



Effects of emersion on acid–base regulation, osmoregulation, and nitrogen physiology in the semi-terrestrial mangrove crab, *Helice formosensis*

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Abstract

Emersion limits water availability and impairs the gill function of water-breathing animals resulting in a reduced capacity to regulate respiratory gas exchange, acid–base balance, and nitrogenous waste excretion. Semi-terrestrial crustaceans such as *Helice formosensis* mitigate these physiological consequences by modifying and recycling urine and branchial water shifting some branchial workload to the antennal glands. To investigate how this process occurs, *Helice formosensis* were emersed for up to 160 h and their hemolymph and urinary acid–base, nitrogenous waste, free amino acids, and osmoregulatory parameters were investigated. Upon emersion, crabs experienced a respiratory acidosis that is restored by bicarbonate accumulation and ammonia reduction within the hemolymph and urine after 24 h. Prolonged emersion caused an overcompensatory metabolic alkalosis potentially limiting the crab's ability to remain emersed. During the alkalosis, hemolymph ammonia was maintained at control levels while urinary ammonia remained reduced by 60% of control values. During emersion, ammonia may be temporarily converted to alanine as part of the Cahill cycle until re-immersion where crabs can revert alanine to ammonia for excretion coinciding with the crabs' observed delayed ammonia excretion response. The presence of high hemolymph alanine concentrations even when immersed may indicate this cycle's use outside of emersion or in preparation for emersion. Furthermore, *H. formosensis* appears to be uniquely capable of actively suppressing its rate of desiccation in absence of behavioral changes, in part by creating hyperosmotic urine that mitigates evaporative water loss.

Keywords Carbon dioxide · Bicarbonate · Ammonia excretion · Alanine-glucose cycle · Free amino acids · Crustaceans

Introduction

Maintenance of acid–base homeostasis and minimizing the toxic potential of ammonia are fundamental physiological processes of all organisms. Incoming acid or alkali loads affect the function of intra- and extracellular proteins due to their pH sensitivity (Somero 1986) and may have substantial consequences when compensatory action cannot maintain homeostasis. The majority of extracellular compensatory

action relies on the carbonate equilibrium and carbonic anhydrase activity (Gilmour and Perry 2009). Carbon dioxide (CO₂) is hydrated by the catalytic action of carbonic anhydrase forming carbonic acid (H₂CO₃) that readily dissociates into a H⁺ and HCO₃⁻ in solutions of physiological pH (ca. pH 7.2–7.8; pK_a = 6.35) and may further dissociate into another H⁺ and CO₃⁻ in alkaline solutions (pK_a = 10.33). Exhalation of CO₂ during respiratory gas exchange and the renal systems ability to resorb and secrete HCO₃⁻ allows the carbonate equilibrium to be manipulated at the respiratory and ionoregulatory level, respectively. In a simplified example, a hyperventilating animal that is rapidly exhaling CO₂ will cause the carbonate equilibrium to shift leftward favouring the formation of CO₂ and H₂O and causing extracellular fluid to become alkaline. As the animal reduces its ventilation rate, CO₂ excretion rates are reduced, and the equilibrium shifts to the right restoring homeostasis by forming HCO₃⁻ that can accept excess H⁺. Generation of ammonia (i.e., combined ammonia gas (NH₃) and ionic ammonium

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(NH_4^+) as a metabolic by-product of amino acid catabolism can cause deleterious effects such as seizures, disrupted branchial gas exchange and ionoregulation (Wilkie 1997), and swimming capabilities (McKenzie et al. 2003, 2009). Consequences of ammonia accumulation in aquatic species reduce branchial excretory capacity (Martin et al. 2011) and immune function (Young-Lai et al. 1991; Le Moullac and Haffner 2000), becoming lethal if not excreted or detoxified (Larsen et al. 2014). As an amphiprotic molecule (pKa ca. 9.3), ammonia also contributes to acid–base regulation acting by accepting or donating a H^+ as NH_3 becomes NH_4^+ and vice-versa or by excretion of NH_4^+ as an H^+ -equivalent. While this non-carbonate buffering system is considered to be a negligible component in many organisms' acid–base compensatory response, some invertebrates have been identified to maintain distinct concentrations of extracellular ammonia and use bi-directional ammonia transport across the gill epithelia, presumably to be used as an acid–base equivalent (Hu et al. 2017; Hans et al. 2018; Weihrauch and Allen 2018; Allen et al. 2020).

Crustacean gills are multifunctional organs that perform the majority of respiratory gas exchange, maintenance of acid–base status, ionoregulation, and ammonia excretion (Henry et al. 2012). In comparison to aerial habitats, water has relatively low oxygen bioavailability meaning that most water-breathing species must maintain high ventilation rates to extract enough oxygen to survive (Claiborne et al. 2002; Gilmour and Perry 2009). Since water-breathers must rapidly ventilate, they do not have the choice to rely upon changes in ventilation rate to adjust acid–base status through ventilatory loss of CO_2 (Claiborne et al. 2002; Gilmour and Perry 2009). Instead, these animals largely rely on ion-exchange processes such as H^+ -excretion by Na^+/H^+ exchangers or HCO_3^- excretion by $\text{Cl}^-/\text{HCO}_3^-$ exchangers to regulate acid–base homeostasis (Goss et al. 1992; Claiborne et al. 2002; Perry et al. 2003; Gilmour and Perry 2009; Fehsenfeld and Weihrauch 2016). Although NH_3 may be diffusively excreted along the partial pressure gradient of NH_3 (ΔP_{NH_3}) aided by Rhesus-proteins across the gill epithelium and into the environment (Nawata et al. 2010), most (ca. 95–99.5%) of systemic and cellular ammonia exists as NH_4^+ due to the pH of biological systems. NH_4^+ transport is typically performed by K^+ -accepting transporters (Na^+/K^+ -ATPase, K^+ -channels, cation/ H^+ exchangers) due to the similar hydration radii and the same molecular charge of the two ions, by ammonium transporters (AMTs) in invertebrates (Chasiotis et al. 2016; Thiel et al. 2017; Durant and Donini 2018), or by vesicular trafficking, where NH_4^+ accumulates in acidified vesicles that can be excreted independent of electrochemical gradients and environmental factors (Weihrauch et al. 1998, 2002; Weihrauch and Allen 2018). In some cases, ammonia excretion over the apical epithelial membrane of the branchial tissue is suggested to

be Na^+ -dependent as often occurs in freshwater fishes and is potentially related to Na^+/H^+ -exchanger activities (Wright and Wood 2012). Thus, environmental conditions that impair gills function or affect ionoregulatory activity may also affect the organism's acid–base and nitrogen homeostasis.

Mangroves are harsh coastal environments whose inhabitants must be capable of surviving dramatic shifts in salinities, temperature, oxygen bioavailability, waste accumulation, and aerial exposure (Abel et al. 1987; Kathiresan and Bingham 2001). Crabs are key-stone mangrove inhabitants and act as ecosystem engineers whose dense populations and burrowing behavior play a crucial role in the environment's nutrient cycling (Kristensen 2008; Penha-Lopes et al. 2009). Semi-terrestrial crabs spend extended periods of time emersed while performing mating rituals and foraging for food, with some species developing lung-like structures and trapping air within their burrows at high tides (Teal and Carey 1967; Burggren 1992; Eshky 1992; Jimenez and Bennett 2015). As gills require moisture to properly function, emersion stress causes impairment of respiratory gas exchange and transport mechanisms, reducing the animal's overall ability to excrete wastes such as CO_2 and ammonia (Morris 2002). Therefore, animals must not only combat acute loss of water availability and search for high humidities (Adamczewska and Morris 2000), but are also required to counteract physiological consequences related to osmotic and pH disruption.

While some terrestrial crabs have generally developed lung-like epibranchial structures (Morris 2002), most semi-terrestrial species continue to rely on trapped moisture within the gill chamber to eliminate CO_2 . Select species such as *Scopimera* and *Dotilla* sand-bubbler crabs use respiratory 'windows' on their legs to perform gas exchange either in place or in addition to their gill function (Maitland 1986). Nitrogen eliminating strategies in terrestrial and semi-terrestrial crabs differ; however, most species are believed to accumulate or detoxify ammonia into temporary stores of less toxic metabolites until the animal can bathe itself in a water-source (Linton et al. 2017). Some semi-terrestrial species use their antennal glands to concentrate urinary ammonia that may be degassed as the urine passes over their mouth and into their branchial chamber as a portion of urine recycling (De Vries et al. 1994). This strategy is exploited by Ocypodoidea fiddler crabs, whereas Grapsoidea crabs are generally unable to shift their reliance from the gills to antennal glands to handle wastes (Tseng et al. 2020). Inability to rely on antennal glands and reduced gill function should cause most *Grapsoidea* crabs to accumulate nitrogenous wastes as ammonia or urea; however, several studies have noted that while the wastes are not detectably excreted it also does not accumulate in the body fluids as expected, although urinary contribution has often been excluded from studies (Wood and Boutilier 1985; Wood et al. 1986).

Helice formosensis, the thick crab, is a burrowing mangrove species native to South-East Asia that undergoes emersion during low tides to forage for food. Unlike other members of the *Helice* family, *H. formosensis* is carnivorous and primarily feeds on other crabs (Mia et al. 2001) inferring that it faces a greater burden of nitrogenous waste excretion than its relatives. When not foraging, the crabs retreat into water-filled burrows where poor water-circulation exposes the animal to its own ammonia-rich waste products (Mchenga et al. 2007). Thick crabs do not possess epibranchial lung-like structures or respiratory ‘windows’ adaptations that would facilitate their lifestyle, making them an interesting case for developing adaptive strategies used by general crab species. *Helice formosensis* has been noted to rapidly recycle urine, a process used by most terrestrial and semi-terrestrial crabs to mitigate physiological consequences related to reduced gill function and dehydration (De Vries et al. 1994). Urine produced by the antennal glands is released from antennal pores and allowed to flow across the anterior surface of the crab where it is either swallowed or recollected into the water filled branchial chamber. The following study aims to characterize how emersion affects the thick crab’s acid–base, osmotic, and nitrogen physiology with a focus on the relative importance of recycled urine to the hemolymph in a crab that is unusually carnivorous amongst semi-terrestrial species.

Methods

Animal husbandry and experimental conditions

Adult male thick crabs (*Helice formosensis*; 16.5 ± 0.4 g) were purchased from local bait shops in Chiayi (嘉義縣) county, Taiwan and transported to the Institute of Cellular and Organismal Biology’s Marine Research Station (Yilan (宜蘭縣) county, Taiwan). Crabs were housed in four aquaria (50 gallons, 15–20 individuals/tank) containing constantly aerated brackish water (15.5 ± 0.1 ppt. salinity, pH 7.99 ± 0.01 , 21.1 ± 0.1 °C, $P_{CO_2} = 52.3 \pm 4.8$ Pa CO_2) maintained on a 12:12-h light:dark cycle and fed cuttlefish three times a week ad libitum. Brackish water was prepared by mixing dechlorinated freshwater with natural seawater. Accumulation of CO_2 within aquaria was monitored daily (see below) and complete water changes in aquaria were performed three times per week 3 h post-feeding to minimize the build-up of ammonia within the tanks. Animals were fasted for 3 days prior to experimentation to minimize the potential effects of dietary nitrogen on results.

Crabs were randomly selected from the housing aquaria and immediately sampled representing immersed control animals. Treatment crabs were randomly selected from the control group, their bodies blotted dry, weight recorded,

and stored in water-free containers for 6, 12, 24, 48, or 160 h at 21 °C temperature and ca. 90–95% relative humidity. Different crabs were used for either whole animal experiments or hemolymph and urine sampling—animals were not repeatedly measured. The mass of crabs after being blotted dry was compared to the mass of the animal at the end of the emersion exposure to determine changes in body mass which were assumed to be primarily caused by evaporative water loss.

Hemolymph and urine carbonate equilibrium

Hemolymph was sampled by puncturing the arthroal membrane with a 25G needle and gas-tight Hamilton syringe. Urine was collected using a syringe following deflection of the antennal pore cover (Wolcott 1991). Sample pH was immediately measured using a micro-pH electrode (InLab-Micro, Mettler-Toledo) attached to a pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany) followed by measurement of total CO_2 using methods described by Lee et al. (2018) and the remaining samples were frozen for further analysis. In brief, 5 μ L of the sample was injected through a septum into a glass chamber containing 0.1 mmol l^{-1} HCl to transform dissolved inorganic carbon within the sample to CO_2 . CO_2 was degassed by the continuous input of N_2 -gas (60 – 80 ml N_2 gas min^{-1} , GFC17, Aalborg, Orangeburg, NY, USA) and passed into a total CO_2 detector (LI-850 infra-red CO_2/H_2O gas analyzer, LI-COR, Lincoln, NE, USA). LI-8 $\times 0$ software (v1.0.2, LI-COR) was used to collect data. Total CO_2 within the samples was determined by comparing data to that of $NaHCO_3$ standards. The partial pressure of CO_2 (P_{CO_2}) within the samples were mathematically determined by inputting the measured pH and total CO_2 into a rearrangement of the Henderson-Hasselbalch equation (Eq. 1) where the solubility of CO_2 ($\alpha = 0.045$ mmol $Torr^{-1}$) and first dissociation constant of carbonic acid ($pK_{a1} = 6.05$) were experimentally determined in *Carcinus maenas* and extrapolated from nomograms supplied by Truchot (1976) at 21 °C and 15 ppt. salinity

$$P_{CO_2} = \frac{TCO_2}{[(1 + \text{antilog}(pH - pK')) * \alpha CO_2]} \quad (1)$$

The concentration of HCO_3^- within the samples was then determined using Eq. 2 applying the same constants

$$[HCO_3^-] = TCO_2 - (P_{CO_2} * \alpha CO_2). \quad (2)$$

The total CO_2 within water samples was similarly determined; however, the carbonate equilibrium was determined using the CO_2SYS software (Pierrot et al. 2006) using dissociation constants of Mehrbach et al. (1973) refit by Dickson and Millero (1987).

Ammonia measurements

Ammonium concentrations in hemolymph, urine, and water samples were determined fluorometrically (360 and 442 nm excitation and emission wavelengths, respectively; Spectramax M5, Molecular Devices) using a borate buffered ortho-phthalaldehyde plate assay that is insensitive to amino acids and proteins (Holmes et al. 1999) as confirmed by cross-analyzing results of deproteinized and non-deproteinized samples (data not shown; deproteinizing sample preparation kit—TCA, ab204708, AbCam, Toronto, Canada). Osmolality of the NH_4Cl standards for each sample type were adjusted with NaCl to account for potential osmotic sensitivity in the assay.

Major ion composition

The major cation composition (Na^+ , K^+ , Mg^{2+} , Ca^{2+}) of samples was determined by flame absorption spectroscopy (Polarized Zeeman Atomic Absorption Spectrophotometer ZA3000 series, Hitachi High-Technologies, Tokyo, Japan) by comparing sample readings to a calibration curve made with standard solutions of individual metal ions. Concentration of HCO_3^- was determined as previously described whereas the mercuric-ferrous cyanide assay was used to spectrophotometrically detect the concentration of Cl^- within the samples (Florence and Farrar 1971). Osmolality of samples was measured using a vapour pressure osmometer (VAPRO Model 5520, Wescor, UT, USA).

Free amino acid composition

Free amino acids from 20 μL samples of hemolymph and urine were extracted in ice cold 50% (v/v) methanol:water solutions spiked with isotopically labeled norvaline internal standards (4 $\mu\text{g}/\text{mL}$) and subsequently derivatized with AccQ●Tag Ultra Derivatization Kit (Waters, MA., U.S.A.; Armenta et al. 2010). The UPLC (Acquity UPLC H-Class System, Waters) was equipped with a BEH C18 column and a FLU detector was used to detect the derivatized amino acids. The free amino acids within samples were quantified by comparison to known standards (WAT088122, Waters).

Whole animal ammonia excretion upon re-immersion

Ammonia excretion rates were determined by adding 300 mL of filtered (0.45 $\mu\text{mol l}^{-1}$) 15 ppt. salinity brackish water into the jars holding animals and measuring the accumulation of ammonia after 30, 60, 90, and 120 min. To account for ammonia that may have been adhered to the jar during the crab's air exposure, samples were also taken immediately after water was added and subtracted from the

final data (time = 0 min). Ammonia concentrations were determined fluorometrically as previously described and excretion rates determined as $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$.

Statistical analysis

Data analysis and statistics were performed using GraphPad Prism 8.4.3 software. Normal distribution and variance in collected data were investigated using the Shapiro–Wilk and Brown–Forsythe tests, respectively. Non-normal data were normalized using a box-cox transformation. Outliers were detected using Grubb's outlier test ($p < 0.05$) and removed from data sets prior to analysis. Normally distributed time-course data with equal variance was tested for statistical significance using one-way ANOVAs and Tukey's post hoc test. Statistical differences between the hemolymph and urine samples at each respective time point were assessed using a two-way ANOVA where time and fluid type (hemolymph or urine) were set as factors and Tukey's post hoc test. If data could not be normalized by transformation, statistical significance was determined using Mann–Whitney U rank comparison tests. Data are presented as the mean \pm S.E.M. Statistical significance was denoted a $p < 0.05$.

Results

Hemolymph of immersed crabs was 7.78 ± 0.02 pH (Fig. 1) with extracellular P_{CO_2} levels of 401 ± 13 Pa (Fig. 2) and 7.38 ± 0.26 mmol l^{-1} HCO_3^- (Fig. 3). The immersed crabs' hemolymph ammonium concentrations were 381 ± 27 $\mu\text{mol l}^{-1}$ NH_4^+ (Fig. 4). Urinary pH (7.83 ± 0.02 ; Fig. 1), P_{CO_2} (360 ± 13 Pa; Fig. 2), $[\text{HCO}_3^-]$ (7.37 ± 0.31 mmol l^{-1} ; Fig. 3), and NH_4^+ (320 ± 45 $\mu\text{mol l}^{-1}$;

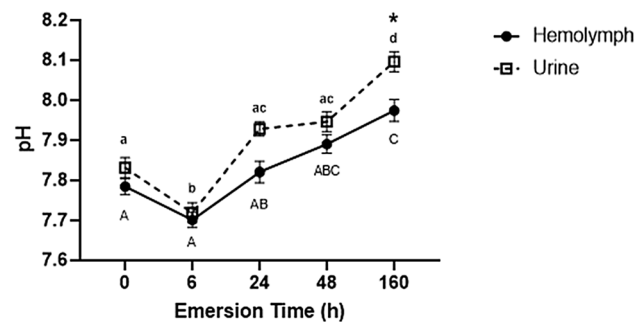


Fig. 1 pH of hemolymph and urine of immersed ($N_H=22$, $N_U=12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H=18$, $N_U=18$), 24 ($N_H=12$, $N_U=12$), 48 ($N_H=6$, $N_U=5$), and 160 ($N_H=12$, $N_U=12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denotes significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

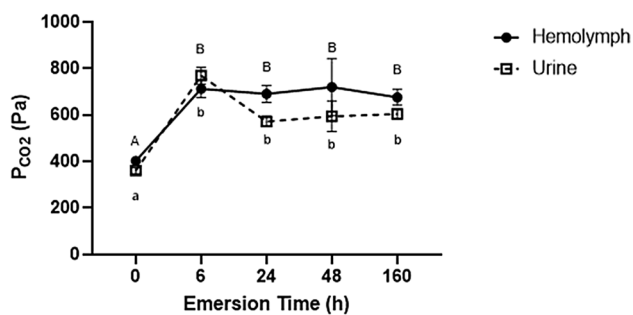


Fig. 2 Partial pressure of CO_2 (P_{CO_2}) within the hemolymph and urine of immersed ($N_{\text{H}}=22$, $N_{\text{U}}=12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_{\text{H}}=18$, $N_{\text{U}}=18$), 24 ($N_{\text{H}}=12$, $N_{\text{U}}=12$), 48 ($N_{\text{H}}=6$, $N_{\text{U}}=5$), and 160 ($N_{\text{H}}=12$, $N_{\text{U}}=12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denotes significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

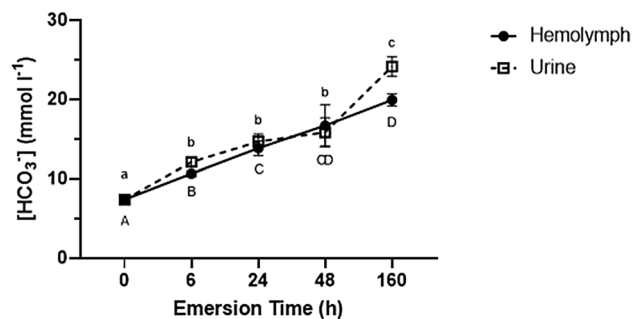


Fig. 3 Concentrations of HCO_3^- within the hemolymph and urine of immersed ($N_{\text{H}}=22$, $N_{\text{U}}=12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_{\text{H}}=18$, $N_{\text{U}}=18$), 24 ($N_{\text{H}}=12$, $N_{\text{U}}=12$), 48 ($N_{\text{H}}=6$, $N_{\text{U}}=5$), and 160 ($N_{\text{H}}=12$, $N_{\text{U}}=12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

Fig. 4) were similar to that of the immersed crabs' hemolymph. Crabs experienced a slight respiratory acidosis after 6 h of emersion as indicated by a 0.84 pH unit reduction in hemolymph pH (Fig. 1, $p=0.0651$) and 77% increase in hemolymph P_{CO_2} (Fig. 2) that is compensated within 24 h by the accumulation of extracellular HCO_3^- (Fig. 3a). Urine parallels the hemolymph changes in pH, P_{CO_2} , and HCO_3^- (Figs. 1, 2, 3) indicating the fluids remain in equilibrium upon immersion. Prolonged emersion caused animals to apparently over-compensate, resulting in a metabolic alkalosis indicated by alkalinization of hemolymph and urine (Fig. 1) caused by the accumulation of HCO_3^- (Fig. 3) while maintaining constant P_{CO_2} (Fig. 2). Ammonia concentrations

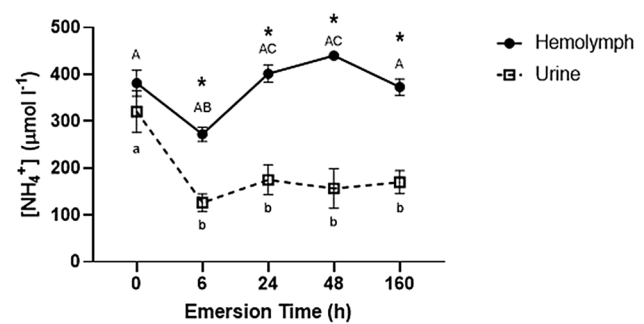


Fig. 4 Concentrations of NH_4^+ within the hemolymph and urine of immersed ($N_{\text{H}}=12$, $N_{\text{U}}=12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_{\text{H}}=12$, $N_{\text{U}}=12$), 24 ($N_{\text{H}}=12$, $N_{\text{U}}=6$), 48 ($N_{\text{H}}=6$, $N_{\text{U}}=5$), and 160 ($N_{\text{H}}=12$, $N_{\text{U}}=12$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denotes significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm S.E.M

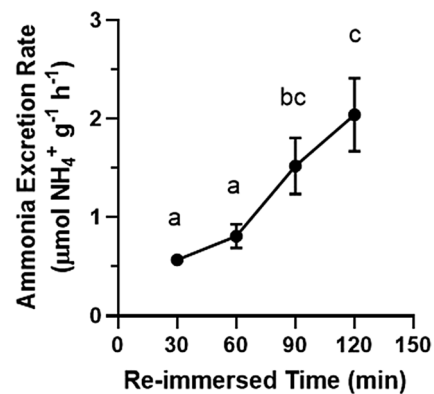


Fig. 5 Whole animal ammonia excretion of crabs ($N=5$) emersed for 160 h immediately following re-immersion. Values are represented as the mean \pm SEM

within the hemolymph were reduced by 29% after 6 h of emersion ($p=0.0685$) but were restored to control levels after 24 h (Fig. 4). Urinary ammonia concentrations were immediately reduced by 60% upon acute emersion and remained constant throughout prolonged emersion (Fig. 4). Whole animal ammonia excretion upon re-immersion was measured in crabs that were emersed for 160 h (Fig. 5) indicating that the crabs progressively increase their ammonia excretion rate rather than immediately releasing ammonia accumulated over time. Excretion rates beginning at $0.57 \pm 0.04 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ upon immediate re-immersion significantly increased by 3.6-fold reaching $2.04 \pm 0.37 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ after 2 h of immersion (Fig. 5).

Hemolymph and urine of immersed and emersed crabs were assessed for 21 amino acids via UPLC (Fig. 6 and

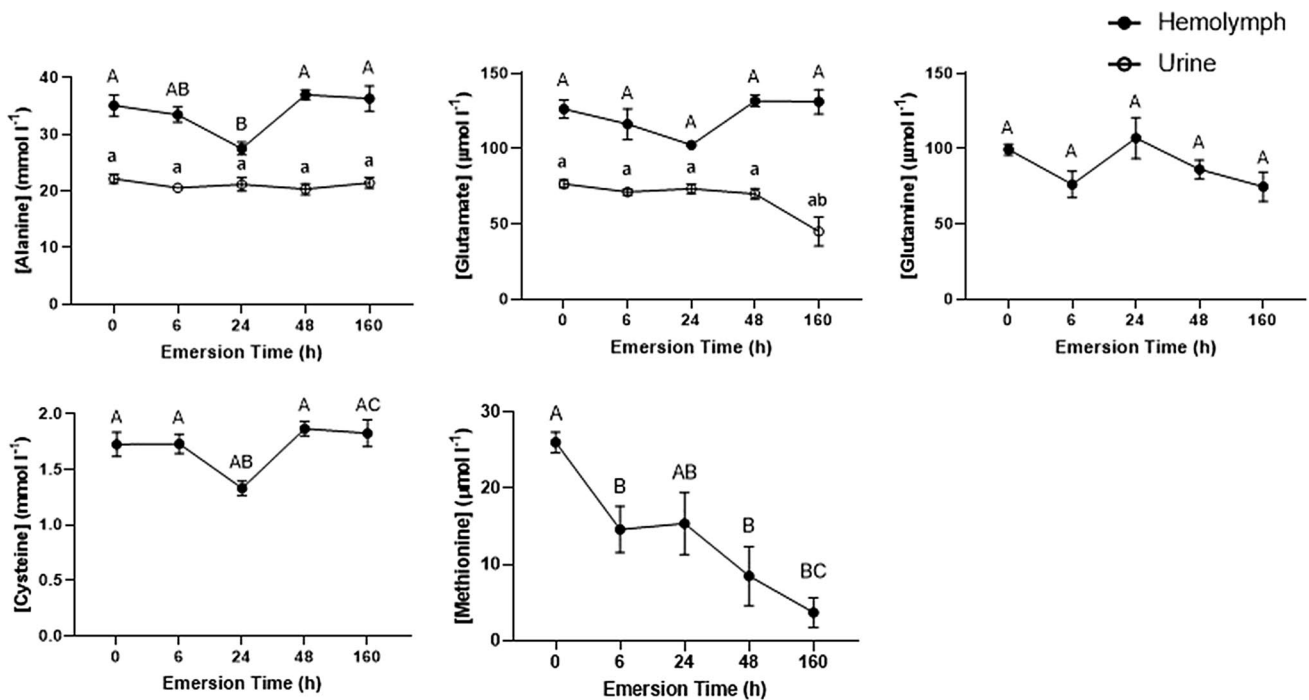


Fig. 6 Predominant free amino acids in the hemolymph ($N=6-12$) and urine ($N=4-12$) of immersed (0-h) or emersed crabs over a 160-h time-course exposure. Where data is not available for urinary concentrations no detectable amino acid presence was found. Upper-

case and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denotes significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

supplemental Figs 1–3). Hemolymph contained detectable concentrations of alanine, cysteine, glutamate, glutamine, glycine, proline, taurine, arginine, asparagine, methionine, valine, isoleucine, serine, threonine, and leucine while histidine, lysine, tyrosine, aspartate, and phenylalanine were undetectable. Urine contained fewer detectable amino acids and lower concentrations of amino acids overall compared to the hemolymph. Alanine was the predominant free amino acid detected in hemolymph and urine, accounting for most of the total free amino acids in both fluids (Fig. 6a). High alanine concentrations of 30–40 and 20 mmol l⁻¹ indicate that the amino acid is the third most abundant osmolyte in immersed and emersed crab's hemolymph and urine, respectively, only being surpassed by Na⁺ and Cl⁻ concentrations (Figs. 6a and 8). Crabs reduced hemolymph alanine concentrations upon acute emersion for up to 24 h reaching 78% of control levels (Fig. 6a). Prolonged emersion (> 24 h) is accompanied by a 22% increase in alanine concentrations returning to control levels. Urinary alanine concentrations remained stable throughout the experiment, consistently containing 10–15 mmol l⁻¹ less alanine than hemolymph (Fig. 6a). Cysteine was the second most abundant free amino acid in the hemolymph, remaining stable throughout all time-points at 1.6–1.8 mmol l⁻¹, but was undetectable in urine (Fig. 6d). Hemolymph glutamate and glutamine glutamate was unchanged throughout the experiments; however, the urine was devoid of glutamine

and glutamate concentrations were significantly reduced upon emersion (Fig. 6b, c). Detected free essential amino acids decreased in samples as crabs were unfed throughout the experiment and fasted for 3 days prior to experimentation (Fig. 7e and supplemental Fig. 1).

Helice formosensis emersed in 15-ppt. brackish water displayed characteristics of strong hyperosmoregulators maintaining 658 ± 5 mOsm kg⁻¹ hemolymph against 410 mOsm kg⁻¹ ambient water (Fig. 8). Urine is maintained hyperosmotic by 40–53 mOsm kg⁻¹ in relation to the hemolymph (Fig. 8) due to increased Na⁺, K⁺, and Mg²⁺ concentrations but not Ca²⁺ or Cl⁻ (Fig. 6). Emersion caused both hemolymph and urine osmolality to increase over time; however, the osmotic difference between the fluids is maintained (Fig. 8). Crabs were able to significantly reduce their rate of evaporative water loss over time from an initial rate of $0.2 \pm 0.02\%$ of their body mass per hour after 6 h of emersion to $0.05 \pm 0.003\%$ of their body mass per hour after 160 h of emersion (Fig. 9).

Discussion

As a relatively unstudied species, there is an absence of information on hemolymph and urine compositions of members of the *Helice* crab family. Hemolymph of immersed *H.*

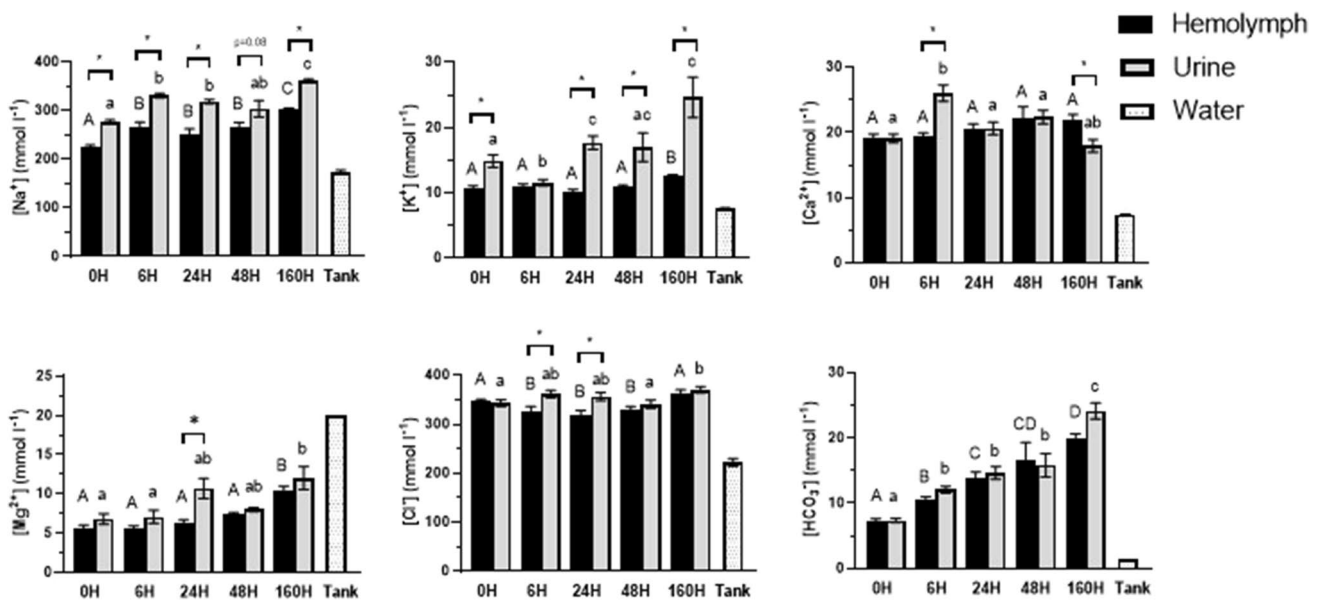


Fig. 7 Major ion composition of immersed (0-h) and emersed crabs' hemolymph ($N=6-12$ for all ions) and urine ($N=5-12$ for all ions) over a 160-h time-course exposure. Tank parameters are included as comparison to the immersed crabs' environmental conditions at 15

ppt salinity. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denotes significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

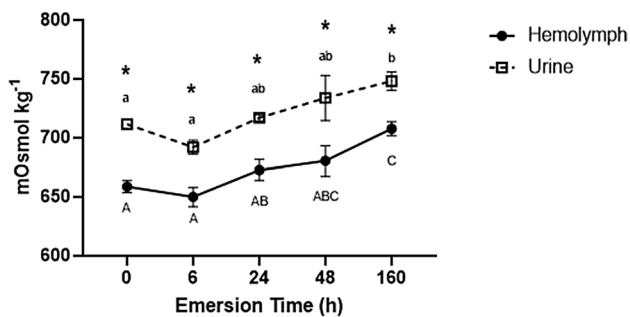


Fig. 8 Osmolality of the hemolymph and urine of immersed ($N_H=22$, $N_U=12$) and emersed crabs over a time-course exposure. Emersed measurements were made after 6 ($N_H=18$, $N_U=12$), 24 ($N_H=12$, $N_U=6$), 48 ($N_H=6$, $N_U=5$), and 160 ($N_H=12$, $N_U=11$) hours of emersion. Uppercase and lowercase letters denote significance of hemolymph and urine osmolality over time, respectively, while asterisk denote significance between the hemolymph and urine osmolality at the same time point. Values are represented as the mean \pm SEM

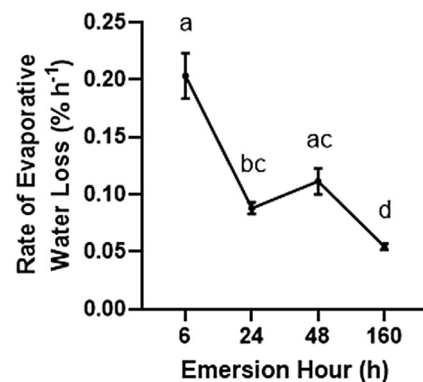


Fig. 9 Rate of evaporative water loss as measured by changes in emersed crabs' total mass over a time course exposure. Emersed measurements were made after 6 ($N=12$), 24 ($N=12$), 48 ($N=12$), and 160 ($N=24$) hours of emersion. Values are represented as the mean \pm SEM

formosensis measured 7.78 ± 0.02 (Fig. 1) and was similar to those of other crab species (e.g., *Carcinus maenas* 7.82 pH and 150 Pa P_{CO_2} (Truchot 1975); *Metacarcinus magister* 7.93 pH and 132.9 Pa P_{CO_2} (Hans et al. 2014); *Callinectes sapidus* 7.78 pH and 270 Pa P_{CO_2} (Henry and Cameron 1982), although the resting levels of *H. formosensis* P_{CO_2} were considerably higher (401 ± 13 Pa P_{CO_2} ; Fig. 2). The comparably high resting levels of P_{CO_2} are balanced by the presence of 7.38 ± 0.26 mmol l^{-1} HCO_3^- (Fig. 3) and

381 ± 27 $\mu\text{mol } l^{-1}$ NH_4^+ (Fig. 4) within the crab's hemolymph. Apart from the uniquely specialized ghost crab, *Ocypode quadrata* (De Vries et al. 1994), few brachyuran crustaceans have had their urine characterized in terms of acid–base and nitrogen composition. Urine collected from immersed *H. formosensis* was similar in pH, P_{CO_2} , HCO_3^- , and NH_4^+ to the hemolymph (pH 7.83 ± 0.02 ; Fig. 1; 360 ± 13 Pa P_{CO_2} ; Fig. 2; 7.37 ± 0.31 mmol $HCO_3^- l^{-1}$; Fig. 3; 320 ± 45 $\mu\text{mol } NH_4^+ l^{-1}$; Fig. 4), suggesting the fluids are in equilibrium and that wastes are not concentrated in urine prior to emersion; however, the apparently

high urinary flow-rate was not determined and could play an important factor in its overall excretory importance (see supplemental video 1).

Helice formosensis remains immersed within its burrows beneath the sediment of the mangrove floor. Studies of the physiochemical properties of the burrow shafts and chambers indicate that *H. formosensis* significantly reduces the salinity of the chamber's water (Mchenga et al. 2007). The current study demonstrates that the thick crab has characteristics of strong hyperosmoregulators (Fig. 8) and can maintain its extracellular osmolality well above ambient levels, likely due to inhabiting environments that naturally fluctuate between 10 and 35 ppt. salinity (Tseng et al. 2020). While most of the crab's hemolymph ion composition reflects that of other hyperregulating crabs, its extracellular K^+ (Fig. 7b) is maintained at $10.7 \pm 0.4 \text{ mmol l}^{-1}$, exceeding the concentrations typically observed even in seawater-dwelling crabs such as *M. magister* ($6.4 \text{ mmol l}^{-1} K^+$ (Hans et al. 2014). Additionally, *H. formosensis*' extracellular NH_4^+ concentrations ($381 \pm 27 \text{ } \mu\text{mol l}^{-1} NH_4^+$ (Fig. 4a)) are also above the average of most brachyuran species *ca.* 150–200 $\mu\text{mol l}^{-1} NH_4^+$ (Fehsenfeld and Weihrauch 2017; Weihrauch et al. 2017). Given that the urine of immersed *H. formosensis* seems to concentrate Na^+ and K^+ while leaving divalent cations (Ca^{2+} , Mg^{2+}) and anions (Cl^- , and HCO_3^-) at the hemolymph level, it is conceivable that the antennal glands may promote the excretion of excess K^+ and is involved in the crabs' osmoregulation as occurs in other crustaceans (Tsai and Lin 2014; Tseng et al. 2020). Future studies would benefit from determining the flow rate of the urine in immersed crabs to calculate the true amounts of ions and osmolytes expelled over time.

While filtrating organs, including antennal glands, generally reabsorb useful ions and molecules it seems that alanine and glutamate are only partially reabsorbed during primary urine production by *H. formosensis* (Fig. 6 and supplemental information). Alanine and glutamate remain present at 22 mmol l^{-1} (Fig. 6a) and $76 \text{ } \mu\text{mol l}^{-1}$ (Fig. 6b) within the urine, respectively, accounting for 63% and 61% of the respected amino acid within the hemolymph while other amino acids are effectively reabsorbed. While the actual amount of alanine and glutamate lost through urine expulsion would depend on the urinary flow rate of immersed crabs, it seems unfavorable for an animal to lose large millimolar levels of potentially useful amino acids. One hypothesis is, that since the crabs are generally immersed within the confines of their burrow chamber, they may be able to reabsorb at least some of the amino acids across their branchial epithelia (Blewett and Goss 2017). Water in the burrow chambers of *H. formosensis* is significantly higher in organic matter than their surroundings, presumably due to detritus and excretion by the crab (Mchenga et al. 2007). While Mchenga et al. (2007) did not measure the presence

of amino acids within the burrows, the current study does suggest the crabs can excrete large levels of amino acids through urine. If excess ammonia is detoxified as alanine, its release into the burrow chamber waters would mitigate the build-up of toxic ammonia while also allowing the crab to reabsorb some degree of the amino acid from the water across its branchial chamber, especially considering amino acid uptake across the gills of brachyuran crabs has recently been discovered (Blewett and Goss 2017). Non-absorbed amino acids may still benefit the crabs and mangrove environment by providing nutrients to the microbes within their burrow chambers. While this study does not provide hard evidence that this process does occur, *H. formosensis* would require a means to reabsorb urinary amino acids through their branchial chamber as part of their urine reprocessing mechanism during emersion (see below). Additionally, maintaining high alanine concentrations within the urine may simply be a strategy used by the crab to be continuously prepared for emersion following tidal movements.

Mangrove crabs are regularly air-exposed during low-tide while they perform energetically demanding tasks such as feeding, digging burrows, or performing mating dances/rituals. While it is unknown how long *H. formosensis* specifically remains emersed, the crabs demonstrated the ability to readily compensate for acid–base disturbances. Despite a significant increase in hemolymph P_{CO_2} (Fig. 2), crabs managed to mitigate a statistically relevant change in hemolymph pH (Fig. 1) through rapid HCO_3^- accumulation (Fig. 3) after only 6-h of emersion. After 24 h of emersion, crabs restored hemolymph pH to near-exact control values, demonstrating a clear ability to fully compensate the observed respiratory acidosis within 24 h. Like immersed crabs, the pH, P_{CO_2} , and HCO_3^- of hemolymph and urine remain in equilibrium when crabs are emersed; however, the urine is no longer lost to the ambient environment and is recycled into the branchial chamber for reprocessing (Supplemental video 1). Urinary CO_2 may be volatilized as urine passes across the ventral body of the crab representing an effective and energetically cheap means of excreting CO_2 for crabs during emersion. Since crabs experience a progressive metabolic alkalosis once emersion passes 24 h, this excretory method may be capable of maintaining constant hemolymph P_{CO_2} (Fig. 2) but is insufficient to prevent build-up of total carbon and protons resulting in a subsequent alkalosis due to HCO_3^- accumulation (Fig. 3).

Upon emersion, changes in the hemolymph of *H. formosensis* are quite different than that of *Cardisoma carnifex*, an air breathing terrestrial lunged crab, that experience an acute metabolic alkalosis and a delayed respiratory acidosis in absence of water (Wood et al. 1986). Upon desiccation, the hemolymph of *C. carnifex* is maintained at about 0.05–0.10 pH units above its hydrated levels while the crabs manage to prevent over-alkalization of its hemolymph by

continuing to gain P_{CO_2} until rehydrated (Wood et al. 1986). The delayed accumulation of CO_2 in *C. carnifex* is likely due to its lungs permitting a greater excretion of CO_2 than the gills of *H. formosensis*. If urinary CO_2 volatilization is sufficient to stabilize hemolymph P_{CO_2} but the crabs do not have an effective means of excreting protons, HCO_3^- accumulation should eventually result in the observed metabolic alkalosis. Given that *H. formosensis* is more likely to experience tidal emersion rather than > 24 h of emersion by choice, the crabs may lack a means of counteracting the process. In the intertidal green shore crab, *Carcinus maenas*, emersion causes an acute respiratory acidosis that requires approximately 115 h to be nearly restored to control levels by accumulation of HCO_3^- (Truchot 1975). In this case, *C. maenas* also maintains a constant level of P_{CO_2} (ca. 660 Pa) while emersed, which is similar to that of *H. formosensis* in this study (Fig. 2a). The more rapid accumulation of HCO_3^- and restoration of hemolymph pH in *H. formosensis* compared to *C. maenas* is presumably due to their degree of terrestrialization that is based on their independence from water (Hartnoll 1988; Greenaway 1999). Crustaceans can be classified between five tiers of terrestrialization (T_1 to T_5) where *C. maenas*, for example, is a T_1 crab that is relatively quiescent upon emersion that survives brief periods of emersion. While *H. formosensis* has not been previously classified, its lifestyle is better described as a T_2 or T_3 crab as it voluntarily emerges for prolonged periods of time but immerses itself in burrows and requires water to breathe and reproduce (Hartnoll 1988; Mia and Shokita 1997; Greenaway 1999; Mia et al. 2001; Mchenga et al. 2007).

Despite being studied in several species, the handling of nitrogenous wastes by semi-terrestrial and terrestrial crustaceans during emersion or desiccative stress has remained poorly understood. With the exception of the uricotelic Robber crab (*Birgo latro*; Morris and Greenaway 1990), all semi-terrestrial and terrestrial brachyuran crustaceans described so far are ammonotelic and cannot rely on branchial excretion of ammonia without access to water. Interestingly, chronically emersed/desiccation stressed crabs manage to prevent the accumulation of hemolymph ammonia despite continued protein catabolism. Therefore, crabs must be capable of either unconventional excretion, such as volatilization of NH_3 , or through temporary detoxification of ammonia through transformation into less toxic metabolites (e.g., urea, uric acid, amino acids) in absence of water.

Volatilization of NH_3 is a prominent strategy used predominantly by T_4 terrestrial crustaceans such as the Atlantic ghost crab (*Ocypode quadrata*) and the purple-backed shore crab (*Geograpsus grayi*) whom either form urine with extremely concentrated ammonia ($100 + \text{mmol l}^{-1}$; De Vries et al. 1994) or alkalizes the ammonia from branchial chamber water (Varley and Greenaway 1994), respectively. These species typically maintain high ($0.9\text{--}1.9 \text{ mmol l}^{-1}$;

Linton et al. 2017) concentrations of hemolymph ammonia to develop high NH_3 partial pressure gradients that are not observed in the hemolymph of *H. formosensis* (Fig. 4a). In NH_3 volatilizing isopods, high hemolymph levels of ammonia are mitigated by temporary detoxification to glutamate that is later catabolized by glutaminase to release ammonia into the pleon fluid for volatilization (Wieser et al. 1969; Wright and O'Donnell 1993; Wright and Peña-Peralta 2005). Given that hemolymph glutamine/glutamate and ammonia levels in *H. formosensis* were not elevated at any point of this study (Figs. 4 and 6b) it is unlikely this crab uses methods similar to isopods or NH_3 volatilizing T_4 crustaceans despite alkalization of its urine (Fig. 1) which would otherwise promote volatilization of NH_3 . With some speculation, it can be reasoned that NH_3 volatilization from within the branchial chamber is also unlikely. The fluid within the branchial chamber will presumably become increasingly similar in composition to the urine as emersion continues since recollected urine is the sole fluid source available to the crab. This means that while branchial chamber fluid will also be slightly alkaline, it will probably be low in ammonia concentration which would not favour volatilization of NH_3 . While these findings do not suggest volatilization occurs to a significant degree, it cannot be completely ruled out without direct measurement, especially considering that *C. maenas* (T_1) has been found to be unexpectedly capable of volatilizing small amounts of NH_3 during bouts of brief emersion (6 h; Simonik and Henry 2014).

An alternative to volatilization is the temporary storage and detoxification of ammonia as non-essential amino acids, urea, or crystalline urate deposits (Linton et al. 2017). Most investigations have failed to identify significant accumulation or excretion of urea or uric acid in crustaceans (Wood et al. 1986; Martin et al. 2011; Linton et al. 2017; Weihrauch et al. 2017); however, several chronically emersed T_{1-3} crabs are capable of temporarily storing nitrogenous wastes as amino acids although the methods differ by species. For example, *Carcinus maenas* was found to maintain control levels of hemolymph ammonia even after 72 h of emersion (Durand and Regnault 1998) but instead accumulate non-essential amino acids (glycine, alanine, and glutamine) in muscle tissue that are reverted to ammonia and released upon return to water (Durand et al. 1999). *Austrothelphusa transversa* (T_3), the Australian arid-zone crab, lives in absence of water for years due to droughts and stores nitrogenous wastes that is gradually re-converted to ammonia once the crab finds water (Macmillen and Greenaway 1978; Linton and Greenaway 1995). While the specific storage molecule has not been found, *C. carnifex* returns its hemolymph ammonia levels to non-desiccated control levels for up to 192 h without water following a transient doubling in hemolymph ammonia after the first 36 h (Wood and Boutilier 1985; Wood et al. 1986). The fact that *H.*

formosensis does not accumulate ammonia within its urine or hemolymph while emersed and even reduces urinary ammonia while maintaining control hemolymph levels (Fig. 4) suggests nitrogenous wastes are stored. This hypothesis is supported by an observed gradually increasing rate of ammonia excretion by the animal following re-immersion (Fig. 5) suggesting the animal requires time to reconvert the storage compounds into ammonia. This counters the usual ‘panic pee’ response associated with crabs where handling stress results in an immediate increase in ammonia excretion rate that decreases over time (Hunter and Kirschner 1986; Weihrauch et al. 1998; Martin et al. 2011). This process is observed in *A. transversa* (Linton and Greenaway 1995) and to a lesser degree *C. carnifex* (Wood et al. 1986), where excretion increases gradually upon re-immersion but it differs fundamentally from crabs like *Cancer pagurus* that are rarely emersed (Regnault 1994). Collectively, it seems that amphibious crabs that temporarily store nitrogen rather than volatilize it, gradually increase ammonia excretion rates upon re-immersion fitting the observations made for *H. formosensis* in this study.

If ammonia detoxification does occur through temporary storage as amino acids in *H. formosensis*, it appears to occur, at least partially, during the production of urine. While hemolymph ammonia remains constant following 24 h of emersion, urinary ammonia is maintained at 40% of immersed levels (approximately $150 \mu\text{mol l}^{-1}$, Fig. 4), indicating the antennal glands reabsorb ammonia. Interestingly, the antennal glands effectively reabsorb free amino acids apart from alanine and glutamate that are only partially reabsorbed (Figs. 6a and b). Reabsorbed ammonia and glutamate could be used by the antennal gland to synthesize glutamine through glutamine synthetase to mitigate ammonia accumulation. This process is used by the mammalian kidney when a metabolic alkalosis occurs (Weiner and Verlander 2013), similar to the physiological state of emersed *H. formosensis*. The peak reabsorption of urinary glutamate occurs after 160 h where the metabolic alkalosis is most severe. Unfortunately, this study did not investigate intracellular amino acid levels and cannot confirm this hypothesis without further investigation. If this process does occur in a similar fashion to the late proximal tubule of the mammalian nephron, it may occur through a similar fashion of K^+ substitution for NH_4^+ through $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters and may be useful to target in future studies (Weiner and Verlander 2011, 2013).

Alanine was found to be present at several magnitudes higher than all other free amino acids (Fig. 6a) in both immersed and emersed crabs reaching up to 37 and 22 mmol l^{-1} in the hemolymph and urine, respectively. During the first 24 h of emersion, hemolymph alanine concentrations decreased from an initial 35 mmol l^{-1} while immersed to 27.5 mmol l^{-1} after 24 h (Fig. 6a). Under desiccating

conditions, vital fluids will progressively become hyperosmotic as water evaporates over time putting greater osmotic stress on the cells. Alanine is a relatively non-toxic amino acid that can be collected from the extracellular space by tissues to counteract osmotic stress. Since emersion is a regular daily stressor for mangrove crabs, this may be another reason that alanine concentrations remain high in immersed crabs. Emersion beyond 24 h caused hemolymph alanine concentrations to increase by 9.5 mmol l^{-1} to remain at about $36\text{--}37 \text{ mmol l}^{-1}$ after 48 and 160 h of emersion (Fig. 6a), potentially as a temporary store of nitrogenous wastes. When ammonia production increases or its excretion/detoxification is impaired (limited water availability, anaerobic metabolism, etc.), many vertebrates produce alanine within extrahepatic tissues as an NH_4^+ carrier that is consumed within the liver as part of the alanine-glucose cycle (also referred to as the Cahill cycle; Felig et al. 1970; Felig 1973). During this cycle, extrahepatic tissues collect and oxidize blood glucose forming pyruvate, which is then transaminated by alanine aminotransferase to form alanine (Felig et al. 1970; Felig 1973). Newly synthesized alanine is exported from the tissue and recirculated until taken up by the hepatic tissue and catabolized as part of gluconeogenesis while the released ammonia is detoxified by the urea cycle (Felig et al. 1970; Felig 1973). The use of this metabolic pathway by aquatic animals is largely unclear and is of minimal importance or absent in most fishes (Mommsen and Walsh 1991; Hemre et al. 2002). This is largely due to the low rate of hepatic gluconeogenesis in most fishes and the low ratio of alanine to ammonia release from fish muscle upon exercise (reviewed by Mommsen and Walsh 1991). To the author’s knowledge, this cycle’s presence and importance in invertebrates are unknown. If a functional alanine-glucose cycle is present, ammonia produced in non-excretory tissues could be detoxified as alanine prior to entering the extracellular space and later collected by tissues as an osmolyte or be catabolized and the ammonia subsequently released across the branchial epithelium. When the animals are re-immersed, the alanine can be catabolized and the ammonia released into circulation to be excreted across the branchial epithelia after a short delay, as appears to occur in *H. formosensis* (Fig. 5). Investigations into the potential use of alanine as a temporary nitrogenous waste storage molecule under environmental conditions limiting ammonia excretion in other crustaceans may help identify the mysterious destination of ammonia as occurs during prolonged exposure of *M. magister* to high environmental ammonia (Martin et al. 2011) or desiccation of *C. carnifex* (Wood et al. 1986).

Regardless of how *H. formosensis* temporarily stores nitrogenous wastes while emersed, the crab is capable of regulating hemolymph and urinary ammonia levels to a fairly constant degree (Fig. 4). Regulation of hemolymph ammonia concentrations has recently been documented in

several invertebrate species including the American horseshoe crab (Hans et al. 2018), the common octopus (Hu et al. 2017), and the shallow hydrothermal vent crab (Allen et al. 2020). It has been hypothesized that this ‘ammonia homeostasis’ may be valuable to animals that are somewhat tolerant to ammonia toxicity as an acid–base equivalent as NH_4^+ may be excreted as a H^+ equivalent or more easily manipulated than free H^+ under some circumstances (Weihrach and Allen 2018; Allen et al. 2020). This process becomes more complicated in an air-exposed animal such as *H. formosensis* as ammonia no longer can be freely excreted as NH_4^+ to the surrounding water and could only be used to reduce the number of free protons if NH_4^+ is incorporated into a metabolite (e.g., production of alanine or glutamine). Future studies investigating whether alanine may be used as an alternative to more documented nitrogen detoxification through urea or uric acid may wish to also consider whether this pathway is linked to terrestrialization in crustaceans.

Water conservation is another major concern for emersed crustaceans, especially in species such as *H. formosensis* that lack lung-like structures and rely on moistening gills to retain their function. Animals that are poor water-conservers would be expected to lose body water due to evaporation at either a constant or increasing rate during prolonged emersion. *Helice formosensis* decreases its rate of evaporative water loss the longer it is emersed (Fig. 9) indicating that the crab is well prepared to deal with prolonged emersion. The capability of *H. formosensis* to suppress their rate of evaporative water loss is interesting given that they were incapable of altering behavioral aspects whilst trapped in jars and would therefore require an active capacity to do so. To the author’s knowledge, amphibious and even true terrestrial crabs (T_{4-5}) are believed to suppress evaporative water loss from their body through behavioral shifts (i.e., aestivation, seeking shade, burrowing, metabolic shifts; Bliss et al., 1978; McGaw et al., 2019; Simonik and Henry, 2014; Wolcott, 1992; Wood et al., 1986), warranting further investigation into the potential of *H. formosensis* to actively suppress evaporative water loss.

It is possible that the reduced rate of evaporative water loss is due in part to the crab’s ability to tolerate elevated osmolality in its extracellular fluid, which increased by $49 \text{ mOsmol kg}^{-1}$ in the hemolymph and $37 \text{ mOsmol kg}^{-1}$ in the urine after 160 h of emersion (Fig. 8). Elevation of the urine osmolality may be particularly important in *H. formosensis*’ emersion response as the urine is directly exposed to the ambient atmosphere. A high osmolality of the urine would reduce or potentially neutralize the evaporative water loss upon contact with the humid environments found in the subtropics (e.g., Taiwan) while still permitting any potential passive loss of waste NH_3 and CO_2 gases to the environment. The process of elevating the urine’s osmolality appears to occur through concentration of Na^+ ,

K^+ , and Mg^{2+} concentrations but not Ca^{2+} or Cl^- (Fig. 7). These mechanisms are crucial in terrestrial isopods such as *Porcellio scaber* and are connected to their previously described mechanism of ammonia volatilization (Wieser et al. 1969; Wright and O’Donnell 1993). As the isopods prepare for NH_3 volatilization, the fluid within pores of the pleon is filled with ions to increase the osmolality up to $8.2 \text{ Osmol kg}^{-1}$ to allow for water vapour absorption to occur (Wright and O’Donnell 1992). Using formulas describe by (O’Donnell and Machin 1988) and data collected in this study, it is unlikely that *H. formosensis* is capable of water vapour absorption; however, the osmolyte-rich urine may be capable of mitigating evaporative water loss while permitting some minor degree of off-gassing of waste NH_3 and CO_2 . This may be part of the reason that the antennal glands of *H. formosensis* poorly resorb Na^+ and have reduced Na^+/K^+ -ATPase activity compared to other semi-terrestrial crustaceans (Tseng et al. 2020). Future studies focusing on elucidating the regulatory mechanisms of crustacean antennal glands are required to more fully interpret the potential of crustaceans to mitigate evaporative water loss.

Understanding physiological adaptations to emersion stress in aquatic species not only helps better understand an animal’s fitness, but also provides insights into the challenges that species must overcome to terrestrialize. Crustaceans are one of the few animal families that have independently terrestrialized several times (Lozano-Fernandez et al. 2016) having found several different strategies to a similar set of physiological challenges. *Helice formosensis* uses a combination of urine recycling and ammonia detoxification to successfully compensate its acid–base and nitrogen homeostasis while emersed. Unlike other investigated crustaceans, it appears that *H. formosensis* may use alanine as a key component to this compensatory response, potentially indicating that at least some crustaceans make use of the alanine-glucose cycle setting their strategies apart from aquatic fishes.

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Author contributions G.J.P.A. designed the study, performed experiments, and wrote the manuscript. M-CW performed some cation measurements and amino acid measurements. Y-CT and DW helped design the experiment, revise the manuscript, and funded the research.

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Availability of data material Data are available at the request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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