Roles of crustacean hyperglycaemic hormone in ionic and metabolic homeostasis in the Christmas Island Blue crab Discoplax celeste

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\textsuperscript{†}Professor Morris died on August 11\textsuperscript{th} 2009 before this work was completed. This paper is dedicated to his memory.

\textbf{Key words:} Discoplax celeste, Christmas Island, crustacean hyperglycaemic hormone (CHH), osmoregulation, season

\textbf{Running header:} CHH and osmoregulation in blue crabs
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. Since many terrestrial decapods experience opposing seasonal demands upon ionoregulatory physiologies, we reasoned that these would make interesting models in which to study the role of CHH upon these phenomena. In particular, those (tropical) species which also undergo seasonal migrations might be especially informative, since not only do we know relatively little regarding the nature of CHHs in terrestrial decapods, but also, 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 which experiences seasonal extremes in water availability, and diurnal and migratory activity patterns, we identified two CHHs in the sinus gland. We firstly biochemically characterised (cDNA cloning) one and functionally characterised (in terms of dose-dependent hyperglycaemic responses and glucose-dependent negative feedback loops) both CHHs. Whole animal in situ branchial chamber $^{22}$NaCl perfusion experiments showed that injection of both CHHs increased gill Na$^+$ uptake in a seasonally dependent manner, and $^{51}$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$^+$/K$^+$-ATPase or V-ATPase activity, suggesting unknown mechanisms of this hormone’s action on Na$^+$ transport across gill epithelia.
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 (Reviews: 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, 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\(^+\) 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, since the insect equivalent of CHH- ion transport peptide (ITP) has long been known to stimulate water uptake in the locust hindgut by opening Cl\(^-\) 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 the 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, since 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, since 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, viz. conservation of water during the dry season vs. 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 (CI), using a variety of approaches. CHHs were firstly isolated and characterized. 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 $^{22}$Na flux and gill Na$^+/K^+$-ATPase and V-ATPase activities. Possible roles of CHH in antennal gland haemolymph(urine filtration were examined by measuring $^{51}$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, it was 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, CI, Indian Ocean (10°29′11″S, 105°40′41″E), and shipped *via* air-freight to the UK within three 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, from The Department of Environment Food and Rural Affairs (DEFRA).

Crabs were maintained at 25°C, L:D 12:12, 80-100% relative humidity in communal plastic terraria, and 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 (SG) were microscopically dissected from ice-anaesthetised *D. celeste* on CI in batches of ~50-100 and immediately snap frozen on dry ice, and stored at -80°C. HPLC purification of 2M acetic acid extracts of SG 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., 1998). 100pmol of peptide dissolved in 100µl *D. celeste* saline (NaCl, 288.7; KCl, 4.6; CaCl$_2$.6H$_2$O, 3.6; MgSO$_4$.7H$_2$O, 2.8; NaHCO$_3$, 1 mmol.L$^{-1}$), or saline only controls, were injected into crabs (n=8) via the arthroic membrane at the base of the fourth walking leg. After 90 min a 200µl haemolymph sample was taken and processed for glucose determination as below.

CHH Time-resolved fluoroimmunoassay (TR-FIA)

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 4-weekly intervals with 5 nmol, 5 nmol 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. IgG purification and biotinylation was performed as detailed in Morris et al. (2010). Antiserum specificity was determined by direct and ‘sandwich type’ ELISAs of HPLC purified SG fractions and by immunohistochemistry of paraffin embedded eyestalks.

TR-FIA

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 TR-FIA was carried out according to the methods detailed in Morris et al. (2010). Before use in the assay, haemolymph samples were purified on 300mg Strata-X (Phenomenex, Torrance, CA, USA) solid phase extraction cartridges, as previously described. Standards were CHHa (50-0.05 fmol/well). All assays were performed in duplicate wells. Time-resolved fluorescence of
Europium was measured on a PerkinElmer Victor \textsuperscript{2} 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 (ratio 1:1) with 0.6 mol.L\textsuperscript{-1} HClO\textsubscript{4} to denature proteins and neutralised with 2.5 mol.L\textsuperscript{-1} K\textsubscript{2}CO\textsubscript{3} (usually 80 µL) followed by storage at -20ºC. Deproteinised haemolymph samples were used to measure glucose and lactate concentrations as detailed in Morris et al. (2010).

**Laboratory experiments**

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

Groups of 8 crabs were injected with 100µl either 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 MIH (peak 6) (0.1, 1, 10 and 100 pmol) and the other with saline. After 90 min, haemolymph was sampled, stored at -20ºC and subsequently assayed for glucose.

**Field experiments**

**Exercise experiments**

On CI 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 (ca. 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 CI 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 either 100µl lactate at 10\textsuperscript{-6} mol.g\textsuperscript{-1}, glucose at 10\textsuperscript{-6} mol.g\textsuperscript{-1} 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. (1991; 2000) and Morris and Ahern (2003).

On CI, infusion experiments were conducted to determine the effects of CHH (CHHa and b) compared to saline (control) on the antennal gland filtration rate using $^{51}$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 the Christmas Island red crab *G. natalis* (using *G. natalis* CHH). The radioactivities of the $^{51}$Cr-EDTA samples were measured using a gamma counter (LKB Wallac Clini Gamma Counter, Wallac). Rates of $^{51}$Cr-EDTA clearance, urine production and fluid resorption within the urinary system were calculated according to Greenaway et al., (1990) and Morris and Van Aardt (1998).

Branchial perfusion experiments were conducted on *D. celeste* and *G. natalis* on CI to determine the effects of CHH (for *D. celeste* CHHa or CHHb was used, for red crabs, *G. natalis* CHH was used) compared to saline (control) on the unidirectional flux of Na$^+$ using $^{22}$NaCl (Perkin-Elmer) as a tracer ($J_{in}$). For *D. celeste*, experiments were conducted in five field seasons on CI which 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 $^{22}$Na samples were measured using a beta scintillation counter (Tri-Carb 2900TR Liquid Scintillation Analyzer, Perkin-Elmer). Samples were also analysed for Na$^+$ concentration using flame atomic absorption spectrophotometry (Avanta Σ AAS). The net rate of Na$^+$ uptake was calculated based on the methods described by Morris and Van Aardt (1998), Morris et al. (1991) and Morris and Ahern (2003).

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

Samples were collected on CI 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 either freshwater collected from the nearby freshwater seepages, 30% seawater (30% SW) or in
some cases 50% seawater (50% SW). Gill Na⁺/K⁺-ATPase and V-ATPase activities were determined in crabs collected directly from the rainforest or after acclimation to either 10% or 50% SW.

**Gill enzyme activities**

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

**Statistical analysis**

Statistical analyses were carried out using MINITAB 15. 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). Parametric tests: \( t \)-tests or one- or two-way analysis of variance (ANOVA) were carried out on data (or transformed data) that exhibited normal distributions. Post hoc testing was carried out using Tukey’s test 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. celeste* SG extracts gave typical separation profiles as shown in Fig. 1A. On the basis of MALDI-TOF MS of the prominent peaks (masses (all MALDI-TOF, M + H⁺): 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 crustacean hyperglycaemic hormone precursor related peptides (CPRPs). Two prominent peaks were markedly hyperglycaemic in the *D. celeste* 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 medulla terminalis, cDNA cloning, degenerate PCR, 3’ and 5’RACE from medulla terminalis mRNA
gave a full length product encoding an ORF corresponding exactly to CHHb and a CPRP (GenBank Accession No. JF894384) (see supplementary material for materials and methods for cloning and sequencing of D. celeste CHH). The mass of the conceptually translated CHHb agreed exactly with that measured by MALDI-TOF (M + H⁺; 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 EIA 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,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. celeste (Fig. 5). Glucose haemolymph levels significantly increased (p=0.0147) in the exercised crabs 20 min post-exercise (1.65 ± 0.42 compared to the controls 0.49 ± 0.11 mmol.L⁻¹). There was much variability seen in the remainder (after 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 ± 0.45 to 21.8 ± 4.72 mmol.L⁻¹ in exercised animals compared to that of the control crabs (0.50 ± 0.30 to 2.02 ± 1.05 mmol.L⁻¹). 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 ± 50.86 compared to the controls 83.2 ± 32.66 mmol.L⁻¹). 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. celeste 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± 27.91 compared to 92.43 ± 4.58 pmol.L⁻¹), 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 since crabs used in this experiment were held in captivity for three 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 (Figs. 6C, 6D). However, lactate levels increased after exercise in all three groups of crabs (Figs. 6E, 6F).

**Branchial infusion and perfusion**

In the dry season (July-August 2009) there was a significant increase in urine production in *D. celeste* infused with CHH compared to 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⁺ uptake (Jₘ) in *D. celeste* demonstrated that there were no significant differences in the effects of CHHa or CHHb when compared to the saline injected crabs (p=0.863) but that the season the crabs were sampled in did have a significant effect on unidirectional Na⁺ uptake (p<0.001). However, this seasonal effect was dependent on which CHH was injected (p<0.001) (Fig. 8A). Thus, the injection of CHHa significantly increased Na⁺ Jₘ in the very dry (pre-wet) season and the injection of CHHb significantly increased Na⁺ Jₘ in the wet season when the crabs were experiencing the driest and wettest conditions respectively (Fig. 8A). Determination of Na⁺/K⁺-ATPase activity in the gills of *D. celeste* following perfusion with CHH demonstrated that there were no significant differences in the effects of CHHa or CHHb when compared to the saline injected crabs (p=0.864) but that the sampling season had a significant effect (p<0.001). However, again this seasonal effect was dependent on which CHH was injected (p=0.034) (Fig. 8B). CHHb significantly elevated Na⁺/K⁺-ATPase activity in the gills of *D. celeste* but this was only the case in the dry season.

In *G. natalis* CHH significantly decreased branchial Na⁺ uptake (Jₘ) in the dry season (p=0.038) and increased Na⁺ uptake (Jₘ) in the wet season (p=0.015) when compared to those crabs injected with saline (Fig. 9). Determination of Na⁺/K⁺-ATPase activity in the gills
of *G. natalis* demonstrated that there was no significant effect of CHH on Na⁺/K⁺-ATPase activity at the gills (*p*=0.849) (Fig. 1, supplementary info).

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

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 ± 45.07 compared to 80.07 ± 11.46 pmol.L⁻¹ 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 ± 0.12 compared to 0.11 ± 0.02 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 (Fig. 2, supplementary info). CHHa appeared to increase the Na⁺/K⁺-ATPase activity in crabs kept in freshwater conditions, compared to 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 Na⁺/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 Na⁺ uptake at the gills in the very dry (pre-wet) season and CHHb significantly increased 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. A likely scenario
therefore could be that CHHa is acting on Na\(^+\) uptake at this very dry time of the year by maximising the amount of 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 in the wet season, the amount of Na\(^+\) by volume (i.e. the urine is more concentrated) will potentially be significantly increased compared to 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 own urine to conserve water in drier periods, which could also increase the rate of Na\(^+\) uptake. Conversely, at the wettest times of the wet season, CHHb could also be acting on Na\(^+\) 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 amounts 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\(^+\) 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\(^+\) 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 potentially 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 current study it was unequivocally shown that when *D. celeste* were acclimated to increasingly saline water regimes that the circulating levels of CHH in the haemolymph increased (Fig. 10). This response could be linked to a down regulatory action of CHH to prevent the crab 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\(^+\)/K\(^+\)-ATPase activity (Fig. 8B, Fig. 1 supplementary material). This was despite the fact Na\(^+\)/K\(^+\)-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 current study were unable to show any significant effect of CHH on V-ATPase activity in *D. celeste* (Fig. 2 supplementary material), 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 (CA) repressor in the eyestalks of several species of crab (Henry, 2006; Henry and Borst, 2006; Henry and Campoverde, 2006). CA plays a significant role in respiratory gas exchange and thus osmoregulation in the gills of freshwater crustaceans. Therefore, it would appear that the action of CHH on CA 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 does not play an important role in water conservation in terrestrial crabs but instead in the regulation of ion uptake (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 (Montagne 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 to *G. natalis* which is much more independent of water and terrestrial in its adult life history. Nethertheless these results clearly suggest that much more work is required to
The seasonal breeding migration undertaken by *D. celeste* not only requires 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 must be 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 current study to see if the same scenario was true in *D. celeste* and thus if this was potentially a universal physiologically relevant process in other terrestrial crabs. Similar results to those recorded by 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 the 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 (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 on not only 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 have 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 to be discovered regarding the evolutionary adaptability of CHH in a wide variety of physiological processes not only in land crabs but in crustaceans in general.

**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHH</td>
<td>crustacean hyperglycaemic hormone</td>
</tr>
<tr>
<td>CI</td>
<td>Christmas Island</td>
</tr>
<tr>
<td>CPRP</td>
<td>crustacean hyperglycaemic hormone precursor-related peptide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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</table>
ACKNOWLEDGEMENTS

We thank the Director of National Parks and the staff of Parks Australia, Christmas Island, for their logistical assistance, hospitality and enthusiasm. Additional thanks go to Jessica Palmer, Ute Postel, Mrinalini, Rosalind Pidcock, Paul Hallas, Amanda Strong, Christiana Anagnostou and Helena Reinardy for assistance in the field.

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REFERENCES


FIGURE LEGENDS

Fig. 1A. HPLC fractionation of an extract from 57 sinus glands from *D. 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* (mmol.L⁻¹) 90 mins post-injection with ~100 pmol of peptide (crab body mass = ~300g). * indicates significant increase compared to saline injected crabs. Kruskal-Wallis ANOVA and Mann-Whitney tests for multiple comparisons used for statistical analysis. Bars represent mean ± SEM (n=8).

Fig. 2. Daily rainfall (mm) recorded in the five field seasons in which sampling was conducted on Christmas Island: the ‘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 ± SEM (n=22-27). Rainfall data were obtained from the Australian Government Bureau of Meteorology (www.bom.gov.au).

Fig. 3. [Glucose] in the haemolymph of *D. celeste* (mmol.L⁻¹) 90 mins post-injection with ~0.1, 1, 10 and 100 pmol of peptide (crab body mass = ~300g). Non-parametric ANCOVA used for statistical analysis. Bars represent mean ± SEM (n=8).

Fig. 4. Typical standard curve for the *D. celeste* CHHα TR-FIA. The working range (limits of the assay) is between 50-0.5 fmol.100µL⁻¹. Standards (duplicates) were diluted from quantified (HPLC purified) SG extract.

Fig. 5. The effect of exercise on circulating A. CHH, B. glucose and C. lactate levels. *Discoplax celeste* 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. * indicates significant increase compared to unexercised crabs. Bars represent mean ± SEM (n=5). Statistical tests were performed by comparing controls with exercised crabs at each timepoint (one-way ANOVA and Tukey’s tests).

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 Aug 2009) and B. wet season (Feb 2012) crabs. * indicates significant increase compared to pre-exercised levels. Bars represent mean ± SEM (n=7) (Student’s t-tests and Mann-Whitney tests). Insert shows glucose (C. dry season, D. wet season) and lactate (E. dry season, F. wet season) levels in the same crabs.

Fig. 7. The effect of CHH on the volume rates of $^{51}$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 U:H ratio of $^{51}$Cr in the urine and haemolymph. * indicates significant elevation compared to saline infused crabs. Bars represent mean ± SEM (n=2-6) (Student’s t-tests).

Fig. 8A. Branchial Na⁺ flux in *D. celeste* infused with either a saline control, CHHα or CHHβ at 100 pmol.crab⁻¹ in different seasons. The branchial chambers were perfused with AU labelled with $^{22}$Na. Flux rates were determined over 120 min. Uptake of $^{22}$Na provided rates of unidirectional influx ($J_{in}$). B. [Na⁺/K⁺-ATPase] in the gills after infusion with either saline or CHH (CHHα or CHHβ). * indicates significant increase in Na⁺ flux in CHHα infused crabs in the pre-wet season, when compared to other seasons, but not when compared to saline.
infused crabs; † indicates significant increase in Na\(^+\) flux in CHHb infused crabs in the wet season, when compared to other seasons, but not when compared to saline infused crabs. In the case of the gill [Na\(^+\)/K\(^+\)-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 used for statistical analysis. The gill [Na\(^+\)/K\(^+\)-ATPase] data were log\(_{10}\) transformed prior to analysis. Bars represent mean ± SEM (n=8).

**Fig. 9.** Branchial Na\(^+\) flux in *G. natalis* infused with either a saline control or CHH at100 pmol.crab\(^{-1}\) in different seasons. The branchial chambers were perfused with AU labelled with \(^{22}\)Na. Flux rates were determined over 120 min. Uptake of \(^{22}\)Na provided rates of unidirectional influx (\(J_{\text{in}}\)). * indicates significant decrease in Na\(^+\) flux in CHH infused crabs in the dry season; † indicates significant increase in Na\(^+\) flux in CHH infused crabs in the wet season. One-way ANOVA and Tukey’s tests for multiple comparisons used for statistical analysis. Unidirection Na\(^+\) uptake data were log\(_{10}\) transformed prior to analysis. Bars represent mean ± SEM (n=8).

**Fig. 10.** [CHH] in the haemolymph of *D. celeste* (pmol.L\(^{-1}\)). Sampling was from crabs taken directly from the rainforest or after acclimation to either 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. Values are means ± SEM (n=16).

**Fig. 11.** The effect of CHH on Na\(^+\)/K\(^+\)-ATPase activity in the gills of *D. celeste* (µmol.mg\(^{-1}\).h\(^{-1}\)). Crabs were injected with either CHHa, CHHb (100 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. * indicates significant increase in gill [Na\(^+\)/K\(^+\)-ATPase] in CHHa infused crabs kept in freshwater conditions, when compared to those infused with CHHb, but not those infused with saline; † indicates significant increase in gill [Na\(^+\)/K\(^+\)-ATPase] in CHHa infused crabs kept in freshwater conditions, when compared to those also infused with CHHa but acclimated to 10% or 50% seawater, but not to those infused with saline. Bars represent mean ± SEM (n=7) (two-way ANOVA and Tukey’s tests).
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6
Fig. 7.

**Discoplax celeste**

- **Saline**
  - Dry: n=6
  - Wet: n=4

- **CHH**
  - Dry: n=5
  - Wet: n=2

**Gecarcoidea natalis**

- **Saline**
  - Dry: n=4
  - Wet: n=4

- **CHH**
  - Dry: n=6
  - Wet: n=6
Fig. 8.
Fig. 9.
Fig. 10.

The figure shows a bar graph representing CHH (pmol/L) levels across different conditions: Forest, CIW, 30% SW, and 50% SW. The graph indicates significant differences among the conditions, with bars labeled for comparisons of ab, abc, bcd, and cd.
Fig. 11.
SUPPLEMENTARY MATERIAL
MATERIALS AND METHODS

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 2 units of DNase 1 (37 °C, 30 min), followed by clean up with TURBO DNA-free (Ambion, Austin, TX, USA) and quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

**cDNA synthesis and rapid amplification of cDNA ends (RACE)**

For 3’ RACE, mRNA, 1–3 µg 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 2 units RNase H.

**Degenerate PCR of *D. celeste* cDNA encoding XO-CHH**

Using available amino acid sequence data for CHH in the Brachyura a set of fully degenerate primers were designed (Table 1). PCR reagents were: 21.5 µL Megamix Blue (Helena Biosciences, Sunderland, UK), 1.25 µL (100 µM) forward and reverse primers (F1, R1 or F1, R2) and 1 µL cDNA template. PCR conditions were : 94 °C 4 min, 35 cycles of 94 °C 30 s, 45 °C 30 s, 72 °C 45 s, final extension at 72 °C for 7 min. The second nested PCR used 1 µL of the first PCR reaction as template with 21.5 µL Megamix Blue, 1.25 µL (100 µM) 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 XO-CHH

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

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 µM) Disco GSP R primer, 1.25 µL (10 µM) 5’ GeneRacer primer (Invitrogen) and 1 µL 5’ RACE cDNA template. Touchdown PCR conditions were used: 94 °C 9 min, 5 cycles of 94 °C 30 s, 64 °C 45 s, 5 cycles of 94 °C 30 s, 62 °C 45 s, 25 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 45 s, final extension at 72 °C for 10 min. The second nested PCR used Disco GSP R_N primer 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 30 s, 58 °C 30 s, 72 °C 45 s, final extension at 72 °C for 7 min. PCR products were electrophoresed and bands 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).
Table 1. Primers used for *D. celeste* sequence identification and 3’ and 5’ RACE PCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. celeste</em> CHH Degenerate</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>CAYCIYTNARACARATHTAYGA</td>
</tr>
<tr>
<td>F1n</td>
<td>GARAARMNCARATHTAYGA</td>
</tr>
<tr>
<td>F2</td>
<td>TGYAARGGNGTNTAYGAYMG</td>
</tr>
<tr>
<td>R1</td>
<td>TCCATRCAYTGICKDAWNAC</td>
</tr>
<tr>
<td>R1n</td>
<td>CATRCAYTGICKDAWNAC</td>
</tr>
<tr>
<td>R2</td>
<td>TTTTARCARTCRCANACRTG</td>
</tr>
<tr>
<td>R2n</td>
<td>TARCARTCRCANACRTG</td>
</tr>
<tr>
<td><em>D. celeste</em> CHH 3’ RACE</td>
<td></td>
</tr>
<tr>
<td>Disco GSP F</td>
<td>AAGGGGTCTATGACAGATCTCCTCCTCCAG</td>
</tr>
<tr>
<td>Disco GSP F N</td>
<td>GACAGATCTCCTCTCCAGCAAGCTGGA</td>
</tr>
<tr>
<td>YNLYRTSYV</td>
<td>GTAACCTCTACCGACCTCCTACACGTC</td>
</tr>
<tr>
<td>LYRTSYVSSA</td>
<td>TCTACCCGACCTCCTACGTGCACCCGCGCC</td>
</tr>
<tr>
<td><em>D. celeste</em> CHH 5’ RACE</td>
<td></td>
</tr>
<tr>
<td>Disco GSP R</td>
<td>GTTCTCCCTGCAGGGCTGAGACGTAG</td>
</tr>
<tr>
<td>Disco GSP R N</td>
<td>AGGCGCTGGAGACGAGGAGGTGCGCTA</td>
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</table>
**Fig. 1.** [Na\(^+\)/K\(^+\)-ATPase] in the gills of *G. natalis* after infusion with either a saline control or CHH at 100 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 ± SEM (n=8).
Fig. 2. The effect of CHH on V-ATPase activity in the gills of *D. celeste* (µmol.mg⁻¹.h⁻¹). Crabs were injected with either CHHa, CHHb (100 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 ± SEM (n=7) (two-way ANOVA and Tukey’s tests).