

RESEARCH ARTICLE

Water relations of an insular pit viper

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ABSTRACT

Colonization of novel habitats often requires plasticity or adaptation to local conditions. There is a critical need to maintain hydration in terrestrial environments having limited water. Atypical populations of Florida cottonmouth snakes, *Agkistrodon conanti*, inhabit continental islands with no permanent sources of fresh water. Here, we report investigations related to how these insular snakes maintain water balance considering the mainland conspecifics are semi-aquatic and typically associate with freshwater mesic habitats. We tested three hypotheses related to water relations of insular populations of cottonmouth snakes compared with those on the mainland. (1) Voluntary drinking of fresh water in free-ranging insular snakes should reflect a relationship to recency of rainfall more strongly than in mainland snakes. (2) Insular snakes will tolerate greater dehydration before drinking than will mainland snakes. (3) Insular snakes will avoid drinking seawater more strongly than will those from the mainland. Between 2001 and 2018, we quantitatively estimated the hydration status of 337 individual cottonmouth snakes from insular populations and 30 cottonmouth snakes from mainland Florida, as judged by the tendency of wild-caught snakes to drink fresh water immediately following capture. We found that insular cottonmouth snakes had a higher incidence of dehydration than did mainland cottonmouth snakes (64% versus 23%), and the hydration status of the insular snakes correlated with patterns of precipitation. We also determined experimentally the dehydration threshold for drinking fresh water in insular (mean±s.d. $-5.64\pm 4.3\%$, $n=34$) and mainland cottonmouth snakes ($-5.74\pm 4.5\%$, $n=21$), and these were not significantly different. Discrimination tests for drinking serially from a graded series of brackish water showed that mainland snakes did not discriminate against the highest brackish value (10.5 ppt or 30% seawater), whereas insular snakes showed a preference for <15% seawater. Naive neonates from insular and mainland cohorts behaved similarly. The preference of insular snakes for fresh water represents an important aspect of the maintenance of water balance that differs from the mainland conspecifics and is likely a habituated or adaptive response to dependence on rainfall.

KEY WORDS: Water relations, Florida cottonmouth, Island, Drinking, Dehydration, Brackish water

INTRODUCTION

Colonization of novel habitats often requires plastic adjustments or adaptation to changes in local conditions (Zimmer, 1998). Water is essential to the survival of all organisms and the need to maintain hydration within a terrestrial environment exerts a selective pressure on all aspects of an organism's life cycle (Bartholomew, 1972). Thus,

invasion of habitats having limitations of water necessitates physiological, morphological and/or behavioral adaptations to maintain water balance. The water requirements of reptiles correlate strongly with the environmental availability of fresh water (Gans et al., 1968; Cohen, 1975; Taplin, 1984; Nagy and Peterson, 1988; Peterson, 1996; Lillywhite et al., 2008a; Guillon et al., 2014). Animals living in arid or semi-arid environments face the significant challenge of maintaining hydration while relying on stochastic water resources that are unevenly distributed across a complex landscape matrix. Those animals are thought to survive by increasing their ability to find and utilize available water, reducing their need for water by minimizing losses, tolerating temporary water imbalances, or some combination of these abilities (Nagy and Peterson, 1988).


Atypical populations of Florida cottonmouth snakes, *Agkistrodon conanti* Gloyd 1969 (Serpentes: Viperidae), inhabit the Cedar Keys and other continental islands in the Gulf of Mexico off the coast of Florida (Carr, 1936) (Fig. 1). Cottonmouth snakes generally are characterized by close associations with mesic freshwater habitats, and they are considered to be the most aquatic species of the genus *Agkistrodon* (Gloyd and Conant, 1990). However, insular populations of cottonmouth snakes are entirely terrestrial, and the populations inhabiting Seahorse Key and Snake Key in the Cedar Keys (Fig. 1) lack access to permanent sources of fresh water (Lillywhite and McCleary, 2008). Precipitation can be patchy or intermittent, and the islands are susceptible to periodic drought. As the majority of other cottonmouth snake populations are semi-aquatic and found in freshwater habitats, these insular populations may provide insight into the important features required for survival and persistence in environments in which fresh water is scarce or absent (Lillywhite et al., 2008b).

One of the principal requirements for water balance of reptiles living in arid or marine environments is the ability to obtain fresh water. Low-latitude deserts are obvious examples of such environments, but islands can also be challenging environments when sources of fresh water are scarce or absent. This is true of islands we investigated where Florida cottonmouth snakes reside. These insular snakes are largely dependent on allochthonous food resources in the form of fish carrion and do not appear to be able to maintain water balance by relying solely on water from food (Wright et al., 2013; Lillywhite, 2017; Lillywhite et al., 2014a, 2018). On very rare occasions, cottonmouth snakes have been observed to enter the ocean (Lillywhite et al., 2008b); however, seawater is thought to be a major barrier to dispersal for insular cottonmouth snakes (Wharton, 1969; Lillywhite et al., 2008b; Lillywhite and McCleary, 2008). Their lack of an extrarenal salt gland makes it very unlikely they can utilize seawater for drinking (Dunson and Mazzotti, 1989). Insular populations of cottonmouth snakes must therefore depend on rainfall to maintain water balance. In contrast, mainland populations of cottonmouth snakes are typically found near standing bodies of fresh water and generally rely on these for hydration and water balance.

The dependence on rainfall for maintaining water balance is problematic because of the stochastic nature of precipitation, and

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Received 27 March 2019; Accepted 5 April 2019

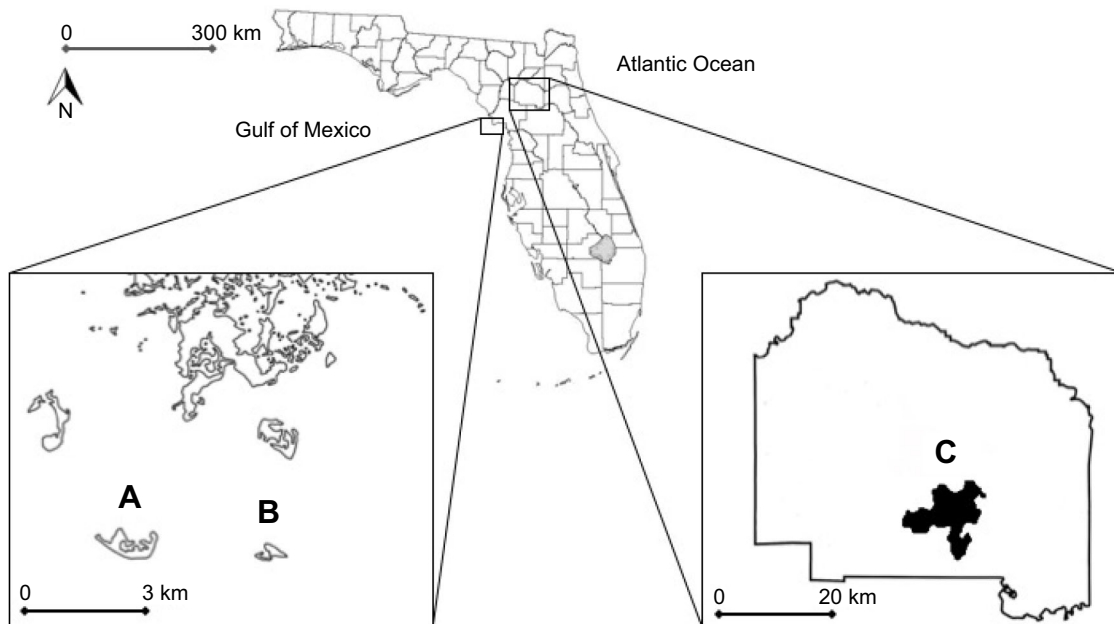


Fig. 1. Aerial view of the study area. Aerial view of the state of Florida, with county boundaries, and insets showing the three study populations consisting of Seahorse Key (A), Snake Key (B) and the mainland population at Paynes Prairie Preserve (C).

insular cottonmouth snakes may therefore experience intermittent dehydration because of periodic drought (Lillywhite and McCleary, 2008). Indeed, foraging behaviors can be suppressed when drought is prolonged and dehydrating snakes remain in refugia beneath the ground (Lillywhite and McCleary, 2008). Knowing the hydration status of snakes in relation to rainfall on islands can provide important insights regarding the dependence of insular snakes on periodic and sometimes unpredictable sources of fresh water.

Here, we report the results of investigations in which we sought to understand how insular cottonmouth snakes maintain water balance, and how the associated behaviors of these snakes might differ from those inhabiting more mesic habitats on the mainland. We tested three hypotheses related to the water relations of cottonmouth snakes. (1) Insular snakes will tend to drink fresh water in a manner that varies with the recency of rainfall, and the pattern of drinking will reflect the variable status of hydration through time. (2) The threshold of dehydration at which snakes first drink fresh water will be greater in insular snakes than in snakes from the mainland. (3) Insular cottonmouth snakes will avoid drinking seawater or brackish water, whereas naive snakes from the mainland will show less avoidance.

MATERIALS AND METHODS

Animals and study site

We tested dehydration and drinking of Florida cottonmouth snakes inhabiting continental islands in the Gulf of Mexico (Levy County, FL, USA) and mainland Florida (Alachua County, FL, USA). Beginning in 2001 and ending in 2018, snakes were captured opportunistically throughout the islands and mainland Florida, placed in secure containers, and then immediately transported to the laboratory at the University of Florida. Following laboratory investigations, all of the snakes were released at their original site of capture.

Seahorse Key and Snake Key are continental islands located ~3.5 km off the coast of Cedar Key, Levy County, FL, USA (Fig. 1). Seahorse Key is the larger of the two islands, with an area of 67 hectares, and has a maximum elevation of 16 m. Snake Key is

15 hectares in size, with an average elevation of 1 m, and is located 2.5 km off the east coast of Seahorse Key (Fig. 1). The primary habitat type for both islands is upland mixed hardwood hammock surrounded by low-lying mangroves and salt marsh. The southern arc of Seahorse Key consists of white sand beaches dotted with mangroves. Seahorse Key and Snake Key are part of the Cedar Keys National Wildlife Refuge, which was established in 1929 to provide nesting habitat for the rookeries of various waterbird species that seasonally nest on the islands (Lillywhite and McCleary, 2008). The islands of the Cedar Keys are recently formed relic sand dunes, and recent patterns of sea level rise in the northern Gulf of Mexico suggest the islands have been isolated from the mainland by seawater for only ~3500–4500 years (Wright et al., 2005).

Paynes Prairie Preserve is a state park in Alachua County, FL, USA. The park is 8500 hectares of mixed lowland habitats. Snakes were captured in the prairie basin, which floods seasonally and is never completely dry. Paynes Prairie Preserve is situated in the center of peninsular Florida, ~100 km northeast of the insular populations at Cedar Keys (Fig. 1).

All experimental methods and procedures were approved by the University of Florida's Institutional Animal Care and Use Committee under studies #Z025, #200903269, #201203269, #201508919 and #201809079. Further, all animals were captured, handled and housed according to Florida Park Service permit #06051712, Florida venomous reptile license #411-133587 and U.S. Fish and Wildlife Service federal special use permit #41511-14-10.

Field hydration status and precipitation patterns

The methods employed here were similar to those used previously in studies of marine snakes reported by Lillywhite et al. (2008a, 2012). Between 2001 and 2018, 337 individual cottonmouth snakes (mass 24–3266 g) were captured from insular populations, returned to the laboratory and tested immediately for drinking as described below (see Table S1 for chronological details). Similarly, between 2016 and 2018, 30 cottonmouth snakes (mass 124–1439 g) were captured from Paynes Prairie Preserve in mainland Florida,

returned to the laboratory and tested for drinking. Upon return to the laboratory, freshly captured snakes were weighed to the nearest 1 g using an Ohaus Scout electronic balance (Ohaus, Parsippany, NJ, USA). Each snake was then placed individually in a Plexiglas cage and provided with access to fresh water (FW, tap water=0 ppt). All snakes were left in individual cages with water for at least 12 h. Snakes were then reweighed to quantify any change in mass. An increase in mass of >1% was considered evidence of drinking. Drinking indicates thirst, which in turn reflects dehydration (Fitzsimons, 1976; Lillywhite et al., 2008a). We consider the percentage increase in mass (i.e. the relative amount of water drunk) as the apparent water deficit that was incurred by prior dehydration in the wild. If a snake defecated before a second measurement of mass could be made, and its mass had not increased by more than 1%, it was excluded from analyses. Snakes that defecated and still experienced an increase of >1% body mass were considered to have drunk but were not included in the calculation of apparent water deficit. Each snake represented an independent binomial drinking trial and was scored with one of two outcomes: (1) drinking or (2) no drinking.

The above procedure allowed us to characterize the status of hydration in individual free-ranging cottonmouth snakes from insular and mainland populations, according to the date of capture. To determine if there was a relationship between the hydration status of cottonmouth snakes and rainfall patterns, we compared the binomial drinking score of snakes with five precipitation parameters, including peak rainfall rate at last rainfall, 3 day rainfall accumulation, 7 day rainfall accumulation, 14 day rainfall accumulation and number of days since the last rainfall of >2.5 mm h⁻¹. We chose 2.5 mm h⁻¹ as our threshold for a 'rainfall event' based on patterns of rainfall in our study area. NASA classifies rainfall with a rate of 2–4 mm h⁻¹ as 'light rain'. A rainfall rate of 2.5 mm h⁻¹ is equivalent to 2.5 l of water falling per square meter of area over the course of 1 h.

Precipitation data were collected from NASA's Tropical Rainfall Measuring Mission (TRMM) satellite monitoring program version 7 and accessed through the Giovanni online data system, developed and maintained by the NASA Goddard Earth Sciences Data and Information Services Center. We caution that the TRMM data may not accurately reflect the precipitation available to cottonmouth snakes from our insular study populations because of the low spatial resolution of precipitation data estimated by satellite. The spatial scale of TRMM is 0.25 deg or ~27 km and covers an area much larger than either of our insular study sites. Therefore, it is possible that a highly localized rainfall event could have fallen outside the land area of Seahorse or Snake Key and therefore erroneously overestimated the rainfall that we considered to be available to cottonmouth snakes.

Dehydration threshold for drinking FW

We measured the dehydration (thirst) threshold for drinking FW in 55 snakes (range 77–2172 g) (Seahorse Key *n*=22, Snake Key *n*=12, mainland *n*=21). Dehydration threshold for drinking is defined here as the relative amount of body mass lost by dehydration at the point when a snake voluntarily drinks FW, expressed as a percentage of the starting hydrated mass. Snakes were not fed during trials. The low metabolic rate of cottonmouth snakes (McCue and Lillywhite, 2002) suggests there is little loss of body mass due to the utilization of stored nutrients within the short time frame of our experiments. Decreases in mass are therefore assumed to result effectively from losses of body water (see discussion in Lillywhite, 2017).

We determined the dehydration threshold for drinking by first allowing a snake unlimited access to FW for at least 24 h prior to testing. After this initial period with *ad libitum* access to FW, a snake was weighed and water was removed from the cage. Snakes were then allowed to dehydrate slowly while exposed to room air at a mean (±s.d.) temperature of 24.7±0.7°C and a relative humidity of 50.2±6.4%. During water restriction, snakes were weighed daily to determine the percentage loss of body mass from the original hydrated condition. When a snake lost between 1% and 3% of the initial hydrated body mass, it was first offered brackish water (BW) of a salinity (23 ppt) found in the waters near Seahorse Key (UF/IFAS Shellfish Aquaculture Extension Program, unpublished data) for 90 min (mean±s.d. 90.9±24 min), immediately followed by a 90 min period (80.1±26 min) with access to FW (tap water=0 ppt). Snakes were weighed before and after each period of access to water to determine whether and when individuals drank, indicated as an increase in mass of >1%. If an individual did not drink, it was allowed to continue to dehydrate and was offered water at decrements in body mass from 1% to 2% until it drank. In many cases, in addition to quantification of amounts of water ingested, snakes were also observed to drink.

All snakes were offered BW before FW as a routine part of the protocol, although no snake was found to drink BW at any time during the dehydration trials. BW was prepared by dissolving commercial Instant Ocean sea salt (Aquarium Systems, Mentor, OH, USA) in tap water at room temperature. All FW provided to the snakes and used to prepare BW dilutions came from ground aquifers that supply the tap water at the University of Florida. Salinity was measured using a digital salinity meter (Ohaus Starter Salinity Pen Meter) to the nearest 0.1 ppt.

Each snake went through both BW and FW trials regardless of the extent of dehydration required to induce drinking. To reduce stress related to experimental procedures, no individuals were allowed to be dehydrated to >–30% change in body mass. Once snakes completed the trials, they were offered water *ad libitum* for at least 24 h and released at their original site of capture, except for a small subset of snakes (*n*=19) that were also tested for their drinking tendencies related to seawater (SW; see below).

SW–BW drinking

After estimating the dehydration threshold for drinking FW in cottonmouth snakes, we determined whether snakes would drink SW or BW dilutions of SW when in a dehydrated state. SW drinking behavior was tested in 54 cottonmouth snakes (mass 72–2039 g) (Seahorse Key *n*=11, Snake Key *n*=15, mainland *n*=28). First, we ensured that snakes were fully hydrated. The mass of each snake was measured and access to FW was subsequently eliminated, allowing each snake to dehydrate in room air until reaching the dehydration threshold for drinking (5–10% body mass loss as determined by dehydration threshold trials described above), which required 3–7 days. In a pilot study of 10 snakes (island *n*=8, mainland *n*=2), dehydrated individuals were offered a series of progressively less-brackish water starting with 100% SW of 35 ppt, then 75% SW, 50% SW, 25% SW and ending with FW of 0 ppt. No snakes drank any dilution other than full FW. We therefore adjusted our test dilutions and began trials using six progressively more dilute waters: 30% SW, 25% SW, 20% SW, 15% SW, 10% SW and FW. Snakes were tested at each SW dilution for 60 min (mean±s.d. 60±9 min) and individuals moved immediately from one SW dilution test period to the next in the same order in succession. The mass of cottonmouth snakes was measured before and after each 60 min period. Drinking was defined as an increase in body mass of >1%. For statistical

analyses of SW-drinking behavior, individuals were only reported to drink from the first test dilution at which mass increased by >1%.

Seawater drinking trials were also conducted on neonate cottonmouth snakes (mass 29–34 g) born in the laboratory and subsequently naive to SW. A single female from each of the mainland and Snake Key populations gave birth in the laboratory to a single litter of neonate snakes (mainland $n=9$, Snake Key $n=7$). The experimental procedure for these neonate snakes was the same as for the adults, except that drinking in these trials was defined as an increase in body mass of >2%. Neonate snakes were observed to wet their skin in the water bowl frequently and some individuals gained >1% body mass from wetting but never >2%. The greater relative gain in mass from wetting of skin in neonates is attributable to a higher surface area to volume ratio compared with adults.

The neonates were included in this investigation because such data can rarely be obtained at present; the population of cottonmouth snakes on Seahorse Key is currently experiencing a significant decline due to the recent abandonment of the island by nesting waterbirds that previously provided them with significant food resources (Sandfoss et al., 2018). Unfortunately, we have neither observed nor captured a single neonate or pregnant female on Seahorse Key in the past 2 years following the abandonment of the nesting rookery. We do not have the option to sample numerous cohorts of neonatal snakes from either insular population because of their protected status in the National Wildlife Refuge and the uncertainties of population decline or recovery at this time. Removing multiple females, or their attended neonates immediately following birth, might stress the adults and behaviorally disrupt their association with areas where nesting bird rookeries might conceivably re-establish (Wharton, 1969). Thus, we were forced to accept limitations of sample size in this study because of both practical and ethical considerations. Limitations in the number of sibling cohorts have been justified in previous studies of this and other species (Cooper and Secor, 2007; Lillywhite et al., 2015).

Statistics

A binomial regression within the generalized linear model framework was used to examine the relationship between the outcome of binomial drinking trials and precipitation parameters. Linear regressions were used to measure the effects of measures of precipitation and body condition index (BCI) on the apparent water deficit of field-caught insular snakes.

The BCI (Jayne and Bennett, 1990; Bonnet and Naulleau, 1995) was calculated for each individual cottonmouth snake to test the effect of body condition on dehydration threshold of drinking. First, we constructed a linear regression of the log-transformed snout–vent length (SVL) and mass at capture of all cottonmouth snakes with length and mass data from field hydration studies ($n=290$) and the individuals that were used in dehydration threshold and SW drinking trials ($n=90$). The resulting residual value for each individual cottonmouth snake was used as its BCI. Snakes with positive BCI values were categorized as being in ‘good’ condition and those with negative scores in as being in ‘poor’ condition. Statistical comparisons of dehydration threshold for drinking between populations were made using the non-parametric Wilcoxon rank sum test (due to small sample sizes <30) and linear regression.

Statistical comparisons of SW drinking behavior were made using the Pearson’s χ^2 test of homogeneity, which compared observed patterns of SW drinking with a predicted even distribution across all test salinities. All statistics were performed in program R (version 3.5.1) and alpha was set at 0.05. Descriptive statistics are reported as means \pm s.d.

RESULTS

Field hydration status and precipitation patterns

The majority of insular snakes (64%, $n=337$) drank within 24 h of capture, indicating thirst and a state of dehydration. Of the snakes from Seahorse Key that drank ($n=193$), the average apparent water deficit, or change in mass as a result of drinking, was $+8.94\pm 8.5\%$, while those from Snake Key included in the analysis ($n=21$) increased in mass on average $+7.56\pm 5.5\%$. Of the cottonmouth snakes captured from the mainland population, 23% ($n=30$) drank and had a mean apparent water deficit of $3.42\pm 2.7\%$ ($n=6$).

The binomial drinking results were compared with precipitation patterns using a generalized linear model (Table 1), which indicates that for insular cottonmouth snakes, the amount of rain in the 7 and 14 days prior to capture has a significant impact on the probability that a given snake will drink. An increase in the amount of rainfall led to a lower probability of drinking in cottonmouth snakes from Seahorse and Snake Key. Additionally, as the number of days since rainfall increased, the probability that a captured insular snake drank increased. Furthermore, the apparent water deficit of insular snakes increased with the number of days since rainfall ($F_{1,288}=6.71$, $P=0.01$) (Fig. 2) and decreased with increasing BCI values of captured individuals ($F_{1,288}=24.34$, $P<0.001$) (Fig. 3).

There were no significant effects of any precipitation parameter on the probability that a mainland cottonmouth snake would drink (Table 1). Direct comparisons between the results of analyses for each population are not informative because the sample size for mainland cottonmouth snakes was considerably smaller and data were collected over a shorter time period than for insular snakes. However, analyses using approximately equal sample sizes (island $n=35$, mainland $n=27$) collected over the same time period (2017 and 2018) demonstrate similar results with the exception of the number of days since rainfall no longer having a significant effect ($z=1.73$, $P=0.083$) on the probability of drinking in the insular populations at the 0.05 level.

Dehydration threshold for drinking FW

We compared the dehydration (thirst) threshold of drinking between insular (mean $-5.64\pm 4.3\%$, $n=34$) and mainland cottonmouth snakes (mean $-5.74\pm 4.5\%$, $n=21$), and found no difference (Wilcoxon $W=369.5$, $P=0.835$) (Fig. 4A). There was considerable inter-individual variation in dehydration threshold in each of the

Table 1. Results of binomial regression within the generalized linear model framework

Parameter	Insular ($n=337$)		(Mainland $n=30$)	
	Estimate	<i>P</i>	Estimate	<i>P</i>
Peak rainfall rate	0.004	0.742	-0.212	0.221
3-day rainfall accumulation	-0.006	0.069	-0.008	0.689
7-day rainfall accumulation	-0.006	0.026*	-0.009	0.384
14-day rainfall accumulation	-0.005	0.006*	-0.014	0.066
Days since last rainfall of >2.5 mm h ⁻¹	0.039	<0.005*	0.126	0.233

Statistical analysis of drinking probability of free-ranging insular cottonmouth snakes during binomial drinking trials in relation to precipitation patterns. The table reports the estimates for each of five precipitation parameters and the corresponding test of significance for each parameter and drinking probability for insular and mainland cottonmouth snakes. Between 2001 and 2018, 337 individual cottonmouth snakes were captured from insular populations (Seahorse Key $n=305$, Snake Key $n=32$). Similarly, between 2016 and 2018, 30 cottonmouth snakes were captured from Paynes Prairie Preserve in mainland Florida and tested for drinking.

*Statistically significant at the 0.05 alpha level.

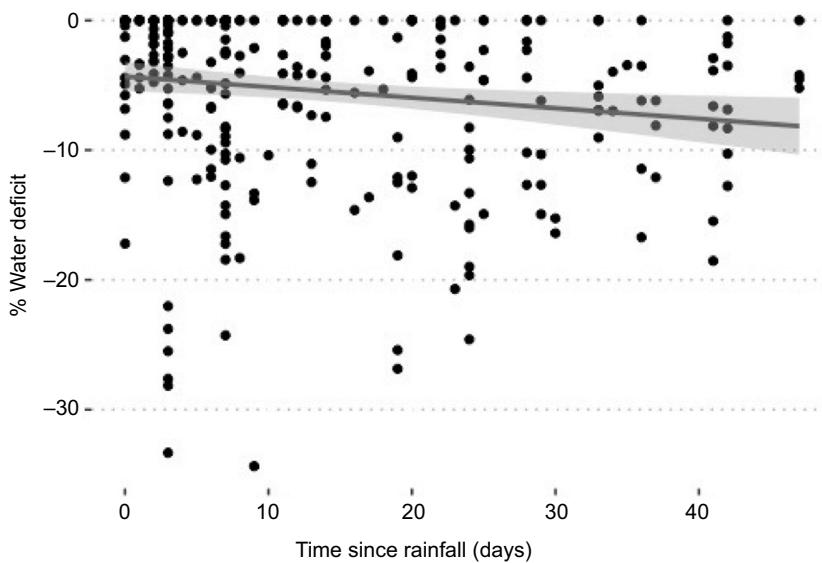


Fig. 2. Scatterplot of number of days since rainfall and apparent water deficit. Results of the field study of the hydration status of insular cottonmouth snakes ($n=290$) showing the linear regression of the number of days since a rainfall of $>2.5 \text{ mm h}^{-1}$ and apparent water deficit of individuals (measured as the percentage change in mass from drinking) ($F_{1,288}=6.71$, $P=0.01$). Solid line indicates line of regression ($y=-0.001x-0.04$, $R^2=0.02$) and gray shading represents the 95% confidence interval.

three populations, with some snakes drinking at $<2\%$ loss of mass while others from the same population did not drink until $>12\%$ loss of mass. There was no effect of body size (SVL; linear regression $F_{1,51}=0.002$, $P=0.956$) on dehydration threshold. However, BCI did affect the dehydration threshold for drinking (Wilcoxon $W=449$, $P=0.039$) with snakes in ‘good condition’ having a smaller mean dehydration threshold (mean $-4.70\pm 4.6\%$, $n=24$) than snakes in poor condition ($-6.56\pm 4.2\%$, $n=28$) (Fig. 4B). Also, we found that females (mean $-4.78\pm 3.4\%$, $n=30$) had a smaller dehydration threshold than did males ($-7.85\pm 5.1\%$, $n=20$) (Wilcoxon $W=418$, $P=0.024$) (Fig. 4C).

No snakes drank BW (23 ppt) during the drinking threshold trials. On average, snakes returned to their fully hydrated mass and increased in body mass by $+3\%$ (mean $3.23\pm 6.4\%$). There was no effect of location (Wilcoxon $W=361$, $P=0.952$), body size (linear regression $F_{1,50}=0.439$, $P=0.511$) or sex (Wilcoxon $W=316.5$, $P=0.751$) on the relative amount of water that was drunk by an individual.

SW drinking behavior

Results from Pearson’s χ^2 tests of homogeneity found that insular snakes did not drink from all SW dilutions equally ($\chi^2=63.1$, d.f.=5, $P<0.005$) (Fig. 5A), while the SW drinking behavior of snakes from

the mainland population did not differ significantly from an equal distribution of drinking across the six SW dilutions ($\chi^2=5.2$, d.f.=5, $P=0.39$) (Fig. 5B). Insular cottonmouth snakes preferentially drank from the less-saline SW dilutions while mainland snakes showed no preference. Results of SW drinking trials in naive neonate snakes from mainland and insular populations showed a pattern similar to that of adult snakes (Fig. 5C,D). All neonates from the mainland population drank from either 30% SW ($n=7$) or 25% SW ($n=2$) dilutions, whereas neonates from Snake Key drank from lower salinity SW dilutions of 20% SW ($n=1$), 15% SW ($n=1$), 10% SW ($n=2$) and FW ($n=3$). No further analyses of SW drinking were performed on the results for neonates because of the limited sample size.

We made several comparisons of change in mass related to SW drinking behavior and found that mainland cottonmouth snakes gained relatively more total mass (mean $+16.2\pm 13.9\%$) during drinking trials than did insular cottonmouth snakes ($+5.9\pm 3.6\%$), and this difference was significant (Wilcoxon $W=153.5$, $P<0.005$). However, the relative mass an individual gained at the dilution where drinking was first recorded, which for mainland snakes was (mean \pm s.d.) $+9.4\pm 9.7\%$ and for insular snakes was $+5.3\pm 4.1\%$, did not differ (Wilcoxon $W=303$, $P=0.29$). In combination, these data show that mainland cottonmouth snakes drank water at more

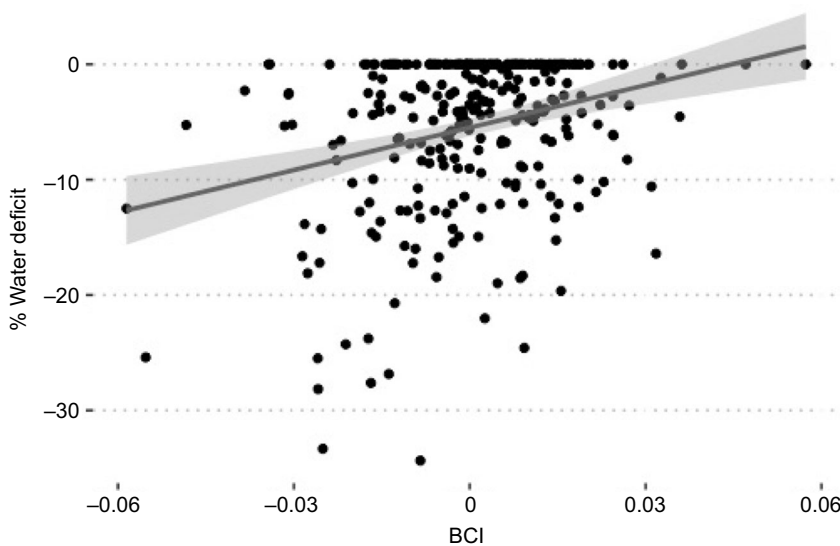


Fig. 3. Scatterplot of body condition index (BCI) and apparent water deficit. Results of the field study of the hydration status of insular cottonmouth snakes ($n=290$) showing the linear regression of BCI and apparent water deficit of individuals (measured as the percentage change in mass from drinking) ($F_{1,288}=24.34$, $P<0.001$). Solid line indicates line of regression ($y=1.23x-0.05$, $R^2=0.08$) and gray shading represents the 95% confidence interval.

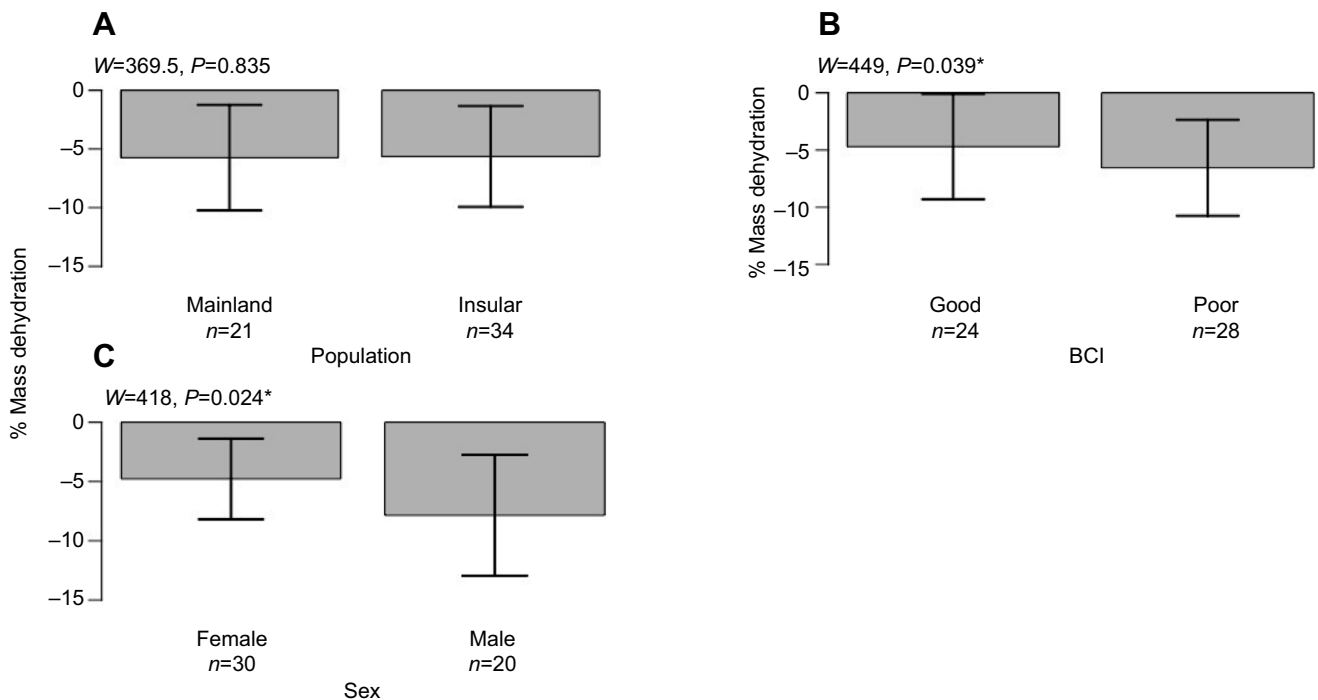


Fig. 4. Bar graph plots of drinking threshold. Drinking threshold is the dehydration threshold for drinking and was defined as the percentage body mass lost to dehydration at which an animal first drinks fresh water. (A) Drinking threshold of mainland ($n=21$) and insular ($n=34$) populations of cottonmouth snakes. (B) Drinking threshold of cottonmouth snakes in good ($n=24$) and poor ($n=28$) condition. (C) Drinking threshold of female ($n=30$) and male ($n=20$) cottonmouth snakes. Data were compared with Wilcoxon rank sum tests. Bar graph plots represent the mean \pm s.d. *Statistical significance at the 0.05 alpha level.

brackish levels of SW and from a greater number of salinity levels than did their insular conspecifics. However, we found no relationship between the percentage mass gained at the dilution where drinking was first observed and an individual's total percentage mass gained (linear regression $F_{1,52}=2.569, P=0.12$). In addition, we did not find an effect of body size (SVL) on the relative mass gained at the dilution where drinking was first observed (linear regression $F_{1,51}=0.10, P=0.75$) or total percentage mass gained (linear regression $F_{1,51}=3.99, P=0.05$). There was no effect of sex on the relative mass gain at the dilution where drinking was first observed (Wilcoxon $W=243, P=0.50$) or total percentage mass gained (Wilcoxon $W=214.5, P=0.98$).

DISCUSSION

Field hydration status and precipitation patterns

Insular habitats without sources of permanent free-standing water challenge inhabitants with dependence on precipitation as sources of water, even while other resources might be abundant. Our observations of insular Florida cottonmouth snakes over many years of field investigations indicate that these populations of snakes, while generally avoiding SW (Wharton, 1969; Lillywhite, et al., 2008b), depend on periodic rainfall to mitigate desiccation. Our data for drinking and hydration status demonstrate the majority (64%) of snakes captured from the two insular populations ($n=337$) were moderately dehydrated to some extent. Episodic drinking often involves ingestion of large quantities of water and is characteristic of ectothermic vertebrates that are dependent on drinking FW in arid or semi-arid environments (e.g. Miller, 1985; Peterson, 1996; Bonnet and Brischoux, 2008; Wright et al., 2013). Thus, it appears that insular cottonmouth snakes spend a great deal of their lives in a dehydrated state, as shown for desert tortoises (Nagy and Medica, 1986; Peterson, 1996), gila monster lizards (Wright et al., 2013) and pelagic sea snakes (Lillywhite et al., 2014a).

Intraspecific variability in the hydration status of insular cottonmouth snakes exposed to the same regime of precipitation is likely related to: (1) differences in the location of snakes relative to uses of underground refugia and access to rainfall; (2) differences in the activity of snakes as this might influence evaporative water losses during the drought interval preceding a particular rainfall event; (3) differences in the size of snakes, whereby larger individuals might not be able to find temporary pools of sufficient size for substantial drinking produced by 'light' rainfall because of mechanical limitations related to mouth size; and (4) differences in BCI, whereby snakes in better body condition, which correlates to some extent with fat reserves (Weatherhead and Brown, 1996), may be buffered against dehydration to a small degree by metabolizing lipids, resulting in a net increase in water. Our results show that free-ranging insular snakes with higher BCI values had a lower apparent water deficit (Fig. 3) at capture.

Limited access to FW is likely to be a major stressor and source of mortality for insular cottonmouth snakes (see Wharton, 1969), while snakes captured from mainland Florida were infrequently thirsty. We found insular snakes to be in a dehydrated state more frequently and to have a larger apparent water deficit than mainland cottonmouth snakes. The observed high rate of dehydration and water deficit in insular cottonmouth snakes could be, in part, the result of climate change. Rates of precipitation in our study area have declined over the past 110 years and are predicted to continue declining (USGCRP, 2017).

There are three routes of water input for animals: preformed water in food, metabolic water produced from fat stores or consumption of a meal, and free water that animals drink or absorb across body surfaces. Overall, metabolic water constitutes a very small quantity of water influx for reptiles and does not exceed their evaporative water loss (Minnich, 1982). Recent work has shown that carnivorous reptiles, including cottonmouth snakes, cannot

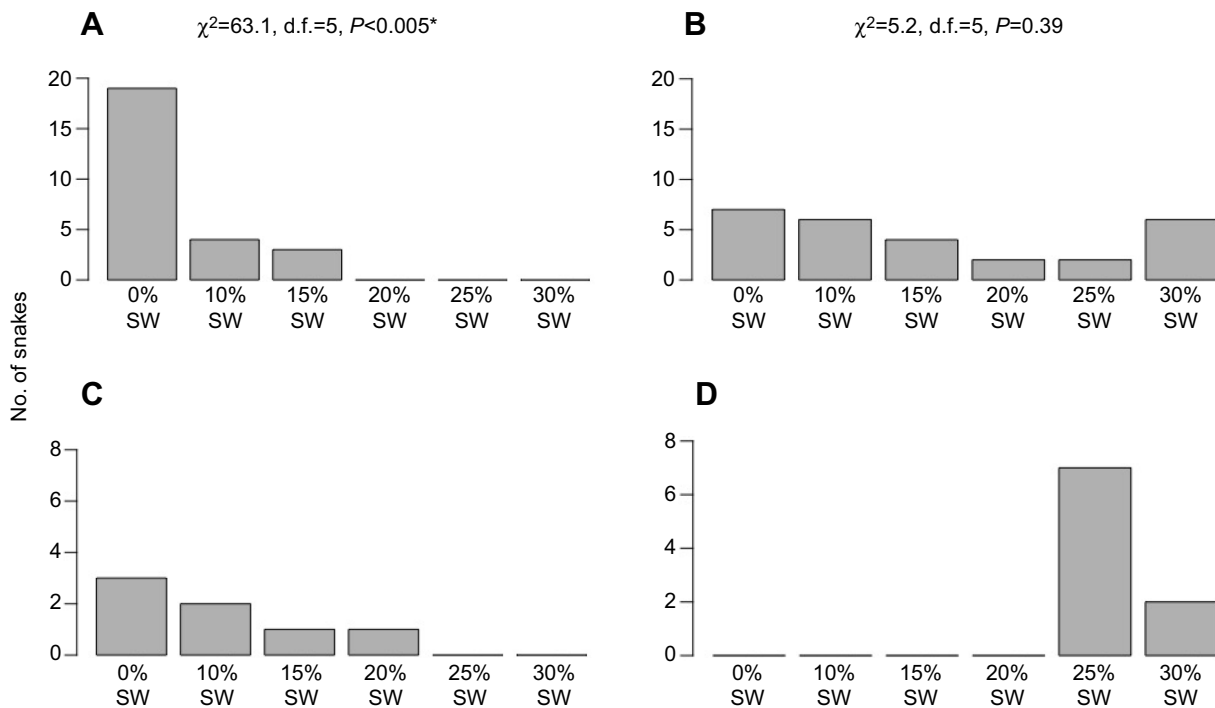


Fig. 5. Bar graph plots of seawater drinking behavior. (A) Results of seawater (SW) drinking trials for cottonmouth snakes ($n=26$) from insular populations. (B) Results of SW drinking trials for cottonmouth snakes ($n=28$) from the mainland. (C) Results of SW drinking trials for naive neonate snakes ($n=7$) from Snake Key. (D) Results of SW drinking trials for naive neonate snakes ($n=9$) from the mainland. Gray bars show the number of snakes that initiated drinking at each SW dilution. Data were compared with χ^2 tests for homogeneity. *Statistical significance at the 0.05 alpha level.

depend on dietary and metabolic water from feeding to maintain water balance and must have access to free-standing FW to avoid desiccation (Lillywhite et al., 2008a; Davis and DeNardo, 2009; Wright et al., 2013; Lillywhite, 2017). Understanding the availability of water to free-ranging snakes is important because a reduction in water availability can influence behavior in the selection of microhabitat (Kotler et al., 1998; Ryan et al., 2016) and level of activity (Lorenzon et al., 1999; Lillywhite and McCleary, 2008). Moreover, the hydration status of individuals strongly influences life history traits including thermal preference (Ladyman and Bradshaw, 2003), growth rate (Lorenzon et al., 1999), reproduction (Seigel and Fitch, 1985; Brown and Shine, 2006) and innate immunity (Moeller et al., 2013; Bruschi and DeNardo, 2017). More severely dehydrated individuals may experience increased osmolality of blood and emaciation that can be life threatening (Brischoux et al., 2017). The persistence and resilience of insular cottonmouth snake populations are difficult to predict owing to stochastic selection pressures that result from drought, intense storms, competition and increasing SW incursion attributable to climate change (Lillywhite et al., 2008b).

Dehydration threshold for drinking FW

Our data for thirst and drinking FW following periods of dehydration in the laboratory demonstrate that cottonmouth snakes drink water at relatively small deficits of body water amounting to a few percent of body mass dehydration (Fig. 4A). This result is similar to observations of drinking behavior in taxa of snakes from mesic habitats (H.B.L., unpublished data), but contrasts with dehydration thresholds for drinking FW in marine file snakes and sea snakes (Lillywhite et al., 2008a, 2012, 2014b, 2015). The tendency for drinking at small dehydration thresholds appears to be consistent with the evolutionary history of cottonmouth snakes

(Guiher and Burbrink, 2008) and suggests a need for water to alleviate further dehydration (that might be tolerated in sea snakes: Lillywhite et al., 2008a, 2014a). Thirst and drinking behaviors were not statistically different between insular and mainland populations (Fig. 4A). However, there was considerable variation of dehydration threshold among snakes in all populations, and some individuals did not drink until they had lost >12% of body mass. This reflects a tolerance for dehydration approaching the mean threshold that is characteristic of file snakes and sea kraits, which are species that often associate with marine and estuarine habitats (Lillywhite et al., 2008a, 2014b). Thus, one could imagine directional selection for dehydration tolerance if insular snakes eventually experience increased association with BW or SW (Lillywhite et al., 2008b).

The reason for a difference in the dehydration threshold for drinking between snakes having good and poor body condition is unclear (Fig. 4B). Given that body condition reflected nutritional status of snakes or even hydration status prior to access to water at the beginning of experiments, the osmolality of body fluids and activation of hormones affecting thirst might have been different in the two groups. We also found females to have a smaller dehydration threshold for drinking than that of male snakes (Fig. 4C). We collected no data on the reproductive status of females during trials and can therefore make no firm statements on the effect of reproduction on water use in cottonmouth snakes. It has been shown in other species of snakes that viviparity as a reproductive strategy can create parent-offspring conflicts over the allocation of water to embryos, particularly in water-limited environments (Dupoué et al., 2014; Bonnet et al., 2017).

SW drinking behavior

Cottonmouth snakes inhabiting islands in the Cedar Keys have access to brackish SW in addition to standing temporary pools of

rainwater. The marine waters surrounding the Cedar Keys are influenced by outflows from two major nearby rivers. Hence, the salinity generally varies from 20 to 30 ppt during the course of annual variation (UF/IFAS Shellfish Aquaculture Extension Program, unpublished data), but can trend considerably lower following periods of heavy rainfall on the mainland. Additionally, cottonmouth snakes could potentially drink from dilute BW in saltmarshes surrounding parts of the islands during periods of heavy rainfall. The close proximity and possible use of dilute SW for the maintenance of water balance seem to have conferred insular cottonmouth snakes with the ability to differentiate the salinity of water for drinking. We found clear differences between insular and mainland populations in their selection of drinking water related to salinity (Fig. 5). This result supports our prediction that cottonmouth snakes residing on islands behaviorally avoid drinking high-salinity SW, even when dehydrated, while mainland snakes might not.

We do not know how drinking 30% SW affects the overall long-term health of snakes. A dilution of 30% SW has a sodium concentration of ~ 140 mOsm l^{-1} which is similar to blood sodium values in the range of normonatremia for snakes: 130–160 mOsm l^{-1} (Dessauer, 1970). Experimentally dehydrated species of sea kraits (*Laticauda* spp.) refuse to drink water at salinities above 30% SW (Lillywhite et al., 2008a) and these amphibious snakes possess a functional salt gland (Kirschner, 1980). It is not known what the upper salinity limit is for drinking BW in mainland snakes, as during SW drinking trials individuals drank at our highest test dilution of 30% SW, but during dehydration threshold studies, described above, no individual ($n=21$) drank from BW of $\sim 66\%$ SW or 23 ppt.

The behavioral avoidance of SW dilutions $>15\%$ SW observed in insular cottonmouth snakes is likely an innate response that avoids deleterious effects of ingestion of salt. No marine snake has ever been experimentally shown to voluntarily ingest full SW, including true sea snakes (Lillywhite et al., 2008a, 2012, 2015). Coastal species of colubrid ‘water snakes’ (*Nerodia* spp.) do not voluntarily drink SW, whereas naive semi-aquatic ‘freshwater’ species drink SW and die as a result (Dunson, 1980). There is limited information on the ability of snakes to detect or avoid SW, but this ability has been demonstrated in several taxa (Dunson, 1980; Kidera et al., 2013). The ability of insular cottonmouth snakes in our study to discriminate relatively small differences (5% SW or 1.75 ppt) in the brackish condition of drinking water invites further investigation into the sensory basis and tolerance for such differences of salinity.

Snakes from the mainland drank a larger relative amount of water during BW drinking trials than did insular snakes. This difference between populations did not exist for relative mass gained during the first drink, which is consistent with previous SW drinking behavior studied in crocodylids (Jackson et al., 1996). There was also no difference in the relative amount of total mass gained from drinking between populations during trials of dehydration threshold. However, mainland snakes were observed to drink from the highest test dilution (30% SW), which could have altered plasma osmolality and possibly prolonged the thirst response, thereby leading to continued drinking during the ensuing SW dilutions comprising the trial. Previously, the volume of BW that was drunk by sea kraits and sea snakes was found to vary inversely with the level of salinity, as we report here for cottonmouth snakes (Lillywhite et al., 2008a, 2012). These data suggest there was possibly a ‘metering’ response that adjusts the water input relative to ingestion of excess salt.

SW drinking trials involving naive neonate snakes produced interesting and contrasting results between insular and mainland individuals, which we must view with caution. The discrimination

and avoidance of salinities $>20\%$ SW for drinking in insular neonates born in captivity (Fig. 5), and therefore totally naive to SW, suggests SW discrimination is not a learned behavior. We cannot, however, rule out maternal effects in influencing the drinking patterns of insular and mainland neonates. We do not fully understand the chemosensory mechanism for salt discrimination and therefore how selection might be acting on this mechanism. A genetic study suggests the cottonmouth snake population on Seahorse Key is relatively inbred and quite isolated (McCleary, 2009).

With respect to the neonate snakes, using a single litter to draw conclusions is potentially flawed, and the drinking results for sibling neonates may not be representative of the population. However, regardless of how one considers the statistical independence of siblings, each snake represents an animal that would be actively experiencing its environment and responding with inherently hard-wired behavior (Arnold, 1981; Cooper, 2008). Therefore, information we learn from the limited groups of siblings to which we had access provides important insight and may stimulate others to investigate these questions further. In the long term, availability of neonates may increase if the nesting rookery of waterbirds is re-established at Seahorse Key and renews growth of the currently dwindling population of cottonmouth snakes (Sandfoss et al., 2018).

We found considerable variation of SW discrimination among adults tested from the mainland population. A quarter (26%, $n=27$) of individuals tested from the mainland population performed at the same level as insular cottonmouth snakes and avoided drinking high-salinity SW dilutions, drinking only FW (Fig. 5B). This variation in drinking behavior in mainland populations lends support to the supposition that variation was historically present in insular populations, and the behavioral avoidance of BW by extant insular cottonmouth snakes is possibly the result of directional selection. Directional selection leading to fixation in island snakes seems a more parsimonious explanation than SW discrimination arising as a result of a novel mutation in insular populations. There is no obvious disadvantage to mainland cottonmouth snakes for maintaining a latent ability to discriminate SW.

Cottonmouth snakes are thought to be the most freshwater viperid species, but other viperids, such as *Crotalus adamanteus*, are observed to enter SW and inhabit coastal areas, including islands (Lillywhite et al., 2008b). Future work should attempt to determine whether other viperids are able to discriminate the salinity of water, and comparisons between coastal and mainland populations might be particularly informative, as demonstrated here. Jackson et al. (1996) found that SW discrimination of drinking in alligatorid species was related in part to habitat of occurrence, although this was not the case for crocodylids. The ability of snakes to maintain water balance and thrive in saline habitats is facilitated by multiple behavioral and physiological adaptations (Zug and Dunson, 1979; Dunson and Mazzotti, 1989; Brischoux et al., 2017), including the reluctance to drink SW (Pettus, 1963). Further study of populations of snakes that have persisted on islands with limited water resources (see, for example, Bonnet et al., 2002) and may be under similar pressures might provide important insights into a process that is poorly characterized by the fossil record (Lillywhite and Martins, 2019).

Acknowledgements

We would like to acknowledge the management and staff of the Cedar Keys National Wildlife Refuge for research permissions. Logistical support was provided by the University of Florida Seahorse Key Marine Laboratory and Nature Coast Biological Station. We thank C. M. Sheehy III, D. Wooten, R. McCleary and others for assistance in collecting cottonmouth snakes over the years. We would also like to thank two anonymous reviewers for their comments and suggestions that greatly improved the paper.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.R.S., H.B.L.; Methodology: M.R.S., H.B.L.; Validation: H.B.L.; Formal analysis: M.R.S.; Investigation: M.R.S., H.B.L.; Resources: M.R.S., H.B.L.; Data curation: M.R.S.; Writing - original draft: M.R.S.; Writing - review & editing: M.R.S., H.B.L.; Visualization: M.R.S.; Supervision: H.B.L.; Project administration: M.R.S., H.B.L.; Funding acquisition: M.R.S., H.B.L.

Funding

Funding was provided by a Society for the Study of Amphibians and Reptiles grant-in-herpetology and a University of Florida Seahorse Key Fellowship awarded to M.R.S. Additional funding was provided by the Ed Stolarz Foundation and the U.S. Fish and Wildlife Service.

Data availability

The associated dataset is publicly available from the figshare digital repository: doi:10.6084/m9.figshare.8040680

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.204065.supplemental>

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