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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%).