Experimental Biology Online - EBO

ISSN 1430-3418

Exp. Biol. Online (1998) 3:10

The ecophysiological significance of lung-air retention during submersion by the air-breathing crabs *Cardisoma carnifex* and *Cardisoma hirtipes*

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Received: 6 August 1998 / Accepted: 28 October 1998

Abstract. The air-breathing crabs *Cardisoma hirtipes* and *C. carnifex* trap 18.8 and 15.5 ml of air respectively within the lumen of the lung (branchial chamber) when submerging, rather than breathe water, and suffer a hypoxic hypercapnia. The role, effect and advantage of trapping air within the lung during submergence was assessed for both species.*Cardisoma*retained at least 85% of the lung air during a 30-min submergence. The mass-specific O_2 uptake rate (MO_2) of air-breathing *C. hirtipes* and *C. carnifex*was reduced by at least 60% during submersion and the crabs additionally consumed 80% or more of the O_2 stored in the trapped air. After 30 min of submergence the large arterial-venous difference in haemolymph partial pressure of O_2 (PO_2) and O_2 content (CO_2) of air-breathing*Cardisoma* was completely removed, consistent with near-zero O_2 transport. Removing the air from the lung lumen did not restore MO_2 but rather deprived the crab of an O_2 store and, in *C. hirtipes*, promoted anaerobiosis. Submergence reduced the haemolymph CO_2 by 50% or more, regardless of the presence of trapped air in their lungs. *C. hirtipes* and *C. carnifex* with a retained air bubble lost Na at only 2.6 and 2.3 μmol g^{-1} h^{-1}, respectively, but at 3.9 and 5.0 μmol g^{-1} h^{-1} without the air bubble. Unidirectional Na uptake in *C. hirtipes* was only 0.90 μmol g^{-1} h^{-1} when air was trapped in the lung but 1.74 μmol g^{-1} h^{-1} when the bubble was removed. In *C. carnifex* these rates were 1.88 and 2.79 μmol g^{-1} h^{-1} respectively. *C. hirtipes* and *C. carnifex* both trap air within the lung and avoid exposing exchange surfaces to water. There is no large respiratory advantage to expelling the air but there are significant ion regulation cost savings in retaining it.

Key words. Air-breathing Œ Amphibious Carbon dioxide Œ *Cardisoma* Œ Crustacean Œ Haemolymph Œ Land crabs Œ Na regulation Œ Oxygen Terrestrial

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Introduction

Land crabs of the genus *Cardisoma* have a distribution that shows they retain a clear dependency on water (Gifford, 1962; Hartnoll, 1988) since their burrows often descend to the local water table (Wood and Boutilier, 1985; Pinder and Smits, 1993; Adamczewska and Morris, 1996; Morris and Adamczewska, 1996). In these crabs the branchiostegal linings of the branchial chambers that hold the gills have become elaborated into a functional lung (Farrelly and Greenaway, 1992, 1993). It has been suggested that *Cardisoma* may be bimodal breathers and use water to facilitate excretion of CO_2 across their gills (e.g. Wood and Randall, 1981; Farrelly and Greenaway, 1994), or that they submerge and breathe water to different extents (e.g. Gifford, 1962; Shah and Herreid, 1978; Cameron, 1981; O'Mahoney and Full, 1984; Burggren *et al*., 1985) because of the need to avoid or compensate for dehydration in air. However, the flooded burrows of*Cardisoma* are generally hypoxic and hypercapnic (Wood and Boutilier, 1985; Pinder and Smits, 1993; Adamczewska and Morris, 1996) and would not facilitate respiratory gas exchange.

Some species of *Cardisoma* have been observed to trap air bubbles within the lung lumen (branchial chamber) and this behaviour appears to be the normal submergence response of *C. hirtipes* (Adamczewska and Morris, 1996) and *C. carnifex* (Morris and Adamczewska, 1996). Cameron (1981) observed that similar air bubbles had to be removed from *C. carnifex* if they were to survive submersion. However, recent investigations of responses of *Cardisoma*, with trapped air, to submersion showed that the respiratory gas exchange rate of *C. hirtipes* in water is less than half that in air (Dela-Cruz and Morris, 1997a). Consequently, *C. hirtipes* and *C. carnifex* submerged *in situ* became internally hypoxic compared to when they breathed air (Adamczewska and Morris, 1996; Morris and Adamczewska, 1996; Dela-Cruz and Morris, 1997a) which they do for the majority of the time (Dela-Cruz and Morris, 1997b). Similarly, the submergence of *C. carnifex* (Morris and Adamczewska, 1996) or of *C. hirtipes* (Adamczewska and Morris, 1996; Dela-Cruz and Morris, 1997a) failed to induce the respiratory alkalosis indicative of gas exchange with water. The effect of submergence on haemolymph partial pressure of CO_2 (PCO_2) may very well be dependent on the extent to which the gills are bathed. Adamczewska and Morris (1996) suggest that the air bubble may provide an oxygen store and prevent diffusive ion losses to freshwater. However, in addition to long-term maintenance of hydration state, periodic access to water is required for nitrogen (NH_3/NH_4^+) excretion. In *C. hirtipes* (Dela-Cruz and Morris, 1997b) and *C. carnifex* (Wood and Boutilier, 1985) nitrogen is stored and excreted in rapid, intense bursts during brief immersion episodes, as found also for the freshwater crab *Potamonautes warreni* (Morris and van Aardt, 1998), limiting the distribution away from water. The apparent aversion of *Cardisoma* to immersion remains puzzling. *C. carnifex* have, however, a greater haemolymph osmotic pressure (by 200 mosmol/l) than *C. hirtipes*, seemingly sustained by periodic visits to seawater (Morris and Adamczewska, 1996). In contrast, *Cardisoma* lose ions when immersed in freshwater (e.g. Wood and Boutilier, 1985; Dela-Cruz *et al*., submitted), creating an extra expense for regulation, and this may be a reason for minimizing submergence. This problem is exacerbated in *C. hirtipes* which has routine access to only freshwater.

The gills of *Cardisoma* may generally be important in ion regulation (Greenaway, 1989; Wolcott, D.L., 1991; Wolcott T.G., 1992). The gills of *C. hirtipes* contain a high-affinity Na uptake system (Greenaway, 1989); *C. hirtipes*, *C. carnifex* and *C. guanhumi* all survive water with a Na concentration as low as 0.5 mmol l^{-1} (Herreid and Gifford, 1963; Greenaway, 1989; Dela-Cruz*et al.*, submitted) but at a considerable greater cost for *C. carnifex* compared with *C. hirtipes* (Dela-Cruz *et al.* submitted) while *C. armatum* is also a hyper-regulator (De Leersnyder and Hoestlandt, 1963). However, some*Cardisoma* at least can manage ion balance without frequent access to open water by using the gills to reprocess urine and reabsorb salts from the urine as it passes over the gills (Dela-Cruz and Morris, 1997b). During submersion some branchial surfaces must be bathed to facilitate the removal of NH_3/NH_4^+ (Dela-Cruz and Morris, 1997b; Morris and van Aardt, 1998). The retention of an air bubble within the lung lumen (branchial chamber) may serve during such short-term submergence to minimize ion efflux. If so, this constitutes a potential behavioural regulation of salt balance and attendant costs, but may limit the surfaces available for respiratory gas exchange with water.

The possibility that retaining air in the lung lumen, rather than ventilating with water, might compromise respiratory gas exchange and transport but minimize ion regulation costs while submerged was investigated in *C. hirtipes* and the more marine *C. carnifex*. Submerged crabs were compared to air-breathing crabs, and in addition the effect of removing the air bubble during submergence was assessed. Respiratory, acid-base and metabolic responses were determined as were the maximum Na loss rate and the unidirectional Na uptake rate in crabs with and without air bubbles trapped within the lung lumen. Specifically the importance of the trapped air in determining O_2 uptake and respiratory gas transport was assessed against the possibility that flooding of the branchial/lung surfaces causes an expensive or even unsustainable ion loss, in turn promoting the adoption of a terrestrial life-style in the*Cardisoma*.

Materials and methods

Gecarcinid crabs, *Cardisoma hirtipes* (Dana) (371Œ457 g) and *C. carnifex* (Herbst) (362Œ462 g) were collected from Christmas Island (Parks Australia permit) and West Island of the Cocos (Keeling) Atoll. The location of these islands in the Indian Ocean, and the situation of the collection sites are described elsewhere (Adamczewska and Morris, 1996; Morris and Adamczewska, 1996). Briefly, *C. hirtipes* were collected around inland freshwater springs and *C. carnifex* adjacent to the central lagoon of the Cocos atoll. The crabs were packed in shredded paper and transported (AQIS (*A*ustralian *Q*uarantine *I*nspection *S*ervice) permit) by aircraft to Sydney. Both species were housed at 25±2°C (the temperature for all experiments) and 85% relative humidity in communal terrariums which contained 2-l water containers that were sufficient to allow the crabs to immerse. Routinely, the water provided was either artificial Christmas Island Water (CIW; Adamczewska and Morris, 1996) for *C. hirtipes* or 10% seawater for *C. carnifex*. The crabs were fed weekly on mixed fruit and dry dog food. The light cycle was 14-h light:10-h dark and all experiments were performed during the light phase.

Effective lung volume and gas composition

The volume of air trapped as a bubble within the lung (for simplicity the vascularized branchiostegal lining of the branchial chamber, which also houses the gills, is referred to as the lung and the space within as the lung lumen) during submersion of *C. hirtipes* and *C. carnifex* was determined by a displacement method (Dela-Cruz and Morris, 1997a). A 15-cm-diameter funnel was attached to a 50-ml measuring cylinder with a water-tight seal and the whole system flooded with water by submergence in a plastic tub. Resting crabs were individually submerged beneath the funnel that collected trapped air into the cylinder. The amount of air was recorded after 5 min and 30 min of submergence. Subsequently, the crab was inverted and agitated vigorously to release all the remaining air in the lung (branchial chambers) into the cylinder.

The PO_2 and PCO_2 within the lung air bubble was determined in both*C. hirtipes* and *C. carnifex* either while air-breathing or subsequent to 30 min of submergence. To sample the lung lumen gas the crabs were bilaterally fitted with short polyethylene catheters which penetrated from the apex of the dorsal surface through the carapace

just into the air-space of the lung, as described by Morris and van Aardt (1998). These catheters had the added function of ensuring that the branchial chambers/lungs were flooded when required, by manually withdrawing all air with a syringe. To sample lung gas at least 5 ml of air was withdrawn into a glass syringe and introduced into serially connected D616 thermostated cells containing either an O_2 or a CO_2 electrode (Radiometer Pacific), and was chased by a second 5-ml sample taken from the other lung. There was never any fluid in either sample. Partial pressures were recorded by a PHM 73 meter (Radiometer) as kPa. The CO_2 electrode was calibrated using humidified precision gas mixtures (Linde Australia, Sydney) and the O_2 using O_2-free gas and air-saturated water.

Oxygen uptake rates

The rate of O_2 uptake for air-breathing crabs was determined by flow-through respirometry using an Applied Electrochemistry O_2 analyser (Morris *et al.*, 1994; Dela-Cruz and Morris, 1997a). The values for *C. hirtipes* are also reported in Dela-Cruz and Morris (1997a) but are compared here with those for *C. carnifex*.

The rate of O_2 uptake for *C. hirtipes* and *C. carnifex* submerged with or without the lung air was determined using a closed respirometry method (Dela-Cruz and Morris, 1997a). Briefly, an individual crab was placed in a cylindrical respirometer and submerged in aerated water for 30 min. Water sampled immediately prior to and after the 30 min was passed over a Radiometer electrode. Rates (MO_2) were calculated as μmol g^{-1} h^{-1} for both*Cardisoma* species. All crabs were fitted with catheters as described above. No attempt was made to determine if there were any small differences in the activity of crabs in air or water since this is integral to the exposure treatments.

Haemolymph measurements

The haemolymph contents and partial pressures of O_2 and CO_2 in *C. hirtipes* and in *C. carnifex* that were either breathing air or submerged for 30 min (CIW and 30% SW respectively), with or without a trapped air bubble within the lung lumen (n=6 for each species), were measured. The carapace of the crabs was drilled a minimum of 24 h prior to experiments to allow haemolymph sampling from the pericardial cavity and from the efferent pulmonary vessel (Farrelly and Greenaway, 1993). Each crab was housed in an individual box with a supply of fresh drinking water for 24 h prior to experimentation. Each crab was sampled for pulmonary (post pulmonary vessel, content and pH only), arterial (pericardial cavity) and venous haemolymph (from the venous sinuses at the base of the last walking leg). The haemolymph samples were immediately analysed for partial pressure and content of O_2 and CO_2, as well as haemolymph pH, using a BMS 3 MK II Blood Micro System thermostated at 25±0.2°C and connected to a PHM73 pH/Blood gas monitor (Radiometer, Copenhagen, Denmark). The electrodes were calibrated daily with humidified gases. The O_2 electrode was calibrated using O_2-free gas and air-saturated water. The CO_2 electrode was calibrated using two humidified gases, one with 0.5% and the other 2.5% CO_2 (precision CIG certified analysis).The pH electrode was calibrated with Radiometer precision buffers of pH 7.410 (S1510) and 6.865 (S1500), accurate to ±0.005 at 25°C.

Haemolymph O_2 contents were measured using the modified Tucker chamber method (Tucker, 1967) as outlined by Bridges *et al.* (1979). The O_2 electrode was maintained at 32°C and connected to an oxygen meter (Strathkelvin model 781). The changes in PO_2 were recorded on a pen recorder (Kipp and Zonen, model BD111). The haemolymph CO_2 content was measured using a Corning 965 analyser (calibrated with HCO_3^- standard, 15 mmol l^{-1}).

An aliquot of the remaining haemolymph samples was mixed (ratio 1:1) with 0.6 mol l^{-1} $HClO_4$ to denature proteins and neutralized with 2.5 mol l^{-1} K_2CO_3. The denatured sample was centrifuged at 10,000 *g* for 10 min and the supernatant was used for l-lactate analysis (Boehringer Mannheim test kit no. 138 084). Whole

haemolymph samples were also analysed for glucose (Sigma Diagnostics test-kit no. 510) and urate concentrations (Sigma Diagnostics test kit no. 685).

Na flux measurements

To determine if the lung air minimized ion loss from the crabs to extra-corporeal water the maximum rate of Na loss was determined in both *C. hirtipes* and *C. carnifex* which had been pre-acclimated for 3 weeks to drinking and immersion in 30% seawater. This elevated haemolymph Na concentration, which was similar in all *C. hirtipes* with a mean 288.9±1.4 mmol l^{-1} and averaged 331.8±3.0 mmol l^{-1} in*C. carnifex*, thereby maximized the Na diffusion gradient to the water (Dela-Cruz *et al.* manuscript in preparation). Maximum rates of Na loss were determined in crabs transferred to, and submerged in, containers with 1.4 l of water with the same salt composition as 30% seawater except for being free of Na and most of the Cl. Four groups of crabs (*n*=6 for each for each species) were assessed; with or without trapped lung air for the two species. The amount of Na appearing in the water was monitored at 5-min intervals for 40 min, and the increase in water Na concentration was determined. Haemolymph was sampled from crabs approximately 2 h prior to experiments so that the apparent permeability constant *K'* (Shaw, 1961; Sutcliffe, 1975; Greenaway, 1981) could be derived for Na. Sampling of the haemolymph prior to the experiment does not alter Na loss rates (Wood and Boutilier, 1985).

Unidirectional Na uptake was determined in *C. hirtipes* and *C. carnifex* previously held for 3 weeks with CIW for drinking and immersion, thus stimulating Na uptake mechanisms (Greenaway, 1989). Crabs were fitted with catheters as described above, and were submerged with or without lung air (*n*=6 for each species) in 1 l of water with the salt composition of CIW but containing Na at 15 mmol l^{-1}. The water was labeled with sufficient ^{22}Na (Amersham, Australia) as to provide 12,000 cpm for a 200-µl sample measured using a Hewlett-Packard scintillation counter. After transferring the towel-dried crabs into the experimental chambers, the water in the containers was sampled at 5-min intervals. Since the crabs invariably added small amounts of Na to the water, the exact start and end values of Na concentration were determined. This facilitated the calculation of unidirectional Na influx,J_{in} (µmol g^{-1} h^{-1}), using the special solution equation of Shaw (1963); $y=y_o \exp[(-Jt)/A]$ where y is the concentration of ^{22}Na in the water at time t, y_o is the concentration of ^{22}Na at time zero and$A$ is the amount of Na in the water. The total Na concentration of haemolymph and water samples was measured by atomic absorption spectrophotometry (AAS; GBC 906, GBC Melbourne). To suppress interference, samples were diluted with 5.9 mmol l^{-1} $CsCl_2$.

Statistical analysis

Data were analysed using SYSTAT 6.0 by ANOVA for fully independent designs and by ANOVAR for repeated measures experiments. Homogeneity of variances was confirmed using Bartlet's χ^2 test. Where ANOVA showed significant differences ($P \leq 0.05$) from control groups, post-hoc testing was by Tukey's HSD or Contrast analysis.

Results

Lung air and O_2 uptake

The total lung volume was 18.8±3.4 and 15.5±1.77 ml in *C. hirtipes* and *C. carnifex* respectively and of this only 7Œ10% was released in the initial 5 min (Fig. 1). In *C. hirtipes* a further 5% was released in the following 25 min, but in both species 85% or more of the air was retained as a trapped bubble while submerged.

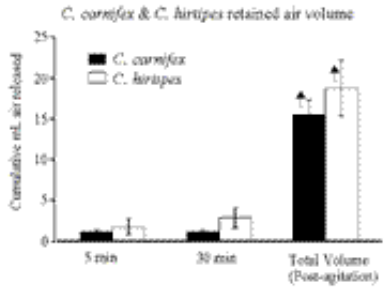

Fig. 1. The cumulative volume of air released from the lung/branchial chambers of *Cardisoma carnifex* and *C. hirtipes* after 5 and 30 min of submergence. The *final column* is the total volume of the lung lumen determined by agitating and inverting the crabs to release air trapped for the full 30 min of submergence. The*arrows* indicate a significant increase in volume, *n*=6 for each species

There were large changes in composition of the lung air during submersion of both species (Fig. 2). In air-breathing *C. carnifex* the lung air was 20.9% depleted of O_2 with respect to atmospheric (PO_2=20.7 kPa; 156 torr), while in *C. hirtipes* the reduction was 11.3%. After 30 min of submergence thePO_2 was markedly reduced to 3.52±0.30 kPa in *C. carnifex* and to 2.79±0.78 in *C. hirtipes*; reductions of 12.88 kPa and 15.68 kPa, or 79% and 85% of the available O_2 stored in the air bubbles, respectively. In addition to extreme hypoxia, submersion promoted a hypercapnia in the lung air (Fig. 2). Interestingly, the PCO_2 of the lung air of air-breathing *C. hirtipes* (0.79±0.09 kPa) and *C. carnifex* (0.83±0.10 kPa) was relatively hypercapnic but after 30 min of submersion, increased to 1.75±0.19 and 1.03±0.14 kPa respectively (Fig. 2).

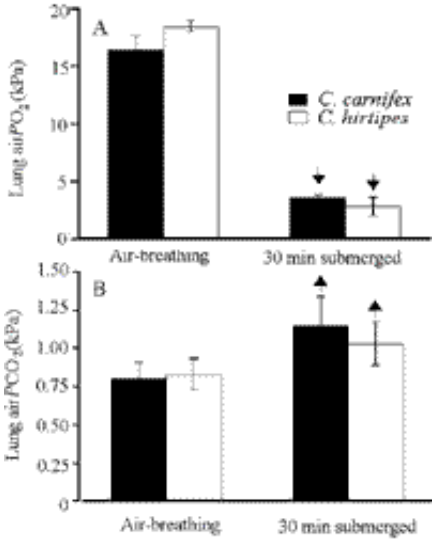

Fig. 2. A The partial pressure of O_2 (PO_2), and**B** CO_2 (PCO_2) of the air retained within the lumen of the lungs in *C. carnifex* and *C. hirtipes* either while breathing air or after 30 min of submergence. The *vertical arrows* indicate a significant change in partial pressure after submergence, *n*=6 for each species in each treatment

The MO_2 of air-breathing crabs was similar in the two *Cardisoma* at 2.45±0.27 μmol g^{-1} h^{-1} in *C. hirtipes* (previously reported in Dela-Cruz and Morris, 1997a) and 2.20±0.15 μmol g^{-1} h^{-1} in *C. carnifex* (Fig. 3). The O_2 uptake of both *C. hirtipes* and *C. carnifex* submerged with lung air decreased to 40% or less of the air-breathing rate (Fig. 3). Removing the air bubble such that the lung lumen was filled with water and the gills completely bathed did not improveMO_2. Taking the lung air volume into account together with the decrease in PO_2 of that air during submersion, the molar amount of O_2 taken from the bubble, and thus the contribution of stored lung O_2 during submersion, could be derived (Fig. 3). While this doubled the available O_2 used in *C. hirtipes* this was still equivalent to only a third of the air-breathing uptake. In *C. carnifex* using the O_2 in the bubble restored the effective MO_2 slightly more but only to 58% of the air-breathing rate and was not significantly improved over that of animals without trapped air bubbles (Fig. 3).

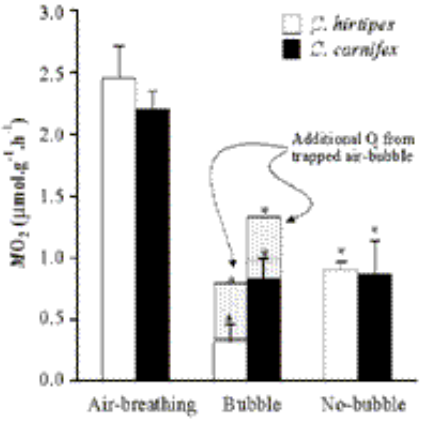

Fig. 3. The mass-specific O_2 uptake rate (MO_2) of *C. hirtipes* and *C. carnifex* while breathing air or submerged, with or without air retained within the lung. The *stippled bars* show the contribution made by O_2 withdrawn from the trapped air during 30 min of submergence, converted to a mass specific rate (i.e. the molar amount withdrawn with respect to time and mass). n=6 for each species in each treatment. * A reduction compared to air-breathing animals. [Note: the data for air-breathing *C. hirtipes* were previously reported by Dela-Cruz and Morris (1997a)]

Haemolymph respiratory gas and metabolites

Submerging the crabs with lung air had significant effects on haemolymph respiratory gas status in both species and these were almost entirely unchanged by the removal of the air from the lung lumen (Table 1). The overall haemolymph CO_2 values were effectively the same in *C. hirtipes* with *C. carnifex*. The pulmonary and arterial haemolymph O_2 content were similar to each other within each species while air breathing. In both *C. hirtipes* and *C. carnifex* approximately 60% of the O_2 was removed during circulation of the haemolymph, 0.35Œ0.4 mmol l^{-1} comparing arterial (C_aO_2) and venous (C_vO_2) O_2 contents. The pulmonary haemolymph O_2 content (C_pO_2) andC_aO_2 of crabs submerged with lung air declined after 30 min and were no longer significantly elevated above C_vO_2 which was 0.24 mmol l^{-1} in *C. carnifex* and 0.38 mmol l^{-1} in *C. hirtipes* and, in both cases, unchanged from the C_vO_2 of air-breathing crabs (Table 1). Submerging crabs with the lung air removed did not assist in maintaining either C_pO_2 or C_aO_2 and in *C. hirtipes* may have lowered CO_2 further, since both C_aO_2 andC_vO_2 appeared to be 0.1 mmol l^{-1} lower in animals without trapped air in the lung lumen.

Table 1. Haemolymph respiratory gas status and pH in *C. hirtipes* and *C. carnifex* either breathing air or after 30 min submerged with trapped lung air, or after 30 min of submergence with all lung air completely removed. Oxygen and carbon dioxide contents (in mmol l^{-1}), and pH, are provided for efferent pulmonary haemolymph as well as for arterial and venous samples. The partial pressures are as kPa for arterial and venous samples (n=6Œ8) (For printing a landscape orientation is also available)

	C_pO_2	C_aO_2	C_vO_2	C_pCO_2	C_aCO_2	C_vCO_2	pH_p	pH_a	pH_v	P_aO_2	P_vO_2	P_aCO_2	P_vCO_2
C. carnifex	(mmol l^{-1})									(kPa)			
Air-breathing	0.67 ± 0.17	0.82 ± 0.18	0.37 ± 0.05 *+	18.58 ± 1.46	18.87 ± 1.11	19.07 ± 1.32	7.591 ± 0.030	7.569 ± 0.047	7.546 ± 0.037	*5.28* ± 1.32	0.77 ± 0.23 +	1.94 ± 0.23	2.05 ± 0.20
Submerged with lung air	0.21 ± 0.05 #	0.39 ± 0.05 #	0.24 ± 0.06	8.53 ± 1.23 #	11.07 ± 1.16 #	8.88 ± 0.75	7.594 ± 0.037	7.591 ± 0.027	7.569 ± 0.041	1.37 ± 0.15 #	1.68 ± 0.32	1.49 ± 0.39	*0.70* ± 0.13 #
Submerged without lung air	0.33 ± 0.05 #	0.30 ± 0.05 #	0.25 ± 0.08	10.88 ± 1.01 #	10.97 ± 1.40 #	11.53 ± 1.34 #	7.581 ± 0.019	7.575 ± 0.030	7.496 ± 0.029	1.32 ± 0.27 #	1.26 ± 0.21	1.80 ± 0.24	1.97 ± 0.29
C. hirtipes													
Air-breathing	0.92 ± 0.09	0.86 ± 0.13	0.36 ± 0.08 *+	15.20 ± 1.8	16.30 ± 1.57	16.70 ± 0.9	7.595 0.034	7.622 ± 0.019	7.599 ± 0.028	*10.23* ± 2.67	0.89 ± 0.05 +	1.95 ± 0.17	2.13 ± 0.17
Submerged with lung air	0.31 ± 0.06 #	0.54 ± 0.08 #	0.38 ± 0.06	11.40 ± 1.31 #	10.77 ± 1.67 #	11.67 ± 1.65	7.628 ± 0.032	7.566 ± 0.031	7.468 ± 0.031	2.06 ± 0.39 #	1.46 ± 0.47	1.08 ± 0.25 #	*1.49* ± 0.28 #
Submerged without lung air	0.30 ± 0.05 #	0.44 ± 0.02 #	0.27 ± 0.02	8.80 ± 0.37 #	8.77 ± 0.63 #	8.93 ± 1.03	7.652 ± 0.026	7.625 ± 0.024	7.549 ± 0.037	1.64 ± 0.12 #	1.84 ± 0.16	0.90 ± 0.16 #	1.24 ± 0.07 #

Different from air-breathing crab of same species

Values in *red italics* indicate differences between *C. carnifex* and *C. hirtipes*

* Different from pulmonary value

+ Different from arterial value

The internal hypoxia apparent in submerged crabs was even more obvious in the PO_2 values. Interestingly, theP_aO_2 in the haemolymph of air-breathing *C. hirtipes* (10.2 kPa) was twice that of *C. carnifex* (5.3 kPa) although the P_vO_2 values were similar (0.89 and 0.77 kPa respectively; Table 1). Submerging the crabs markedly reduced P_aO_2 to 2.1 kPa or less, which removed any significant arterial-venous PO_2 difference. As evidenced by the O_2 content values, removing the air bubble at the onset of emergence did not facilitate any improvement in haemolymph oxygenation.

Submergence of *Cardisoma* reduced the haemolymph CO_2 (Table 1), despite the developing hypercapnia in the air of the lung lumen, especially in *C. carnifex* in whichP_aCO_2 declined from 1.95 to 1.08 kPa. Consequently, submerged crabs had CO_2 values that were only 46Œ59% those of air-breathing animals in *C. carnifex* and 66Œ75% in *C. hirtipes* (Table 1). There was no evidence that submergence with the lung lumen (branchial chamber) completely flooded elicited any significant improvement of CO_2 excretion sinceP_aCO_2 remained at 1.80 and 0.90 kPa in *C. carnifex* and *C. hirtipes* respectively (Table 1). There was an apparent further decline

inCCO_2 in *C. hirtipes* with the trapped air removed, to 53Œ58% of that in air-breathing crabs but this seemed independent of PCO_2.

Despite the relatively large changes in CO_2 associated with submergence, with or without a trapped air bubble, there were no significant changes in haemolymph pH in either species of *Cardisoma* (Table 1). Neither were there differences in the pH of haemolymph comparing pulmonary, arterial and venous samples for either species under each of the three different conditions.

The concentration of l-lactate in the haemolymph remained low (1.12Œ1.32 mmol l^{-1}) and was essentially the same in the two *Cardisoma* when submerged with a trapped air bubble in the lung (Table 2). However, l-lactate increased to 3.5 mmol l^{-1} in *C. hirtipes*, but not *C. carnifex*, when submerged without air within the lung. The haemolymph of *C. hirtipes* contained more urate but less glucose than that of *C. carnifex* (Table 2). In addition, submerging *C. carnifex* without trapped air in the lung induced a marked increase in haemolymph glucose compared to crabs submerged for 30 min with air trapped within the lung.

Table 2. Haemolymph concentration of l-lactate, urate and glucose in *C. hirtipes* and *C. carnifex* after 30 min of submergence, either with air within the lung lumen or with that air removed (*n*=6 for each species)

	l-lactate (mmol l^{-1})	**Urate (mmol l^{-1})**	**Glucose (mmol l^{-1})**
C. hirtipes			
Submerged with lung air	1.12 ± 0.15	*0.116* ± 0.153	*0.85* ± 0.19
Submerged without lung air	3.46 ± 0.81#	*0.073* ± 0.073	*1.05* ± 0.22
C. carnifex			
Submerged with lung air	1.32 ± 0.37	*0.028* ± 0.065	*1.19* ± 0.35
Submerged without lung air	0.87 ± 0.41	*0.030* ± 0.083	*1.83* ± 0.31#

#Effect of removing lung air

Values in *red italics* indicate a difference between corresponding values in the two species

The influence of lung air on Na flux in submerged crabs

In submerged *C. hirtipes* and *C. carnifex* with a retained air bubble the Na loss rate to Na-free water was only 2.6±0.55 and 2.3±0.34 µmol g^{-1} h^{-1} but when the air bubble was removed the rate increased to 3.9±0.42 and 5.0±0.48 µmol g^{-1} h^{-1} respectively (Fig. 4). While the loss rate was not significantly greater in *C. carnifex* than in *C. hirtipes* the magnitude of the increase was larger (Fig. 4). The apparent permeability constant *K*' can be calculated as [ion loss rate (in mmol l^{-1} haemolymph) per hour divided by the haemolymph concentration (in mmol l^{-1})]; the extracellular/haemolymph space was taken as 33.2% for *C. hirtipes* and 24.7% for *C. carnifex* (Dela-Cruz and Morris, unpublished). The value of *K*' and thus apparent permeability was approximately doubled by bubble removal in *C. carnifex* from 0.032±0.005 to 0.067±0.009 but in *C. hirtipes K*' was 0.032±0.012 in animals with trapped air and similarly 0.039±0.004 after 30 min of lung (branchial chamber) flooding.

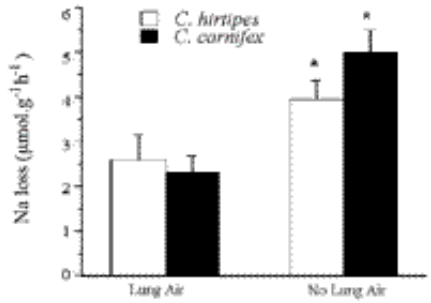

Fig. 4. The maximum Na loss rate, as μmol g^{-1} h^{-1}, from *C. hirtipes* and *C. carnifex* submerged with or without an air bubble retained within the lung. * An increase in loss associated with removal the retained air bubble. Both *Cardisoma* species were acclimated to drinking water of 30% seawater to maximize loss rates, *n*=6 for each species in each treatment

Unidirectional Na uptake (*J*in) was determined in*Cardisoma*acclimated to freshwater (Fig. 5). The Na uptake rate of*C. hirtipes* submerged with lung air was only 0.90±0.30 μmol g^{-1} h^{-1} compared to the 1.74±0.54 μmol g^{-1} h^{-1} crabs submerged without the trapped air. Similarly, *C. carnifex* had aJ_{in} of 1.88±0.61 μmol g^{-1} h^{-1} with air in the lung but 2.79±0.88 μmol g^{-1} h^{-1} with the air bubble removed (Fig. 5). However, there were considerable differences in the rates of individuals which masked both the difference between species ($F_{1,19}$=3.56, *P*=0.07) and the effect of bubble removal ($F_{1,19}$=2.62, *P*=0.12).

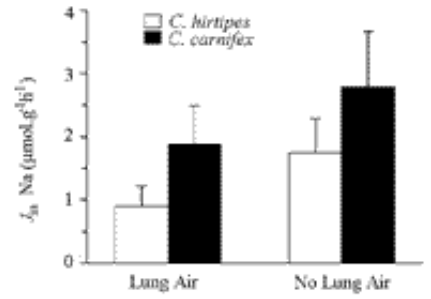

Fig. 5. The unidirectional Na uptake rate (J_{in}), as μmol g^{-1} h^{-1}, by *C. hirtipes* and *C. carnifex* submerged with or without an air bubble retained within the lung. ANOVA provided *P*=0.07 for the difference between species and *P*=0.12 for the effect of bubble removal. Both*Cardisoma* species were acclimated to drinking CIW to maximize uptake rates, *n*=6 for each species in each treatment

Discussion

Respiratory and acid-base consequences of submergence and trapped lung air

C. hirtipes and *C. carnifex* retain most of the air within the lung lumen during submersion, confirming previous field (Adamczewska and Morris, 1996; Morris and Adamczewska, 1996) and laboratory observations (Dela-Cruz and Morris, 1997a). ThePO_2 and PCO_2 in the lung air of *C. hirtipes* and *C. carnifex* were indistinguishable from the PO_2 of 18.7 kPa and PCO_2 of 0.66 kPa previously obtained for "branchial chamber air" in *C. carnifex* (Wood and Randall, 1981). The large hypoxic hypercapnia that develops within the lung lumen signifies there was little gas exchange between the air space and the water, and minimal ventilation of the chamber. Both species showed greatly reduced but nonetheless appreciable O_2 uptake from the water, possibly facilitated by the lowered PO_2 within the lung air space. The MO_2 of immersed *C. hirtipes* (2.45 μmol g^{-1} h^{-1}; see also Dela-Cruz and Morris, 1997a) and *C. carnifex* (2.20 μmol g^{-1} h^{-1}) were also similar to previous values for these species (Wood and Randall, 1981) and to values for other obligate air-breathing crabs (Adamczewska and Morris, 1994 for review figure). Submersion reduced the MO_2 of *C. carnifex* by 60% and similarly by nearly 80% in *C. hirtipes*, as previously reported for the latter species (Dela-Cruz and Morris, 1997a). Flooding the lung failed to improve MO_2, perhaps because of minimal ventilation during submergence. This may not be true for all*Cardisoma* since O'Mahoney and Full (1984) found that *C. guanhumi*, in which the lung air was removed, maintainedMO_2 in air and water at equal rates but only at the expense of a 300% increase in ventilation. Conversely, clearly obligate land crabs, such as *Gecarcinus lateralis*, reducedMO_2 in water by almost 50% compared to in air (Taylor and Davies, 1981, 1982; O'Mahoney and Full, 1984). Thus, both *C. hirtipes* and *C. carnifex* appear more committed to breathing air than *C. guanhumi*. Expelling the air would not improve the respiratory status of the two species

and in fact the lung air store added appreciably to the O_2 available during the 30-min submergence.

The maximum PaO_2 in air-breathing *C. hirtipes* was high but consistent with previous field determinations (Adamczewska and Morris, 1996) while greater than laboratory values (Farrelly and Greenaway, 1994; Dela-Cruz and Morris, 1997a). The arterial PO_2 in air-breathing*C. carnifex* was 50% of that in *C. hirtipes* suggesting relatively impaired diffusive uptake of O_2. However, in both *C. hirtipes* and *C. carnifex*, the pulmonary and arterial haemolymph of air-breathing crabs was apparently 100% saturated with O_2 (Burggren and McMahon, 1981; Farrelly and Greenaway, 1994; Adamczewska and Morris, 1996; Morris and Adamczewska, 1996; Dela-Cruz and Morris, 1997a). The similarity in pulmonary and arterial values suggests that either the gills were little perfused or, perhaps, that the gills and lungs were functioning similarly in O_2 uptake from air.

Field studies of *C. carnifex* (Morris and Adamczewska, 1996), and both field and laboratory studies of *C. hirtipes* (Adamczewska and Morris, 1996; Dela-Cruz and Morris, 1997a) showed that 30 min of submergence rapidly depletes haemolymph O_2. In the current study, the pulmonary and arterialCO_2 both declined to near theC_vO_2 after 30 min under water, which contrasts with *Gecarcinus lateralis* in which both arterial and venous haemolymph O_2 content decreased to similar extent (Taylor and Davies, 1982). The reduction in lung airPO_2 to 3 kPa or less meant that the maintenance of P_aO_2 was impossible. The gradient for inward diffusion of O_2 from the lung lumen into the haemolymph declined from 8.27 to 0.79 kPa in *C. hirtipes* and from 11.22 to 2.15 kPa in *C. carnifex*. Consequently, P_vO_2 must decline to sustain any inward diffusion of O_2 but eventually failed in *Cardisoma* since the arterial-venous difference declined to zero. The haemocyanin-bound O_2 (Hc-O_2) has been previously calculated to provide O_2 for approximately 5 min in a 400-g *C. hirtipes* (Adamczewska and Morris, 1996) and 8 min in 300-g *C. carnifex* (Morris and Adamczewska, 1996). These stores in crabs submerged for 30 min with trapped air bubbles (Fig. 3) were depleted at a rate of 0.23 and 0.31 μmol g^{-1} h^{-1} *C. hirtipes* and *C. carnifex*, respectively, effectively supplementing the MO_2 which was still well below the air-breathingMO_2, of *C. hirtipes* especially. In the absence of any appreciable anaerobiosis in *C. hirtipes*, Adamczewska and Morris (1996) concluded that the crabs become relatively hypometabolic; a very different strategy to the threefold to fourfold elevation in ventilatory work suggested by O'Mahoney and Full (1984) for submerged *C. guanhumi*.

Submerging *C. hirtipes* and *C. carnifex* without trapped air, such that the water bathed the entire branchial structure, failed entirely to relieve the submergence hypoxia. This is quite at odds with both Cameron (1981) and O'Mahoney and Full (1984), who suggested that this allowed respiratory exchange with water. Nonetheless, neither *C. hirtipes* or *C. carnifex* showed any improvement in either the haemolymph PO_2 or consequently the O_2 content. Conversely, the CO_2 data for *C. hirtipes* support the suggestion that removing the air bubble actually exacerbates the hypoxia, since haemolymph lactate concentrations increased to 3.5 mmol l^{-1}. The effect of submergence on haemolymph metabolites has been shown to be variable between species and depend on circumstance. For example, in *C. hirtipes* neither urate nor the l-lactate concentration increased in crabs submerged in the field (Adamczewska and Morris, 1996) whereas urate, but not l-lactate, did increase during submergence in the laboratory (Dela-Cruz and Morris, 1997a). Similarly, in the current study lactate increased in *C. hirtipes* only when the air bubble was removed from the lung lumen. Field determinations of *C. carnifex* also showed urate to be unaltered in the haemolymph during submersion, but, in contrast to *C. hirtipes*, there were some increases in haemolymph l-lactate levels although urate was unchanged by submersion (Morris and Adamczewska, 1996). In both field and laboratory determinations the submersion of *C. hirtipes* promoted a hypoglycaemia whereas *C. carnifex* showed evidence of a hyperglycaemia (Morris and Adamczewska, 1996) which was evident in the present study only once the air bubble was removed. Thus, in *C. hirtipes* removal of the lung air, and therewith an O_2 store that was otherwise used during submergence, made a crucial difference in the recruitment of anaerobiosis, which does not normally occur in *C. hirtipes*, although it was observed by Shah and Herreid (1978) for *C. guanhumi*. The lactacidosis in *C. carnifex* measured in crabs submerged in the field caused Morris and Adamczewska (1996) to conclude that this species differs from *C. hirtipes* in that it maintains its metabolic rate by supplementary anaerobiosis. However, there was no such anaerobiosis in the present laboratory

study of *C. carnifex*. Given that 30 min of submergence has proven to be close to the aerobic limit for both species, and the extremely low O_2 content in the haemolymph, it seems reasonable that small differences, for example in temperature, may determine the duration of submergence that can be attained without initiating anaerobiosis.

Haemolymph acidosis can arise because of accumulation of respiratory CO_2 as well as through metabolic acidosis. The haemolymph PCO_2 and CCO_2 were generally not different comparing *C. hirtipes* and *C. carnifex*, and the PCO_2 values in air-breathing crabs were essentially the same as previously obtained values for *C. carnifex* (1.92 kPa, Randall and Wood, 1981; 1.90 kPa, Morris and Adamczewska, 1996) and for *C. hirtipes* (1.81 kPa, Farrelly and Greenaway, 1994; >1.60 kPa, Adamczewska and Morris, 1996). Submergence of *C. hirtipes* and *C. carnifex*, despite air trapped within the lung, induced a haemolymph hypocapnia in both species, suggesting some facilitation of CO_2 excretion by bathing water. Laboratory studies of *C. carnifex* showed a 2.5-fold increase in ventilation volume when in water (McMahon and Burggren, 1988 for review) and as a consequence reduced haemolymph PCO_2 and CCO_2, and thus a respiratory alkalosis (Cameron, 1981), in common with nearly every species able to inhabit air and water (McMahon and Burggren, 1988). However, this was not the case for either *C. hirtipes*, which exhibited a marked metabolic alkalosis (Adamczewska and Morris, 1996), or *C. carnifex* voluntarily submerged in the field, which showed a respiratory acidosis (Morris and Adamczewska, 1996). This provides further evidence that submerged *Cardisoma* ventilate very little and that gas exchange is primarily with a progressively hypoxic-hypercapnic air bubble. Excretion of CO_2 was therefore severely impaired in submerged *Cardisoma*, thus the gradient for outward diffusion into the lung lumen from the haemolymph declined from 1.11 to 0.46 kPa in *C. carnifex*, and in *C. hirtipes* changed from an outward 1.16 kPa to an inward gradient of 0.67 kPa, as a result of the hypercapnia in the lung air.

The acid-base status, the influence of submersion, the effect of removing the trapped air bubble from the lung and the differences between *C. carnifex* and *C. hirtipes* are best appreciated with the aid of a pH/HCO_3^- diagram (Fig. 6). Under normal circumstances *C. carnifex* exist in a state of relative respiratory acidosis compared with *C. hirtipes* (Fig. 6). This cannot be ascribed to higher CO_2 production rates in *C. carnifex* since the two species have similar respiration rates. Neither can it be due a relatively hypercapnic lung lumen; thus, *C. carnifex* must experience relatively greater diffusion resistance to CO_2 excretion, consistent with the apparent relative resistance to inward O_2 diffusion (above). Thus, *C. hirtipes* is better than *C. carnifex* at breathing air. The submergence of *C. carnifex* and *C. hirtipes* promoted some metabolic acidosis which in *C. carnifex* especially was compensated by a respiratory alkalosis (Fig. 6). The source of the metabolic acid is not obvious but the pH changes were slight. *C. hirtipes* submerged in the field experienced net ion loss (Greenaway, 1989; Adamczewska and Morris, 1996) and changes in strong ion concentrations within the haemolymph could account for the slight acidosis. The relative hypocapnia that occurred in both species after 30 min submerged indicates that some improved CO_2 excretion to the water occurs, even with retained air in the lung. Similarly, the compensatory respiratory alkalosis in *C. carnifex* is a consequence of improved CO_2 excretion. That this compensation is less apparent in submerged *C. hirtipes* indicates relatively less facilitation of CO_2 excretion and perhaps less contact of the exchange surfaces with water. Flooding the lung/branchial chamber of *C. carnifex* did little to further enhance the compensatory respiratory alkalosis, whereas in *C. hirtipes* the alkalosis was really apparent only once the lumen was flooded and a larger epithelial surface area was bathed (Fig. 6).

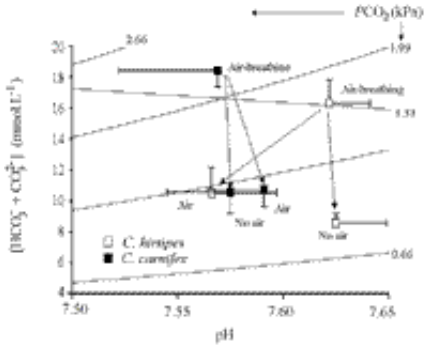

Fig. 6. The pH/HCO3- diagram for *C. hirtipes* and *C. carnifex* in each of the three treatments: air-breathing, submerged with air retained in the lung (bubble) and with the air removed (no bubble). The slope of the non-bicarbonate buffer line was from the data of Dela-Cruz and Morris (1997) and the PCO_2 isopleths derived from the Henderson-Hasselbalch relationship using a pK'=6.025 and an αCO_2=0.2267 μmol l^{-1} Pa^{-1}

Thus, the air bubble retained by the two *Cardisoma* investigated does not hinder O_2 uptake by submerged crabs, since its removal does not alleviate the internal hypoxia, but instead it may provide an important O_2 reservoir. Under laboratory conditions expelling the air bubble may assist some CO_2 excretion, but apparently not under field circumstances, and to a greater extent in *C. carnifex* than in *C. hirtipes*.

Immersion avoidance and lung air retention as mechanisms to minimize ion loss

Under field conditions *C. hirtipes* lost Ca and Mg while submerged (Adamczewska and Morris, 1996) and in the laboratory lost salt to freshwater (Greenaway, 1989; Dela-Cruz and Morris, 1997a). Adamczewska and Morris (1996) suggested that the trapped lung air, by reducing contact between the water and permeable branchial and branchiostegal epithelia, could mitigate ion losses to freshwater and lower regulative costs in *C. hirtipes*, and thereby explain the propensity to avoid prolonged submersion. Similarly, *C. carnifex* given only fresh water lost K, Ca and Mg (Wood and Boutilier, 1985) but salt regulation is perhaps less problematic for *C. carnifex* since it can visit the ocean to replace salts lost to lower salinity water in the burrows (Morris and Adamczewska, 1996).

C. hirtipes and *C. carnifex* submerged in Na-free CIW in the laboratory lost Na 33% and 54% (respectively) more rapidly when the air trapped within the lung lumen (branchial chamber) was removed. There was thus a greater effect on Na loss rates in *C. carnifex*, possibly since the Na concentration gradient was 13% greater than for *C. hirtipes*. The rates were high compared to freshwater crabs, e.g. *Potamonautes warreni* at 0.22 μmol g^{-1} h^{-1} (Morris and van Aardt, 1998) and 0.07Œ0.20 μmol g^{-1} h^{-1} *Holthuisana transversa* (Greenaway, 1981), and represent significant Na losses which are greatly exacerbated by loss of the protecting air bubble, which must mean a marked increase in energetic requirements. However, the crabs used in the Na loss measurements were pre-acclimated to 30% SW to emphasize any effect of the air bubble on loss rates. The loss rates in freshwater-acclimated crabs are much lower, 0.12 and 0.52 μmol g^{-1} h^{-1} for *C. hirtipes* and *C. carnifex* respectively, due to reduced permeability (Dela-Cruz, Morris and Greenaway, unpublished) and the actual consequential costs may be lower.

The salt regulation costs during immersion, e.g. compensatory Na uptake, can only be reduced by decreased permeability. Exact measurements of whole-animal permeability are often difficult to obtain since they depend not only on the diffusion resistance of the epithelia but also on their surface area, but estimates of K' can provided good estimates of apparent Na permeability (Mantel and Farmer, 1983). The value of K' increased when the air bubbles were removed, in *C. carnifex* by 107% and in *C. hirtipes* by 22%, implying that the greater K' of crabs submerged without trapped branchial air must be due to the increased exposure of the gills and/or branchiostegites to water.

The unidirectional Na uptake measurements on CIW-acclimated *Cardisoma* cannot be directly compared to the loss rates since acclimation salinity also alters J_{in} (Dela-Cruz *et al.* manuscript in preparation). However, the uptake rates were highest in animals in which the lung and gills were flooded. A higher rate of uptake in *C. carnifex* than in *C. hirtipes* is consistent with compensation needed to accommodate the increased Na loss rate and apparent permeability. It is difficult to determine to what extent these data indicate true compensation. It

seems probable that in crabs with trapped lung air that part of, or even all, the gills would be in contact with water, hence the Na exchange in submerged crabs, and that expelling the air simply increases the surface area bathed to include the lung also, thereby increasing the surface involved both in diffusive ion loss and in compensatory pumping.

Neither species of *Cardisoma* investigated seems to be a truly amphibious crab since they both appear to eventually asphyxiate under water (Adamczewska and Morris, 1996; Morris and Adamczewska, 1996). *C. hirtipes* and*C. carnifex* naturally trap air within the lung lumen, rather than expose the lining and the gills to water. This trapped air acts as an O_2 store and a CO_2 repository. Expelling the trapped air fails to significantly improve any aspect of respiration and indeed removes a usable O_2 store. In many previous studies of *Cardisoma* the crabs were probably able to breathe air facultatively at the water surface. *Cardisoma* submerged in freshwater lose Na but when the air is removed from the lung lumen (branchial chamber) Na loss, permeability and Na uptake are increased. Retention of trapped air has the major effect of reducing Na turnover and associated pumping costs. Under normal humid conditions in tropical and sub-tropical habitats the only identifiable reason for *Cardisoma* to be so closely associated with water is for the excretion of nitrogen (Dela-Cruz and Morris, 1997b). These conclusions need to be extended and tested by investigation of other *Cardisoma* species and by further field determinations under natural conditions.

Acknowledgements. We would like to thank Agnieszka Adamczewska, Peter Greenaway and Stuart Linton for assistance in collecting animals. We also thank Professor Peter Greenaway for helpful discussions and Dr. Adamczewska for critical reading of the manuscript. Thanks go also to the Conservators and Staff of Parks Australia North, on Christmas Island and on Cocos Island for their hospitality and help. This work and a Post-Graduate Scholarship to J.D.-C. were supported by ARC grants to S.M.

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Online publication: November 6, 1998
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