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Salt and water balance in the land crabs of Christmas Island: a review

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Abstract. Work conducted on the land crabs of Christmas Island has advanced our knowledge of the ecophysiology of ion and water balance not only in terrestrial crabs, but across the Crustacea. For instance, studies undertaken on endemic Christmas Island species, such as *Discoplax celeste*, which is unique in its evolutionary position and ecology as a freshwater/land crab, have allowed us to increase our understanding of the mechanisms that have underpinned the evolution of these animals from the sea to the terrestrial environment. Recent studies have focused on understanding the neuroendocrine control of osmoregulation in terrestrial crabs on Christmas Island, with crustacean hyperglycaemic hormone being confirmed to have significant roles in the control of seasonal osmoregulation in gecarcinids.

Key words. Christmas Island, osmoregulation, physiology, crab, crustacean

INTRODUCTION

The ability to control internal osmolarity is fundamental to the success of all crabs, and enables these crustaceans to colonise not only the full spectrum of aquatic habitats (i.e., marine, brackish and freshwater environments) (Mantel & Farmer, 1983; Pequeux, 1995; Freire et al., 2008; McNamara & Faria, 2012), but also to successfully invade the terrestrial realm (Greenaway, 1988). There is perhaps nowhere that this is more obvious than Christmas Island (CI), in the Indian Ocean. This relatively small (135 km^2), remote island of volcanic origin is renowned for the diversity of the terrestrial crabs that inhabit it (Hicks et al., 1990; Gray, 1995) (separate comprehensive discussions of the landscape and natural history, fauna and flora of Christmas Island are included in this Special Supplement of the Raffles Bulletin of Zoology and the reader is directed to these for further detail). Recent surveys on CI have put the number of terrestrial crab species at 20 (including four endemics), and that of marine species at over 100 (Ng et al., 2010; Orchard, 2012), with this number likely to be revised upwards with further taxonomic study. Since the first survey of the natural history of the island, which was conducted in collaboration with the British Museum (Andrews, 1900), the island's land crabs have attracted the interest of ecophysiologists and evolutionary biologists, with the resultant body of work contributing to many of the major advances in our understanding of land crab physiology, including in the fields of respiratory gas exchange (i.e., Greenaway et al., 1988; Adamczewska & Morris, 1998, 2000), acid base balance (i.e., Adamczewska & Morris, 1996), nitrogenous excretion (i.e., Greenaway & Morris, 1989; Greenaway & Nakamura, 1991), exercise physiology (i.e., Adamczewska & Morris, 1994a, 1994b; Morris et al., 2010), digestion (i.e., Greenaway & Raghaven, 1998; Linton & Greenaway, 2004), olfaction (i.e., Krieger et al., 2010) and osmoregulation (i.e., Morris & Ahern, 2003; Turner et al., 2013).

Maintaining salt and water balance is problematic, and thus is a significant limiting factor for crabs when living in the terrestrial environment. The reasons for this are twofold: firstly, in aquatic crustaceans it is the gills that are used for salt transport. In terrestrial species these are no longer in contact with water, removing the ability for adjustments in ion content to be made via this method (McNamara & Faria, 2012). Secondly, the fact that these crabs are no longer submerged in an aquatic medium, but instead surrounded by air, means that maintaining water balance is also difficult due to evaporation (Greenaway, 1988). Osmoregulatory mechanisms in terrestrial species have thus been modified to cope with these changes. Land crabs have evolved through several routes (exemplified by several extant lineages) from the fully marine to terrestrial habitat, via the intertidal or through a variety of brackish and freshwater life histories (Little, 1983, 1990). Land crabs, therefore, are truly useful and interesting subjects for the study of the evolution of the physiological mechanisms, such as osmoregulation, that have allowed the terrestrial invasion to take place. CI with its abundance of species that occupy various positions along this continuum of forms, together with the phenomenal numbers of some species present on the island (for example, the population of the endemic CI red crab, *Gecarcoidea natalis*, has been estimated at 43.7 million (Adamczewska & Morris, 2001)) has facilitated the study of the ecophysiology of these unique organisms.

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Work conducted on the land crabs of Christmas Island has significantly contributed to our understanding of osmoregulation in terrestrial crabs (for reviews see Morris (2002) and Greenaway (2003)). The aims of this review are to summarise the current knowledge in the field of CI land crab osmoregulation and suggest directions for future research.

ION AND WATER BALANCE IN CRUSTACEANS

There are obvious osmoregulatory challenges encountered by crabs when living in the terrestrial environment, specifically the need to avoid desiccation as well as the loss of salt from the body (Greenaway, 1988; Morris, 2001, 2002). In aquatic crabs, the gills are the primary site of salt transport, although the mechanisms for this are different when marine species are compared to those that reside in freshwater environments (Pequeux, 1995; Freire et al., 2008). Freshwater crabs are able to supplement branchial pumping with the utilisation of the antennal glands to reclaim salts from the urine. However, due to the large difference in salt concentration between the crab's tissues and the external freshwater medium, the gills of freshwater crabs must work 'harder' than those of marine species to maintain body salt concentration and prevent salt loss (Morris, 2001). At the gills, euryhaline marine crabs utilise apical membrane branchial Na^+/H^+ and Cl^-/HCO_3^- exchange (Lucu, 1990) powered by a basal membrane Na^+/K^+-ATPase to maintain salt balance (Lucu & Towle, 2003; Tsai & Lin, 2007). However, this is not a stand-alone mechanism and there are fundamentally important linkages between salt balance, CO_2 excretion, acid-base balance and nitrogen excretion in the gills of aquatic crustaceans (Henry, 1988; Morris, 2001; Weihrauch et al., 2004; Freire et al., 2008). In the case of CO_2 excretion, this occurs as HCO_3^- through the apical Cl^-/HCO_3^- exchanger (Truchot, 1983; Henry & Wheatly, 1992; Wheatly & Henry, 1992). However, this process is facilitated by carbonic anhydrase (CA) which at the basal membrane assists the dehydration of HCO_3^- to CO_2 which then diffuses into the cytoplasm where an additional CA facilitates the rehydration to HCO_3^- and H^+ (Henry, 1984a, 1988). This provides the counterions for the Cl^- and Na^+ exchangers involved in salt balance as well as managing acid-base balance (Fig. 1).

In decapod freshwater crustaceans however, rather different physiologies are used. Firstly, the antennal gland plays a significant role in osmoregulation. In freshwater crabs (e.g., *Potamon* spp., *Metopaulias depressus*, *Pseudothelphusa jouyi*, *Holthuisana transversa* and *Potamonautes warreni*), it has been shown that salts and water are reabsorbed at the antennal gland (Shaw, 1959; Thompson, 1970; Harris, 1975; Greenaway, 1980, 1981; Morris & Van Aardt, 1998). In addition, it has been demonstrated in the crayfish, *Pacifastacus leniusculus*, that the antennal gland utilises Na^+/K^+-ATPase and CA to reclaim urinary salts, resulting in urine which is hypo-ionic *before* it is passed into the gill chambers (Wheatly & Henry, 1987; Wheatly & Toop, 1989). In freshwater, NaCl concentration is lower than that of the gill epithelium, meaning that straightforward apical exchange of H^+ and Na^+ is no longer possible at the gills. Using the Chinese mitten crab, *Eriocheir sinensis*, as a model

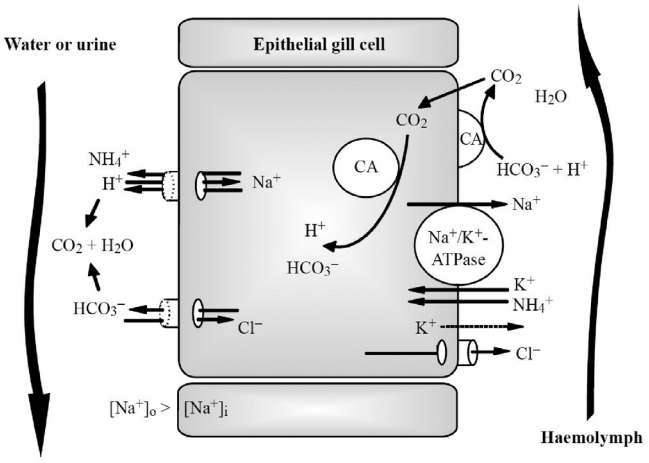

Fig. 1. Model of gill epithelial ion exchange mechanisms typical for a marine euryhaline crab, but also the basic model for terrestrial crustaceans of marine ancestry (after Morris, 2001).

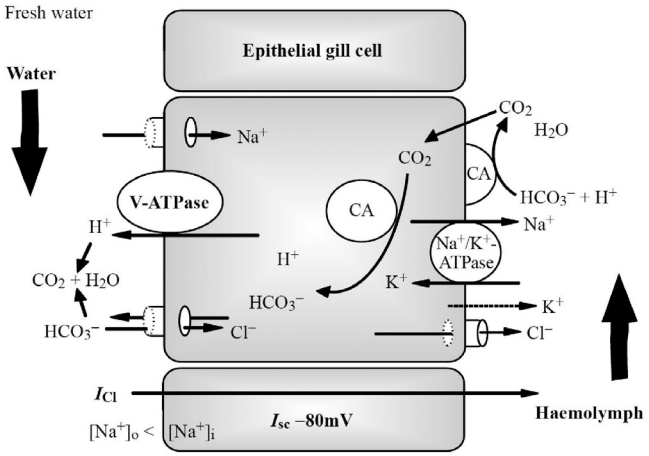

Fig. 2. Generalised model for branchial transport mechanisms in freshwater decapods (after Morris, 2001).

(Onken & Graszynski, 1989; Onken et al., 1991; Zeiske et al., 1992; Riestenpatt et al., 1994; Onken & Putzenlechner, 1995; Riestenpatt et al., 1995; Onken & Riestenpatt, 1998; Onken, 1999), it is now known that an electrogenic force (now known to be a vacuolar (V) type H^+-ATPase, or V-ATPase) is necessary to drive Cl^- transport, pumping H^+ into the water independently of Na^+ uptake. This V-ATPase provides for electrogenic uptake of Cl^- in exchange for HCO_3^- (Onken et al., 1991; Onken & Putzenlechner, 1995). The HCO_3^- is provided by CA facilitating CO_2 excretion while NH_4^+ can substitute for K^+ in the basal ATPase and for H^+ in the apical exchange (Henry, 1984a, 1984b) (Fig. 2). Perhaps, even more so in freshwater crabs, it is clear that the maintenance of salt balance, CO_2 excretion, acid-base balance and nitrogen excretion in crustaceans relies on all of these processes.

In land crabs the gills are retained, but are reduced in number, as well as having a reduced planar gill surface area compared to those of water-breathing crabs (Farrelly & Greenaway, 1992). In these crabs the gills were originally thought to play a role in gas exchange, but it is now known that they are the organs used primarily for ion regulation and urine reprocessing rather than respiration (Greenaway,

1989; Adamczewska & Morris, 1996; DelaCruz & Morris, 1997a, 1997b; Morris & Dela-Cruz, 1998; Morris, 2005) and are thus used as a filtration-reabsorption system, 'similar to that of the vertebrate kidney' to control urinary salt loss (Fig. 3) (Morris, 2001).

It is now understood that essentially the same ion-exchange mechanisms of their aquatic ancestors are utilised in land crabs, but are employed instead to facilitate exchanges within their own urine (Morris, 2001). The gills as a site of ion uptake are retained in terrestrial crabs, which results in a very dilute final excretory product 'P' (Wolcott & Wolcott, 1985). In general terms, land crabs with a marine origin conserve water and salts by extrarenal urinary reprocessing and by decreasing urinary output (Greenaway, 1988), i.e., *Cardisoma guanhumi* (Harris, 1977; Wolcott & Wolcott, 1984; Wolcott, 1991), *Ocypode quadrata* (Wolcott & Wolcott, 1985), *Birgus latro* (Harris & Kormanik, 1981; Greenaway & Morris, 1989; Greenaway et al., 1990; Morris et al., 1991; Taylor et al., 1993; Morris et al., 2000; Greenaway, 2001), *Gecarcinus lateralis* (Harris, 1977; Wolcott, 1991; Wolcott & Wolcott, 1991), *Geograpsus grayi* (Varley & Greenaway, 1994), *Gecarcoidea lalandii* (Harris & Kormanik, 1981), *Discoplax celeste* (DelaCruz & Morris, 1997b; Dela-Cruz, 1998), *Cardisoma carnifex* (Harris & Kormanik, 1981; Dela-Cruz, 1998), and *Gecarcoidea natalis* (Greenaway, 1994; Morris, 2001; Taylor & Greenaway, 2002; Greenaway, 2003; Morris & Ahern, 2003). These species can redirect their primary urine from the antennal gland to the gill chambers where salt reabsorption takes place at the gills producing a final hypo-ionic excretory product 'P' (Wolcott & Wolcott, 1985). This is very dilute, containing less than 30% of the ions measured in the primary urine and less that 5% of the ions measured in the haemolymph (Morris, 2001). In addition, some terrestrial crabs also utilise the ion exchange mechanisms of the antennal gland to reclaim urinary salts (DeVries & Wolcott, 1993; DeVries et al., 1994), a process which is similar to that employed by freshwater crustaceans.

DISCOPLAX CELESTE: THE PERFECT CRUSTACEAN MODEL TO STUDY OSMOREGULATION?

Previously misidentified on CI as *Discoplax hirtipes* (Dana, 1851) (and before that as *Cardisoma hirtipes* – for a full discussion see Ng & Davie, 2012), this crab has been recognised as a distinct new species endemic to CI, the Christmas Island blue crab, *Discoplax celeste* (Ng & Davie, 2012). Unlike the other two most obvious and abundant land crab species on CI, *G. natalis* and *B. latro*, *D. celeste* is restricted in its distribution (especially during the dry season (April–October)) to areas of freshwater seepages and springs, usually well inland (>1 km from and 150 m above the ocean) (DelaCruz & Morris, 1997a, 1997b; Morris, 2005). Due to the underlying geology (Barrett, 2001), these areas of freshwater and thus established populations of *D. celeste* are found at only a few places on the island. When the wet season begins (November–March) *D. celeste* disperse over a wider area (DelaCruz & Morris, 1997a, 1997b), and only when there

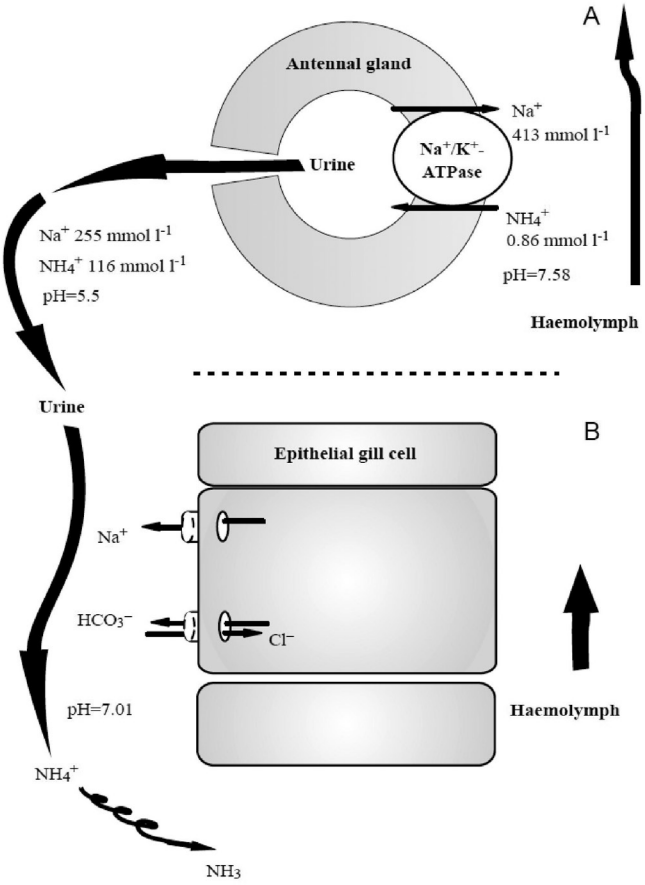

Fig. 3. Two part model: A, the antennal gland and B, the branchial epithelial layer (after Morris, 2001).

is an adequate supply of standing water (February–March) do they undertake their breeding migration to the ocean.

We now know that *D. celeste* is an ammoniotelic species and it is the need for long term nitrogen, ion and water balance that drives this crab into association with freshwater, therefore explaining the local and seasonal variations in the distribution of this species (Greenaway, 1989; Adamczewska & Morris, 1996; DelaCruz & Morris, 1997b). When this crab submerges in freshwater the gills are exposed to an enormous dilution gradient (~400 times lower than that of the crab's tissues) yet *D. celeste* is still able to maintain salt balance in solutions containing concentrations as low as 0.12 mmol.L^{-1} Na^+ (Greenaway, 1989). This is due to the fact that although *D. celeste* has a high Na^+ permeability (1.609 µmol.g^{-1}.h^{-1}), this is compensated by a high Na^+ affinity uptake mechanism (Km = 0.264 mmol.L^{-1}) (Greenaway, 1989), and also that the stored nitrogen is excreted in rapid, intense bursts during brief immersion episodes (DelaCruz & Morris, 1997b). However, it was noted by the same authors that although *D. celeste* manages Na^+, Cl^- and K^+ in the same way as other species of gecarcinid, Mg^{2+} and Ca^{2+} are conserved using similar methods to those of freshwater crabs, i.e., that they are reabsorbed in the antennal gland/bladder structure. The importance of behaviour in the osmoregulatory ability of this crab was also highlighted—the salts lost by *D. celeste* in their urine are passed into the burrow water which remains as a large reservoir of ions that potentially the crab can later draw on (Greenaway, 1989).

Urine flow rates in *D. celeste* have been found to be three times higher than those of other gecarcinid species (but still less than marine crabs), as well as other species of freshwater crustaceans (Greenaway, 1989). These high urine flow rates mean that the amount of salt lost in the urine is much greater compared to these other species. A high production rate of concentrated urine is very energetically expensive to produce. Therefore, although *D. celeste* is able to successfully osmoregulate in a freshwater environment, it appears to be doing so at the very limits of its sodium regulatory system (Greenaway, 1989). In addition, this crab is also able to maintain salt balance when supplied with only freshwater or seawater to drink (Greenaway, 1989). These factors indicate that this species occupies a unique position in evolutionary terms and can be considered an intermediary between marine and freshwater species (Greenaway, 1989). *Discoplax celeste* therefore makes an excellent model species for studies of the evolution of osmoregulation and ultimately the evolution of life on land (Little, 1983, 1990; Morris, 2001). See section on 'Hormonal control of osmoregulation' for further details of the control of osmoregulation in this species.

BIRGUS LATRO

The Robber Crab or Coconut Crab, *B. latro*, is the largest land arthropod, weighing up to 4 kg (Drew et al., 2010). Currently listed as data deficient on the International Union for Conservation of Nature Red List, this once widely distributed crab was previously common throughout the islands of the Indo-Pacific as well as on large land masses including Madagascar and the tropical north-east coast of Australia. *Birgus latro* is now only present on remote islands and rapidly declining throughout its range primarily due to overharvesting for human consumption and as a result of habitat loss (Drew et al., 2010). However, a significant population is still present on CI and this species is found all over the island (Hicks et al., 1990; Orchard, 2012). This crab is classed as a true terrestrial crab and does not require periodic immersion in fresh or saltwater for the purposes of osmoregulation (Hartnoll, 1988; Greenaway, 2003).

When compared to gecarcinids, in *B. latro* it is the gills in the branchial chambers that play the greater role in ion and water balance rather than the antennal gland, with water reabsorption also occurring in the gut (Greenaway et al., 1990; Morris et al., 1991). Ion uptake at the gills is driven by ATPases (Morris et al., 1991). It is apparent that the ion-transport system in the gills of *B. latro* is reminiscent of those of marine species and thus demonstrates the evolutionary route that this species has taken. *Birgus latro* is also able to select drinking water of a suitable salinity to regulate body osmolarity although it is the physiological up or down regulation of ion uptake from the urine, instead of any kind of behavioural mechanism, by which this crab ultimately regulates its ion uptake (Taylor et al., 1993). Field studies of free-ranging *B. latro* on CI have also demonstrated this species' ability to be a competent osmoregulator under natural conditions and have confirmed that the drinking of saline water is not necessary for maintaining salt and water balance (Greenaway, 2001).

In *B. latro*, dopamine and cAMP both decrease branchial Na^+/K^+-ATPase activity as well as ion (Na^+ and Cl^-) uptake (Morris et al., 2000). However, this inhibition of ion uptake in *B. latro* is in contrast to what is seen in similar pharmacology-based experiments in aquatic brachyurans (for a recent review see McNamara & Faria (2012)). However, this mechanistic control makes sense in the context of the ecology of these crabs, which despite their ability to cope in very dry conditions will at no time of the year be completely restricted from the permanent freshwater seepages on CI (except by geographical distance) from which they can take up ions. This coupled with the fact that these crabs are able to reabsorb ions from their own urine via the gills explains the evolution of a mechanism to down-regulate ion uptake in this way (Morris et al., 2000).

GECARCOIDEA NATALIS

The Christmas Island red crab, *G. natalis*, is endemic to CI and is widely distributed throughout the island (Hicks et al., 1990; Adamczewska & Morris, 2001; Orchard, 2012). *Gecarcoidea natalis* is also classified as a true terrestrial crab as this species is independent of water (fresh or saline) for osmoregulatory purposes (Morris, 2001). This species participates in a spectacular annual breeding migration to the ocean, and the osmoregulatory system has been shown to be able to cope successfully with the energetic demands of exercise that this entails (Greenaway, 1994). Upon arrival at the shoreline these crabs are observed to 'dip' into the ocean before the commencement of breeding activities, thus replenishing lost salts (Hicks, 1985; Greenaway, 1994).

In terms of control of osmoregulation via biogenic amines, in this species, in contrast to what has been observed in *B. latro*, dopamine appears to stimulate the up-regulation of branchial chloride transport and increases the amount of urine produced (Taylor & Greenaway, 2002). Serotonin also stimulates Na^+ uptake at the gills, but the exact mechanism for this varies depending on the salinity of the available drinking water. When crabs are given freshwater to drink, serotonin stimulates Na^+ uptake via increased gill Na^+/K^+-ATPase activity, whereas dopamine has no effect. Serotonin stimulation also appears to be independent of cAMP and therefore is in contrast to what is observed in marine species where the control of branchial ion uptake is normally via a dopamine/cAMP pathway. In comparison, when *G. natalis* are given 50% seawater to drink, serotonin and dopamine appear to control ion uptake via the control of branchial leak permeability (the rate of ion pumping, as well as gill Na^+/K^+-ATPase activity, remains unchanged) (Morris & Ahern, 2003). Furthermore, serotonin appears to increase urine production in the dry season in *G. natalis* (when urine production rates were lowered), whereas dopamine had no effect (Morris & Ahern, 2003). Thus it appears that the specific effects of monoamines on osmoregulation in *G. natalis*, as well as the overall control of osmoregulation in land crabs, is far more complex than previously thought and has led directly to the work carried out in recent years to more fully understand the neuroendocrine control of osmoregulation in terrestrial crabs (see next section 'Hormonal control of osmoregulation').

HORMONAL CONTROL OF OSMOREGULATION

Crustacean hyperglycaemic hormone (CHH) has recently been shown to have osmoregulatory effects in CI land crabs, including on a seasonal basis (Turner, 2010; Turner et al., 2013). Hormones in the CHH family are known to have pivotal roles in various physiological processes, including the control of energy metabolism, moulting and reproduction (see Webster et al. (2012) for a recent review) and there is a growing body of evidence implicating involvement of CHH in ionic homeostasis in decapod crustaceans (Charmantier-Daures et al., 1994; Charmantier et al., 1999; Chung et al., 1999; Townsend et al., 2001; Serrano et al., 2003; Chung & Webster, 2006). Therefore CHH seemed a prime candidate for investigating the hormonal control of osmoregulatory process in these crabs.

Crustacean hyperglycaemic hormone is able to adjust gill Na^+ uptake in a seasonally dependent manner in gecarcinids (Turner et al., 2013). In *G. natalis* there is a clear downregulation of Na^+ uptake in the dry season and for both *D. celeste* and *G. natalis* an upregulation of Na^+ uptake in the wet season following injection with CHH. Therefore, it seems that CHH is important in driving the response to high water throughput in the wet season. It is imperative that land crabs do not become depleted of salts, something that can easily happen if urine and 'P' production rates are high at this time of year. The similar observations recorded for these closely related land crab species suggest a universal ion uptake mechanistic effect of CHH amongst terrestrial crabs. CHH was also shown to increase urine production at the antennal gland on a seasonally specific basis in *D. celeste*, but had no effect on urine production in *G. natalis*, perhaps as a result of the contrasting life histories of these 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 throughout its adult life history (Turner et al., 2013).

Phylogenetic analyses of CHH suggests that this hormone may have had a fundamental role in the invasion of land by decapod crustaceans (Table 1, Fig. 4). When compared with the phylogeny produced from the analysis of moult inhibiting hormone (MIH) (Fig. 5.), which is also in the CHH-superfamily (Webster et al., 2012), it is clear that while CHH has diverged several times during the evolution of the Decapoda, MIH has remained highly conserved (Fig. 5). Thus it appears that different selection pressures may have shaped the evolution of CHH and MIH. It is well recognised that CHH sensu stricto exhibits many pleiotropic functions (Webster et al., 2012), whereas the physiological function of MIH is constrained to that of involvement with the control of the timing of ecdysis (Nakatsuji et al., 2009). Recent molecular phylogenetic evidence has confirmed the position of ITP at the base of the CHH family in terms of ecdysozoan evolution (Montagne et al., 2010) and 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). Indeed, the present extant land crab fauna have evolved through a number of routes; moving to a terrestrial habitat directly from the ocean, through the intertidal, or through a variety of brackish and freshwater habitats (Greenaway, 1988). Consequently, in line with their evolutionary route into a terrestrial existence, land crabs have evolved a number of mechanisms to cope with the physiological demands of life on land, including those to deal with salt transport and ion regulation. Therefore it is possible that the divergent evolution of CHH as well as the conserved evolution of MIH reflect this.

Despite work demonstrating the involvement of CHH in osmoregulation in land crabs, as yet the exact osmoregulatory mode of action of CHH in these crabs has not been identified: intriguingly this hormone appeared to have no effect on gill Na^+/K^+-ATPase or V-ATPase activity, suggesting unknown mechanisms for this hormone's action on Na^+ transport across gill epithelia in terrestrial crabs (Turner et al., 2013). Nevertheless, these results clearly suggest that much more work is required to examine the hormonal controls on osmoregulation in land crabs, with one obvious avenue for future research being to compare and contrast the situation in the anomurans with that in the brachyurans.

CONCLUSIONS

Work conducted on the land crabs of CI has undoubtedly advanced our knowledge of the ecophysiology of ion and water balance not only in terrestrial crustaceans, but in crustaceans as a whole. For instance, studies undertaken on CI species, such as *D. celeste* with its unique evolutionary position and ecology have allowed us to increase our understanding of the mechanisms that have underpinned the evolution of these animals, including the routes they have taken to enable them to successfully exploit the terrestrial landscape (Little, 1990).

It is imperative that we continue to learn all we can about these unique animals, especially as the number of described and/or endemic CI species continues to rise (Orchard, 2012). It appears likely that when these kinds of studies are extended to investigate the patterns and processes of osmoregulation in additional (endemic) species of CI land crab, our knowledge and understanding of crustacean osmoregulation will be further enhanced. With the application of new molecular and analytical techniques we have the tools available that will enable us to learn much more about the molecular mechanisms that underpin the ecophysiology and ultimately drives the ecology of these crabs. However, work must continue with some urgency due to the unfortunate fact that many of these species are at risk from the challenges imposed on the CI ecosystem by anthropogenic influences, for example, habitat destruction (Morris-Pocock et al., 2012), increased numbers of invasive species (Thomas et al., 2010; Green et al., 2011), as well as global climate change (Marshall & McCulloch, 2001). It is a worrying thought that it appears possible that further species of land crab could potentially be lost forever before their biology has been studied by a new generation of carcinologists. The prevention of this scenario must continue to be taken extremely seriously and

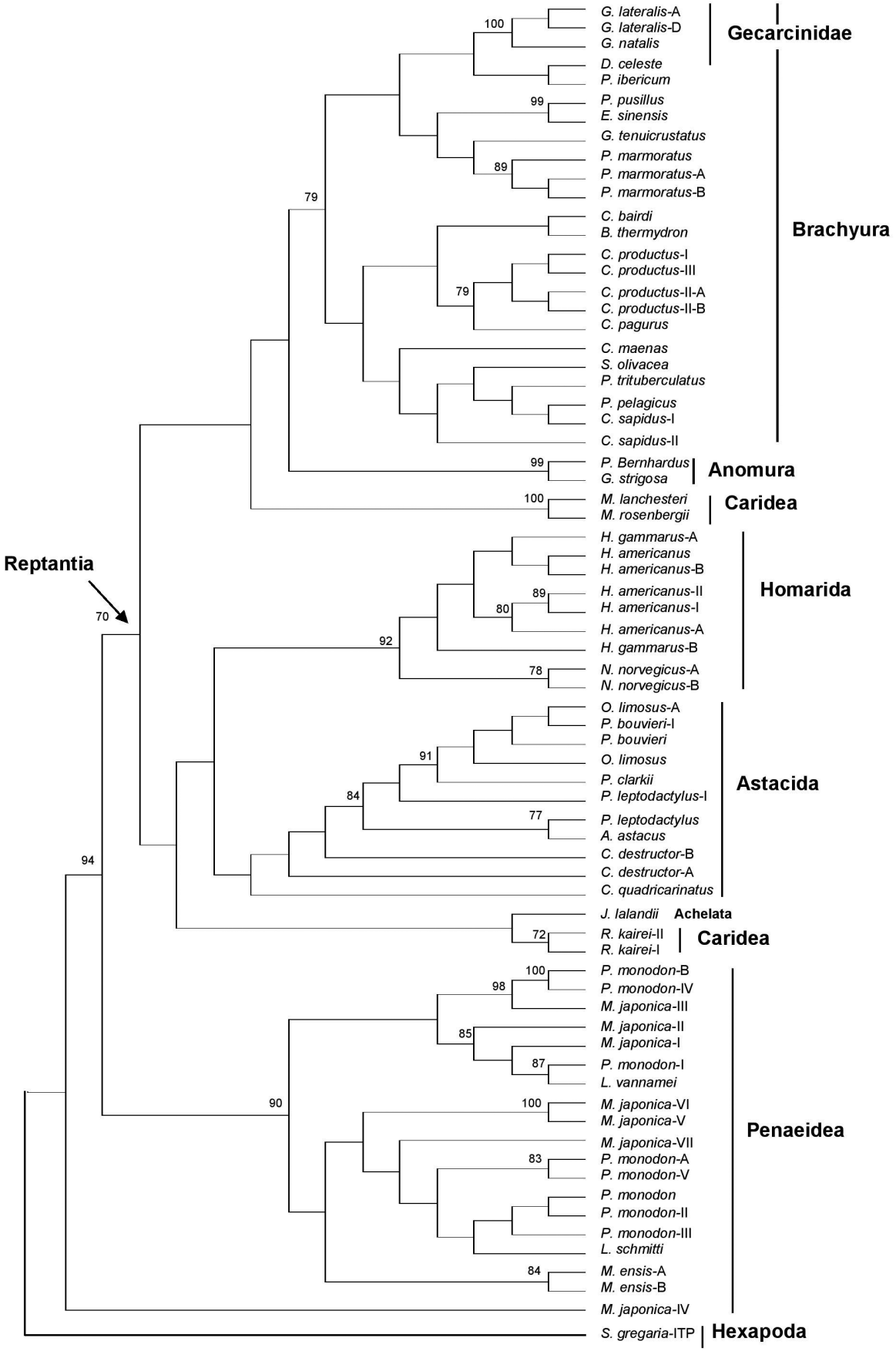

Fig. 4. Phylogeny of crustacean hyperglycaemic hormones (CHH) based on maximum-likelihood analysis of the CHH amino acid data set (39 taxa, 71 sequences, 78 residues (Table 1)) using a JTT+I+G model of protein evolution (tree shown is 50% majority rule consensus tree). Maximum-likelihood tree reconstruction was performed using PhyML (Abascal et al., 2005). The best-fit model for protein evolution was determined using ProtTest 1.3 (Jones et al., 1992; Guindon & Gascuel, 2003). The locust *S. gregaria* ITP was assigned as the outgroup. Sequences were acquired from GenBank or directly sequenced (Turner, 2010). See Turner (2010) for methodologies used. Numbers at nodes are bootstrap values (100 replicates). Only bootstrap values ≥70 are shown.

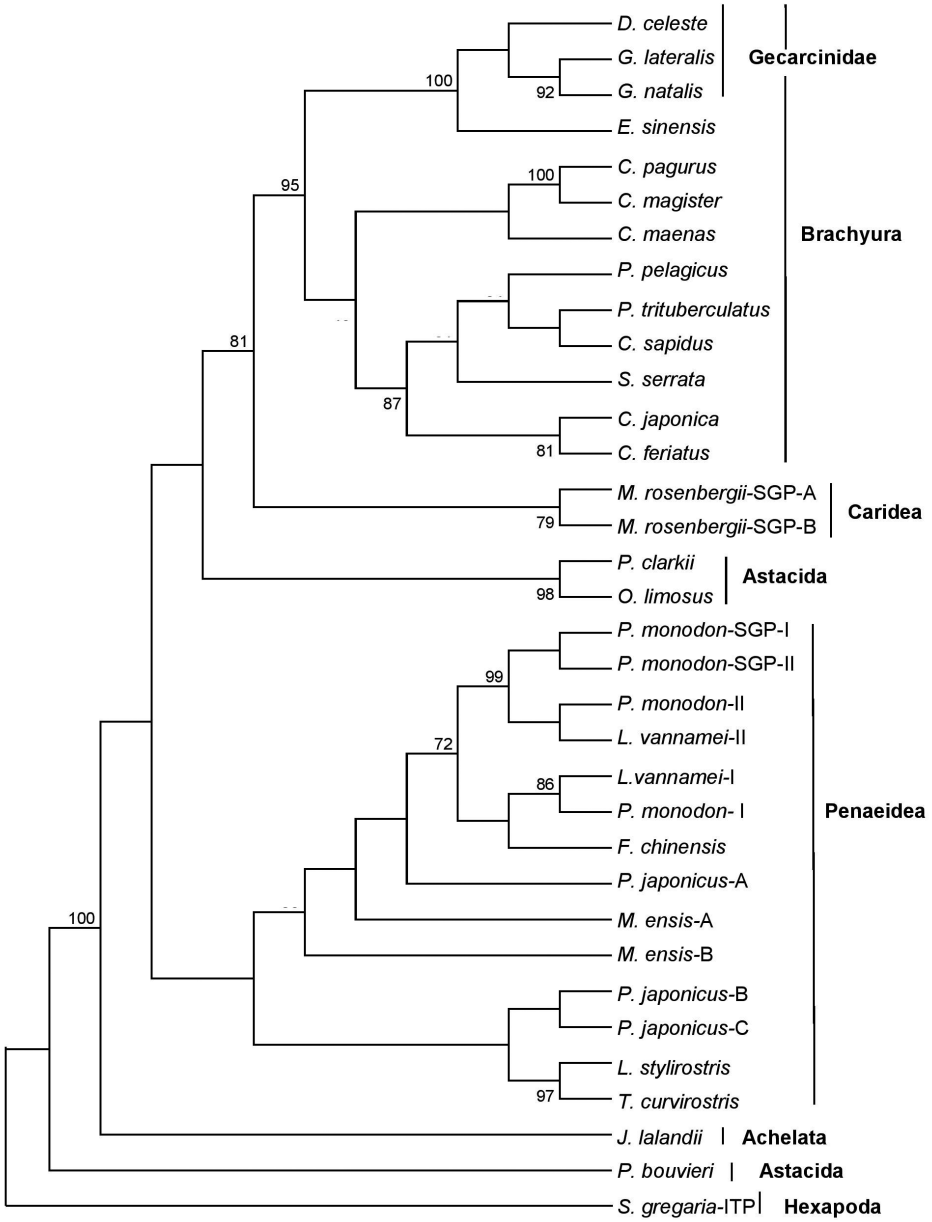

Fig. 5. Phylogeny of crustacean moult inhibiting hormones (MIH) based on maximum-likelihood analysis of the MIH amino acid data set (26 taxa, 34 sequences, 114 residues) using a JTT+I+G model of protein evolution (tree shown is 50% majority rule consensus tree). Maximum-likelihood tree reconstruction was performed using PhyML (Abascal et al., 2005). The best-fit model for protein evolution was determined using ProtTest 1.3 (Jones et al., 1992; Guindon & Gascuel, 2003). The locust *S. gregaria* ITP was assigned as the outgroup. Sequences were acquired from GenBank or directly sequenced (Turner, 2010). See Turner (2010) for methodologies used. Numbers at nodes are bootstrap values (100 replicates). Only bootstrap values ≥70 are shown.

efforts must continue to conserve and protect not only the crabs, but the whole CI ecosystem for future generations.

ACKNOWLEDGEMENTS

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Table 1. Amino acid sequences used in this study.

Species	Sequence Identifier	GenBank Accession Number
Arthropod: Crustacean		
Astacus astacus	Asa-CHH	P83800
Bythograea thermydron	Byt-CHH	AF241264
Callinectes sapidus	Cas-CHH-I	Q6QJL8
	Cas-CHH-II	ACH85179
	Cas-MIH	P55321
Chionoecetes bairdi	Chb-CHH	O61389
Cancer magister	Crm-MIH	ACG50068
Cancer pagurus	Cap-CHH	P81032
	Cap-MIH	CAC39425
Cancer productus	Crp-CHH-I	ABQ41269
	Crp-CHH-II-A	ABQ41270
	Crp-CHH-II-B	ABQ41271
	Crp-CHH-III	ABQ41272
Carcinus maenas	Cam-CHH	P14944
	Cam-MIH	Q27225
Charybdis feriatus	Chf-MIH	O96605
Charybdis japonica	Chj-MIH	ACD11361
Cherax destructor	Chd-CHH-A	P83485
	Chd-CHH-B	P83486
Cherax quadricarinatus	Chq-CHH	AAZ03612
Discoplax celeste	Dic-CHH	AEM45614
	Dic-MIH	AEM45616
Eriocheir sinensis	Ers-CHH	ABA42603
	Ers-MIH	ABC68517
Fenneropenaeus chinensis	Fec-MIH	AAL55258
Galathea strigosa	Gas-CHH	ABS01332
Gecarcinus lateralis	Gel-CHH-A	ABF48652
	Gel-CHH-B	ABF58090
	Gel-MIH	DQ473354
Gecarcoidea natalis	Gen-CHH	ABL09570
	Gen-MIH	(Webster et al., unpublished)
Grapsus tenuicrustatus	Grt-CHH	AER27832
Homarus americanus	Hoa-CHH-A	CAA38611
	Hoa-CHH-B	CAA38612
	Hoa-CHH-I	2105187A
	Hoa-CHH-II	2105187B
	Hoa-CHH	Q25154
Homarus gammarus	Hog-CHH-A	Q3HXZ6
	Hog-CHH-B	Q3HXZ5
Jasus lalandii	Jal-CHH	P56687
	Jal-MIH	P83220
Litopenaeus schmitti	Lss-CHH	P59685
Litopenaeus stylirostris	Lis-MIH	AF312976
Litopenaeus vannamei	Liv-CHH	CAA68067
	Liv-MIH-I	AAR04348
	Liv-MIH-II	AAR04349
Macrobrachium lanchesteri	Mal-CHH	AAC36310
Macrobrachium rosenbergii	Mar-CHH	AAL40915
	Mar-SGP-A	AAL37948
	Mar-SGP-B	AAL37949
Marsupenaeus japonicus	Maj-CHH-I	O15980
	Maj-CHH-II	Q9U5D2
	Maj-CHH-III	BAA13481
	Maj-CHH-IV	P55847

Species	Sequence Identifier	GenBank Accession Number
	Maj-CHH-V	O15981
	Maj-CHH-VI	P81700
	Maj-CHH-VII	O15982
	Maj-MIH-A	P55847
	Maj-MIH-B	BAD36757
	Maj-MIH-C	BAE78494
Metapenaeus ensis	Mee-CHH-A	AAD45233
	Mee-CHH-B	AAF63028
	Mee-MIH-A	AAC27452
	Mee-MIH-B	AAL33882
Nephrops norvegicus	Nen-CHH-A	Q6WGR4
	Nen-CHH-B	AAQ22392
Orconectes limosus	Orl-CHH	CAA56674
	Orl-CHH-A	1802398A
	Orl-MIH	P83636
Pachygrapsus marmoratus	Pam-CHH-A	Q6Y5A6
	Pam-CHH-B	AAO27805
	Pam-CHH	AAM21927
Pagurus bernhardus	Pab-CHH	ABE02191
Penaeus monodon	Pem-CHH	AAC84143
	Pem-CHH-I	O97383
	Pem-CHH-II	O97384
	Pem-CHH-III	O97385
	Pem-CHH-IV	O97386
	Pem-CHH-V	O97387
	Pem-CHH-A	AAD03606
	Pem-CHH-B	AAD03607
	Pem-MIH-I	AAR89516
	Pem-MIH-II	AAR89517
	Pem-SGP-I	BAB69829
	Pem-SGP-II	BAB69830
Pontastacus leptodactylus	Pol-CHH	Q1RN83
	Pol-CHH-I	AAS45406
Portunus pelagicus	Pop-CHH	ABM74398
	Pop-MIH	ABM74397
Portunus trituberculatus	Pot-CHH	ACB46189
	Pot-MIH	ABZ04547
Potamon ibericum	Poi-CHH	Q2VF26
Procambarus bouvieri	Prb-CHH	P55845
	Prb-CHH-I	AAB2553
	Prb-MIH	Q10987
Procambarus clarkii	Prc-CHH	BAA89003
	Prc-MIH	P55848
Ptychognathus pusillus	Ptp-CHH	AER27833
Rimicaris kairei	Rik-CHH-I	ACS35346
	Rik-CHH-II	ACS35347
Scylla olivacea	Sco-CHH	AAQ75760
Scylla serrata	Scs-MIH	AY083797
Trachypenaeus curvirostris	Trc-MIH	AF312978
Outgroup **Arthropod: Insect**		
Schistocerca gregaria	Scg-ITP	Q26491

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