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## RESEARCH ARTICLE

# Intraspecific investigation of dehydration-enhanced innate immune performance and endocrine stress response to sublethal dehydration in a semi-aquatic species of pit viper

Mark R. Sandfoss<sup>1,\*‡</sup>, François Brischoux<sup>2</sup> and Harvey B. Lillywhite<sup>1</sup>

## ABSTRACT

Sublethal dehydration can cause negative physiological effects, but recent studies investigating the sub-lethal effects of dehydration on innate immune performance in reptiles have found a positive correlation between innate immune response and plasma osmolality. To investigate whether this is an adaptive trait that evolved in response to dehydration in populations inhabiting water-scarce environments, we sampled free-ranging cottonmouth snakes ( $n=26$  adult cottonmouths) from two populations inhabiting contrasting environments in terms of water availability: Snake Key ( $n=12$ ), an island with no permanent sources of fresh water, and Paynes Prairie ( $n=14$ ), a flooded freshwater prairie. In addition to field surveys, we manipulated the hydration state of 17 cottonmouths (Paynes Prairie  $n=9$ , Snake Key  $n=8$ ) in a laboratory setting and measured the response of corticosterone and innate immune performance to dehydration with the aim of identifying any correlation or trade-offs between them. We measured corticosterone of cottonmouths at a baseline level and then again following a 60 min stress test when at three hydration states: hydrated, dehydrated and rehydrated. We found that innate immune performance improved with dehydration and then returned to baseline levels within 48 h of rehydration, which agrees with previous research in reptiles. Despite the frequent exposure of cottonmouths on Snake Key to dehydrating conditions, we did not find cottonmouths inhabiting the island to show a greater magnitude or more prolonged immune response compared with cottonmouths from Paynes Prairie. We also found a positive association between dehydration and corticosterone values.

**KEY WORDS:** Cottonmouth, Physiological trade-offs, Corticosterone, Natural antibodies, Island snakes, Drinking

## INTRODUCTION

Water is essential to the survival of all living organisms. The availability of water in the environment varies significantly across time and space, and anthropogenic climate change is altering global patterns of precipitation – which can be the primary source of fresh water for many animal populations (e.g. island species: Sandfoss and Lillywhite, 2019; desert species: Peterson, 1996; marine species: Lillywhite et al., 2019; Rash and Lillywhite, 2019). The

implications of a changing climate, including reduced rainfall, increased drought and increased desertification in parts of the world (IPCC, 2021), are predictably an increase in the frequency and/or magnitude of dehydration that animals experience (Kearney et al., 2013; McKechnie, 2019; Rozen-Rechels et al., 2019; Riddell et al., 2021). Sub-lethal dehydration can cause a multitude of negative physiological effects that reduce individual health (Bouby and Fernandes, 2003), alter cognition (Wilson and Morley, 2003), reduce activity (Davis and DeNardo, 2009; Kearney et al., 2018), affect thermoregulatory behavior (Lorenzon et al., 1999; Ladyman and Bradshaw, 2003), impair locomotion (Moore and Gatten, 1989) and lower reproductive fitness (Dupoué et al., 2020; Bourne et al., 2021). The response of animals to dehydration involves multiple physiological and behavioral adjustments (selected reviews and examples: Schmidt-Nielsen, 1964, 1983; Shoemaker and Nagy, 1977; Costa, 1995; Silanikove, 1994; McCormick and Bradshaw, 2006).

Recent studies investigating the sub-lethal effects of dehydration on innate immune performance (see Glossary) in reptiles have found a positive correlation between immune response (see Glossary) and plasma osmolality (Moeller et al., 2013, 2017; Bruschi et al., 2017; Bruschi and DeNardo, 2017; Bruschi et al., 2019, 2020). The mechanisms behind this relationship are unknown, but the observed increase in innate immune performance with dehydration has been hypothesized to be an adaptive trait that evolved to prevent infection when individuals are in a vulnerable state (Moeller et al., 2013). However, the majority of research on this topic has been limited to a small number of species inhabiting arid environments (but see Bruschi et al., 2019, 2020), and the generality of this phenomenon requires additional study. Laboratory investigations of water balance in reptiles have found variations in performance correlate with habitat (Gans et al., 1968; Krakauer et al., 1968; Dmi'el, 1972; Elick and Sealander, 1972). Therefore, comparative studies of populations inhabiting differing environments might provide further insights into the generality of dehydration-enhanced innate immune performance in reptiles, although the limitations of inferring adaptation from two-population studies are well understood (Garland and Adolph, 1994). In addition to the nervous and endocrine systems, the immune system is a mechanism for communication within an organism and proper immune responses are vital for fending off pathogens and survival (Zimmerman, 2020). And the immune system is heavily involved in the repair and maintenance of organisms. A better understanding of the relationship between hydration state and enhanced innate immune performance will improve our ability to assess the impacts of global climate change on reptile populations. Innate immune performance indicates the ability of an organism to resist infection by a pathogen and involves a variety of resistance mechanisms that recognize a pathogen and mount a response.

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Innate immunity is present in all individuals at all times and does not improve with repeated exposure or change based on the type of pathogen.

Investigations of innate immune performance benefit from considering the interaction between the immune response and the endocrine stress response (see Glossary) which have been energetically linked for decades (Lochmiller and Deerenberg, 2000). The primary stress response involves physiological and behavioral changes initiated by the activation of two different systems that together work to mediate a coordinated response: the sympathetic nervous system, which releases catecholamines, and the hypothalamus–pituitary–adrenal (HPA) axis, which results in the release of glucocorticoid hormones (Sapolsky et al., 2000). Historically, a simplified model of the stress response has been presented that describes short-term increases in hormones as an adaptive response, while chronic increases in catecholamines and especially glucocorticoids were interpreted as maladaptive and indicative of an individual's poor health and inability to appropriately respond to a stressor. However, this generalized description of a stress response is an over-simplification and ignores the complex effects of stress and glucocorticoids on physiology.

For the purposes of this study, we consider a stressor to simply be a perturbation that disrupts or disturbs an animal's homeostatic balance (Sapolsky et al., 2000; Romero and Wingfield, 2015). Therefore, the primary function of a stress response is to allow individuals to ameliorate the effects of a stressor (Sapolsky et al., 2000; Wingfield, 2005; Romero et al., 2009; Wingfield et al., 2011), and the exact role of the endocrine system in responding to the challenge of dehydration remains unclear in reptiles (Bentley, 2002; McCormick and Bradshaw, 2006; Bruschi et al., 2020; but see Bradshaw, 1997).

Here, we investigated the release of glucocorticoids in response to dehydration and physical disturbance. Dehydration alters osmotic balance and can be considered a stressor, although the response to and tolerance of dehydration varies by species and can be affected by several factors such as life history (Perotti et al., 2011; Strachan et al., 2015), diet (Sabat and Martinez del Rio, 2002; Sabat et al., 2009), reproductive status (Brusch et al., 2020), age (Wilson et al., 2001; Weldon et al., 2016), environmental conditions (Dmi'el 1998; Lillywhite et al., 2015; Dupoué et al., 2017) and presence of additional stressors (Velasco et al., 2019; Rozen-Rechels et al., 2019).

Investigation of the acute stress response (see Glossary) in reptiles has primarily focused on measuring the rapid release of the glucocorticoid corticosterone when a stressor is encountered (McEwen and Wingfield, 2003; Romero et al., 2009). The magnitude of a stress response is often characterized by changes in the level of corticosterone when resting versus acute levels in response to a stressor. And while it is difficult to directly measure resting levels of corticosterone in an individual because of the disturbance caused by the collection of a sample (Romero and Reed, 2005), the initial measurement of corticosterone, taken as quickly as possible upon encountering an animal in the field, is often referred to as the 'baseline sample'. Dehydration is expected to increase baseline corticosterone in response to osmotic stress and the presumed mineralocorticoid role of corticosterone in maintaining water balance (de Kloet et al., 2000; Bentley, 2002; McCormick and Bradshaw, 2006; Bruschi et al., 2020). Corticosterone has been shown to respond to osmotic challenge in vertebrates and plays a role in both ion and water transport via mechanisms regulating salt retention and  $\text{Na}^+$  reabsorption (McCormick and Bradshaw, 2006). It is expected that dehydrated individuals will have higher

maximum acute values of corticosterone because of already elevated baseline levels. However, those species that can tolerate moderate levels of dehydration might not induce a stress response but rather stimulate physiological defense mechanisms (Stahlschmidt et al., 2011). Investigations of stress using measures of corticosterone often report results to be context dependent (e.g. Sandfoss et al., 2020). Studies of dehydration and values of corticosterone have found an association between baseline values, but not for measures of corticosterone during acute stress responses, and this trend appears to hold true across many vertebrate species (e.g. Cain and Lien, 1985; Parker et al., 2004; Hogan et al., 2007; Giorgi Barsotti et al., 2019; Dezetter et al., 2021), but not all (e.g. Dupoué et al., 2014; Moeller et al., 2017). If circulating corticosterone levels increase in response to dehydration, it might provide an explanation for the enhanced innate immune performance observed in dehydrated individuals of some reptile species (Moeller et al., 2013, 2017; Bruschi et al., 2017, 2019, 2020; Bruschi and DeNardo, 2017). It is generally thought that elevated levels of corticosterone have initial enhancing effects on innate immune performance, but, if prolonged, can depress immune responses (Dhabhar, 2014).

If enhanced innate immune performance in response to dehydration is an adaptive trait, then we might predict reptile populations living in water-limited environments to show a greater immune response relative to animals inhabiting mesic environments with constant access to fresh water. However, if dehydration-enhanced innate immune performance is a wide-spread trait present in all reptiles, we would expect similar responses in populations regardless of environmental conditions. Florida cottonmouth snakes (*Agkistrodon conanti* Gloyd 1969, Serpentes: Viperidae) are characterized by a close association with mesic freshwater habitats and are considered a more aquatic species of the genus *Agkistrodon*. However, atypical populations can be found inhabiting continental islands in the Gulf of Mexico, where they are entirely terrestrial and lack access to permanent sources of fresh water (Wharton, 1969; Lillywhite and Sheehy, 2019; Sandfoss and Lillywhite, 2019). Precipitation can be patchy or intermittent (Fig. S1), and even after significant rainfall events there are no significant pools of standing fresh water, leaving snakes with the opportunity to drink only from small, natural depressions such as cupped leaves and tree roots or ingesting water from their own body coils (Lillywhite and Sheehy, 2019). Field investigations of hydration status have found insular cottonmouths experience dehydration at a much greater rate than mainland cottonmouths (Sandfoss and Lillywhite, 2019). Insular populations of cottonmouths that persist in this fresh water-limited environment may provide insight into the important features required for adaptation to arid environments, and potentially respond to dehydration in a contrasting manner to cottonmouth snakes from mainland populations with access to permanent sources of fresh water.

Our objectives here were to: (1) characterize how the state of hydration affects the endocrine stress response and innate immune performance of Florida cottonmouth snakes and (2) determine whether the availability of fresh water in the environment alters the direction or magnitude of this relationship by studying individuals from contrasting environments – an insular population that is fresh water limited and a mainland population inhabiting a mesic environment. Furthermore, to investigate how differences in seasonal changes in water availability and frequency of dehydration affect free-ranging cottonmouths, we supplemented laboratory experiments with sampling of snakes *in situ* throughout the active season.

## Glossary

### Endocrine stress response

The endocrine stress response is part of a complex physiological and behavioral response to a stressor and is initiated by the activation of two different systems that work together to mediate a coordinated response: the sympathetic nervous system, which releases catecholamines, and the hypothalamus–pituitary–adrenal (HPA) axis, which results in the release of glucocorticoid hormones. These pathways are present across vertebrates but there is substantial temporal and species variation in the regulation of these pathways. Consequently, even though the anatomy of the stress response is highly conserved, the activation and functional output is not highly conserved and individual and population-level stress responses can be context dependent.

### Acute stress response

As part of the acute stress response, the glucocorticoid corticosterone is released with the aim of ameliorating the effects of disturbance. Corticosterone has been frequently used in studies of stress to characterize the stress response of an animal.

### Immune response

The immune system works to protect organisms from disease. An immune response involves a complex system of structures and mechanisms that have evolved to protect organisms from disease. The function of these components is divided up into non-specific mechanisms, which are innate to an organism, and adaptive responses, which are responsive to specific pathogens.

### Innate immune performance

Innate immunity is the first line of defense against pathogens. Innate immune defenses include physical barriers and cells that provide a non-specific response against invading pathogens. Innate immune responses are rapid and require no previous exposure to a pathogen. Reptiles have a well-developed, broad innate immune response thought to be the primary method of protection from pathogens.

## MATERIALS AND METHODS

### Animals and study location

All experimental methods and procedures were approved by the University of Florida's Institutional Animal Care and Use Committee (study #201809079). Animals were captured, handled and housed according to Florida venomous reptile license #411-133587 and US Fish and Wildlife Service federal special use permit #41511-14-10.

The two study populations consisted of a mainland population at Paynes Prairie (Alachua County, FL, USA) and an insular population of cottonmouths on Snake Key (Levy County, FL, USA). Snake Key is a 15 ha continental island that lies ~6 km from mainland Florida. The habitat of the island consists primarily of mixed upland hardwood hammock and extensive mangrove stands, surrounded by brackish water containing sea grass, sand flats and oyster beds. 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 ~3500–4500 years (Wright et al., 2005). Paynes Prairie preserve is an 8500 ha state park in Florida composed of mixed lowland habitats. Snakes were captured in the prairie basin, which floods seasonally and is never completely dry. Paynes Prairie is situated in the center of peninsular Florida and is located ~100 km northeast of the coast and Snake Key.

### Field hydration state

Between January and September 2019, 26 cottonmouths (Paynes Prairie,  $n=14$ , 6 males, 8 females, body mass range 125–790 g) and Snake Key ( $n=12$ , 5 males, 6 females, 1 undetermined, body mass range 235–1497 g) were opportunistically captured following sunset and a blood sample was collected at the initial time of capture ('baseline'). In a subset of 18 snakes (Snake Key  $n=8$ ,

Paynes Prairie  $n=10$ ), a standardized stress test was then conducted in the field that consisted of confinement in an opaque 5 gallon (~19 l) bucket for 60 min (Holding et al., 2014; Sandfoss et al., 2020), at which time a second or 'acute stress' blood sample was collected.

Blood collection involved the restraint of animals in a clear plastic tube while a ~1 ml blood sample was drawn from the caudal vein using a 25-gauge heparinized needle. Time to collection and time of day at each blood collection were recorded, and the body temperature of individuals was measured using a temperature probe (14-649-81, Traceable Products™, Webster, TX, USA) inserted into the cloaca. Blood samples were kept cool (~4°C) until transported back to the laboratory (<4 h) where they were centrifuged (3000 g for 10 min) and plasma was stored at –80°C until analyses were performed.

Cottonmouths were transported back to the laboratory at the University of Florida and mass and snout–vent length (SVL) were measured. State of hydration at capture was determined in two ways: blood plasma osmolality (in mosmol kg<sup>-1</sup>) and relative change in mass following 24 h with access to fresh water. Osmolality was measured in duplicate from a 10 µl sample of plasma using an osmometer (Vapro2 osmometer, Elitech group). Relative change in mass of cottonmouths between initial mass and mass following access to water was also used as a measure of hydration state. Snakes that gained >1% in relative mass were determined to have drunk, while those individuals that did not gain >1% relative mass were conservatively judged not to have drunk. Those individuals that defecated urates or feces without still gaining >1% in relative mass were excluded from analyses (Snake Key  $n=1$ , Paynes Prairie  $n=2$ ). An increase of 1% in relative mass is sufficient to confirm drinking as water is the only manner in which snakes can gain mass in captivity and snakes that soak themselves in water dishes are unable to gain >1% in relative mass (see methods of Sandfoss and Lillywhite, 2019). Drinking is a reliable physiological response to dehydration (Rash and Lillywhite, 2019). Following drinking trials, all animals were released at their original site of capture.

### Laboratory dehydration and stress test

Between March and August of 2019, we captured 17 non-gravid cottonmouths from Snake Key (5 males, 3 females, mean±s.d. mass 812.1±339.9 g, range 402–1497 g) and Paynes Prairie (4 males, 5 females, mass 606.8±225.1 g, range 362–989 g) and transported individuals back to the laboratory at the University of Florida. Wild-caught cottonmouths were acclimated to the laboratory for at least 30 days (mean±s.d. 91±35 g) prior to the start of the dehydration experiment. Snakes were housed individually in Plexiglas enclosures with access to water *ad libitum* and were fed a fish meal once a week (Spanish Sardines, Raffield Fisheries Inc.) until experimental trials began in September 2019. Animals were fasted for 2 weeks prior to drinking trials to avoid changes in mass due to digestion and defecation. The temperature and humidity of rooms for housing and experimental trials were recorded daily and maintained between 23 and 25°C with a relative humidity of 40–60%. The health of snakes in captivity was monitored via body condition, which was calculated as an index (BCI) of standardized residuals from a linear regression of mass and SVL. Only snakes in relatively good body condition (BCI>–0.10) (Brusch and Denardo, 2017) were used for experimental trials (Paynes Prairie  $n=9$ , mean±s.d. BCI=–0.003±0.06; Snake Key  $n=8$ , BCI=0.010±0.07).

To directly test the effects of dehydration on innate immune performance and corticosterone values in cottonmouths, we



experimentally manipulated the hydration state of individuals from both populations and collected blood samples before and after a stress test. The stress test was modified from Moeller et al. (2017) and consisted of confining an individual in a 5 gallon (~19 l) plastic bucket and gently tapping them on the top of the snout with a rubber stopper once every 60 s over the course of a 60 min period. We measured innate immune performance and stress response of cottonmouths from both populations at three successive hydration states: (1) hydrated, (2) dehydrated and (3) rehydrated. Blood samples were collected from individuals at each of the three treatment hydration levels at the start (baseline) and end (acute) of stress tests and samples were analyzed for corticosterone levels and osmolality of plasma. Innate immune performance was only measured from samples collected at baseline.

Hydration state was determined via changes in body mass and was supported by measures of osmolality of blood plasma samples (Fig. 1). Fasted snakes were first tested in a hydrated state, which followed >30 days of free access to fresh water. To dehydrate snakes, water bowls were removed from cages and snakes were allowed to slowly dehydrate at room temperature until they experienced a -20% change in body mass from the hydrated state. This level of dehydration is sub-lethal (H.B.L., unpublished data) but well beyond the thirst threshold of cottonmouths (-5.5%; Sandfoss and Lillywhite, 2019). No snakes showed clinical signs of extreme dehydration during trials (e.g. lethargy, loss of righting response). The mass of each snake was measured every 1–3 days and until individuals reached the set state of dehydration. Twenty-four hours after it was determined that an individual had reached a dehydrated state, a baseline blood sample was collected and a stress test was performed with an acute blood sample being collected at the termination of the stress test, as described above. All stress tests and blood collections were performed between 09:00 h and 13:00 h at a room temperature of 24–25°C. Following the stress test at the dehydrated state, water bowls were returned to the cages and snakes were allowed 48 h to

rehydrate before a final stress test and blood collection were conducted at the rehydrated state. Rehydration was confirmed by an increase in mass of >1% relative to body mass at the dehydrated state.

### Collection of blood samples

Approximately 1 ml of blood was taken from the caudal vein of snakes using a heparinized 1 ml syringe with a 25-gauge needle. Blood samples were collected in lithium heparinized tubes and stored at 4°C until processing (<24 h). Samples were centrifuged within 120 min of collection at 3000 g for 10 min to separate plasma from blood cells and aliquots were stored at -80°C until analysis.

### Innate immune assay

We assessed innate immune response using a modified version of the *in vitro* assay developed by Matson et al. (2005). The assay provides general information regarding performance of the innate immune response, which acts as the first line of defense in reptiles and is considered the most ecologically relevant measure of immune response (Moeller et al., 2013; Rios and Zimmerman, 2015; Zimmerman, 2020). The assay was developed to measure non-cellular innate compounds involved in both agglutination and lysis of foreign red blood cells. We modified the Matson et al. (2005) protocol by adding 20 µl of 2% sheep blood (#SBH100, Hemostat Laboratories, Dixon, CA, USA) suspension to serially diluted plasma samples (1:1 to 1:1024, 20 µl in each well) plated in duplicate. Hemolysin was added to half of the final row of wells on the plate as a positive control. Plates were incubated for 90 min at 22°C, which is within the active body temperature range of cottonmouths (H.B.L., unpublished data). The incubation periods for agglutination and lysis and scoring procedures were as in Matson et al. (2005). Dilution experiments have verified that any elevated immune scores found during dehydration are not the result of increased immune protein concentration (Moeller et al., 2013).

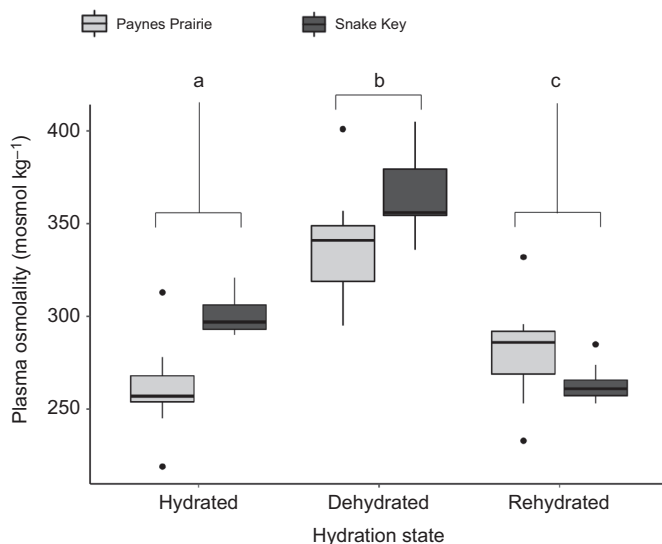
### Corticosterone assay and measurement of plasma osmolality

Plasma corticosterone was determined by radioimmunoassay, as described in Lormée et al. (2003). The minimum detectable corticosterone concentration was 0.28 ng ml<sup>-1</sup>, and the intra- and inter-assay coefficients of variation were 9.96% and 15.36%, respectively (samples were assayed in duplicate, in three assays). Baseline and acute stress samples from the same individuals within treatment were always run in the same assays, while treatments were randomly distributed between assays.

Plasma osmolality (mosmol kg<sup>-1</sup>) was measured from 10 µl aliquots on a Vapro2 osmometer (Elitech group). The machine was calibrated before each session of assays (two sessions in total) and no significant deviation was detected using standards (290 mosmol kg<sup>-1</sup>) within measurement sessions. These 17 runs with standards (290 mosmol kg<sup>-1</sup>) yielded a (mean±s.d.) measurement error of 2.0±1.2%.

### Statistical analyses

Analysis of variance (ANOVA) tests were used to investigate the effects of plasma osmolality, population and their interaction on field measures of corticosterone (baseline, acute, change in corticosterone) and innate immune performance. Covariates of sex, body mass, time to blood collection, time of day of blood collection, Julian date of field sampling, and body temperature during blood sampling were individually added to models



**Fig. 1. Plasma osmolality of cottonmouth snakes from the two locations under different hydration states.** Boxplot (median, upper and lower quartiles and 1.5× interquartile range) of plasma osmolality measured in baseline blood samples collected across three hydration states during laboratory trials in cottonmouths from Paynes Prairie ( $n=11$ ) and Snake Key ( $n=9$ ). Different letters represent significant differences between hydration state at an alpha level of 0.05.

and the AICcmodavg package (<https://CRAN.R-project.org/package=AICcmodavg>) was used to select the model with the lowest AICc value. If the two lowest scoring models had AICc values within 1 point, then the model with the fewest number of terms was selected.

We used linear mixed models with the R package lme4 (Bates et al., 2015) to test for effects of plasma osmolality, sex, time to blood collection, acclimation period (number of days), body mass, population and the interaction of osmolality and population on laboratory measures of corticosterone (baseline, acute and change from baseline to acute) and innate immune performance. Individual snake was included as a random factor. Correlation of fixed effects for each model was calculated using the r2glmm package (Nakagawa and Schielzeth, 2013). Data were inspected for heterogeneity and heteroskedasticity prior to analyses. Statistical significance was set at an alpha level of 0.05. All statistical tests were performed using R (v.3.6.0).

## RESULTS

### Field experiment

Over a sampling period of 195 days, we measured plasma osmolality of 26 cottonmouths in the field from Snake Key and Paynes Prairie (Fig. S1). The average value of plasma osmolality for free-ranging individuals sampled on Snake Key ( $n=12$ , mean $\pm$ s.d.  $344.1\pm 42.0$  mosmol  $\text{kg}^{-1}$ ) was significantly higher (Student's  $t$ -test,  $t=-2.67$ , d.f.=14.5,  $P=0.028$ ) relative to that of snakes sampled in the field at Paynes Prairie ( $n=14$ ,  $309.1\pm 18.2$  mosmol  $\text{kg}^{-1}$ ). Of the 26 individuals captured in the field, 12 snakes (Paynes Prairie  $n=5$ , Snake Key  $n=7$ ) were brought back to the laboratory and a drinking trial was conducted. Six of the seven cottonmouths captured on Snake Key drank water during trials, while none of the snakes captured on Paynes Prairie drank. The plasma osmolality of snakes that drank during drinking trials ( $n=6$ ,  $366.5\pm 43.9$  mosmol  $\text{kg}^{-1}$ ) was significantly higher (Student's  $t$ -test,  $t=-2.34$ , d.f.=8.5,  $P=0.045$ ) than that of cottonmouths that did not drink ( $n=6$ ,  $317.5\pm 27.5$  mosmol  $\text{kg}^{-1}$ ).

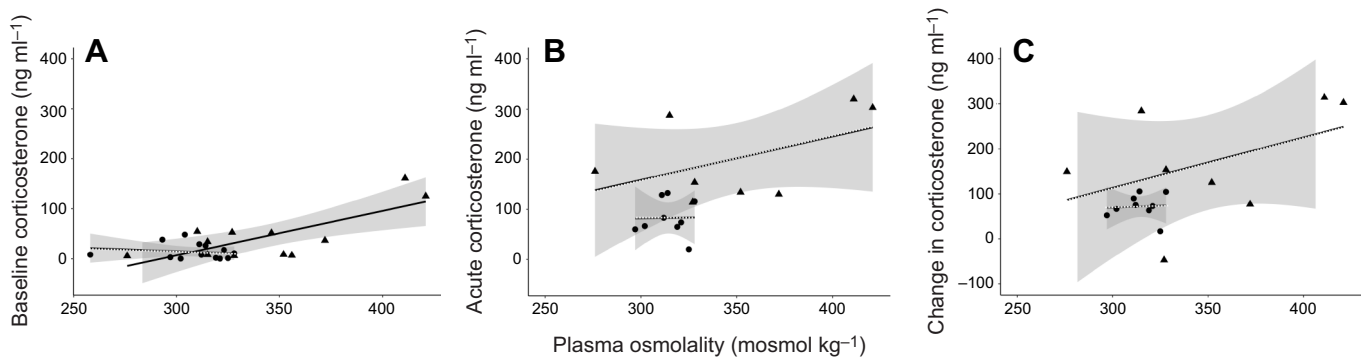
The model with the lowest AICc value for field measures of baseline corticosterone (Table S1) found a significant, positive effect of plasma osmolality ( $F_{1,12}=28.17$ ,  $P<0.001$ ) and identified a significant interactive effect of population and plasma osmolality ( $F_{1,12}=6.21$ ,  $P=0.028$ ) (Fig. 2A). Covariates of body mass

( $F_{1,12}=1.75$ ,  $P=0.210$ ) and time to blood collection ( $F_{1,12}=0.11$ ,  $P=0.743$ ) were included in the model of best fit but were non-significant (Table S1). An ANOVA for field measures of acute corticosterone identified significant effects of plasma osmolality ( $F_{1,10}=19.08$ ,  $P=0.001$ ) (Fig. 2B), population ( $F_{1,10}=8.42$ ,  $P=0.016$ ) and body temperature ( $F_{1,10}=10.93$ ,  $P=0.008$ ), and the lowest AICc model (Table S1) also included non-significant effects of sex ( $F_{1,10}=1.30$ ,  $P=0.282$ ) and the interaction between plasma osmolality and population ( $F_{1,10}=0.74$ ,  $P=0.409$ ). We found that the change in corticosterone values from baseline to acute samples was significantly greater in individuals from Snake Key than in those from Paynes Prairie ( $F_{1,9}=17.99$ ,  $P=0.002$ ) (Fig. 2C), and was significantly affected by time to baseline blood draw ( $F_{1,9}=12.37$ ,  $P=0.007$ ), Julian date of capture ( $F_{1,9}=14.16$ ,  $P=0.004$ ), sex ( $F_{1,9}=5.43$ ,  $P=0.045$ ) and plasma osmolality ( $F_{1,9}=41.94$ ,  $P<0.001$ ), but the interaction between population and plasma osmolality was not statistically significant ( $F_{1,9}=0.70$ ,  $P=0.424$ ).

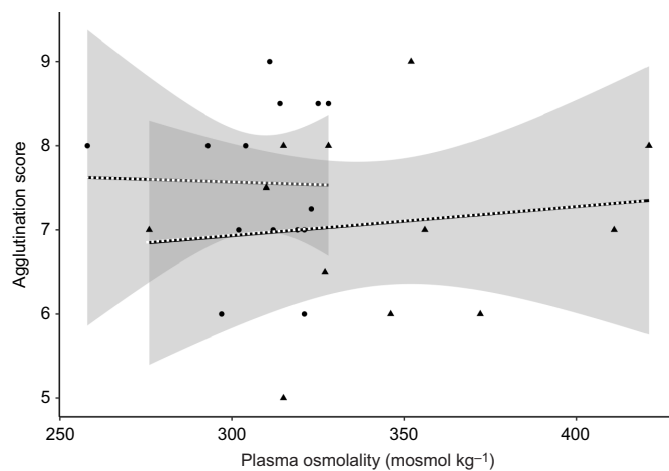
An ANOVA of agglutination scores from baseline blood samples collected from cottonmouths in the field found no effect of plasma osmolality ( $F_{1,15}=0.35$ ,  $P=0.565$ ), population ( $F_{1,15}=2.97$ ,  $P=0.105$ ), or their interaction ( $F_{1,15}=0.77$ ,  $P=0.394$ ) (Fig. 3), and the final model also included body temperature ( $F_{1,15}=0.27$ ,  $P=0.610$ ) (Table S1). Lytic activity was not observed during innate immune assays and may be the result of weak complement activity in this species (Matson et al., 2012; Sandfoss et al., 2020).

### Laboratory experiment

Individuals from Paynes Prairie were dehydrated to a mass of  $-23.7\pm 2.1\%$  relative to their hydrated mass in  $22.6\pm 3.7$  days and cottonmouths from Snake Key were dehydrated to  $-21.1\pm 0.7\%$  over the course of  $41.4\pm 10.4$  days. Time required for dehydration was affected by body mass ( $F_{1,13}=5.75$ ,  $P=0.032$ ), population ( $F_{1,13}=61.88$ ,  $P<0.001$ ), and their interaction ( $F_{1,13}=17.39$ ,  $P=0.001$ ) (Fig. S2). Following rehydration, snakes from Paynes Prairie recovered to an average of  $93.3\pm 9.0\%$  of their hydrated mass and snakes from Snake Key recovered to  $91.6\pm 3.6\%$ , which did not differ significantly between populations (Student's  $t$ -test,  $t=0.78$ , d.f.=14.3,  $P=0.447$ ). Plasma osmolality, however, differed significantly across the three experimental hydration states ( $F_{2,47}=39.71$ ,  $P<0.001$ ) (Fig. 1). Change in mass during



**Fig. 2. Plasma osmolality and corticosterone levels of cottonmouths in the field.** Scatterplot of the relationship between plasma osmolality and (A) baseline corticosterone (Paynes Prairie,  $n=14$ , 6 males, 8 females:  $r^2=0.03$ ,  $F_{1,12}=0.3$ ,  $P=0.571$ ; Snake Key,  $n=12$ , 5 males, 6 females, 1 undetermined:  $r^2=0.55$ ,  $F_{1,10}=12.5$ ,  $P=0.005$ ), (B) acute corticosterone (Paynes Prairie,  $n=9$ , 5 males, 4 females:  $r^2<0.01$ ,  $F_{1,7}=0.1$ ,  $P=0.961$ ; Snake Key,  $n=8$ , 5 males, 2 females, 1 undetermined:  $r^2=0.25$ ,  $F_{1,6}=1.9$ ,  $P=0.212$ ) and (C) change in corticosterone from baseline to acute stress blood samples (Paynes Prairie,  $n=9$ , 5 males, 4 females:  $r^2<0.01$ ,  $F_{1,7}=0.03$ ,  $P=0.860$ ; Snake Key,  $n=8$ , 5 males, 2 females, 1 undetermined:  $r^2=0.19$ ,  $F_{1,6}=1.4$ ,  $P=0.276$ ). Statistically significant regression lines are solid, non-significant regression lines are dashed and shading represents the 95% confidence interval (CI). Cottonmouths from Paynes Prairie are represented with circles and those from Snake Key with triangles.



**Fig. 3. Plasma osmolality and agglutination of cottonmouths in the field.** Scatterplot of the relationship between plasma osmolality and baseline agglutination scores (Paynes Prairie,  $n=14$ , 6 males, 8 females:  $r^2<0.01$ ,  $F_{1,12}=0.01$ ,  $P=0.932$ ; Snake Key,  $n=12$ , 5 males, 6 females, 1 undetermined:  $r^2=0.02$ ,  $F_{1,10}=0.2$ ,  $P=0.682$ ). Non-significant regression lines are dashed and shading represents the 95% CI. Cottonmouths from Paynes Prairie are represented with circles and those from Snake Key with triangles.

dehydration was highly correlated with plasma osmolality values ( $F_{1,49}=29.68$ ,  $r^2=0.38$ ,  $P<0.001$ ) (Fig. S3).

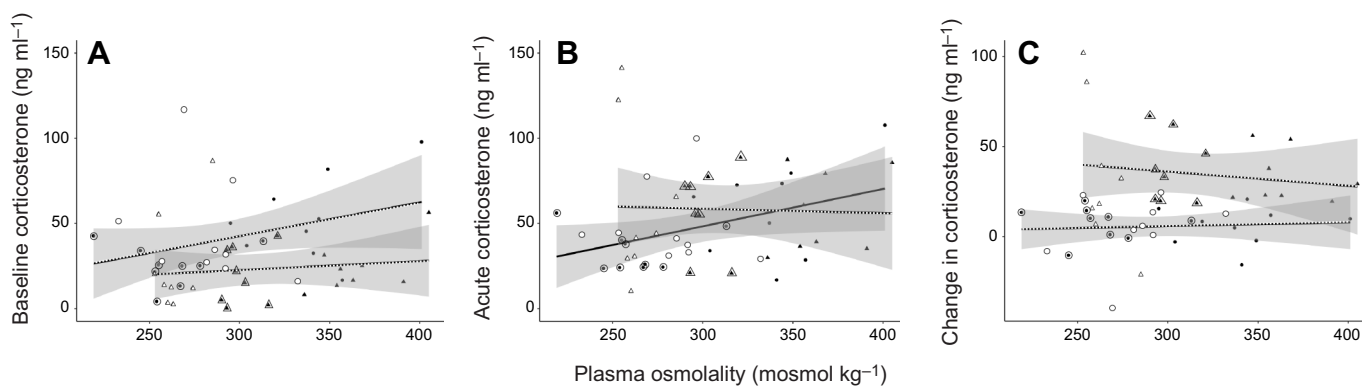
The results of the mixed model for baseline corticosterone found increased plasma osmolality ( $F_{1,33}=4.77$ ,  $P=0.036$ ,  $R^2_{GLMM}=0.126$ ) to lead to a significant increase in baseline corticosterone values during the laboratory experiment (Fig. 4A, Table 1). There were no other strong effects identified by the model for baseline corticosterone (Table 1). We tested for the same effects, minus time to blood collection, on measures of acute corticosterone in the laboratory and found significant differences between populations ( $F_{1,38}=4.79$ ,  $P=0.035$ ,  $R^2_{GLMM}=0.111$ ) and the interaction between population and plasma osmolality ( $F_{1,33}=5.07$ ,  $P=0.031$ ,  $R^2_{GLMM}=0.132$ ), while plasma osmolality alone was marginally significant ( $F_{1,33}=3.17$ ,  $P=0.084$ ,  $R^2_{GLMM}=0.087$ ) (Fig. 4B, Table 1). Next, we tested for effects on the net change in values from baseline to acute samples and found no significant results, while the length of acclimation time prior to the laboratory

experiment marginally increased corticosterone values ( $F_{1,12}=4.29$ ,  $P=0.061$ ,  $R^2_{GLMM}=0.267$ ) (Fig. 4C, Table 1).

An ANOVA for innate immune performance found a significant positive effect of plasma osmolality on agglutination values during laboratory dehydration ( $F_{1,33}=13.94$ ,  $P<0.001$ ), but no significant difference was found between populations ( $F_{1,33}=0.01$ ,  $P=0.928$ ), based on sex ( $F_{1,13}=0.02$ ,  $P=0.886$ ) or body mass ( $F_{1,13}<0.001$ ,  $P=0.928$ ). Baseline hydrated measures of innate immune performance improved as animals became dehydrated and this effect was lost following rehydration (Fig. 5).

## DISCUSSION

During laboratory trials, we successfully manipulated the hydration state of cottonmouths to directly test the response of corticosterone and innate immune performance to changes in plasma osmolality. Our results provide further evidence that sub-lethal dehydration can affect values of corticosterone and innate immune performance in reptiles. Dehydrated snakes showed an increase in baseline corticosterone which could be the result of osmotic stress and/or the role of corticosterone in osmoregulation (Duggan and Lofts, 1978; Bentley, 2002; McCormick and Bradshaw, 2006; Bruschi et al., 2020). Similar studies in other species of reptile have found contradicting results. Investigations of dehydration and reproduction in Children's python (*Antaresia childreni*: Bruschi et al., 2020) and the European adder (*Vipera berus*: Dezetter et al., 2021) found baseline corticosterone values to increase with water deprivation in agreement with our findings. In contrast, Dupoué et al. (2014) and Moeller et al. (2017) did not find baseline values of corticosterone to correlate with hydration status in *A. childreni* and *Heloderma suspectum*, respectively. Our study differs from theirs in methodology as we controlled for temperature in both field and laboratory analyses, which is an important factor for ectothermic animals, as snakes can alter thermoregulatory behavior based on hydration status (e.g. Ladyman and Bradshaw, 2003). In this study, we found body temperature of field animals to have a significant effect on measures of acute corticosterone. But this may not be sufficient to explain the difference in our findings. Additionally, Dupoué et al. (2014) dehydrated animals in the laboratory to a level of  $-10\%$  body mass and did not measure plasma osmolality. It is possible that the level of dehydration that animals experienced in their study may not have been sufficient to trigger a physiological



**Fig. 4. Plasma osmolality and corticosterone levels of cottonmouths in the laboratory.** Scatterplot of the relationship between plasma osmolality and (A) baseline corticosterone (Paynes Prairie:  $r^2=0.10$ ,  $F_{1,25}=2.92$ ,  $P=0.100$ ; Snake Key:  $r^2=0.01$ ,  $F_{1,22}=0.30$ ,  $P=0.591$ ), (B) acute corticosterone (Paynes Prairie:  $r^2=0.15$ ,  $F_{1,25}=4.45$ ,  $P=0.045$ ; Snake Key:  $r^2<0.01$ ,  $F_{1,22}=0.03$ ,  $P=0.864$ ) and (C) change in corticosterone values between baseline and acute samples (Paynes Prairie:  $r^2<0.01$ ,  $F_{1,25}=0.11$ ,  $P=0.744$ ; Snake Key:  $r^2=0.02$ ,  $F_{1,22}=0.42$ ,  $P=0.525$ ) measured in 17 cottonmouths from Snake Key ( $n=8$ , 5 males, 3 females) and Paynes Prairie ( $n=9$ , 4 males, 5 females). Cottonmouths from Paynes Prairie are represented with circles and those from Snake Key with triangles. Symbols that are outlined in black indicate snakes during the hydrated state, solid symbols indicate snakes during the dehydrated state, and open symbols represent rehydrated individuals. Non-significant regression lines are dashed and shading represents the 95% CI.

**Table 1. Summary table of results from linear mixed models on measures of baseline corticosterone, acute corticosterone and change in corticosterone values for cottonmouths from two populations sampled during laboratory dehydration and stress response experiments**

Model	Parameter	Estimate±s.d.	F	d.f.	P-value	R <sup>2</sup> GLMM
Baseline corticosterone	Sex	-3.75±5.2	0.516	13	0.485	0.038
	Body mass	0.03±0.0	2.23	12	0.161	0.159
	Time to blood collection	0.01±0.0	0.37	42	0.546	0.009
	Acclimation time	-0.18±0.2	1.28	12	0.28	0.098
	Population	-16.08±19.9	0.656	38	0.423	0.017
	Osmolality	0.13±0.1	4.77	33	0.036*	0.126
	Population×Osmolality	0.09±0.1	2.04	34	0.162	0.056
Acute corticosterone	Sex	-4.18±6.3	0.44	12	0.52	0.036
	Body mass	0.02±0.0	1.13	12	0.308	0.087
	Acclimation time	0.07±0.2	0.13	12	0.724	0.011
	Population	-46.94±21.5	4.79	39	0.035*	0.111
	Osmolality	0.12±0.1	3.17	33	0.084	0.087
	Population×Osmolality	0.15±0.1	5.07	33	0.031*	0.132
Change in corticosterone	Sex	-2.01±4.1	0.24	13	0.63	0.018
	Body mass	-0.002±0.0	0.02	12	0.88	0.002
	Time to blood collection	0.01±0.0	0.51	43	0.478	0.012
	Acclimation time	0.26±0.1	4.29	12	0.061	0.267
	Population	-22.06±17.8	1.53	38	0.224	0.039
	Osmolality	-0.02±0.1	0.2	34	0.655	0.006
	Population×Osmolality	0.03±0.1	0.3	35	0.586	0.009

\*Statistical significance.

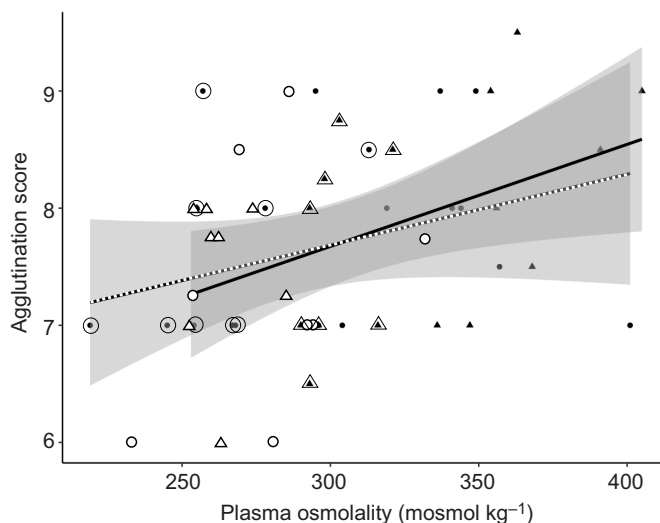
stress response and activate the HPA axis, resulting in increased levels of circulating corticosterone (Sapolsky et al., 2000; Wingfield et al., 2011).

Elevated values of baseline corticosterone have been associated with a marine lifestyle across several species of birds (Brischoux et al., 2015), but we found both insular and mainland populations to have similar values of baseline corticosterone in the laboratory, and snakes from both populations experienced an increase in baseline values when dehydrated. Field measures of baseline corticosterone

were also higher in individuals with higher values of plasma osmolality, particularly for cottonmouths sampled on Snake Key. While we found statistical differences at the population level for field measures of baseline corticosterone, this is probably due to the relatively low values of plasma osmolality observed in free-ranging cottonmouths sampled at Paynes Prairie (<329 mosmol kg<sup>-1</sup>). During the laboratory experiment, snakes from Paynes Prairie experienced a wider range of plasma osmolality (295–401 mosmol kg<sup>-1</sup>), which did result in a weak, positive relationship between plasma osmolality and baseline corticosterone values.

We found the influence of dehydration on field and laboratory measures of acute corticosterone to be significant but variable. Values of acute corticosterone measured in cottonmouths from Snake Key appeared to be weakly associated with plasma osmolality during both field and laboratory trials, whereas acute levels of corticosterone measured in cottonmouths from Paynes Prairie had a strong positive relationship with plasma osmolality for values measured during the laboratory but not field trials. This difference in population-level response is difficult to explain. Field measures of acute corticosterone in cottonmouths from Paynes Prairie were relatively constant across values of plasma osmolality, but sample size was limited, and the range of osmolality values observed in free-ranging mainland cottonmouths was small. Previous studies of Children's pythons (Dupoué et al., 2014) and Gila monsters (Moeller et al., 2017) found water-deprived individuals had higher values of acute corticosterone relative to hydrated individuals.

Elevated values of acute corticosterone that we observed in relation to dehydration in the laboratory appear to be the result of elevated baseline values of corticosterone, as increased dehydration did not cause a significant change in the magnitude of the stress response (delta change in corticosterone values between baseline and acute samples) for either population. This may be a strategy of all cottonmouths to avoid the negative consequences of increased corticosterone release, which can lead to further increases in energy expenditure and water loss, particularly when animals are already in a vulnerable state (Parker et al., 2003; Dupoué et al.,



**Fig. 5. Plasma osmolality and agglutination of cottonmouths in the laboratory.** Scatterplot of the relationship between plasma osmolality and agglutination scores (Paynes Prairie,  $n=9$ , 4 males, 5 females:  $r^2=0.08$ ,  $F_{1,25}=2.28$ ,  $P=0.144$ ; Snake Key,  $n=8$ , 5 males, 3 females:  $r^2=0.21$ ,  $F_{1,22}=5.81$ ,  $P=0.025$ ) measured across three hydration states. Cottonmouths from Paynes Prairie are represented with circles and those from Snake Key with triangles. Symbols that are outlined in black indicate snakes during the hydrated state, solid symbols indicate snakes during the dehydrated state, and open symbols represent rehydrated individuals. Non-significant regression lines are dashed and shading represents the 95% CI.



2018). Suppression of a greater magnitude stress response to avoid negative fitness costs has been observed in several species of birds (Wingfield and Hunt, 2002), and organisms that frequently experience osmotic stress during dehydration may suppress the stress response to maintain organismal fitness (Wingfield and Sapolsky, 2003). In this study, we found the opposite to be the case, as cottonmouths from Snake Key consistently had a larger magnitude stress response relative to individuals from Paynes Prairie regardless of hydration state.

We found our stress test to produce a significant increase in circulating corticosterone values in both field and laboratory animals. Interestingly, maximum acute corticosterone values measured in the field were significantly higher than values measured in snakes undergoing stress tests in the laboratory. This may be a function of several factors as our ANOVA model for field stress response of cottonmouths identified an array of significant terms including temperature, time of year and sex. In our study, individuals with longer acclimation periods in the laboratory had a more pronounced stress response. This agrees with our anecdotal observations that Florida cottonmouths appear more irritable with time in captivity (M.R.S. and H.B.L., personal observations). Laboratory settings are simplified environments and lack many of the abiotic and biotic conditions that snakes can experience in the wild, making it difficult to replicate stressors that are naturally present. Moeller et al. (2017) was not able to induce a significant increase in corticosterone values for hydrated *Gila* monsters in the laboratory despite a set stress protocol that induced a strong stress response in dehydrated individuals, rehydrated individuals and individuals in the field. Claunch et al. (2017) used various tests of disturbance in a laboratory setting on *Crotalus oreganus* and found no difference in values of baseline corticosterone for disturbed and undisturbed snakes. Here, we were clearly able to induce a consistent stress response in animals within both laboratory and field settings, but the field responses were of greater magnitude.

We found dehydration to enhance innate immune performance, in agreement with previous research in reptiles (Moeller et al., 2013; Bruschi and DeNardo, 2017; Bruschi et al., 2017, 2019). While two of these studies found changes in lysis ability rather than agglutination, we found agglutination to follow a similar pattern of enhanced innate immune performance to dehydration, whereby innate immune performance increased with dehydration and then returned to baseline levels within 48 h of rehydration. Despite the frequent exposure of cottonmouths on Snake Key to dehydrating conditions, we did not find cottonmouths inhabiting the island to show a greater magnitude or more prolonged immune response compared with cottonmouths from Paynes Prairie. The lack of differences in the immune response between the two populations suggests enhanced innate immune performance when dehydrated might be a conserved physiological trait in reptiles and not necessarily an evolved adaptive trait in organisms inhabiting water-limited habitats. Earlier studies have suggested dehydration-based increases in innate immune performance might have evolved to prime individuals that experience frequent dehydration and are in a physiologically stressful state to fight infections (Moeller et al., 2013, 2017). However, more recent studies investigating the phenomenon in snakes inhabiting mesic environments (Bruschi et al., 2019, 2020) have found the same pattern of dehydration-enhanced innate immune performance as in those species from arid environments, which combined with our results here, suggests alternative hypotheses should be considered. An important step towards determining the universality of this

phenomenon is to identify the as yet unknown underlying cellular mechanisms responsible. It is not clear why we did not find a relationship between innate immune performance and osmolality in cottonmouth snakes sampled in the field.

Interestingly, our results conflict with the dehydration–glucocorticoid–immunity cascade which presumes that a dehydration-induced elevation in plasma corticosterone will lead to a decrease in immune response. Physiological trade-offs are complex and often involve multiple physiological pathways and mechanisms that are not well understood and can be context dependent. Depression in innate immune performance due to increased glucocorticoids is often presented in the context of chronic stress (Dhabhar, 2014), and the lack of immune suppression in cottonmouths suggests that the magnitude and/or duration of dehydration that individuals experienced during laboratory trials did not replicate ‘sustained’ or ‘chronic stress’ to the degree that would cause a depression in immune response; however, this requires further investigation. Previous studies of insular cottonmouths found chronic stress, related to the loss of food resources (Sandfoss et al., 2018), did not increase baseline levels of corticosterone but did dampen the magnitude of the acute stress response and led to reduced innate immune performance (Sandfoss et al., 2020).

Observed measures of plasma osmolality of snakes from the field showed considerable variation for both populations. And the values of plasma osmolality we observed in free-ranging snakes were within the levels of dehydration achieved during laboratory dehydration trials, suggesting our experimental design provided an ecologically relevant test of response to dehydration in cottonmouths. Field data collected here, and in a previous study (see Sandfoss and Lillywhite, 2019), demonstrate that cottonmouths from Snake Key experience dehydration more frequently and to a higher degree than do individuals from Paynes Prairie. Cottonmouths sampled on Snake Key had, on average, higher values of plasma osmolality, and most individuals captured on the island drank while no cottonmouths from Paynes Prairie did. This difference in field hydration state is thought to be primarily driven by the reliance of insular snakes on rainfall to maintain water balance (Sandfoss and Lillywhite, 2019).

It is apparent that cottonmouth snakes from Paynes Prairie and Snake Key inhabit environments that differ in terms of freshwater availability, but we found only slight differences in their response to dehydration. Interestingly, during dehydration trials, we found individuals from Snake Key required twice as long without water to achieve our threshold for dehydration (80% relative hydrated body mass) compared with cottonmouths from Paynes Prairie. Water loss for reptiles primarily occurs via evaporative water loss across the surface of the skin, and results from a comparative study of evaporative water loss in the closely related species of *Agkistrodon contortrix* and *Agkistrodon piscivorus* suggest habitat influences rates of evaporative water loss (Moen et al., 2005). We did not directly measure evaporative water loss rates in this study; however, given the extreme difference in time to dehydration observed during laboratory trials, it might be suggested that individuals from Snake Key have developed behavioral and/or physiological mechanisms for resisting dehydration in contrast to snakes from Paynes Prairie. Despite any adaptations that cottonmouths from Snake Key may have developed to reduce rates of water loss, snakes from the two populations responded in a similar manner to sub-lethal dehydration once reached. Studies of free-ranging reptiles have found species inhabiting arid environments have developed strategies to avoid dehydration (King and Bradshaw, 2010), tolerate extremely high values of plasma osmolality (Peterson, 1996), or a combination of the two.

This study appears to be the first investigation of intraspecific differences in endocrine response and immune performance to sublethal dehydration in reptiles. We found dehydration to have a strong effect on both corticosterone values and immune performance, but the response of cottonmouths from our two study populations was not consistent across field and laboratory investigations. Differences between laboratory and field findings are thought to be the result of limited dehydration experienced by free-ranging cottonmouths from Paynes Prairie. Our findings add to a growing body of literature on the complex relationship between dehydration and values of corticosterone across vertebrate species (e.g. Cain and Lien, 1985; Parker et al., 2004; Hogan et al., 2007; Dupoué et al., 2014; Moeller et al., 2017; Giorgi Barsotti et al., 2019; Dezetter et al., 2021). Clearly, this topic requires further investigation across a wider range of species and habitats to improve our understanding of the interaction and trade-offs between hydration, innate immune performance and stress physiology, especially if we are to characterize the response of populations to anthropogenic climate change.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: M.R.S.; Methodology: M.R.S., H.B.L., F.B.; Validation: F.B.; Formal analysis: M.R.S., F.B.; Resources: M.R.S., F.B.; Writing - original draft: M.R.S.; Writing - review & editing: M.R.S., F.B., H.B.L.; Supervision: H.B.L.; Funding acquisition: M.R.S., F.B.

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#### Data availability

Data are publicly available from figshare: <https://doi.org/10.6084/m9.figshare.20419161>

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