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RESEARCH ARTICLE

When less means more: dehydration improves innate immunity in rattlesnakes

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ABSTRACT

Immune function can vary based on availability of resources, and most studies of such influences have focused on the co-investment of energy into immune and other physiological functions. When energy resources are limited, trade-offs exist, which can compromise immunity for other functions. As with energy, water limitation can also alter various physiological processes, yet water has received little consideration for its possible role in modulating immune functions. We examined the relationship between immunocompetence and hydration state using the western diamond-backed rattlesnake (*Crotalus atrox*). This species is known to undergo substantial seasonal fluctuations in water availability with extreme limitations during the hot-dry season. We collected blood samples from free-ranging *C. atrox* to compare osmolality and innate immune function (lysis, agglutination and bacterial growth inhibition) during the milder and relatively moister early spring season, the hot-dry season and the hot-wet season. To isolate effects of dehydration from other possible seasonal influences, we complemented this field study with a laboratory study in which we withheld food and water from individually housed adult *C. atrox* for up to 16 weeks. We collected blood samples from each snake as it dehydrated and collected a final sample after the snake was given water *ad libitum* at the end of the experiment. Our results demonstrate that *C. atrox* experience significant dehydration during the hot-dry season, and that, in general, innate immune function is highly correlated with osmolality, whether natural or artificially manipulated.

KEY WORDS: Hydration, Immune function, Osmotic stress, Immunocompetence, Water limitations

INTRODUCTION

The vertebrate immune system is a host defense that consists of numerous structural, biological and chemical components that are responsible for defending the host against a wide array of invasive pathogens. It can be roughly divided into one component that is innate and another that is adaptive (Murphy, 2011). The immune system is typically only fully activated when needed, suggesting that there are costs associated with an immune response (Hasselquist and Nilsson, 2012), including costs of production (Klasing and Leshchinsky, 1999), costs of maintenance (Råberg et al., 1998) and substantial costs associated with reacting to foreign pathogens (Klasing, 2004). In addition, there is evidence that immune defenses fluctuate throughout the year owing to changes in environmental cues, differing threats of disease over time, and balancing immune function with other physiological processes (Nelson et al., 2002).

Much of the current focus on dynamic immune function has examined the theory that immune defenses compete with other physiological functions (e.g. growth, reproduction) for energetic resources, creating associated life-history trade-offs. Stimulated immune responses have been shown to increase resting metabolic rates (Martin et al., 2003), decrease growth rates (Parmentier et al., 1996), lower reproductive success (Bonneaud et al., 2003) and reduce parental care (Råberg et al., 2000).

Although the vast majority of work on the dynamics of immune function has focused on trade-offs for energetic resources, immunocompetence can also be affected by non-energetic resources such as vitamins and carotenoids (Hartley and Kennedy, 2004). However, our understanding of which non-energetic mechanisms or resources can directly modulate the immune system and the magnitude of their effects on immunity is much less understood (Viney et al., 2005). Water is a fundamental, non-energetic resource that has received only limited consideration for its role in possibly modulating immune functions. Water is essential for life and is the main constituent of cells, tissues and organs (Lang and Waldegger, 1997). It can greatly influence the fitness of an organism because of its compulsory role as a solvent for other macronutrients (Jéquier and Constant, 2010), modulation of cell-to-cell signaling (Grandjean and Campbell, 2004) and capacity to maintain the function of all tissues and organs in an organism (Ritz and Berrut, 2005). Clearly, the importance of water balance to homeostasis in most vertebrates cannot be overstated; in some taxa, an absence of water can be lethal within days (Popkin et al., 2010).

Even before lethal limits of water deprivation are reached, dehydration can cause severe impairment of metabolism (Gerich et al., 1973), alter cognitive function (Wilson and Morley, 2003), damage an organism at the level of membranes (Potts, 1994; Prestrelski et al., 1993) and impair locomotor performance (Titon et al., 2010). Therefore, many animals maintain plasma osmolality within a small osmotic range (300 mOsm kg^{-1} ±5%), even during periods during which they do not drink (Ramsay and Thrasher, 1984). Juxtaposed to this, some animals adapted to living in xeric environments can tolerate extreme variation in osmolality [toads: 250–370 mOsm kg^{-1} (Johnson and Propper, 2000); tortoises: 290–400 mOsm kg^{-1} (Nagy and Medica, 1986); lizards: 280–350 mOsm kg^{-1} (Davis and DeNardo, 2009); and birds: 325–425 mOsm kg^{-1} (Williams et al., 1991)] and appear to function normally. However, impacts on immune function may be inconspicuous yet still significant to the survival of the organism.

Given the importance of water across physiological systems, it seems logical to expect that hydric state would have a considerable impact on immune function as well. In support of this, fruit flies (*Drosophila melanogaster*) with challenged immune systems did not survive desiccation for as long as flies with unchallenged immune

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systems (Hoang, 2001). However, as the flies were also subject to starvation during the trials, a direct link between immune challenge and desiccation resistance is confounded by energy limitation. Interestingly, in Gila monsters (*Heloderma suspectum*), a species that regularly goes for extended periods without food and naturally experiences dehydration during seasonal drought, aspects of innate immune function are enhanced when animals are dehydrated (Moeller et al., 2013). Clearly, it appears that water can influence immune function, but further work is needed to better understand this newly discovered and poorly explored relationship. The interaction between hydration state and immune function will be of increasing importance as we attempt to predict the impacts of global climate change that, in addition to warming average temperatures, is expected to alter rainfall patterns in many areas.

Accordingly, we examined the relationship between immunocompetence and hydration state using an abundant predator of the Sonoran Desert – the western diamond-backed rattlesnake (*Crotalus atrox*). As with the Gila monster, these rattlesnakes experience substantial seasonal fluctuations in water availability and regularly tolerate months-long periods without food. Because of these similarities between the two species, we hypothesized that (1) extended drought leads to a measurable yet well-tolerated increase in osmolality in this species and (2) increased osmolality within this tolerated range will result in improved immune function. We predicted that (1) rattlesnakes in the wild will show elevated plasma osmolality during the hot-dry season compared with other seasons and (2) elevated plasma osmolality, whether naturally in the wild or as a result of water deprivation in the laboratory, will proportionally enhance plasma-based assessments of innate immune function.

MATERIALS AND METHODS

Study animals

The western diamond-backed rattlesnake (*Crotalus atrox* Baird and Girard 1853) is a large-bodied (typical adult size in the Sonoran Desert: snout–vent length=700–1100 mm, mass=200–800 g; Taylor and DeNardo, 2005), desert-dwelling reptile that is an important predator of the Sonoran Desert. In central Arizona, this species is known to experience substantial seasonal (mid-May through mid-July) droughts when rainfall and standing water are typically non-existent. *Crotalus atrox* is considered a binge-feeding species, often surviving by consuming large meals at infrequent intervals. As *C. atrox* naturally can go months without feeding, this species is a very good candidate for studying the effects of dehydration on physiological performance without results being confounded by concerns of an effect due to starvation (see below for validation of this assumption).

Field-based experiment

To determine the extent to which *C. atrox* naturally dehydrate during the dry season and whether innate immune function differs across seasons, we collected blood samples (see details below) from a total of 60 individual *C. atrox* between March and August 2015 in and around Apache Junction, AZ, USA. We did not determine the sex of the snakes to reduce handling time; however, we did abdominally palpate all snakes to ensure no pregnant animals were included. All blood samples for the field study were collected between 19:00 and 21:00 h. To avoid sampling the same animals twice, snakes were marked on their back and rattle segments using a permanent marker. Twenty *C. atrox* were bled during the milder and relatively moister early spring season (March-April), 19 during the hot-dry season (mid-May through early July) and 21 during the hot-wet season (mid-July through August). For all samples, plasma osmolality was determined using a vapor pressure osmometer (±3 mOsm kg^{-1}; model 5600; Wescor Inc., Logan, UT, USA). Samples were run in triplicate as described in Davis and DeNardo (2009). Additionally, we performed a suite of plasma-based immune function assays on each of the samples (see details below).

Laboratory-based experiment

To better assess the effect of dehydration on immune function independent of other possible seasonal effects, we conducted a laboratory-based study on *C. atrox* where we serially manipulated the snake's hydration state and collected blood samples for determination of osmolality and assessments of immune function. Between October 2014 and May 2015, we collected 36 adult *C. atrox* from in and around Gold Canyon, AZ, USA, and transported them to Arizona State University, Tempe, AZ, USA, where they were housed within individual cages (51×40×13 cm) of a purposefully designed snake rack system (Freedom Breeder, Turlock, CA, USA) for at least 2 weeks to habituate to captivity. During this period, snakes were fed two adult mice, had free access to water and were provided with a thermal gradient (25 to 35°C) via a sub-surface heating element. Snakes that arrived in poor body condition or refused to eat in captivity were not used for this study. To quantify condition, we used a body condition index (BCI) based on standardized residuals from a linear regression using mass and SVL, and we excluded animals with low (<−1) BCI indices (n=4 or 11% of captured animals). Snakes used in the laboratory study were abdominally palpated to ensure they were not pregnant, and this was confirmed by the females not producing offspring while in our possession, which covered the normal birthing period. Pilot studies showed that *C. atrox* held in captivity allow their osmolality to fluctuate considerably (290–335 mOsm kg^{-1}) even when provided *ad libitum* access to water (G.A.B. and D.F.D., unpublished data). In order to begin the study with all animals at a similar, normosmotic state (290–305 mOsm kg^{-1}), we withheld food and water from all snakes for 8–10 days and then offered them water *ad libitum*. Twenty-four hours later, access to water was removed, and a blood sample was collected to determine plasma osmolality. Each snake that was within the target osmolality range (n=29) was moved to a 5-gallon bucket within an environmental chamber (males=15, females=14, SVL=688–996 mm, mass=380–599 g). As innate immune function is sensitive to metabolic rate (Tieleman et al., 2005) and the metabolic rate of *C. atrox* is correlated with temperature (Beaupre and Duvall, 1998), the chamber was held at 26.5°C to approximate the species' preferred temperature based on several field and laboratory studies (Beck, 1996; Pappas et al., 2004; Rubio, 1998; Taylor et al., 2004). Blood from this first bleed was also used for assessing immune function under hydrated conditions. Snakes that had osmolalities outside the desired range were not used for this study (n=3).

Snakes used in the study were randomly assigned to one of two treatment groups: 21 snakes had food and water withheld for 16 weeks, while eight snakes were given *ad libitum* access to water but no food for 16 weeks. The former group was used to serially evaluate immune function at different hydration states, while the latter group was used to confirm that up to 16 weeks of food deprivation did not alter immune function in this binge-feeding species. Snakes were again bled six (second bleed), 10 (third bleed) and 16 weeks (fourth bleed) after their first bleed. All blood samples for the laboratory study were collected between 08:00 and 12:00 h. At each time of each bleed, snakes were also weighed. After 16 weeks, the snakes were provided water *ad libitum* and a final blood sample was collected 72 h later (fifth bleed). Snakes (n=7)

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that showed clinical signs of dehydration (lethargy, slow righting reflex) or reached a maximum osmolality of 380 mOsm kg^{-1} before 16 weeks without water had their fourth blood sample taken at that time, were provided water, and then had their blood sampled a final time (fifth bleed) 3 days later. To meet the requirements of the statistical modeling, the data from these animals were not included in the analyses presented, but their results were consistent with those from the animals that had their fourth sample at 16 weeks.

Sample collection

A 0.7 ml blood sample was drawn primarily from the caudal vein using a heparinized 1 ml syringe. However, if we were unable to obtain sufficient blood from the caudal vein, we collected samples using cardiocentesis. After blood collection, the snake was either returned to its enclosure (laboratory studies) or released at the site of capture (field study). Total time for capture, restraint and collection was typically less than 5 min and did not exceed 10 min for both laboratory and field studies. Blood samples from the laboratory study were immediately centrifuged at 4000 ***g*** for 3 min to separate plasma from blood cells. Plasma osmolality was immediately measured and the remaining plasma was aliquoted (~50 µl) into separate vials and frozen at −80°C to be used later to evaluate immune function (see below). Blood samples from the field study were stored on ice for no more than 12 h before plasma was separated and stored at −80°C. All samples were used within 75 days of being frozen at −80°C.

Immune function assays

We examined the relationship between immunocompetence and hydration state using several plasma-based assays to assess innate immune function. While snakes possess both innate and adaptive components (Glinski and Buczek, 1999; van Hoek, 2014; Zimmerman et al., 2010), we focused on innate immunity as the lack of any established assay protocols limits the evaluation of the adaptive immune components of most reptiles, including rattlesnakes. Agglutination and lysis assays were used to evaluate the involvement of complement and native immunoglobulins (natural antibodies) in reacting to a novel antigen, sheep red blood cells (SRBC; SBH050, Hemostat Laboratories, Dixon, CA, USA), and thus serve as a standard measure of constitutive innate humoral immunity (Matson et al., 2005). Briefly, 20 µl of each plasma sample were serially diluted from 1:2 to 1:2048 with phosphate buffered saline (PBS) along a row of a 96-well plate. We then added 20 µl 1% SRBC to each well. Plasma was not added to the final column, where the first four wells contained only 20 µl PBS and 20 µl 1% SRBC to serve as a negative control (0% lysis) and the bottom four wells contained 20 µl ACK lysing buffer (Lonza, Basel, Switzerland) and 20 µl 1% SRBC to serve as a positive control (100% lysis). Plates were incubated at 26.5°C, the same temperature at which the snakes were maintained, for 90 min and then placed at room temperature (~25°C) for 20 min, after which point they were scanned at 600 dots per inch using a flat-bed scanner (Hewlett-Packard Co., ScanJet 3670) for agglutination images. Plates remained at room temperature for an additional 70 min and were then centrifuged for 5 min (700 ***g***, Sorvall, Newtown, CT, USA), after which the supernatant was aspirated into a clean 96-well plate. We then measured absorbance using a microplate reader (405 nm, BioTek Instruments, Winooski, VT, USA) to calculate lysis scores. Hemolytic-complement activity was expressed in CH_{50} units ml^{-1} plasma, where 1 CH_{50} unit equals the reciprocal of the dilution of plasma required to lyse 50% of the SRBC.

We also conducted bacterial killing assays (BKA) to determine the ability of *C. atrox* plasma to inhibit the growth of a microorganism (French and Neuman-Lee, 2012). For these assays, we used two species of gram-negative bacteria, *Escherichia coli* and *Salmonella enterica*, that are known pathogens of snakes (Jacobson, 2007) and thus provide ecological relevance. In brief, we combined 1:8 or 1:4 plasma dilution with CO_2-independent medium plus 4 nm L-glutamine, 10^5 colony-producing units of *E. coli* (lot 483-478-1, ATCC 8739, MicroBioLogics, St Cloud, MN, USA) or 10^6 colony-producing units of *S. enterica* (lot 501-13-1, ATCC 51741, MicroBioLogics), and agar broth on a 96-well microplate. We calculated absorbance using a microplate reader (300 nm, BioTek Instruments) immediately and after 12 h of incubation at 37°C. Percent bacterial growth inhibited was calculated as the mean number of colonies for each sample, which were run in triplicate, divided by the mean number of colonies for the positive control (triplicate wells containing only medium and bacteria), multiplied by 100. Together, these four assays provided a detailed, comparative assessment of how *C. atrox* plasma-based innate immune function responds to dehydration.

Plasma dilution experiment

Dilution experiments were conducted to verify that elevated immune scores during dehydration were not simply the result of increased immune protein concentration as a result of reduced blood volume when dehydrated. We randomly selected plasma sample sets from eight snakes in the laboratory study. We repeated all immune function assays using an aliquot from the first bleed, an aliquot from the fourth bleed, and a fourth bleed aliquot that we diluted with Nanopure water (2.0 to 5.4 µl) to reduce its osmolality to match the osmolality of the first bleed (e.g. if an animal's first bleed was 303 mOsm kg^{-1} and its fourth bleed was 387 mOsm kg^{-1}, we added 3.8 µl Nanopure to 20 µl fourth bleed plasma to create a fourth bleed sample with an osmolality the same as that of the first sample, 303 mOsm kg^{-1}). We verified the osmolality of these diluted samples before running immune function assays.

Plasma degradation experiment

Degradation experiments were conducted to explore any possible effects of separating plasma from whole blood, freezing samples and storage time on our immune assays. We collected blood samples (as described above) from three captive adult *C. atrox* not being used for this study. We performed all immune assays on the freshly collected whole blood as well as freshly separated plasma from these samples. We aliquoted additional plasma into separate vials and froze them at −80°C to be used for repeating all immune assays after being frozen for 1, 2, 3, 24, 77 and 115 days.

Statistical analysis

To test the effect of food deprivation and time on immune function, we ran a repeated-measures ANOVA (rmANOVA) on scores from snakes given *ad libitum* access to water but no food for 16 weeks (n=8). We tested for compound symmetry to ensure linearity of sample change over time and a homogeneous relationship among samples (epsilon=0.5<Greenhouse–Geisser epsilon=0.81). We also used rmANOVAs to test for the effect of plasma degradation on all immune scores. To test the effect of dehydration (i.e. increased osmolality) on innate immune function, we performed a linear mixed-effect model on scores from the first four bleeds of all snakes – those deprived of food and water (n=14) and those deprived just of food (n=8) – with treatment (access to water or not) and bleed (time) as fixed effects, and individual as a random effect. To identify the optimal set of explanatory variables for our statistical model, we also included

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parameters on sex, SVL and mass after checking for collinearity using a correlation matrix. To avoid variables with a variance inflation factor (VIF) greater than 3, we used residuals from BCI indexes in place of SVL and mass. However, after stepwise removable of insignificant variables using the change in Akaike's information criterion (ΔAIC) and model weights (Arnold, 2010; Zuur et al., 2010), we continued with a model that used individual ID, treatment and bleed. To test the effect of acute rehydration, we ran a separate linear mixed-effect model on scores from these animals using only their fourth and fifth bleeds. For field-collected samples, we performed ANOVAs to examine the differences in immune function scores and osmolality among the different times of year. We also used an ANOVA to test for the effect of osmolality and dilution on immune function scores. A *post hoc* Tukey's HSD test was used when interaction terms were insignificant and after ANOVAs to determine which of the groups were significantly different. All statistical analyses were completed in R with the packages nlme and multcomp (Hothorn et al., 2008; Pinheiro et al., 2014; R Core Team, 2015). Significance was set at α=0.05.

Ethical statement

All work was conducted with approval from the Arizona State University Institutional Animal Care and Use Committee (protocol 15-1409R). Field research was conducted with the approval of the Arizona Game and Fish Department (permit SP706936).

RESULTS

Field-based experiment

Plasma osmolality of free-ranging *C. atrox* (n=60) ranged from 277 to 436 mOsm kg^{-1}. Seasonally, osmolality was highest during the hot-dry season ($F_{2,57}$=16.01, $P\leq$0.01), with levels during spring and the hot-wet season being similar to each other (Fig. 1). Lysis ($F_{2,57}$=5.98, $P\leq$0.01) and agglutination ($F_{2,57}$=14.01, $P\leq$0.01) scores were also significantly higher during the hot-dry season (Fig. 1). *Salmonella enterica* BKA scores were significantly higher ($F_{2,57}$=3.96, P=0.02) in the hot-dry season compared with spring, though hot-dry scores were not significantly different from scores during the hot-wet season. There were no significant differences in *E. coli* BKA scores ($F_{2,57}$=1.56, P=0.22) during the three sampling periods.

Laboratory-based experiment

Not surprisingly, all snakes lost mass over the 16-week duration of the study – snakes without access to food lost 17.12±9.63 g, while snakes without access to food or water lost 72.50±5.26 g. Because mass loss was highly correlated with change in osmolality, mass loss was not included in the statistical models evaluating the effect of hydration state on immune function. Similar to the field study results, we found a strong association between osmolality and innate immune performance (Fig. 2). As plasma osmolality increased (i.e. snakes dehydrated) over 16 weeks without food or water, we found a significant effect of the time by treatment interaction (compared with the eight snakes that had water but no food for 16 weeks) on osmolality ($F_{3,60}$=27.31, P<0.01), lysis ($F_{3,60}$=19.44, P<0.01), agglutination ($F_{3,60}$=3.98, P=0.01), *E. coli* inhibition ($F_{3,60}$=3.85, P=0.01) and *S. enterica* inhibition ($F_{3,60}$=3.68, P=0.02). When the dehydrated snakes were given access to water, which drastically decreased osmolality (i.e. snakes rehydrated), there was a significant reduction in lysis ($F_{1,20}$=31.68, P<0.01), *E. coli* inhibition ($F_{1,20}$=13.67, P<0.01) and *S. enterica* inhibition ($F_{1,20}$=21.15, P<0.01). However, there was no significant reduction in agglutination score ($F_{1,20}$=3.3537, P=0.08) after rehydration. In

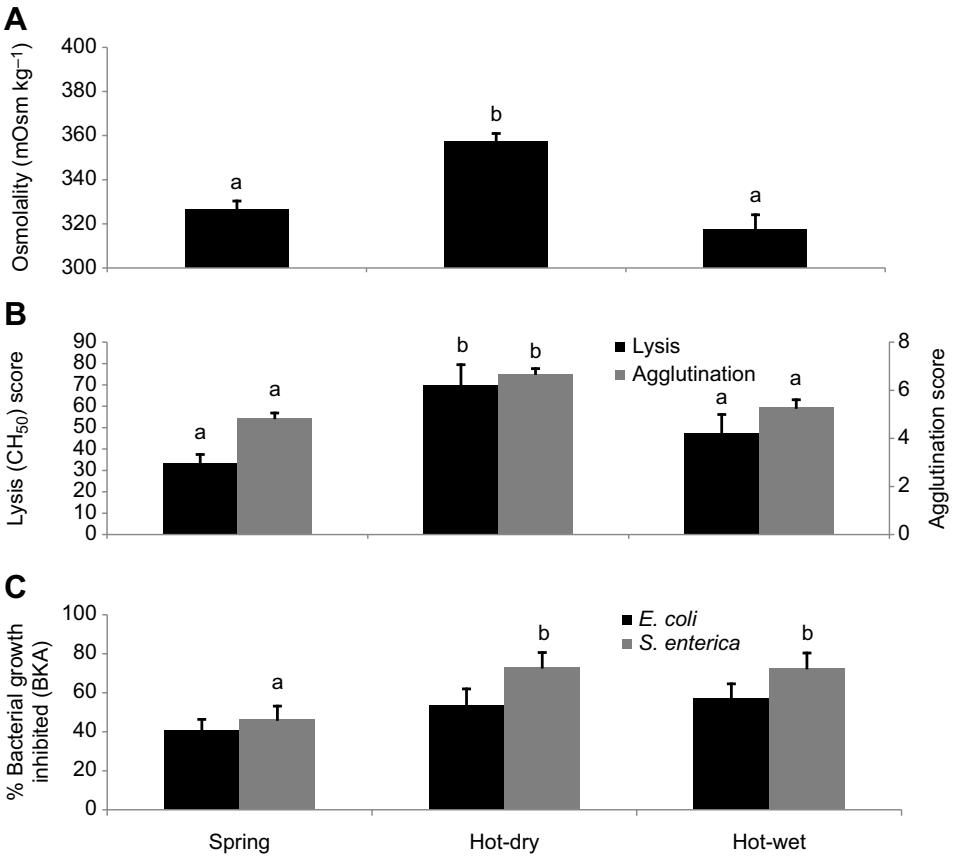

Fig. 1. Seasonal fluctuations in osmolality and immune performance in free-ranging rattlesnakes. Average osmolality (A) and immune scores [B: lysis and agglutination; C: *Escherichia coli* and *Salmonella enterica* bacterial killing ability (BKA)] from free-ranging *Crotalus atrox* during the spring (n=20), hot-dry season (n=19) and hot-wet season (n=21). Osmolality was highest during the hot-dry season, as were lysis and agglutination scores. *Salmonella enterica* BKA scores were higher in the hot-dry and hot-wet seasons, while *E. coli* scores did not significantly differ across seasons (P>0.05). Groups that share the same letter did not have statistically significant differences in means. Error bars represent ±1 s.e.m.

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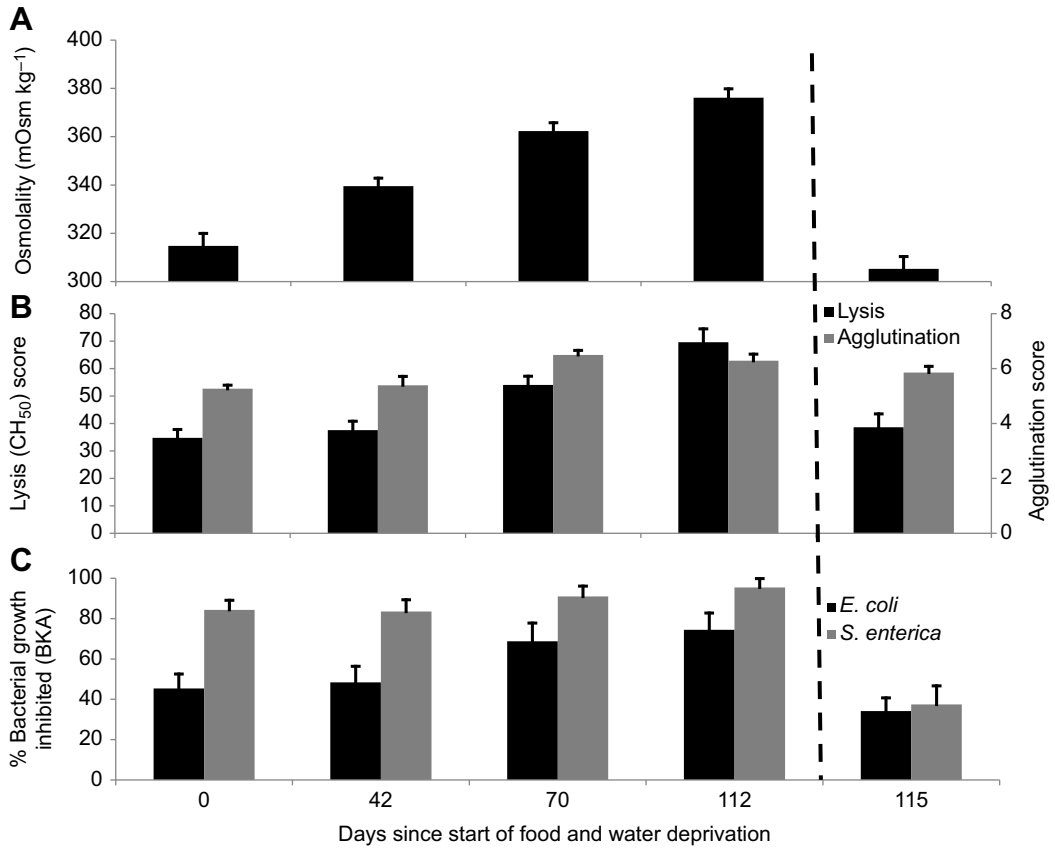

Fig. 2. Fluctuations in osmolality and immune performance in captive rattlesnakes held without food and water. Average osmolality (A) and immune scores (B: lysis and agglutination; C: *E. coli* and *S. enterica* BKA) in captive *Crotalus atrox* (n=14) during a 16-week period without water and 72 h after being given *ad libitum* access to water. There was a significant time by treatment interaction effect (P<0.05) on osmolality and all immune scores for the first 16 weeks and a similar significant interaction effect on osmolality and all scores except agglutination at acute rehydration. Dashed line represents when dehydrated animals were given water *ad libitum*. Error bars represent ±1 s.e.m.

contrast to the water-deprived snakes, osmolality and immune scores did not significantly differ (P>0.05) over time in the eight animals deprived of food but given access to water (Fig. 3) for 16 weeks.

Plasma dilution experiment

Despite reduction of osmolality to that of the first blood sample, diluted samples from the fourth bleed, when the snakes were most dehydrated, had immune function scores that were not significantly different (P>0.05) from unaltered aliquots of the same samples. The diluted fourth bleed samples retained higher immune scores than the normosmotic (first bleed) samples in lysis ($F_{2,14}$=33.13, P<0.01), agglutination ($F_{2,14}$=13.59, P<0.01), *E. coli* inhibition ($F_{2,14}$=5.01, P=0.02) and *S. enterica* inhibition ($F_{2,14}$=8.66, P<0.01).

Plasma degradation experiment

We did not detect a significant difference (P>0.05) in immune function scores between fresh, whole blood and plasma samples. Immune scores of the plasma did not significantly differ (P>0.05) over time after being frozen (−80°C) for up to 115 days.

DISCUSSION

Animals living in environments with predictable, seasonal water restrictions show a remarkable suite of behavioral and physiological adaptations to survive when there is no access to free-standing water. Because hyperosmotic conditions are often poorly tolerated, most of these strategies entail maintaining water balance through either conserving water or using alternate water sources. Water can be conserved through behavioral [e.g. altered activity patterns (Bissonette, 1978) or using more thermally suitable microclimates (Whitford and Steinberger, 2010)] or physiological means [reducing water use for the elimination of nitrogenous waste; e.g. uric acid (Braun, 1999), lengthy loops of Henle (Khalil and Tawfic, 1963)]. Alternate water sources include internal water reservoirs [e.g. the rumen in camels (Mousa et al., 1983) or the urinary bladder in toads (Ruibal, 1962), tortoises (Peterson, 1996) and Gila monsters (Davis and DeNardo, 2007)], dietary water (that present in food; Morgart et al., 2005) or metabolic water (as a byproduct of metabolism; Rutkowska et al., 2016). However, fewer species can tolerate considerable variation in osmolality. Our field data demonstrate that during the seasonal drought (mid-May through mid-July), *C. atrox* experience periods of considerable dehydration (Fig. 1). Their tolerance of hyperosmolality (277–436 mOsm kg^{-1}) is greater than most other species known to use this strategy [camel: 310–352 mOsm kg^{-1} (Bekele et al., 2013); Gila monster: 280–350 mOsm kg^{-1} (Davis and DeNardo, 2009)], but not as high as has been documented for tortoises (up to 562 mOsm kg^{-1}; Peterson, 1996).

During periods of elevated plasma osmolality, whether naturally in the wild or via manipulation in the laboratory, rattlesnakes had enhanced innate immune function. These results are consistent those of with Moeller et al. (2013), who found that lysis and agglutination scores increased during periods of dehydration in Gila monsters, *Heloderma suspectum*. Rattlesnakes in the laboratory also had enhanced *E. coli* and *S. enterica* inhibition capabilities when

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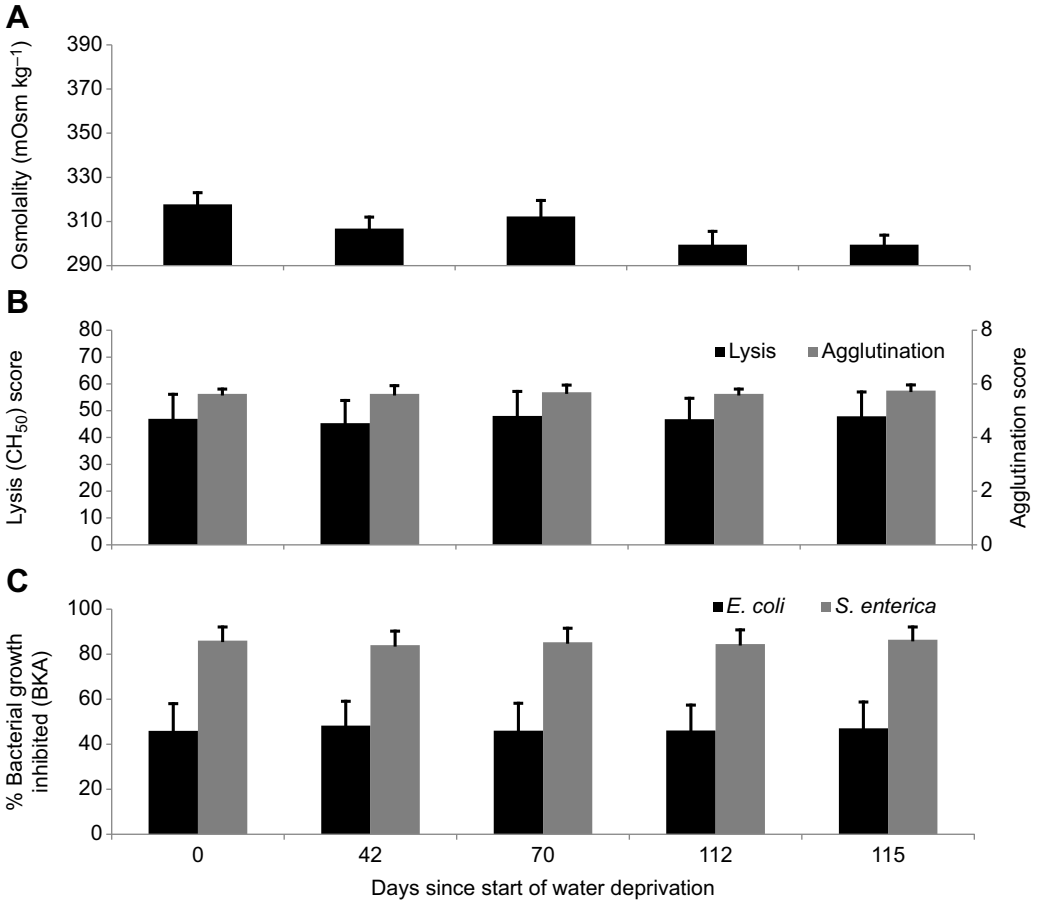

Fig. 3. Fluctuations in osmolality and immune performance in captive rattlesnakes held without food. Average osmolality (A) and immune scores (B: lysis and agglutination; C: *E. coli* and *S. enterica* BKA) in captive *Crotalus atrox* (n=8) during a 16-week period with no food and *ad libitum* access to water. There was no significant main effects or time by treatment interaction effect (P<0.05) on osmolality or any immune scores for the entire study. Error bars represent ±1 s.e.m.

dehydrated. In contrast, while wild rattlesnakes had higher *S. enterica* inhibition in the hot-dry season compared with the spring, scores did not decrease during the hot-wet season, when water was once again available and osmolality decreased. Furthermore, *E. coli* inhibition in wild snakes was not significantly different across the three seasons sampled. In the laboratory experiment, we controlled for factors thought to impact *C. atrox* performance, including temperature and food availability (Beck, 1996). These confounding factors could not be controlled in the field and might explain discrepancies between field and laboratory-based results.

Validating that our laboratory results represented the effects of dehydration, immune performance did not change over time in rattlesnakes that were deprived of food, but had water available *ad libitum*, for 16 weeks. This finding may seem contrary to much of the current literature on energetic factors that impact immune performance (Berger et al., 2005; Brace et al., 2015; Husak et al., 2016); however, *C. atrox*, like most vipers, are binge feeders, being well adapted to eating large, widely spaced meals (Beck, 1995). By starting with animals in good body condition and feeding them two large meals before the start of the experiment, these snakes had ample energy stores to support their relatively limited energy demands, especially considering that the laboratory housing greatly limited their movement. Thus, as we expected, there was no effect of a 16-week food deprivation on immune performance in captive *C. atrox*.

The results from our dilution of plasma samples from dehydrated snakes indicate that elevated immune scores associated with dehydration were not simply the result of dehydration causing increased concentration of immune factors within the plasma. This suggests that plasma proteins associated with innate immunity are upregulated (in terms of number or activity) during periods of dehydration. While innate immune performance gradually increased as animals gradually dehydrated over 16 weeks, it rapidly decreased after animals were given access to water and subsequently rehydrated quickly. This suggests that plasma proteins responsible for our findings rapidly disassociate or become ineffective. The lysis assay specifically measures the involvement of complement (Matson et al., 2005), a highly regulated and crucial systemic effector mechanism synthesized primarily in the liver (Ricklin et al., 2010). As complement proteins have relatively short half-lives (1–60 min) (Mollnes et al., 2007), the rapid return to baseline immune scores we detected upon rehydration is expected. Although the specific mechanisms involved in bacterial inhibition are unknown, our inhibition scores followed the same trends as lysis, providing evidence of additional, innate proteins capable of dynamic changes in response to dehydration and rehydration. Furthermore, we did not detect a performance difference between whole blood and plasma in our degradation experiments, which suggests that a non-cellular mechanism is responsible for our results. As with many plasma proteins, complement is primarily synthesized in the liver (Colten and Strunk, 1993), and we suggest that future research examine other important effector molecules secreted by hepatic cells and found in blood plasma such as β-defensins and cathelicidins (García et al., 2001; Zanetti, 2004), both of which may provide a mechanistic explanation for our findings.

Innate immune function provides a rapid, broadly reactive response using general effector mechanisms that are often sufficient to control infections. Innate immunity, however, also has an integral role in informing the adaptive immune system to make an overwhelming, tailored response. Complement has long been known as an important bridge between innate and adaptive immune responses (Carroll, 2004; Dunkelberger and Song, 2010), and recent research suggests a similar roll of both cathelicidins and β-defensins (Kao et al., 2004; Wolk et al., 2004). Given the interconnected relationship between the innate and adaptive branches, it is reasonable to suspect that adaptive responses will be enhanced as well, and future research should explore this area.

In addition to understanding the proximate mechanisms behind our findings, it is also appropriate to consider ultimate mechanisms that might explain the perhaps initially counter-intuitive positive relationship between dehydration and innate immunity. Dehydration creates a homeostatic imbalance, which may leave the animal vulnerable to disease. Accordingly, it would be advantageous to increase innate defenses (such as complement) to defend the body from such threats. The classic dogma of immune function is that it exists to ward off harmful pathogens; however, recent evidence suggests that immunocompetence may also play a major role in maintaining physiological (Kotas and Medzhitov, 2015; Marques et al., 2016) and neurological (D'Acquisto, 2016) homeostasis.

The question remains: why not upregulate these plasma proteins, and therefore decrease vulnerability, all the time? Although humans typically maintain their plasma osmolality within a narrow range (285–295 mOsm kg^{-1}) (Verbalis, 2003), recent studies have revealed that some tissues experience hyperosmotic stress, which may contribute to acute and chronic inflammatory disorders (e.g. Brocker et al., 2012). Some diseases such as cystic fibrosis (Neuhofer, 2010), inflammatory bowel disease (Vernia et al., 1988) and arthritis (Yoon et al., 2011) are marked by both hyperosmolality and a measurable increase in inflammatory cytokines that typically result in an upregulation of the same innate effector proteins we believe are involved in our study (i.e. complement, cathelicidins and β-defensins). We suspect that organisms capable of elevating innate immune proteins during periods of dehydration do so while running an increased risk of auto-immune disorders similar to those seen in humans under long-term hyperosmotic stress. Constantly elevating concentrations of innate immune proteins may be mediated by long-term costs from autoimmunity owing to oxidative stress (Bertrand et al., 2006) and chronic inflammation (Sorci and Favire, 2009).

Immune function can vary based on life stage (Schwanz et al., 2011), season (Buehler et al., 2008) or an animal's ecology (French et al., 2009). It is suspected that much of this variability is a result of balancing resources among immunity and other physiological functions (French et al., 2007; Toomey et al., 2010; Nebel et al., 2012). Interestingly, our results, and those of Moeller et al. (2013), indicate that, unlike what is seen with energy limitation, water limitation enhances immune function. Additionally, in species with relatively low energetic and water demands (e.g. reptiles), the effect of water limitation is more immediate than that of food limitation. However, in some of these same species, energy and water investment into reproduction is enormous, with reproductive output often representing more than a third of their pre-reproductive body mass. It would be valuable to explore whether the effects of food and water deprivation on immune function are similar during times of very limited and extensive resource demands. As the availability of resources is often limited during some periods of the year and availability of resources is expected to be affected by global climate change, it is important to further expand our understanding of how resources influence immune function and how resource demand by the organism influences these relationships.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
G.A.B. IV and D.F.D. designed the study, collected the samples, and wrote the manuscript. G.A.B. IV conducted all assays and performed statistical analyses.

Author contributions metadata
Conceptualization: G.A.B., D.F.D.; Methodology: G.A.B., D.F.D.; Formal analysis: G.A.B.; Investigation: G.A.B., D.F.D.; Writing - original draft: G.A.B.; Writing - review & editing: G.A.B., D.F.D.

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