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Comparison of the osmoregulatory capabilities among three amphibious sea snakes (*Laticauda* spp.) in Taiwan

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Abstract

Background: The three species of amphibious sea snakes (*Laticauda semifasciata*, *L. laticaudata*, and *L. colubrina*) in Taiwan were described as having different habitat affinities from terrestrial to marine. In this study, the osmoregulatory capabilities of three species were compared to test if their capabilities were associated with different habitat affinities.

Results: The sea snakes were transferred from a terrestrial environment to freshwater (FW) or seawater (SW) for 1 week, and then, Na⁺/K⁺-ATPase (NKA) activities of the salt gland (sublingual glands) and kidneys, the water content of the muscles, the body fluid osmolality, and Na⁺, Cl⁻, and K⁺ concentrations were measured. Results showed that the body fluid osmolality, Na⁺ and Cl⁻ levels, and muscle water content of most marine species, *L. semifasciata*, remained relatively constant, and the NKA activity of its salt gland was approximately threefold higher than those of *L. laticaudata* and *L. colubrina*. In both *L. semifasciata* and *L. laticaudata*, NKA activities of the salt glands were higher in SW than in FW; however, no significant change was found in *L. colubrina* (the most terrestrial species).

Conclusions: This study suggests that the NKA activity of the sublingual gland is associated with salt excretion, and the three species possess different osmoregulatory strategies which are associated with their habitat affinities.

Keywords: Sea snake; Salt gland; Osmoregulation; Na⁺/K⁺-ATPase

Background

Secondary marine animals are groups that originated from freshwater (FW) or terrestrial habitats and have recolonized the marine environment (Vermeij and Dudley 2000; Willmer et al. 2004). When a marine invasion occurs, salinities of marine environments pose a strong physiological barrier to these animals. The osmolality of seawater (SW) is appropriately 1,000 mOsm/kg; however, most terrestrial vertebrates maintain their body fluids at appropriately 250 to 450 mOsm/kg. In hypertonic marine environments, vertebrates experience challenges of dehydration and salt accumulation. For successful habitation, various osmoregulatory tissues and organs have evolved in marine vertebrates in response to osmotic challenges. Salt glands are specialized organs for salt secretion in marine vertebrates including birds, reptiles, and elasmobranches

(Schmidt-Nielsen 1958, 1960; Oguri 1964). Nasal salt glands and rectal salt glands were respectively described in marine birds and marine elasmobranches (Burger 1965; Peaker 1971). In reptiles, several kinds of salt gland are found: lachrymal glands in sea turtles and terrapins (Dunson 1970; Hudson and Lutz 1986), nasal glands in marine iguanas (Dunson 1969), lingual glands in crocodilians (Taplin and Grigg 1981), and sublingual and premaxillary glands in marine snakes (Dunson et al. 1971; Dunson and Dunson 1973, 1979). In marine teleosts, the gills are the major organ for salt excretion (Evans et al. 2005).

The physiological mechanisms of salt secretion were explicitly studied in the gills of marine teleosts (Marshall 2002; Evans et al. 2005; Hwang and Lee 2007) and in the salt glands of marine birds and elasmobranches (Riordan et al. 1994; Silva et al. 1997; Shuttleworth and Hildebrandt 1999; Hildebrandt 2001). In the gills of marine teleosts, mitochondrion-rich cells (MRCs) actively secrete internal Cl⁻ and Na⁺. Three ion transport proteins were found to play critical roles in salt secretion: Na⁺/K⁺-ATPase (NKA),

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the $\text{Na}^+/\text{K}^+ / 2 \text{Cl}^-$ cotransporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR)-like Cl^- channel (Marshall 2002). Basolateral NKA pumps Na^+ out of MRCs, and the accumulated Na^+ is extruded by an electrochemical gradient through a paracellular pathway. The Na^+ gradient of MRCs drives the NKCC to carry Na^+ , K^+ , and 2 Cl^- into MRCs. Then, the accumulated Cl^- in MRCs diffuses out of cells via the Cl^- channel on the apical membrane (Marshall 2002; Evans et al. 2005; Hwang and Lee 2007). In the salt glands of marine birds and elasmobranchs, the three transport proteins of NKA, NKCC, and CFTR were also identified in secretory principal cells, and their mechanism is similar to that of the fish gills (Hildebrandt 2001).

The sublingual gland of sea snakes secretes a concentrated salt solution after salt loading, suggesting that it is the salt gland of the sea snakes (Dunson et al. 1971). The sublingual gland lies on the ventrolateral surface of the tongue sheath, and its morphology is similar to those of the other salt glands in marine birds and elasmobranchs (Babonis et al. 2009). Recently, NKA and the NKCC were immunolocalized to the basolateral membrane of principal cells in the sublingual gland of three species of the sea snakes (*Laticauda* spp.), suggesting that the mechanism of salt secretion in sea snakes is similar to that of the other marine vertebrates (Babonis et al. 2009). The three species of amphibious sea snakes (Elapidae: Laticaudinae) are distributed at Orchid Island (*Lanshu* in Chinese) and Green Island (*Ludao* in Chinese) off the southeast coast of Taiwan. They were described as having different habitat affinities in previous studies (Lillywhite et al. 2008, 2009). *Laticauda semifasciata* (Reinhardt) is the most marine species. On the contrary, *L. colubrina* (Schneider) is the most terrestrial species (Shetty and Shine 2002). *L. laticaudata* (Linnaeus) has an intermediate habitat affinity. Lillywhite et al. (2008) measured the dehydration rates of the three species in air and found that the rate in *L. colubrina* (the most terrestrial species) was lower than those in the other two species. Moreover, the skin permeability to water also differed among these species (Lillywhite et al. 2009), suggesting that their water regulation is associated with their habitat affinities (Lillywhite et al. 2008, 2009). However, the osmoregulatory capabilities of the three species in response to osmotic challenges have not been fully investigated. In this study, we attempted to further examine their osmoregulation and test if there is an association between their physiological performances and habitat affinities. NKA activities (which are a parameter for the activity of salt-secreting organs) of the salt gland and kidney, body fluid osmolality, ion concentrations, and muscle water content were analyzed in the three species after subjecting them to hypertonic (SW) and hypotonic (FW) challenges.

Methods

Animal collection and experimental protocols

Three species of adult male sea snakes were mainly collected from Orchid Island, Taiwan, and a few *L. laticaudata* and *L. colubrina* were collected from Green Island, Taiwan, in August to September 2009 and April to August 2010. The animals were captured by hand in shallow coastal inlets and were transported to the laboratory of National Taiwan Normal University, Taipei, Taiwan. The body length and weight of the three species were respectively 104.2 ± 1 cm and 532.3 ± 18 g for *L. semifasciata*, 91.4 ± 1 cm and 165.0 ± 7 g for *L. laticaudata*, and 89.7 ± 1.7 cm and 200.6 ± 11 g for *L. colubrina*. The animals were kept in plastic tanks ($36.5 \times 23.3 \times 15.6$ cm, one individual per tank). Each tank contained a plastic basin ($15 \times 20 \times 8$ cm) filled with dechlorinated tap water for drinking and soaking during 1 week of acclimation. Then, five to eight individuals of each species were initially sampled (representing the pretransfer condition), and the remainder were randomly transferred to FW (dechlorinated tap water) or 32 ppt SW (prepared using artificial sea salt, Instant Ocean™ powder, Crystal Sea, Baltimore, MD, USA). The salinity of the water was monitored with a refractometer (PAL-06S, Atago, Tokyo, Japan). During FW or SW acclimation, the animals were confined to the water but could expose their head to breathe. Five to eight individuals of each species were sampled after 7 days of acclimation in FW or SW. In a preliminary study, several individuals were acclimated to FW or SW for up to 14 days, and we found that some of them became inactive probably due to starvation and osmotic stress. Therefore, we chose 7 days of acclimation for sampling. The animals were not fed, and the water temperature was kept at 27°C with a 12-h light and 12-h dark photoperiod during the experimental period. In this study, all animals were treated in accordance with the protocols approved by the Animal Care and Use Committee of National Taiwan Normal University (permit no. 93013).

Tissue sampling

The animals were anesthetized with ice and euthanized by decapitation, and their body fluid was collected from the opening of vessels into microtubes for analysis of osmolality and ionic concentrations. The sublingual gland and kidneys were excised and stored at -80°C for NKA activity measurements. A lateral trunk muscle was excised for water content measurement.

Measurement of body fluid osmolality and ionic concentrations

The collected body fluid was immediately centrifuged at $6,000 \times g$ for 5 min, and aliquots of fluid were stored at -20°C for the analysis of osmolality and ionic concentrations. The osmolality was determined with a vapor pressure

osmometer (Wescor Model 5520, Logan, UT, USA), and Na^+ , Cl^- , and K^+ concentrations were determined by a biochemical analyzer (Vitro 5.1 FS, Johnson & Johnson, New Brunswick, NJ, USA).

Water content of the muscle

Surface water and blood on the muscle tissues were cleaned off with tissue paper. The water content was determined by calculating the percentage weight loss after drying the tissues at 100°C for 3 days (Huang et al. 2010).

Protein extraction from the salt gland and kidneys

Salt gland tissues and kidneys were separately homogenized in a homogenizing medium consisting of 25 mM Tris-HCl (Bio-Rad, Hercules, CA, USA), 0.25 mM sucrose (Merck, Darmstadt, Germany), 20 mM EDTA (Sigma, St Louis, MO, USA), and 0.4% sodium deoxycholate (Sigma, pH 7.4), which contained protease inhibitors of 3.31 mM antipain (Sigma), 2.16 mM leupeptin (Sigma), and 63.86 mM benzamide (Sigma) in an aprotinin saline solution (5 to 10 trypsin inhibitor units/ml, Sigma) using an ultrasonic processor. The protease inhibitor and homogenizing medium volumetric ratio was 1:200. Homogenates were first centrifuged at 4°C and 6,000×g for 15 min and then centrifuged at 4°C and 20,000×g for 20 min. Fresh supernatants were immediately analyzed for NKA activity. Then, 5 μL of the supernatant was further diluted to 50 μL with deionized water. An aliquot of 10 μL of this mixture was further diluted to 800 μL with deionized water. This diluted supernatant was well mixed with 200 μL of protein assay solution (Bio-Rad). Total protein levels were determined with a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 595 nm.

NKA activities of the salt gland and kidneys

The method of measuring NKA activity was as described in a previous study (Tsai and Lin 2007). Briefly, NKA activity was assayed in a ouabain-containing reaction medium of 20 mM imidazole (Sigma), 130 mM NaCl (Merck), 10 mM MgCl_2 (Merck), and 1 mM ouabain (Sigma) at pH 7.4 and ouabain-free reaction medium of 20 mM imidazole, 100 mM NaCl, 30 mM KCl (Merck), and 10 mM MgCl_2 at pH 7.4. The mixture was supplemented with 100 μL of an ATP stock solution (25 mM Na_2ATP , Sigma) and incubated at 37°C for 15 min. Ten microliters of protein extraction was added to 400 μL of reaction medium. The reaction was stopped by adding 200 μL of an ice-cold 30% trichloroacetic acid (TCA) stock solution (Merck). The reaction mixture was centrifuged at 1,640×g and 4°C for 10 min, and the supernatant (500 μL) was collected. The NKA activity was calculated as the difference in the inorganic phosphate (Pi) content between the ouabain-containing and

ouabain-free reaction solutions. The Pi concentration was determined by Botting's color reagent (560 mM H_2SO_4 , 8.1 mM ammonium molybdate tetrahydrate, and 176 mM FeSO_4 , all from Sigma) with a spectrophotometer. Ice-cold Botting's color reagent (1,000 μL) was added to the supernatant (500 μL) and incubated in a 20°C water bath for 20 min, and the absorbance of the solution was measured at 700 nm. The NKA activity was expressed as μmol Pi/mg/protein/h.

Statistical analysis

All values are presented as the mean ± standard error of the mean (SEM). Values were analyzed by a *t* test or one-way analysis of variance (ANOVA) and Tukey's *post hoc* honest significant difference (HSD) test conducted using JMP 7 (SAS Institute, Cary, NC, USA). Differences were considered statistically significant for *p* values of <0.05.

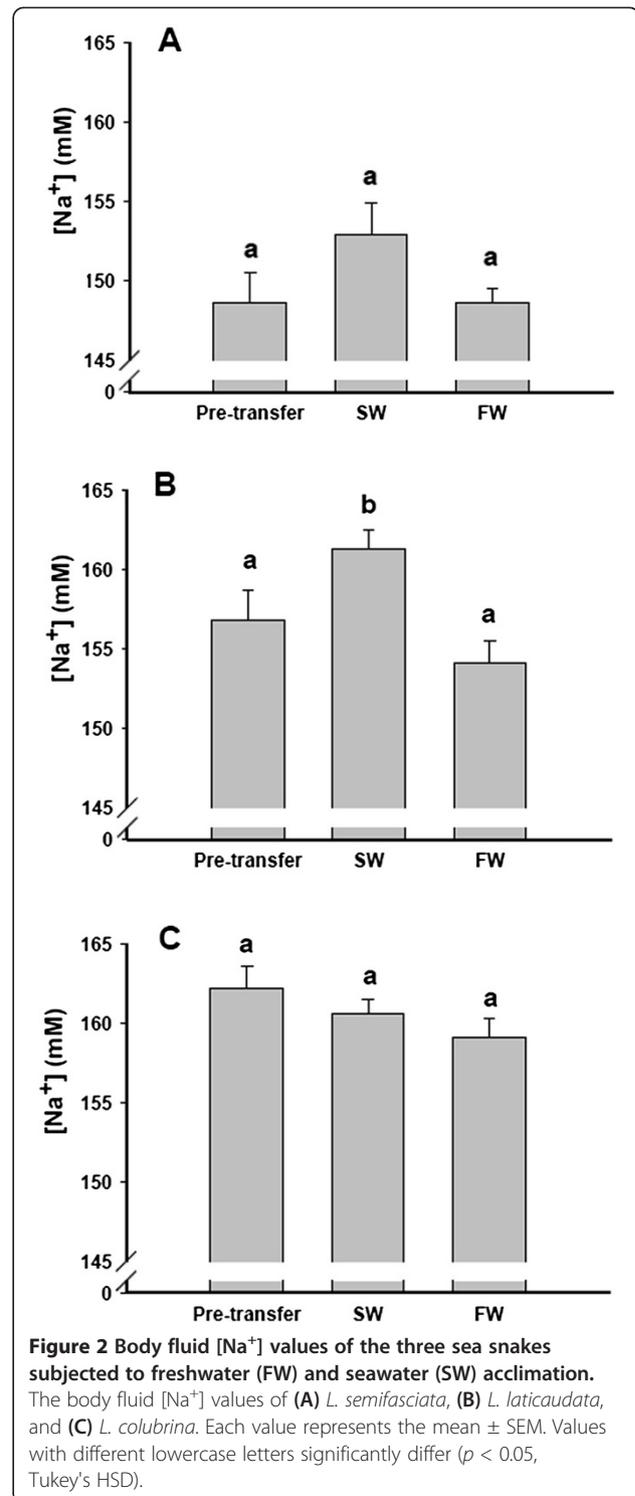
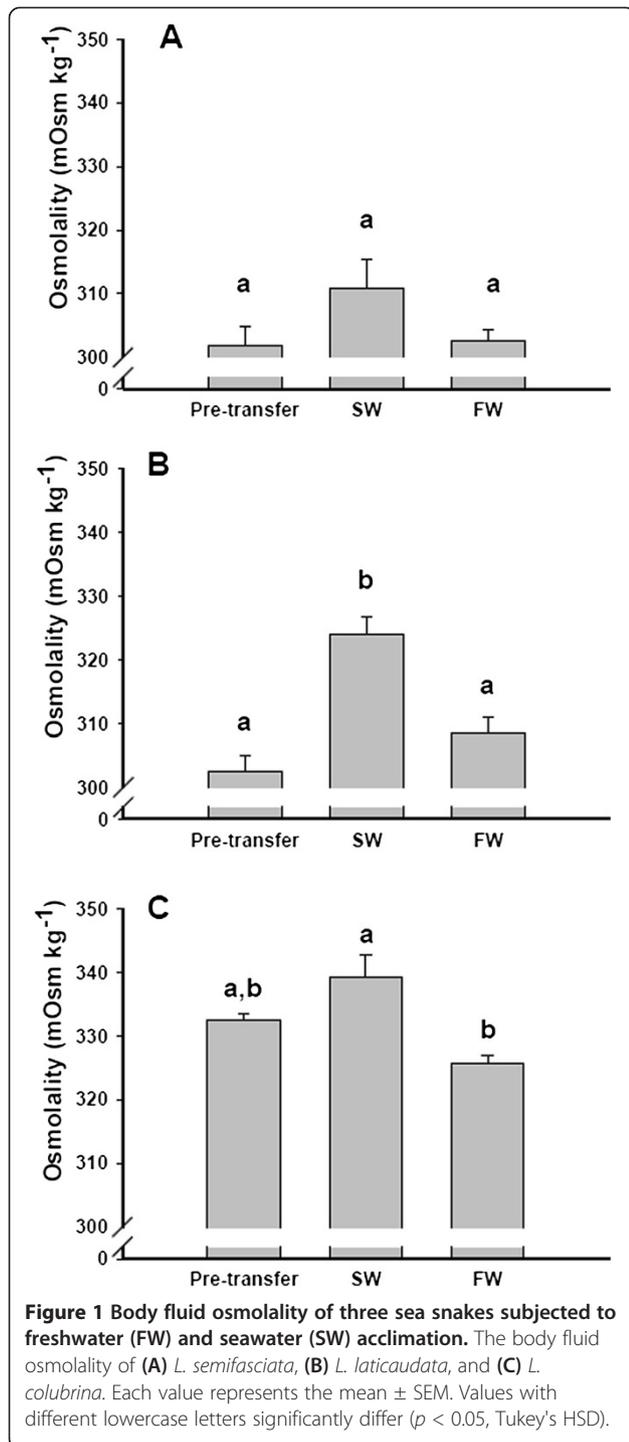
Results

Body fluid osmolality

In the most marine species, *L. semifasciata*, the body fluid osmolality was 301.9 ± 3 mOsm/kg before transfer (Figure 1A), and no significant change was found after FW or SW acclimation (302.5 ± 2 mOsm/kg in FW and 310.8 ± 4 mOsm/kg in SW; $F_{2,21} = 2.16$, $p = 0.14$, ANOVA). In *L. laticaudata*, the osmolality was 302.4 ± 3 mOsm/kg before transfer (Figure 1B), and it significantly increased to 324.1 ± 3 mOsm/kg ($p < 0.001$, Tukey's HSD) after SW acclimation but did not significantly change after FW acclimation (308.4 ± 3 mOsm/kg; $p = 0.18$, Tukey's HSD). In the most terrestrial species, *L. colubrina*, the osmolality was significantly higher than those of the other two species before transfer ($p < 0.001$, Tukey's HSD) at 332.4 ± 1 mOsm/kg (Figure 1C), and it slightly increased to 339.2 ± 3 mOsm/kg ($p = 0.1$, Tukey's HSD) after SW acclimation and slightly decreased to 325.7 ± 1 mOsm/kg ($p = 0.08$, Tukey's HSD) after FW acclimation. A significant difference was found between the FW and SW groups ($p < 0.05$, Tukey's HSD).

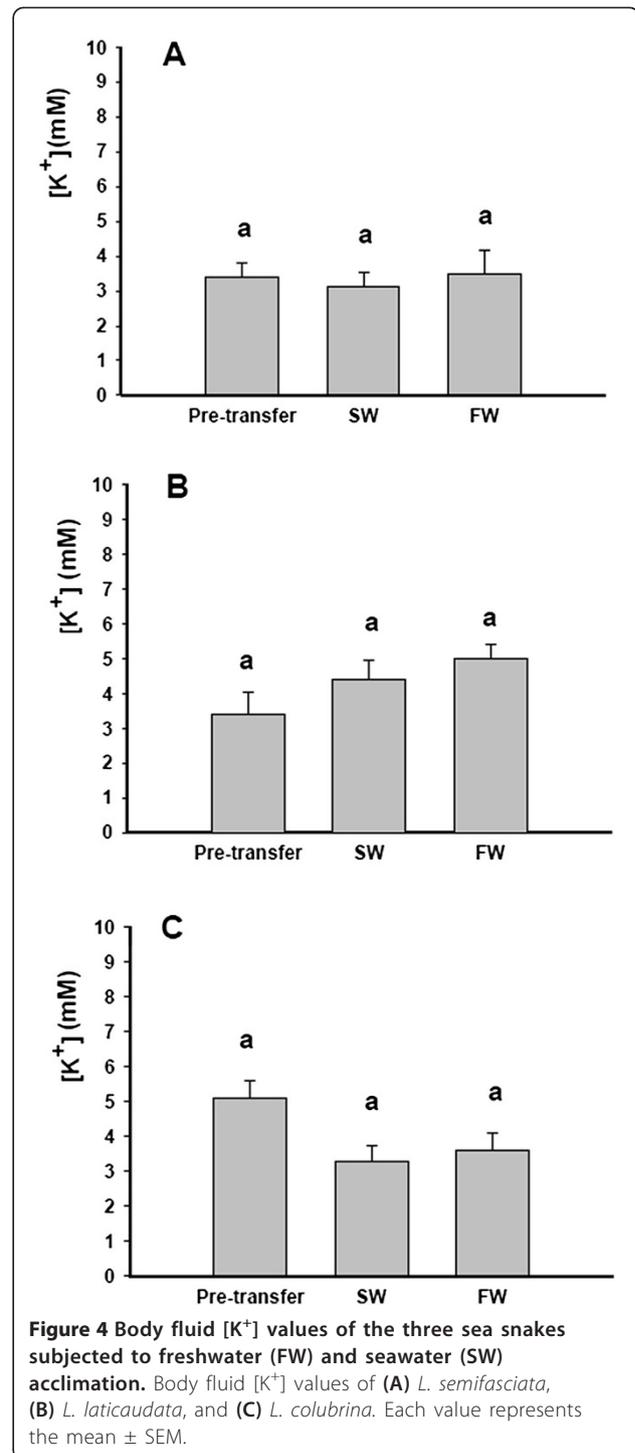
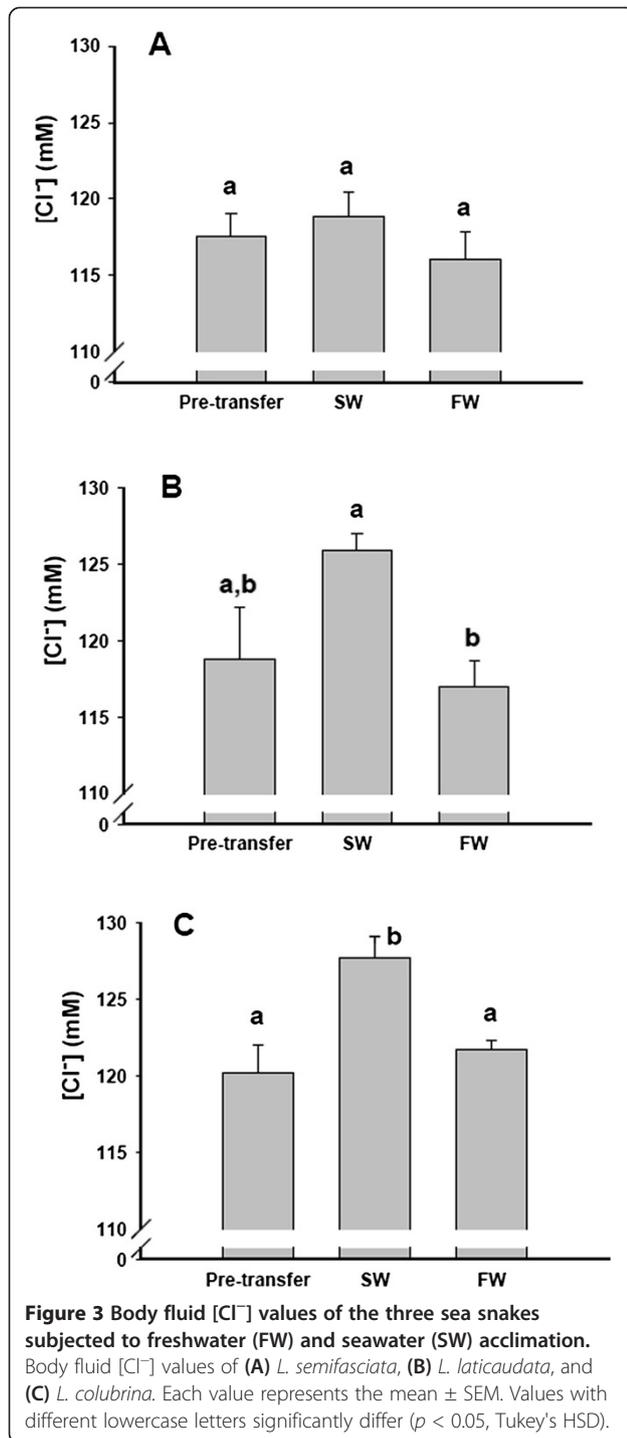
Body fluid ion concentrations

In the most marine *L. semifasciata*, body fluid [Na^+], [Cl^-], and [K^+] levels were 148.6 ± 2 , 117.5 ± 2 , and 4.6 ± 1.2 mM, respectively, before transfer (Figures 2A, 3A, and 4A). After transfer to FW or SW, no significant change was found in these ionic concentrations (Na^+ , $F_{2,21} = 2.06$, $p = 0.15$; Cl^- , $F_{6,34} = 0.85$, $p = 0.44$; K^+ , $F_{2,19} = 0.21$, $p = 0.81$, ANOVA). In *L. laticaudata*, the body fluid [Na^+] was 156.8 ± 2 mM before transfer (Figure 2B), and a significant difference was found between the FW (153.1 ± 1 mM) and SW (161.3 ± 1 mM) groups ($p < 0.05$, Tukey's HSD). Body fluid [Cl^-] was 118.8 ± 3 mM before transfer (Figure 3B), and a significant difference was found



between the FW (117 ± 2 mM) and SW (125.9 ± 1 mM) groups ($p < 0.05$, Tukey's HSD). Body fluid $[K^+]$ was relatively constant, and no significant change was found after FW or SW acclimation ($F_{2,21} = 2.02$, $p = 0.16$, ANOVA; Figure 4B). In the most terrestrial *L. colubrina*, $[Na^+]$ was 162 ± 1 mM before transfer (Figure 2C), and no significant change was found after FW or SW

acclimation ($F_{2,16} = 1.64$, $p = 0.22$, ANOVA). Body fluid $[Cl^-]$ of *L. colubrina* was 120.2 ± 2 mM before transfer (Figure 3c), and it significantly increased after SW acclimation (127.7 ± 1 mM, $p < 0.05$, Tukey's HSD), whereas no significant change was found after FW (121.7 ± 0.6 mM) acclimation ($p = 0.7$, Tukey's HSD). Body fluid $[K^+]$

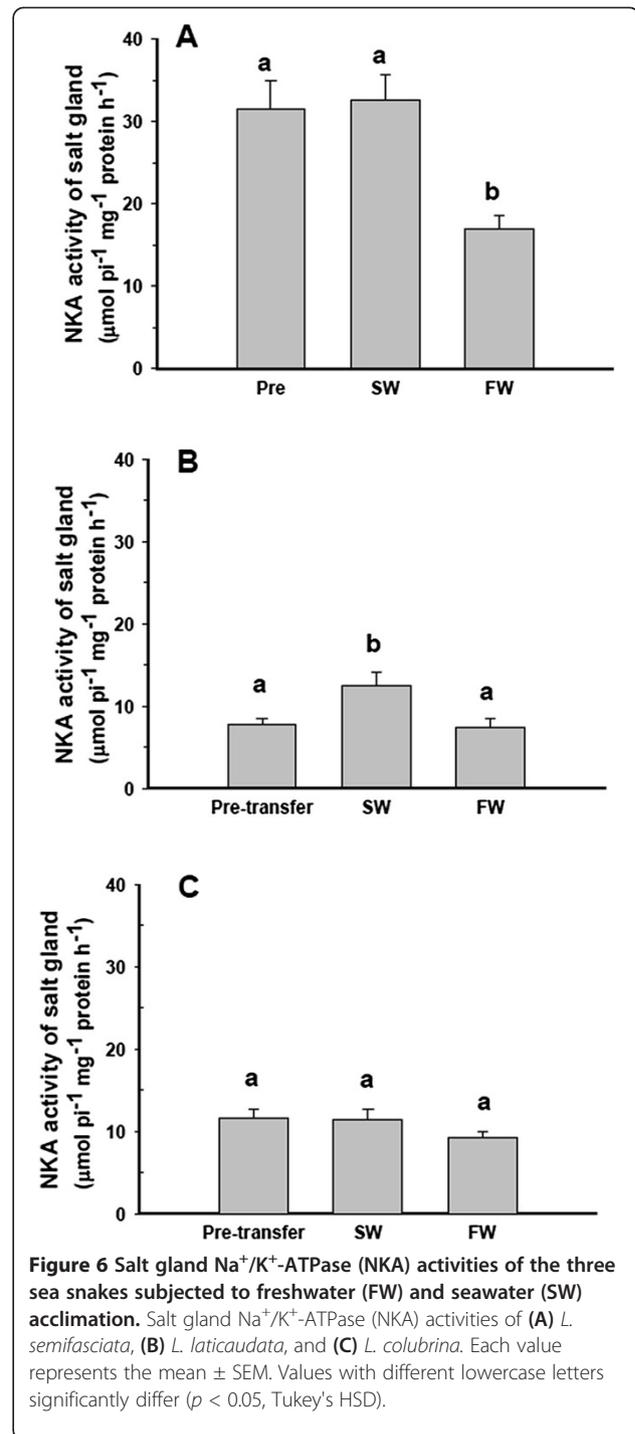
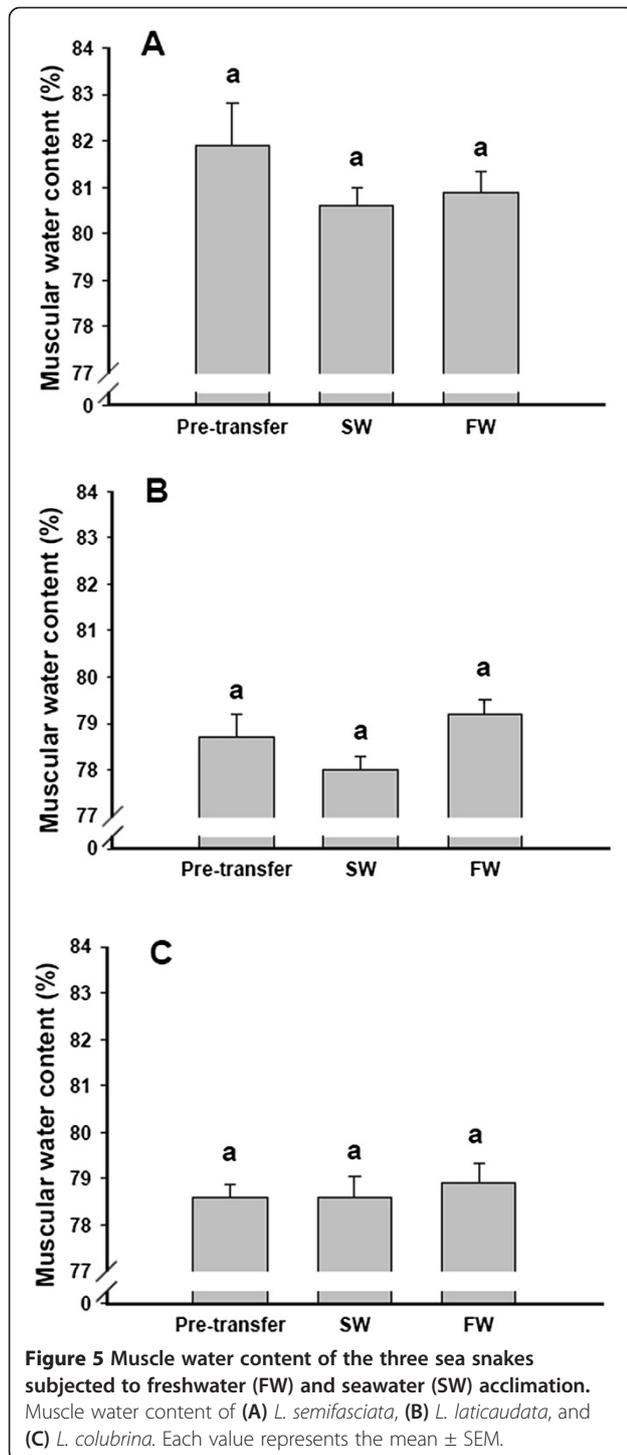


was 5.9 ± 0.3 mM before transfer (Figure 4C), and no significant change was found after FW or SW acclimation ($F_{2,16} = 1.19$, $p = 0.33$, ANOVA).

Muscle water content

Muscle water content among the three species was similar, at 80% to 81% for *L. semifasciata*, 78% to 79%

for *L. laticaudata*, and 78.6% to 78.9% for *L. colubrina*. No significant change was found after FW or SW acclimation in the three species (*L. semifasciata*, $F_{2,18} = 1.34$, $p = 0.84$, ANOVA, Figure 5A; *L. laticaudata*, $F_{2,18} = 2.83$, $p = 0.09$, ANOVA, Figure 5B; *L. colubrina*, $F_{2,16} = 0.19$, $p = 0.83$, ANOVA, Figure 5C).



NKA activities of the salt gland

In *L. semifasciata*, NKA activity of the salt gland before transfer was significantly higher than those of the other species ($31.6 \pm 3 \mu\text{mol Pi/mg/protein/h}$; $p < 0.001$, Tukey's HSD, Figure 6A). It did not significantly change after SW acclimation ($32.6 \pm 3 \mu\text{mol Pi/mg/protein/h}$; $p = 0.9$, Tukey's HSD). However, it significantly decreased to

$17.0 \pm 2 \mu\text{mol Pi/mg/protein/h}$ after FW acclimation ($p < 0.05$, Tukey's HSD). The NKA activity of *L. laticaudata* was $7.1 \pm 0.5 \mu\text{mol Pi/mg/protein/h}$ before transfer (Figure 6b). It did not significantly change after FW acclimation ($7.5 \pm 1 \mu\text{mol Pi/mg/protein/h}$; $p = 0.9$, Tukey's HSD), but it significantly increased to $12.5 \pm 2 \mu\text{mol Pi/mg/protein/h}$ after SW acclimation ($p < 0.05$, Tukey's HSD). In *L. colubrina*, the NKA activity of the

salt gland was $11.6 \pm 1 \mu\text{mol Pi/mg/protein/h}$ before transfer (Figure 6c). However, no significant difference was found after FW or SW acclimation ($F_{2,15} = 1.89$, $p = 0.18$, ANOVA).

NKA activities of the kidneys

The NKA activity of the kidneys was lower than that of the salt glands in all species before transfer (*L. semifasciata*, $3.4 \pm 0.4 \mu\text{mol Pi/mg/protein/h}$, $p < 0.001$, *t* test; *L. laticaudata*, $3.4 \pm 0.6 \mu\text{mol Pi/mg/protein/h}$, $p < 0.001$, *t* test; *L. colubrina*, $5.1 \pm 0.5 \mu\text{mol Pi/mg/protein/h}$, $p < 0.001$, *t* test). In addition, no significant change was found after FW or SW acclimation in the three species (*L. semifasciata*, $F_{2,18} = 0.21$, $p = 0.81$, ANOVA, Figure 7A; *L. laticaudata*, $F_{2,21} = 1.42$, $p = 0.26$, ANOVA, Figure 7B; *L. colubrina*, $F_{2,15} = 3.29$, $p = 0.07$, ANOVA, Figure 7C).

Discussion

The sea snakes are usually subdivided into two subfamilies (the Hydrophiidae and Laticaudinae) which originated from terrestrial elapid ancestors. However, the two subfamilies do not form a monophyletic group; instead, they represent two separate marine invasions in evolutionary history (Keogh 1998). The Hydrophiidae is comprised of fully marine species. However, the Laticaudinae (including one genus and eight species) is comprised of amphibious sea snakes (Cogger and Heatwole 2006) that forage in the sea but return to land to digest food, shed their skins, rest, and reproduce (Heatwole 1999; Brischox et al. 2007; Brischox and Bonnet 2009).

The three sea snakes (*L. semifasciata*, *L. laticaudata*, and *L. colubrina*) in Taiwan are also amphibious species, and they prefer to rest on coastal reefs with freshwater fountains (Liu et al. 2012). A previous study (Lillywhite et al. 2008) measured the dehydration rates of the three species when they were out of water and found no difference between *L. semifasciata* and *L. laticaudata*, whereas that of the *L. colubrina* was significantly lower than those of the other two species. Surprisingly, the dehydrated sea snakes were found to drink diluted SW but not full-strength SW. The salinities of the water they chose to drink also differed; *L. semifasciata* drank 30% SW, *L. laticaudata* drank 20% SW, and *L. colubrina* drank the most diluted SW (10%). This finding explains why these species prefer to stay on coastal reefs with freshwater fountains. In the present study, we further found differences in their osmoregulatory abilities. In the most marine species, *L. semifasciata*, osmolality, $[\text{Na}^+]$, and $[\text{Cl}^-]$ were relatively constant after FW and SW acclimation (Figures 1, 2, and 3). However, in *L. laticaudata* and *L. colubrina*, osmolality, $[\text{Na}^+]$, and $[\text{Cl}^-]$ significantly increased after SW acclimation, and values of these parameters in the SW groups were significantly higher than those in the FW groups (Figures 1, 2, and 3). These

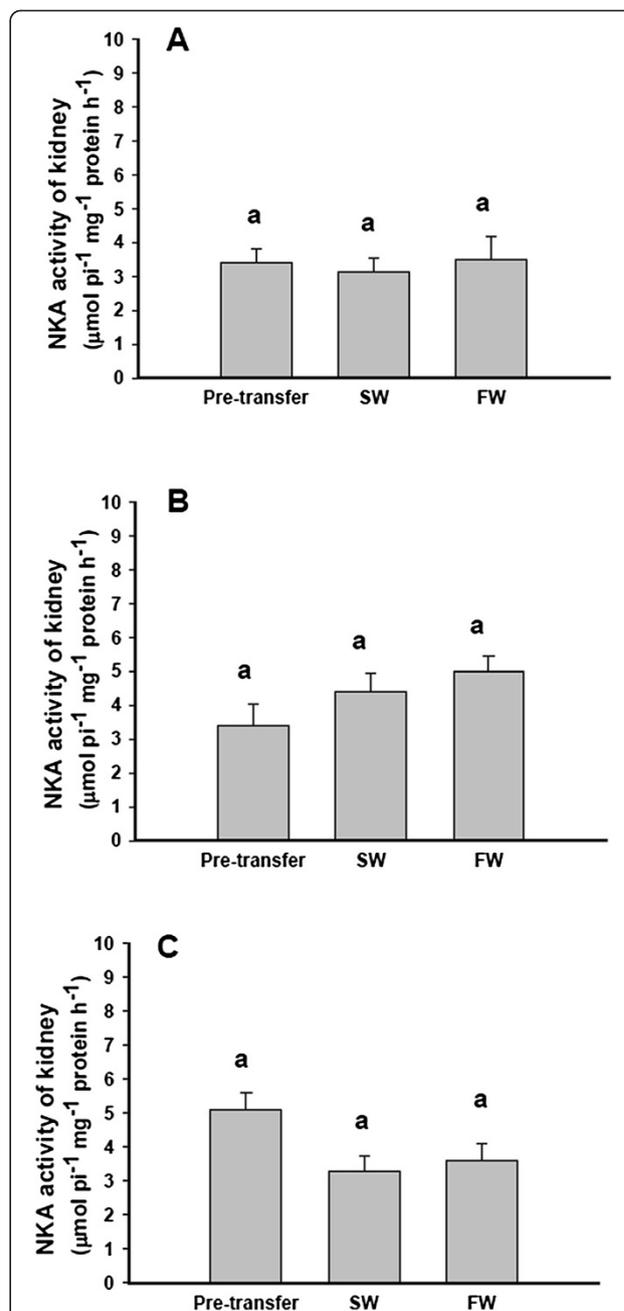


Figure 7 Renal Na^+/K^+ -ATPase (NKA) activities of the three sea snakes subjected to freshwater (FW) and seawater (SW) acclimation. Renal Na^+/K^+ -ATPase (NKA) activities of (A) *L. semifasciata*, (B) *L. laticaudata*, and (C) *L. colubrina*. Each value represents the mean \pm SEM.

results suggest that the three sea snakes have different osmoregulatory capabilities or strategies to respond to osmotic challenges.

Compared to euryhaline teleosts (Jensen et al. 1998; Kelly and Woo 1999; Lin et al. 2004a, b, 2006; Scott et al. 2006; Chew et al. 2009; Huang et al. 2010), fluctuations in body fluid osmolality and ionic concentrations were relatively

small in the three sea snakes subjected to salinity challenges. Changes in osmolality are about 50 to 100 mOsm/kg in teleosts, whereas they were only about 20 mOsm/kg in these sea snakes (Figure 1). In teleosts, the problem of osmotic and ionic gradients is exacerbated by the presence of the gill epithelium, a large, thin tissue used for efficient gas exchange and passive movement of water and salts (Evans et al. 2005). In contrast, the sea snakes are air breathers, and their skin is impermeable to Na^+ and slightly permeable to water (Dunson and Robinson 1976; Lillywhite et al. 2009). Therefore, the body fluid osmolality and ionic composition were relatively stable in these sea snakes subjected to salinity changes.

NKA plays a critical role in salt secretion by branchial MRCs of marine teleosts (Hwang and Lee 2007). It is well documented that euryhaline teleosts regulate branchial NKA activity in response to salinity changes (Marshall 2002; Evans et al. 2005). In euryhaline teleosts such as salmon (Tipsmark et al. 2002), eel (Wilson et al. 2007), tilapia (Lin et al. 2004a), and spotted green pufferfish (Lin et al. 2004b), branchial NKA activity was reported to increase in response to hypertonic acclimation. In euryhaline elasmobranchs such as the bull shark *Carcharhinus leucas* and Atlantic stingray *Dasyatis sabina*, NKA activity of the rectal salt gland was significantly higher in SW than in FW (Piermarini and Evans 2000; Pillans et al. 2005). In sea snakes, NKA activities of the salt gland in several species were reported in early studies (Dunson and Dunson 1974). However, little is known about the regulation of NKA activity in the sea snakes subjected to salinity changes. In an early report (Dunson and Dunson 1975), no significant difference was found in salt gland NKA activity of the yellow-bellied sea snake *Pelamis platurus* acclimated to SW and FW. In this study, we found that NKA activity was higher in SW-acclimated than in FW-acclimated *L. semifasciata* and *L. laticaudata* (Figure 6), suggesting that NKA is critical and upregulated for salt secretion by the sublingual salt gland. Interestingly, responses of salt gland NKA activities to salinity changes varied among the three species, suggesting that they might have different strategies to cope with osmotic challenges.

In the most marine *L. semifasciata*, the NKA activity of the salt gland was approximately threefold higher than those of the other species before transfer, and it was downregulated after transfer to FW (Figure 6A) but did not significantly change after transfer to SW. We suggest that this species has high NKA activity and a high salt-secreting capability to cope with SW challenges, and this capability is associated with its marine affinity. With a high salt-secreting capability, it can drink 30% seawater (Lillywhite et al. 2008) and excrete redundant salt. On the other hand, it can still maintain its osmolality and ionic levels in FW, indicating

that hypotonic water is not a limiting factor of its distribution.

In the most terrestrial *L. colubrina*, NKA activity was lower than that of the *L. semifasciata* and did not respond to SW or FW acclimation, implying that *L. colubrina* might have a low capability to excrete salt (Figure 6C). However, its osmolality and ionic concentrations remained relatively constant after acclimation. Since this species has a lower skin permeability to water than the other two species (Lillywhite et al. 2009), it might also have low permeability to salt and use a passive strategy to cope with osmotic challenges instead of an active regulatory strategy. In the wild, it probably drinks low-salinity water from freshwater fountains and stays on land a longer time to prevent salt loading. In addition, only $[\text{Cl}^-]$ but not $[\text{Na}^+]$ was elevated in *L. colubrina* after transfer to SW, suggesting that *L. colubrina* might have a lower permeability to Na^+ than to Cl^- .

Due to lacking Henle's loop, marine teleosts, elasmobranchs, and reptiles cannot produce highly concentrated urine (Hill et al. 2004). In marine reptiles, early studies suggested that the kidneys of the saltwater crocodile *Crocodylus porosus* and olive sea snake *Aipysurus laevis* are the primary site of sodium and water reabsorption rather than salt excretion (Benyajati et al. 1985; Yokota et al. 1985; Kuchel and Franklin 1998). In addition, a recent study on *L. semifasciata* and other marine snakes showed that the distribution and abundance of the NKA protein and messenger RNA in the kidneys did not significantly change after salinity acclimation (Babonis et al. 2011). In the present study, the renal NKA activity of the kidneys was lower than that of the salt gland and did not respond to salinity changes (Figure 7), supporting the kidneys not being involved in salt secretion in sea snakes.

Conclusions

This study suggests that the NKA activity of the sublingual gland is associated with salt excretion, and the three species possess different osmoregulatory strategies which are associated with their habitat affinities.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YCC carried out the most experiments and drafted the manuscript. MCT participated in the design of the study. JRT carried out a portion of the NKA activity assays. HCL participated in the design of experiments. LYL conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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