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Effect of Salinity on the Survival, Ions and Urea Modulation in Red-eared Slider (*Trachemys scripta elegans*)

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Abstract To understand the tolerance to salinity and osmoregulation of the introduced *Trachemys scripta elegans*, the salinity stress of four groups (salinity 5‰, 15‰, 25‰ and control group) were conducted. Inorganic ions, osmotic pressure, glucose and aldosterone of blood and urine in *T. s. elegans* (BW: 125.60 ± 19.84 g) were analyzed at 30 d, 60 d and 90 d stress. The results showed that: 1) inorganic ions concentration of blood and urine increased with ambient salinity, which indicated that high influx of ions was combined with higher outflow when exposed to saline water in *T. s. elegans*. However, blood aldosterone decreased with increasing salinity, which indicated that an increased sodium intake resulting in a diminished aldosterone production. However, with elapsed time, inorganic ions in urine decreased, which indicated that inorganic ions in blood would be accumulated, and Na^+ and Cl^- in the plasma inevitably build up to harmful levels, at last death was happening when *T. s. elegans* was exposed to salinity 25 during 90 d salinity stress; 2) blood osmotic pressure increased as ambient salinity increased, it would reach 400 mOsm/kg in the group of salinity 25, which was about 1.5 fold of the control group. Higher blood osmotic pressure was due to both higher blood ions and urea concentrations. There may be another mechanism to avoid an excess of NaCl together with an important loss of water using one of the end-products of nitrogen metabolism; 3) blood glucose in each group except the group of salinity 5 decreased with time elapsed and with salinity increased. Therefore, we can conclude that *T. s. elegans* is an osmoregulator that limits the entry of Na^+ and Cl^-, but can also tolerate certain degrees of increases in plasma Na^+ and Cl^-. When ambient salinity was lower than 15‰, *T. s. elegans* can increase blood osmotic pressure by balancing the entry of NaCl with the secretion of aldosterone decreased, and by accumulating blood urea for osmoregulation effectors, and survive for at least three months. These results could provide theoretical basis for salinity tolerance and the invasion on physiological mechanism for *T. s. elegans*.

Keywords *Trachemys scripta elegans*, salinity stress, osmotic pressure, ion modulation, blood aldosterone

1. Introduction

The Red-eared Slider (*Trachemys scripta elegans*), a freshwater turtle originally from the eastern United States and northeastern Mexico (van Dijk *et al.*, 2011), is commonly sold as pets worldwide, and as a result it is considered as an invasive species in Africa, Asia, Europe and Australia (Luiselli *et al.*, 1997; O'Keeffe, 2005). Now, *T. s. elegans* has been listed as one of the 100 most dangerous invasive species because it can displace native species through predation, hybridization, introduction of pathogens, and/or competition for resources (Cadi and Joly, 2003, 2004; ISSG/SSC, 2001).

Many studies have shown that *T. s. elegans* is a very robust species that is tolerant to harsh environmental conditions. When submerged in cold water (3°C), it can survive without oxygen for up to 18 weeks (Ultsch and Jackson, 1982). Such conditions occur during overwintering in ice-covered rivers and ponds where the water becomes hypoxic and the turtles bury themselves in

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anoxic mud (Ultsch, 1989). The magnitude of metabolic depression can be 10%– 20% of the normoxic rate and can be further decreased to 0.1% due to Q_{10} effects of temperature (Jackson and Heisler, 1982; Jackson *et al*., 2001). Additionally, studies in China have shown that the anti-nitrite stress and starvation endurance of *T. s. elegans* were stronger than native *Mauremys sinensis* (Wei *et al.*, 2012; Zhang *et al.*, 2011). Gibbons *et al* (1979) reported that *T. s. elegans* was able to live in saline ponds (less than 10 ‰ salinity) on Kiawah and Capers Islands, South Carolina, and achieved unusually larger body sizes. During field studies in Hainan Province, China, *T. s. elegans* was often found in the low salinity (0.1‰–26 ‰) estuary portions of the Nandujiang River (Liu, 2011). These studies indicate that the invasive *T. s. elegans* has the ability to invade both in freshwater and saline habitats. This raises two questions for clarification: (1) what is the suitable salinity range for *T. s. elegans* ? and (2) what are the primary factors that enable *T. s. elegans* to inhabit the brackish water?

One of the major difficulties encountered by reptiles living in high salinity water is ionic and osmotic regulation: the osmolarities of their body fluids are about 1/3 of seawater, and their kidneys cannot process urine hypertonic to the plasma (Prange, 1985). The solution to this problem in many marine reptiles, such as the sea turtle *Caretta caretta*, is the development of salt glands (Prange and Greenwald, 1980). And adaptations to salinity for the estuarine turtle are different (Gilles-Baillien, 1970; Dunson and Seidel, 1986). The diamondback terrapin, *Malaclemys terrapin*, is known to have a functional salt gland and can also retain high concentrations of urea to osmoregulate. It has shown levels of 309 mOsm/kg mean serum osmotic concentration in fresh water and as high as 459 mOsm/kg when in sea water (Gilles-Baillien, 1970). *Pseudemys nelsoni*, another species of estuarine turtle, does not possess salt glands, but appears to possess the water-conserving and sodium-excluding mechanisms (Dunson and Seidel, 1986). This leads us to remark a question that how freshwater *T. s. elegans* without salt glands can tolerant saline water. The mechanism and physiological adaptation of salinity on *T. s. elegans* are unclear. Therefore, variation of plasma and urine concentration is of greatest interest in turtles, that inhabit a wide range of osmotic environments.

In this paper, we analyzed the inorganic ion concentrations in blood and urine, blood osmotic pressure, and glucose and aldosterone levels in turtles under different salinity regimes (salinity 5‰, 15‰, 25‰ and control group) at 30 d, 60 d and 90 d stress. Understanding the salinity tolerance and osmoregulation in *T. s. elegans* based our experiment is very important for us to evaluate the ability of this species to invade different habitats.

2. Materials and Methods

2.1 Experimental animals All juveniles of *T. s. elegans* were bought from a local turtle farm in Hainan Province, China. Subsequent experiments were all performed in a laboratory at Hainan Normal University. Individuals were acclimated in four cement pools (190 cm × 65 cm × 32 cm) half filled with fresh water for 30 d. Ninety-two healthy individuals (BW: 125.60 ± 19.84 g) were divided into four groups (salinity: 5‰, 15‰, 25‰, freshwater group and control group), adjusted the experimental salinity we needed by sea salt.. Numbers were painted on the carapace of each individual for identification. Turtles were fed on a diet of food specialized for turtles each Tuesday and Friday, mixed with the same saline water as their surrounding environment. After 24 h, the remaining food was extracted and weighed, and the water in the pools was changed. The salinity of the experimental groups was measured and adjusted to the proper salinity level each day. Ceramic tiles were placed in each pond for shelter and basking. Water temperature in pools was maintained near 25°C and turtles received 12–15 h of light daily.

2.2 Experimental procedure All of the following protocols were approved by the Animal Research Ethics committee of Hainan Provincial Education Centre for Ecology and Environment, Hainan Normal University. At 30 d, 60 d and 90 d, blood and urine samples were collected from six turtles in each group. Urine samples were collected from the bladders by inserting a piece of rubber tubing half-way into the cloaca, followed by applying suction with a 22 gauge hypodermic syringe without a needle which connected to the rubber tubing. The urine was centrifuged and the supernatant frozen to analyze K^+, Na^+, Cl^-, and urea concentrations using a DIMENSION automatic biochemical analyzer (Siemens, German). All samples that appeared to have fecal contamination were discarded.

Individuals were weighted and then sacrificed by cervical dislocation after 10–20 minutes freezing for anaesthesia. A 5–7 ml blood sample without anticoagulant was collected from each turtle and stored in a freezer to analyze the concentration of urea, uric acid, glucose, Na^+, Ca^{2+}, K^+, Cl^-, Mg^{2+} and osmotic pressure using a DIMENSION RxL automatic biochemical analyzer

(Siemens, German) and aldosterone concentration using a GC-911 γ radioimmunoassy instrument (Zhongjia, China).

2.3 Statistical analyses All experimental data were analyzed using SPSS 16.0. Two-way analysis of variance was run to examine the influence of environmental salinity and exposure time on the osmotic and chemical properties of blood and urine. The significant difference between different groups was treated using a Duncan multi-comparison (one-way ANOVA). A 95% level of confidence was used to represent a significant difference between the samples.

3. Results

3.1 Survival rate in *Trachemys scripta elegans* under different salinity stress Table 1 summarizes the survival rate of turtles at different salinity levels. In the experimental group with the salinity (25‰), 1–2 individuals died at each of the time frames. No individuals died in the other groups.

3.2 Effects of ambient salinity on blood osmotic pressure and inorganic ions concentration of *Trachemys scripta elegans* In each sampling time frame, there was no significant difference in blood potassium (K^+) level ($P > 0.05$). However, the level of blood sodium (Na^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) and chlorine (Cl^-) in the group of salinity 25 was significantly higher than that in the control group ($P < 0.05$). As for osmotic pressure in blood, there was a significant difference between each group in each time frame ($P < 0.05$). In the control group, the blood osmotic pressure was 250–260 mOsm/kg, increasing 160% to 400–440 mOsm/kg in the group of salinity 25.

Two-way ANOVA showed that the main effect of ambient salinity in blood Na^+, Cl^-, and osmotic pressure were significantly different between each group ($P < 0.05$), however, no significant difference was found in blood K^+ ($F_{3,46} = 1.718$; $P = 0.176$). However, significant differences were found in blood Mg^{2+} levels between different sampling times ($P < 0.05$). Furthermore, there was a significant interaction between sample time and ambient salinity of osmotic pressure in blood ($F_{6,46} = 3.394$; $P = 0.007$; Table 2)..

3.3 Effects of ambient salinity on biochemical indexes in urine of *Trachemys scripta elegans* Table 3 summarizes the results obtained for Na^+, K^+, Cl^- and urea concentration in urine. As ambient salinity increased, the concentrations of K^+, Na^+ and Cl^- increased in urine, and there was a significant difference between each group in these inorganic ions concentration ($P < 0.05$). In the control group, the concentration of K^+, Na^+ and Cl^- was 2.52 mmol/l, 19.28 mmol/l and 19.46 mmol/l, respectively; these values increase approximately ten-fold to 33.32 mmol/l, 146.15 mmol/l and 183.53 mmol/l, respectively, in the group of salinity 25. Urea levels, increased as a result of increased salinity stress, especially comparing the control group with the group of salinity 5 ($P < 0.05$). In the control group, the urea concentration was 22 mmol/l, and increased 2-fold to 48 mmol/l in the group of salinity 5.

Two-way ANOVA showed that the main effect of sample time on urine Na^+ and Cl^- concentrations were significantly different between groups ($P < 0.05$). As time elapsed, the concentration of Na^+ and Cl^- in urine decreased significantly ($P < 0.05$), however, no significant differences were found in K^+ and urea levels between different sampling times ($P > 0.05$). As for the main effect of ambient salinity, there were significant differences in urine K^+, Na^+, Cl^- and urea ($P < 0.05$), except the urea in the group of salinity 5 and salinity 15 ($P > 0.05$). Furthermore, there was a significant interaction between sample time and ambient salinity in urine Na^+, Cl^- and urea ($P < 0.05$).

3.4 Effects of ambient salinity on blood glucose, aldosterone , urea and uric acid content in *Trachemys scripta elegans* As ambient salinity increased, the concentration of blood urea and uric acid increased significantly. the groups of control and salinity 5 in blood uric acid at each sampling time frame, and the groups of salinity 15 and 25 in blood urea at 60 d and 90 d stress (Table 4). As for blood glucose and aldosterone, there was a tendency to increase with ambient salinity, but decrease when the ambient salinity reach to 15‰.

Two-way ANOVA showed that the main effect of sample time in blood urea was significantly different between each group ($P < 0.05$). As time elapsed, the concentration of blood urea increased significantly ($P < 0.05$). No significant difference existed in blood aldosterone and uric acid and glucose between different sampled times, but there was a tendency to decrease with more time ($P > 0.05$). However, there was significant difference in the main effect of ambient salinity in blood aldosterone of different groups and in blood glucose between the group of salinity 5 and the other groups, almost with the tendency of increased and then decreased as ambient salinity increased ($P < 0.05$). Also, there was significant difference in the main effect of ambient salinity in blood urea and uric acid of different groups (*P*

Table 1 The numbers of deaths in *Trachemys scripta elegans* under different salinity stress.

Time	Control group (n = 23)	Salinity 5‰ (n = 23)	Salinity 15‰ (n = 23)	Salinity 25‰ (n = 23)
0–30 d	0	0	0	2
30–60 d	0	0	0	2
60–90 d	0	0	0	1

Table 2 Effects of ambient salinity on blood osmotic pressure and inorganic ions concentration of *Trachemys scripta elegans*.

	Salinity	K^+ (mmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Ca^{2+} (mmol/L)	Mg^{2+} (mmol/L)	Osmotic pressure (mOsm/kg)
One-way ANOVA							
30d	Control (n = 3)	4.16 ± 0.12	125.60 ± 0.85^a	96.13 ± 2.32	2.23 ± 0.03^a	1.31 ± 0.07^a	261.40 ± 3.71^a
	5‰ (n = 3)	4.09 ± 0.15	131.90 ± 1.75^b	123.37 ± 25.82	2.22 ± 0.01^a	1.40 ± 0.05^a	304.22 ± 9.66^b
	15‰ (n = 3)	3.70 ± 0.39	135.80 ± 1.76^b	105.60 ± 0.59	2.37 ± 0.14^a	1.44 ± 0.04^a	323.01 ± 1.60^c
	25‰ (n = 3)	4.88 ± 0.24	186.93 ± 1.90^c	148.73 ± 1.37	3.04 ± 0.06^b	1.94 ± 0.08^b	433.68 ± 1.71^d
	$F_{3,8}$	3.927	303.780	3.173	23.744	22.504	191.559
	P-value	0.054	0.000	0.085	0.000	0.000	0.000
60d	Control (n = 5)	4.52 ± 0.26	120.55 ± 0.89^a	90.92 ± 1.51^a	2.19 ± 0.05^a	1.53 ± 0.03^a	257.69 ± 1.50^a
	5‰ (n = 6)	4.11 ± 0.52	133.03 ± 1.08^{ab}	101.8 ± 1.06^b	2.18 ± 0.16^a	1.57 ± 0.08^a	300.52 ± 7.06^b
	15‰ (n = 5)	4.11 ± 0.39	148.10 ± 3.38^b	118.46 ± 4.16^c	2.65 ± 0.06^{ab}	1.86 ± 0.09^a	356.70 ± 1.16^c
	25‰ (n = 6)	4.87 ± 0.62	185.48 ± 14.54^c	140.87 ± 3.93^d	3.30 ± 0.50^b	2.34 ± 0.21^b	406.63 ± 4.46^d
	$F_{3,18}$	0.588	13.614	59.000	3.764	9.460	218.249
	P-value	0.630	0.000	0.000	0.028	0.000	0.000
90d	Control (n = 6)	4.47 ± 0.34	121.23 ± 2.14^a	87.50 ± 1.95^a	2.46 ± 0.07^a	1.68 ± 0.05^a	258.93 ± 5.96^a
	5‰ (n = 6)	3.86 ± 0.19	129.72 ± 0.61^b	96.37 ± 0.27^a	2.39 ± 0.03^a	1.72 ± 0.07^a	295.04 ± 6.17^b
	15‰ (n = 6)	3.89 ± 0.40	145.55 ± 2.78^c	117.28 ± 3.43^b	2.84 ± 0.13^b	1.97 ± 0.08^b	360.28 ± 1.37^c
	25‰ (n = 6)	4.02 ± 0.19	181.85 ± 2.42^d	137.63 ± 5.57^c	3.49 ± 0.16^c	2.37 ± 0.09^c	415.46 ± 13.65^d
	$F_{3,20}$	0.914	155.125	43.100	21.824	20.768	73.703
	P-value	0.452	0.000	0.000	0.000	0.000	0.000
Two-way ANOVA							
Main effect: Day							
30d (n = 12)		4.21 ± 0.17	145.06 ± 7.40	118.46 ± 8.19	2.47 ± 0.11	1.52 ± 0.08^a	330.58 ± 19.30
60d (n = 22)		4.42 ± 0.23	146.73 ± 6.43	112.79 ± 4.31	2.58 ± 0.16	1.82 ± 0.09^b	329.24 ± 12.38
90d (n = 24)		4.06 ± 0.15	144.59 ± 4.94	109.70 ± 4.35	2.79 ± 0.10	1.93 ± 0.07^b	332.43 ± 13.10
$F_{2,46}$		0.880	0.196	2.280	2.342	13.367	0.123
P-value		0.421	0.823	0.114	0.107	0.000	0.885
Main effect: Salinity							
Control(n = 14)		4.43 ± 0.17	121.83 ± 1.02^a	90.59 ± 1.32^a	2.30 ± 0.05^a	1.54 ± 0.04^a	258.93 ± 2.43^a
5‰ (n = 15)		4.01 ± 0.21	131.48 ± 0.68^b	103.96 ± 5.14^b	2.27 ± 0.07^a	1.59 ± 0.05^a	299.07 ± 4.02^b
15‰ (n = 14)		3.90 ± 0.22	144.37 ± 2.07^c	115.20 ± 2.41^c	2.67 ± 0.08^b	1.82 ± 0.07^b	351.02 ± 4.15^c
25‰ (n = 15)		4.53 ± 0.27	184.32 ± 5.61^d	141.15 ± 2.80^d	3.33 ± 0.20^c	2.27 ± 0.10^c	415.57 ± 6.04^d
$F_{3,46}$		1.718	66.252	45.216	14.098	27.684	276.452
P-value		0.176	0.000	0.000	0.000	0.000	0.000
Interaction: Day × Salinity							
$F_{6,46}$		0.441	0.429	2.022	0.165	0.354	3.394
P-value		0.848	0.856	0.081	0.985	0.904	0.007

Note: a,b,c,d Analyzed as a One-way ANOVA and data with different superscript small letters are significantly different ($P < 0.05$).

Table 3 Effects of ambient salinity on biochemical indexes in urine of *Trachemys scripta elegans.*

Time	Salinity	Urine K^+ (mmol/L)	Urine Na^+ (mmol/L)	Urine Cl^- (mmol/L)	Urine Urea (mmol/L)
One-way ANOVA					
30d	Control (n = 6)	2.52 ± 0.16^a	19.28 ± 1.70^a	19.46 ± 1.28^a	22.04 ± 0.29^a
	5 ‰ (n = 6)	12.57 ± 0.79^b	67.07 ± 3.31^b	63.57 ± 2.76^b	48.43 ± 1.66^b
	15 ‰ (n = 6)	23.60 ± 1.14^c	145.88 ± 13.95^c	145.42 ± 20.93^c	57.90 ± 0.71^c
	25 ‰ (n = 6)	33.32 ± 1.92^d	146.15 ± 6.94^d	183.53 ± 4.65^d	56.35 ± 1.93^c
	$F_{3,13}$	137.708	51.349	35.458	239.001
	P-value	0.000	0.000	0.000	0.000
60d	Control (n = 7)	2.73 ± 0.10^a	19.44 ± 1.22^a	22.60 ± 2.46^a	23.09 ± 1.08^a
	5‰ (n = 8)	14.61 ± 0.96^b	66.59 ± 1.28^b	66.09 ± 1.00^b	57.29 ± 1.08^b
	15‰(n = 6)	23.88 ± 1.03^c	94.45 ± 2.77^c	129.20 ± 9.08^c	57.96 ± 3.10^b
	25‰(n = 6)	35.55 ± 0.80^d	142.52 ± 4.93^d	153.03 ± 4.38^d	54.73 ± 2.52^b
	$F_{3,23}$	282.455	387.505	170.569	85.327
	P-value	0.000	0.000	0.000	0.000
90d	Control (n = 6)	2.61 ± 0.06^a	19.67 ± 1.71^a	21.93 ± 0.95^a	23.48 ± 2.05^a
	5‰ (n = 6)	14.59 ± 0.32^b	59.12 ± 1.91^b	53.75 ± 1.70^b	59.95 ± 1.27^b
	15‰ (n = 6)	26.50 ± 0.85^c	86.37 ± 3.64^c	102.98 ± 1.74^c	57.77 ± 2.59^b
	25‰ (n = 5)	34.25 ± 1.32^d	108.48 ± 3.12^d	157.00 ± 4.77^d	48.52 ± 2.01^c
	$F_{3,19}$	343.635	197.957	547.907	69.608
	P-value	0.000	0.000	0.000	0.000
Two-way ANOVA					
Main effect: Day					
	30d (n = 17)	17.74 ± 2.92	95.08 ± 14.29^a	104.06 ± 17.05^a	45.32 ± 3.87
	60d (n = 27)	18.24 ± 2.88	75.36 ± 10.74^b	85.82 ± 12.58^b	47.11 ± 3.82
	90d (n = 23)	18.85 ± 2.85	66.66 ± 8.02^c	80.74 ± 12.08^b	47.38 ± 3.72
	$F_{2,55}$	2.262	28.161	3.076	1.092
	P-value	0.082	0.000	0.001	0.343
Main effect: Salinity					
	Control (n = 18)	2.63 ± 0.06^a	19.47 ± 0.84^a	21.56 ± 1.15^a	22.94 ± 0.78^a
	5‰ (n = 17)	14.25 ± 0.52	64.04 ± 1.37^b	61.28 ± 1.68^b	56.66 ± 1.02^b
	15‰ (n = 17)	24.72 ± 0.63^c	106.34 ± 7.35^c	124.72 ± 7.54^c	57.88 ± 1.36^b
	25‰ (n = 15)	34.52 ± 0.73^d	132.14 ± 5.22^d	162.49 ± 4.23^d	53.09 ± 1.51^c
	$F_{3,55}$	701.830	357.288	284.090	231.771
	P-value	0.000	0.000	0.000	0.000
Interaction: Day × Salinity					
	$F_{6,55}$	1.185	11.971	3.076	3.399
	P-value	0.328	0.000	0.011	0.006

< 0.05) except between the group of salinity 15 and 25 in blood urea and the group of control and salinity 5 in blood uric acid. Furthermore, there was a significant interaction between sample time and ambient salinity in blood urea (P < 0.05).

4. Discussions

In our study, we found that *T. s. elegans* juveniles (BW: 125.60 ± 19.84 g) can survive in ambient salinity of 15‰ for nearly three months. These results are in agreement of studies by other researchers (Gibbons *et al.*,1979; Shu *et al.,* 2012). The ability to tolerate salinity in *T. s. elegans* appears better than other freshwater turtles (e.g., *P. sinensis* [~300g] can survive in 50% seawater [that is about 17‰ salinity] for 7 d (Lee *et al.*, 2006).

4.1 Increase in blood osmolarity and Na^+ and Cl^- concentration Turtles that reside in freshwater (10 –40 mOsm/kg) maintain their plasma osmolarities hyperosmotic to the external medium, such as that in *A. spinifera* is 252–282 mOsm/kg (Seidel, 1975), *Mauremys*

Table 4 Effects of ambient salinity on blood glucose, aldosterone, urea and uric acid content in *Trachemys scripta elegans.*

	Salinity	Blood glucose (mmol/L)	Blood aldosterone (ng/L)	Blood urea (mmol/L)	Blood uric acid (μmol/L)
One-way ANOVA					
30d	Control (n = 3)	3.43 ± 0.23	338.90 ± 15.00^a	5.20 ± 0.55^a	59.67 ± 4.84^a
	5‰ (n = 3)	5.17 ± 0.77	372.36 ± 3.26^b	10.70 ± 0.74^a	63.67 ± 1.20^a
	15‰ (n = 3)	4.00 ± 0.21	297.84 ± 2.58^c	23.55 ± 1.12^b	74.33 ± 0.88^b
	25‰ (n = 3)	4.00 ± 0.15	261.95 ± 3.52^d	41.19 ± 4.89^c	114.00 ± 2.31^c
	$F_{3,8}$	2.981	36.310	39.292	79.612
	P-value	0.096	0.000	0.000	0.000
60d	Control (n = 6)	3.40 ± 0.13	335.09 ± 1.58^a	7.36 ± 0.51^a	59.83 ± 1.47^a
	5‰(n = 6)	4.07 ± 0.25	375.29 ± 6.40^b	16.66 ± 3.47^b	60.83 ± 1.62^a
	15‰(n = 6)	3.45 ± 0.17	296.85 ± 21.74^c	36.99 ± 3.10^c	75.33 ± 1.17^b
	25‰(n = 6)	3.60 ± 0.52	258.30 ± 8.69^d	34.74 ± 2.28^c	105.83 ± 2.04^c
	$F_{3,20}$	0.972	20.642	26.609	178.249
	P-value	0.425	0.000	0.000	0.000
90d	Control (n = 6)	3.36 ± 0.16^a	332.76 ± 11.94^a	8.86 ± 1.47^a	59.33 ± 1.61^a
	5‰ (n = 6)	3.90 ± 0.38^a	363.21 ± 4.75^a	18.06 ± 2.77^b	60.00 ± 1.33^a
	15‰ (n = 6)	2.42 ± 0.28^b	282.85 ± 2.77^b	45.86 ± 2.75^c	76.17 ± 0.71^b
	25‰ (n = 6)	2.07 ± 0.15^b	238.41 ± 16.43^c	42.75 ± 4.02^c	101.67 ± 1.64^c
	$F_{3,20}$	10.293	27.354	35.726	96.499
	P-value	0.000	0.000	0.000	0.000
Two-way ANOVA					
Main effect: Day					
	30d (n = 12)	4.15 ± 0.26^a	317.76 ± 13.01	20.16 ± 4.31^a	77.92 ± 6.59
	60d (n = 22)	3.63 ± 0.15^a	316.42 ± 11.18	24.66 ± 2.89^b	75.46 ± 3.95
	90d (n = 24)	2.94 ± 0.21^b	304.31 ± 11.08	29.75 ± 3.60^c	74.29 ± 3.70
	$F_{2,46}$	12.439	1.845	7.661	2.561
	P-value	0.000	0.170	0.001	0.088
Main effect: Salinity					
	Control (n = 14)	3.39 ± 0.09^a	334.91 ± 5.62^a	7.44 ± 0.70^a	59.60 ± 1.16^a
	5‰ (n = 15)	4.22 ± 0.25^b	369.87 ± 3.40^b	16.02 ± 1.83^b	61.07 ± 1.33^a
	15‰ (n = 14)	3.20 ± 0.21^a	291.06 ± 7.57^c	37.85 ± 2.70^c	75.46 ± 0.71^b
	25‰ (n = 15)	3.06 ± 0.30^a	251.07 ± 7.58^d	39.24 ± 2.17^c	105.80 ± 1.61^c
	$F_{3,46}$	7.935	57.915	75.048	318.011
	P-value	0.000	0.000	0.000	0.000
Interaction: Day × Salinity					
	$F_{6,46}$	2.148	0.168	2.643	1.901
	P-value	0.066	0.984	0.028	0.100

leprosa, previously as *Clemmys leprosa*, 362 mOsm/kg (Minnich, 1979), *P. sinensis*, 285 mOsm/kg (Lee *et al.*, 2006). Our results for *T. s. elegans* are similar, with individuals maintaining a plasma osmolarity of 250–261 mOsm/kg. As a result, freshwater turtles must experience hypoosmotic and hypoionic stresses due to the continuous influx of water and loss of electrolytes. Excess water could be removed by excreting copious amounts of dilute urine, and salt loss could be minimized through the diet. However, when exposed to 15‰ water, the osmoregulatory problem would be reversed, and *T. s. elegans* would experience hyperosmotic and hyperionic stress (approximately 513 mOsm/kg). This would result in the turtle losing water and gaining electrolytes. Prange (1985) showed that reptilian kidneys do not function well for osmoregulatory purpose, as they continue to conserve monovalent ions when the animals are confronted with hyperosmotic stress. Therefore, levels of Na^+ and Cl^- in the plasma slowly build up to harmful levels. Turtles that live in the ocean and often possess salt glands to maintain

their plasma osmolarities at lower values than that of seawater. For example, the plasma osmolarity in *Caretta caretta* is 316–465 mOsm/kg (Prange, 1985) and that in *Chelonia mydas* is 390 mOsm/kg (Prange and Greenwald, 1980).

In our study, when *T. s. elegans* were subjected to salinity stress, a significant increase in the blood osmotic pressure was observed over time and appears to be due exclusively to an increase in Na^+ and Cl^- concentrations. This suggests that during this first step of adaptation to saline environments, a progressive entry of NaCl takes place. At 30 d stress, the Na^+, Cl^-, and K^+ concentration in urine increased rapidly with the ambient salinity, which indicates that the entry of NaCl is balanced through a renal route. However, Peterson and Greenshields (2001) have found that the cloacal bursae in many aquatic turtles (including *T. s. elegans*) have been speculated to function primarily in buoyancy control and secondarily in ion transport and nesting. It is unclear and needs to be studied if there is an extrarenal route to balance the entry of NaCl in *T. s. elegans*. As time passed, the Na^+ and Cl^- concentration in urine of the 15‰ and 25‰ salinity group decreased, resulting in harmful levels of Na^+ and Cl^- in the blood. These results indicate that *T. s. elegans* is adapted to environments with salt levels less than 15‰. This phenomenon in our study is different from that in the terrapin going from fresh water to 50% sea water during which an increase in Na^+ and Cl^- concentrations in its blood occurred but this entry of NaCl was rapidly balanced, probably through the intermediary of the orbital salt gland, since a steady-state of concentration is obtained in the blood after only 3 d (Gilles-baillien, 1973).

Gilles-Baillien (1973) found that after *M. terrapin* was transferred from freshwater to 50% seawater, the plasma osmolarity increased from 309 mmol/l to 355 mOsm/kg, plasma Na^+ increased form 129 mmol/l to 156 mmol/l and plasma Cl^- from 88 mmol/l to 113 mmol/l. Also, Dantzler and Schmidt-Nielsen (1966) found that *Pseudemys scripta* had a concentration of 260 mOsm/kg when in fresh water and 340 mOsm/kg during water deprivation. Thus, judging from our results that the plasma osmolarity of *T. s. elegans* increases from 250 mOsm/kg to 410 mOsm/kg, blood Na^+ increases form 120 mmol/l to 185 mmol/l and blood Cl^- from 90 mmol/l to 141 mmol/l when transferred from freshwater to 25‰, the capacities of ion- and osmoregulation in these turtles are comparable.

4.2 Increase in urea contents in response to increased salinity If the osmolarity of the extracellular fluid (as reflected by that of the plasma) increases, water efflux from the cell would occur, leading to cell shrinkage and molecular crowding (Burg *et al.*, 2005). Theoretically, increases in plasma electrolyte concentrations should result in an increase in plasma volume at the expense of cell volume regulation, which involved osmolytes like urea (one of the end-products of nitrogen metabolism). Comparing urine and blood composition of *T. s. elegans* individually in our study additional conclusion can be drawn: the urea concentration is always higher in the urine than in the corresponding blood whatever the salinity the animals are living in, and independently of the fact that the urine is hypo-osmotic or isosmotic to the blood. So, there may be two factors on the blood urea concentration increased. One is maybe a reabsorption from urine probably at the level of the bladder where urea accumulate, which proposed by Gilles-Baillien (1970) who found that urea accumulation in *M. terrapin* exposed to seawater was not a result of increased urea production, but a consequence of increased urea retention in the urinary bladder. And the other is maybe the increase in the rate of urea synthesis. Lee *et al.* (2006) studied that increases in urea contents in tissues of *P. sinensis* on days 3, 4 and 6 indicated an increase in the rate of urea synthesis, and the overall rate of urea synthesis increased 1.4 fold during the 6 d. Also, the capacity of *P. sinensis* to retain urea is apparently limited, and the urea excretion rate returned back to the control level as the plasma urea concentration increased, which is coincident with our results. However, the recent study on an important excretory route for urea in *P. sinensis* has shown that it is well adapted to aquatic environments, including brackish swamps and marshes, can excrete urea mainly through the buccophyaryngeal cavity of mouth instead of the kidney during immersion (Ip *et al.*, 2012).

Indeed, ureogenesis allows the transfer from fresh water to seawater in the diamondback terrapin; moreover, in a dehydrated desert tortoise, *Gopherus agassizii,* the increase in the osmolarity of the blood is principally due to an increase in urea concentration (Dantzler and Schmidt-Nielsen, 1966). It is also interesting to note that blood urea also plays a part in the terrestrial tortoise, *Testudo hermanni hermanni* Gmelin, in the course of hibernation (Gilles-Baillien, 1969). Furthermore, additional experimental support is required before it is known whether nitrogen catabolism of *T. s. elegans*, which is at one and the same time uricotelic and ureotelic, is affected by the salinity of the external medium. For that purpose the percentages contributed by urea, uric acid and ammonia should be established taking account of the possible transfer of urea into the blood and accumulation

of uric acid and ammonia in the bladder, and also of the rate of urine production in various conditions of salinity.

4.3 Decrease in blood aldosterone and glucose in response to increased salinity Corticosterone is known to be the major secretory product with a mineralocorticoid action of reptilian adrenocortical tissue (Sandor, 1972) and a direct intervention of aldosterone in the salt-water balance of some reptiles is now confirmed. Bradshaw *et al*. (1976) note that considerable variability in aldosterone may exist between reptilian species and the extent to which aldosterone is apparently involved in the regulation of sodium balance. Uva *et al*. (1982) studied a terrestrial chelonian and showed that an increased sodium intake resulting in a diminished aldosterone production and in reverse a loss of sodium by diuresis is a very potent stimulus for aldosterone secretion. This finding is coincident with our result that blood aldosterone decreased with ambient salinity increased, and firmly supports the hypothesis that aldosterone is directly involved in hydromineral regulation, at least in chelonians.

Some researchers have showed that when the animals are subjected to stress, blood glucose quickly increases. In fish, blood glucose is intermediated by catecholamines that stimulate glycogenolysis in the liver (Mommsent *et al.*, 1999). Blood glucose values of *Colossoma macropormum* reared at 0 g/l was 85.0 ± 7.5 mg/dl and was significantly lower than in the higher salinity levels, which ranged from 206.0 ± 11.0 mg/dl to 251.0 ± 12.0 mg/dl (Fiúza *et al*, 2013). In *T. s. elegans*, the blood glucose in the groups of salinity 10‰ and 20‰ is significantly higher than that of control group (Shu *et al.*, 2012). However, in our study, there is an increase in blood glucose only in the group of salinity 5 than the other groups, the same phenomenon was observed in Imanpoor *et al.* (2012)'s study in which goldfish exposed to different salinities (0, 6‰, 12‰) and temperature (23°C, 27°C and 31°C) exhibited the unchanged plasma glucose at 45 d stress.They believed that it may be contributed by that blood glucose was analyzed after a long period of salt exposure (Imanpoor *et al.*, 2012).

5. Conclusion

In conclusion, *T. s. elegans* is an osmoregulator that limits the entry of Na^+ and Cl^-, but can tolerate certain degrees of increases in plasma Na^+ and Cl^-. Together with the contribution from increased plasma urea concentration, the consequential increase in plasma osmolarity in turtles exposed to brackish water represents a strategy to decrease osmotic water loss. When the ambient salinity was lower than 15‰, *T. s. elegans* could increase blood osmotic pressure by balancing the entry of NaCl with the secretion of aldosterone decreased, and by accumulating blood urea for osmoregulation effectors, and survive for at least three months.

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