摘要

淡水蟹為高張調節者，在低離子含量的環境中維持其體液滲透壓。觸角腺與鰓為十足目甲殼類中負責滲透壓調節的器官。其中，鰓直接接觸外界，為一表面積高度特化且具有鈉鉀幫浦與氫離子幫浦的器官。鈉鉀幫浦（Na^+,K^+-ATPase， NKA）與氫離子幫浦（V-type H^+-ATPase，VHA）在高張調節的螃蟹中，被視為離子運輸的驅動力來源。另外，十足目甲殼類的排泄器官－觸角腺，可能具有維持其體液滲透壓的功能。雖然觸角腺在結構上及功能上與哺乳動物的腎臟相似，但目前對於螃蟹觸角腺滲透壓調節的功能並不清楚。淡水蟹為研究螃蟹高張調節合適的模型。拉氏清溪蟹（*Candidiop otamon rathbunae*）為典型淡水蟹，在本實驗被選擇作為高張調節者模型。本實驗目的有二：一、探討觸角腺是否具有離子或滲透壓調節能力；二、氫離子幫浦在離子吸收上扮演的角色。本研究的第一個部份，以去離子水設定為離子壓力的環境。螃蟹於實驗室條件馴養兩週後轉移到去離子水中一週。測量其鈉鉀幫浦活性與表現量，以及其尿液及血液滲透壓及離子組成。觸角腺鈉鉀幫浦的活性轉移後的第一天最高隨後降低。而鰓上鈉鉀幫浦活性在轉移至去離子水四天後最高。觸角腺鈉鉀幫浦表現量轉移到去離子水後下降而

鰓上的鈉鉀幫浦表現量則上升。離子組成的數據顯示觸角腺吸收鈣離子、鉀離子以及氯離子，但是排出鈉離子與鎂離子。鈉鉀幫浦被標定在迷路細胞的基底膜上。顯示迷路細胞是主要負責離子調節的位置。第二個部份的研究目的為測試氫離子幫浦在離子吸收功能中所扮演的角色。利用快速增殖cDNA端點技術找出拉氏清溪蟹氫離子幫浦表現的序列。結果顯示拉氏清溪蟹具有兩種不同型的氫離子幫浦-B 次單位（L from 以及S form）。其mRNA表現量在低氯環境中顯著高於低鈉環境。顯示其氫離子幫浦的功能為負責氯離子吸收。由本實驗的結果推測，淡水蟹觸角腺的功能主要為離子調節而非滲透壓調節。由本研究的結果，我提出一個新的氯離子吸收模型：位於基底膜的氫離子幫浦與頂膜的HCO_3^-/Cl^-交換者以及細胞質內的carbonic anhydrase一同作用，負責鹼的排除以及氯離子吸收。

Abstract

Freshwater crabs are hyperosmoregulators that maintain hemolymph osmolality in environments with low ion content. Antennal gland and gills are the two candidates for osmoregulation of decapod crusdaceans. Gills have direct contact with surrounding water. In gills of hyperosmoregulatory crabs, Na^+,K^+ -ATPase (NKA) and V-type H^+-ATPase (VHA) in gills are considered as the driving force of ion uptake from ambient water. The antennal gland, the excretory organ of decapod crustaceans, may also maintain osmolality of crab hemolymph. Although the structure and function of the antennal gland are similar to mammalian kidneys, the osmoregulatory ability of crabs remains unclear. The freshwater crab, *Candidiopotamon rathbunae*, was chosen for this study because it is a good model for studying hyperosmoregulation in crustaceans. The purposes were to investigate the iono- or osmo-regulatory ability of the antennal gland and the function of VHA in ion absorption. Deionized water (DW) was the ionic stressor. I recorded NKA activity, ionic composition, and osmolality of hemolymph and urine. In DW, NKA activity of the antennal gland and gills were highest on the first and fourth days, respectively. Increased NKA activity indicates increased ion uptake rate. *Candidiopotamon rathbunae* produced isosmotic, but not isoionic, urine. Ionic concentrations of hemolymph in DW remained constant in the first four days. Ion

composition of urine showed the antennal gland reabsorbed Ca^{2+}, K^{+}, and Cl^{-}, but secreted Na^{+} and Mg^{2+}. The NKA was then localized in the basolateral membrane of labyrinthine cells. To test VHA function in osmoregulation, I cloned two isoforms of VHA B-subunit from gills of *C. rathbunae* (L and S forms). The mRNA of both isoforms of VHA increased in gill 5 and gill 9 in crabs acclimated in low Cl^{-} artificial freshwater for 96 hours, but not in those acclimated in the low Na^{+} artificial water. Therefore, VHA participated in Cl^{-} uptake in *C. rathbunae*. The antennal gland in this freshwater crab has the ability to regulate ions but not osmolality. Based on these results, I propose a new model of Cl^{-} uptake in freshwater crabs: VHA in the basolateral membrane is responsible, with apical HCO_3^{-}/Cl^{-} exchanger and cytoplasmic carbonic anhydrase, for base secretion and Cl^{-} uptake.

Introduction

Osmoregulation in brachyuran crabs

There are more than 6700 species of branchyuran crabs that inhabit a wide spectrum of salinities with various physiological adaptations (Ng *et al.*, 2008). They can be found in deep sea, intertidal, terrestrial, and in freshwater habitats (Henry, 1994), and salinity in these habitats ranges from more than 1000 mOsm in marine or intertidal habitats to less than 10 mOsm in fresh water (Charmantier *et al.*, 2009).

Classified by their osmoregulatory pattern, the crabs can be defined as osmoconformers, hyper-isosmotic regulators, and hyper-hyposmoregulators (Reviewed by Charmantier *et al.*, 2009). Osmoconformers keep hemolymph osmolality isotonic to ambient water. These are mostly marine stenohaline crabs living in a narrow range of salinities. Hyper-hyposmoreglators keep hemolymph osmolality relatively stable whether in hypo- or hypertonic conditions. Hyper-isosmotic regulators are crabs living in brackish and fresh water. They keep hemolymph osmolality higher than ambient salinities when they are in low salinity habitats and maintain hemolymph osmolality to ambient salinities when in full strength seawater.

Hyperosmoregulation in crabs

When inhabiting in low salinity environments, the hemolymph osmolality of euryhaline or freshwater crabs is higher than the salinity of ambient water. These crabs gain water by osmosis and lose ions through a concentration gradient on their body surface if they have a direct contact with ambient water. Knowledge of hyperosmoregulation in brachyuran crabs is mostly from the studies of euryhaline crabs, including *Noehelice granulata* (Luquet *et al.*, 2002; Onken *et al.*, 2003; Genovese *et al.*, 2005; Schein *et al*., 2005; Bianchini *et al.*, 2008; Tresguerres *et al.*, 2008), *Eriocheir sinensis* (Onken and Putzenlechner, 1995; Onken, 1999; Onken *et al.*, 2000), *Carcinus maenas* (Shaw, 1961; Taylor *et al.*, 1977; Towle *et al.*, 2011), *Uca* spp. (Lin, *et al.*, 2002), *Scylla paramamosain* (Chung and Lin, 2006), and other species (Tsai and Lin, 2007). These studies show that some primary and secondary active transporter proteins in cells of the osmoregulatory organs are involved in reducing the osmolality gradient and permeability of a hyperosmoregulatory crab.

Although freshwater crabs are usually strong hyperosmoregulators, they are less studied. For example, crabs from the superfamily Potamoidea maintain osmolality higher than ambient and within a narrow range (Shaw, 1959; Harris, 1975; Onken and McNamara, 2002; Kirschner, 2004), even when under a steep osmolality gradient in fresh water. These species do not return to marine environments for breeding. They inhabit freshwater ecosystems throughout their life history and have

a phylogenetic relationship distinct from marine crabs (Cumberlidge and Ng, 2009). The osmolality of body fluid in freshwater crabs is from 400 mOsm in *Dilocarcinus pagei* (Onken and McNamara, 2002) to 540 mOsm in *Potamon edulis* (Harris, 1975). This value is lower than those in their euryhaline relatives (about 600 mOsm) in hypotonic environments, but higher than those in freshwater crayfish, *Procambarus clarkii* (about 400 mOsm), a very successful freshwater crustacean (Sarver *et al.*, 1994). Freshwater crabs have low urine production, a strategy to reduce ion loss during excretion (Shaw, 1959). In freshwater crabs, reducing hemolymph osmolality, urine production, and ion permeability are suggested mechanisms for conserving ions in freshwater system (Mantel and Farmer, 1985; Charmantier *et al*., 2009).

In low salinities, ion loss through diffusion should be compensated by active ion uptake. The compensatory mechanisms for ion uptake through the osmoregulatory organ are the main physiological adaptations for the hyperosmoregulatory species (Kirschner, 2004).

Osmoregulatory organs in crustaceans

Osmolality is controlled by a few osmoregulatory organs including the digestive tract, antennal gland, and gills (Charmantier *et al*., 2009). These organs are candidate osmoregualtory organs in decapod crustaceans. The digestive tract, with its cuticle lining, may have

difficulty in osmoregulating during the inter-molting period (Chung and Lin, 2006; Charmantier *et al.*, 2009). For this reason, the gills and the antennal gland are the main osmoregulatory organs during the inter-molting period.

Gills

Gills in brachyuran crabs are a multifunctional organ responsible for gas exchange, nitrogen waste excretion, and osmoregulation (Freire *et al.*, 2008; Charmantier *et al.*, 2009). The phyllobranchiate gills in brachyuran crabs are a series of paired lamellae in which afferent and efferent vessels are connected by marginal canals (Goodman and Cavey, 1990). Gills consist of two epithelial cell layers separated by hemolymph space which is supported by the pilaster cells. Each gill is covered by the cuticle, an extracellular matrix deposited at the apical side of the epidermal cell. This cuticle is selectively ion-permeable.

There are two types of gill in brachyuran crabs: the respiratory and osmoregulatory (Taylor and Taylor, 1992; Lucu and Towle, 2003; Tsai and Lin, 2007). Epithelium of 1-5 μm is specialized for gas exchange in the anterior gills while that of 10-20μm is in the posterior gills (Freire *et al.*, 2008).

The cells responsible for osmoregulation in brachyuran crabs are similar to the mitochondria-rich cells in fish gills and the renal tubule

cells in mammals (Freire *et al.*, 2008). Their membrane-bounded transporters and exchangers for ionic uptake are mostly powered by Na^+,K^+-ATPase (from now on NKA) in the basolateral membrane (Luku and Towle, 2003; Kirschner, 2004, Freire, *et al.*, 2008). The anterior-gas exchange and posterior-osmoregulation gill is widely seen in most brachyuran crabs (Lucu and Towle, 2003; Freire *et al.*, 2008).

Although this gill differentiation is supposed to exist in most crabs, opposite functional arrangement was found in the freshwater crab, *Candidiopotamon rathbunae* (Tseng, 2008). The posterior gills have thin epithelium, low NKA abundance and activity, and are considered the gas exchange gills. The anterior gills, however, have the typical characteristics for ionocytes: cells thicker than posterior gills, and basolateral folding, apical microvilli, and numerous mitochondria. The NKA activity, as an indicator of osmoregulatory ability, is upregulated in anterior gills by osmotic stress. The NKA abundance and activity in anterior gills is greater than that in posterior gills. Both structural and functional analysis of gills of *C. rathbunae* show this unusual arrangement (Tseng, 2008).

Antennal gland

The excretory organ of decapod crustaceans, the antennal gland, may also play a role in osmoregulation (Freire *et al.*, 2008). The structure and

function of the antennal gland is similar to the kidney in mammals, implying potential for osmoregulation (Rigel, 1968; DeVries, *et al.*, 1994). The function of the coelomosac, labyrinth, and nephridial canal in crayfish is respectively similar to the glomorulus, proximal tubule, and distal tubule in mammals (Peterson and Loizzi, 1974). In crabs, the nephridial canal is absent in the antennal gland (Schmidt-Neilson *et al.*, 1968).

The terrestrial crab, *Ocypode quadrata*, produces iso-osmotic urine with high NKA activity, implying osmoregulatory ability in this species (DeVries, 1994). In the marine crab, *Carcinus maenas*, the antennal gland is an additional site of ion loss at low salinities (Cameron and Batterton, 1978). However, function of the antennal gland in the freshwater crabs is still unclear.

Crabs produce isotonic or slightly hypotonic urine compared to the hemolymph when in euryhaline or freshwater conditions (De Vries *et al.*, 1994; Onken and McNamara, 2002). Isotonic urine is another way for terrestrial crabs to lose ions, but they can reprocess urine in the gill chamber by active uptake mechanisms (Taylor and Greenaway, 2002). Whien crabs are hyperosmoregulating in fresh water, their urine osmolality remains nearly isotonic to the hemolymph (Mantel and Farmer, 1985; Charmantier *et al.*, 2009). Urine flow rate is low to conserve ions when *Potamonautes warreni* (Morris and Van Aardt,

1998), *Potamon niloticus* (Shaw, 1959), and *Potamon edulis* (Harris and Michaleff, 1971) are in fresh water.

Freshwater crabs living in an ion poor environment should minimize ion loss through excretion and diffusion, but they produce isotonic urine in a way that loses ions. Is the antennal gland another way to lose ions? These crabs may also regulate ion compositions in the hemolymph, but not through osmolality.

Molecular mechanisms in gills of hyperosmoregulation

A suggested hyperosmoregulation model was proposed in reviews by Morris (2001) and Kirschner (2004). They suggest that the two driving forces for ion uptake in hyperosmoregulatory crabs are vacuolar-type H^+ ATPase (VHA) and Na^+, K^+-ATPase (NKA) respectively in the apical and basolateral membranes of osmoregulatory gill epithelium. These driving forces provide power for ion uptake through secondary transport. The concentration gradient generated by NKA through the epithelium Na^+ channel (ENaC) in apical membrane uptakes Na^+. Exchange with HCO_3^- from carbonic anhydrase (CA) in the cytoplasm uptakes Cl^-.

There are not enough ions for a concentration gradient to uptake ions in freshwater system (Parks *et al.*, 2008). The VHA serves as another driving force by generating an electrical gradient for ion uptake, mainly for Cl^- (Onken *et al.*, 1991; Onken and Putzenlechner, 1995). VHA

coupled with Cl^- uptake was obtained from the studies on the strong hyperosmoregulatory crab, *E. sinensis*. Tsai and Lin (2007) suggest that VHA in the apical region is more important in hyposmotic environments, such as fresh water, but it remains unclear in true freshwater crabs.

Na^+,K^+-ATPase

The main driving force for NaCl uptake in hyperosmoregulation is NKA (Morris, 2001; Lucu and Towle, 2003; Freire *et al.*, 2008). Osmotic stress leads to a rise in NKA expression in both abundance and activity of crab gill (Chung and Lin 2006; Li *et al.*, 2006; Tsai and Lin, 2007). Since NKA in osmoregulatory organs is sensitive to environmental change, an up-regulation of NKA in crab gills in low salinities is an indication of ion uptake (Lucu and Towle, 2003).

V-type H^+-ATPase

VHA is a multifunctional protein mainly for acid-base regulation (Tresgurres *et al.*, 2005; 2006) and ion uptake (Wieczorek *et al.*, 1999). The apically located VHA is another driving force for ion uptake in hyperosmoregulating (Onken and Putzenlechner, 1995; Morris, 2001; Tsai and Lin, 2007; Freire *et al.*, 2008; Tresguress *et al.*, 2008; Charmantier *et al.*, 2009). Apical VHA is found in euryhaline crabs in

low salinities, for example, *Erioicheir sinensis* (Onken and Putzenlechner, 1995; Tsai and Lin, 2007) and *Neohelice granulata* (Tresguress *et al.*, 2008). Increasing VHA activity, but not increasing abundance, is also found in anterior gills of *Uca formosensis* acclimated in 5 g salt/L (Tsai and Lin, 2007). These studies show apical VHA is involved in ion uptake in hyperosmoregulating crabs.

VHA is reported, however to be on the basolateral side of epithelial cells in gills of freshwater animals (Piermarini and Evans, 2001; Katoh *et al.*, 2003) such as killifish, *Fundulus heteroclitus* under low ionic stress (Kato *et al.*, 2003). Basolateral VHA is involved in ion uptake in an indirect way by being coupled with cytoplasmic carbonic anhydrase (CA) for base extrusion and Cl^- uptake in the freshwater stingray, *Dasyatis sabina* (Piermarini and Evans, 2001).

There is a difference in the ions VHA is responsible for in crustaceans: Na^+ uptake in crayfish (Zare and Greenaway, 1998) and Cl^- uptake in crabs (Onken and Putzenlechner, 1995; Tresgurres *et al.*, 2008). In his review, Kirschner (2004) suggested VHA is responsible for Na^+ uptake in hyperregulating crabs. It is unknown in freshwater species.

Onken and McNamara (2002) found asymmetrical epithelia in the posterior gill of *Dilocarcinus pagei*. In this species, Na^+ and Cl^- are absorbed by thick and thin epithelia, respectively (Onken and McNamara, 2002; Weihrauch *et al.*, 2004). Weihrauch *et al.* (2004) found that NKA,

but not VHA activity, is significantly higher in the posterior than in anterior gills. The specific inhibitor for VHA, concanamycin, has a major effect when applied to the hemolymph side (Weihrauch *et al*., 2004). Their results suggest that VHA is located at the basolateral membrane fo gills of the freshwater crab, *D. pagei*.

Candidiopotamon rathbunae

In Taiwan, more than 42 freshwater crabs are described (Shih and Ng, 2011). These freshwater crabs can be found in paddy farms, streams, and wetlands. They are suggested to be hyperosmoregulators (Kirschner, 2004). *Candidiopotamon rathbunae* is a widely distributed endemic crab in Taiwan (Liu and Li, 2000; Shih *et al.*, 2006). It spends most of its time in stream water (Liu and Li, 2000; Li, 2005) and is considered as a hyperosmoregulator well-adaptive to freshwater environment.

Purposes

The purpose of the present study is to propose an iono-regulatory model for the gills and antennal gland of the freshwater crustacean, *C. rathbunae*. Specifically, I examined the osmotic response of the antennal gland to deionized water (DW) acclimation. I also examined VHA function in Na^+ or Cl^- uptake by the low-Na^+ and low-Cl^- acclimation. I hypothesized that antennal gland functions in ionic regulation, but not in

osmotic regulation, and that VHA is sensitive to low-Cl^- condition.

Material and methods

Experimental design

There were two hypothese in my experiment include (1) that the antennal gland is an ionregulatory organ and t(2) VHA is responsible for Na^+ or Cl^- uptake. To show the antennal gland regulates ions, I examined the antennal gland structure and localization of NKA. The deionized water (DW) was treated as an ionic stressor because of its low ion content. I monitored NKA activity and abundance in the crabs acclimated in DW for 7 days. Ionic composition and osmolality of urine and hemolymph were measured during DW acclimation. The NKA expression from antennal gland was compared to that from osmoregulatory gills. To determine whether VHA plays a role in Na^+ or Cl^- uptake, its expression should be responsive to either low-Na^+ or low Cl^- condition. I cloned full length sequence of VHA B-subunit of gill 5 of *C. rathbunae*. Artificial freshwater (AFW), artificial low-Na^+ water (LNW), and artificial low-Cl^- water (LCW) were used as ionic stressors. The expression of VHA among different organs was examined with primers specific for *C. rathbunae* VHA B-subunit. I also recorded VHA B-subunit abundance from crabs acclimated for 96 hr in AFW, LNW, and LCW and measured responses of VHA expression in osmoregulatory and non-osmoregulatory gills.

Candidiopotamon rathbunae collection

Candidiopotamon rathbunae is an endemic species to Taiwan. The phylogeny on Taiwan Island have been studied by Shih *et al.* (2006). They suggested that there are six phylogenetic groups in Taiwan, correlating with their geographical distribution. In central Taiwan, two clades separated by the Dajia River. North of this river is the NW group and south of this river is the W group. Unlike other freshwater crabs, *C. rathbunae* does spend more time under water. They live under water, occasionally surfacing for air. I collected crabs from Shijaolin, Dongshi Township and Wushikeng, Heping Township in Taichung City, Taiwan. Both streams are branches of the Da-an River and are at similar latitudes. Crabs were raised in a 75cm (l) x 30cm (w) x 12cm (h) plastic containers (volume: 27L) separated into 12 cells by black plastic. Every two cells share an air-operated filter. Each cell had one crab. Crabs were acclimated in Tunghai University tap water (Table 1) for 2 weeks before each experiment. The light cycle was light:dark 12hr:12hr at 24°C. Crabs were fed frozen *Artemia* three times a week. The day after feeding, water was replaced with new tap water. Each cell had 3 cm height of air space, preventing crabs from easily accessing air. This setup make the crabs difficult to access air for blocking the urine reprocess.

Acclimation regime

Antennal gland function

After acclimation in tap water for two weeks, crabs were transferred to deionized water (DW) (Millipore). Six individuals were transferred to a 27-L plastic container separated to 6 cells by black plastic plates. Each cell was aerated. Each cell contained one crab. Crabs were physically isolated from each other. Ten liters of DW was replaced every day of the experiment to maintain the ion environment. Ionic contents of DW recorded by atomic spectrophotometer (Z-5000, Hitachi, Japan) and spectrophotometer (U-2001, Hitachi, Japan).

VHA function

Crabs acclimated in tap water for 2 weeks were transferred to artificial water (AFW), artificial low-Na^+ water (LNW), and artificial low-Cl^- water (LCW). Ionic concentrations of AFW were referenced by the tap water of Tunghai University and water from Shijaolin and Wushikeng (Table 1). Crabs were acclimated for 96 hr. Then I took samples of the total RNA from gill 5 and 9 as osmo- and non-osmoregulatory gills, respectively for further experiments.

Muscle water content

I measured muscle water content in crabs acclimated to deionized water for a physiological base line. If crab could maintain stable muscle water content, it was considered successfully acclimated to deionized water. To collect fresh muscle samples, a crab was anesthetized on ice for 5-10 minutes. Then, I used a scalpel to carefully remove the claw carapace. I removed the claw muscle and dehydrated it on a tinfoil plate at 95 ºC for 3 days or until there was no further change in weight. The period for dehydration (3 days) was determined by weight unchanged. Water content is calculated as (fresh weight – dry weight) / fresh weight and presented as a percentage (%).

Protein extraction

For protein extraction, I followed the procedures of Tsai and Lin (2007) with modifications described bellow. Paired gill 5 and antennal gland were shredded and immediately immersed in an ice-cold homogenizing medium (pH = 7.4) which contained 25 mmol l^{-1} Tris-HCl, 0.25 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 0.4% sodium deoxycholate, and 1 mmol l^{-1} PMSF with 100:1 protease inhibitor (3.31 mmol l^{-1} antipain, 2.16mmol l^{-1} leupeptin, 63.86 mmol l^{-1} benzamidine in aprotinin saline solution containing 5-10 trypsin inhibitor unit ml^{-1}. After short shredding, tissues were homogenized by ultrasonic processor (Sonics, Newton, CT, USA) on ice for 20 seconds at 2 Watts. The homogenate

was centrifuged at 4ºC and 6,000 *g* (Hitachi, Japan) for 10 minutes. This first centrifuging removed debris, chitinous tissue, and cell nuclei. The supernatants were further centrifuged at 4ºC and 21,500 *g* for 20 minutes. The supernatant was crude protein which I used for tests of enzymatic activity and for western blotting. Protein concentration was measured by spectrophtometry (U-2001 Spectrophotometer, Hitachi, Japan) at 595 nm with detergent compatible kit (BioRad, Hercules, CA, USA).

NKA activity

The protocol for measuring of Na^+,K^+-ATPase activity was modified from Holliday (1985) and Tsai and Lin (2007). I added 5 µl protein to 400µl reaction medium. I also added 100 µl ATP stock solution (25 mmol. l^{-1} Na_2ATP). This mixed solution was incubated at 30ºC for 15 min. The incubation reaction was stopped by adding 200 µl of 30% ice-cold trichloroacetic acid (Merck, Germany). Then the mixture was centrifuged at 4ºC and 1,640 *g*. The 500 µl aliquot was incubated with 1000 µl ice-cold Boting's color reagent (560 mmol l^{-1} H_2SO_4, 8.10 mmol l^{-1} $(NH_4)_2MoO_4$, and 176 mmol l^{-1} $FeSO_4$) at 20ºC, 20 minutes before measuring for inorganic phosphate concentration by colorimetry at 700 nm (U-2001, Hitachi, Japan.) Two groups of reaction medium were used: ouabain group containing 20 mmol l^{-1} imidazole, 130 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, and 1 mmol l^{-1} ouabain and a ouabain-free group

containing 20 mmol l^{-1} imidazole, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 30 mmol l^{-1} KCl. The enzyme specific activity for Na^+,K^+-ATPase was defined as the rate of inorganic phosphate released.

Western blot

Aliquots of 20 μg and 10 μg total protein from gill 5 and the antennal gland, respectively, were used in the western blotting. The aliquot of proteins was run on 5% upper and 10% lower polyacrylamide gels. After fractioned by electrophoresis, samples were electroblotted to a PVDF membrane (Millipore, USA) through the semi-dry method (Trans-Blot SD, Semi-Dry Transfer Cell, Bio-Rad, USA). The membrane was blocked by 5% non-fat milk for 1 hour. It was incubated with primary antibody α5, mouse monoclonal against the α-subunit of an avian sodium pump (Developmental Studies Hybridoma bank, Iowa, USA) or primary antibody β-actin, monoclonal antibody (Chemicon, USA) for 1 hour at room temperature. Then it was incubated with the second antibody, goat anti-mouse, IgG, with horseradish peroxidase conjugate (Zymed, USA) for 1 hour at room temperature. The abundance was luminated by Western Lightning Plus-ECL system (PerkinElmer, USA) and measured in Fujifilm LAS-1000 digital camera (Fujifilm, Japan). The intensity was analyzed by Image Gauge 4.0 (Fujifilm, Japan). Relative abundance was calculated as intensity of α5 over β-actin.

Paraffin section

Gill 5, gill 9, and the antennal gland were fixed in Bouin's solution (Sigma, USA) for 24 hours. After fixation, tissue was washed with 70% ethanol until Bouin's solution was thoroughly removed. Then the tissue was dehydrated with 80%, 90%, 95%, and 100% ethanol for 15 minutes each. After dehydration, the ethanol solution was displaced by ethanol:xylene as 3:1, 1:1, 1:3 for 20 minutes each befor the samples were twice soaked in xylene for 20 minutes. Xylene was replaced by paraffin. This tissue was stored over night at 60ºC. Tissue was embedded into paraffin on a hot plate. The paraffin block was cooled and hardened at room temperature. Sections of 5μm in thickness were prepared by Leica RM 2125RT (Leica, Germany). I collected sections near the central region for the antennal gland. The efferent vessel of a gill was also sectioned for corresponding orientation.

Immunohistochemistry

Sections were immersed in xylene for 6 minutes to remove paraffin before immersed in 100% EtOH and 95% EtOH for 2 minutes, respectively. Slides were placed into double deionized water (Millipore) for 10 minutes for rehydration. Sections were incubated with 3% hydrogen peroxide for 5 minutes and three times, rinsed with PBS-Tween

20 for 2 minutes. Sections were incubated with the primary antibody, α5 (Developmental Studies Hybridoma bank, Iowa, USA) 1:10000 in PBS-Tween 20 for 1 hour at room temperature (Control group, in PBS-Tween 20 only). After incubating with the primary antibody, the sections were rinsed with PBS-Tween 20 for 2 minutes for three times and incubated with the secondary antibody, HRP-conjugated, for 30 minutes and then rinsed by PBS-Tween 20 for 2 minutes for three times. Then they were incubated in aminoethyl cabazol (AEC) single solution chromogen (Invitrogen, USA) for 30 minutes and rinsed with double deionized water for 2 minutes. The sections were stained with hematoxylene (Sigma, USA) for 5 minutes and washed by double deionized water. Each plate was sealed by clear mount (Invitrogen, USA). Sections were photographed by light microscopy (Nikon, Japan).

Urine and hemolymph collection

After accliamtion in DW for 1, 4, and 7 days, crab urine and hemolymph were collected by cannulation. The 29-gauge needle penetrated the pericardial cavity to collect hemolymph. The hemolymph was centrifuged at 20,000 *g* at 4ºC for 20 minutes. Urine was carefully collected from nephropore with a 29-gauge needle. The bladder of a crab is fragile. Urine was checked by its coagulation property. Coagulation is

an indication of hemolymph contamination in the urine samples. Contaminated samples were discarded.

I measured concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} and Cl^- in each hemolymph and urine sample.

Osmolality measurement

Osmolality of hemolymph and urine was measured by an osmometer (Wescor 5500 vapor pressure osmometer, Wescor, USA). Aliquots of 10 μL were used. Samples from crabs acclimated to freshwater, and artificial water, artificial low-Na^+ water, and artificial low-Cl^- water were lower than the minimum (100 mOsm/kg) of standard curve of the osmometer. Therefore they were measured by atomic absorptive spectrum (Z-5000, Hitachi, Japan).

Ion concentrations in hemolymph and urine

The concentration of cations was measured by atomic absorption spectrophotometer (Z-5000, Hitachi, Japan). Water samples were diluted by double deionized water (Millipore). I measured Na^+, K^+, Ca^{2+}, and Mg^{2+} in FW, DW, artificial water, artificial low-Na^+ water, artificial low-Cl^- water, hemolymph and urine. These were diluted 51-fold before measuring Na^+ and Mg^{2+} and 21-fold before measuring other cations. The standards for the ions were Na^+: 0, 0.2, 0.4, and 0.8 mg/L; K^+: 0, 0.25,

0.5, and 1 mg/L; Ca^{2+}: 0, 0.5, 1, and 2 mg/L; and Mg^{2+}: 0, 0.1, 0.2, and 0.4 mg/L.

The Cl^- was measured by ferricyanide method (Franson, 1985). Water samples and hemolymph and urine were diluted to appropriate dilution. Aliquots of 1,000 μL diluted water samples were added 200 μL 100 mM $NH_4Fe(SO_4)_2$ (Merck, Germany) in 6N HNO_3 (Union Chemical Work Ltd., Taiwan) and 100 μL of 1mM $Hg(SCN)_2$ (Merck, Germany) in 95% ethanol. Concentration of Cl^- was measured by spectrophometer (U-2001, Hitachi, Japan) at 600 nm. The standards for Cl^- were 0, 0.25, 0.5, 1, and 2 mg/L.

RNA extraction

Gill 5 and Gill 9 from ice-anesthetized crabs were cut off by RNase free condition. Total RNA was extracted by TRIZol reagent (Invitrogen, USA). Tissues were immersed immediately into 400 μL TRIzol reagent on ice and briefly cut. Tissues were homogenized by homogenizer and added to 600 μL TRIZol reagent. Homogenates were incubated for 10 minutes at room temperature. Then, 200 μL chloroform was added while the homogenates were gently mixed and incubated for 10 minutes at room temperature as the RNA. Homogenates were centrifuged at 4°C and 14,900 *g* for 15 minutes. The aqueous layer was isolated. 500 μL isopropanol was added before incubating in ice for 15 minutes and

centrifuging at 4°C and 14,900 *g*, for 15 minutes. The pellet was retained and the supernatant was removed. The pellet was washed with 70% EtOH and by air-dried before being resolved in DEPC-treated water. The quality and concentration of the total RNA was measured by SSP-3000 Nanodrop spectrophotometer (Infinigen, CA).

mRNA isolation

Total RNA extracted by TRIZol was further purified for mRNA purification by Microfast mRNA isolation kit (Invitrogen, USA). First, 20μg total RNA was resuspended in 1mL binding buffer provided by the kit. Heated aliquots for 5 minutes at 65ºC then cooled the tube in ice for 2 minutes. The aliquots was added to 25mg oligo (dT) and incubated for 1 hour at room temperature. The tube was then centrifuged at 4,000*g* for 5 minutes at room temperature three times. Supernatants were removed each time. The pellet was resuspended in 300μL binding buffer and transferred to a spin column to purify mRNA. With the spin column, it washed with Binding Buffer at 4,000*g* for 10 seconds 3 times. Then, I washed it with low salt wash buffer to remove SDS and rRNAs as the methods described above. To the spin column, I added 100 μL elution buffer and centrifuged it at 4,000*g* for two times. Resulting aliquots were retained and added to 10 μL of 2mg/mL glycogen carrier, 30 μL 2M sodium acetate, and 600 μL 100% EtOH and then solidified at –80°C.

After thawing, the tube was centrifuged at 20,000*g* at 4ºC for 15 minutes to produce the mRNA pellet. This pellet was washed by 70% EtOH and air-dried. The mRNA was disolved in elution buffer and stored at –80ºC.

Rapid amplified cDNA ends (RACE) PCR

The RACE PCR was done with the SMARTer RACE cDNA amplification kit (Clontech, USA). For 5'-RACE, 2.75 μL mRNA from gill 5 was added to 1 μL 5'-CDS Primer A before incubating at 72ºC for 3 minutes and 42ºC for 2 minutes. Then, I added 1 μL SMARTer IIA oligo, 4 μL buffer mix (2 μL 5X first-strand buffer, 1 μL 20 mM DTT, and 1 μL 10 mM dNTP), 0.25 μL RNase inhibitor (40U/μL), and 1 μL SMARTScribeTM reverse transcriptase (100U). For 3'-RACE, I added 3.75 μL mRNA, but no SMARTer IIA oligo. I incubated the tube at 42ºC for 90 minutes and then heated at 70ºC for 10 minutes. These procedures anchored detectable sequences on 5' and 3' ends respectively for universal primer mix (provided by kit). The 5' and 3' RACE ready cDNA are used for further experiments.

cDNA synthesis

For cDNA synthesis, about 1 μg of total RNA was reverse-transcribed in a final volume of 20 μl containing 0.5 mM dNTPs, 2.5 μM oligo(dT), 5 mM dithiothreitol, 40 units of an RNase inhibitor, and 200 units of

SuperScript III RT (Invitrogen, USA) for 1 h at 50°C. Then I incubated it at 70°C for 15 min.

PCR

The PCR solution had 1 unit of proTaq (PROTECH, Taiwan), 0.5 mM dNTPs, 10 mM Tis-HCL, 50 mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, and 0.5-1μg cDNA in a 50 μL aliquot. PCR began at 95ºC for 3 minutes followed by 30 cycles of 95ºC for 60 seconds, 60ºC for the specific primer and 50ºC for the degenerate primer for 30 seconds, and 72ºC for 60 seconds. The PCR product was stored at 4°C.

Cloning

I cut the PCR product with gel purification. Purified DNA was inserted into the vector provided by the RBC TA cloning kit (GeneMark, Taiwan). The 5'-RACE and 3'-RACE products were insert into the vector with T4 DNA ligase at 4ºC overnight for ligation. Then vectors were incubated with competent cells, DH5α (strain of competent cell), for 30 minutes on ice, at 42ºC for 45 seconds, and then chilled on ice for 2 minutes. The cells were recovered with 500 μL SOC medium at 37ºC for 60 minutes. After recovery, the cells were spread on LB plate with 5mM of ampicillin, X-gal, and IPTG. After 16-24 hours of incubation in 37ºC, white colonies were selected for further sequencing.

Quantitative RT-PCR

Total RNA are extracted from gill 5 and gill 9 of a crab acclimated in LNW, LCW, and AFW for 96 hr by TRIZol (Invitrogen, USA). 1 μg total RNA are reverse transcripted with super script III (Invitrogen, USA) at 50ºC for 30 minutes. The cDNA made from above was diluted 10 times for quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR), fluorescence stained by SyBR Green (Roche, Germany), was 1 cycle of 95ºC for 10 minutes, then 40 cycles of 95ºC for 15 seconds, 60ºC (annealing temperature for all primers) for 10 seconds and 72ºC for 10 seconds.

Primers for qRT-PCR are list at Table 5. Target genes are VHA subunit B L form, S form, and NKA respectively. The internal control is arginine kinase (Weihrauch *et al.*, 2001). qRT-PCR data was quantified by ΔΔCt method. The products were checked by melting curve analysis and electrophoresis.

Data analysis

Data are shown as mean ± S.D. Statistical differences among different times was determined by one-way ANOVA (SAS ver. 9.0) for each organ. Comparison of mRNA expression in gill 5 and gill 9 of VHA B-subunit A and B isoforms, NKA, and arginine kinase were analyzed by *t*

test. Goups were considered significantly different if $P<0.05$.

Results

Muscle water content and Osmolality in hemolymph and urine

Muscle water content in chelip was recorded after 0, 1, 4, and 7 days acclimation in DW (Table 3). It remained in a stable state of about 80% (one-way ANOVA: $F_{3,18}$=1.34, P>0.05). The hemolymph osmolality (Table 3) was 560±24 mOsm/kg H_2O in FW group and after one day of acclimation in DW, it decreased to 500±20 mosm/kg H_2O, and remained constant for the rest of the seven days in DW (one-way ANOVA: $F_{3,29}$=15.97, P<0.05; Duncan's Multiple Range Test: *P*<0.05) ranging from 500±20 to 492±28 mOsm/kg H_2O. Osmolality changes in urine (Table 3) were similar to that in hemolymph, but were significantly lower on the 4th and 7th days in DW than on the 1st day (one-way ANOVA: $F_{3,30}$=15.89, P<0.05; Duncan's Multiple Range Test: *P*<0.05). On the first day in DW, the osmolality in urine was 562±25 mOsm/kg H_2O. After one day of acclimation in DW, osmolality in urine was significantly lower (511±20 mOsm/kg H_2O) Osmolality in urine on the 4th and 7th days were 481±27 and 471±38 mOsm/kg H_2O, respectively, and significantly lower than the 1st day of acclimation in DW. The U/H ratio was around 1 (one-way ANOVA: $F_{3,22}$=2.21, P>0.05) (Table 3). Therefore *Candidiopotamon rathbunae* produced isosmotic urine regardless of the osmolality change.

Ion concentrations in hemolymph and urine

Sodium concentration was constant in both hemolymph (one-way ANOVA: $F_{3,29}$=1.20, P>0.05) and urine (one-way ANOVA: $F_{3,29}$=0.76, P>0.05) within 7 days acclimated in DW. The U/H ratio for Na^+ was 1.25±0.15 mM in the FW group. This ratio was greater than 1, indicating that Na^+ was secreted into urine. Potassium concentration remained constant in hemolymph (one-way ANOVA: $F_{3,29}$=1.48, P>0.05), urine (one-way ANOVA: $F_{3,29}$=0.61, P>0.05), and U/H ratio (one-way ANOVA $F_{3,29}$=0.97, P>0.05) (Table 4). The U/H ratio for K^+ was 0.76±0.29 in FW (Table 4). This is an indication that K^+ was reabsorbed from the urine. Calcium concentration in hemolymph was 15.28±1.17 mM in FW and remained constant during the experiment (one-way ANOVA: $F_{3,29}$=1.53, P>0.05) (Table 4). Calcium concentration in urine was 4.38±1.54 mM in the FW group (Table 4). On the first day of acclimation, calcium was significantly lower than the FW group (one-way ANOVA: $F_{3,29}$=3.86, P<0.05; Duncan's Multiple Range Test: *P*<0.05) (Table 4). On the 4th day and 7th day in DW, urine calcium concentrations recovered to 10.05±3.46 mM and 9.20±1.88 mM, respectively, and they were similar to the FW control. U/H ratio of Ca^{2+} was 0.54±0.26 in FW and significantly lower than that from the first day acclimated in DW (one-way ANOVA $F_{3,29}$=3.81, P<0.05; Duncan's

Multiple Range Test, $P<0.05$). This ratio of recovered to the FW levels on the 4th day and 7th day (Table 4). Magnesium concentration in hemolymph was 1.91±0.92 mM in FW group. It remained constant during 7 days of acclimation to DW (one-way ANOVA: $F_{3,29}=2.52$, $P>0.05$) (Table 4). There was also no significant difference in magnesium concentration in urine (one-way ANOVA: $F_{3,29}=1.36$, $P>0.05$) (Table 4). The U/H ratios of magnesium on the 4th and 7th days were significantly higher than FW (one-way ANOVA: $F_{3,29}=2.83$, $P>0.05$; Duncan's Multiple Range Test: $P<0.05$) (Table 4). U/H ratio increased with acclimation to DW, implying accumulation of Mg^{2+} in urine. There was, however, no significant difference in magnesium concentration in urine among different days. This may be due to the greater variation in urine magnesium concentration.

Chloride concentrations (Table 4) in crabs from FW group were 332.39±27.36 and 291.44±37.06 mM in hemolymph and urine, respectively. On the 7th day of acclimation to DW, hemolymph chloride decreased significantly (one-way ANOVA: $F_{3,29}=4.34$, $P<0.05$; Duncan's Multiple Range Test: $P<0.05$). Chloride concentration in urine decreased significantly on the first day of acclimation to DW (332.04±44.03 mM; one-way ANOVA: $F_{3,29}=7.64$, $P<0.05$; Duncan's Multiple Range Test: $P<0.05$). The U/H ratio of chloride was 0.90±0.10 in FW and decreased

on the first day of acclimation to DW (one-way ANOVA: $F_{3,29}$=3.03, P<0.05; Duncan's Multiple Range Test: P<0.05).

Antennal gland structure and NKA localization

I examined four parts of the antennal gland under light microscope. The coelomosac (Figure 1) was mainly in the central part of the antennal gland. Coelomic cells had large vacuoles and small nuclei. This type of cells was fragile because of the highly vacuolated cell. The foot process could not be observed in this resolution.

The labyrinthine cells were distributed widely throughout the antennal gland. Labyrinth was a coiled system and with dense cytoplasm. It was adjacent to the coelomic cells, but was not always in contact with coelomic cells. Nuclei of the labyrinthine cells were on the apical side. Microvilli on the apical side were observed. In summary, the labyrinthine cells were showed a typical type of ionocyte.

Hemocytes were scatterd between coelomic and labyrinthine cells. This area is considered the hemolymph sinus. Apical to the labyrinthine cells is the urine space. It is easily identified by the lack of hemocytes in this space.

Localized throughout the antennal gland tissue (Figure 2A), NKA widely distributed on the basolateral membrane of labyrinthine cells not only in the center (Figure 2B) and in outer part (Figure 2C) of the

antennal gland. There was no specific binding within the negative control (Figure 2D-F) and no detectable signals in coelomic cells were observed.

NKA activity

In freshwater, NKA specific activity in the antennal gland (Figure 3) was similar to that in gill 5 (Figure 4). After DW acclimation, at day 1 was significantly higher than fresh water in NKA activity between antennal gland and gill 5. In the antennal gland, there was a significant difference among the four groups (one-way ANOVA: $F_{3,39}$=5.68, P<0.05; Duncan's Multiple Range Test: P<0.05) (Figure 3). In gill 5 (Figure 4), NKA activity was significantly higher on day 4 of acclimation to DW (one-way ANOVA: $F_{3,39}$=2.91, P<0.05; Duncan's Multiple Range Test: P<0.05).

NKA abundance

There was a distinct band at 110 kDa blotted by α5. Relative abundance in gill 5 (Figure 5) significantly increased after one day of acclimation in DW and remained constant thereafter (one-way ANOVA: $F_{2,25}$=1.01, P>0.05). In the antennal gland (Figure 6), relative abundance decreased significantly after one day of acclimation to DW and remained constant thereafter (one-way ANOVA: $F_{2,19}$=0.85, P>0.05).

VHA sequences

Primers used in PCR are listed in Table 5. The full length of VHA was obtained through RACE PCR using degenerate primers HATF2 and HATR4. Specific primers were designed from the above sequence: CrHA1F and CrHA1R for 5' RACE and 3' RACE, respectively.

There were two distinct fragments from 5' RACE, but only one distinct fragment from 3' RACE (Figure 7). Sequences for these two fragments confirmed existence of two isoforms of VHA B-subunit in *C. rathbunae* (Figure 8). The longer form had 2620 base pairs and was designated as the L form while the shorter form had 2545 base pairs and designated the S form. Translation by the open reading frame of L form started and ended at 116 and 1736 bps (Figure 9), respectively. Translation of the S form started and ended at 41 and 1681 bps, respectively. The two isoforms translated the same protein sequence. Length of the protein translated by the L and S form was 546 amino acids (Figure 9). Use of BLAST in NCBI showed a high similarity among VHA B-subunits (Figure 9).

Expression of two VHA isoforms in different organs

Total RNA from gills 4-9, antennal gland, fore-gut, hind-gut, heart, and muscle was extracted and reverse transcripted to cDNA. Specific primers for arginine kinase, CrAK1F, and CrAK1R were designed from

the sequence obtained through cDNA made by gill 5 by degenerate primers AKF51 and AKR31 (Weihrauch *et al.*, 2001). To distinguish between isoforms, primer pairs of VHAQ3F/3R and VHAQ4F/4R were designed from sites unique to each isoform (Table 5). PCR fragment shows that there was only one PCR product for each primer pair. Both A and B isoforms and arginine kinase were expressed in all organs examined (Figure 10).

VHA expression in LNW and LCW

The specific primers for quantitative real-time PCR were designed (Table 5): CrAK1F/1R for arginine kinase, CrNKAQ1F/Q1R for NKA, VHAQ3F/Q3R for VHA S isoform, and VHA Q4F/Q4R for VHA L isoform. Ion concentrations were recorded on the 96 hr acclimation to different conditions (Table 2).

Relative abundance of VHA L form was lower in the LNW group than in the LCW group in both gill 5 (t=11.36, P<0.05, n=5) (Figure 11A) and gill 9 (t=3.77, P<0.05, n=5) (Figure 11D). Relative abundance of S form increased in the LNW group compared to the LCW for both gill 5 (t=3.90, P<0.05, n=6) (Figure 11B) and gill 9 (t=2.84, P<0.05, n=6) (Figure 11E).

NKA relative abundance was higher in LCW than in LNW (t=7.37, P<0.05, n=5). In both LCW and LNW, NKA was up-regulated (Figure

12C). For gill 9, there was no significant difference in the relative abundance of Na^+ between LNW and LCW (t=0.59, P>0.05, n=6). Although gill 9 is suggested as a respiratory gill, I recorded increasing NKA expression in LNW and LCW (Figure 11F). This shows that NKA was upregulated during ionic stress both in osmo- or non-osmoregulatory gill. In the osmoregulatory gills, NKA was higher than LNW.

Discussion

This is the first study of the function of the antennal gland from a true freshwater crab encountering ionic stress. The results show that the antennal gland cannot regulate osmolality, but instead ion composition of urine. Tseng (2008) studied the osmoregulation in the gills of *C. rathbunae* and revealed the novel functional arrangement of these gills (Tseng, 2008). There are nine gills arranged in the gill chamber of *C. rathbunae*. Epithelium thickness distinctly varies between gills 4-6 and gills 7-9. Gills 1-3 are too small to contribute a significant function. The anterior gills, gills 4-6 are the osmoregulatory gills. The thickness of their epithelium is about 7μm. The posterior gills, gills 7-9, have thin epithelial cells and are the gas exchange gills. Their epithelium is about 3 μm thickenss.

In this study, I examined the role of the antennal gland in osmoregulation in FW crabs. Since none of the U/H ratios of measured ions equaled to 1, the ionic composition changed after filtration and solute reabsorption took place in the antennal gland. Although osmolality decreased after DW acclimation, ionic compositions were relatively constant in hemolymph than in urine. The antennal gland may regulate and maintain ion concentrations in body fluid in freshwater crabs by regulating urine composition.

The role of VHA in Cl^- uptake was also investigated. The mRNA expression of VHA increased on the fourth day of acclimation to low-Cl^-

conditions, an indication that VHA is responsive to ambient Cl^- condition. In *C. rathbunae*, however, VHA is localized in gill basolateral membranes (Tseng, 2008). This is not consistent with the previous studies about apical VHA with Cl^- uptake. The VHA in basolateral membrane of osmoregulatory gills may works with the apical anion exchanger for Cl^- uptake and base extrusion.

Structure of *C. rathbunae* antennal gland

The structure of antennal gland in *C. rathbunae* is similar to that described in a study of *Uca mordax* (Schmidt-Nielson *et al.*, 1968). Similar to *U. mordax* (Schmidt-Neilson *et al.*, 1968)) *C. rathbunae* does not have a nephridial canal in the antennal gland. My study of structure and immunocytochemistry showed two major cell types in the antennal gland of *C. rathbunae*: the coelomic cells and the labyrinthine cells.

In *C. rathbunae*, coelomic cells had no NKA immunoreactive activity. This is in agreement with the ultrafiltration role of coelomosac (Peterson and Loizzi, 1974; Khodabandeh *et al.*, 2005). In the antennal gland, coelomosac is well conserved in function and structure (Reviewed by Freire *et al.*, 2008). Coelomic cells, also known as the podocytes (Schmidt-Neilson *et al*., 1968; Mantel and Farmer, 1985), have their pedicel is in contact with the blood sinus. This cell type is also found in the Bowman's capsule in mammals (Peterson and Loizzi 1974). The coelomosac in the antennal gland of *C. rathbunae* is similar to that

in *U. mordax* (Schmidt-Nielson *et al.*, 1968) and *Homarus gammarus* (Khodabandeh *et al.*, 2005).

The other main cell type in the antennal gland is the labyrinthine cell. The labyrinthine cells in *C. rathbunae* have apical microvilli. This is similar to the ionocytes described previously in *U. mordax* (Schmidt-Neilson, 1968), *Homarus gammarus* (Khodabandeh *et al*., 2005a), and *Astacus leptodactylus* (Khodabandeh *et al.*, 2005b). Fixation by Bouin's solution produced a well-preserved and antigenicity of tissue to show the basolaterally localized NKA in the labyrinthine cells. These labyrinthine cells are distributed throughout the antennal gland and are increasingly concentrated from distal to the central part of antennal gland. In crayfish and lobsters, NKA is localized on the labyrinth, nephridial canal, and bladder. In the antennal gland of *C. rathbunae*, NKA is specifically localized in the labyrinthine cells. Since NKA is considered a driving force for ion uptake and solute reabsorption by the antennal gland (Sarver *et al.*, 1994), the labyrinthine cells are clearly the main sites for solute reabsorption.

Muscle water content

Muscle water content is an indicator of the physiological homeostasis of an organism and the capacity of isosmotic intracellular regulation (IIR) (Freire 2008b; Foster *et al.*, 2010). In *C. rathbunae*, muscle water content remained

stable at about 80% during the DW acclimation. This value is similar to that for *D. pagei* (Foster *et al.*, 2010), but higher than that in some amphibious freshwater species: 74.7% in *Holthuisana transversa* (Greenaway, 1980), 74% for *Potamonautes warreni* (Morris and Van Aardt, 1980), 78.4% for *Potamon niloticus* (Shaw, 1958). In *C. rathbunae*, stable muscle water content indicates a good capacity of IIR during the DW acclimation.

Osmolality in hemolymph and urine

When acclimated in fresh water, *C. rathbunae* has a higher hemolymph osmolality than *D. pagei* (Augusto *et al.*, 2007; Foster *et al.*, 2010). Hemolymph osmolality in *C. rathbunae*, however, is similar to that of *P. warreni*, a species which spends most of its time in terrestrial habitat (Morris and Van Aardt, 1995). Compared with crayfishes which is a species well adapted to freshwater and that has low hemolymph osmolality, *C. rathbunae*, freshwater shrimp (*Macropodus potiuna* and *M. brasiliense*; Freire *et al.*, 2003), and freshwater crabs (*Potamon niliticus* and *Potamonautes warreni*; Shaw, 1959; Morris and Van Aardt, 1995) have a high hemolymph osmolality. The steep gradient in freshwater crustaceans may result from not only the low permeability of gills and integuments but also in efficiency of ion uptake mechanisms (Mantel and Farmer, 1985).

Isosmotic urine, found in *C. rathbunae* during the DW acclimation, is also

found in the land crabs *Gecarcoidea natalis* (Taylor and Greenaway, 2002) and *Potamon edulis* (Harris and Michaleff, 1971). Freshwater crabs may have other mechanisms to conserve ions, such as reducing urine production (Shaw, 1959; Morris and Van Aardt, 1998) or urine reprocessing via the gill chamber (Taylor and Greenaway, 2002).

Ion concentrations

All ions measured in hemolymph remained stable during the DW acclimation. Although ion concentrations were not equal between urine and hemolymph, osmolality remained the same. In urine, Na^+, K^+, and Ca^{2+} remained constant, but not Mg^{2+} and Cl^-. Ionic concentration in hemolymph was more stable than that in urine, indicating that the antennal gland can conserve ion for hemolymph (Morris and Van Aardt, 1998). This stability was also observed in the U/H ratio.

In fresh water, the U/H ratio for Na^+ was 1.25 indicating that Na^+ is secreted into urine rather than reabsorbed. This value remains constant during DW acclimation. In *Potamonautes warreni*, the U/H ratio for Na^+ and Cl^- is close to 1 (Morris and VanAardt, 1998). If the urine production rates are equal, *C. rathbunae* should lose more Na^+ through urine excretion. Since *C. rathbunae* is considered an freshwater species well-adapted to low ionic environment, an alternative explanation for Na^+ conservation is needed in the future study.

During acclimation to DW, Cl^- decreased in both hemolymph and urine. In normal conditions, the U/H ratio of Cl^- is 0.9, close to 1. This means Cl^- concentration are closed in hemolymph and urine. After DW acclimation, the U/H ratio decreased to 0.65 because the concentration decreased in urine, but not in hemolymph. The reduction of Cl^- in urine implies more Cl^- was reabsorbed through the antennal gland.

In freshwater U/H ratio of K^+ was 0.76. The U/H of K^+ is higher than 1 in another freshwater species, *Potamonautes warenii* (Morris and Van Aardt, 1998) and in the estuarine species, *Carcinus maenas* (Cameron and Batterton, 1978; De Vries, 1994). *Potamonautes warenii* is amphibious. It may have urine reprocessing mechanisms for ion conservation. *Carcinus maenas* is a weak osmoregulator and may lose K^+ through excretion (Cameron and Batterton, 1978). In *C. rathbunae*, retaining potassium in body fluid is important for the ion uptake by, NKA during the ionic stress like DW (Lucu and Towle, 2003).

Calcium is important for calcification of crustacean carapace (Wheatly, 1997). In fresh water, the U/H ratio in *C. rathbunae* is about 0.5 in fresh water. The ratio shows Ca^{2+} was actively reabsorbed from urine. This is similar to that found in previous studies of estuarine crabs (Cameron and Batterton, 1978; De Vries, 1994; Morris and Ahern, 2003) but not of a freshwater crab (Morris and Van Aardt, 1998). The recovery on the 4th day may be due to increasing ion uptake resulting from high NKA activity.

Magnesium levels remained constant in both hemolymph and urine but not in U/H ratio. Magnesium is also secreted to urine in *Ocypode quadrata*, *Potamon edulis*, and *Potamonautes warreni* (De Vries, 1994; Morris and Van Aardt, 1998; Morris and Ahern, 2003). Magnesium is suggested for the exchange of Na^+ in the bladder (Holliday, 1980).

NKA abundance and activity

I chose gill 5 of *C. rathbunae* to represent osmoregulatory gills, following conclusions by Tseng (2008). Gill 5 with its intermediate epithelium is considered as having the osmoregulatory ability. Low ion content or saline environment triggers activity or abundance of the membrane-bound NKA (Lucu and Filk, 1999; Lin *et al*., 2002) as an indication of increasing ion uptake in response to osmolality stress (Lucu and Towle, 2003; Kirschner, 2004).

The NKA activity in the antennal gland responded faster than the osmoregulatory gills on the first day acclimated in DW. The highest value of NKA in Gill 5 was found on the 4th day of acclimation to DW. Upon encountering the ionic stress of DW, *C. rathbunae* increase by the NKA activity in antennal gland first for enhancing the ion reabsorption in antennal gland. On the fourth day, the function of ion uptake is transferred to the osmoregulatory gill in which ion uptake from ambient water is increased. On the 7th day, NKA activity, however, recovered to control level in both gill 5 and antennal gland on

the seventh day.

Abundance of NKA increased in gill 5, but decreased in the antennal gland. It is different from Tseng's 2008 study of *C. rathbunae*. Tseng found no difference in NKA abundance during DW acclimation. This inconsistency may result from the method I used to obtain readings the internal control. I considered relative abundance of NKA in the FW group was treated as the internal control. The relative abundance is presented in times of internal control. In Tseng (2008) calculated abundance of NKA based or intensity over α5 and β-actin, calibrating each membrane by a known protein used in the previous membrane. The intensity in Tseng's (2008) results show large variation in the freshwater group (control group). In addition, I used 2 watts in an attempt to reduce protein degradation during homogenization.

The antennal gland of *C. rathbunae* produced isotonic urine but not isoionic urine. Asymmetry of ions in urine and hemolymph indicates ionic regulatory ability by the antennal gland. Freshwater crab antennal glands, however, cannot regulate their osmolality (Charmantier *et al.*, 2009) but can regulate ion uptake. Upon encountering ionic stress, the crabs first use ions their bodies, then they uptake more ions from the environment.

VHA sequence in *C. rathbunae*

Another driving force for electrogenic ion uptake in freshwater species is

VHA (Kirshner, 2004; Tsai and Lin, 2007; Charmantier *et al.*, 2009). Apical VHA is found in euryhaline species in hyperosmoregulatory conditions (Onken and Putzlenchner, 1995; Tsai and Lin, 2007; Tresguerres *et al*., 2008). VHA is considered the ionic uptake power provider in low ion environment, such as fresh water. There are, however, exceptions in freshwater species. VHA is localized in the basolateral membrane. In killifish (Katoh *et al.*, 2003) and stingray (Piermarini and Evans, 2001), basolateral VHA, localized by the heterologous antibody is also found in *C. rathbunae* (Tseng, 2008). The function of basolateral VHA on ion uptake will be discussed below.

Two isoforms of VHA B-subunit were cloned from *C. rathbunae* gills. The mRNA sequences showed high similarity to other crabs. Translated protein sequences are highly similar to VHA B-subunit forms L and K in *Carcinus maenas* (Weihrauch *et al*., 2001). These protein sequences show strong similarity to the isoform found in human brain. This kind of VHA B-subunit is considered organelle acidification and is not consistent with previous studies of VHA and ion uptake in fresh water reviewed in (Morris, 2001). It is, however, consistent with Tseng (2008) who found basolateral VHA in osmoregulatory gills.

VHA isoforms expression in different conditions

In gill 5, mRNA abundance of NKA from crabs in low-Na^+ condition did not increase as much as in low-Cl^- condition. This observation is consistent

with the protein abundance of crabs acclimated to ion-poor environments. Relative abundance of NKA mRNA also increased in the nonosmoregulatory gill, gill 9. This shows that mRNA expression, not activity of NKA, was induced (Tseng, 2008).

In gill 5 and gill 9, mRNA abundance was upregulated when crabs were acclimated in low-Cl^- condition. This supported my hypothesis that Cl^- uptake is driven by VHA in *C. rathbunae*. The Cl^- uptake mechanism in hyperosmoregulatory crabs, VHA is localized in the apical membrane of *E. sinensis* by electrophysiology (Onken and Putzenlenchner, 1995) and *N. granulata* (Tresguerres *et al.*, 2008) and *Uca fomosensis* (Tsai and Lin, 2007) by immunolocalization. Apical VHA did not directly participate in Cl^- uptake, but via the electrical gradient made by apical VHA (Freire *et al.*, 2008; Charmantier *et al.*, 2009). Apical VHA is coupled with the apical Cl^-/HCO_3^- exchanger and cytoplasmic carbonic anhydrase (Genovese *et al.*, 2005; Tresguerres *et al.*, 2008). Suggesting that VHA is powering the Cl^-/HCO_3^- exchanger for Cl^- uptake in hypotonic environments. Tsai and Lin (2007) suggest apical VHA is a unique feature for crabs adapted to ion-poor environments, such as fresh water.

Although apical VHA is suggested in hyperosmoregulatory crabs, Tseng (2008) found basolateral VHA in *C. rathbunae*. I found that mRNA abundance was induced by low-Cl^- condition and may have participated in Cl^- uptake

mechanisms. Therefore VHA may be responsible for Cl^- uptake in the freshwater crab, *C. rathbunae*. While the basolateral VHA may not have a direct role in osmoregulation, but mRNA expression was still induced by low Cl^- condition in both nonosmo- and osmoregulatory gills in *C. rathbunae*. Basolateral VHA is responsible for base excretion and is coupled with Cl^- uptake in freshwater fish (Piermarini and Evans, 2001; reviewed by Tresguerres *et al.*, 2006). Basolateral VHA is also suggested as responsible by an electrophysiological study of *D. pagei*. Concanamycin has a stronger effect on internal hemolymph-like saline than on external site (Weihrauch *et al.*, 2004) implying that the basolateral VHA in the freshwater crab, *D. pagei*. In addition, VHA-dependent Cl^- uptake coupled with the apical Cl^-/HCO_3^- exchanger is suggested for the role of basaolateral VHA in *C. ratubunae* in Cl^- uptake via apical Cl^-/HCO_3^- exchanger and also in base excretion.

Conclusion

I. Antennal gland in *Candidiopotamon rathbunae* contained of coelomosac and labyrinth.

II. NKA was localized in the basolateral membrane of the labyrinthine cells, the major site for ion reabsorption.

III. Isotonic and anisoionic urine in the freshwater crab, *Candidiopotamon rathbunae* indicates ionoregulatory ability of the antennal gland.

IV. There were two isoforms of VHA-B subunit in gills of *C. rathbunae*. These isoforms were also expressed in selected nonosmo- and osmoregulatory tissues.

V. Isoforms of VHA B-subunit were related to crab phylogeny and habitat.

VI. VHA was responsible for Cl^- uptake and may be coupled with apical Cl^- /HCO_3^- exchanger.

Prospectives

I. Function of different cell types in the antennal gland should be investigated.

II. The function in calcium homeostasis of antennal gland during the molt cycle should be tested.

III. Reprocessing of isotonic urine when freshwater crabs are exposed to air.

IV. The HCO_3^-/Cl^- exchanger and carbonic anhydrase involved in osmoregulation in a freshwater crab need more evidence to prove.

V. Crustacean hyperglycemic hormone (CHH) may induce VHA expression of a freshwater crab during osmotic stress.

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