Dehydration enhances innate immunity in a semiaquatic snake from the wet-dry tropics

George A. Brusch IV1 | Keith Christian2 | Gregory P. Brown3 | Richard Shine3,4 | Dale F. DeNardo1

1School of Life Sciences, Arizona State University, Tempe, Arizona
2Research Institute for the Environment and Livelihoods, Charles Darwin University, Darwin, NT, Australia
3School of Life and Environmental Sciences, University of Sydney, Sydney, NSW, Australia
4Biological Sciences, Macquarie University, Sydney, NSW, Australia

Correspondence
George A. Brusch IV, School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501.
Email: gbrusch@asu.edu

Funding information
Directorate for Biological Sciences, Grant/Award Numbers: 1311230, 1606367; Arizona State University, Grant/Award Number: College of Liberal Arts & Sciences Graduate Excell

Abstract
Dehydration is considered a physiological challenge, and many organisms live in environments that undergo periods of reduced water availability that can lead to dehydration. Recent studies have found a positive relationship between dehydration and innate immune function in animals adapted to xeric or semixeric environments. To explore the generality of this relationship, we examined the impact of dehydration on innate immune performance in water pythons (Liasis fuscus), a semiaquatic snake from the wet-dry tropics of Australia. We collected blood samples from male and female water pythons held in the laboratory without food and water for 4 weeks. We also collected blood from free-ranging snakes throughout the Austral dry-season. We evaluated plasma osmolality and innate immune function (agglutination, lysis, and bacterial-killing ability) and found that increased osmolality, whether manipulated in the laboratory or as a result of natural water limitation, resulted in enhanced aspects of innate immune performance. Counter-intuitively, snakes in the wild became more hydrated as the dry season progressed, suggesting the dehydrated snakes move to water sources periodically to rehydrate. Comparing our data with those from previous studies, we suspect species divergence in the level of dehydration (i.e., hyperosmolality) that triggers enhanced immune capabilities.

KEYWORDS
hydration, immunocompetence, Liasis fuscus, osmotic stress, water limitations, water python

1 | INTRODUCTION

Water is a fundamental resource to sustain life, yet water availability can be limited. In many environments, the uneven distribution of precipitation throughout the year results in seasonal droughts: >66% of the earth’s land surface endures 65 or more consecutive days per year without any measurable rainfall (Hao, Singh, & Xia, 2018). To cope with water scarcity, many organisms exhibit water-conserving behavioral, morphological, or physiological adaptations to maintain water balance (Takei, 2000). Alternatively, other organisms tolerate dramatic fluctuations in plasma osmolality and endure bouts of dehydration (i.e., hyperosmolality) until water becomes available (Brusch & DeNardo, 2017; Peterson, 1996). Although the latter strategy is well-documented, we have only a limited understanding of the behavioral and physiological implications of nonlethal dehydration. Moderate levels of dehydration negatively impact the duration of surface activity (Davis & DeNardo, 2009), cellular homeostasis (Häussinger, 1996), thermoregulation (Montain, Latzka, & Sawka, 1999), and metabolism (Gerich, Penhos, Gutman, & Recant, 1973). Recent work has begun to examine the relationship between dehydration and immune function. Notably, aspects of immunity are often enhanced in response to dehydration, at least for the xeric-adapted squamate reptiles studied to date (Brusch, Billy, Blattman, & DeNardo, 2017; Moeller, Demare, Davies, & DeNardo, 2017). As effective functioning of the immune system is vital for fending off pathogens and thus survival (Zuk & Stoehr, 2002), a better
understanding of the relationship between hydration state and immune function will be valuable for predicting organismal impacts of altered rainfall patterns associated with global climate change (Dai, 2013; Mukherjee, Mishra, & Trenberth, 2018).

Accordingly, we examined the relationship between dehydration and immune function in water pythons (Liasis fuscus), a semiaquatic snake that has been the focus of extensive ecological research (Brown, Shine, & Madsen, 2002; Madsen & Shine, 1996a, 1996b, 1998a, 1998b, 1999a, 1999b, 2000a, 2000b, 2002; Stahlschmidt, Shine, & DeNardo, 2012a,b). While frequently in or around water, water pythons also spend extended periods of time away from water, particularly to forage and during seasonal drought when water sources are limited. Does such reduced accessibility lead to dehydration and, if so, is the extent of dehydration sufficient to enhance innate immunity, as it does in species that face a greater degree of water deprivation? We tested the hypotheses that (a) despite the presence of some water sources during drought, free-ranging water pythons experience dehydration during the dry season and (b) when dehydrated, water pythons have enhanced innate immunity.

2 | METHODS

All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee (protocol #16-1495R), the University of Sydney Animal Ethics Committee (protocol #2016/997), the Charles Darwin University Animal Ethics Committee (protocol #A16010), and the Northern Territory Parks and Wildlife Commission (permit #58507).

2.1 | Study species and site

Water pythons are large (adult snout-vent length [SVL] >2 m) semiaquatic snakes found throughout the wet-dry tropics of northern Australia (Wilson & Swan, 2013). They are mostly nocturnal and primarily feed on dusky rats (Rattus colletti; Shine & Madsen, 1997). Snakes used for this study were captured from Beatrice Hill Farm and Harrison Dam, which are located 7 km apart on the Adelaide River floodplain (approximately 55 km southeast of Darwin, Northern Territory [NT, Australia]). This area experiences high average daily temperatures year-round (≈32°C) but the vast majority of the high annual precipitation (≈1400 mm) occurs during the wet season from December to March (Shine, 1992). Mating and egg development occur during the dry season (July–August), and eggs are typically oviposited 1–2 months later (Madsen & Shine, 1996b). Water pythons may meet their water demands during the extended dry season by utilizing remnant natural water holes or anthropogenic sources (e.g., dams and cattle ponds; Warfe et al., 2011).

2.2 | Field study

To determine whether a natural reduction in water availability during the Austral dry-season leads to dehydration and whether any naturally occurring hyperosmolality correlates with plasma-based immune function, we collected blood samples (see below) from 60 water pythons during a 5-week period in the middle of dry-season (June and July 2016) when, in most years, there is no rainfall (Australian Government Bureau of Meteorology, http://www.bom.gov.au) yet water pythons are reproducing actively (Stahlschmidt et al., 2012a). Upon capture, we determined mass (by placing the snake in a tared bag and hanging it from a spring scale (±2 g, model 40300; Pesola AG, Schindellegi, Switzerland), sex (by probing the cloaca caudally), and SVL (using a cloth tape). We also collected a blood sample for determination of osmolality and immune function (see details below), then marked each snake on its back with pink nail polish to avoid duplicative sampling. We temporarily stored the collected blood in a cooler with ice until return to the lab.

2.3 | Laboratory study

To assess the effect of dehydration on immune function independent of other environmental effects, we conducted a laboratory-based study where we deprived water pythons of water to induce progressive dehydration and serially collected blood samples for determination of osmolality and assessments of immune function. In early June 2016, we collected 15 adult water pythons (male = 8, female = 7) from Beatrice Hill Farm and Harrison Dam, and transported them to the nearby University of Sydney’s Tropical Ecology Research Facility, Middle Point, NT. Mass, sex, and SVL of each snake were recorded, and females were abdominally palpated to confirm they were not gravid as gravidity can affect both hydration and immune function in a sympatric python species (Brusch et al., 2017). Snakes were housed individually in translucent containers (87 × 60 × 29.5 cm) with lids and held at ambient temperature with natural light from rooftop windows. Snakes were initially provided ad libitum access to water to ensure they began the experiment in a normosmotic state. A blood sample was collected from each snake, 6 days after capture. Snakes were then held without food and water, being bled 7, 16, and 28 days after their initial blood sampling. After the Day 28 sampling, the snakes were provided water ad libitum and a final blood sample was collected 72 hr later. All snakes were checked daily and weighed twice per week. No snake lost more than 15% of its initial body mass or showed clinical signs of dehydration (e.g., lethargy, loss of skin turgor). As with the field study, we used the blood samples to determine plasma osmolality and conduct immune function assays. Snakes were released at their sites of captures upon completion of the study.

2.4 | Collection of blood samples

We used heparinized 1-ml syringes with a 25-G × 1.6 cm (5/8 inch) needle to collect a 0.8 ml blood sample via cardiocentesis. After blood collection, we either returned the snake to its container (laboratory) or marked and released it at its capture site (field). Total time for capture, restraint, and collection was typically less than 5 minutes and did not exceed 8 minutes for both lab and field portions. We
immediately centrifuged the blood samples from captive snakes at 3,000 rpm for 3 minutes to separate plasma from blood cells. We aliquoted plasma (~50 µl) into separate vials that were frozen at ~80°C until we used them within 35 days to measure plasma osmolality and evaluate immune function.

2.5 | Determination of osmolality and assays of immune function

For all samples, we determined plasma osmolality using a vapor pressure osmometer (±3 mOsm/kg, model 5100C; Wescor Inc., Logan, Utah). We ran samples in triplicate as described in Davis and DeNardo (2009).

We used several plasma-based assays to assess innate immune function and examine the relationship between immunocompetence and hydration state. Agglutination and lysis assays evaluated the reaction of natural antibodies and complement to a novel antigen, sheep red blood cells (sRBC, SB050; Thermo Fisher Scientific, Scoresby, Victoria, Australia), and thus serve as a measure of innate immunity (Matson, Ricklefs, & Klasing, 2005). In brief, we serially diluted 20 µl of each plasma sample from 1:2 to 1:2,048 with phosphate-buffered saline (PBS) along a row of a 96-well plate. We then added 20 µl 1% sRBC to each well. We did not add plasma to the final column; the top four wells contained only 20 µl PBS and 20 µl 1% sRBC (negative control, 0% lysis) and the bottom four wells contained 20 µl ammonium-chloride-potas- sium (ACK) lysing buffer (Lonza, Basel, Switzerland) and 20 µl 1% sRBC (positive control, 100% lysis). We incubated the plates at 37°C for 90 min and placed at room temperature, ~25°C, for 20 min after which point we scanned the plates at 600 dots per inch using a flat-bed scanner (ScanJet 3670; Hewlett-Packard Co.) for agglutination images. Plates remained at room temperature for an additional 70 min and then we centrifuged them for 5 min (500 rpm, Sorvall; Newtown, CT) after which we aspirated the supernatant into a clean 96-well plate. We measured the absorbance values measured using a microplate spectrophotometer (405 nm; Bio-Rad, Hercules, CA) to calculate lysis scores. Hemolytic-complement activity was expressed in CH50 units (ml plasma)⁻¹, where 1 CH50 unit equals the reciprocal of the dilution of plasma required to lyse 50% of the sRBC.

We also used bacterial killing assays outlined in French and Neuman-Lee (2012) to assess the ability of blood to inhibit the growth of a gram-negative bacteria, Escherichia coli. Briefly, we combined 1:4 plasma dilution with CO2-independent media plus 4 nM l-glutamine, 10⁶ colony-producing units of E. coli (Lot#483-306-1, ATCC 8739; MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated absorbance using a microplate reader (300 nm; Bio-Rad) at 0 hr and after 12 hr of incubation at 37°C. We calculated percent bacterial growth inhibited as one minus the mean absorbance for each sample, which we ran in triplicate, divided by the mean absorbance for the positive control (triplicate wells containing only media and bacteria), multiplied by 100.

2.6 | Statistical analysis

Using the data from the laboratory experiment and linear mixed effect models, we tested the effect of dehydration (first 28 days without water) and rehydration (72 hr after water was provided ad libitum) on plasma osmolality and innate immune function. We used time (days since start of water deprivation) as a fixed effect and individual as a random effect. To identify the optimal set of explanatory variables for our statistical model, we also included sex, date, location of capture, SVL, and mass after checking for collinearity using a correlation matrix. To avoid variables with a variance inflation factor >3, we used residuals from a body condition index (linear regression using SVL and mass) in place of SVL and mass. We used a stepwise removal of insignificant variables with ΔAIC and model weights (Arnold, 2010; Zuur, Ieno, Walker, Saveliev, & Smith, 2009) and continued with a model that used sex, nested within individual, and time. We used a post hoc Tukey’s HSD test to identify significant differences among time periods. We also performed linear regressions using the profiles among individuals to explore the relationship between osmolality and immune scores, independent of time.

For field-collected samples, we first determined if there was a sex effect on mass, SVL, osmolality, or immune scores using Student’s t tests. We then performed similar linear regressions using the field-collected data to explore both the (a) relationship between osmolality and immune score and (b) changes in plasma osmolality throughout the dry season. We checked the data to ensure they met the assumptions for parametric testing, and we used transformations where necessary. We completed all statistical analyses in R with the packages “nlme” and “multcomp” (Hothorn, Bretz, & Westfall, 2008; Pinheiro, Bates, DebRoy, & R Core Team, 2016; R Core Team, 2015). We set significance at α = 0.05.

3 | RESULTS

3.1 | Field study

On average, females were larger than males (SVL: t₅₂ = 2.06; p = 0.041; mass: t₅₈ = 2.80; p < 0.001, n = 60, male = 34, female = 26), but there were no significant sex-based differences in osmolality or immune scores (p > 0.05). Plasma osmolality ranged from 279 to 326 mOsm/kg and decreased (F₁₃,₄₆ = 2.13, p = 0.031, R² adj = 0.13) as the dry season progressed (Figure 1). We found a positive relationship between osmolality and lysis (F₁,₅₈ = 19.4, p < 0.001, R² adj = 0.24) and between osmolality and bacterial-killing ability (F₁,₅₈ = 16.3, p < 0.001, R² adj = 0.21; Figure 2b,c), but there was no significant relationship between osmolality and agglutination (F₁,₅₈ = 0.97, p = 0.338; Figure 2a).

3.2 | Laboratory study

Osmolality progressively increased (i.e., the animals dehydrated) over the 28 days when snakes were held without water (all p < 0.05), and
subsequently decreased 3 days after snakes were provided with water (Figure 3; Table S1). Similar to results from the field study, the laboratory study showed a positive relationship between osmolality and lysis ($F_{1,73} = 8.02; p = 0.005, R^2_{adj} = 0.09$) and between osmolality and bacterial killing ability ($F_{1,73} = 16.56; p < 0.001, R^2_{adj} = 0.17$), but not between osmolality and agglutination ($F_{1,73} = 0.72, p = 0.40$). However, lysis and bacterial killing were significantly higher only after 28 days without water compared to all other time points (all $p < 0.05$; Figure 4b,c; Table S2). Our models generated the same conclusions: a significant time effect on plasma osmolality ($F_{4,56} = 151.8; p < 0.001$), lysis ($F_{4,56} = 4.45, p = 0.003$) and bacterial killing ($F_{4,56} = 8.47, p < 0.001$). We did not detect any significant effect of time on agglutination scores ($F_{4,56} = 1.82, p = 0.14$; Figure 4a; Table S2).

4 | DISCUSSION

Plasma osmolality was quite variable among snakes throughout the dry season, and, contrary to our prediction, average osmolality actually decreased in free-ranging water pythons over a 2-month period as the dry season progressed (Figure 1). While there are permanent water sources at Harrison Dam and at Beatrice Hill during the dry season, pythons in this population move up to 3 km from these permanent water sources to the drying floodplains to follow their primary food source, the dusky rat (Madsen & Shine, 1996b). We expected that this seasonal migration away from water would increase plasma osmolality as the dry season progressed. However, the variable hydration states among snakes during the dry season, including a lower average osmolality later in the dry season, suggests that these pythons periodically return to water, despite the distance, to rehydrate. An alternate explanation for varying and even decreasing osmolality as the dry season progresses is that the pythons are gaining hydric benefits from eating the rats, which are >70% water. Squamates living in seasonally xeric environments can obtain water through dietary and metabolic sources (heat monitor; Green, Dryden, & Dryden, 1991; stripe-tailed goanna; Thompson, Bradshaw, & Withers, 1997; desert iguana; Minnich & Shoemaker, 1970). However, other studies in lizards (Wright, Jackson, & DeNardo, 2013) and in snakes (Lillywhite, 2017) have found that animals that eat infrequently, and do not alter their diet during dry times of the year, do not obtain a hydric benefit (i.e., reduced plasma osmolality) from consuming meals. Another
potential explanation is that brush fires burned extensive areas in and around each site during our study (18 June, 23 June, 29 June, 1 July, & 15 Jul; G. Brusch, personal observation). Such fires are common (Woinarski, Williams, Price, & Rankmore, 2005) and frequently burn large swaths of the grassland every year (Spessa, McBeth, & Prentice, 2005). Snakes may have moved to water sources to flee the fire, as occurs in other ectotherms (Grafe, Döbler, & Linsenmair, 2002; Huntley et al., 1984; Withgott & Amlaner, 1996). If so, we may have captured snakes in July that were rehydrated and returning to the burnt floodplain to take advantage of postfire, reduced vegetative cover and increased prey availability (Payne, Ritchie, Kelly, & Nimmo, 2014). Repeated tracking and sampling of radiotelemetered pythons would provide valuable insight into the movement patterns of this species and their relationship to water balance, prey consumption, and fire.

In our laboratory experiment, pythons held without access to water experienced a progressive increase in osmolality over time (Figure 3). At 4 weeks, mean osmolality (331 mOsm/kg) slightly exceeded the upper limit of measured field osmolalities (325 mOsm/kg). These results suggest that our laboratory study modeled natural water scarcity and that animals in the wild do not exceed 4 weeks without access to water, supporting the idea that water pythons periodically return to distant water resources during the 2-month dry season.

We found that dehydration, whether due to natural fluctuations in water availability or manipulation in the lab, enhanced aspects of innate immunity. Specifically, we saw a significant increase in lytic and bacterial-killing ability in both the laboratory and field portions of the study (Figures 2 and 4). We did not detect any significant increases in agglutination, however. These results are consistent with findings of a positive relationship between hyperosmolality and innate immune performance in other squamates (Gila monsters; Moeller, Butler, & DeNardo, 2013; rattlesnakes; Brusch & DeNardo, 2017; Children’s pythons; Brusch et al., 2017). As in those studies, we found that rehydration rapidly returned immune metrics to baseline values (Figure 4), suggesting that the innate molecules responsible for the positive influence of dehydration on immune function rapidly disassociate or become ineffective upon rehydration. The complement suite of proteins are likely candidates, as they have short half-lives (≤1 hr; Mollness et al., 2007), are crucial systemic effectors (Ricklin, Hajishengallis, Yang, & Lambris, 2010), and are involved in both lysis (Matson et al., 2005) and bacterial killing (French & Neuman-Lee, 2012). The cellular mechanisms responsible for dehydration-based immune enhancement remain unknown and are in need of further study.

Because dehydration negatively impacts cognitive function (Wilson & Morley, 2003), locomotor performance (Moore & Gatten, 1989), and major physiological systems (Popkin, D’Anci, & Rosenberg,
it may seem surprising that dehydration has a positive effect on innate immunity. Dehydration clearly creates a homeostatic imbalance, leaving an organism vulnerable to pathogens. Increasing immune defenses, especially less energetically costly molecules such as complement proteins (McDade, Georgiev, & Kuzawa, 2016), can defend the body from pathogens that may be costlier to combat during later stages of infections (Lochmiller & Deerenberg, 2000). Water pythons are frequently wounded as they capture and ingest dusky rats, a behavior which correlates with higher levels of natural antibodies (Madsen, Ujvari, Nandakumar, Hasselquist, & Holmdahl, 2007). Although we did not detect any significant changes in agglutination scores, increased dehydration in foraging pythons may offer protection from infectious bites via other mechanisms, such as higher circulating levels of complement proteins. It is important to address an obvious alternative explanation: as the animals dehydrate, their blood volume decreases and, as a result, innate molecules become more concentrated. Previous work to address this possibility showed that diluting plasma samples from dehydrated animals to adjust for the decreased volume, still resulted in higher immune metrics compared to plasma samples from hydrated animals (Brusch & DeNardo, 2017; Moeller et al., 2013), suggesting that dehydration leads to an upregulation (either in total number or activity) of innate molecules. Although we did not conduct a similar experiment for our study, we predict that a similar upregulation would be detected in dehydrated water pythons.

Comparing our data (pooled from our field and laboratory studies) with similarly pooled data from other experiments that used analogous methods on a sympatric species (Children’s pythons; Brusch et al., 2017) and a xeric-adapted species (western diamond-backed rattlesnake; Brusch & DeNardo, 2017), reveal some interesting comparisons (Figure 5). Water pythons have the smallest range of plasma osmolality of the three species (279–343 mOsm/kg). Children’s pythons are intermediate (279–354 mOsm/kg), and rattlesnakes have the largest range (277–436 mOsm/kg). All three species show a positive relationship between osmolality and lytic scores (water pythons; F_{1,133} = 31.62, p < 0.001, R^2_{adj} = 0.19; Children’s pythons; F_{1,82} = 54.28, p < 0.001, R^2_{adj} = 0.39; rattlesnakes; F_{1,150} = 60.43, p < 0.001, R^2_{adj} = 0.28; Figure 6a–c) and between osmolality and bacterial-killing ability (water pythons; F_{1,133} = 28.48, p < 0.001, R^2_{adj} = 0.17; Children’s pythons; F_{1,82} = 24.19, p < 0.001, R^2_{adj} = 0.22; rattlesnakes; F_{1,150} = 45.17, p < 0.001, R^2_{adj} = 0.23; Figure 5a–c and Figure 5d-f). In addition, there appears to be a species-specific plasma osmolality above which both lysis and bacterial-killing abilities are always elevated. This threshold is low for water pythons (~310 mOsm/kg), intermediate for Children’s pythons (~325 mOsm/kg), and high for rattlesnakes (~360 mOsm/kg). Children’s pythons occur in the same area as water pythons (the study by Brusch et al., 2017 was conducted at Beatrice Hill) but are typically found in dry woodlands and rocky outcrops (Wilson & Swan, 2013) and may be adapted to longer periods without access to water (compared to semiaquatic water pythons). Similarly, the study by Brusch and DeNardo (2017) used rattlesnakes from the Sonoran Desert, where the snakes often experience no rainfall for >3 months during annual seasonal droughts (and can survive for 16 weeks without water before showing signs of clinical dehydration). This variation in dehydration tolerance among species and the

**FIGURE 5** Relationships between plasma osmolality (mOsm/kg) and immune scores. (a–c) lysis (CH50), (d–f) percent bacteria (*Escherichia coli*) killed in pooled data from free-ranging and laboratory held water pythons (*Liasis fuscus*; a,d; this study), Children’s pythons (*Antaresia childreni*; b,e; Brusch et al., 2017), and western diamond-backed rattlesnakes (*Crotalus atrox*; c,f; Brusch & DeNardo, 2017) . Gray circles represent individual blood samples, and the line of best fit is in black. Statistical analysis and results described in the text.
coinciding variable osmolalities at which immune scores are all elevated suggest that enhanced immune abilities are triggered by species-specific physiological set points rather than absolute osmolality values per se. Future studies should consider the natural history of the animal and look for osmotic thresholds related to dehydration-induced enhancement of immune function. These comparisons stress the importance of taking the natural history of organisms into account when conducting ecological and physiological experiments and when predicting animal survival under resource-limited conditions.

ACKNOWLEDGMENTS

The authors thank all members of the Shine lab at Middle Point, the staff at the Australian Academy of Sciences (especially S. Owen), and faculty and staff at Charles Darwin University for their assistance. G. A. B. IV wishes to particularly thank the Christian-Gibb family for their generosity. Finally, we wish to thank Drs. M. Greenlees and K. Gibb for their contributions. This work was supported by the National Science Foundation Graduate Research Fellowship (grant #1311230), National Science Foundation East Asia & Pacific Summer Institute Fellowship (grant #1606367), and Arizona State University's College of Liberal Arts & Sciences Graduate Excellence Fellowship for First Generation Students for G. A. B. IV.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

DATA ACCESSIBILITY

The datasets supporting this article can be accessed at https://doi.org/10.6084/m9.figshare.7066619.v1

ORCID

George A. Brusch http://orcid.org/0000-0001-7740-6066
Gregory P. Brown http://orcid.org/0000-0002-2924-9040

REFERENCES

Madsen, T., & Shine, R. (2000b). Silver spoons and snake body sizes: Prey availability early in life influences long-term growth rates of


