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Physiological and behavioral responses to salinity in coastal Dice snakes

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ABSTRACT

Secondarily marine tetrapods have evolved adaptations to maintain their osmotic balance in a hyperosmotic environment. During the transition to a marine habitat, the evolution of a euryhaline physiology likely encompassed successive changes in behavior and physiology that released organisms from regular access to fresh water. Deciphering these key steps is a complicated task. In this study, we investigated a species of freshwater natricine snake in which some populations are known to use marine environments. We experimentally subjected 30 adult Dice snakes (*Natrix tessellata*) from a population inhabiting the Black Sea coast to three salinities corresponding to freshwater (~0.1‰), brackish water (~15.0‰), and full-strength seawater (~34.0‰) in order to investigate their physiological (variation of body mass, osmolality) and behavioral (activity, drinking behavior) responses to salinity. Our results show that coastal Dice snakes from the study population are relatively tolerant to salinity close to that recorded in the Black Sea, but that prolonged exposure to full-strength seawater increases osmolality, stimulates thirst, decreases the activity of snakes and may ultimately jeopardize survival. Collectively with previously published data, our results strongly suggest specific physiological adaptations to withstand hyperosmolality rather than to reduce intake of salt, in coastal populations or species of semi-aquatic snakes. Future comparative investigations of Dice snakes from populations restricted to freshwater environment might reveal the functional traits and the behavioral and physiological responses of coastal *N. tessellata* to life in water with elevated salinity.

1. Introduction

Because seawater is hyperosmotic to body fluids, secondarily marine species of vertebrates tend to gain salt and/or lose water across permeable surfaces (Dunson, 1978; Schmidt-Nielsen, 1983). As a consequence, marine tetrapods have to regulate the osmolarity of body fluids to survive in seawater (Schmidt-Nielsen, 1983). Owing to their developed salt-excreting organs (kidneys in mammals and salt glands in reptiles sensu *lato*, Ortiz, 2001; Peaker and Linzell, 1975), secondarily marine vertebrates are believed to maintain water balance even without access to fresh water (Houser et al., 2005; Randall et al., 2002).

However, this widely held opinion has been recently challenged in some lineages of marine tetrapods (Lillywhite et al., 2008a, 2014a). For example, investigations of the main lineages of marine-adapted snakes (i.e., Laticaudinae, Hydrophiinae, Acrochordidae) suggest that they frequently live in a dehydrated state and cannot maintain their water balance without access to fresh water (Lillywhite et al., 2008a, 2012, 2014a, 2014b, 2015). Interestingly, dehydration rates in seawater seem to be dependent on the degree of reliance on the ancestral terrestrial environment, both within and across phylogenetic lineages (Brischoux et al., 2012; Lillywhite et al., 2009, 2012, 2014a, 2014b). Current data suggest that availability of fresh water and/or ability to excrete excess salt are pivotal to the invasion of marine environments by tetrapods (Dunson and Mazzotti, 1989; Lillywhite et al., 2008b; Roe et al., 1998).

According to scenarios of transitions to marine life, both behavioral and physiological changes have allowed organisms to gradually become independent from regular access to fresh water and to eventually thrive in saline environments. Such behavioral adjustments include frequent and obligate drinking of fresh water, which allows dehydrated and/or hyperosmotic individuals to periodically restore their osmotic balance (Bonnet and Brischoux, 2008; Combs et al., 1992; Davenport and Macedo, 1990; Lillywhite et al., 2014a). The reliance on drinking fresh water is likely associated with discrimination and selectivity of water's salinity (e.g., avoidance of drinking highly brackish water, Davenport and Macedo, 1990; Jackson et al., 1996; Lillywhite et al., 2008a, 2012; see also Kidera et al., 2013). On the other hand, physiological adjustments include features such as reduction of salt gain and/or water loss through permeable surfaces (Babonis et al., 2011; Dunson and

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Robinson, 1976; Dunson and Stokes, 1983; Lillywhite et al., 2009), increased resistance to hypernatremia (Brischoux et al., 2013; Brischoux and Kornilev, 2014), and modification of the thirst threshold that triggers drinking behaviors (Lillywhite et al., 2015). Ultimately, the evolution of a euryhaline physiology should involve the co-option of an unspecialized or mucus-secreting gland and its subsequent development as a gland for excreting salt (Babonis and Brischoux, 2012).

Despite the seemingly straightforward character of these scenarios, deciphering the key steps that have accompanied the evolution of a euryhaline physiology during the transition from land to sea remains a complicated task. This is especially true for the relative role of the behavioral versus physiological adjustments during early evolutionary steps. Indeed, fossils tend to lack detailed information related to the behavioral and physiological adjustments that likely occurred in early transitional forms. In this context, studies of extant species that are tolerant to marine environments (e.g., salinity), but lack essential features of marine tetrapods (i.e., salt glands) may present a significant opportunity to investigate the early steps in the evolution of a euryhaline physiology (Brischoux and Kornilev, 2014).

Snake lineages provide a powerful study system with which to elucidate the evolutionary steps that allowed coping with the osmotic challenges linked to the transition to marine life (Brischoux et al., 2012). The diversity of snakes represents associations with a remarkable gradient of habitats and behaviors that allows investigating groups that are tolerant to marine environments but cannot be considered as marine (Murphy, 2012). In this study, we examined such a species. The Dice snake (*Natrix tessellata*) is a typical semi-aquatic freshwater natricine that lacks a salt gland and occurs over most of Western Eurasia (Mebert, 2011). Although this species relies primarily on bodies of fresh water to forage for fish and amphibians, some populations use brackish and/or saline habitats, thereby offering the possibility to investigate a key intermediate condition between freshwater and marine life (Brischoux and Kornilev, 2014; Lillywhite et al., 2008b). A previous examination of a coastal Dice snake population in Bulgaria has shown that individuals forage in the Black Sea and can have elevated plasma sodium concentrations (Brischoux and Kornilev, 2014).

In this study, we experimentally exposed *N. tessellata* from this coastal population to water of three salinity levels, corresponding to typical fresh water, brackish water, and seawater, to assess physiological and behavioral responses to salinity. Our aims were to determine 1) the influence of external water salinity on osmolality of the blood, 2) the behavioral and morphological responses to salinity, and 3) the manner by which drinking is invoked to maintain osmotic balance.

2. Materials and methods

2.1. Study species and housing

The Dice snake, *Natrix tessellata* (Laurenti, 1768), is a typical semi-aquatic natricine species (Mebert, 2011). Although *N. tessellata* predominantly inhabits freshwater habitats, a few populations thrive in saline environments (see Mebert, 2011 and references therein). In this study, we examined individuals from one such population from the Bulgarian Black Sea coast (Naumov et al., 2011; Brischoux and Kornilev, 2014).

We captured 30 female *N. tessellata* from around "Poda" coastal wetlands near Burgas, Bulgaria (for a description of the study site see Brischoux and Kornilev, 2014). Upon capture, each individual was measured with a flexible ruler (snout-vent length [SVL] and total length [TL], ± 0.5 cm), and weighed with a Pesola spring scale (± 1 g). Snakes were brought to the laboratory (< 10 km), and blood was sampled to obtain plasma osmolality values from free-ranging individuals. Snakes were housed individually in transparent plastic boxes (30 × 20 × 15 cm) with a perforated cover to minimize evaporation while providing sufficient ventilation. Each box was filled with 2 cm of water (see details below) and a small rock (2 × 8 × 8 cm) was provided for resting while snakes remained in permanent contact with water. Snakes were not fed during the experiment. They were kept outside in the shade under natural conditions.

2.2. Experimental design

Our experiment consisted of three successive stages:

1) Because a preliminary investigation has shown that some wild-caught *N. tessellata* from Poda wetlands displayed hypernatremia (Brischoux and Kornilev, 2014), boxes were first filled with freshwater for 48 h to allow snakes to drink ad libitum and to restore osmotic balance. This preparatory stage, hereafter termed "Baseline", was intended to establish baseline plasma osmolality among all the animals in the experimental groups. At the end of these two days, blood samples were taken and snakes were randomly allocated to the experimental groups.

2) In the second stage (hereafter termed "Experimental treatment"), we subjected the snakes to one of three salinity levels (freshwater, brackish water or full-strength seawater = "FW", "BW" and "SW", respectively) for six days. This duration corresponded to that used in similar studies (e.g., Babonis et al., 2011). Water was prepared by dissolving sea salt (obtained from a local salt extraction facility) in tap water. We prepared salinities of 0.16 ± 0.005‰ (tap water) for FW, 14.77 ± 0.38‰ for BW and 33.97 ± 0.76‰ for SW. Salinity was measured using a calibrated real-time conductivity meter (Testo 240, Testo AG, Germany). At the end of the Experimental treatment, blood was sampled, and snakes were subjected to the last stage of the experiment.

3) In the last stage (hereafter termed "Recovery"), each treatment water was replaced by freshwater (tap water) for two days in order to allow snakes to drink ad libitum and to restore their osmotic balance. At the end of this stage, final blood samples were taken, and snakes were then released at the site of capture.

During all stages, the salinities of water were recorded daily to assess potential changes linked to evaporation (i.e., indicated by an increase in the salinity of the water) or to approximate the direction of diffusion of water and salt (e.g., a decrease in treatment salinity should indicate absorption of salt). Each individual was weighed daily (± 1 g) after being gently dried with paper towels and allowed to air-dry. During this daily manipulation, we also assessed the condition of each individual as follows. Snakes were described as in "good condition" when they were moving energetically, tried to evade manipulation, and had perceptible muscular strength; they were considered as "weak" if they displayed low muscular strength and no tendency to engage in escape behaviors.

2.3. Sampling of blood and osmolality assays

All blood samples were collected via cardiocentesis, using a 1 ml syringe and a 30-gauge heparinized needle. Collected blood (~200 µl representing < 0.1% of a snake's body mass) was placed in a 0.675 ml microcentrifuge tube and centrifuged for 3 min at 2000 G. The plasma was separated and stored at − 18 °C in sealed microtubes until analysis (~5 months). Plasma osmolality ($mOsm \cdot kg^{-1}$) of the samples was determined at the "Plateau Ecophysiologie" of the LIENSs laboratory (UMR 7266, University of La Rochelle) with a Micro-Osmometer Autocal Type 13 (Hermann Roebling Messtechnik, Germany). A separate subsample (n = 9 randomly distributed across treatments and experimental stages) measured in duplicates allowed to calculate a mean measurement error of 1.5% (range 0–3.9%).

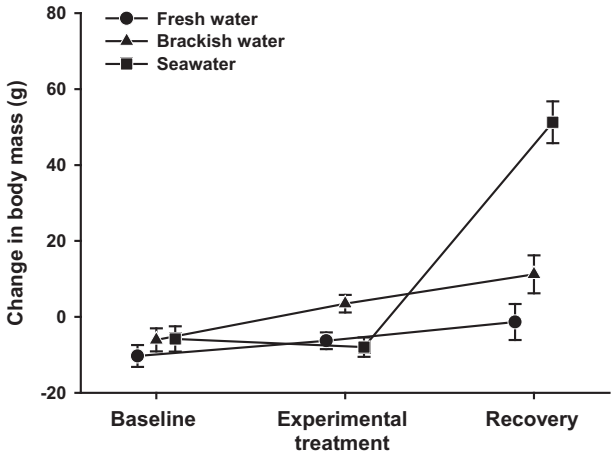

Fig. 1. Change in body mass for the three Experimental treatments throughout the experiment. Changes were calculated relatively to initial body mass. Data are presented as mean ± s.e.m. (n = 10 in each groups).

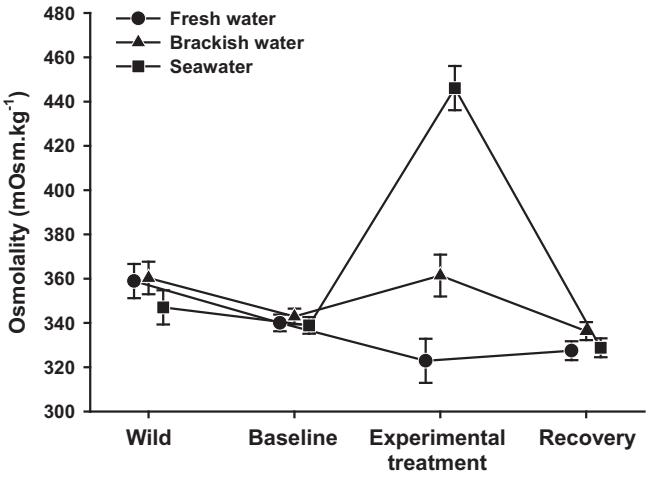

Fig. 2. Change in osmolality for the three Experimental treatments throughout the experiment. Data are presented as mean ± s.e.m. (n = 10 in each groups).

3. Results

3.1. Variation of body mass

Changes in body mass (relative to initial body mass at capture) throughout the experiment were strongly linked to the stage and to the Experimental treatment (repeated measures ANOVA, effect of the stage: $F_{2,25} = 13.31$, $p = 0.0001$; effect of the Experimental treatment: $F_{2,50} = 1.39$, $p = 0.26$; and their interaction: $F_{4,50} = 20.34$, $p < 0.0001$, Fig. 1). Post-hoc tests (Fisher's LSD) showed that variation of body mass was similar between groups at the end of the Baseline stage (all $p > 0.34$, Fig. 1), that body mass was significantly different between BW and SW at the end of the Experimental treatment ($p = 0.04$, all other $p > 0.06$, Fig. 1), and that body mass was significantly different between the three groups after Recovery (all $p < 0.02$, Fig. 1). Body mass of FW individuals did not vary through time (all $p > 0.08$), while BW and SW individuals gained body mass after Recovery ($p < 0.002$, all other $p > 0.06$).

3.2. Osmolality

Osmolality throughout the experiment varied according to the stage and to the Experimental treatment (repeated measures ANOVA, effect of the stage: $F_{2,25} = 9.45$, $p = 0.001$; effect of the Experimental treatment: $F_{3,75} = 34.10$, $p < 0.0001$; and their interaction: $F_{6,75} = 30.15$, $p < 0.0001$, Fig. 2). The Baseline stage significantly lowered plasma osmolality for all snakes compared with the initial condition in the field (ANOVA: $F_{1,57} = 8.34$, $p = 0.005$, mean value: 353.8 ± 23.1 $mOsm.kg^{-1}$ versus 341.1 ± 11.0 $mOsm.kg^{-1}$, Fig. 2). Each Experimental treatment produced a specific osmotic response (Fig. 2). Full-strength SW produced a very strong increase in plasma osmolality (up to a mean value of 457.0 ± 55.7 $mOsm{\cdot}kg^{-1}$, Fig. 2). An increase in plasma osmolality was also noticeable in the BW snakes, where osmolality at the end of the Experimental treatment reached values similar to those of wild-caught individuals (ANOVA between plasma osmolality measured in the field versus at the end of the Experimental treatment, restricted to BW individuals: $F_{1,18} = 0.014$, $p = 0.91$, mean value of 361.4 ± 20.8 $mOsm{\cdot}kg^{-1}$, Fig. 2). In FW snakes, plasma osmolality continued to decrease after six days in fresh water (ANOVA between plasma osmolality measured at the beginning and at the end of the treatment, restricted to FW individuals: $F_{1,17} = 7.16$, $p = 0.016$, mean value of 322.6 ± 11.8 $mOsm{\cdot}kg^{-1}$, Fig. 2). The Recovery stage induced a strong decrease of plasma osmolality in both SW and BW snakes, down to values found in FW individuals (ANOVA: $F_{2,26} = 1.58$, $p = 0.22$, mean value: 330.6 ± 12.9 $mOsm{\cdot}kg^{-1}$, Fig. 2).

We did not find any relationship between snout-vent length or body mass and the variation of osmolality during the Experimental treatment (all $p > 0.52$), neither for FW nor BW individuals when analyzed separately (all $p > 0.56$). For the SW group, however, we found a marginal negative relationship between snout-vent length and the variation of osmolality during the Experimental treatment ($r^2 = 0.35$, $p = 0.07$, Fig. 3) and a negative relationship between body mass and variation of osmolality during the Experimental treatment ($r^2 = 0.39$, $p = 0.05$, Fig. 3).

We did not find any relationship between snout-vent length or body mass and the variation of osmolality during Recovery (all $p > 0.14$), even when Experimental treatments were analyzed separately (all $p > 0.24$). However, we found a significant negative correlation between the decrease of osmolality and the increase of body mass during Recovery for all individuals ($r^2 = 0.61$, $p < 0.0001$, Fig. 4), but not within Experimental treatments (all $p > 0.20$).

3.3. Variation of salinity in the treatment water

Changes in the salinity of water varied according to the stage and to the Experimental treatment (repeated measures ANOVA, effect of the stage: $F_{2,19} = 4.29$, $p = 0.03$; effect of the Experimental treatment: $F_{2,38} = 33.19$, $p < 0.0001$; and their interaction: $F_{4,38} = 27.38$, $p < 0.0001$, Fig. 5). Post-hoc tests (Fisher's LSD) show that there was a significant decrease of water salinity in the SW group during the Experimental treatment and a significant increase during Recovery (all $p < 0.0001$, Fig. 5). At the end of the Recovery, changes in the salinity of water in the SW group was significantly higher than were changes recorded in the BW and FW groups (all $p < 0.0001$, Fig. 5). Moreover, salinity change at the end of Recovery in the BW group was higher than at the end of the Experimental treatment ($p = 0.02$, Fig. 5).

3.4. Condition of snakes

All individuals from the FW and BW treatments remained in good condition throughout the experiment, while three individuals from the SW group became progressively weaker and lethargic during the Experimental treatment (difference between Experimental treatments: $\chi^2 = 6.66$, df = 2, $p = 0.03$). We found that weak individuals had significantly higher osmolality than individuals in good condition (ANOVA between individuals in good and weak conditions, restricted to SW individuals, $F_{1,8} = 27.40$, $p < 0.001$, 426.57 ± 28.08 $mOsm{\cdot}kg^{-1}$ versus 528.00 ± 28.05 $mOsm{\cdot}kg^{-1}$, Fig. 6). Interestingly, weak individuals were marginally smaller than individuals that remained in good condition (ANOVA between individuals in good

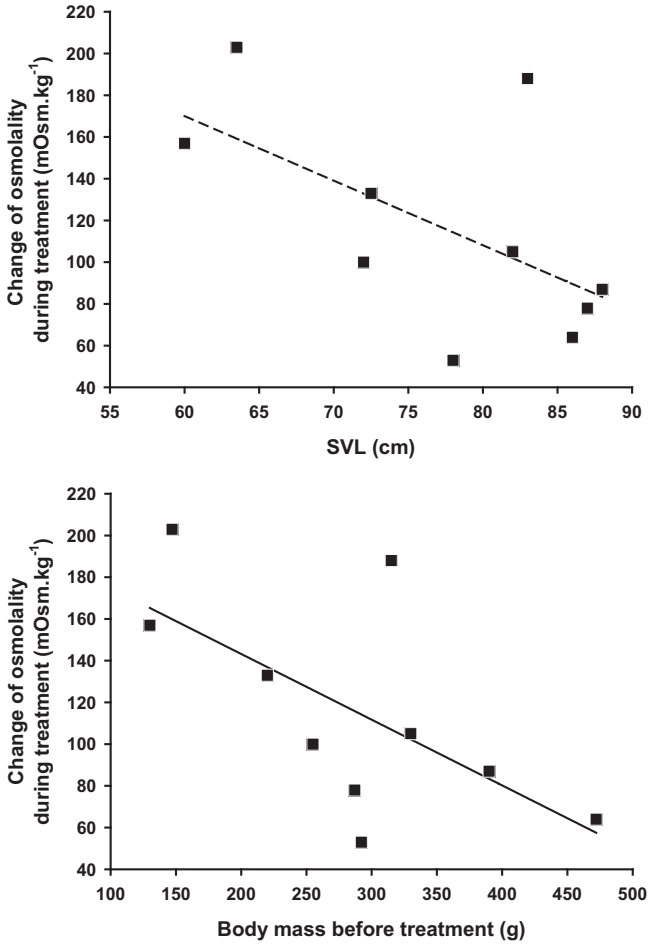

Fig. 3. Relationships between the variation of plasma osmolality at the end of the Experimental treatment stage and the body size (A, snout-vent length, SVL) and body mass (B) of individuals in the seawater group (n = 10).

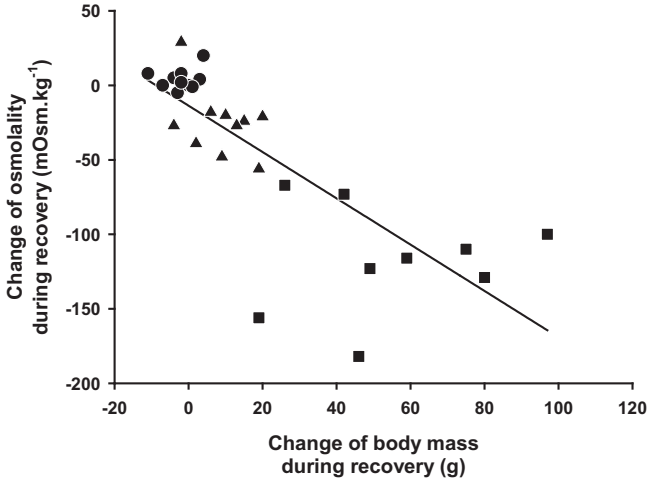

Fig. 4. Relationship between the variation of plasma osmolality and the change of body mass at the end of the Recovery stage (n = 30). Symbols represent snakes from the different Experimental treatments.

and weak conditions, restricted to SW individuals, $F_{1,8} = 4.14$, $p = 0.07$, SVL = 80.8 ± 6.7 cm versus 68.8 ± 12.4 cm). Unfortunately, one weak individual died < 15 min after being transferred to FW, upon which it was drinking copiously. It was one of the smaller snakes from this group (SVL = 63.5 cm) and had the highest osmolality value recorded at the end of the treatment (555 mOsm·kg^{-1}, Fig. 6).

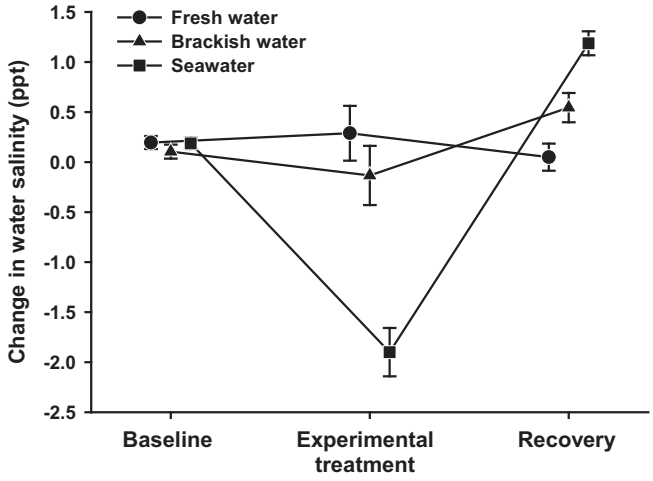

Fig. 5. Change in water salinity for the three Experimental treatments throughout the experiment. Data are presented as mean ± s.e.m. (n = 10 in each group).

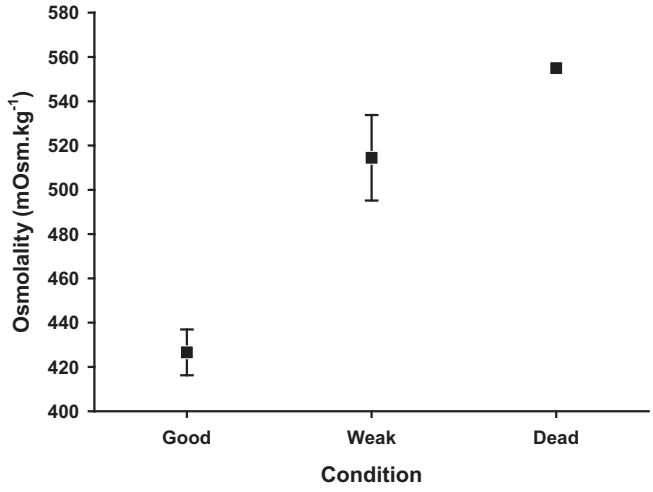

Fig. 6. Plasma osmolality of snakes from the SW group in relation to the condition of these individuals (n = 7, n = 2 and n = 1 respectively for good, weak and dead).

4. Discussion

Our results generally support the tolerance of coastal Dice snakes to marine environments insofar as individuals from the studied population display some resistance to prolonged contact with salt water, especially water with salinity similar to the Black Sea. Nevertheless, coastal Dice snakes suffer negative effects of prolonged contact with full-strength seawater (see also Koleva et al., 2017). Below we review the functional traits and the behavioral and physiological responses to increased salinity highlighted by our results.

At the end of the Experimental treatment, during which individuals from the BW and the SW groups were in prolonged (six days) contact with relatively elevated salinity, losses of body mass were very small and similar among groups (Fig. 1), whereas osmolality of the blood greatly increased, especially in the SW group (Fig. 2). The limited decreases of body mass we recorded suggest that individuals did not lose significant body water. Conversely, the significant decreases we detected in the salinity of the water during the Experimental treatment in the SW group indicate that snakes gained salt from the water (Fig. 5, see also Babonis et al., 2011; Dunson and Robinson, 1976; Dunson and Stokes, 1983). Interestingly, such asymmetrical directions of diffusion of water and salt through the integument have been recorded in both marine and freshwater snakes (Babonis et al., 2011; Dunson, 1978; Dunson and Robinson, 1976; Dunson and Stokes, 1983). Furthermore,

Table 1
Osmolality in natricine species caught in the wild, and subjected to treatments similar to the current study. Survival rates (%) are indicated between brackets. Values for *N. tessellata* are from the current study.

Species	Wild	0% SW	50% SW	100% SW
Natrix tessellata	353.8 ± 23.1	322.6 ± 11.8 (100%)	361.4 ± 20.8 (100%)	457.0 ± 55.7 (90%)
Nerodia fasciata[a]	–	298.2 ± 15.4 (100%)	367.5 ± 48.2 (60%)	338.6 ± 48.4 (60%)
Nerodia clarkii[a]	–	304.6 ± 14.4 (100%)	300.8 ± 9.3 (100%)	308.4 ± 8.1 (100%)
Nerodia clarkii[b]	390	–	–	482 ± 47 (100%)

Note that *N. fasciata* is a strictly freshwater species, while *N. clarckii* (Salt marsh snake) is restricted to brackish salt marshes along the Gulf of Mexico.
[a] Data from Babonis et al. (2011) in which 5 individuals per treatment per species were maintained for 7 days in experimental conditions.
[b] Data from Dunson (1980) in which 5 individuals were maintained 27 days in 35‰ sea water.

salt gain did not appear to be linearly related to the salinity of the water. Indeed, during the Experimental treatment, plasma osmolality increased 5.8 times more in the SW group relative to the BW group (osmolality increase of ~20 mOsm·kg^{-1} in the BW group versus ~116 mOsm·kg^{-1} in the SW group) despite the difference in salinity between treatment water being 2.3 (14.8 versus 34.0‰). This may indicate that susceptibility to salt gain does not increase linearly with increasing osmotic difference between the body fluids and the surrounding water.

At the end of the Experimental treatment, we observed that individuals from both the BW and the SW groups drank fresh water, sometimes frantically and almost immediately after being given the opportunity (i.e., while still being transferred). Freshwater intake was also confirmed by the subsequent gain of body mass (Fig. 1). The ingestion of water was likely a response to restore osmotic balance (Brischoux et al., 2013; Lillywhite et al., 2008a, 2012, 2014a, 2014b) as illustrated by the rapid return to normosmolality in both groups (Fig. 2). It is important to add that no frantic drinking behavior was observed while snakes were in the BW and SW treatments. During the short Recovery stage, both groups apparently flushed excess salt as suggested by the increase in salinity of the water (Fig. 5), either through the production of urates and urination following the copious drinking of fresh water, and/or through diffusion of salt through permeable surfaces. Yet, it is noteworthy that individuals from the SW group were likely to be in considerable hyperosmolality as they drank an average of 20% of their body mass by the end of the Recovery stage (54.8 ± 24.6 g of fresh water drunk). Drinking fresh water reached 26% of body mass in the most extreme case: a 281 g individual drank 97 g of fresh water. In comparison, individuals from the BW group drank an average of 4.6% of their body mass at the end of the Recovery stage (8.8 ± 8.3 g of fresh water). Two individuals (20%) from the BW group did not drink fresh water during Recovery stage, whereas 100% of the individuals of the SW group drank. The significant relationship between the change of body mass during recovery (proxy of the amount of fresh water drunk) and the variation of osmolality during recovery across treatments (Fig. 4) indicates that the snakes did not recover normosmolality by absorbing water through permeable surfaces. Instead, they drank fresh water, as we have observed directly (FB & YVK, pers. obs.) and as it is suggested by the variation of body mass we measured (see above; Lillywhite et al., 2008a). This relationship further indicates that the amount of fresh water drunk likely played a role in regulating osmolality and in flushing the excess salt.

The morphology of snakes (body mass and to a lesser extent body size) evidently influenced the rate of salt gain only in the SW group (Fig. 6). This further strengthens the conclusion that snakes did not directly drink seawater but rather gained salt through permeable surfaces such as the integument and the mucous membranes of the mouth and cloaca (Babonis et al., 2011; Dunson and Robinson, 1976; Dunson and Stokes, 1983), and that smaller and thinner individuals were more susceptible to salt gain, as predicted by their larger surface area to volume ratio. This size effect of salt gain and susceptibility to increased osmolality was also noticeable in relation to the condition of snakes. Indeed, although all individuals from the FW and BW treatments remained in good conditions throughout the experiment, three individuals from the SW group became progressively weaker during the Experimental treatment, and these weak individuals were smaller than the snakes that remained in good condition. This reinforces the notion that increased osmolality and/or dehydration linked to prolonged contact with sea water can have high costs and may ultimately jeopardize survival (Babonis et al., 2011; Dunson, 1980).

Despite the fact that the period of our Experimental treatment likely represents a duration that is longer than a typical foraging bout of an individual Dice snake in the Black Sea, collectively our results show that coastal Dice snakes from our study population are relatively resistant to salinity close to those recorded in the Black Sea (i.e., ~14‰). Free-ranging individuals have already been observed to display hypernatremia (up to 195.5 mmol·l^{-1}) without detected negative effects on physiology and behavior (Brischoux and Kornilev, 2014). In addition, in the BW group fresh water did not automatically elicit drinking behavior, conversely to the SW group (see also Brischoux et al., 2013; Lillywhite et al., 2008a, 2012).

A literature review of change in osmolality in other natricine snakes subjected to similar experimental treatments gives additional insights to our results (Table 1, see also Babonis et al., 2011; Dunson, 1980). Indeed, relatively modest increase in osmolality in a strictly freshwater species (*Nerodia fasciata*) maintained in full-strength SW induces high rates of mortality (Table 1, Babonis et al., 2011, see also Dunson, 1980 for *Nerodia sipedon* and *Regina septemvittata*). Conversely, in a species known to occur in marine environments (Salt marsh snake *Nerodia clarckii*), high deviations of osmolality toward elevated values (e.g., > 450 mOsm·kg^{-1}, Table 1) did not appear to jeopardize survival even during prolonged exposure to SW (up to 27 days, Table 1, Dunson, 1980). Collectively with our data on coastal Dice snakes, such information strongly suggests specific physiological adaptations to withstand hyperosmolality, rather than to reduce intake of salt, in coastal populations or species of semi-aquatic, freshwater snakes. Interestingly, other coastal populations of Dice snakes are known to occur along the coasts of low salinity seas such as the Caspian Sea and the Sea of Azov (~12‰, Tuniyev et al., 2011). However, there are also coastal populations of this species that thrive in much more saline waters, notably the Adriatic Sea (38–39‰, Jelić and Lelo, 2011; Mebert, 2011) and the Ionian Sea (35–37‰, Ioannidis and Mebert, 2011). Future studies might usefully assess whether these populations display specific physiological adaptations (e.g., increased resistance to hyperosmolality) or behavioral responses (e.g., frequent and obligate freshwater drinking) to withstand such salinity. It is important that future studies investigate resistance to salt gain in individuals from freshwater populations in order to fully evaluate the functional traits and the behavioral and physiological responses of this coastal population to life in water with elevated salinity.

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