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A Shift to an Ion Regulatory Role by Gills of a Semi-Terrestrial Crab, *Ocypode stimpsoni*

Jyuan-Ru Tsai[1] and Hui-Chen Lin[1,2,*]

[1]*Department of Life Science, Tunghai University, Taichung 407, Taiwan*

[2]*Center for Tropical Ecology and Biodiversity, Tunghai University, Taichung 407, Taiwan*

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Jyuan-Ru Tsai and Hui-Chen Lin (2012) A shift to an ion regulatory role by gills of a semi-terrestrial crab, *Ocypode stimpsoni*. *Zoological Studies* **51**(5): 606-618. *Ocypode stimpsoni* is a highly terrestrial species of the Ocypodidae that faces challenges of both gas exchange and hypotonic stress when on land. The aim of this study was to investigate the impacts of terrestrial modifications on the ion regulatory mechanism of the gills in this semi-terrestrial crab. Our results showed that the lungs were the air-breathing organ and may take the place of anterior gills for gas exchange. Epithelial cells in the anterior gills changed from a thin epithelium for gas exchange to an intermediate type epithelium for ion regulation. We also found significantly higher Na^+, K^+-ATPase activity in the anterior gills when crabs were transferred to 3‰ and 5‰ diluted seawater. The ion regulatory proteins in both the anterior and posterior gills showed similar localization, an indication of the involvement of the anterior gills in ion regulation. Cell thicknesses and Na^+, K^+-ATPase activity suggest that the anterior gills can assist with ion regulation in acclimation to dilute seawater. We concluded that the presence of the lungs caused the gills to be modified for ion regulation. http://zoolstud.sinica.edu.tw/Journals/51.5/606.pdf

Key words: Air-breathing organ, Terrestrial adaptation, Lung, Ion regulation, *Ocypode stimpsoni*.

Crabs live in diverse habitats from ocean, marine euryhaline, intertidal, and bimodal environments to freshwater and terrestrial ones. To resolve problems of hemodilution and dehydration during freshwater and terrestrial invasions, strategies for water and ion conservation are important. The gills are a multifunctional organ in brachyuran crabs. Generally, most brachyuran crabs have 9 pairs of gills with distinct morphological differences specialized for both gas exchange and ion regulation (Barra et al. 1983, Péqueux 1995, Luquet et al. 2000). In addition, air-breathing crabs differ from marine crabs in their well-developed air-breathing modifications which are key to terrestrial adaptation (Morris 2002). Morphological changes were correlated with the tradeoff between gas exchange and ion regulation in the gills. This implies that air-breathing crabs should have reduced gill numbers and surface area from water loss and lamella collapse (Taylor and Taylor 1992, Takeda et al. 1996). The accessory air-breathing organ, the lung, is also an important modification. The structure and type of lungs differ among species, but the function of the lung is for enhancing gas exchange (Taylor and Taylor 1992, Maina and West 2005). The presence of the lung, an air-breathing organ, compensates for the loss of respiratory areas of the gills (Greenaway 1984). It also implies a physiological plasticity for ion regulation by the gills (Innes and Taylor 1986, Santos et al. 1987, Greenaway and Farrelly 1990, Farrelly and Greenaway 1992 1994 2005).

Osmoregulatory and ion regulatory mechanisms in crustaceans show great diversities related to their environments (Greenaway 1994, Péqueux 1995, Morris 2001). Marine euryhaline crabs use

*To whom correspondence and reprint requests should be addressed. Tel and Fax: 886-4-23500461. E-mail:hclin@thu.edu.tw

their posterior gills to maintain ion and solute homeostasis by increasing the activity of Na^+, K^+-ATPase (NKA), a well-studied ion transporting protein (Lucu and Flik 1999, Chung and Lin 2006). But evidence showed that increased NKA activity was found in the posterior gills in brachyuran crabs and also in the anterior gills of *Cyrtograpsus angulatus* for ion regulation during short-term acclimation to dilute seawater environments (Mañanes et al. 2002). The functional differentiation between the anterior and posterior gills deserves further investigation.

Two major models were proposed for the active absorption of ions by freshwater and seawater crabs (Morris 2001). In the seawater model, sodium influx is driven by the basolateral NKA coupled with an apical Na^+/H^+ exchanger (NHE) (Towle and Weihrauch 2001). Chloride influx is driven by cytoplasmic carbonic anhydrase (CA) coupled with an apical anion exchanger (AE) and a $Na^+/K^+/2Cl^-$ cotransporter (NKCC) (Towle and Weihrauch 2001). Recent studies showed that the apical V-type H^+-ATPase (VHA) is the key for freshwater adaptation in brachyuran crabs (Weihrauch et al. 2001, Morris 2001, Kirschner 2004). In the freshwater model, sodium influx is driven by an electrogenic force caused by apical VHA, and the chloride influx is also driven by cytoplasmic CA through the apical AE (Morris 2001, Towle and Weihrauch 2001).

In central Taiwan, *Ocypode* crabs are abundant and predominately distributed in the intertidal zone. This genus has a highly modified branchial chamber and other specialized structures in the gills, and it is the most terrestrial species in the Ocypodidae (Greenaway 1999). The present study focused on the tradeoff between gas exchange and ion regulation in the gills, with special emphasis on the effects of the presence of an extra-branchial air-breathing organ on the ion regulatory mechanisms in *O. stimpsoni*. However, the structure of the modified branchial chamber was not clear in the semi-terrestrial crab *O. stimpsoni*. The 1st aim of this study was to investigate the fine structure of the modified branchiostegite (lung) and the effect of the lung on functional differentiation of the gills. According to our previous study, we also found physiological divergence when crabs were acclimated to 5‰ diluted seawater for 7 d. *Ocypode stimpsoni* had no significant increase in NKA activity in the posterior gill (gill 6) (Tsai and Lin 2007). The 2nd aim of the study was to measure NKA activity in all gills and in a time-course experiments to clarify the ion regulatory strategy. In addition, we found a certain degree of variation among the 12 brachyuran crabs (Tsai and Lin 2007). Some of the euryhaline and intertidal crabs, like the semi-terrestrial crab *O. stimpsoni*, had an apical VHA but were not tolerant of a freshwater environment. Third, we looked for a more-complete ion regulatory mechanism in the gill epithelia.

MATERIALS AND METHODS

Animals and acclimation

Males of *Ocypode stimpsoni* in the intermolt stage with carapaces of 30 ± 5 mm wide were collected by hand from sandy habitats in the Gaomei wetland (24°19'25.6"N, 120°33'7.9"E), Taichung County, Taiwan. Crabs were kept in plastic containers (L 74 × W 50 × H 25 cm) with about 5-cm-deep aerated artificial seawater (35‰), and several plastic platforms were provided for the crabs to freely emerge from the water. Crabs were fed 2 or 3 times per week with frozen dried shrimp. Room temperature was kept at 25 ± 3°C with a 14-h light: 10-h dark photoperiod. After acclimation for at least 1 wk, crabs were randomly assigned to 3‰ and 5‰ diluted seawater treatment for measurements of Na^+, K^+-ATPase activity. The sampling time intervals were 0, 1, 4, and 7 d after transfer. Artificial seawater was made by mixing aerated local tap water with Coralife Scientific Grade Marine Salt (Central Garden & Pet Company, CA, USA).

Histological study

After 7 d of acclimation in 35‰ seawater, the crabs were kept on ice and quickly sacrificed by destroying the dorsal ganglia. The lungs and gills were carefully removed, immediately immersed in a fixative solution, and incubated for 15-18 h at 4°C. The fixative solution was 4% paraformaldehyde, 5% glutaraldehyde, and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4). Tissues were washed in phosphate-buffered saline (PBS) until the color of the waste PBS turned from light-yellow to transparent. The osmolality of both the fixative solution and the PBS were adjusted to the hemolymph osmolality by adding solid sucrose (Merck KGaA, Darmstadt, Germany). All tissues were dehydrated in a 50%, 75%, and 95% ethanol series, followed by 50%, 75% and 100% LR White™ Resin (London Resin Company Ltd.,

London, UK). Finally, tissues were embedded in a gelatin capsule and incubated in an oven at 50°C for 22 h. Semi-thin sections (0.6 μm) were stained with 1% toluidine blue, observed by a light microscope (E600, Nikon), and photographed with a digital camera (D1, Nikon). For ultrastructure observations, ultrathin sections (80-90 nm) were post-stained with 2% uranyl acetate for 15-18 h. Images were observed and photographed on a transmission electron microscope (H-7000, Hitachi).

Immunofluorescence and immunohistochemical (IHC) studies

Gills were removed and fixed in 4% para-formaldehyde in 0.1 M PB for 4-6 h on ice. For the immunofluorescence studies, gills were washed in PBS 3 times for 20 min each, perfused in 30% sucrose, and then embedded in O.C.T. compound (Tissue-Tek® O.C.T. Compound 4583, Sakura Finetechnical Co., Ltd., Japan) for cryosectioning. For the IHC studies, gills were washed 3 times for 20 min each, dehydrated in a graded ethanol and xylene series, and then embedded in paraffin. Sections (5-8 μm) were incubated with antibodies, and localization of each protein was observed with a confocal microscope (LSM 510, Zeiss) or a light microscope (E600, Nikon). Negative control experiments on each slide which applied PBS instead of the primary antibody showed no specific staining in our results.

Antibodies

The mouse monoclonal antibody (mAb) against the α-subunit of the avian sodium pump (α5) and the mouse mAb against the human NKCC (T4) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). The antibody of VHA was a rabbit polyclonal antibody (pAb) against the rat VHA A-subunit (Wako Pure Chemical Industries, Japan). The antibody of the NHE was a rabbit pAb against the dace NHE and was kindly provided by Prof. Hirose (Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan). For the IHC studies, we stained gill sections with a commercial kit containing a secondary antibody HRP/Fab polymer conjugate and aminoethyl carbazole (AEC) single solution chromogen (PicTure-Plus™, Zymed, USA). To reduce cross reactions of the antibodies when double staining in the immunofluorescence studies, we used a highly cross-absorbed Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) antibody (Molecular Probes, USA) and a Cy3-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, USA).

Protein extraction

After acclimation for 7 d in 5‰ diluted seawater (DSW), crabs were quickly sacrificed, and the gills were cut into small pieces, placed in homogenizing medium, and homogenized with an ultrasonic processor (Sonics & Materials, Inc, USA). The homogenizing medium was premixed with a protease inhibitor cocktail containing 2 mM antipain, 1 mM leupeptin, 10 mM benzamidine, and 5-10 mM aprotinin (all proteinase inhibitors were purchased from Sigma). The homogenate was first centrifuged at 4°C and 6000 xg for 15 min, and then the supernatant was centrifuged at 4°C and 20,160 xg for 20 min. The final supernatant was used to measure the NKA activity and run Western blotting to detect the antibody specificity. The assay for determining the protein concentrations was conducted according to a protocol described in Tsai and Lin (2007) and used Bio-Rad protein assay (500-0002, Bio-Rad). The absorbance was read at 695 nm on a spectrophotometer (U-2001, Hitachi).

NKA activity

NKA activity was assayed by adding the protein extract to 400 μL of reaction medium (the ouabain-free group contained 20 mM imidazole, 100 mM NaCl, 30 mM KCl, and 10 mM $MgCl_2$ at pH 7.4; the ouabain group contained 20 mM imidazole, 130 mM NaCl, 10 mM $MgCl_2$, and 1 mM ouabain at pH 7.4). The ouabain group had no KCl to ensure that no K ion was available for pumping before NKA was fully inhibited by ouabain. This protocol was modified from Tsai and Lin (2007). The reaction was begun by adding 100 μL of the ATP stock solution (25 mM Na_2ATP) and incubating it at 30°C for 15 min, and it was stopped by adding 200 μL of an ice-cold trichloroacetic acid solution (30% w/v). After centrifugation at 1640 xg and 4°C for 10 min, an aliquot of 500 μL of the supernatant was taken, and the inorganic phosphate concentration was measured using a colorimetric method by adding 1 ml ice-cold Bonting's color reagent (560 mM H_2SO_4, 8.10 mM ammonium molybdate, and 176 mM $FeSO_4$). The color was allowed to develop in a water bath at

20°C for 20 min. The concentration was measured at 700 nm (U-2001 Spectrophotometer, Hitachi). The enzyme-specific activity of NKA was defined as the difference between the level of inorganic phosphate liberated in the reaction medium in the presence and absence of ouabain.

Statistical analysis

All results from this study are expressed as the mean value ± standard deviation (S.D.). Differences in NKA activity among the 3‰, 5‰, and 35‰ salinity treatments and sampling times were assessed by a two-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. Differences in the thicknesses of the 2 types of lamellar cells were assessed by a two-way ANOVA followed by Duncan's multiple-range test. SAS software (vers. 9.1; SAS, Cary, NC, USA) was used.

RESULTS

Modifications in the branchial chamber

Ocypode stimpsoni has only 5 major gill pairs, named gills 3-7 according to their position. The smallest anterior gill, gill 3, was not examined in this study. The branchial chamber was surrounded by a branchiostegal membrane (under the dorsal carapace, defined as the branchiostegal lung) and by an epibranchial membrane (above the thoracic wall and facing the branchiostegal membrane, defined as the epibranchial lung), and the dorsal-anterior parts of these 2 types of lungs were connected to each other (Fig. 1A). These branchial membranes were well developed in *O. stimpsoni*. The inner linings had evolved complex, evaginated, 3-dimensional tubular systems (without an opening) facing the branchial chamber with numerous air channels (Fig. 1B). Tissue sections showed that the tubular systems were filled with numerous branching air sacs (Fig. 2A). The inner sides of the air sacs were supported by connective tissue, and epidermal cells were located around the outer side of the air sacs (Fig. 2B-D). A similar arrangement was found on the branchial chamber side (Fig. 2B), through the middle of the lung (Fig. 2C), then to the carapace side (Fig. 2D) of the lung. These 2 cell types were distinguished by the shape of their nuclei and the location of the cells. The oval-shaped nucleus of epidermal cells could be easily distinguished from the round-shaped nucleus of connective tissues. Hemocytes were found in the large blood sinus between the epidermal cells and connective tissues (Fig. 2B, C). The large hemolymph sinus branched into a small hemocoel on the outer side of the air sac just between the epidermal cells and cuticles. The ultrastructure of the hemolymph-gas barrier in the air sacs showed a very thin cuticle and epithelial cells (Fig. 2E, F) ranging 40-200 nm, which was about 1/100 to 1/5 the thickness of the posterior gill.

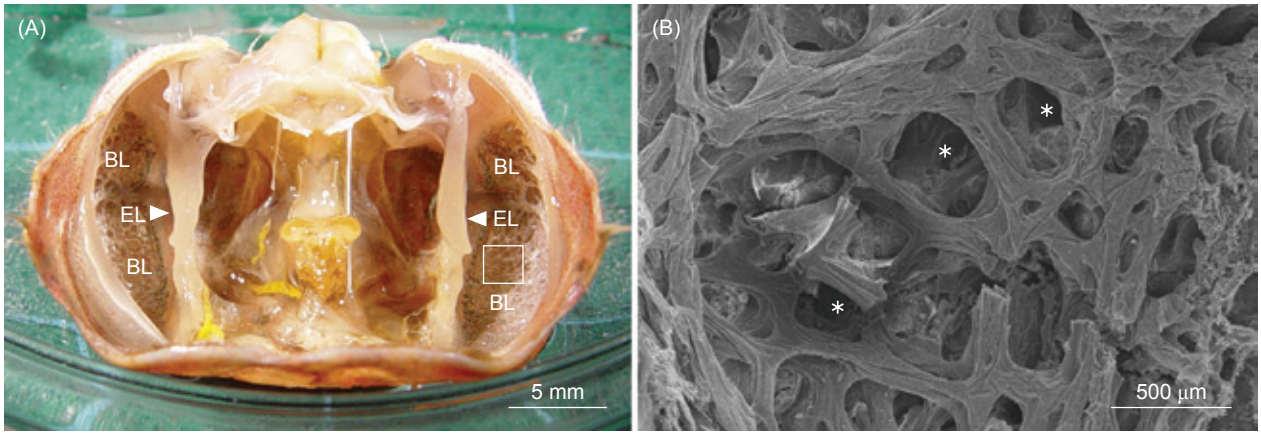

Fig. 1. Gross anatomy of the lung of *O. stimpsoni*. (A) An inside-out view of the branchial chamber showing the 2 parts of the lung. The branchiostegal lung (BL) is located on the dorsal side of the branchial chamber and is attached to the carapace. The epibranchial lung (EL, arrowhead) is evaginated from the lower side of the branchial chamber and faces the branchiostegal lung. (B) Higher magnification of the BL from the square in figure A. The lung structure shows a complex tubular system with air channels (asterisk).

Structure of the gill epithelia

In all gill lamellae, there were 2 major types of cells: flat epithelial cells and pillar cells (as defined by Goodman and Cavey 1990). Hemocytes and connective tissue were often found in the center of the lamella (Fig. 3A-D). Pillar cells were funnel-shaped, and the bottom of a pillar cell was always connected to another pillar cell on the opposite side in the gill lamellae (Fig. 3A-D). The flat epithelial cells had more-uniform thicknesses than did pillar cells. The thicknesses of the flat cells in gills 4-7 were measured from the top of the apical microvilli, through the nucleus, to the basal lamina.

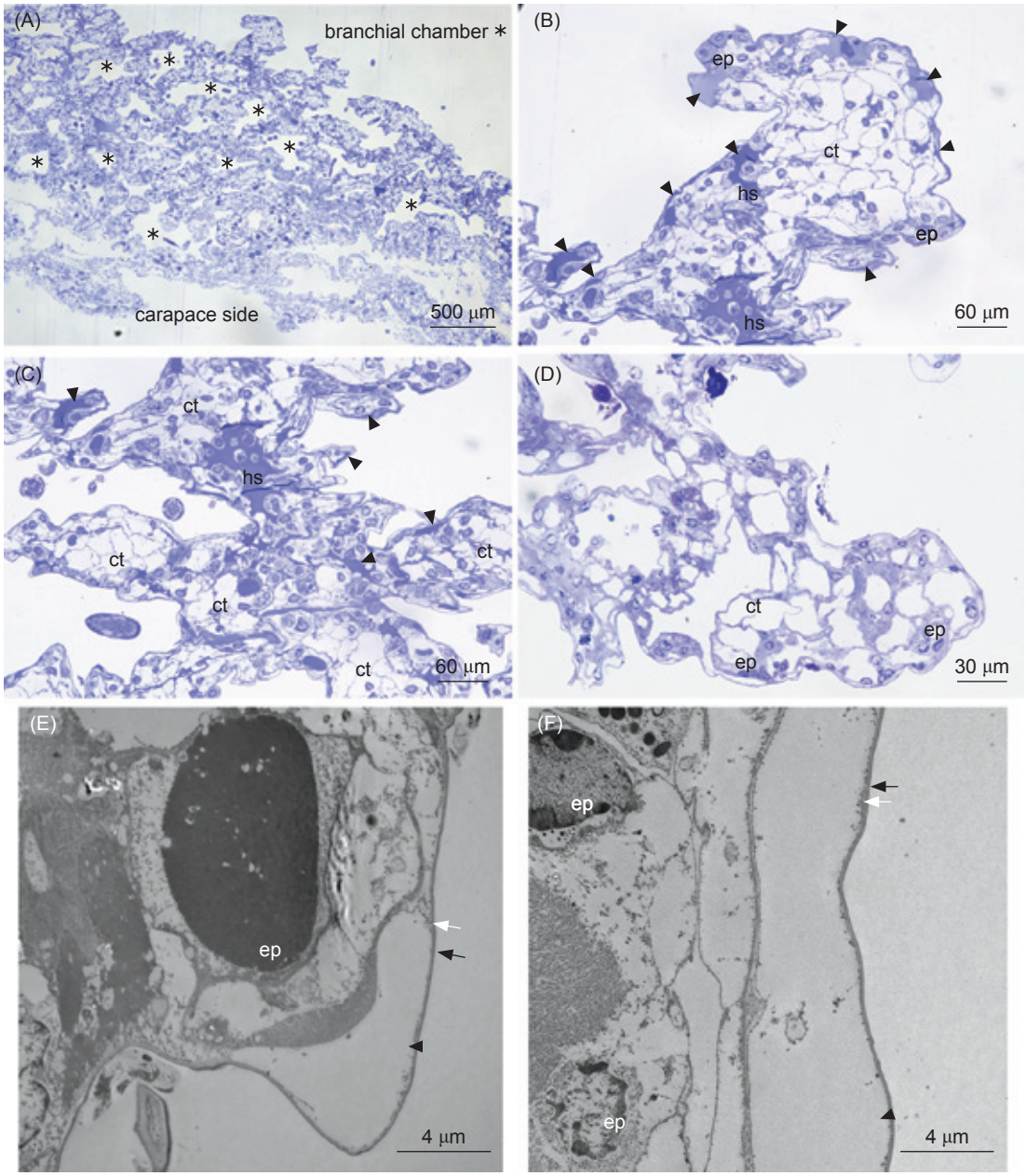

Fig. 2. Structure of the branchiostegal lung (BL) of *O. stimpsoni*. (A) Cross-section of the BL showing the branching tubular system with air channels (asterisks). The air channels are continuously distributed from the branchial chamber (B), through the middle of the lung (C), to the carapace side (D). Higher magnification sections of the tubular system show the loose connective tissue (ct) surrounded by epidermal cells (ep). A large hemolymph sinus (hs) is located inside the middle of the tubular system, and branches into a small hemocoel in the outer side of the tubular system and the end air sac. (E, F) Hemolymph-gas barrier composed of a thin cuticle (black arrows) and flanges of epidermal cells (white arrows) in the margin of the air sac. Asterisk, air channel; arrowhead, hemocoel.

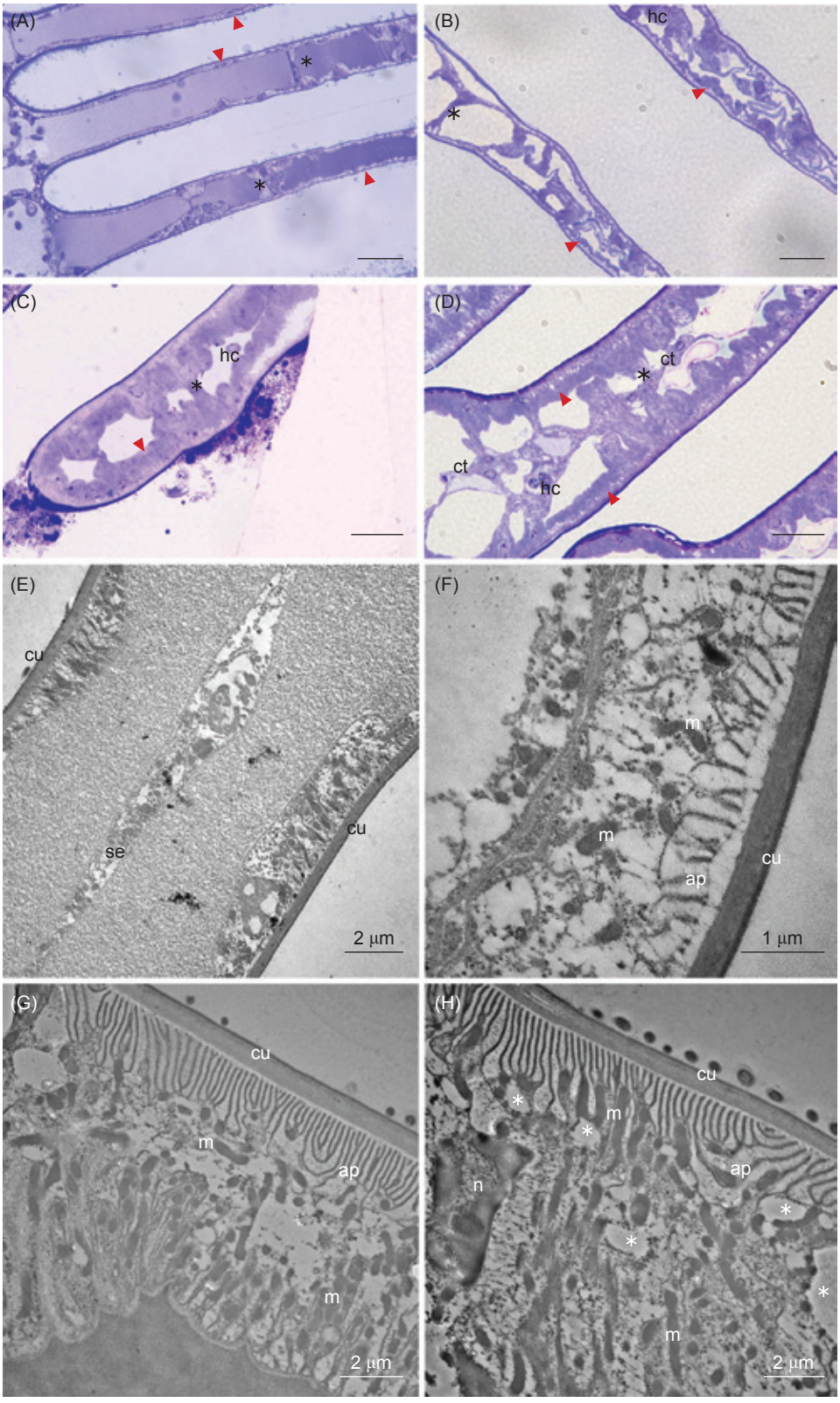

Fig. 3. Structure of epithelial cells in gills of *O. stimpsoni*. (A-D) Semi-thin sections of gills 4 (A), 5 (B), 6 (C) and 7 (D). Two types of cells were found in each gill lamella: pillar cells (∗) and flat epithelial cells (arrowhead). Scale bar = 20 μm. (E-H) Ultrastructure of gills 5 (E, F) and 6 (G, H). Both gills 5 and 6 had apical microvilli (ap) and mitochondria (m). hc, hemocyte; ct, connective tissue; cu, cuticle; se, septum; n, nucleus.

Pillar cells were thicker than flat cells (Table 1). The thickness of the epithelial cells in the anterior gills (gill 4 and 5) was 4.3-5.5 μm, and that in the posterior gills (gill 6 and 7) was 16.1-23.1 μm. Sections of both the anterior and posterior gills showed features of ion regulatory epithelia with numerous mitochondria, apical microvilli, and basolateral folding (Fig. 3E-H). Both flat and pillar cells in the gill lamella had the same features of ion regulatory epithelia.

NKA activities in the gills

In the gills, NKA activities were significantly higher in the posterior gills (gills 6 and 7), and NKA activities did not change in gills 4, 6, and 7 when *O. stimpsoni* was transferred from 35‰ to 5‰ diluted seawater for 7 d (Fig. 4A) (similar to our results in Tsai and Lin 2007). However, NKA activities increased in all gills (including both the anterior and posterior gills) after the crabs were transferred from 35‰ to both 3‰ (Fig. 4A) diluted seawater for 4 d and 5‰ diluted seawater for 4 d (Fig. 4B). When crabs were transferred to 5‰ diluted seawater for 7 d, the NKA activity decreased to that of the control level (35‰) seawater except in gill 5 (Fig. 4A). NKA activities in the 35‰ and 5‰ diluted seawater groups for 7 d in figure 4A and 4B were the same measurements.

Localization of ion regulatory proteins in the gills

The specificity of the antibodies is shown in figure 5. NKA, the NHE, and VHA were detected with a single band pattern in gills with respective molecular weights of 100, 70, and 65-70 kDa. Two bands of the NKCC were detected with a molecular weight of about 150-160 kDa. The protein abundance of the NKA increased in 3‰ extremely

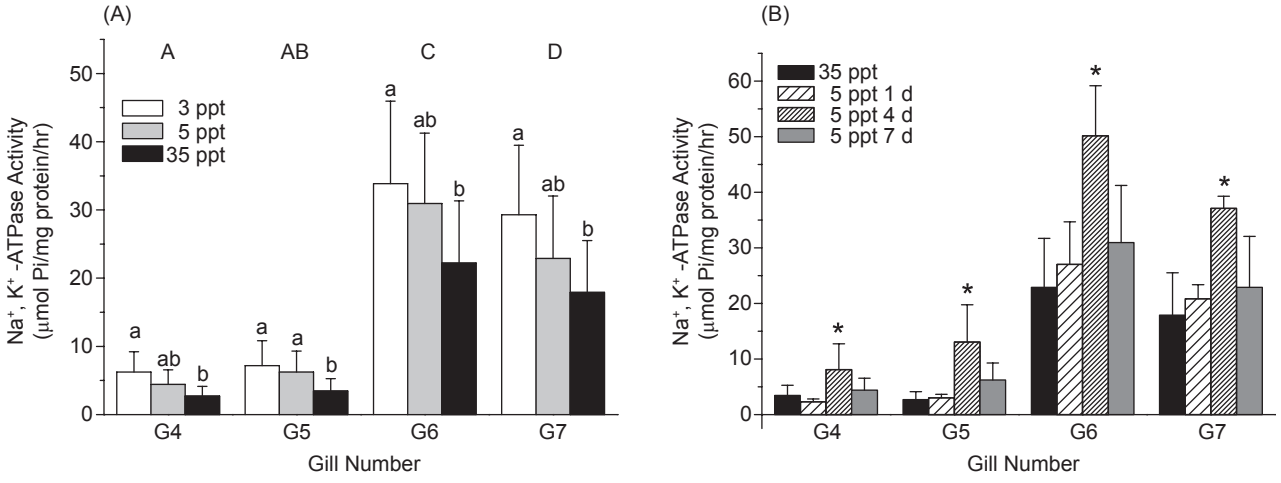

Fig. 4. Na^+, K^+-ATPase (NKA) activity in gills 4 to 7 of *O. stimpsoni*. (A) NKA activities in salinity acclimation groups. NKA activity increased in all gills after acclimation in 3‰ extremely diluted seawater (EDSW) for 4 d. There was no significant difference between crabs transferred to 5‰ diluted seawater (DSW) with the control group for 7 d, except in gill 5. Black bars indicate the 35‰ seawater (SW) treatment as the control group. Black, gray, and dark-gray bars respectively indicate crabs transferred to 5‰ DSW for 1, 4, and 7 d. Gill 6 had the highest NKA activity of all gills, followed by gill 7 (grouped by capital letters). (B) Time-course measurements of NKA activities after crabs were transferred to 5‰ SW for 1, 4, and 7 d. NKA activities in all gills had significantly increased (star) on the 4th day after transfer. These results were analyzed by a two-way ANOVA followed by Duncan's multiple-range test.

Table 1. Thicknesses of the 2 types of lamellar cells in each gill

	Gill 4	Gill 5	Gill 6	Gill 7
Flat cells	4.30 ± 1.35^a	5.51 ± 1.58^a	8.21 ± .93^b	12.74 ± 2.95^c
Pillar cells	13.50 ± 3.01^c	12.37 ± 2.53^d	16.13 ± 2.44^d	23.07 ± 6.67^e

Values are the mean ± standard deviation. All values were determined by ImagePro Plus software (vers. 4.5). Superscript letters indicate groupings by Duncan's multiple-range test ($p < 0.05$).

diluted seawater (EDSW) compared to that in 35‰ seawater (SW) treatment. The protein abundance of VHA did not significantly differ between the EDSW and SW treatments (Fig. 5B).

NKA was located in the basolateral membranes of the 2 types of epithelial cells (Fig. 6A). The NHE, NKCC, and VHA were located on the apical membrane and subapical space (Figs. 6B, 7). No difference in the localization of the ion transporters was found between the anterior and posterior gill epithelia or between the cell types in the gill lamella.

DISCUSSION

Structural modifications for terrestrial adaptation

In brachyuran crabs, the gills are an organ with multiple functions, including at least respiration, ion regulation, ammonia excretion, and acid-base balance. Most brachyuran crabs have at most 9 pairs of gills with different degrees of morphological modifications. For example, the gills can be further divided into anterior gills specialized for gas exchange and posterior gills for ion regulation (Barra et al. 1983, Péqueux 1995, Luquet et al. 2000, Lin et al. 2002, Chung and

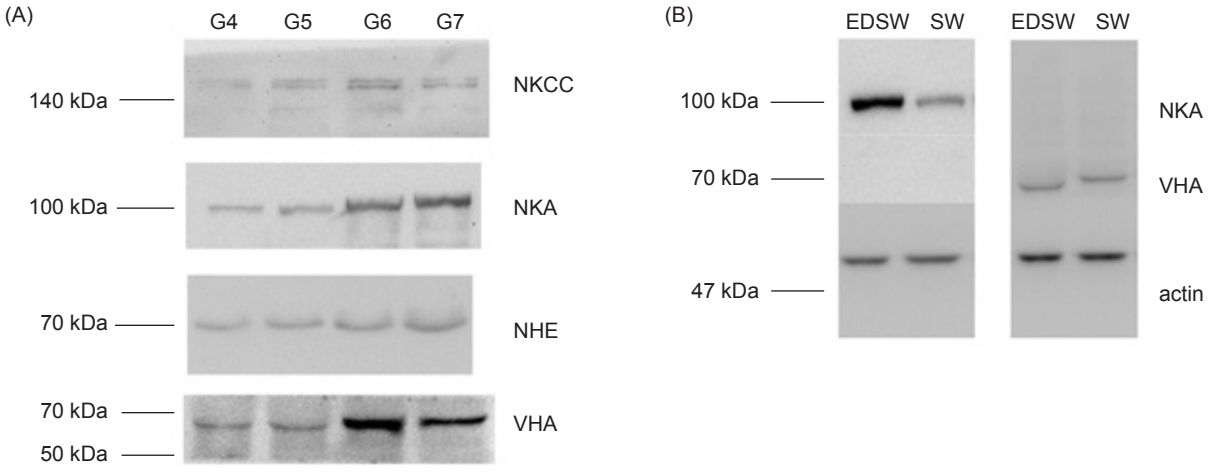

Fig. 5. (A) Abundance of ion regulatory proteins in gills 4-7. Two bands of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) were detected with a molecular weight of about 150-160 kDa. A single band of Na^+, K^+-ATPase (NKA) was detected with a molecular weight of 100 kDa. A single band of the Na^+/H^+ exchanger (NHE) was detected with a molecular weight of 70 kDa. A single band of V-type H^+-ATPase (VHA) was detected with a molecular weight of about 65-70 kDa. The total protein concentration of each lane was 15 μg. (B) Abundances of NKA and VHA in gill 6. The protein abundance of NKA increased in 3‰ extremely diluted seawater (EDSW). The protein abundance of VHA did not significantly differ between the salinities.

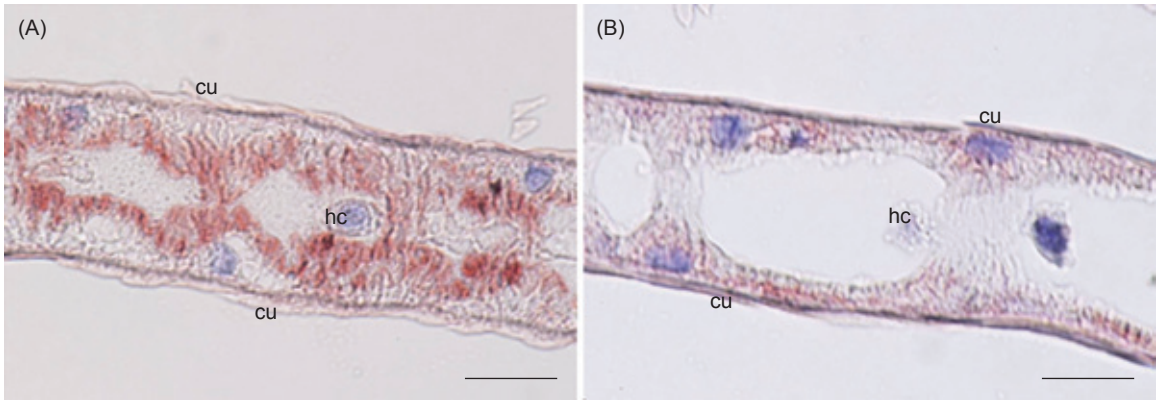

Fig. 6. Localization of Na^+, K^+-ATPase (NKA) and V-type H^+-ATPase (VHA) in the gill epithelia. (A) NKA was located in the basolateral membrane of the epithelia. (B) VHA was located in the apical membrane and subapical region. cu, cuticle; hc, hemocyte. Scale bar = 20 μm.

Lin 2006). For gas exchange, epithelial cells in the anterior gills (gills 1-5) are very thin (1-5 μm). For ion regulation, epithelial cells in the posterior gills (gills 6-9) are thick (10-20 μm) with a greater amount of mitochondria and membrane folding that in turn increases the blood-gas diffusion interface (Péqueux 1995). Such functionally differentiated gills are very common in marine and euryhaline crabs like *Carcinus maenas* (Lucu and Flik 1999), *Scylla paramamosain* (Chung and Lin 2006), *Cyrtograpsus angulatus* (Mañanes et al. 2002), *Chasmagnathus* (*Neohelice*) *granulatus* (Halperin et al. 2000, Luquet et al. 2000 2002), and 4 *Uca* species (Lin et al. 2002). However, *Ocypode stimpsoni* has only 5 conspicuous gill pairs. Gills 6 and 7 have typical ion regulatory epithelial cells with apical microvilli, mitochondria, basolateral folding, and thick cells, while gills 4 and 5 have a cell type that is between gas exchange and ion regulatory cells (Fig. 3). Well-developed apical microvilli were found in flat cells of gills 4 and 5, and cell thicknesses (at 4.3-5.5 μm) were also relatively thicker than those in typical respiratory gill epithelia (at 1-5 μm) (Taylor and Taylor 1992, Péqueux 1995).

The dehydration stress of terrestrial life may be related to the reduction in gill numbers/areas, and their various modifications, including the thickened cuticle, nodules, and lung-like structure (Taylor and Taylor 1992, Farrelly and Greenaway 1992). The characters for terrestrial adaptation such as lamellar interdigitation and nodules (pers. observ.) were also found in 2 other Ocypodids, *O. cordimanus* and *O. ceratophthalmus* (Greenaway and Farrelly 1984). It is likely that the modifications of the gills and branchial chamber in air-breathing crabs are all for terrestrial adaptation, but this suggestion is only based on data from the Ocypodidae family. It was proposed that the reduced gill number is a kind of structural economy for the complex lung structure (Farrelly and Greenaway 1987). The interdigitation of the gill lamellae may be a strategy for structural economy in the branchial chamber.

Lung structure in brachyuran crabs

The specialized lung structure of air-breathing crabs has been extensively studied, especially in terrestrial/land crabs. A number of species were reported to have a specialized lung structure or a modified circulatory system. Different types of lungs in different crabs are summarized in table 2, including *O. cordimanus* (Ocypodoidea) and *Mictyris longicarpus* (Mictyridae) in the Ocypodoidea; *Geograpsus grayi* (Grapsodae), *Geo. crinipes* (Grapsodae), *Cardisoma hirtipes* (Gecarcinidae), *Gecarcoidea natalis* (Gecarcinidae), and *Hemigrapsus nudus* (Varunidae) in the Grapsoidea; and *Holthuisana transversa* (Parathelphusidae) in the Potamoidea (Taylor and Greenaway 1984, Greenaway and Farrelly 1984 1990, Farrelly and Greenaway 1987 1992). Common characteristics of these lungs are an increased surface area in the branchial chamber and a very thin gas/blood diffusion distance. The average thickness of the gas/blood distance in both the branchiostegal and epibranchial lungs of *O. stimpsoni* was about 40-200 nm, which is similar to observations in *H. transversa*, another air-breathing crab (Taylor and Greenaway 1979). Furthermore, the diameter of the hemocoel sinus (or lacunar bed) was in the same range as that of other crab species. Lungs can be classified into

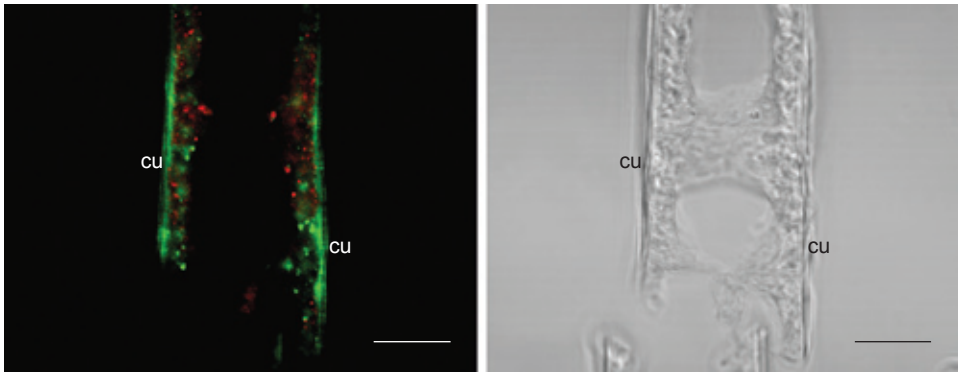

Fig. 7. Localization of the Na^+/H^+ exchanger (NHE) and the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) in the gill epithelia. Left: The red color indicates localization of the NHE, and the green color indicates localization of the NKCC. The NKCC and NHE were both located near the apical region. Right: Light microscopic image of the same tissue slice. cu, cuticle. Scale bar = 20 μm.

several types according to their complexity, shape, and phylogenetic relationships (Table 2). Complex 3-dimensional specializations are mostly found in the Ocypodidae and Mictyridae. Although the most complex lung structures are only found in the Ocypodoidea, *Uca* species show a simple smooth lung type similar to those in the Grapsoidea (pers. observ.). Farrelly and Greenaway suggested that the complex lung system may be a response to evolutionary changes in the portal circulatory system. These diverse lung structures are the results of convergent evolution (Farrelly and Greenaway 1993). By comparing the lung types and gill numbers with phylogenetic relationships among brachyuran crabs, we found that *Ocypode* species had compact lungs with reduced gill numbers, whereas the others had expanded branchial volumes with normal gill numbers. The highly modified branchiostegite (lung) replaced the respiratory function which was believed to be carried out by anterior gills in *O. stimpsoni*. However, some terrestrial/bimodal species of brachyuran crabs such as *Neohelice* (*Chasmagnathus*) *convexus* and *Chiromantes dehaani* do not have highly modified branchiostegites and maintain the 9 pairs of gills (unpubl. data). These different lung structures may reflect different strategies of terrestrial adaptation and may correspond to terrestriality of the crab (Morris 2002). The evolutionary diversity of lung structures deserves more effort to elucidate the entire picture of terrestrial adaptation in decapod crustaceans.

The tradeoff between gas exchange and ion regulation

In previous studies on marine and euryhaline crabs, the ability to maintain ionic homeostasis under decreasing environmental salinity was attributed to increases in NKA activity, especially in the posterior gills (Péqueux 1995). NKA activities in the posterior gills were relatively higher than those in the anterior gills during seawater acclimation (Fig. 4). This indicates that the posterior gills are a major site of the ion regulatory mechanism in daily life. However, the increased NKA activity in the anterior gills of *O. stimpsoni* demonstrated that the anterior gills also have the ability to assist with ion regulation in EDSW (3‰). Other studies also revealed physiological plasticity in the anterior gills of crabs. The euryhaline crab *Cyrtograpsus angulatus* increases NKA activity in anterior gills when facing a diluted seawater

Table 2. Lung types and properties in brachyuran crabs

Superfamily	Family	Habitat*	Species	Lung type**			Reference
				Cp/Ex	Sm/Ev/Iv/Un	2D/3D	
Ocypodoidea	Ocypodidae	T	*Ocypode ceratophthalmus*	Cp	Ev	3D	3
		T	*Ocypode cordimanus*	Cp	Ev	3D	1
		T	*Ocypode stimpsoni*	Cp	Ev	3D	This study
		BI	*Uca coarctata*	Cp	Sm	2D	3
		IT	*Uca arcuata*	Cp	Sm/Un	2D	5
		IT	*Macrophthalmus banzai*	Cp	Sm/Un	2D	5
	Mictyridae	IT	*Mictyris longicarpus*	Cp	Iv	3D	1, 3
Grapsoidea	Grapsidae	BI	*Hemigrapsus nadus*	Ex	Sm	2D	2
		T	*Geograpsus grayi*	Ex	Sm	2D	3
	Gecarcinidae	T	*Discoplax hirtipes* [a]	Ex	Sm	2D	3
		T	*Cardisoma carnifex*	Ex	Sm	2D	3
		T	*Gecarcoidea natalis*	Ex	Sm	2D	2
	Varunidae	BI	*Neohelice granulatus* [b]	Ex	Sm	2D	4
		BI	*Neohelice convexus* [b]	Ex	Sm/Un	2D	5
Potamoidea	Parathelphusidae	Fw/BI	*Holthuisana transversa*	-	Sm	-	2, 3

*Habitat: T, terrestrial; IT, intertidal; BI, bimodal; Fw, freshwater. [a]Past genus name: *Cardisoma*; [b]past genus name: *Chasmagnathus*. **Lung type: Cp, compact; Ex, expanded; Sm, smooth; Ev, evaginated; Iv, invaginated; Un, unclear lung type, indicating that no high-quality data were obtained by paraffin section; 2D: the lung extended in a 2-dimensional direction; 3D, the lung extended in a 3-dimensional direction; -, data not available. References: 1, Farrelly and Greenaway 1987; 2, Greenaway and Farrelly 1990; 3, Farrelly and Greenaway 1993; 4, Halperin et al. 2000; 5, unpublished data from Tsai.

environment (Mañanes et al. 2002). The semi-terrestrial crab *Chasmagnathus* (*Neohelice*) *granulatus* also shows an instantaneous increase in NKA activity in both the anterior and posterior gills upon exposure to 2‰ seawater (Castilho et al. 2001). In our observation, not every terrestrial/bimodal crab has the modified branchiostegite (lung). In *O. stimpsoni*, we found that the modified lung played a role of anterior gills in respiration, and the anterior gills shifted to assist with ion regulation under salinity stresses. *Neohelice* sp. and *Chiromants* sp. are also highly terrestrial crabs but maintain 9 pairs of gills with a smooth branchiostegite (pers. observ.). Furthermore, the main ion regulatory sites are the anterior gills, not the posterior gills, in the true freshwater crab *Candidiopotamon rathbunae* (Tseng 2008, Wang 2011). One must be very cautious in drawing binary conclusions on the functional differentiation of gills in various crabs, and the functional differentiation of gills may be related to modifications in the branchial chamber (lung and numbers of gill pairs).

Possible transitions of the ion regulatory mechanism in air-breathing crabs

NKA activity is an indication of an active ion regulatory ability when crabs face ionic stress (Lin et al. 2002, Chung and Lin 2006). Differences in air-breathing strategies may reflect a physiological continuum from aquatic to terrestrial environments, and all variations in different crab species might be in transitional states in the evolution of air-breathing crabs (Morris 2002). With the juxtaposition in lung types, we found that there were at least 2 ways for crabs to invade land. Members of the Ocypodoidea have highly complex lung types with decreased gill numbers, while members of the Grapsoidea have evolved an expanded branchial chamber so as to retain most of their gills (Table 2). In addition, rearrangement of the branchial chamber provides another means of maintaining ion homeostasis and compensating for the smaller number of gill pairs. Aside from modifications of the lungs and gills, the presence of the portal circulatory system in brachyuran crabs may have been the key factor for later terrestrial adaptation (Taylor and Greenaway 1984, Farrelly and Greenaway 1993). The combination of the specialized portal system and the development of an air-breathing organ, the lung, may have facilitated the functional switch in the gills.

A proposed model of ion regulation in *Ocypode stimpsoni* gills

This study provides direct evidence of the localizations of the 2 important transporters, NHE and NKCC (or NCC), in the gill epithelium (Fig. 8). Ion regulatory models of brachyuran crabs were mostly derived from electrophysiological studies (Morris 2001, Freire et al. 2008). Localizations of the NKCC (or NCC) and NHE provide additional support for apical Na^+ ion uptake. The apically located NKCC is for Na^+ uptake and was only proposed to be effective in brackish water (Morris 2001, Kirschner 2004), but no direct evidence was presented. Our results provide more-direct evidence for the Na^+ uptake pathway in the crab gill epithelium. Furthermore, we discovered that apical VHA was colocalized with the apical NHE (Fig. 8). An acid-trapping model was proposed in which the apically located NHE may also be linked to ammonia excretion (by the Rh protein) in the euryhaline crab *Carcinus maenas* (Weihrauch et al. 2004 2009). The apical NHE/VHA/Rh transporter package is for ammonia excretion in aquatic crustaceans, including crayfish and terrestrial crabs (Morris 2001, Kirschner 2004, Weihrauch et al. 2004). But the role of the NHE between ion regulation and ammonia excretion is hard to dissociate in an ion-transporting epithelium. In the freshwater model, apical VHA was thought to be the driving force of ion flow (Morris 2001, Kirschner 2004). *Ocypode stimpsoni* could not

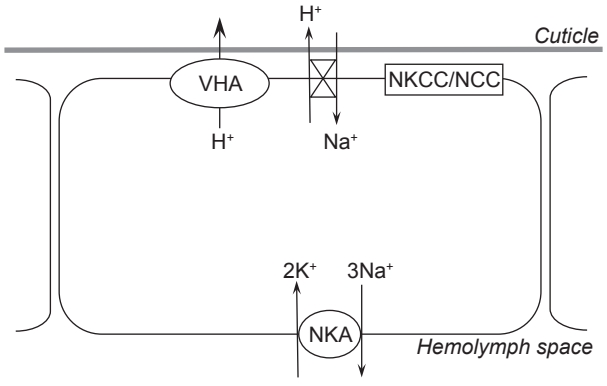

Fig. 8. Proposed model of active ion uptake in the semi-terrestrial crab *O. stimpsoni*. In general conditions, Na^+ influx through the apical $Na^+/K^+/2Cl^-$ cotransporter (NKCC) (or Na/Cl cotransporter (NCC)) and Na^+/H^+ exchanger (NHE) was normally powered by basolateral Na^+, K^+-ATPase (NKA). In a diluted seawater environment, V-Type H^+-ATPase (VHA) may be involved in providing the electrogenic force.

survive in fresh water for over 12 h (pers. observ.). Our results suggest that apical VHA might provide an additional driving force for ion regulation and also have other roles, such as facilitating ammonia excretion in ion-transporting epithelia.

This study revealed a trade-off between gas exchange and ion regulation in *Ocypode stimpsoni*. The lung structure is responsible for gas exchange, whereas all of the gills are for ion regulation. The physiological function of the intermediate-type anterior gills is to assist with ion regulation when crabs are transferred to a diluted seawater environment.

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