1</sup> in the wet season). There was also a significant effect of sampling season on haemolymph glucose concentration ( $P<0.001$ ); however, here the highest levels were recorded in the wet season (0.55 $\pm$ 0.12 mmol l<sup>-1</sup>,

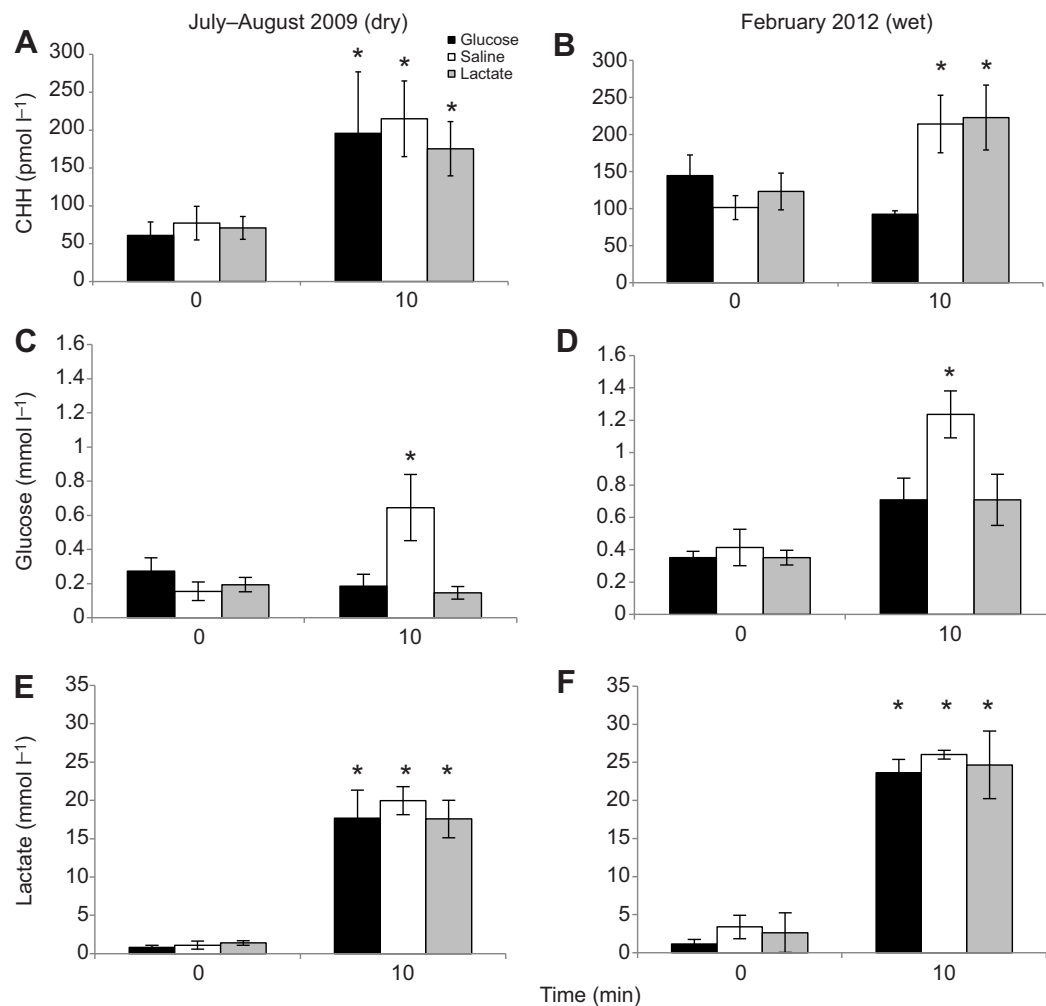


Fig. 6. The effect of exercise on haemolymph CHH levels in *D. celeste* previously injected with metabolites (glucose, lactate) or control saline. Haemolymph samples were taken before and after 10 min exercise periods, for (A) dry season (July and August 2009) and (B) wet season (February 2012) crabs. Asterisks indicate a significant increase compared with pre-exercised levels. Bars represent means  $\pm$  s.e.m. ( $N=7$ ) (Student's *t*-tests and Mann-Whitney tests). (C–F) Glucose [(C) dry season, (D) wet season] and lactate [(E) dry season, (F) wet season] levels in the same crabs.

compared with  $0.11 \pm 0.02 \text{ mmol l}^{-1}$  in the dry season). However, when *D. celeste* were acclimated to increasing salinities, the amount of CHH in the haemolymph increased with salinity ( $P < 0.001$ ; Fig. 10).

No effect of CHH ( $P = 0.231$ ) was found on gill V-ATPase activity (supplementary material Fig. S2). CHHa appeared to increase the  $\text{Na}^+/\text{K}^+$ -ATPase activity in crabs kept in freshwater conditions, compared with those with access to 10% seawater ( $P = 0.0027$ ) or 50% seawater ( $P = 0.0005$ ; Fig. 11). However, there was no overall effect of CHH on  $\text{Na}^+/\text{K}^+$ -ATPase activity.

#### DISCUSSION

This study is the most comprehensive to date investigating the osmoregulatory capacities of CHH in crustaceans and is the first to investigate this on a seasonal basis. It is also one of the few field-based studies investigating the physiological effects of CHH *in vivo*.

It has long been recognised that the cue for the seasonal migration of *D. celeste* on Christmas Island is the wet season rains, with the crabs setting out on their seaward journey some weeks after the start of the monsoon (Gibson-Hill, 1947; Hicks et al., 1990; Morris, 2005). For the few months prior to the seasonal rains the crabs experience the driest conditions and thus must limit their daily

activities to dawn and dusk and to those areas where a freshwater supply remains constant. It seems relevant then, that the two forms of CHH identified in this study had a significant seasonally synchronised osmoregulatory effect in *D. celeste*: CHHa significantly increased  $\text{Na}^+$  uptake at the gills in the very dry (pre-wet) season and CHHb significantly increased  $\text{Na}^+$  uptake at the gills in the wet season (Fig. 8A). In the pre-wet season, the crabs are experiencing some of the driest conditions and are therefore in a 'water conservation mode', minimising the amount of 'P' and thus ions lost. Therefore, a likely scenario could be that CHHa is acting on  $\text{Na}^+$  uptake at this very dry time of the year by maximising the amount of  $\text{Na}^+$  taken up from the very small amounts of urine being filtered from the haemolymph into the branchial chambers. Due to the fact that the amount of urine being produced at this much drier time of year is significantly smaller than that in the wet season, the amount of  $\text{Na}^+$  by volume (i.e. the urine is more concentrated) will potentially be significantly increased compared with that of larger amounts of dilute urine produced in the wet season. Furthermore, it is possible, as alluded to previously (Taylor and Greenaway, 2002; Morris and Ahern, 2003), that CHH could act on the 'drinking mechanism' in these crabs; the crabs are capable of drinking their

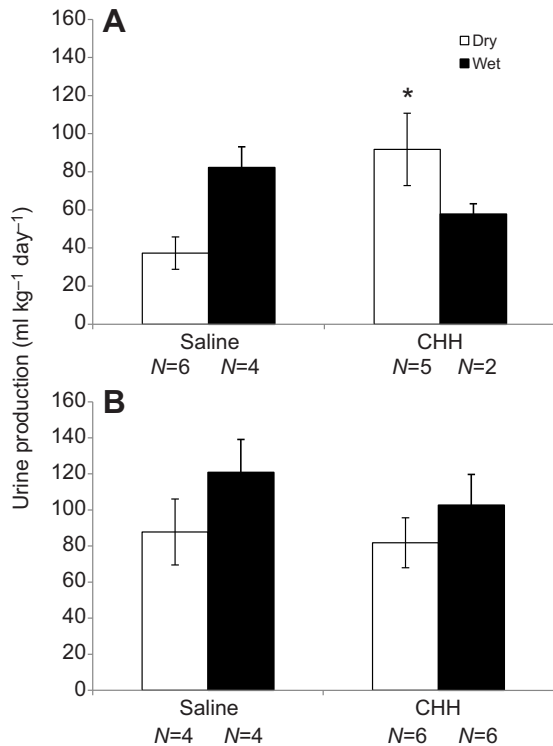


Fig. 7. The effect of CHH on the volume rates of <sup>51</sup>Cr-EDTA clearance from the haemolymph and urine flow in (A) *D. celeste* and (B) *G. natalis*. Crabs were infused with either CHH or a saline control. Urine production was determined over 24 h using the appropriate urine:haemolymph ratio of <sup>51</sup>Cr. The asterisk indicates a significant elevation compared with saline-infused crabs (Student's *t*-test). Bars represent means  $\pm$  s.e.m.

own urine to conserve water in drier periods, which could also increase the rate of Na<sup>+</sup> uptake. Conversely, at the wettest times of the wet season, CHHb could also be acting on Na<sup>+</sup> uptake. At this time of the year large amounts of urine are produced by *D. celeste*, a significant proportion of which is excreted as 'P', meaning that large numbers of ions could also be lost from the crab. Of primary importance for land crabs is the need to keep salt loss to a minimum, thus potentially explaining the increased rate of Na<sup>+</sup> uptake by CHHb from a much larger volume of urine in the wet season. The similar observations recorded for the closely related land crab *G. natalis* (Fig. 9) suggest a universal ion uptake mechanistic effect of CHH amongst terrestrial crabs. In this species the results mirrored those seen in *D. celeste* in the wet season: in CHH-injected crabs, Na<sup>+</sup> uptake increased during the wet season. During their breeding migration it is of paramount importance that salt loss is minimised, especially as the crabs have to conquer the twin dangers of dehydration due to increased aerobic exercise and potential salt depletion as a result of monsoon downpours. When the crabs reach the lower terraces where they will carry out their breeding activities, both males and females first 'dip' in the ocean to replenish these lost salts (Hicks et al., 1990) and later females spend up to a minute submerged in the sea whilst they are releasing their fertilised eggs (Greenaway, 1989). In the present study it was unequivocally shown that when *D. celeste* were acclimated to increasingly saline water regimes, the circulating levels of CHH in the haemolymph increased (Fig. 10). This response could be linked to a downregulatory action of CHH to prevent the crab from being swamped with dangerously high levels of salts at times such as this.

In both *D. celeste* and *G. natalis*, CHH had no significant effect on gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Fig. 8B, supplementary material Fig. S1). This was despite the fact that Na<sup>+</sup>/K<sup>+</sup>-ATPase has previously been shown to be instrumental in driving ion uptake at the gills in *G. natalis* (Morris and Ahern, 2003) as well as in other terrestrial crabs, including *Birgus latro* (Morris et al., 2000; Greenaway, 2003). It is well established that it is V-ATPase that plays a significant role in ion regulation in the gills of freshwater crustaceans, including the freshwater crayfish *Cherax destructor* (Onken and Graszynski, 1989), *E. sinensis* (Onken et al., 1991; Onken and Putzenlechner, 1995; Riestenpatt et al., 1995) and the South American true freshwater crab *Dilocarcinus pagei* (Weihrauch et al., 2004). However, further investigations in the present study were unable to show any significant effect of CHH on V-ATPase activity in *D. celeste* (supplementary material Fig. S2), thus indicating that the mode of action of CHH on the gills to facilitate ion uptake remains to be fully characterised in terrestrial crabs. Recent work has provided evidence for a carbonic anhydrase repressor in the eyestalks of several species of crab (Henry, 2006; Henry and Borst, 2006; Henry and Campoverde, 2006). Carbonic anhydrase plays an important role in respiratory gas exchange and thus osmoregulation in the gills of freshwater crustaceans. Therefore, it would appear that the action of CHH on carbonic anhydrase activity in terrestrial crabs is a promising route for further enquiry.

Interestingly, CHH also appeared to influence urine production and antennal gland filtration rate in *D. celeste* on a seasonal basis, with an increase in urine production being recorded in the dry season (Fig. 7). It has been previously suggested that the antennal gland plays an important role not in water conservation but instead in the regulation of ion uptake in terrestrial crabs (Harris, 1977). This seems to make sense, as for many land crabs the main osmoregulatory challenge is that of salt, as opposed to water loss (Freire et al., 2008), with the alteration of urine filtration rate (Wolcott, 1992) in *B. latro* (Greenaway et al., 1990; Taylor et al., 1993) and *G. natalis* (Greenaway, 1994; Morris and Ahern, 2003) being recognised as one of several mechanisms for the control of salt loss in terrestrial crustaceans (Morris, 2001). Furthermore, this result has obvious correlations with the action of ITP in insects (Phillips and Audsley, 1995; Phillips et al., 1998), which is perhaps not surprising due to the fact that ITP is at the base of the CHH family in terms of ecdysozoan evolution (Montagné et al., 2010). Indeed this finding appears to support the hypothesis that ionic and water regulation could be the ancestral function of CHH/ITP family peptides (Toullec et al., 2006). The observation that no effect of CHH was seen on urine production rates in *G. natalis* concurs with the contrasting life history strategies of the two species: *D. celeste* spends much of its life in association with freshwater, and therefore faces rapid water influx and salt loss compared with *G. natalis*, which is much more independent of water and terrestrial in its adult life history. Nevertheless, these results clearly suggest that much more work is required to examine the specific neurohormonal controls on the osmoregulatory capabilities of the antennal gland in terrestrial crabs, including the role of ATPases.

CHH has a well-studied role in the regulation of energy metabolism in many crustacean species (for a review, see Webster et al., 2012). Therefore, it is possible that any seasonal response to the control of salt and water balance could be integrated with those of metabolic homeostasis. It was an important part of the experimental design to establish whether the hormonal control of energy metabolism in these crabs behaved in a manner similar to that of the many other species of crustaceans investigated to date. Initial experiments demonstrated that the SGs of *D. celeste* contained the two functionally active CHHs

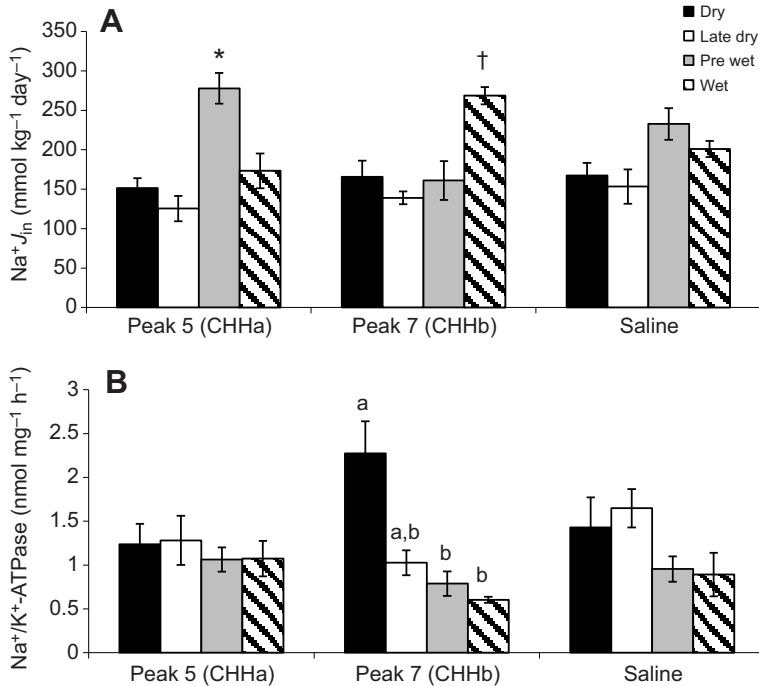


Fig. 8. (A) Branchial Na<sup>+</sup> flux in *D. celeste* infused with a saline control, CHHa or CHHb at 100 pmol crab<sup>-1</sup> in different seasons. The branchial chambers were perfused with artificial urine labelled with <sup>22</sup>Na. Flux rates were determined over 120 min. Uptake of <sup>22</sup>Na provided rates of unidirectional influx ( $J_{in}$ ). (B) [Na<sup>+</sup>/K<sup>+</sup>-ATPase] in the gills after infusion with either saline or CHH (CHHa or CHHb). The asterisk indicates a significant increase in Na<sup>+</sup> flux in CHHa-infused crabs in the pre-wet season, when compared with other seasons, but not when compared with saline-infused crabs; the dagger indicates a significant increase in Na<sup>+</sup> flux in CHHb-infused crabs in the wet season, when compared with other seasons, but not when compared with saline-infused crabs. In the case of the gill [Na<sup>+</sup>/K<sup>+</sup>-ATPase] data, bars with different letters indicate significant differences within the CHHb data set (however, these were not significantly different from saline-infused crabs). Two-way ANOVA and Tukey's tests for multiple comparisons were used for statistical analysis. The gill [Na<sup>+</sup>/K<sup>+</sup>-ATPase] data were log<sub>10</sub>-transformed prior to analysis. Bars represent means ± s.e.m. (N=8).

(CHHa and CHHb) (Fig. 1). However, whilst it was shown that CHHa and CHHb were able to induce significant levels of hyperglycaemia in the haemolymph of *D. celeste* (Fig. 1B), despite repeated attempts using molecular (5'/3' RACE) and protein sequencing techniques, the sequence of CHHa remained incomplete. Notwithstanding this, after exercise, levels of CHH, glucose and lactate in the haemolymph of *D. celeste* increased significantly (Fig. 5) and showed similar profiles of release over time compared with those produced by other exercised terrestrial crabs (Morris et al., 2010). Although CHH titres appeared to be significantly higher in *D. celeste* compared with those of *G. natalis*, this is probably due to species-specific differences in haemolymph CHH concentrations. Large differences in CHH titre have been previously recorded between taxa; for instance, lobsters have resting CHH levels an order of magnitude lower than those of crabs (i.e. Chang et al., 1998). Furthermore, of the two Christmas Island terrestrial crabs, *D. celeste* is by far the more active (Hicks et al., 1990; Morris, 2005), which might be reflected in differences in CHH titre when comparing these species.

The seasonal breeding migration undertaken by *D. celeste* requires not only an adequate supply of carbohydrate to power the crabs' muscles for the several days of continuous walking that must be undertaken, but also that this energy store is closely and economically controlled. Previous work has demonstrated that haemolymph glucose

levels influence CHH levels *via* negative feedback loops (Santos and Keller, 1993; Glowik et al., 1997) and that in *G. natalis* these have a seasonally specific application (Morris et al., 2010). Experiments were undertaken in the present study to see whether the same scenario was true in *D. celeste* and thus whether this was potentially a universal physiologically relevant process in other terrestrial crabs. Results similar to those recorded by Morris et al. (Morris et al., 2010) were noted, with high haemolymph glucose levels in the wet season apparently restricting CHH release in exercising crabs (Fig. 6). Conversely, in the dry season when the crabs were mostly inactive, glucose-injected crabs showed a significant increase in haemolymph CHH concentration after exercise. This uncoupling of the negative feedback loop could be important for the regulation of energy, i.e. glycogen stores, during the migration, especially when the crabs are returning to the rainforest, having carried out their very energetically demanding activities such as mating and, in the case of male crabs, digging and defending burrows from competitors. Furthermore, the crabs still have access to food, i.e. leaf litter, at this time of year, so to release more CHH (for the purpose of ensuring a release of glucose) would be unnecessary. However, as well as regulating energy supply during the actual migration itself, an alternative hypothesis could be that any effect(s) of CHH on osmoregulation are indirect and instead concern the metabolic regulatory properties, specifically of fuel

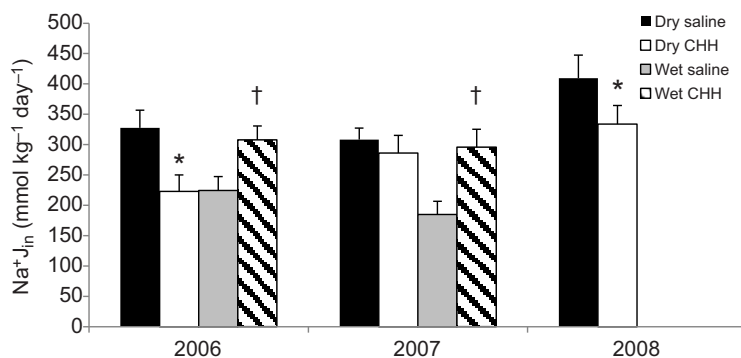


Fig. 9. Branchial Na<sup>+</sup> flux in *G. natalis* infused with either a saline control or CHH at 100 pmol crab<sup>-1</sup> in different seasons. The branchial chambers were perfused with artificial urine labelled with <sup>22</sup>Na. Flux rates were determined over 120 min. Uptake of <sup>22</sup>Na provided rates of unidirectional influx ( $J_{in}$ ). Asterisks indicate a significant decrease in Na<sup>+</sup> flux in CHH-infused crabs in the dry season; daggers indicate a significant increase in Na<sup>+</sup> flux in CHH-infused crabs in the wet season. One-way ANOVA and Tukey's tests for multiple comparisons were used for statistical analysis. Unidirectional Na<sup>+</sup> uptake data were log<sub>10</sub>-transformed prior to analysis. Bars represent means ± s.e.m. (N=8).



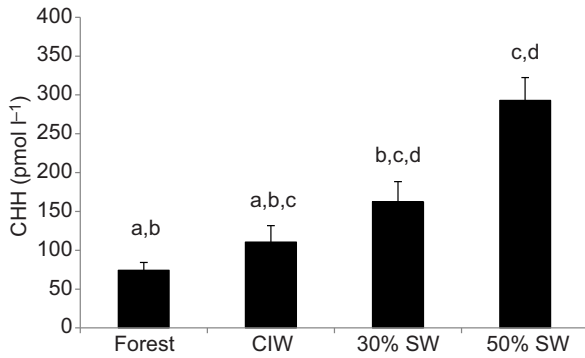


Fig. 10. [CHH] (pmol l<sup>-1</sup>) in the haemolymph of *D. celeste*. Sampling was from crabs taken directly from the rainforest or after acclimation to Christmas Island water (CIW), 30% seawater (30% SW) or 50% seawater (50% SW). Bars with different letters indicate significant differences. One-way ANOVA followed by Tukey's tests for multiple comparisons were used for statistical analysis. Bars represent means  $\pm$  s.e.m. ( $N=16$ ).

(glucose) provision by this hormone (Fanjul-Moles, 2006; Morris et al., 2010). Thus, CHH could potentially increase ion uptake and thus osmoregulatory capacity at the gills by increasing the availability of metabolisable energy to the ion-exchange pumps at the gills through increased glycogenolysis (Spanings-Pierrot et al., 2000).

It has long been known that CHH is a pleiotropic hormone and the adaptive significance of CHH has been demonstrated by the results of this study. It has been shown that CHH has specific seasonal effects not only on salt and water balance but also on energy provision and the regulation of metabolism in *D. celeste*, especially in the context of the physiologically demanding breeding migration. Whilst phylogenetics indicate that freshwater land crabs such as *D. celeste* are some of the most recently evolved crustaceans, it is the evolution of the control of osmoregulatory mechanisms that has allowed the colonisation of land by aquatic crustaceans. The results of this study suggest that CHH has had a pivotal role in this evolutionary strategy; however, it is certain that there is much more

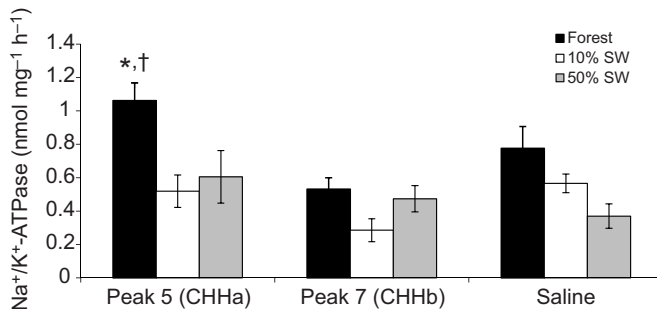


Fig. 11. The effect of CHH on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity ( $\mu\text{mol mg}^{-1} \text{h}^{-1}$ ) in the gills of *D. celeste*. Crabs were injected with CHHa, CHHb ( $100 \text{ pmol crab}^{-1}$ ) or a saline control after being collected directly from the rainforest or acclimated for 14 days to either 10% or 50% seawater. The asterisk indicates a significant increase in gill [Na<sup>+</sup>/K<sup>+</sup>-ATPase] in CHHa-infused crabs kept in freshwater conditions compared with those infused with CHHb, but not with those infused with saline; the dagger indicates a significant increase in gill [Na<sup>+</sup>/K<sup>+</sup>-ATPase] in CHHa-infused crabs kept in freshwater conditions compared with those also infused with CHHa but acclimated to 10% or 50% seawater, but not compared with those infused with saline (two-way ANOVA and Tukey's tests). Bars represent means  $\pm$  s.e.m. ( $N=7$ ).

to be discovered regarding the evolutionary adaptability of CHH in a wide variety of physiological processes not only in land crabs, but also in crustaceans in general.

## APPENDIX

### Cloning and sequencing of *D. celeste* CHH

#### Tissue preparation and RNA extraction

For cDNA cloning, RNA was extracted from a single medulla terminalis using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by treatment with two units of DNase I (37°C, 30 min) and clean up with TURBO DNA-free (Ambion, Austin, TX, USA), and was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

#### cDNA synthesis and RACE

For 3' RACE, mRNA, 1–3  $\mu\text{g}$  total RNA was reverse transcribed using Superscript III RT (Invitrogen) (50°C, 50 min) using the Gene Racer 3' oligo(dT) adapter primer. For 5' RACE, mRNA was dephosphorylated, decapped, ligated to a 5' RACE RNA oligo (Invitrogen) and reverse transcribed using Superscript III using random primers according to the manufacturer's instructions. Following reverse transcription, the sample was treated (37°C, 20 min) with two units of RNase H.

#### Degenerate PCR of *D. celeste* cDNA encoding X-organ CHH

Using available amino acid sequence data for CHH in the Brachyura, a set of fully degenerate primers were designed (Table A1). PCR reagents were: 21.5  $\mu\text{l}$  Megamix Blue (Helena Biosciences, Sunderland, UK), 1.25  $\mu\text{l}$  ( $100 \mu\text{mol l}^{-1}$ ) forward and reverse primers (F1, R1 or F1, R2) and 1  $\mu\text{l}$  cDNA template. PCR conditions were: 94°C for 4 min, 35 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 7 min. The second nested PCR used 1  $\mu\text{l}$  of the first PCR reaction as template with 21.5  $\mu\text{l}$  Megamix Blue and 1.25  $\mu\text{l}$  ( $100 \mu\text{mol l}^{-1}$ ) forward and reverse primers (F1n, R1n or F2, R1n or F1n, R2n or F1n, R2 or F2, R1 or F1n, R2 or F1, R2n or F1n, R2n or F2, R2n or F2, R2). PCR conditions were the same as shown above. PCR products were electrophoresed on agarose gels, and bands of expected sizes were excised and extracted using a Perfectprep gel purification kit (Eppendorf, Hamburg, Germany).

#### 3' and 5' RACE PCR of *D. celeste* cDNA encoding X-organ CHH

Using sequence information obtained from degenerate PCR, gene-specific primers (GSPs) were designed for 3' and 5' RACE PCR. An additional set of GSPs were also designed to ensure the entire untranslated region of CHH was sequenced (primers YNYRTSYV and LYRTSYVSSA). For 3' RACE, nested PCRs were performed as follows. PCR reagents were: 12.5  $\mu\text{l}$  AmpliTaq Gold Master mix (Roche, Branchburg, NJ, USA), 9  $\mu\text{l}$  water, 1.25  $\mu\text{l}$  ( $100 \mu\text{mol l}^{-1}$ ) Disco GSP F or YNYRTSYV, 1.25  $\mu\text{l}$  ( $10 \mu\text{mol l}^{-1}$ ) 3' GeneRacer primer (Invitrogen) and 1  $\mu\text{l}$  3' RACE cDNA template (prepared as detailed above). Touchdown PCR conditions were used: 94°C for 9 min, five cycles of 94°C for 30 s, 62°C for 45 s, five cycles of 94°C for 30 s, 60°C for 45 s, 25 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The second nested PCR used 1  $\mu\text{l}$  of the first PCR reaction as template with 44  $\mu\text{l}$  Megamix Blue, 2.5  $\mu\text{l}$  ( $100 \mu\text{mol l}^{-1}$ ) Disco GSP F<sub>N</sub> or LYRTSYVSSA and 2.5  $\mu\text{l}$  ( $10 \mu\text{mol l}^{-1}$ ) 3' nested GeneRacer primer (Invitrogen). PCR conditions were: 94°C for 4 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were electrophoresed and bands were extracted as described earlier.

Table A1. Primers used for *Discoplax celeste* sequence identification and 3' and 5' RACE PCR

	Primer name	Sequence
<i>D. celeste</i> CHH degenerate	F1	CAYCCYITNGARAARMGNCARATHAYGA
	F1n	GARAARMGNCARATHAYGA
	F2	TGYAARGGNGTNTAYGAYMG
	R1	TCCATRCAYTGICKDAWNAC
	R1n	CATRCAYTGICKDAWNAC
	R2	TTRTARCARTCRTRCANACRTG
	R2n	TARCARTCRTRCANACRTG
	<i>D. celeste</i> CHH 3' RACE	Disco GSP F
Disco GSP F <sub>N</sub>		GACAGATCTCTCTCAGCAAGCTGGA
YNLYRYSYV		ATAACCTCTACCGCACCTCTACGTC
LYRYSYVSSA		TCTACCGCACCTCTACGTCCTCCAGCGCC
<i>D. celeste</i> CHH 5' RACE	Disco GSP R	GTTCTCCCTGCAGGCGCTGGAGACGTAG
	Disco GSP R <sub>N</sub>	AGGCGCTGGAGACGTAGGAGGTGCGCTA

CHH, crustacean hyperglycaemic hormone; GSP, gene-specific primer.

5' RACE PCR was performed on cDNA prepared from reverse transcription of ligated mRNA (as described above). PCR reagents were: 12.5 µl AmpliTaq Gold Master mix, 9 µl water, 1.25 µl (100 µmol l<sup>-1</sup>) Disco GSP R, 1.25 µl (10 µmol l<sup>-1</sup>) 5' GeneRacer primer (Invitrogen) and 1 µl 5' RACE cDNA template. Touchdown PCR conditions were used: 94°C for 9 min, five cycles of 94°C for 30 s, 64°C for 45 s, five cycles of 94°C for 30 s, 62°C for 45 s, 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The second nested PCR used Disco GSP R<sub>N</sub> and 5' nested GeneRacer primer (Invitrogen), with 1 µl of the first PCR reaction as template. PCR conditions were: 94°C 4 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products were electrophoresed and bands were extracted as described earlier.

#### Cloning and sequencing of PCR products

Purified PCR products were ligated into a PCR 4-TOPO vector and transformed (TOP-10F', Invitrogen) according to the manufacturer's instructions. Plasmid DNA from positive clones containing inserts of correct sizes were purified (FastPlasmid Mini kit, Eppendorf), quantified (NanoDrop-1000) and sequenced commercially (MWG Biotech, London, UK).

#### LIST OF SYMBOLS AND ABBREVIATIONS

CHH	crustacean hyperglycaemic hormone
CPRP	crustacean hyperglycaemic hormone precursor-related peptide
ITP	ion transport peptide
$J_{in}$	unidirectional flux
MALDI-TOF MS	matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
MIH	molt-inhibiting hormone
RACE	rapid amplification of cDNA ends
SG	sinus gland
TR-FIA	time-resolved fluorimmunoassay

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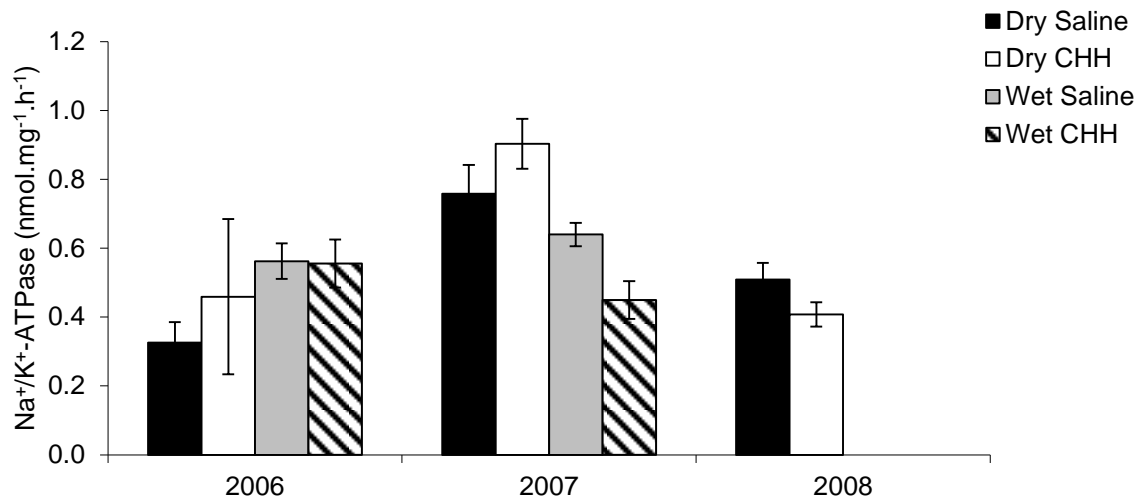
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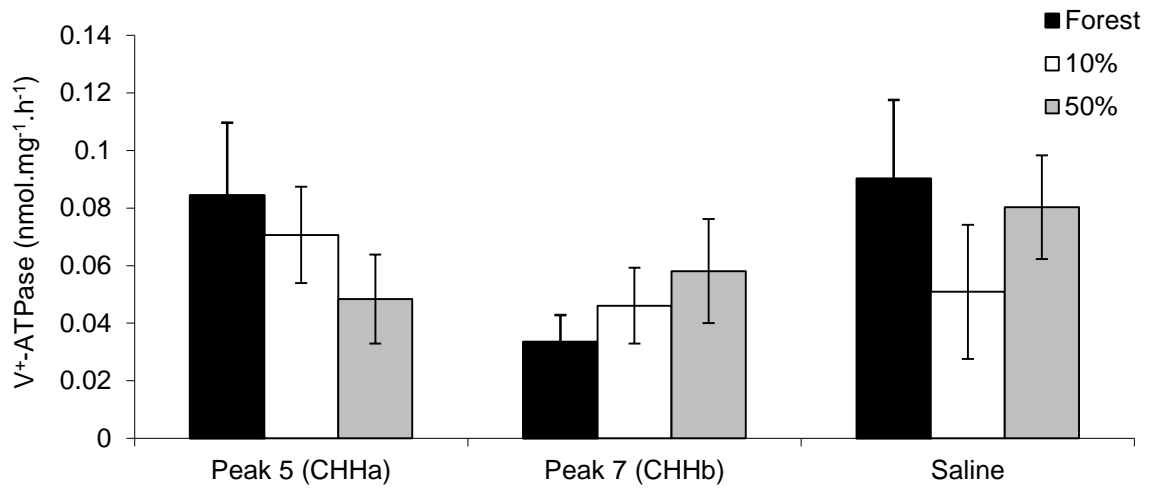
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**Fig. 1.**  $[Na^+/K^+-ATPase]$  in the gills of *G. natalis* after infusion with either a saline control or CHH at  $100 \text{ pmol.crab}^{-1}$  in different seasons. One-way ANOVA and Tukey's tests for multiple comparisons used for statistical analysis. The data were square-root transformed prior to analysis. Bars represent mean  $\pm$  SEM (n=8).



**Fig. 2.** The effect of CHH on V-ATPase activity in the gills of *D. celeste* ( $\mu\text{mol.mg}^{-1}.\text{h}^{-1}$ ). Crabs were injected with either CHHa, CHHb ( $100 \text{ pmol.crab}^{-1}$ ) or a saline control after being collected directly from the rainforest or acclimated for 14 days to either 10% or 50% seawater. Bars represent mean  $\pm$  SEM ( $n=7$ ) (two-way ANOVA and Tukey's tests).