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Review

Ammonia excretion in aquatic and terrestrial crabs

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Summary

The excretory transport of toxic ammonia across epithelia is not fully understood. This review presents data combined with models of ammonia excretion derived from studies on decapod crabs, with a view to providing new impetus to investigation of this essential issue. The majority of crabs preserve ammonotely regardless of their habitat, which varies from extreme hypersaline to freshwater aquatic environments, and ranges from transient air exposure to obligate air breathing. Important components in the excretory process are the $Na^+/K^+(NH_4^+)$-ATPase and other membrane-bound transport proteins identified in many species, an exocytotic ammonia excretion mechanism thought to function in gills of aquatic crabs such as *Carcinus maenas*, and gaseous ammonia release found in terrestrial crabs, such as *Geograpsus grayi* and *Ocypode quadrata*. In addition, this review presents evidence for a crustacean Rhesus-like protein that shows high homology to the human Rhesus-like ammonia transporter both in its amino acid sequence and in its predicted secondary structure.

Key words: ammonia excretion, ammonia transporter, crab, exocytosis, Rhesus-like protein, Na^+/K^+-ATPase.

The ammonia problem

Ammonia[†] (i.e. the total of NH_3 and NH_4^+: T_{Amm}) is highly toxic in most animals. Hydrated NH_4^+ and K^+ ions have the same ionic radius (Knepper et al., 1989) and due to their K^+-like behavior, ammonium ions affect the membrane potential for example, in the giant axon of *Loligo pealei* (Binstock and Lecar, 1969) and of mammalian neurons (Cooper and Plum, 1987). In mammals, elevated ammonia causes major damage in the central nervous system, including changes in blood–brain barrier morphology (Laursen and Diemer, 1997). In addition, elevated ammonia levels in mammals have been related to Alzheimer disease (Alzheimer Type II astrocytosis) due to toxic accumulation of glutamine in astrocytes, which leads to cell swelling and cell death (for review see Butterworth, 2002). Also, in microglia and astroglioma cell lines, ammonia affects major functional activities, such as phagocytosis and endocytosis. Ammonia also modifies the release of cytokines and increases the activity of lysosomal hydrolases (Atanassov et al., 1994, 1995). Marcaida et al. (1992) speculated that ammonia toxicity is mediated by excessive activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the brain. As a consequence, cerebral ATP depletes while intracellular Ca^{2+} increases with subsequent increases in extracellular K^+ and finally cell death.

In crustaceans, for example in the lobster *Homarus americanus* (Young-Lai et al., 1991) and the crayfish *Pacifastacus leniusculus* (Harris et al., 2001), elevated ammonia levels in low-salinity media disrupt ionoregulatory function. Exposure of the green shore crab *Carcinus maenas* to 1 mmol l^{-1} total ammonia leads to increased ion permeability and salt flux across the gill; higher concentrations reduce both variables (Spaargaren, 1990). In fish, branchial gas exchange and oxidative metabolism are disturbed by excess ammonia (Wilkie, 1997).

An effective ammonia detoxification or excretion system is, therefore, essential to maintain cellular functions, and to keep cellular and body fluid ammonia levels within a tolerable range. In most species, including mammals (Cooper and Plum, 1987), fish (Wood et al., 2002) and aquatic crabs (Cameron and Batterton, 1978; Weihrauch et al., 1999), the ammonia concentration of the body fluids is typically low (50–400 µmol l^{-1}; Table 1). Concentrations exceeding 1 mmol l^{-1} total ammonia (NH_3+NH_4^+) are usually toxic to mammalian cells (Hrnjez et al., 1999). In crustaceans,

[†]In this review, NH_3 refers to molecular ammonia, NH_4^+ to ammonium ions, and ammonia to the sum of both.

Table 1. *The concentration of ammonia in the hemolymph of selected crab species*

Species	Ammonia (mmol l^{-1})	Source
Aquatic		
Callinectes sapidus	0.83	Kormanik and Cameron (1981)
Cancer pagurus	0.22	Regnault (1994)
	0.08	Weihrauch et al. (1999)
Carcinus maenas	0.08	Durand and Regnault (1998)
Eriocheir sinensis	0.12	Weihrauch et al. (1999)
Maia squinado	0.05–0.07	Durand et al. (2000)
Necora puber	0.12	Durand and Regnault (1998)
Air-breathing		
Austrothelphusa transversa	0.3–0.5	Linton and Greenaway (1995)
Cardisoma carnifex	1.6	Wood et al. (1986)
Discoplax hirtipes	1.66–2.31	Dela-Cruz and Morris (1997)
Gecarcoidea natalis	1.46	Greenaway and Nakamura (1991)
Geograpsus grayi	1.92	Greenaway and Nakamura (1991)
	1.18–2.07	Varley and Greenaway (1994)
Ocypode quadrata	0.86	De Vries et al. (1994)
Potamonautes warreni	0.2–0.3	Morris and van Aardt (1998)

environmental exposure of ammonia is lethal at relatively low doses. For instance, LC_{50} after 96 h of exposure was determined in the crayfish *Orconectes nais* at 186 µmol l^{-1} NH_3 (Hazel et al., 1982), in the Sao Paulo shrimp *Penaeus paulensis* at 19 µmol l^{-1} NH_3 and 0.307 mmol l^{-1} total ammonia (Ostrensky et al., 1992) and in the redtail prawn *Penaeus penicillatus* 58 µmol l^{-1} NH_3 and 1.39 mmol l^{-1} total ammonia (Chen and Lin, 1992).

Mammals accrue ammonia both from metabolism and as an influx to the hepatocytes from the gastrointestinal tact. This ammonia is detoxified in the urea cycle, an energy-consuming process, by incorporation into the less-toxic urea. Crustaceans are largely ammonotelic, aquatic species exclusively so, and in water excrete their nitrogenous waste directly to the environment as highly soluble ammonia.

Origin of ammonia in crustaceans

The vast majority of the excreted ammonia originates from the catabolism of proteins and amino acids. There is little evidence to support nitrogenous excretion as amino-nitrogen because consumable amino acids released by crustaceans are more likely a result of passive lost *via* amino-acid-permeable structures (Dagg, 1976) and *via* feces (Claybrook, 1983). Some additional ammonia is produced in reactions involving purine and pyrimidine bases (Claybrook, 1983) (Fig. 1). However, ammonia derived *via* this uricolytic pathway is considered to contribute only a small portion of the total ammonia excreted compared with the predominant production from amino acids (Hartenstein, 1970; Schoffeniels and Gilles, 1970). Ammonia derives, for the most part, from deamination of glutamine, glutamate, serine and asparagine by the specific enzymes glutaminase, glutamate dehydrogenase, serine dehydrogenase and asparaginase, respectively (King et al., 1985; Krishnamoorthy and Srihari, 1973; Greenaway, 1991, 1999).

Organs of ammonia excretion in aquatic crabs

In aquatic crabs, primary urine is formed *via* ultrafiltration in the antennal gland, which is thought to play the key role in regulation of body water and divalent cations (e.g. Mg^{2+}, Ca^{2+}; Mantel and Farmer, 1983), but not to contribute significantly to the excretion of nitrogenous waste products (Regnault, 1987). For instance, in the blue crab *Callinectes sapidus*, <2% of total ammonia is excreted in the urine *via* the antennal gland system (Cameron and Batterton, 1978).

The main site for ammonia excretion by aquatic crabs is the phyllobranchiate gill (Claybrook, 1983; Kormanik and Cameron, 1981; Regnault, 1987), featuring a single-cell-layered epithelium covered by an ion-selective cuticle (Avenet and Lignon, 1985; Lignon, 1987; Onken and Riestenpatt, 2002, Weihrauch et al., 2002). The gills of aquatic crabs are multifunctional organs. In addition to their function in excretion of nitrogenous waste products, they are also responsible for respiratory gas exchange (Burnett and McMahon, 1985), regulation of acid–base balance (Henry and Wheatly, 1992) and osmoregulatory ion transport (Towle, 1981; Lucu, 1990, Riestenpatt et al., 1996, Towle and Weihrauch, 2001). Several transporters and enzymes putatively linked and involved in ammonia transport have been shown to be present in the branchial epithelium of crabs, as summarized in Fig. 2 and Table 2.

Ammonia excretion in aquatic crabs

In solution, both forms of ammonia, non-ionic ammonia (NH_3) and the ammonium ions (NH_4^+) exist in a pH-dependent equilibrium. As a weak base (pK ≈9.48 at 20°C and NaCl=250 mmol l^{-1}; Cameron and Heisler, 1983) and at a physiological pH of pH 7.8, 98% of total ammonia exists in the ionic form NH_4^+, whereas only 2% is present as non-ionic NH_3. However, the higher lipid solubility of NH_3 makes it more diffusible through phospholipid bilayers. Kormanik and Cameron (1981) reported that ammonia excretion of seawater adapted blue crabs *Callinectes sapidus* occurred mainly by diffusion of non-ionic NH_3. An excretion mechanism based predominately on NH_3 diffusion is not likely, however, because membrane permeability of NH_3 is much lower than that of CO_2 (Knepper et al., 1989). Indeed, some plasma membranes of animal epithelia are relatively impermeable to NH_3 as shown for frog oocytes (Burckhardt and Frömter, 1992), the renal proximal straight tubules (Garvin et al., 1987) and colonic crypt cells (Singh et al., 1995). Accordingly, other authors have obtained experimental evidence for at least partial excretion of ammonia in its ionic form (NH_4^+) in *Callinectes sapidus* (Pressley et al., 1981) and *Carcinus maenas* (Lucu, 1989; Siebers et al., 1995).

Studies on isolated perfused gills of several aquatic crabs

showed that ammonia can be excreted actively against a 4–8-fold inwardly directed ammonia gradient across both the anterior and the posterior gills to a similar degree despite their different morphological and physiological characteristics (Copeland and Fitzjarrell, 1968; Goodmann and Cavey, 1990; Weihrauch et al., 1998, 1999; Towle and Weihrauch, 2001) (Fig. 3). Under physiologically relevant conditions, the potential for active branchial ammonia excretion is significantly greater in the marine *Cancer pagurus* than in freshwater-acclimated Chinese mitten crabs *Eriocheir sinensis*, despite the much larger ionic conductance of *Cancer pagurus* gills (~250–280 mS cm^{-2}) compared with that of *Eriocheir sinensis* gills (~4 mS cm^{-2}) (Fig. 4). It is noteworthy that the posterior gills of *Carcinus maenas* (thought to play the dominant role in osmoregulatory NaCl uptake) and also the anterior gills (thought to be primarily responsible for gas exchange) are equally capable of active ammonia excretion (Weihrauch et al., 1999).

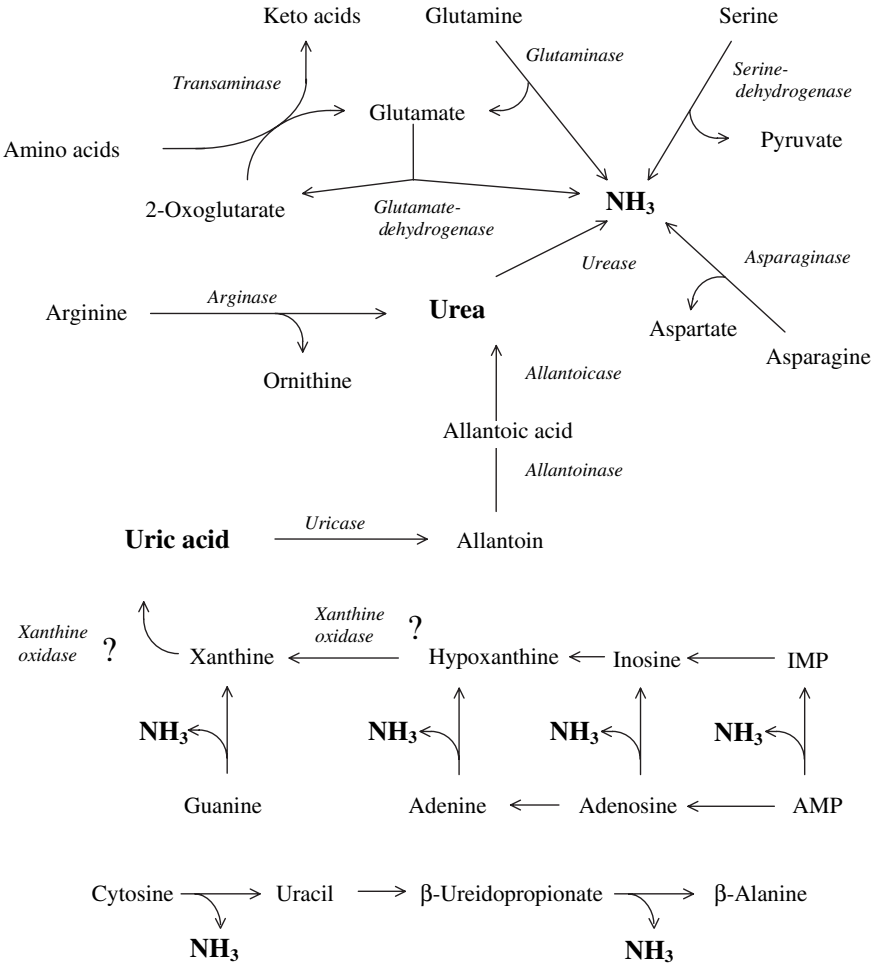

Fig. 1. Metabolic formation of nitrogen excretion products in Crustacea. Modified after Claybrook (1983). Existence of xanthine oxidase is unclear, as indicated by '?'.

Ecological relevance of active ammonia excretion in aquatic crabs

The finding that at least three different crab species with disparate ionic regulatory requirements can actively excrete ammonia raises issues about the metabolic costs involved. The ability to excrete ammonia against a gradient has significant ecological implications with regard to habitats that would, thus, be available to these crabs. Traditionally, ammonia excretion in aquatic animals has been considered to be a passive process driven by diffusion along the partial pressure gradient of NH_3 (P_{NH_3}). Such a model requires environmental concentrations to be kept low, normally by bacterial nitrification of ammonia to nitrite and nitrate. This view is probably justified in pelagic animals colonizing the water column of aquatic habitats because, according to Koroleff (1983), the amounts of NH_4^+ rarely exceed 5 µmol l^{-1} in oxygenated, unpolluted seawater.

By contrast, benthic and interstitial animals are often faced with higher ambient ammonia concentrations. High ammonia is especially prevalent in anoxic, deep stagnant water and pore water during periods of high mineralization following collapse of phytoplankton blooms. For example, several investigations of pore water composition in the North Sea showed considerable concentrations of ammonia in a range between 100 and 300 µmol l^{-1}, but also up to 2500 µmol l^{-1} in 4–9 cm sediment depth (Enoksson and Samuelsson, 1987; Lohse et al., 1993).

Like most aquatic crab species, *Carcinus maenas*, *Cancer pagurus* and *Eriocheir sinenis* are benthic-living animals, hiding under stones or burying themselves in the sediment for long periods, for example, during low tide or in the winter season. Under conditions where crabs are situated at sites with low rates of ambient water exchange, plus the fact that the animals produce and excrete metabolic ammonia, the concentration of the ambient ammonia can reach high values.

Considering hemolymph ammonia concentrations of ~100 µmol l^{-1} (Weihrauch et al., 1999; Table 1) of which less than 5 µmol l^{-1} exist in the gaseous form NH_3, these crabs may encounter ambient NH_3 and/or NH_4^+ concentrations exceeding those in their hemolymph. While NH_3 diffuses along its partial pressure gradient across the exposed epithelia, NH_4^+ follows its electrochemical gradient by either paracellular diffusion or NH_4^+ permeable channels and transporters (see Table 2). An adaptive protection against net ammonia influxes (i.e. an active mechanism for excretion of metabolic ammonia against an inwardly directed gradient, tolerances for high hemolymph ammonia concentrations or efficient detoxification mechanisms) must, therefore, have evolved.

Branchial ammonia excretion mechanisms in aquatic crabs

In the blue crab *Callinectes sapidus,* ammonia excretion

rates are correlated with Na^+ absorption (Pressley et al., 1981). The same result was obtained both for the Chinese crab *Eriocheir sinensis* (Péqueux and Gilles, 1981) and for the shore crab *Carcinus maenas* (Lucu et al., 1989). Studies employing membrane vesicles from gill epithelia (Towle and Hølleland, 1987) and isolated, perfused gills (Lucu et al., 1989) indicated that NH_4^+ substitutes for K^+ in activation of the ouabain-sensitive Na^+/K^+-ATPase. In gill sections from *Callinectes sapidus*, this Na^+/K^+-ATPase was demonstrated to be located in the basolateral membranes of the branchial epithelial cells (Towle and Kays, 1986; Towle et al., 2001). Complete or partial cDNA sequences for the α-subunit of Na^+/K^+-ATPase from crab gills have been published in GenBank (see Table 2) thus confirming both its presence in branchial epithelia and its similarity to α-subunits of other species.

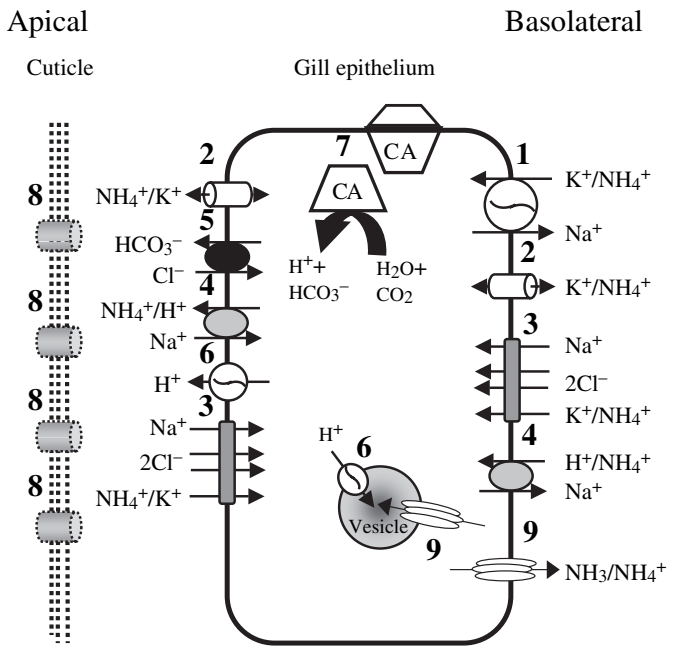

Fig. 2. Transporters and enzymes putatively involved in the process of ammonia excretion in crabs. Note: composition and localization of transporters may vary between crab species. (1) Na^+/K^+-ATPase; (2) K^+ channels; (3) $Na^+/K^+/2Cl^-$ co-transporter; (4) Na^+/H^+ exchanger; (5) HCO_3^-/Cl^- exchanger; (6) V-type H^+-ATPase; (7) Carbonic anhydrase (CA); (8) amiloride-sensitive cation-permeable channel-like structures of the cuticle; (9) Rhesus-like protein (RhCM), putative ammonium transporter with unknown localization. For further information please refer to Table 2.

Recently, Masui et al. (2002) showed that the branchial Na^+/K^+-ATPase from *Callinectes danae* is synergistically stimulated by NH_4^+ and K^+, increasing its catalytic activity by up to 90%. Masui et al. (2002) came to the conclusion that the two ions bind to different sites of the branchial Na^+/K^+-ATPase. This observation was also attributed to the branchial Na^+/K^+-ATPase of the freshwater shrimp *Macobrachium olfersii* by Furriel et al. (2004), who suggested for this species that at high NH_4^+ concentrations the pump exposes a new binding site for NH_4^+ which, after binding to NH_4^+, modulates the activity of the Na^+/K^+-ATPase independently of K^+ ions.

In the marine crab *Cancer pagurus*, active branchial excretion of ammonia is completely inhibited by ouabain, a specific inhibitor of the Na^+/K^+-ATPase (Weihrauch et al., 1999), suggesting this pump is the only driving force for excretion. However, in the gills of *Carcinus maenas* acclimated to brackish water, both gradient-driven (Lucu et al., 1989) and active ammonia excretion (Weihrauch et al., 1998) are only partially inhibited by ouabain, consistent with a second active mechanism responsible for branchial ammonia extrusion in this species.

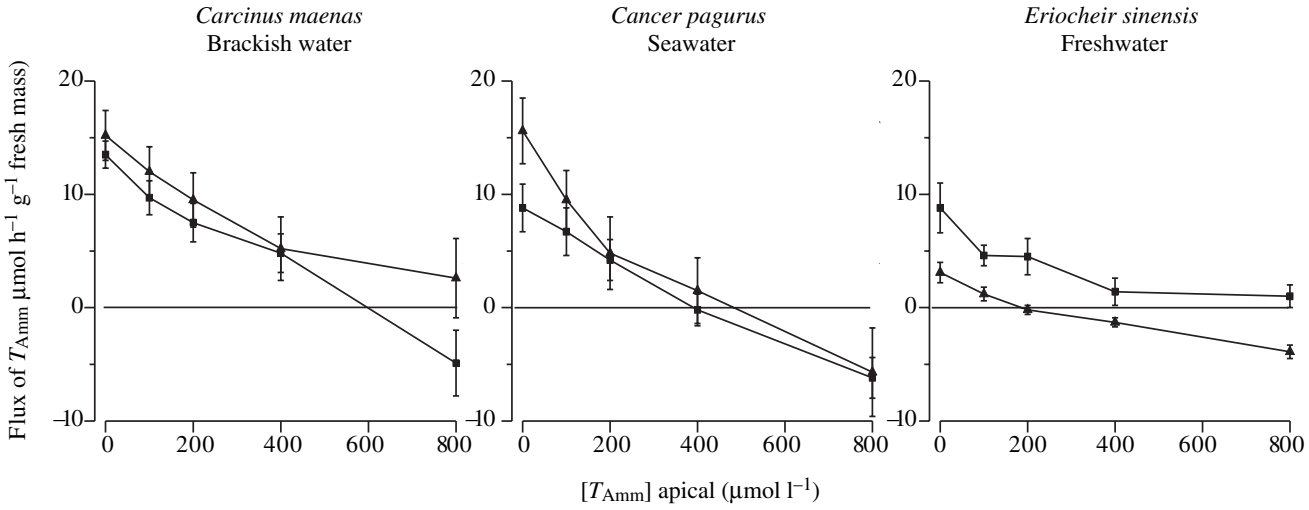

Fig. 3. Fluxes of total ammonia (T_{Amm}) across anterior (triangles) and posterior (squares) gills of seawater-adapted *Cancer pagurus*, brackish water-adapted *Carcinus maenas*, and freshwater-adapted *Eriocheir sinensis*. Gills were perfused with salines containing 100 µmol l^{-1} NH_4Cl. Concentrations of NH_4Cl in the bathing saline increased stepwise from 0 to 800 µmol l^{-1}. Positive and negative values represent net effluxes and influxes from/into perfusate, respectively. Data represent means ±S.E.M. *Carcinus maenas*: *N*=7 (anterior) and *N*=9 (posterior gills). *Cancer pagurus*: *N*=7 (anterior) and *N*=8 (posterior gills). *Eriocheir sinensis*: *N*=7 (anterior) and *N*=6 (posterior gills). (Modified after Weihrauch et al., 1999.)

The presence of an apically located amiloride-sensitive Na^+/NH_4^+ exchanger, transporting NH_4^+ from the epithelial cell into the ambient medium in exchange for Na^+, has been suggested for *Callinectes sapidus* (Pressley et al., 1981) and for *Carcinus maenas* (Lucu et al., 1989; Siebers et al., 1995). Indeed, branchial mRNA expression of a Na^+/H^+-antiporter, putatively transporting also NH_4^+ ions, was demonstrated in *Carcinus maenas* (Towle et al., 1997) and in *Eriocheir sinensis* (Weihrauch and Towle, 2000). However, experiments employing the isolated cuticle from *Carcinus maenas* have shown that cuticular Na^+ and NH_4^+ conductances (G_{cut}) are inhibited by apically applied amiloride in a dose-dependent manner, with an inhibitor constant K_{amiNa+}=0.6 μmol l^{-1} for sodium ions and K_{amiNH_4+}=20.4 μmol l^{-1} for ammonium ions, respectively (Onken and Riestenpatt, 2002; Weihrauch et al., 2002). Differences in K_{amiNH_4+} and K_{amiNa+} are not understood yet. One can speculate that amiloride blocks the passage of cations in the cuticle in a mechanical way like a plug, rather than by blocking a general cation-binding side. Passage of smaller ions (like Na^+) that carry a larger coat of water

Table 2. *Type and location of ion transporters in various species of crab*

Protein/transporter	Species	Localization	References	GenBank*
Na^+/K^+-ATPase	*Callinectes sapidus*		1, 2	AF327439
	Callinectes danae		3	AY035550
	Carcinus maenas		1, 4	AF548369
	Chasmagnathus granulatus	General	5	AF409119
	Dilocarcinus pagei	Basolateral	6	AF375957
	Eriocheir sinensis		7, 8	
	Hemigrapsus nudus		9	
	Pachygrapsus marmoratus		10	
	Uca sp.		11, 12	
K^+ channel	*Carcinus maenas*	Basolateral Apical	4	
$Na^+/K^+/2Cl^-$ cotransporter	*Callinectes sapidus*	Unknown	13	AF190129
	Carcinus maenas	Apical (?)	4	AY035548
	Chasmagnathus granulatus	Unknown	8	AF548368
	Eriocheir sinensis	Unknown		AF301160
Na^+/H^+ exchanger	*Callinectes sapidus*	Apical (?)	14	
	Carcinus maenas	Apical (?)	15	U09274
	Eriocheir sinensis	Unknown	8	AF301159
Cl^-/HCO_3 exchanger	*Carcinus maenas*	Apical (?)	16	–
V-Type H^+ ATPase	*Callinectes sapidus*	Unknown	17	AF189780
	Cancer irroratus	Unknown	17	AF189781
	Carcinus maenas	Vesicular	17	AF247971
	Chasmagnathus granulatus	Unknown	17	AF189783
	Dilocarcinus pagei	Unknown	17, 18	AF409118
	Eriocheir sinensis	Apical		AF189782
	Pachygrapsus marmoratus	Unknown		AF375958
Carbonic anhydrase	*Callinectes sapidus*	Cytoplasmatic and membrane-bound	19, 20, 21	
	Carcinus maenas	Unknown	22, 23	
	Chasmagnathus granulatus	Unknown	8	
	Eriocheir sinensis			
Cation-channel-like structures	*Carcinus maenas*	Cuticle	24–26	
Rh-like protein (putative ammonia transporter)	*Carcinus maenas*	Unknown	27	AF364404

*All cDNA sequences submitted by the authors of the present paper.

References for source data: (1) Towle and Kays (1986); (2) Towle et al. (2001); (3) Masui et al. (2002); (4) Riestenpatt et al. (1996); (5) Luquet et al. (2002); (6) Onken and McNamara (2002); (7) Péqueux et al. (1984); (8) Weihrauch and Towle (2000); (9) Corotto and Holliday (1996); (10) Schleich et al. (2001); (11) D'Orazio and Holliday (1985); (12) Lin et al. (2002); (13) Towle and Weihrauch (2001); (14) Burnett and Towle (1990); (15) Towle et al. (1997); (16) Lucu (1989); (17) Weihrauch et al. (2001a); (18) Onken and Putzenlechner (1995); (19) Henry (1988); (20) Böttcher and Siebers (1993); (21) Henry et al. (2003); (22) Vitale et al. (1999); (23) Lopez Mananes et al. (2000); (24) Lignon (1987); (25) Onken and Riestenpatt (2002); (26) Weihrauch et al. (2002); (27) Weihrauch et al. (2001b).

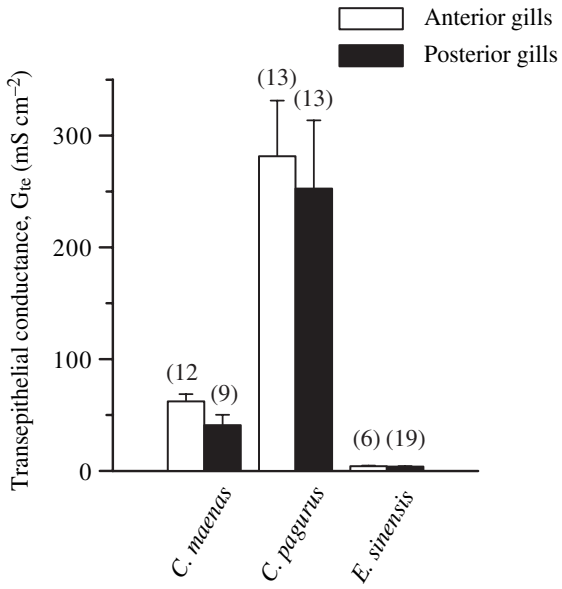

Fig. 4. Transepithelial conductance in isolated half lamellae of gills of seawater-adapted *Cancer pagurus*, brackish water-adapted *Carcinus maenas*, and fresh water-adapted *Eriocheir sinensis*. For the electrophysiological measurements, half lamellae of anterior and posterior gills were mounted in a modified Ussing chamber. Data represent means ± S.E.M. with the number of experiments given in brackets. (From Weihrauch et al., 1999.)

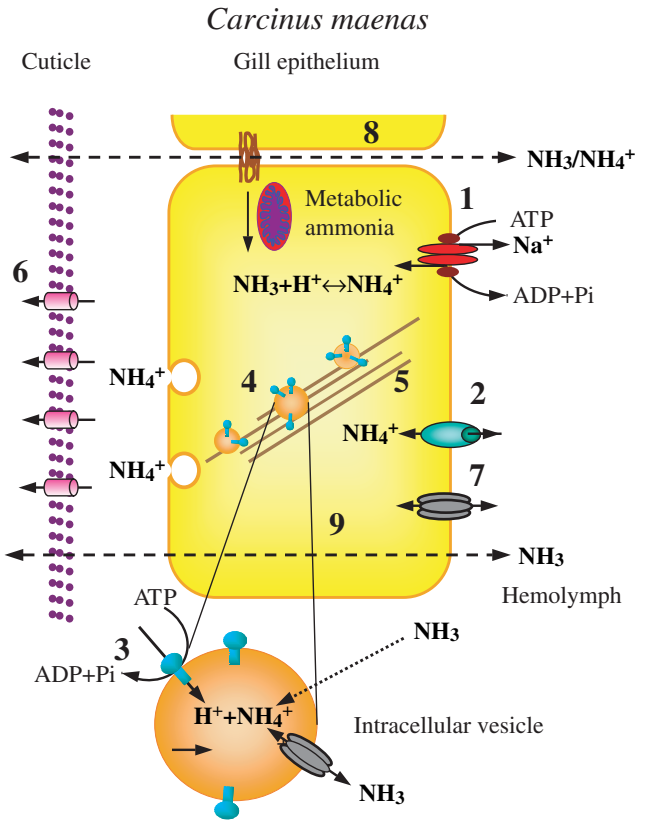

Fig. 5. Proposed hypothetical model of active ammonia excretion across gills of the shore crab *Carcinus maenas*. According to this model, NH_4^+ is pumped across the basolateral membrane by Na^+/K^+-ATPase (1) or traverses the membrane *via* Cs^+-sensitive channels (2). Dissociation of cytosolic NH_4^+ to H^+ and NH_3 is accompanied by diffusion of NH_3 into vesicles acidified by V-type H^+-ATPase (3). The ammonia-loaded vesicles (4) then are moved *via* microtubules (5) to the apical membrane where vesicles fuse with the external membrane, releasing NH_4^+ into the subcuticular space. The NH_4^+ then is believed to diffuse across the cuticle, *via* amiloride-sensitive structures (6). Role and localization of the putative ammonia transporter (RhCM) identified in *Carcinus maenas* gill epithelium (GenBank Accession number: AF364404) are presently uncharacterized (7). Paracellular ammonia diffusion (8) and non-ionic transcellular diffusion of NH_3 (9) is considered to be low under physiologically meaningful transepithelial ammonia gradients (Weihrauch et al., 1998). (Modified after Weihrauch et al., 2002.)

molecules is, therefore, possibly easier to block out by lower amiloride concentrations than K^+ or NH_4^+ ions, which carry only about half the number of water molecules around their core. However, according to these observations, some of the results obtained by applying amiloride to crab gills (apical) should be interpreted with caution and with special attention to the concentration of this particular inhibitor.

Further studies on the branchial ammonia excretion mechanism in *Carcinus maenas* employing the K^+ channel blocker Cs^+ (10 mmol l^{-1}) revealed that basolateral (but not apical) K^+ channels play a role in the excretory process (Weihrauch et al., 1998). In addition, experiments inhibiting the branchial V-Type H^+-ATPase by adding bafilomycin A_1 resulted in a reduction of active ammonia transport by 66%, identifying the H^+-ATPase as the second active component in the excretory mechanism of the shore crab (Weihrauch et al., 2002). While in *Eriocheir sinensis* a V-Type H^+-ATPase has been localized to the apical membrane of the gill epithelium (Onken and Putzenlechner, 1995), in *Carcinus maenas* this pump was found predominantly in the cytoplasm, probably associated with vesicles (Weihrauch et al., 2001a). This latter finding led to the suggestion (Weihrauch et al., 2002) of a vesicular ammonia-trapping mechanism, in which cellular NH_3 diffuses into acidified vesicles to be transformed into its membrane-impermeable ionic form, NH_4^+. For a directed excretion, these NH_4^+-loaded vesicles would then be transported to the apical membrane for exocytotic release. Such an excretion mechanism was supported by data showing total inhibition of active ammonia excretion by blockers of the microtubule network, including colchicine, thiabendazole and taxol (Weihrauch et al., 2002). The resulting hypothetical model of the ammonia excretion in *Carcinus maenas* is described in detail in Fig. 5.

For crabs (such as the partially limnic Chinese crab *Eriocheir sinensis*) that utilize a proton gradient across the apical membrane of the epithelial cell to accomplish NaCl uptake from highly diluted media, it is likely that NH_3 diffuses across the apical membrane along its partial pressure gradient, as shown in freshwater rainbow trout *Oncorhynchus mykiss* (Wilson et al., 1994).

Recently, Weihrauch and others (D. Weihrauch, unpublished data) have sequenced a full-length cDNA coding

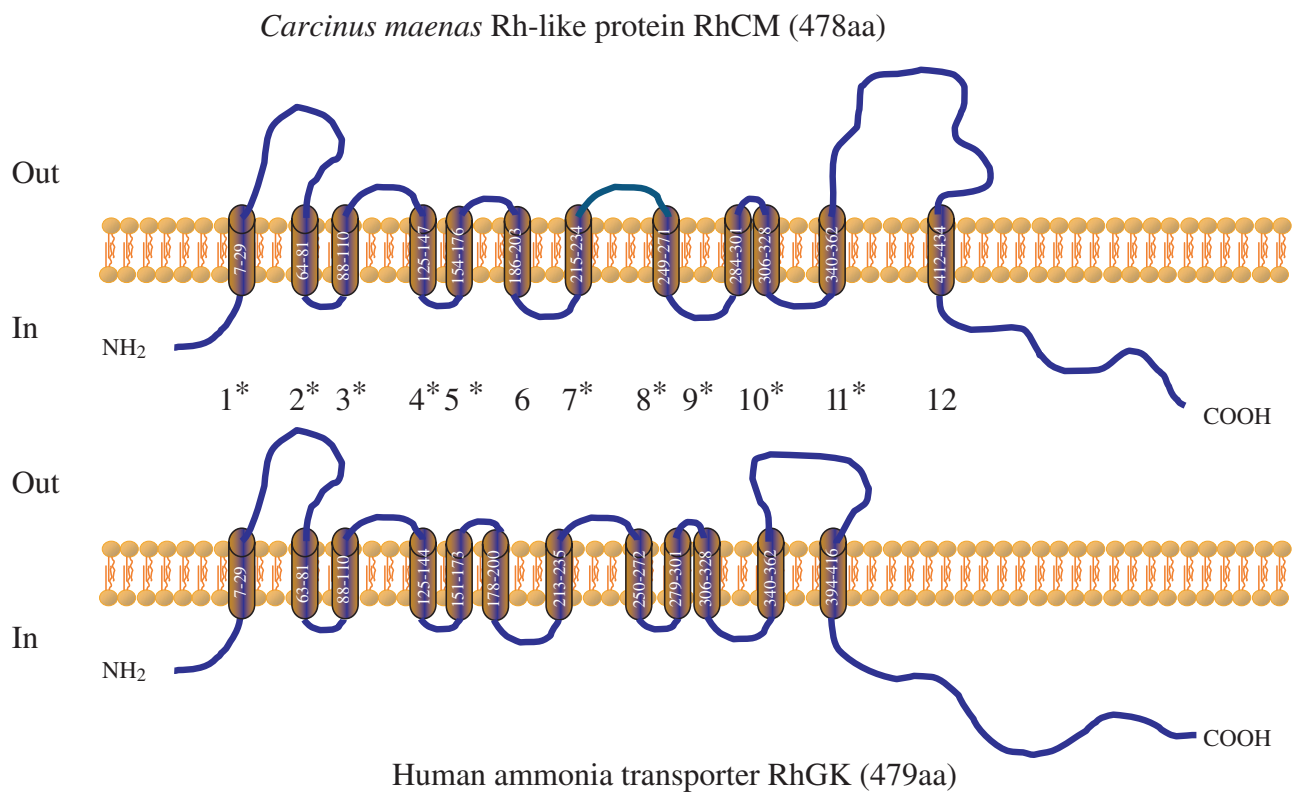

Fig. 6. Localization of the 12 predicted transmembrane domains http://biowb.sdsc.edu (TMHMM) of putative *Carcinus maenas* (RhCM, GenBank Accession number: AF364404) and human (RhGK, also called PDRC2, GenBank Accession number: AF081497) ammonia transporter. Asterisk indicates identical predicted sites of transmembrane domains in RhCM and the human ammonia transporter RhGK.

for a Rhesus-like protein from *Carcinus maenas* gills (GenBank Accession number: AF364404), named RhCM (Rhesus-related protein from *Carcinus maenas*). In mammals Rhesus-related proteins, such as RhGK, have been shown to mediate ammonia (NH_3/NH_4^+), but not K^+ or amino acid transport when functionally expressed in yeast mutants lacking endogenous ammonia transporters (triple ΔMep mutant; Marini et al., 2000). However, for this novel Rhesus-like ammonia transporter the detailed transport characteristics (such as mode of transport or kinetics) have not yet been defined. A comparison of the deduced secondary structure of the amino acid sequence of RhCM, and the human ammonia transporter RhGK, showed that 10 out of 12 predicted transmembrane domains are positioned at identical sites of the sequence (Fig. 6). The localization and role of RhCM in branchial ammonia excretion need to be investigated in detail in further studies. One can speculate that the putative ammonia transport of crabs is not localized in the apical membrane of the gill epithelium, because here ammonia (NH_3/NH_4^+) permeable structures would be of disadvantage allowing ammonia influxes when the animals are exposed to high external concentrations. The human Rhesus-like ammonia transporter RhGK (identical to RhCG), has been described to be localized in the distal tubule and the collecting duct of the kidney in co-localization with a V-type H^+-ATPase (Eladari et a., 2002). RhCG expressed in *Xenopus* oocytes facilitates a highly specific NH_3 diffusion *via* a complex electrogenic NH_4^+ transport (Bakouh et al., 2004). In addition, Eladari et al. (2002) suggested a secondary active mode of ammonia transport in the distal tubule by acid trapping. According to this assumption, RhGK would promote the transmembrane passage of NH_3. A similar mechanism would be plausible in the gills of the shore crab *Carcinus maenas*, however, RhCM would be co-localized with the H^+-ATPase within the membranes of intracellular vesicles to support the proposed vesicular acid-trapping mechanisms. Also, a basolateral localization cannot be excluded, where the putative ammonia transporter might serve as an overflow valve, transporting ammonia back in to the hemolymph, when crabs are exposed to high external ammonia concentrations. Under this condition, intracellular ammonia concentrations might rise to toxic levels due to a passive influx from the apical side, while the Na^+/K^+-ATPase is actively pumping NH_4^+ from the hemolymph space into the cytoplasm. Ammonia directed back into the hemolymph, *via* RhCM, could probably be buffered, at least for a short term, by incorporation into proteins, for instance glutamine or hemocyanins.

The high degree of conservation with ammonia transporters found in fungi, bacteria and archaebacteria (~20%), as well as the striking homology to mammalian ammonia transporters (>40%), led to the suggestion that proteins of the Rh-family play a universal role in ammonia transport (Fig. 7).

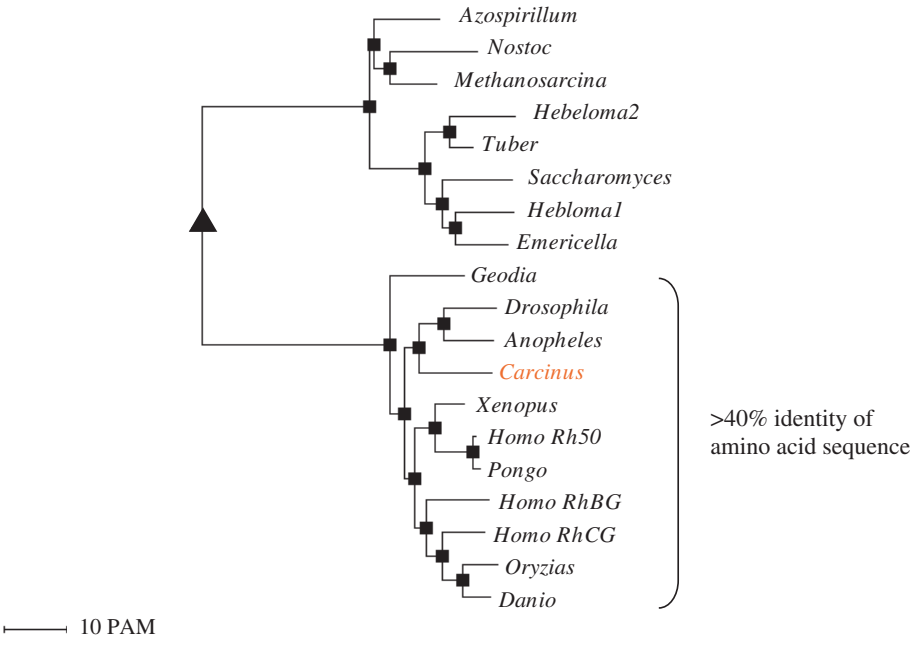

Fig. 7. Phylogenetic tree of published ammonia transporters. GenBank Accession numbers and type of ammonia transporter are given in parentheses. *Anopheles gambiae* (EAA01247, Rh-like protein); *Azospirillum brasilense* (AAC38548, AMT/MEP family); *Carcinus maenas* (AF364404, Rh-like protein); *Danio rerio* (AAM90586, Rh-like protein); *Drosophila melanogaster* (AF64673, Rh-like protein); *Emericella nidulans* (AAL73117, AMT/MEP family); *Geodia cydonium* (CAA73029, Rh-like protein); *Hebeloma cylindrosporum* 1 (AAK82417, AMT/MEP family); *Hebeloma cylindrosporum* 2 (AAM21926 AMT/MEP family); *Homo sapiens* 50 KDa (CAA45883, Rh-like protein), *Homo sapiens* RhBG (AAL05978, Rh-like protein); *Homo sapiens* RhCG (AAH30965, Rh-like protein); *Methanosarcina acetivorans* (AAM07268, AMT/MEP family), *Nostoc* sp. (BAB72949, AMT/MEP family); *Oryzias latipes* (BAB13346, Rh-like protein); *Pongo pygmaeus* (AAG00305, Rh-like protein), *Saccharomyces cerevisiae* (P53390, AMT/MEP family), *Tuber borchii* (AAL11032, AMT/MEP family); *Xenopus laevis* (BAB13345, Rh-like protein). Tree was constructed with Multalin (Corpet, 1988). PAM, percent accepted mutations (a measure of phylogenetic distance).

Ammonia excretion in terrestrial crabs – consequences of air exposure

The mechanisms and processes by which air-breathing crustaceans excrete nitrogenous waste into the terrestrial habitat have been subject to considerable scrutiny (Greenaway, 1988, 1991; Wolcott, 1991, 1992; O'Donnell and Wright, 1995). Terrestrial crabs appear to tolerate considerably greater hemolymph ammonia loads than do aquatic species (Table 1). Conversely, Wolcott (1992) points out that diluting ammonia to non-toxic levels in the urine might require an unsustainable water loss in a land crab.

The probability that branchial NH_4^+ excretion is linked to sodium transport, both through apical ion exchangers and the basal membrane Na^+/K^+-ATPase, is of special importance to air-breathing crabs. In all land crabs examined to date, the gills have become adapted for reabsorption of salt from primary urine directed through the branchial chamber (Wolcott and Wolcott, 1985, 1991; Morris, 2001), allowing diffusive NH_3 loss and NH_4^+ extrusion in exchange for required ions from the urine. Ocypodid crabs seem exceptional in utilizing the antennal gland for increasing urinary ammonia, although the gills are required to complete the excretory process (DeVries and Wolcott, 1993). Generally, while the gills are initially bathed with a fluid isosmotic with the hemolymph, osmotic concentration may decline by as much as 90% (Wolcott and Wolcott, 1985; Varley and Greenaway, 1994; Greenaway, 1999; Morris et al., 2000; Taylor and Greenaway, 2002; Morris and Ahern, 2003). Even very euryhaline aquatic species do not experience the same range of osmo-concentration as occurs in the extra-branchial fluid of some land crabs.

In terrestrial arthropods as a whole, the primary nitrogenous excretory products are generally purines, whereas in land crabs various mechanisms are employed to permit the continued excretion of ammonia. The single known exception is the terrestrial anomuran *Birgus latro*, which is purinotelic excreting urate (Greenaway and Morris, 1989) and guanine (Greenaway, 2003). The reasons for the general persistence of ammonotely may be found by examining a continuum of extant species in the transition from aquatic to land crab.

Ammonia excretion and air exposure of aquatic crabs

Exposure to air of the aquatic crabs *Cancer pagurus* and *Cancer productus* caused hemolymph ammonia to increase by 25 $\mu mol\,h^{-1}$ (Regnault, 1992) and 26 $\mu mol\,h^{-1}$ (deFur and McMahon, 1984), respectively. However, in *Cancer pagurus* this rate of accumulation was between 15 and 30% of the rate expected by Regnault (1992) on the basis of basal aquatic rates, leading to the suggestion of nitrogen storage in tissues, to avoid toxic hemolymph ammonia loading. Many crustaceans store nitrogen (N) as solid urate (for review see Greenaway, 1999) but this seems to be formed primarily as a result of diet rather than being of any large significance in NH_3/NH_4^+ detoxification (Linton and Greenaway, 1997a) – except possibly under desiccating conditions in land crabs (below). In any case, urate formation was ruled out as a significant contribution in *Cancer pagurus* (Regnault, 1992). Ammonia excretion during air exposure of *Cancer pagurus* was only 4% of the normal aquatic rate (170–190 $\mu mol\,kg^{-1}\,h^{-1}$) but when re-immersed they exhibited a very large (50-fold within 5 min) but transient increase to 8860 $\mu mol\,kg^{-1}\,h^{-1}$ (derived from Regnault 1994). Thus, nitrogen storage as NH_4^+, or as some readily oxidized form, is apparently a normal response to transient air exposure, as is the subsequent pulsatile clearance

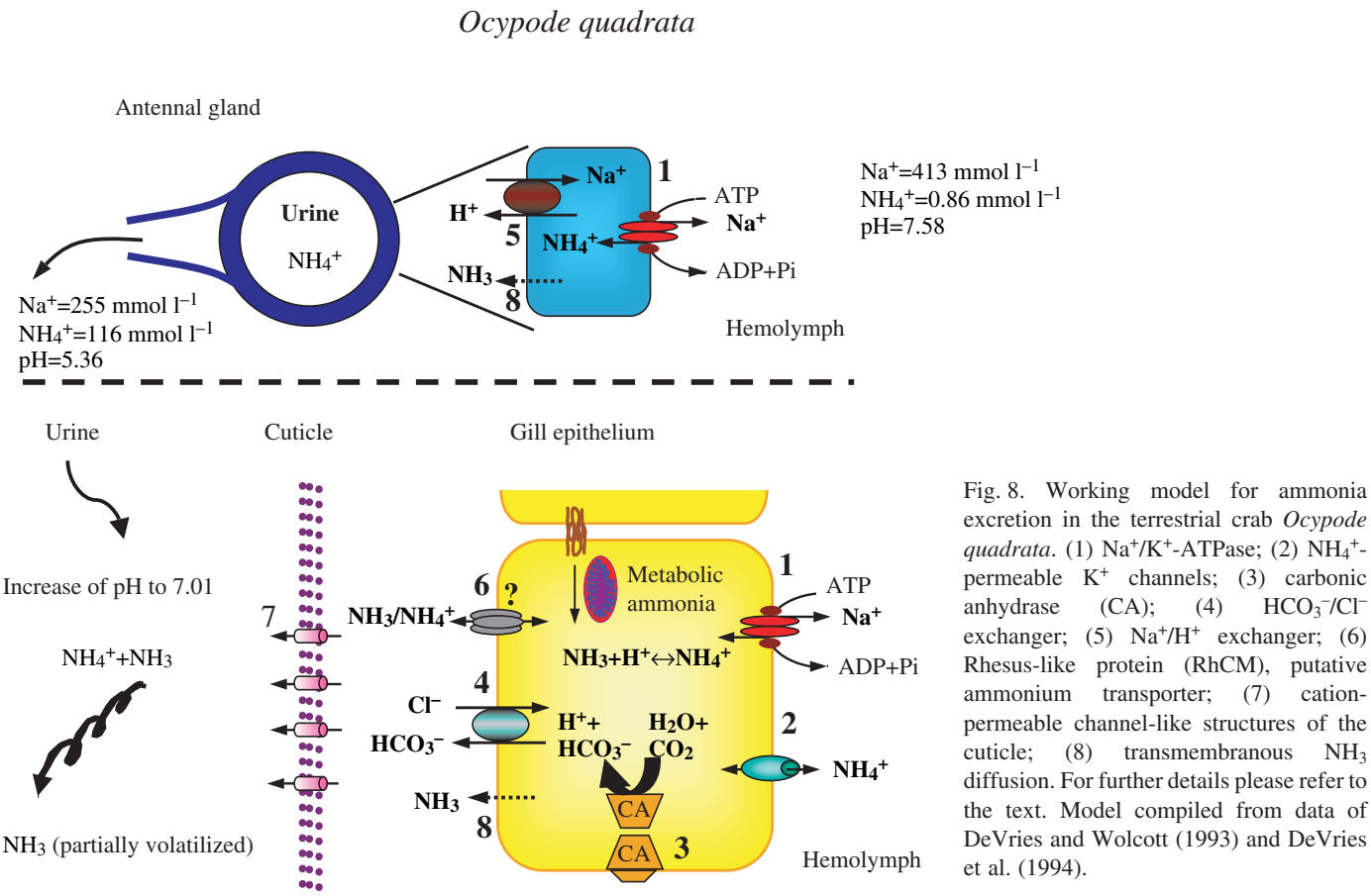

Fig. 8. Working model for ammonia excretion in the terrestrial crab *Ocypode quadrata*. (1) Na^+/K^+-ATPase; (2) NH_4^+-permeable K^+ channels; (3) carbonic anhydrase (CA); (4) HCO_3^-/Cl^- exchanger; (5) Na^+/H^+ exchanger; (6) Rhesus-like protein (RhCM), putative ammonium transporter; (7) cation-permeable channel-like structures of the cuticle; (8) transmembranous NH_3 diffusion. For further details please refer to the text. Model compiled from data of DeVries and Wolcott (1993) and DeVries et al. (1994).

of ammonia on re-immersion. The possibility that urate can be so rapidly mobilized to NH_4^+ seems unlikely and expensive. However, the vesicular sequestration of NH_4^+ (see above) has hitherto not been considered in this role.

Ammonia excretion in land crabs that immerse

A similar 'storage–excretion' is seen in diverse air-breathing crabs (e.g. *Potamonautes warreni*, Morris and van Aardt, 1998; *Austrothelphusa transversa,* Linton and Greenaway, 1995; *Discoplax hirtipes*, Dela-Cruz and Morris, 1997; *Cardisoma carnifex,* Wood et al., 1986). [Note: *Cardisoma hirtipes* has been revised to *Discoplax hirtipes* and *Holthuisana to Austrothelphusa* (Davie, 2002).] *Discoplax hirtipes* excretes 99% of its waste as ammonia, but when it is breathing air the

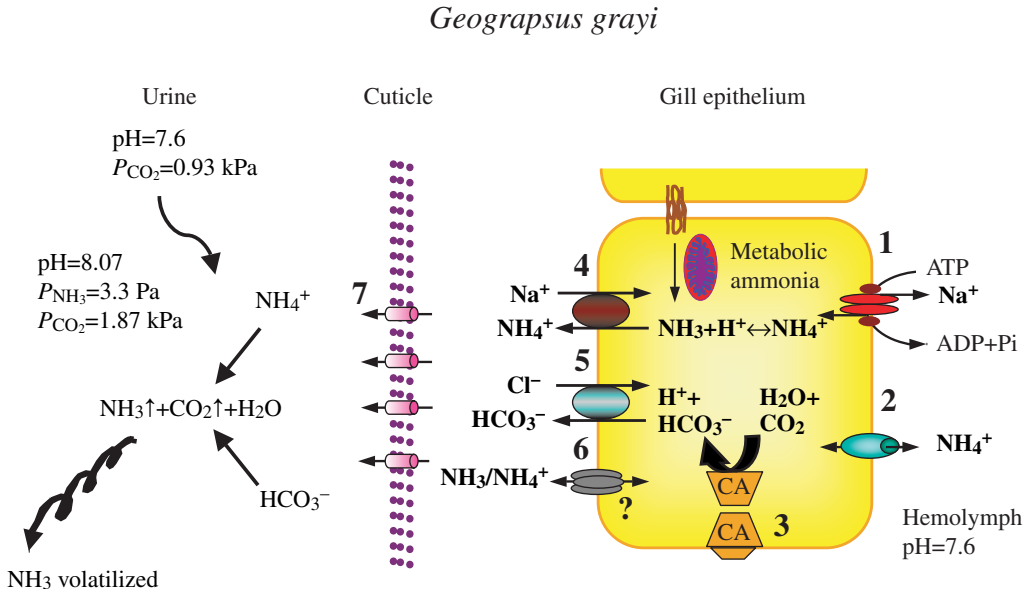

Fig. 9. Working model for ammonia excretion in the terrestrial crab *Geograpsus grayi*. (1) Na^+/K^+-ATPase; (2) NH_4^+-permeable K^+ channels; (3) carbonic anhydrase (CA); (4) $Na^+/H^+/NH_4^+$ exchanger; (5) HCO_3^-/Cl^- exchanger; (6) Rhesus-like protein (RhCM), putative ammonium transporter; (7) cation-permeable channel-like structures of the cuticle. For further details please refer to the text. Model compiled from data of Varley and Greenaway (1994).

rate of nitrogen loss in the urinary flow is only 0.2 μmol kg^{-1} h^{-1} and NH_3 is volatilized at a very slow rate (0.4 μmol kg^{-1} h^{-1}). On re-immersion, the ammonia excretion rate is transiently elevated to 1100 μmol kg^{-1} h^{-1} (compared with the normal 300 μmol kg^{-1} h^{-1}). *Potamonautes warreni* also does not excrete while in air but, on return to water, excretes ammonia across the gills at 4900 μmol kg^{-1} h^{-1} (compared with the normal rate in water of 70 μmol kg^{-1} h^{-1}). Artificially irrigating the gills of air-breathing *Potamonautes warreni* sustained ammonia excretion (Morris and van Aardt, 1998). Gill irrigation appears to be a ubiquitous activity following excursions into the terrestrial environment (Dela-Cruz and Morris, 1997). The requirement to re-immerse, albeit briefly, to accomplish ammonia excretion *via* the ancestral branchial mechanisms may ultimately limit the duration of air-breathing in these amphibious species. However, *Austrothelphusa transversa* can spend many months without access to water (Greenaway and MacMillan, 1978) and can forgo ammonia excretion during that time (Linton and Greenaway, 1995). Linton and Greenaway (1995) suggested that the near-cessation of nitrogen excretion in *A. transversa* implied reduced nitrogen catabolism and temporary nitrogen storage. The speed and brevity of the excretion pulse in *P. warreni* showed that wastes stored during terrestrial forays are rapidly excreted on return to water. However, it seems unlikely that this store is accumulated as NH_4^+/NH_3 within the gill epithelium because hemolymph levels remain low and pH (and therefore P_{NH_3}) remains unchanged (Adamczewska et al., 1997) although the required enzymes may be present (Linton and Greenaway, 1998). Further study is required to determine the storage product but an accessible intermediate is implicated (such as glutamine rather than, for example, urate). Apical H^+/NH_4^+ exchange and V-ATPase-driven cell alkalization have been suggested as likely mechanisms of transbranchial ammonia transport (Linton and Greenaway, 1995), but experimental evidence is required to confirm this. Again, the involvement of a Rhesus-related ammonia transporter needs to be evaluated.

Ammonia excretion in terrestrial crabs

Gecarcinid land crabs recycle their urine over the branchial surfaces, producing a dilute fluid 'P' (Wolcott and Wolcott, 1985; for review Morris, 2002). *Discoplax hirtipes*, a crab that immerses from time to time, can reduce the NaCl concentration of the urine by 90% (Dela-Cruz and Morris, 1997) but this ion pumping does not allow ammonia excretion while in air. For example, while the NH_4^+ content of 'P' of *Discoplax hirtipes* is significantly elevated (5 mmol l^{-1}) compared with the hemolymph (Table 1), the rates of urinary and 'P' flow slow down to almost zero when the animals are in air (Dela-Cruz and Morris, 1997). Thus, ammonia excretion becomes severely limited by urine flow and consequent 'P' production rates.

In *Gecarcoidea natalis*, a gercarcinid land crab that does not routinely immerse or have access to pools of water, the primary urine contains 0.36 mmol NH_4^+ l^{-1}, which is less than in the blood (Table 1). However, reprocessed 'P' contains up to 10.8 mmol l^{-1} (Greenaway and Nakamura, 1991), and this is sufficient to excrete up to 68% of the total nitrogenous output because 'P' production was ~450 μl kg^{-1} h^{-1}. This flow rate is much greater than the 3 μl kg^{-1} h^{-1} in *Discoplax hirtipes*, which is unable to sustain ammonia excretion in air (Dela-Cruz and Morris, 1997). The rate of 'P' production in *Gecarcinus lateralis* was >900 μl kg^{-1} h^{-1}, which facilitated an excretion rate of 20 μmol kg^{-1} h^{-1} (Wolcott, 1991), compared with the 25 μmol kg^{-1} h^{-1} rate in *Gecarcoidea natalis* (Greenaway and Nakamura, 1991). These authors (Greenaway and Nakamura, 1991; Wolcott, 1991) concluded that acid trapping in the 'P' was not involved and, thus, the outward gradient across the gill epithelia is not favorable for gaseous NH_3 diffusion. In addition, Wolcott (1991) measured the urine pH of *Gecarcinus lateralis* and *Cardisoma guanhumi*, and in both crabs found it to be greater than that of the hemolymph. However, branchial Na^+/H^+ exchange would assist in NH_3 diffusion through reciprocal pH changes of intracellular and extra-corporeal fluids. The gills of *Gecarcoidea natalis* are highly active in Na^+ transport and NH_4^+ might easily substitute for K^+ in the basal Na^+/K^+-ATPase (Morris, 2001; Morris and Ahern, 2003) but the necessary active transport of NH_4^+ across the apical membrane into the 'P' remains unresolved. A net exchange of Na^+ for NH_4^+ (e.g. Pressley et al., 1981) would facilitate salt reclamation and nitrogen excretion, but would be hampered as the external Na^+ declined. The possibility of exocytotic mechanisms and/or involvement of a Rhesus-related ammonia transporter needs to be evaluated in the excretory processes of these species.

While this branchial system allows routine excretion of ammonia to air, it also seems to make NH_4^+ excretion dependent on the urinary flow rate, as well as on the extent of ion re-absorption. For example, in *Gecarcoidea natalis*, urine and 'P' flow can decline to zero under dry season conditions (Morris and Ahern, 2003) and so this mode of NH_4^+ clearance becomes inoperable. In *Cardisoma guanhumi* the fluid retained within the abdominal flap (~13.5 mmol l^{-1}) is contiguous with that in the branchial chamber (~6.5 mmol l^{-1}) and further NH_4^+ excretion may occur *via* unknown mechanisms (Wolcott, 1991) but, even so, this would be unavailable to land crabs in the dry season. Purine is stored in large amounts in connective tissue cells throughout the bodies of some land crabs (Linton and Greenaway, 1997b). In *Gecarcoidea natalis* this stored purine is normally synthesized *de novo*, from excess dietary nitrogen (Linton and Greenaway, 1997a). Recent data, including enzyme activities and nitrogen utilization (Linton and Greenaway, 1998, 2000) have lead to the suggestion of a storage–excretion function for the urate accumulated by *G. natalis* (Greenaway, 2003). However, this seems likely to be infrequently called upon (Linton and Greenaway, 1997a) and evidence is required to show that waste amino N is incorporated as well as dietary N.

Other lineages of terrestrial crabs have not been investigated to the same extent as the gecarcinids, but at least one air-breathing ocypodid, *Ocypode quadrata*, has been shown to sustain ammonia excretion (DeVries and Wolcott, 1993).

O. quadrata also recycles the urine to produce 'P', which can be as little as 10% of the osmotic strength of the primary urine (Wolcott and Wolcott, 1985). However, the mechanism of NH_4^+ excretion is quite different from that of gecarcinids because the concentration in the primary urine of the ghost crab is extraordinarily high. For example, in *O. quadrata* (DeVries and Wolcott, 1993; DeVries et al., 1994) this reaches 116–212 mmol l^{-1}, and in *O. ceratopthalma* and *O. cordimanus* (under field conditions) >40 and 27 mmol l^{-1}, respectively (S. Morris, unpublished). The primary urine of *Ocypode quadrata* is unusually acidic (pH 5.36±0.21), providing an 'acid-trap' for NH_4^+. On passage over the gills, the pH is increased (pH 7.01±0.24) and Cl^- (but not Na^+) is reclaimed, such that the alkalinization of the fluid promotes significant NH_3 volatilization (~71 µl kg^{-1} h^{-1} in control crabs). While pH 7 is not alkaline, the increase in pH is quite effective. For example, at an ammonia concentration of 116 mmol l^{-1} and pH 5.4 for primary urine (DeVries & Wolcott, 1993), if the pH is increased to pH 7 it is possible to estimate P_{NH_3} using the pK and solubility for NH_3 provided by Kormanik and Cameron (1981) as used by Varley and Greenaway (1994). In the primary urine P_{NH_3}=11.6 Pa whereas at pH 7 the P_{NH_3}=460 Pa, which is a 40-fold increase in potential diffusive gradient. In view of the concomitant increase in the fluid CO_2 concentration and the uptake of Cl^-, the most obvious candidate for the net base excretion is transport by an apical HCO_3^-/Cl^- exchanger (DeVries & Wolcott, 1993). Reclamation of urinary Na^+ appears to be accomplished within the antennal gland (DeVries et al., 1994). These authors (DeVries et al., 1994) report high activity of Na^+/K^+ATPase in the antennal gland of *O. quadrata* for which NH_4^+ may substitute for K^+ in the basal membrane exchange. Furthermore, apical Na^+/H^+ antiporters in the antennal gland may sustain both Na^+ reclamation and acidification of urine to promote NH_4^+-trapping (Fig. 8).

Study of the more-terrestrial grapsid, *Geograpsus grayi*, has revealed further modifications of the NH_3/NH_4^+ excretory system (Greenaway and Nakamura, 1991; Varley and Greenaway, 1994). *G. grayi* is a highly active carnivorous land crab and also reprocesses the urine to reclaim salts *via* branchial uptake (Greenaway and Nakamura, 1991) but, unlike *Ocypode* sp., does not employ ion reclamation within the antennal gland (Varley and Greenaway, 1994). Clearly *Ocypode* and *Geograpsus* represent separate radiations in to the terrestrial habitat, but with superficially analogous ammonia excretion physiology. *G. grayi* volatilizes NH_3 from the limited volume of 'P' within the branchial chamber and, thereby, increases the effective NH_4^+ capacity of the fluid, which may achieve concentrations in excess of 80 mmol l^{-1} compared with <1 mmol l^{-1} in the urine (Varley and Greenaway, 1994). However, *G. grayi* manages a rate of ammonia excretion comparable to that of aquatic crabs in water (107–220 µmol kg^{-1} h^{-1}; Greenaway and Nakamura, 1991; Varley and Greenaway, 1994). Gaseous ammonia contributes ~78% of this total excretion in a discontinuous process over 3 h to 3 days (Varley and Greenaway, 1994) although urine flow is apparently limited, restricting fluid available for 'P' formation. The pH of this fluid (pH 8.07) is higher than that of the hemolymph (pH 7.66–7.59) and at the same time the CO_2 content (36 mmol l^{-1}) is considerably greater than that of the hemolymph (13.7–17.2 mmol l^{-1}). Amiloride reduced NH_4^+ efflux by 83% in this system and reduced unidirectional Na^+ uptake. Thus, NH_3 volatilization is achieved by raising the fluid pH towards the pK, such that gaseous NH_3 becomes 8% of the total ammonia, creating a diffusive gradient (P_{NH_3} ~3.3 Pa) into the convective air stream. Varley and Greenaway (1994) discussed the difficulties in transporting NH_3/NH_4^+ outward into this fluid in the absence of 'acid-trapping' and proposed a net excretion reaction $NH_4^+ + HCO_3^- \rightarrow H_2O + CO_2\uparrow + NH_3\uparrow$. The volatilization of NH_3 and CO_2, together with formation of water, all contribute to lowered ionic strength in the extrabranchial fluid. Again, apical NH_4^+ transport, possibly mediated by a putative Rhesus-related ammonia transporter protein, needs to be investigated in further studies. This mechanism in *G. grayi* effectively increases the functional volume of the 'P' offering some escape from the dependency on regular and significant urine flow rate. Thus, there are significant increases in the amount of ammonia excreted per unit volume of 'P', while retaining the advantages of ammonia excretion, thereby allowing a more terrestrial habit (Fig. 9). At the same time, the system remains potentially limited by the supply of Na^+ and Cl^- in a lowered supply of urine.

The most successful terrestrial animals have abandoned NH_3 as an N-excretory vehicle in favor of urea or purines. The anomuran *Birgus latro* is the only identified purinotelic crab (Greenaway and Morris, 1989) but is sympatric with several species of gecarcinids that retain NH_3 excretion. Excreting purines allows greater flexibility of urinary water flow independently of urine reprocessing and salt reclamation, but significant energetic advantages may accompany ammonotely.

Implications

Decapod crustaceans exhibit a wide variety of ammonia excretion mechanisms and consequently provide good models for general investigation of nitrogen excretion. The debate as to whether ammonia is lost *via* NH_3 diffusion or by NH_4^+ transport remains active, but the answer may be both or either depending on circumstance and species. The ability to move ammonia against its gradient is obviously essential. There is a clear continuum of increased terrestriality accompanied by managed and active excretion with lowered water loss. This continuum represents multiple transitions onto land and is underpinned by phylogenetic differences. The increased application of molecular and post-genomic methodologies to the question will reveal, for example, the role of Rhesus-related proteins and vesicular transport systems in the physical extrusion of ammonia.

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