Clarifying the Dehydration Cascade:

The Relationship Between Water, Stress, and Immune Function in Squamates

by

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ABSTRACT

There is considerable recent interest in the dynamic nature of immune function in the context of an animal's internal and external environment. An important focus within this field of ecoimmunology is on how availability of resources such as energy can alter immune function. Water is an additional resource that drives animal development, physiology, and behavior, yet the influence hydration has on immunity has received limited attention. In particular, hydration state may have the greatest potential to drive fluctuations in immunity and other physiological functions in species that live in water-limited environments where they may experience periods of dehydration. To shed light on the sensitivity of immune function to hydration state, I first tested the effect of hydration states (hydrated, dehydrated, and rehydrated) and digestive states on innate immunity in the Gila monster, a desert-dwelling lizard. Though dehydration is often thought to be stressful and, if experienced chronically, likely to decrease immune function, dehydration elicited an increase in immune response in this species, while digestive state had no effect. Next, I tested whether dehydration was indeed stressful, and tested a broader range of immune measures. My findings validated the enhanced innate immunity across additional measures and revealed that Gila monsters lacked a significant stress hormone response during dehydration (though results were suggestive). I next sought to test if life history (in terms of environmental stability) drives these differences in dehydration responses using a comparative approach. I compared four confamilial pairs of squamate species that varied in habitat type within each pair—four species that are adapted to xeric environments and four that are adapted to more mesic environments. No effect of life history was detected between groups, but hydration was a driver of some

measures of innate immunity and of stress hormone concentrations in multiple species. Additionally, species that exhibited a stress response to dehydration did not have decreased innate immunity, suggesting these physiological responses may often be decoupled. My dissertation work provides new insight into the relationship between hydration, stress, and immunity, and it may inform future work exploring disease transmission or organismal responses to climate change.

I dedicate this work to my parents, Sylvia and Charles, who planted the seed of aspiration in my well-nourished heart when it was small and who taught me in different ways the importance of education and finding a career that makes you happy; to my sisters, Alina and Joan, who each constantly show me how to pursue what you love, despite wrong turns, delays, and dead ends; and to the rest of my family for always supporting and encouraging me, no matter what I try to do.

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CHAPTER ONE:

Introduction

You stumble as you cross the dry wash, filling your boots with more sand. The wash seems familiar, so you turn left, hoping it's the right direction. They call this place the maze for a reason—each canyon reveals more of the same red sand and creosote bushes. You've been lost and out of water for over a day and the heat is unrelenting. Everything starts happening more slowly. The sand sucks at your feet with each slow step and when you look down, you become dizzy and almost fall over. If your head stopped aching, maybe you could think straight and have a better chance of finding your way back. Exhausted and worried about these symptoms of dehydration, you sit down in the shade to rest. You hear movement under a nearby bush and look over to see a snake coiling up along a prey path. Its calm and unworried presence almost taunts you; how does this animal live in a place with so little water?

Finding enough water to survive can be a fierce challenge for many animals that live in dry (xeric) habitats. Humans and most other mammals need regular access to water, whether it is available as liquid, in food, or through the breakdown of tissue. The main strategy of mammals living in xeric habitats is to avoid dehydration, as most can't deal with high levels of solutes in the blood or low blood volume. When dehydration occurs, it can be stressful for mammals, increasing the production of stress hormones; this conclusion is often extended to include all vertebrates (with a few exceptions). However, some stress hormones can make things worse by raising metabolism and thus increasing

water loss. Chronic stress can eventually have negative effects on other body systems, such as the immune system.

Immunity is an important body function that keeps organisms healthy in a world filled with pathogens. Innate immunity is the body's first defense against intruders. It is a non-specific response that attacks any invaders that appear to be "non-self." This includes white blood cells that attack foreign material in the body, cellular enzymes that attack bacteria, and antimicrobial peptides, lytic proteins, and natural antibodies found in blood plasma that clump and destroy foreign cells. We know that the amount of energy or micronutrients that are available can affect immunity. However, water is an important resource that has not been well studied as a factor affecting immune function.

Many reptiles that live in xeric habitats must survive through hot summer months in which there is little or no rainfall. While some reptiles get a water benefit from food, others do not, and must tolerate periods of dehydration every year that may last weeks or months. If an animal is dehydrated regularly, minimizing the stress associated with this state would be beneficial. Thus, reptiles living in xeric habitats are good models in which we can test questions about the relationship between stress, dehydration, and the immune system. Specifically, we can test how dehydration affects animals that must tolerate it regularly. We can also compare the responses these species have to those of other reptile species that live in wetter habitats. Such research will provide insight into how habitats may have helped shape a species' immune and stress responses.

Dissertation Summary

In Chapter 2, I focus on two main assumptions: 1) dehydration is a chronic stressor that negatively affects immune function in most vertebrates; and 2) energy influences innate immunity more than water. I measured innate immunity in Gila monsters that were hydrated, dehydrated, and rehydrated, as well as in various stages of digestion. I found that dehydration increased two main measures of innate immunity: the ability of natural antibodies to identify and target foreign cells (agglutination) and the ability to destroy those cells (lysis). Additionally, I found that this increase was not solely due to the concentration of immune molecules as body water was lost. Further, an animal's hydration state was more important to this innate immune response than was the digestive state of the animal (though animals still had fat stores). My next step was to directly test if stress hormones increased during dehydration.

In chapter 3, I focus on the idea of dehydration as a potential stressor. I measured levels of corticosterone (a hormone that increases in circulation during stress) in Gila monsters when they were hydrated, dehydrated, and rehydrated. I also validated my previous innate immunity measurements and added measurements of bacterial killing ability of the bacteria *Escherichia coli* as well as relative numbers of white blood cells in circulation. Results confirmed my previous findings that dehydration increases various measures of innate immunity. White blood cell differential was not affected by hydration state. Dehydration also did not cause a significant stress response in Gila monsters, though the findings were close to significant. These results suggest that dehydration may not be stressful and that dehydration can increase certain measures of innate immunity. Such

findings then beg the question: is this lack of a stress response related to Gila monsters being adapted to a xeric habitat?

In chapter 4, I expand my view of the dehydration response to address the potential evolutionary drivers involved. Specifically, I tested whether the environment in which a species lives, in terms of water availability, has affected its response to dehydration. I ran the same assays as I used in Chapter 2 on seven additional squamate (lizard or snake) species. Each of the four pairs I used had 2 species from the same taxonomic family. One of each of these species was from a more xeric habitat, while the other was from a wetter or more mesic one. The response of each species was measured across hydric states, and the overall responses of species were compared between xeric and mesic groups. Results showed that there were no differences between species from the different environments, but that overall, hydric state affected nearly all measures of innate immunity and corticosterone concentration, most of which increased during dehydration. These results suggest that while environmental water availability may not be a driver of the dehydration response, dehydration positively affects stress levels and immune function in various squamate species. What's more, increased stress does not decrease immune function. Thus, it may be a common strategy for physiologically challenging conditions not to compromise important functions like immunity. In other words, physiological stress and immunity in these species may be decoupled.

In sum, my dissertation tests assumptions related to dehydration in vertebrates and it tests one potential evolutionary driver of the observed variation in responses. Understanding the dynamic relationship between dehydration, stress, and immunity will provide deep

insight into how two major physiological systems (endocrine and immune) interact in vertebrates. Such knowledge may help us understand natural disease transmission dynamics, better manage domesticated animal populations, and it may enable us to better predict how animals may respond to a changing climate. Future research can build on my dissertation by focusing on mechanisms, investigating how the endocrine and immune systems are decoupled in some reptiles. Additional work can focus on a broader scale, investigating the ecological relevance of these findings for free-ranging animals.

CHAPTER TWO:

The Effect of Hydration State and Energy Balance on Innate Immunity of a Desert Reptile

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Abstract

Immune function is a vital physiological process that is often suppressed during times of resource scarcity due to investments in other physiological systems. While energy is the typical currency that has been examined in such trade-offs, limitations of other resources may similarly lead to trade-offs that affect immune function. Specifically, water is a critical resource with profound implications for organismal ecology, yet its availability can fluctuate at local, regional, and even global levels. Despite this, the effect of osmotic state on immune function has received little attention.

Using agglutination and lysis assays as measures of an organism's plasma concentration of natural antibodies and capacity for foreign cell destruction, respectively, we tested the independent effects of osmotic state, digestive state, and energy balance on innate immune function in free-ranging and laboratory populations of the Gila monster, *Heloderma suspectum*. This desert-dwelling lizard experiences dehydration and energy resource fluctuations on a seasonal basis. Dehydration was expected to decrease innate immune function, yet we found that dehydration increased lysis and agglutination abilities in both lab and field studies, a relationship that was not simply an effect of an increased concentration of immune molecules. Laboratory-based differences in digestive

state were not associated with lysis or agglutination metrics, although in our field population, a loss of fat stores was correlated with an increase in lysis.

Depending on the life history of an organism, osmotic state may have a greater influence on immune function than energy availability. Thus, consideration of osmotic state as a factor influencing immune function will likely improve our understanding of ecoimmunology and the disease dynamics of a wide range of species.

Introduction

Immune function is vital to an animal's survival and its subsequent fitness (Lochmiller and Deerenberg 2000; Hanssen et al. 2004), yet is greatly influenced by sufficient availability of resources. Resources can become limited as a result of reduced intake or increased demand, oftentimes to simultaneously support multiple physiological functions (French et al. 2007). As resource intake and demand are condition-dependent and may change temporally, immune function can vary based on environmental context (French et al. 2009) or season or life stage (Buehler et al. 2008; Schwanz et al. 2011). The importance of such factors to alterations of immune function and resulting survival has led to the recent surge of interest in ecoimmunology.

Currently, ecoimmunology research pays considerable attention to the balance of energy investment in immune defense versus other physiological functions (e.g., reproduction (French et al. 2007; French et al. 2007b), sensory ability (Toomey et al. 2010), exercise

(Nebel et al. 2012). While energy allocation among physiological processes plays a critical role in life history trade-offs, immune function, like many other physiological functions, is not influenced solely by energetic resources. Non-energetic resources such as vitamins and trace elements can also modulate immune function (Wintergerst et al. 2007).

Water, another non-energetic resource, can greatly influence organisms due to its role in biochemical or physiological processes or traits, including cellular volume and composition (Hochachka and Somero 2002), heat shock protein production (Benoit et al. 2010), plasma hormone concentrations (El-Nouty et al. 1980), and membrane permeability (Sawyer and Schisgall 1956), as well as organismal development (Lorenzon et al. 1999). Thus, most organisms must actively osmoregulate to maintain normal physiological function. While some taxa maintain plasma osmolality at extremes (e.g., some arthropod hemolymph: 165 mOsm/kg; Woodring 1974, some marine taxa: ~1000 mOsm/kg; Yancey et al. 2002), terrestrial vertebrates typically maintain osmolality between 250 and 300 mOsm (Bradley 2009). However, to cope with water constraints, some species temporarily tolerate hyperosmotic states that can increase blood osmolality 20% or more (e.g., desert tortoise; Peterson 1996). Seasonal dehydration occurs in various species (Bradshaw 1986; Preest and Pough 1989), in a wide range of biomes, from deserts (Peterson 1996; Davis and DeNardo 2010) to pelagic habitats (Lillywhite et al. 2008), but dehydration can also occur on a daily basis (e.g., spotted salamander, *Ambystoma maculatum,* Pough and Wilson 1970) or over the course of several days (e.g., camel, *Camelus dromedaries,* Siebert and Macfarlane 1975).

Changes in osmotic state affect numerous aspects of organismal function, so it is reasonable to suspect that hydration may also influence immune function. However, to our knowledge, the interaction of osmotic state with immune function has received minimal attention. Intense exercise can decrease immune response of humans (Nieman 2007) and periods of drought can decrease immune response of kangaroos (King and Bradshaw 2010), suggesting that hydric state negatively affects immune function. However, these situations likely involve simultaneous changes in energy and water balance; thus, these findings may result from a decreased investment in immune function due to either limited energy availability or physiological changes associated with osmotic stress. Due to this confound of energetic and hydration state in previous studies, the distinct effects of water balance and energy availability on immune function remain unclear.

We tested the separate effects of water balance, digestive state, and energy balance on immune function in the Gila monster, *Heloderma suspectum*, a desert-dwelling reptile that annually undergoes a period of food and water deprivation during the hot, dry season in the Sonoran Desert. As this species' behavior seems to be more limited by hydric rather than energetic budgets (Davis and DeNardo 2009; Christian D. Wright unpublished observations), we hypothesized that while both hydric state and energy balance would affect immune function, hydric state would have the greater influence on immune function. Specifically, we predicted that innate immunity would be suppressed by a decrease in energetic state and by dehydration, and that this suppressed function would be more pronounced due to dehydration than to compromised energetic state. We collected plasma samples from free-ranging Gila monsters throughout the active season and

laboratory-housed Gila monsters with differential access to food and water, and tested for relationships between immune function and plasma osmolality, digestive state, and fluctuation of fat stores. In particular, we assessed innate immune function using an *in vitro* assay to examine lysis and agglutination capacity of the plasma. Agglutination of foreign red blood cells is a measure of the concentration of natural antibodies (molecules that are produced prior to antigen exposure and assist in foreign particle removal and complement-mediated lysis), whereas lysis assesses the plasma's ability to destroy foreign cells by rupturing them (Matson et al. 2005). While this assay is not intended to capture an individual's overall immunocompetence, it provides information regarding investment in innate immunity prior to an immune challenge and is useful when assessing innate immunity within populations (Matson et al. 2006), especially if sampling time points may be limited. Additionally, while reptiles have both innate and adaptive components of the immune system, the adaptive response is often much slower in ectotherms (by days to weeks; Zimmerman et al. 2010); thus, measurements of innate immunity (which acts as the first line of defense) may be the most ecologically relevant, especially if individuals have to deal with acute infection.

Materials and Methods

Study Animals

The Gila monster is a large-bodied, desert-dwelling lizard that has storage capacities that provide water reserves (in the urinary bladder) that can last up to 3 months and energy

reserves (in coelomic and tail fat bodies) that last even longer (Beck 2005; Davis and DeNardo 2007). These reserves enable Gila monsters to balance resource acquisition and expenditure over abnormally long periods relative to other lizards. Despite these extensive storage capacities, Gila monsters experience seasonal resource imbalances that can alter behavior patterns. For example, dehydration causes Gila monsters to reduce surface activity during the hot, dry season (Davis and DeNardo 2009).

Sample Collection

Blood samples were drawn from the caudal vein using heparinized 1 ml syringes. Plasma was separated from cells via centrifugation at 3000 rpm for 3 minutes and frozen at −80°C within 1 hour, with the exception of samples for the degradation trials (see below) and field samples. Field samples were collected at the beginning of each month of the active season (April through September) from 17 radio-tagged, free-ranging Gila monsters (9 females and 8 males). One female was gravid, so separate analyses were run including and excluding this individual. Due to reduced activity during certain periods, not all animals were sampled every month. Blood samples were collected within 5 minutes of capture and whole blood was placed on ice within 1 hour of sampling. As some trips to the field were longer than others, samples remained on ice for anywhere from 3 hours to 2 days before plasma was separated and frozen at −80°C.

Field-Based Correlation Between Hydration State and Immune Function

In 2010, we collected plasma samples from 17 free-ranging Gila monsters from a population in the Arizona Upland subdivision of the Sonoran Desert, roughly 30 km NNE

of Tucson. Adult Gila monsters (mean body mass = 396 g; range = 243 – 552 g) were implanted with 13 g radiotransmitters (model SI-2, Holohil Systems Ltd., Ontario, Canada; see Davis and DeNardo (2009) for procedure description) and tracked at least weekly. To evaluate energy balance, variation in tail volume (±1 ml) was recorded using water displacement whenever a plasma sample was acquired from an animal. Serial tail volume measurements provide an effective index of changes in an individual's stored energy over time because Gila monsters store energy reserves caudally (Bogert and Del Campo 1956; Beck 2005) and tail volume is not influenced by fecal elimination or water intake (Davis and DeNardo 2007). We determined plasma osmolality and performed agglutination and lysis assays on aliquots of each field sample.

Laboratory Trials

All other studies were conducted in the laboratory in 2010 and 2011 using a long-term captive population of wild-caught Gila monsters (mean mass = 497 g; range = 420 – 602 g). These Gila monsters were typically housed in individual cages (35 × 75 × 12.5 cm; Freedom Breeder, Turlock, California, USA) where they had continuous access to water, refugia, and a thermal gradient (25 to 35°C). For dilution trials (see below), animals were housed as described, but with no access to water. During the initial dehydration trial (see below), animals were housed in individual cages (24 × 36 × 13 cm; United States Plastic Corporation, Lima, Ohio, USA) with a modified screen top within an environmental chamber held at a constant 30°C, which approximates the species' preferred body temperature (Beck 2005). During this trial, the animals had no access to water or refugia. All laboratory Gila monsters were maintained in good body condition on a diet of adult

mice, but were fasted for at least 14 days before the beginning of each trial to attain a post-absorptive state and thus standardize digestive state. All females were non-reproductive, as confirmed using ultrasound. Digestive and degradation trials involved 10 animals (each trial used the same three females and seven males), and the laboratory dehydration trials involved a subset of six of these animals (one female and five males). Four Gila monsters were used in the dilution trial (one female and three males), two of which were also used in all other laboratory trials. Gila monsters used in multiple trials had at least 2 weeks of water access between trials.

Laboratory Dehydration Trial

For initial dehydration trials, six animals had their bladders drained via transurethral catheterization as described in Davis and DeNardo (2007). They were then housed within an environmental chamber (30°C air temperature, affluent air at 2°C dew point) in individual containers. To monitor plasma osmolality, each animal was bled (0.15 ml) within 2 hours of catheterization, once more during the first week (0.07 ml), and twice per week (0.07 ml) thereafter until the animal reached a state of moderate dehydration (plasma osmolality of 305–335 mOsm). At this point, a second 0.15 ml sample was obtained. Plasma continued to be monitored twice per week until the animal showed clinical signs of severe dehydration (e.g., lethargy, plasma osmolality above 340 mOsm). Once severely dehydrated, a third 0.15 ml blood sample was taken, and the animal was provided with water to rehydrate. Two final 0.15 ml blood samples were taken 24 and 48 hours post-rehydration. We used the initial, moderately dehydrated, severely dehydrated,

24-hour post rehydration, and 48-hour post rehydration samples in agglutination and lysis assays to evaluate the effect of hydration state on innate immunity.

Plasma Dilution Trial

Dilution trials were conducted to test whether any differences in immune function due to hydration state of the animal could be explained purely by plasma osmolality during assaying. An initial 0.15 ml blood sample was collected from four laboratory animals for osmolality and lysis and agglutination analyses. Animals were held without food or water, and body masses were closely monitored as a rough indicator of water loss (since energy loss over this time would be minimal). Additionally, blood samples (0.07 ml) were taken twice per week to monitor plasma osmolality. Once an animal had lost 15% of its initial body mass or had reached a plasma osmolality of over 320 mOsm (reaching moderate to severe dehydration according to clinical signs shown by some Gila monsters in this hydration state), a final 0.15 ml blood sample was collected from the animal. An aliquot of plasma (60 µl, with the exception of one 30 µl aliquot from a sample that required a repeated osmolality analysis) from each of these dehydrated state samples was then diluted with nanopure water (5 to 10 µl) to generate a sample from a dehydrated animal but with an osmolality similar to that of the animal's plasma when in a hydrated state. We used the initial hydrated (20 µl plasma), the dehydrated (20 µl plasma), and the diluted dehydrated (15.4-16.8 µl plasma with 3.2-4.6 µl nanopure to make 20 µl total) samples from each individual to compare osmolality, lysis, and agglutination.

Digestive State Trial

Effects of digestive state were assessed using 10 adult Gila monsters. After an initial blood sample (0.07 ml, day 0) was collected, each animal was fed a mouse meal equal to 20.0 ± 0.5% of its body mass. Additional 0.07 ml blood samples were then collected at day 2 (representing peak metabolic investment into digestion), day 5 (non-peak investment into digestion), day 12 (post-absorptive with minimal to no investment into digestion), and day 20 (when animals were definitively post-absorptive), based on Christel, DeNardo, and Secor (2007).

Sample Degradation Trial

As the blood samples for the field component of this study were initially collected for a separate study for which immune molecule degradation was not a concern, time elapsed prior to freezing of samples was not recorded. To test for possible effects of sample storage time on the immune assays, we collected blood samples from 10 Gila monsters and refrigerated the samples immediately at 2°C to mimic conditions that some field samples experienced. Whole blood aliquots were removed from the refrigerated sample 0 (immediately), 2, 5, and 15 days later, plasma was separated from aliquots, and plasma was frozen at −80°C until analyzed. These sample points represent a range of degradation times, including those our laboratory samples were subjected to (immediately: 0 days), those which our field samples were subjected to (0 to 2 days), as well as time points (5 and 15 days) beyond the degradation time experienced by any of our samples.

Sample Processing: Determination of Plasma Osmolality

Plasma osmolality was determined for all samples except those from the digestive state and sample degradation trials. During the latter trials, water was provided throughout the trial in order to assure a normosmotic state and thus enable us to tease apart the effects of digestive state or degradation from those due to dehydration. Osmolalities were determined in triplicate using a vapor pressure osmometer (±6 mOsm/kg; model 5500xr; Wescor, Inc., Logan, Utah, USA) as described in Davis and DeNardo (2007).

Sample Processing: Natural Antibody Agglutination and Lysis Assay

To assess natural antibody agglutination and lysis of all samples from all trials, we followed the protocol of Matson et al. (2005) with several modifications. 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. Plasma was not added to the PBS in the final row in the well to act as a negative control. We then added 20 µl of 50% heparinized whole sheep blood (SBH050, HemoStat Laboratories, Dixon, California, USA) diluted 1:100 to each well. Each plate was gently vortexed, covered with Parafilm, and because temperature can significantly affect lysis and agglutination scores (Butler et al. 2013), we incubated for 90 minutes in a 29°C water bath to approximate the mean diurnal temperature of free-ranging Gila monsters during the active season (Beck 2005). To improve visualization, we then tilted the plates at 45º for 20 minutes at room temperature (Matson et al. 2005), after which they were scanned at 600 dots per inch using a flat-bed scanner (Hewlett-Packard Co., ScanJet 3670) for agglutination images. The plates were

then left flat at room temperature for 70 minutes, after which they were rescanned for lysis images (see Matson et al. (2005) for scoring procedures). Scanned images of the plates were visually scored by KTM and MWB independently. Each scorer selected the well along each row in which definitive agglutination (denoted by preventing the formation of a dense RBC pellet) or lysis (denoted by the lack of compartmentalization of red pigment) had occurred; both KTM and MWB's scores were repeatable (see Lessells and Boag (1987) for repeatability analysis procedure) for both agglutination ($R = 0.99$, $P < 0.0001$) and lysis ($R = 0.96$, $P < 0.0001$). Thus, we used average values for all subsequent analyses. To determine repeatability of samples among plates, we also ran aliquots of six samples on each of five different plates, and these samples were significantly repeatable for both agglutination ($R = 0.83$, $P < 0.0001$) and lysis ($R = 0.88$, $P < 0.0001$).

Details Regarding Sample Degradation

We found that agglutination and lysis abilities of plasma degraded when the samples were kept on ice and not quickly frozen. It is unlikely that these differences were due to changes in osmolality of the plasma, as the samples were stored in air-tight containers, yielding minimal opportunity for evaporation to occur, and our dilution results indicate agglutination and lytic capabilities were not altered by concentration effects. Therefore, it is likely that the natural antibodies and/or complement proteins themselves were degrading when not frozen. As this difference manifested within just 48 hours, we recommend the centrifugation and freezing of plasma on the same day, if not within a

few hours, of collection. However, the specific rate of sample degradation on a finer scale (hours) has yet to be examined, so we cannot provide a more specific recommendation.

Statistical Analyses

To test for the effects of season, body condition, and osmolality on lysis and agglutination in animals from the field, we performed a mixed model analysis with either osmolality or change in tail volume as a covariate, month of capture as a fixed effect, and individual as a random effect. Since animals from the laboratory dehydration trials dehydrated at different rates and were therefore at different hydration states on different days, we tested for effects of hydric state on either lysis or agglutination using two different mixed models, with either the continuous variable of osmolality or the class variable of hydration state (hydrated, 270–300 mOsm; moderately dehydrated, 305–335 mOsm; dehydrated, 340+ mOsm, rehydrated, 1 day post-drinking; rehydrated, 2 days post-drinking) as the independent variable, individual as a random effect, and either lysis or agglutination score as the dependent variable. To directly test the effects of plasma osmolality on lysis and, separately, agglutination, we performed repeated measures analyses of variance (rmANOVAs) on scores from hydrated, dehydrated, and diluted plasma samples from each of four individuals. We also used rmANOVAs to test for the effects of digestive state and degradation on either lysis or agglutination scores. All statistics were performed using SAS 9.2 (Cary, NC) and least squares means (LSMeans) are reported ± standard error.

Results

Field Correlations

Month of sampling did not affect lysis ($F_{7,43} = 1.55$, $P = 0.18$) or agglutination ($F_{7,43} = 1.66$, $P = 0.15$) scores in free-ranging Gila monsters. When the single gravid female was removed from the analysis, month of sampling still did not significantly affect lysis ($F_{7,39} = 1.49$, $P = 0.20$) or agglutination ($F_{7,39} = 1.54$, $P = 0.18$). Change in tail volume was negatively related to lysis ($F_{1,21} = 7.74$, $P = 0.011$), yet this relationship was no longer statistically significant with the removal of the gravid female from the analysis ($F_{1,18} = 3.76$, $P = 0.068$). No significant relationship was detected between tail volume and agglutination ($F_{1,21} = 3.61$, $P = 0.071$ with gravid female; $F_{1,18} = 1.32$, $P = 0.27$ without gravid female). Sex did not significantly affect lysis ($F_{1,43} = 0.00$, $P = 0.99$) or agglutination ($F_{1,43} = 0.00$, $P = 0.98$) in free-ranging Gila monsters even when the gravid female was removed from analysis (lysis, $F_{1,39} = 0.07$, $P = 0.79$; agglutination, $F_{1,39} = 0.06$, $P = 0.81$). Elapsed time prior to freezing was not recorded for the field samples and thus was not included in the analysis. Despite the potential for increased variability associated with sample degradation, plasma osmolality was positively related to both lysis ($F_{1,43} = 6.40$, $P = 0.015$) and agglutination ($F_{1,43} = 7.83$, $P = 0.0077$) scores (Figure 2.1), and the statistical significance of the results persisted even when a gravid female was excluded ($F_{1,39} = 6.17$, $P = 0.017$ for lysis; $F_{1,39} = 7.62$, $P = 0.0087$ for agglutination). These findings support a strong association between hydric state and innate immunity even with the confound of varying (and unknown) degradation time.

Laboratory Dehydration Trial

Gila monsters took 28 ± 7 days to reach clinical dehydration, but returned to a fully hydrated state within 24 hours of drinking. Plasma osmolality over the course of dehydration and rehydration was positively related to both lysis ($F_{1,29} = 34.35$, $P < 0.0001$) and agglutination ($F_{1,29} = 22.56$, $P < 0.0001$) ability when osmolality was a continuous covariate. Sex did not affect lysis ($F_{1,29} = 0.06$, $P = 0.81$) or agglutination ($F_{1,29} = 0.00$, $P = 0.99$). Similarly, when samples were analyzed as a class variable based on dehydration state, there was a significant effect of dehydration state on both lysis ($F_{4,26} = 4.65$, $P = 0.0057$) and agglutination ability ($F_{4,26} = 8.63$, $P = 0.0001$), but no effect of sex on lysis ($F_{1,26} = 0.06$, $P = 0.81$) or agglutination ($F_{1,26} = 0.00$, $P = 1.0$). In both analyses, lytic and agglutination ability were positively related to dehydration state (Figure 2.2).

Plasma Dilution Trial

Plasma treatment (hydrated animal, dehydrated animal, diluted plasma from dehydrated animal) significantly affected both lysis ($F_{2,4} = 12.12$, $P = 0.020$) and agglutination ($F_{2,4} = 7.75$, $P = 0.042$) scores. For both metrics of immune function, hydrated samples had statistically lower performance than both dehydrated and diluted samples (Figure 2.3). Lysis data were normally distributed ($P = 0.57$), but agglutination data were not ($P = 0.026$), thus we suggest applying this result with caution. Effects of sex were not tested because we used only one female and three males, which would lead to erroneous statistical conclusions.

Digestive State Trial

Neither lysis ($F_{4,34} = 0.30$, $P = 0.88$) nor agglutination ($F_{4,34} = 0.94$, $P = 0.45$) ability changed with digestive state (Figure 2.4). Sex did not affect analysis for either lysis ($F_{1,8} = 1.39$, $P = 0.27$) or agglutination ($F_{1,8} = 2.95$, $P = 0.12$).

Sample Degradation

Samples degraded over time when left in the refrigerator prior to freezing, with both lysis ($F_{3,24} = 44.52$, $P < 0.0001$) and agglutination ($F_{3,24} = 19.10$, $P < 0.0001$) scores decreasing (Figure 2.5; see Additional file 1 for further discussion). Post-hoc tests of LSMeans revealed that agglutination values were significantly lower after 5 and 15 days of refrigeration (both $P < 0.0001$), and lysis scores were significantly lower after 2, 5, and 15 days of refrigeration (all $P < 0.013$). Sex affected lysis ($F_{1,8} = 11.05$, $P = 0.0105$) and agglutination ($F_{1,8} = 12.42$, $P = 0.0078$), but there was no significant interaction between sex and day for either lysis ($F_{3,24} = 0.84$, $P = 0.49$) or agglutination ($F_{3,24} = 3.74$, $P = 0.025$). Agglutination of samples from females was greater than that of males on days 2 ($P = 0.0472$) and 15 ($P < 0.0001$; LSMeans F: 3.75, SE: 0.329; M 1.75, SE: 0.215). Due to the low number of females in our analysis (3 females to 7 males), we did not interpret these sex differences, but encourage including sex as a factor in future studies of sample degradation.

Discussion

Previous studies have examined the effect of dehydration on some physiological systems (Lorenzon et al. 1999; Hochachka and Somero 2002), but exploration of the effects of dehydration on the immune system has been relatively rare. Our data demonstrate that osmotic state can alter immune function, and does so in a surprising fashion; despite our expectations of a decreased immune response during times of dehydration, we consistently observed that Gila monsters have more robust innate immune function during bouts of dehydration. Specifically, our results from both field and laboratory experiments show a strong, positive correlation between plasma osmolality and lysis and agglutination capacity of plasma on both continuous and discrete scales of hydration state (Figures 2.1 and 2.2, respectively). Though isolated assessments of immune function do not fully represent the suite of alterations in immunity that may be occurring within an organism, as different combinations of protective measures can achieve the same level of defense (Matson et al. 2006), the increased response of plasma during dehydration indicates that the innate humoral immune system exhibits increased reactivity during these times. These findings provide a new context that will likely enhance the general understanding of the influences of resource limitations on the physiology and ecology of a wide range of species.

A corresponding increase in immune response and osmolality may serve to prime the immune system with a more robust first line of defense during times of the greatest osmotic stress, when mounting a more expensive response may be impractical. In organisms that do not tightly regulate plasma volume, such a response could be solely a

function of the concentration of immune molecules (e.g., natural antibodies) within the plasma. In dehydration trials, osmolality increased with a concurrent decrease in body mass, indicative of decreasing plasma volume (and potentially a concentrating effect). However, diluting dehydrated samples back to the osmolality of hydrated samples did not decrease the plasma capacity for agglutination and lysis to the levels expected if concentration were the sole influencing factor. This suggests that increased immune function is not strictly bound by the increases in solute concentrations associated with dehydration, but is rather augmented by an additional physiological effect that may be employed during times of osmotic stress.

While this study did not explicitly identify the mechanistic reasons behind the relationship between osmolality and immune function, it did lead to several functional hypotheses. The increase in agglutination and lysis ability could be due to an increased investment in less energetically expensive, innate immune defenses (Lochmiller 2000) during times of osmotic stress. While the data regarding the role of osmolality and osmotic stress in immune cell function are currently lacking, it is reasonable that immune cells are negatively affected by dehydration just like other cell types (Franca et al. 2006). Thus, an investment in proteins that can perform basic immune functions during periods of osmotic stress while reducing investment in cellular processes (e.g., phagocytosis, cell-mediated immune responses) could be evolutionarily adaptive. Indeed, shifts in utilization of different components of the immune system based on the environment have been documented previously in fish, with innate immune function being utilized more readily at lower temperatures, whereas acquired immunity is relied upon at higher temperatures (Magnadóttir 2006). The correlation of lysis and agglutination scores within

a sample (r = 0.93, $P < 0.0001$) may support the use of basic immune functions during osmotic stress. While this correlation is not uncommon, these measures are not always positively correlated (Buehler et al. 2011); additional immune assessments may help clarify the importance of this finding.

The purpose of the increased immune function we observed also remains to be experimentally investigated; for example, it is possible that these higher metrics provide increased protection against pathogens that are more prevalent during the dry season, or that the individual is reallocating resources to a less effective, but physiologically cheaper, method of immune defense during a stressful period. While immune function and water balance may be physiologically linked (e.g., non-specific protein kinases are involved in signaling pathways for both immune function and cell volume regulation (Choe and Strange 2009); keratinocytes contribute to both immune defense and water conservation (Kattan and Lillywhite 1989; Drake et al. 2008)), we still lack adequate understanding of how the interaction of immune function and hydration affects organismal ecology.

Osmolality-based differences in immune function may have profound ecological effects, through, for example, alteration of disease transmission. In some species, communal conditions may occur during times of osmotic stress, creating ample opportunity for disease transmission, such as in animals that use limited subterranean refugia or those that live in areas where preferred habitats become increasingly patchy during the dry season (Crump and Pounds 1989; Longo et al. 2010). Many desert tortoise populations in the Mojave Desert have declined due to increased mortality rates resulting from upper

respiratory tract infections (Brown et al. 1994). Additionally, the disease-causing fungus *Batrachochytrium dendrobatidis* associated with amphibian declines appears to more strongly decrease population size during dry seasons that have an increased number of dry spells (Longo et al. 2010). Estimates of the force of infection (i.e., probability of transmission) may be altered if the hot, dry season (during which animals experience osmotic stress) affects immune function, either severely increasing or decreasing rates of transmission, thereby making individuals more susceptible to infection on a seasonal cycle.

For species that experience regular or prolonged periods of dehydration, any effects of increased osmolality on immune function could affect individual survival. Gila monsters have high rates of injury in some populations (at a natural and an urban site, scarring was observed in roughly 32% and 71% of adult individuals, respectively; Jon R. Davis unpublished observations), likely making immunity an important investment even during periods of dehydration. Additionally, in the wild, dehydration bouts are frequently nested within season, and seasonal investment in immune function may be adaptive if there is a predictable seasonal variation in pathogen prevalence or virulence. However, the lack of a monthly effect in our field study, as well as the rapid reduction in innate immune function after rehydration in the lab demonstrates that the variation in innate immunity associated with hydration state is not a result of seasonal adjustments in immune strategies.

While our results are directly relevant to animals that dehydrate seasonally (including periods of drought and other resource-limited seasons, such as winter, when many

animals are dormant), these results may also be relevant to animals that experience changes in hydration state on shorter time scales, as was observed during rehydration trials. Thus, fluctuations in osmotic state may be an important factor to include in estimates of disease transmission or general efforts to understand the ecoimmunology of various species. Further investigation regarding how short-term variation in hydration state may affect immune function in a more ecologically relevant context (e.g., during wound healing) will increase our understanding of the implications of these results.

In contrast to our finding that immune function varied with hydration state, we found no evidence that digestive state affected immune function. Immune responses are energetically expensive (Dantzer 2004) and can result in an increased metabolic load (Ardia et al. 2012). However, an immune response is frequently accompanied by the induction of anorexia, which may allow for the utilization of physiological pathways that enhance immune function (Adamo et al. 2010), highlighting the context-dependent relationship between energy acquisition and immune function. While food intake has been shown to affect the immune response in other lizards during times of high energy demand (French et al. 2007b), Gila monsters may be less sensitive to food intake because their life history includes frequent extended bouts of aphagia and thus they rely heavily on energy stores to support physiological functions. Such species may be more likely to maintain a constant investment in immune function as long as adequate energy stores exist.

Changes in tail volume provide us with a metric for relative energy stores in individual Gila monsters. An increase in tail volume, indicative of increased energy stores and

positive energy balance, was negatively associated with lysis, but not agglutination. While the exclusion of a gravid female made this finding non-significant ($P = 0.068$), the suggested trend of decreasing lysis with increasing tail volume may indicate a trade-off between innate and cell-mediated immune responses, as potential differences in costs and benefits may alter the advantage of these respective responses with ecological context (Lochmiller and Deerenberg 2000; Magnadóttir 2006). Further research to test this idea is needed and must be reconciled with the lack of effect on agglutination and the potential influence of reproductive states. The lack of correlation between lysis or agglutination and change in tail volume, in conjunction with the finding that changes in energetic state over shorter time periods (i.e., in the lab feeding trial) did not affect agglutination or lysis, suggests that ecologically relevant variation in energetic intake or balance has a minimal effect on these measures of innate immunity, at least in this species. Because we detected differences in immune function due to osmotic state, future studies evaluating various immune metrics during periods of positive and negative energy balance as well as during different hydration states will help clarify how apparently complex immune strategies (Matson et al. 2006) are associated with both energy and water availability.

Conclusions

According to the present understanding of ecoimmunology, availability of energetic resources is a primary factor influencing the immune response of individuals across numerous taxa. However, using both laboratory and field studies, we show that osmotic

state can have a greater influence on immune function than digestive state or fluctuations in the extent of energy stores. It remains unclear whether dehydration-induced shifts occur differently between specific components of the immune system or whether the entire immune system is systemically suppressed or enhanced, and studies to test this possibility are greatly needed. Overall, this study emphasizes the need for further research into the effects of hydric state on immune function; this relationship may have important implications for understanding the causes of individual variation in ecoimmunology, as well as changes in rates of disease transmission in some species.

Figure 2.1 Agglutination and lysis capacities of free-ranging Gila monsters. Agglutination and lysis scores as a function of (A, B) plasma osmolality, (C, D) month of sampling, and (E, F) change in tail volume in free-ranging Gila monsters. Individuals with a greater plasma osmolality (i.e., more dehydrated) had greater agglutination and lysis scores. Change in tail volume (a measure of energy balance) was negatively related to lysis ($F_{1,21} = 7.74$, $P = 0.011$), but after removing a gravid female from analysis, this was non-significant ($F_{1,18} = 3.76$, $P = 0.068$), a change denoted with a dashed line. Change in tail volume had no significant relation ($F_{1,21} = 3.61$, $P = 0.071$) to agglutination. There was no effect of month on either agglutination or lysis ability (all $P > 0.05$). No individual was disproportionately influential (all Cook's D < 4/n) in these analyses. Raw data are presented, uncorrected for individual. LSMeans with error bars (SE) from the mixed model are presented for month of sampling.

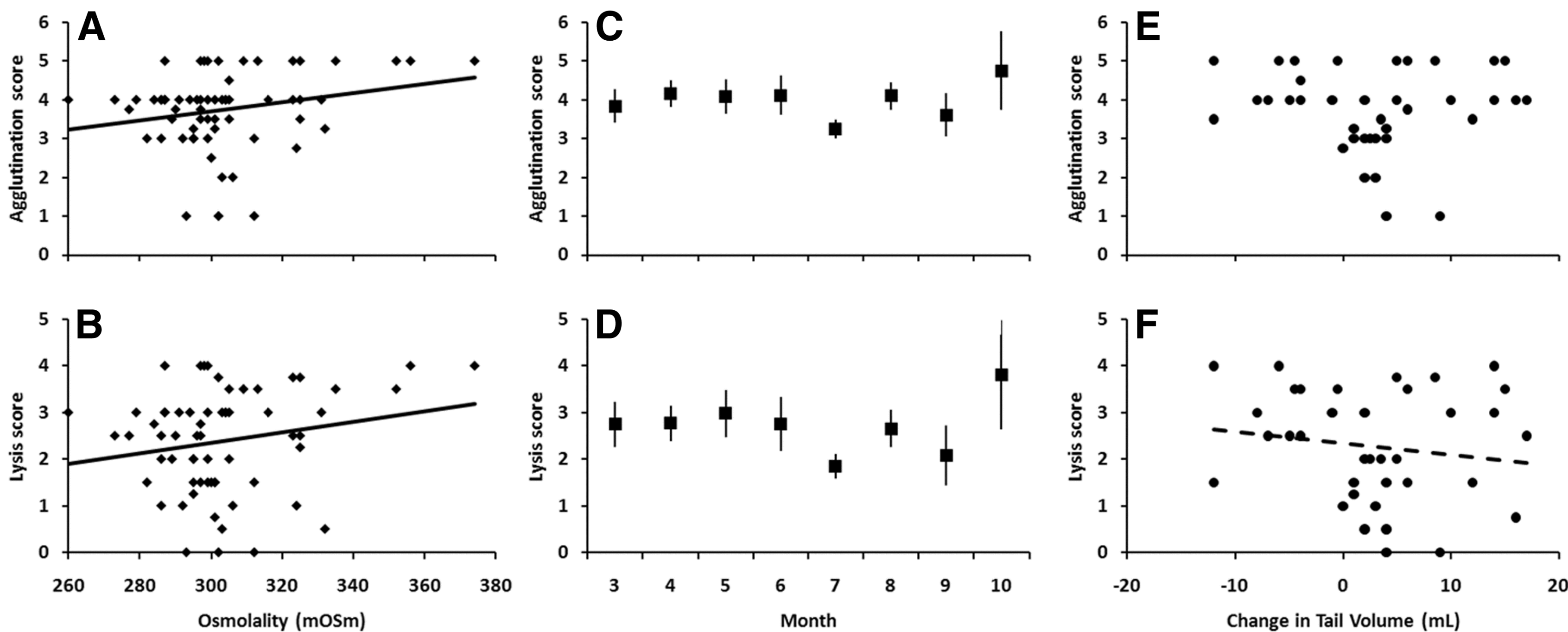


Figure 2.2. Agglutination and lysis capacities of captive Gila monsters as a function of hydration state. Individuals had more robust (A) agglutination and (B) lysis scores as they dehydrated, with immune function decreasing to (for agglutination) and even beyond (for lysis) baseline samples post-drinking. Here, hydration state is defined as: hydrated (270–300 mOsm); moderately dehydrated (305–335 mOsm); severely dehydrated (340+ mOsm), 1 day post-drinking (285–295 mOsm); 2 days post-drinking (255–285 mOsm). Groups that share the same letter have LSMeans that are not statistically different ($P > 0.05$). Bars represent LSMeans, with error bars showing SE.

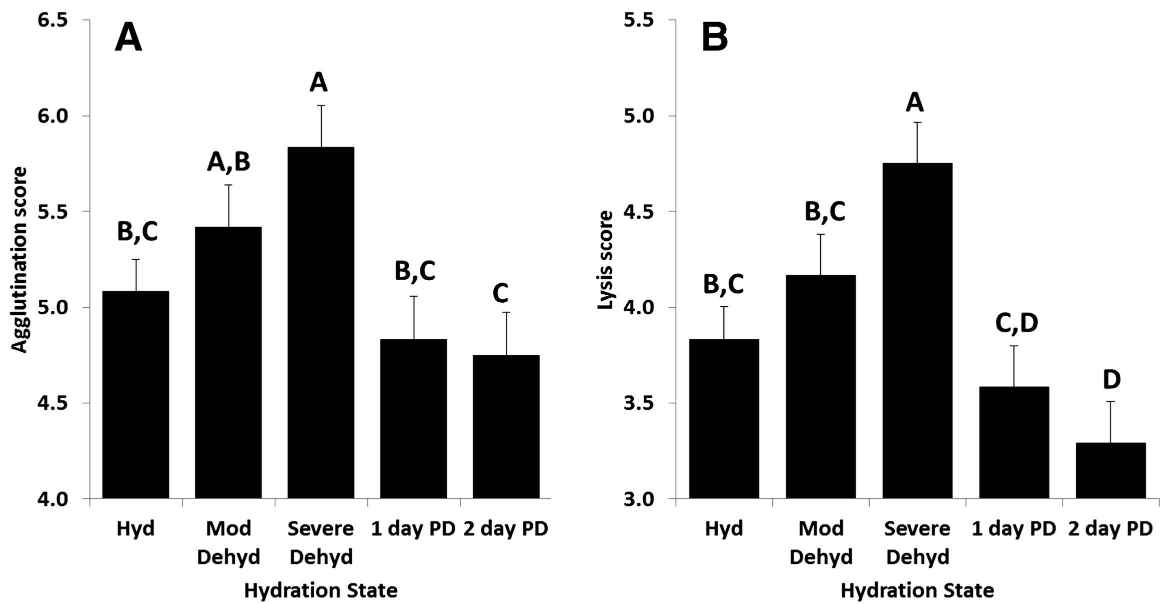


Figure 2.3. Effects of dehydration and dilution on agglutination and lysis capacities of captive Gila monsters. (A) Agglutination and (B) lysis scores of plasma from captive Gila monsters tested in both hydrated and dehydrated states, as well as plasma from Gila monsters in the dehydrated state that was diluted with nanopure water by 16 to 23% to match the osmolality of the individual's hydrated plasma sample. The hydrated state samples had significantly lower agglutination and lysis scores relative to both non-manipulated and diluted dehydrated samples. Groups that share the same letter have LSMeans that are not statistically different ($P > 0.05$). Bars represent LSMeans, with error bars showing SE.

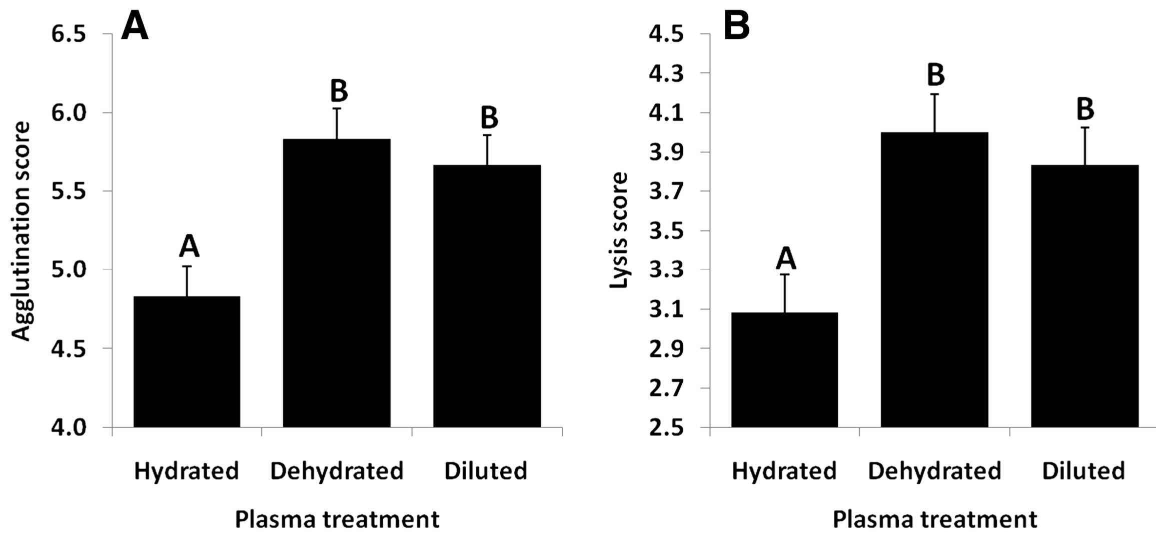


Figure 2.4. Effects of digestive state on agglutination and lysis capacities. (A) Agglutination and (B) lysis scores from captive Gila monsters as a function of digestive state. Individuals were given a meal (approximately 20% of body mass) immediately after blood collection on day 0. Neither agglutination nor lysis scores significantly varied ($P > 0.05$) over the duration of meal absorption. Bars show LSMeans and error bars represent SE.

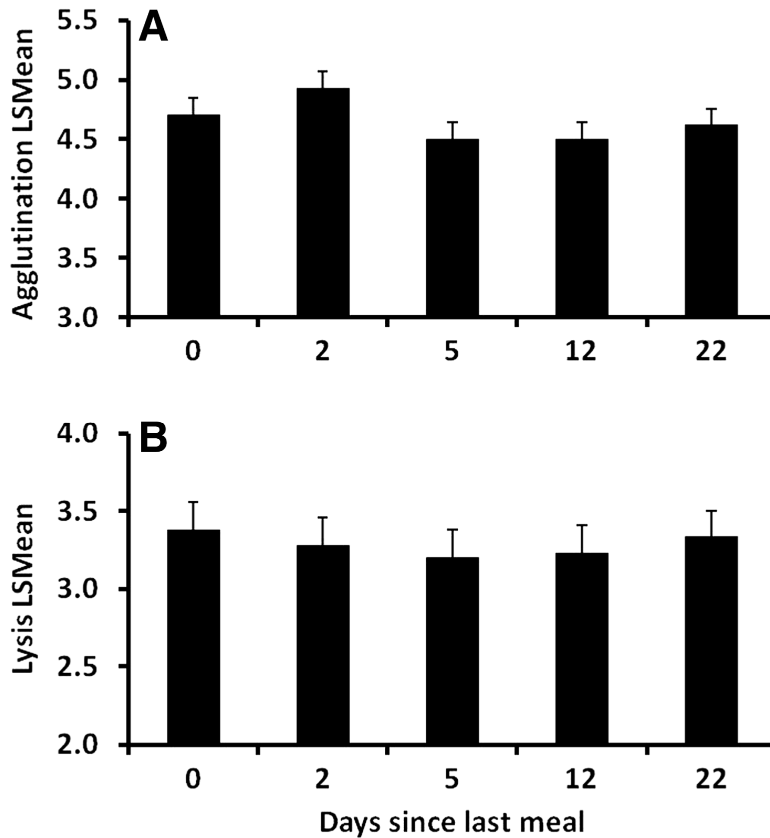
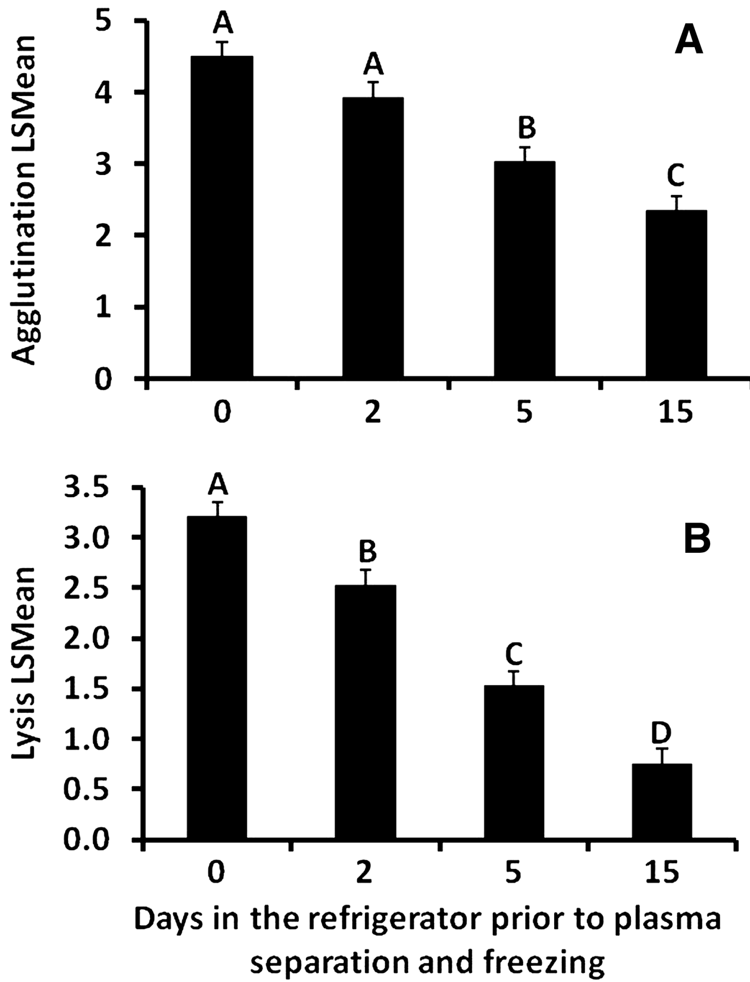


Figure 2.5. Degradation of agglutination and lysis capacities over time. Number of days in the refrigerator prior to plasma separation and freezing significantly reduced both (A) agglutination and (B) lysis scores. Groups that share the same letter have LSMeans that are not statistically different (all $P > 0.05$), although agglutination scores on days 0 and 2 approached significance ($P = 0.058$). Bars show LSMeans and error bars represent SE.



CHAPTER THREE:

Is Dehydration Stressful in a Xeric-Adapted Reptile?

Abstract

The challenge of dehydration often induces an increase in plasma glucocorticoid (e.g., cortisol or corticosterone; CORT) concentrations, a response that can negatively impact immune function. However, data clarifying the interactions of this response, which I term the "vertebrate dehydration cascade," are lacking, as each step of the cascade has not been well studied within a single species. Additionally, there is a lack of data supporting either of these physiological steps among xeric-dwelling species where substantial dehydration can be a predictable, seasonal occurrence. Previously, I demonstrated that Gila monsters (*Heloderma suspectum*), which inhabit xeric environments, have enhanced measures of innate immunity when dehydrated. This suggests that, in this species, dehydration may not induce a glucocorticoid response. To explore this possibility, I examined multiple measures of innate immunity and the glucocorticoid response in captive and free-ranging Gila monsters at various hydration states. The results show that dehydration does not cause a substantial increase in baseline plasma glucocorticoids, and I validate that dehydration improves various measures of innate immune function in this species. These findings support the hypothesis that physiological responses to dehydration depend heavily on an organism's ecology. More research on the effects of dehydration on the glucocorticoid response and immunity will help clarify the interactive roles they play in response to hydric challenges and whether adaptations to the environment influence these interactions.

Introduction

Water imbalance in organisms, even at non-lethal levels, affects a variety of biological functions. Dehydration reduces productivity (e.g., in plants; Farooq et al. 2009), alters blood chemistry (Hamadeh et al. 2006), and causes other alterations in physiology or behavior (Olsson 2005; Dupoué et al. 2014). These adjustments may slow water loss (e.g., through modified activity patterns; Davis and DeNardo 2010), alter growth patterns (Lorenzon et al. 1999; Doğramacı et al. 2014), alter protein production and gene transcription (Benoit et al. 2010; Junttila et al. 2013), or otherwise assist in prevention or tolerance of dehydration (e.g., through accumulation of osmolytes; Hare et al. 1998; Watanabe et al. 2002).

Regardless of whether such alterations occur, some species tolerate considerable variation in hydration state (i.e., dehydration or hypohydration) as measured by increased plasma solute concentrations and mass loss due to water loss (in the dromedary or Arabian camel (*Camelus dromedarius*), Schmidt-Nielsen et al. 1956; in the desert tortoise (*Gopherus agassizii*), Peterson 1996; in various dormant insects, Danks 2000). Furthermore, despite documented detrimental effects of dehydration (e.g., increases in oxidative stress (Franca et al. 2006), decreases in energy availability in the cells of specific organs (e.g., ATP in the liver; Churchill and Storey 1995), and decreases in aerobic performance (Barr 1999)) some species have improved immune performances during dehydration, such as was found in the Gila monster (Moeller et al. 2013). I predict that such a counterintuitive response is possible due in part to the absence of a substantial glucocorticoid response to dehydration in species that experience predictable seasonal

droughts. While it is unclear if increased immune function during dehydration improves fitness or chances of survival, it is likely that an interaction with the endocrine system is an important piece in this puzzle.

Endocrine responses are often an integral part of an organism's response to dehydration (Sebaai et al. 2002; Maresh et al. 2004). In particular, corticosterone (CORT; cortisol in humans) may be produced as a result of dehydration (Parker et al. 2004; King and Bradshaw 2010), and this hormone affects immune function in various ways (Webster Marketon and Glaser 2008; Martin 2009; Stier et al. 2009). For example, CORT may have a depressive effect on the synthesis or release of immune-promoting molecules, or may stimulate or depress the proliferation of B and T cells, depending on physiological conditions (Sapolsky et al. 2000). In general, acute stress seems to have more enhancing effects, while chronic stress seems to have more depressive effects, though response may also depend on concentration (Sapolsky et al. 2000). Release of CORT is often thought to be a part of a stress response, the organism's reaction to an unpredictable stressor (Koolhaas et al. 2011). An increase of CORT in dehydrated animals is widespread in previously studied vertebrates (e.g., in mice: Tsuchida et al. 2004, Roberts 2011, Bekkevold et al. 2013; in rats, Sebaai et al. 2002, Arnhold et al. 2007; in rabbits, Kallaras 2004; in quail, Cain and Lien 1985; in chicken, Klandorf 1984; in cattle, Parker et al. 2004, etc.). Additionally, the decrease of immune function in vertebrates with chronically elevated levels of CORT has also been repeatedly shown (reviews in McEwen et al. 1997; Sapolsky 2000). Because these two responses are well established, a linkage between them is somewhat assumed—namely, dehydration increases CORT, which decreases immune function. However, the study of the three components of this

relationship (dehydration, the stress response, and immunity), which I term the "vertebrate dehydration cascade," have not been well studied in the same individuals of a single species. Additionally, the separate steps of the dehydration cascade have not been studied extensively in vertebrates that naturally experience seasonal drought.

The release of CORT in response to dehydration is often expected, but it is not a universal response to dehydration among vertebrates. While the CORT response to dehydration is found in some animals that are not domesticated (e.g., bullfrogs, Belenkii 1991), it is lacking in others (e.g., Children's pythons; Dupoué et al. 2014). This first step of the vertebrate dehydration cascade also may not be observed in humans (Malarkey et al. 1993; Hoffman et al. 1994; but see Maresh et al. 2006) or Awassi sheep (*Ovis aries*; Jaber et al. 2004; Hamadeh et al. 2006; but see Ghanem et al. 2008). Clearly, there must be underlying factors causing this varied response.

One major underlying factor that may influence whether dehydration elicits a glucocorticoid stress response may be environmental conditions. Birds that experience unpredictable or harsh (potentially stressful) conditions may be able to avoid either exhibiting or responding physiologically to an increase in glucocorticoids, at least during part of the reproductive season (Wingfield et al. 1995; Wingfield and Hunt 2002). Similarly, there is some evidence that suggests animals that annually experience prolonged bouts of dehydration may not respond to the condition as a stressor (which would potentially result in glucocorticoid production or decreased immune function). Dupoué and colleagues (2014) did not see elevated baseline CORT levels in Children's pythons (*Antaresia childreni*) that were deprived of water for 52 days. In many parts of

their range in northern Australia, these snakes experience drought from April to November. Hamadeh and colleagues (2006) observed decreased baseline levels of CORT in dehydrated Awassi sheep ewes. Awassi sheep are well adapted to arid regions, as they originate from the Syro-Arabian Desert, which is a mix of steppe and true desert habitat with large swaths of dry desert and occasional oases. Aside from these studies, such data on CORT changes during controlled dehydration in xeric-dwelling species is lacking.

To further examine how dehydration affects both immunity and CORT release in a xeric-dwelling species, I measured innate immunity and plasma CORT concentrations concurrently in Gila monsters (*Heloderma suspectum*), across hydric states. I tested these measures in both captive and free-ranging Gila monsters to address innate immunity and CORT responses under controlled conditions, as well as under conditions of natural variation. I previously found that Gila monsters, which experience prolonged but predictable seasonal dehydration (Davis and DeNardo 2009; Davis and DeNardo 2010), have increased innate immunity during periods of moderate dehydration (Moeller et al. 2013), and this might be explained in part by a lack of a substantial CORT response to dehydration. I took blood samples from captive and free-ranging Gila monsters in hydrated and dehydrated states to: 1) test the repeatability of my previous findings of increased innate immunity (i.e., hemolysis and hemagglutination tests; Moeller et al. 2013) during mild dehydration, 2) examine additional measures of innate immunity including bacterial killing ability and white blood cell differentials, and 3) determine CORT levels prior to and after a stress treatment across hydric states. Together, these tests provide a simultaneous look at stress hormone and innate immune function responses to dehydration at levels experienced during a typical seasonal drought.

Specifically, I hypothesize that a hormonal stress response would be maladaptive in drought-adapted species. Thus, I predicted that in Gila monsters, dehydration would have little or no effect on the CORT response, while positively affecting multiple measures of innate immune function.

Methods

Study Species and Overview

The Gila monster is a large-bodied lizard that is found in southwestern desert scrub habitats of North America, such as the Sonoran Desert, which receives rain during the winter, early spring, and the late summer monsoon period, but typically receives no rain for 2-3 months during the hot, dry season in late spring and early summer. Prior to the monsoon season, free-ranging Gila monsters must typically survive, without any standing water or rainfall, the 60- to 80-day hot, dry period (with average highs ranging from 28 to 38˚C and average overnight lows ranging from 12 to 22˚C; based on data for April through June from 1961 to 1990 in Tucson, AZ; U.S. Climate Data). Further challenging water balance during this seasonal drought, these lizards do not acquire a hydration benefit from their food (Wright et al. 2013). In order to tolerate this period, Gila monsters store fluid in the urinary bladder that they reabsorb to slow the rate of dehydration (Davis and DeNardo 2007). Despite this adaptation, Gila monster plasma osmolality typically increases up to 20% by the end of the seasonal drought (from ~300 mOsm to ~360 mOsm in natural populations; Davis and DeNardo 2010). To test the effects of seasonal

dehydration on innate immune function and glucocorticoid response, I collected blood samples from a captive population of Gila monsters that were sequentially hydrated, dehydrated, and then rehydrated, as well as from seasonally hydrated and dehydrated free-ranging animals (which experience these states in April and June, respectively; Davis and DeNardo 2010).

Captive Gila Monster Experiment

I used 8 captive adult Gila monsters obtained from the Arizona Game and Fish Department (permits #SP577864, SP684760) to examine the effects of hydration state on immune and stress responses. Animals in a post-absorptive state (i.e., fasted for at least two weeks) were maintained in individual cages (75 X 35 X 13 cm; Freedom Breeder, Turlock, CA, USA) in an environmental chamber at 30°C from two weeks prior to the start of the experiment until the experiment was completed. Each animal was sampled when in hydrated, dehydrated, and rehydrated states (as described below).

Three different hydration states were reached by regulating access to water and monitoring blood plasma osmolality. Animals were provided with water *ad libitum* during the two weeks they acclimated to their experimental environment. Water was then removed from each animal's cage immediately prior to collecting an initial 0.7 ml blood sample from each (as described below). After the initial sample, animals were given a 30-minute stress treatment during which the animal's movement was limited and it was repeatedly prodded gently (mostly on the head, along the back, on the toes of the rear feet, and on the tail) with a foam square (2" by 2" by 1") attached to the end of a wooden dowel. Animals were prodded for 10 of every 30 seconds of the 30-minute treatment.

Thirty minutes is an adequate stress treatment time to elicit a significant increase in CORT in other reptile species (Langkilde and Shine 2006), and three times the stress treatment times used for many smaller lizard species (e.g., 10 minutes; Woodley and Moore 2002). I collected a 0.1 ml blood sample after the stress treatment. Each blood sample was collected from the caudal vein using a heparinized 1 ml syringe with a 25g X 1.59 cm (5/8 inch) needle. Within one day of taking this hydrated state blood sample set from an individual, the individual's urethra was catheterized (as described in Davis and DeNardo 2007) to remove any water stored in the urinary bladder. After catheterization, the animal was assessed using ultrasound imaging to ensure that all bladder contents had been removed. The animal then entered the dehydration phase.

During dehydration, animals were checked daily and weighed every two to seven days. Blood samples (0.06 ml, as described above) for monitoring hydration state were collected on the second week, and once a week thereafter. If an animal was approaching a state of moderate dehydration (>330 mOsm; often the extent of dehydration in the wild), on the next planned monitoring day, a dehydrated state blood sample set (0.7 ml initial, 0.1 ml post-stress-treatment) was collected. This ensured that the dehydrated blood sample set was collected at least 4 full days after a monitoring bleed to avoid any potential effects of acute stress from the bleeding process. All samples were collected within 3 minutes of the start of handling to avoid a handling-associated elevation of plasma corticosterone (Romero and Reed 2005), the primary stress response hormone in reptiles. Any initial sample that could not be collected within three minutes was rescheduled for four days later. Once an individual was sampled during a state of dehydration, it was given access to water *ad libitum.* Seven days after the dehydrated

state sample, I collected the rehydrated state blood sample set (0.7 ml initial and 0.1 ml post-stress-treatment). Each blood sample was placed on ice immediately after it was drawn. Samples were processed within six hours of collection and stored for later assaying (see Sample Assays, below).

Free-Ranging Gila Monster Study

To provide ecological relevance to my findings in captive animals, I collected initial and post-stress-treatment blood samples from 11 adult Gila monsters (4 females, 7 males) from a field site in the Arizona Upland subdivision of the Sonoran Desert in Pinal County, AZ, roughly 30 km NNE of Tucson. The site is characterized by a series of dry stream beds, with thick surrounding brush largely composed of catclaw acacia (*Senegalia greggii*), mesquite trees (*Prosopis* spp.), and various species of cactus. I found Gila monsters by walking up and down a two-mile section of the main wash while looking in the wash and under bushes.

As with the captive animals, blood samples from free-ranging animals were used to evaluate whether hydration state correlated with innate immune function and plasma corticosterone concentrations. Samples were collected during times when free-ranging individuals are characteristically in hydrated and dehydrated states: in April and in late May to June, respectively (Davis and DeNardo 2010). Though the samples were not evenly distributed between these two seasons, they were relatively balanced by animal hydric state (5 hydrated, 6 dehydrated). An animal was only included in the study if the initial blood sample was obtained within three minutes of first interaction (Romero and Reed 2005). Blood samples were placed on ice immediately after collection. To avoid

repeatedly sampling the same individual, a photograph of the dorsal pattern, which is unique to each individual, was taken. Within 12 hours of collection, blood samples were processed and stored for later assaying (see Sample Assays, below).

Sample Preparation and Assays

All initial blood samples were analyzed for hydration state (hematocrit, plasma osmolality), innate immune function (agglutination and lysis assays, bacterial killing assays, and white blood cell profiles), and baseline plasma CORT concentration. Due to limited volume, the post-stress-treatment blood samples were used only to determine plasma CORT concentration (i.e., CORT responsiveness). To process each initial sample, two drops of whole blood were used to create two blood smears for cell profiling, and whole blood was used to fill two capillary tubes to measure hematocrit (packed cell volume). The remaining sample was then centrifuged and plasma was separated into 50 μl aliquots. All aliquots were stored at -80°C for later assays. Post-stress-treatment blood samples were centrifuged and the plasma separated and frozen at -80°C for later assays.

Sample Processing: Determination of Hydration State

Plasma osmolality was analyzed using vapor pressure osmometry (±6 mOsm/kg; model 5100C; Wescor, Inc., Logan, Utah, USA). Prior to use, the osmometer was calibrated, and calibration was monitored throughout use, as described in Davis and DeNardo (2007), with one modification: Each sample's osmolality was determined in triplicate, using 8 μl of plasma for each run.

To measure hematocrit, which often changes with hydration state, two capillary tubes were filled three-quarters of the way with whole blood, then sealed with clay and centrifuged at 4000 rpm for 90 seconds. Hematocrit was measured as the percentage of all blood components made up by the packed red blood cells.

Sample Processing: Agglutination and Lysis Assays

I performed agglutination and lysis assays on all initial samples following Moeller et al.'s (2013) modified version of the protocol from Matson et al. (2005). Briefly, I serially diluted 20 µl of each initial plasma sample along a 96-well plate from 1:2 to 1:2048 with phosphate-buffered saline, using the final row for a negative control. Fifty percent heparinized sheep blood (SBH050, HemoStat Laboratories, Dixon, California, USA) was diluted 1:100 and 20 µl were added to each well. Each plate was then covered with paraffin film, gently vortexed, and incubated for 90 minutes in a water bath at 29°C (the active season mean diurnal body temperature of Gila monsters; Davis and DeNardo, 2010). I tilted the plates at a 45° angle for 20 minutes at room temperature to improve visualization before scanning them (Hewlett-Packard Co., ScanJet 3670) for agglutination analysis. I incubated the plates at room temperature for an additional 70 minutes before rescanning them for lysis images. Plate images were visually scored independently by two individuals. Scores were repeatable (Intraclass Correlation Coefficient reliability analysis: 98% repeatable) and averaged for statistical analysis.

Sample Processing: Bacterial Killing Ability

Bacterial killing assays (BKA) were conducted using plasma from initial samples, following methods in French and Neuman-Lee (2012). Briefly, using autoclaved instruments in a sterilized laminar fume hood, frozen initial plasma samples were thawed and 2 µl were immediately pipetted onto 96-well round-bottom microplates. None of the samples had been thawed previously and all were run in duplicate. Negative-control wells were run to ensure no contamination occurred by adding 6 µl phosphate-buffered saline and 18 µl CO_2-independent media plus 4 mM L-glutamine (no bacteria). Positive-control wells were made with 6 µl of working bacteria solution, which consisted of 10^4 colony-forming units of *Escherichia coli* (NO. 8739, ATCC, Manassas, VA), along with 18 µl of media. Sample wells also contained 6 µl working bacteria solution and 16 µl media, so that all wells had a final volume of 24 µl. The microplates were vortexed gently for 1 minute, then incubated at 37°C (optimal temperature for *E. coli* growth) for 30 minutes to allow bacterial killing to occur. After incubation, the plates were vortexed gently for 1 minute, then 125 µl sterile tryptic soy broth (Sigma-Aldrich NO. T8907; 15 g broth/500 ml nanopure water) was added to each well using a multichannel pipette. The plates were gently vortexed again, for an additional minute. Then absorbance was read using a microplate reader (BioRad xMarkTM Microplate Absorbance Spectrophotometer) at 300 nm. Following the reading, the plates were incubated at 37°C for 12 hrs, then gently vortexed for 1 minute, then the plates were read again. Changes in sample absorbance over the 12-hour bacterial growth phase were compared to the positive controls, which represent 0% bacterial killing. All absorbance differences were converted to percentage values.

Sample Processing: Corticosterone Assays

Samples were assayed for corticosterone in duplicate using enzyme-linked immunoassay kits (Enzo Life Sciences, Farmingdale, NY). Validation of kits was performed by demonstrating parallelism of a serially diluted plasma sample (4-64x dilution) with a standard curve.

I diluted 25 µl of plasma with assay buffer containing steroid displacement reagent before assaying. The average intra-assay coefficient of variation was 19.5% and the average inter-assay coefficient of variation was 2.3%. The average assay sensitivity was 6.4 pg/ml.

Sample Processing: Blood Cell Profiles

Blood smears were created in duplicate on 1mm thick, 75 x 25 mm frosted slides (VWR, Radnor, PA) by smearing one drop of blood on the slide with a cover slip. Slides were dried and then stored in a vacuum-sealed chamber with an air desiccator (indicating Drierite; W.A. Hammond Drierite Co., Xenia, OH). Within one month, they were treated with 100% methanol for a minute and were stored again for up to six months. Slides were stained by submerging them in a 1:15 dilution of Giemsa-Wright stain (with nanopure water) for 60 minutes, and were then rinsed in two separate nanopure water baths for 5 minutes each. Using a light microscope (BX60, Olympus Optical Co., Tokyo, Japan), blood smears were visually scanned at 400x magnification to determine the number of heterophils, lymphocytes, monocytes, and basophils in the first 100 white blood cells seen. Cell types were identified according to descriptions in Cooper-Bailey et al. (2011).

Statistical Analyses

All statistical analyses were performed in R (3.0.3; R Core Team, 2014). For the captive animals, I used repeated-measures analyses of variances (rmANOVAs) to validate differences in osmolality between treatment groups and to test for the effect of hydration state on hematocrit, innate immune function, and stress indicators using the *nlme* R package (Pinheiro et al. 2013). Post-hoc tests were completed using the *multcomp* R package (Hothorn et al. 2008) to test differences between groups. Sphericity of data was validated when possible using Mauchley's tests and if sphericity was not met, F values were modified using Greenhouse-Geisser corrections from the *car* R package (Fox and Weisberg 2011). Normality was also assessed using visual inspection of qq-plots.

The study of free-ranging animals was limited to observance of two hydric states, so I used Welch's t-tests to assess differences between hydration treatment groups in osmolality, hematocrit, innate immune function, and stress indicators. Normality of data was visually assessed using qq-plots and equality of variances was determined using F tests. Hematocrit had unequal variances that transformations did not correct, but I still used a Welch's test as it is robust to unequal variances. Bacterial killing ability was problematic with group sample sizes of 3 and 5, and because a few of the samples maxed out bacterial killing. Thus, the data had abnormal distribution and unequal variances and neither could be achieved. There is no good non-parametric equivalent for data that fails to meet both these assumptions, but in a Welch's test the findings were non-significant. Post-stressed CORT and delta CORT (the difference between baseline and post-stressed

CORT levels) were both abnormal and unequal, but normality and equal variances were achieved after log transformation.

For both captive and free-ranging animals, paired t-tests were used with log-transformed CORT data to compare baseline and post-stress levels, assessing the effectiveness of stress treatments. All plots were made using Graph Pad Prism (GraphPad Software, Inc., La Jolla, CA).

Results

Plasma Osmolality

All hydric states had significantly different plasma osmolality (Figure 3.1; Table 3.1). Captive animals in a dehydrated state had a significantly higher osmolality than when they were hydrated and rehydrated ($F_{2,12} = 300.24$; $P < 0.001$). The hydrated and rehydrated states also differed ($P = 0.026$), with the rehydrated state having a lower osmolality than the hydrated state. Similar to captive animals, free-ranging animals had significantly higher osmolality when dehydrated than when hydrated ($t(9) = -9.53$; $P < 0.001$).

Agglutination and Lysis Assays

Agglutination and lysis activity both increased when animals were dehydrated (Figure 3.2). Agglutination was highest during dehydration in captive animals ($F_{2, 14} = 12.19$; $P = 0.0016$; Figure 3.2; Table 3.1) and in free-ranging animals ($t(9) = -2.86$; $P = 0.021$;

Figure 3.3; Table 3.1). Lysis also increased during dehydration in captive animals ($F_{2,12}$ = 31.48; $P < 0.001$; Figure 3.2; Table 3.1) as well as in free-ranging animals (t (9) = -2.80; P = 0.021; Figure 3.3; Table 3.1).

Bacterial Killing Assays

Bacterial killing ability was highest in the dehydrated state for captive animals ($F_{2, 14}$ = 7.84; P = 0.0052; Figure 3.2; Table 3.1) but there was no difference among states in free-ranging animals (t (2) = -1.45; P = 0.28; Figure 3.3; Table 3.1).

Corticosterone Assays

In captive animals, baseline CORT did not vary among hydric states, though there was a near-significant difference, with the highest CORT in dehydrated states ($F_{2,14}$ = 2.99; P = 0.083; Figure 3.2; Table 3.1). However, post-stress-treatment CORT levels were significantly different among hydration states ($F_{2,14}$ = 35.14; $P < 0.001$), with higher post-stress-treatment CORT in dehydrated animals (Figure 3.2; Table 3.1). The hydrated and rehydrated states did not have significantly different post-stress-treatment CORT levels (P = 0.41; Figure 3.2; Table 3.1). Delta CORT, a direct measure of CORT reactivity, showed similar results, and was significantly higher during dehydration than the other two hydration states ($F_{2,14}$ = 13.01; $P < 0.001$). Baseline and post-stress-treatment samples did not differ when animals were hydrated (P = 0.13)), but post-stress-treatment CORT was significantly higher than baseline when in a dehydrated state (t (7) = -4.21; $P < 0.005$) and when in a rehydrated state (t (7) = -2.47; $P < 0.05$; Figure 3.2; Table 3.1).

Baseline CORT did not vary between hydrated and dehydrated free-ranging animals ($t\ (9) = -1.94$; $P = 0.089$; Figure 3.3; Table 3.1). Post-stress-treatment CORT levels varied significantly ($t\ (9) = -3.78$; $P < 0.005$), with dehydrated animals having higher levels (Figure 3.3; Table 3.1). Post-stress-treatment samples had higher CORT levels than baseline in hydrated ($t\ (4) = -6.060$; $P < 0.005$) and dehydrated free-ranging animals ($t\ (5) = -6.16$; $P < 0.005$).

Hematocrit

Hematocrit was significantly different among hydration states in captive animals, with higher packed cell volume in the dehydrated group ($F_{2,14} = 16.34$; $P < 0.001$; Figure 3.2; Table 3.1). In free-ranging animals, hematocrit of hydrated and dehydrated animals did not differ significantly, though results were near significant ($t\ (9) = -2.20$; $P = 0.083$; Figure 3.3; Table 3.1).

White Blood Cell Profiles

Hydric state in captive animals did not affect percentages of heterophils ($P = 0.33$) or lymphocytes ($P = 0.91$), or the heterophil to lymphocyte (H/L) ratio ($P = 0.70$; Figure 3.2; Table 3.1). Monocyte percentages were also not affected by hydration ($P = 0.17$; Table 4.3) but basophils were more common when hydrated compared to the other two treatment groups ($F_{2, 14} = 6.94$; $P < 0.001$). In free-ranging animals, there were no significant differences between groups for percentages of heterophils ($P = 0.314$), lymphocytes ($P = 0.078$), or basophils ($P = 0.99$), or the H/L ratio ($P = 0.15$; Figure 3.3; Table 3.1).

Discussion

My results from both free-ranging animals and experimentally dehydrated animals confirm the counterintuitive previous finding that dehydration can enhance some aspects of innate immune function. The results also show that dehydration does not evoke a substantial baseline change in glucocorticoids. Thus, in some drought-adapted species, dehydration does not necessitate an increase in the production of the stress hormone corticosterone.

Corticosterone

Most studies of the effects of hydric state on stress show that CORT increases with dehydration. This trend appears to hold true across many species, including mice (Bekkevold et al. 2013, Tsuchida et al. 2004), rats (Sebaai et al. 2002; Arnhold et al. 2007), quail (Cain and Lien 1985), cattle (Parker et al. 2004; Hogan et al. 2007), and, under some conditions, humans (Hoffman et al. 1994; Maresh et al. 2006). However, most of these species have been domesticated and/or do not regularly experience dehydration in the wild and therefore may not have adapted to coping with a dehydrated state.

Based on the established trend among amniote vertebrates, one might assume that the first step of the vertebrate dehydration cascade—an increase in CORT production—would be increased in dehydrated Gila monsters. However, if this were the case, one would expect to find a decrease in immune function in dehydrated Gila monsters, as glucocorticoids like CORT usually inhibit immune function (Sapolsky et al. 2000; though

there are exceptions, Martin et al. 2011). Thus, contrary to the trend, but in accordance with my previous result of enhanced immune function in dehydrated Gila monsters (Moeller et al. 2013), I predicted that baseline levels of CORT would not be affected by hydric state in the Gila monster. My data show no significant effect of dehydration on baseline CORT levels. However, my near-significant results have fairly low power (0.582 among baselines of captive animals; 0.55 among baselines of free-ranging animals). Thus, the data indicate that dehydration causes a minimal if any increase in plasma CORT concentrations in Gila monsters, suggesting that dehydration to naturally experienced levels is not a highly stressful event.

Interestingly, some other xeric-dwelling species lack a baseline hormonal stress response during dehydration, with no dehydration-induced increase in baseline levels of CORT in Children's pythons (Dupoué et al. 2014) and Awassi sheep ewes (Hamadeh et al. 2006); in many related studies on other species, the test subjects avoid dehydration symptoms altogether during drought periods of average length for the area (King and Bradshaw 2010; Brown et al. 2011). In cases where dehydration does occur, avoiding release of glucocorticoids associated with stress would be beneficial as CORT release can increase energy expenditure and water loss (Parker et al. 2003), compounding resource imbalances. While the trend across taxa is interesting, currently there are insufficient data that test whether a species' habitat has an effect on vulnerability to dehydration.

My captive animal experiment removes any effect of seasonality, and thus demonstrates that the results are related to hydration state. The mechanism behind an absent or minimal increase in baseline corticosterone in dehydrated Gila monsters is unclear; however,

conditions that are predictably challenging may provide an opportunity to evolve physiological compensations for these challenges (Martin 2009). Specifically, dissociation of dehydration and CORT release could be an adaptation to challenging xeric environments. Several studies of birds have shown that stress responses can be suppressed to avoid negative effects on fitness (Wingfield et al. 1995; Holberton et al. 1996; Wingfield and Hunt 2002). In Arctic birds, dissociation of CORT production and a weather-based stressor was hypothesized to modulate the stress response through insensitivity to the hormonal cascade resulting from acute stressors (Wingfield and Hunt 2002). Further testing of the direct effects of CORT release during dehydration would help us to understand whether any CORT dissociation has occurred in Gila monsters.

Hydrated captive animals lacked an increase of CORT after stressful perturbation ($P = 0.114$). Treatments were successful among dehydrated Gila monsters, as well as in hydrated animals that were free-ranging, thus I feel the treatment was adequate. Why CORT release did not increase significantly in hydrated captive animals is unknown, but is likely due to physiological differences or coping mechanisms associated with regular disturbances experienced during captivity rather than an issue with methodology.

Interestingly, despite the fact that dehydration did not induce a significant increase in baseline CORT levels, dehydration was not without an effect on CORT release. Dehydration induced a greater CORT response to a stressful perturbation (e.g., prodding over a 30-minute stress treatment). I only found one other study that examined stress-induced plasma CORT concentrations in dehydrated animals: Dupoué and colleagues (2014) had similar results in the Children's python. They hypothesized this effect may

enable dehydrated animals to compensate for inactivity with a hyper response to stressful stimuli. However, dehydration-associated inactivity has not yet been documented in this species and the benefit of a hyper response in inactive animals is uncertain. Clearly, more research on the interaction among dehydration, CORT release, and metabolic effects are needed to understand the observed patterns.

Immune Function

These immune response results verify my previous findings that dehydration enhances agglutination and lysis in the Gila monster (Moeller et al. 2013). Additionally, using captive animals, I show that another estimate of innate immune response, bacterial killing ability, also increases with dehydration in this species. I suspect that the insignificant enhancement in bacterial killing ability in samples from free-ranging animals may be a result of weaker statistical tests due to smaller sample size or the lower average osmolality of the dehydrated animals, suggesting a lesser state of dehydration.

Agglutination and lysis assays measure levels of natural antibodies and complement activity, respectively (Demas et al. 2011). While bacterial killing ability also addresses these immune functions, it does so in a more ecologically relevant way, assessing response to a pathogen that may be encountered naturally in the wild (French and Neuman-Lee 2012). Thus, these findings support increased immune function of dehydrated Gila monsters that could be beneficial in an ecologically relevant context.

Increased immunity during dehydration may boost an organism's defenses against pathogens during times when other physiological systems could experience functional

challenges. Osmotic stress may decrease function of more expensive, adaptive immune responses. Thus, energetically cheaper innate immunity may increase instead as a strategy to limit vulnerability of the immune system, though this hypothesis remains untested. The mechanisms by which dehydration increases immune function also warrant further study, as it was previously shown that increased plasma concentration of immune molecules is not the sole cause (Moeller et al. 2013). I posit that this response of the immune and endocrine systems to dehydration, which goes against the general expected pattern of the vertebrate dehydration cascade, may be an adaptation of species that regularly experience bouts of dehydration. Testing of additional related species that live in either xeric or mesic environments will help address this hypothesis.

Overall, the interplay of immunity, stress, and dehydration is an area of study that is relatively unexplored, but promises important insight into differences in physiological responses to hydric challenges. Focusing on these responses will be a critical area of study as we try to understand current and potential effects of climate change on species' survival.

Table 3.1. Mean (± SEM) values for all physiological assessments made on captive and free-ranging Gila monsters at different hydration states. HYD: hydrated, DHY: dehydrated, and RHY: rehydrated. Significant differences among hydration states within captive animals and within free-ranging animals are shown using superscript letters, in descending order of value (i.e., "a" is highest).

	Captive Gila Monsters			Free-ranging Gila Monsters	
	HYD	DHY	RHY	HYD	DHY
Osmolality (mOsm)	282.8^{b} ± 1.6	343.8^{a} ± 3.0	274.8^{c} ± 2.4	292.0^{b} ± 1.5	317.8^{a} ± 2.3
Agglutination Score	4.47^{b} ± 0.47	5.53^{a} ± 0.50	4.38^{b} ± 0.54	3.10^{b} ± 0.41	4.58^{a} ± 0.32
Lysis Score	2.78^{b} ± 0.23	4.00^{a} ± 0.25	2.63^{b} ± 0.25	1.80^{b} ± 0.37	3.29^{a} ± 0.38
BKA (% killing)	38.96^{b} ± 16.26	81.33^{a} ± 12.44	29.08^{b} ± 14.54	71.72 ± 18.39	98.49 ± 0.92
Baseline CORT (ng/ml)	3.25 ± 1.57	6.75 ± 2.21	2.37 ± 0.55	1.62 ± 0.60	3.90 ± 1.01
Stressed CORT (ng/ml)	3.10^{b} ± 0.58	45.64^{a} ± 17.35	6.67^{b} ± 2.84	3.13^{b} ± 0.88	21.22^{a} ± 9.62
Hematocrit (% packed cells)	26.7^{b} ± 1.5	32.4^{a} ± 1.4	26.2^{b} ± 1.5	29.1 ± 2.0	33.6 ± 0.6
H/L Ratio	1.12 ± 0.13	1.24 ± 0.15	1.18 ± 0.08	0.65 ± 0.04	0.91 ± 0.14
Heterophil Percent	38.38 ± 2.54	41.38 ± 2.37	42.50 ± 2.11	33.60 ± 1.21	37.17 ± 3.04
Lymphocyte Percent	36.50 ± 3.19	35.63 ± 2.92	36.50 ± 1.27	52.00 ± 2.17	43.50 ± 3.59
Basophil Percent	11.63 ± 1.79	6.50 ± 1.85	6.50 ± 1.89	7.80 ± 1.93	7.83 ± 2.56

Figure 3.1. Captive and free-ranging Gila monster osmolalities across hydric states. Gila monsters had significantly different osmolalities when they were hydrated (HYD), dehydrated (DHY), and rehydrated (RHY). Mean osmolality is shown for both captive (solid circles) and free-ranging (open circles) animals. Error bars represent standard error and letters depict significant differences among hydration states.

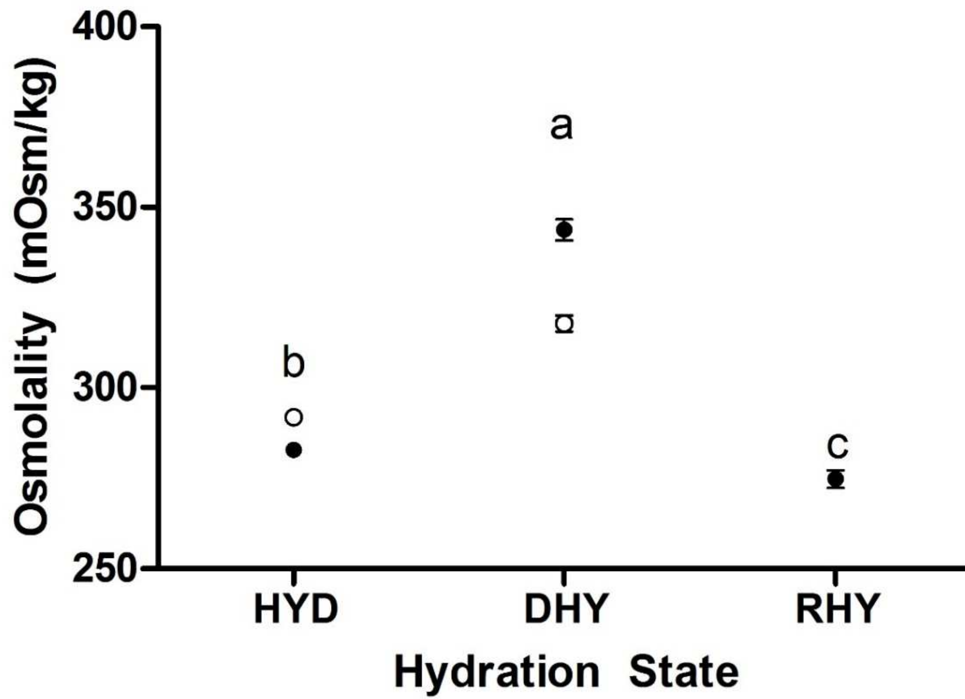


Figure 3.2. Immune and stress hormone levels for captive Gila monsters across hydric states. Dehydrated (DHY) captive Gila monsters had more robust agglutination (A), lysis (B), and bacterial killing ability (C), as well as higher plasma corticosterone (CORT) concentration after stress treatment (D, solid bars), and hematocrit (E) compared to when these same individuals were hydrated (HYD) and rehydrated (RHY). In contrast, baseline plasma CORT (D, open bars) and heterophil to lymphocyte (H/L) ratio did not change with hydration state. Letters above bars represent significant differences among hydration states, while asterisks depict significant difference in CORT pre- and post-stress (* = P <0.05; *** = P <0.001). ns: no significant differences.

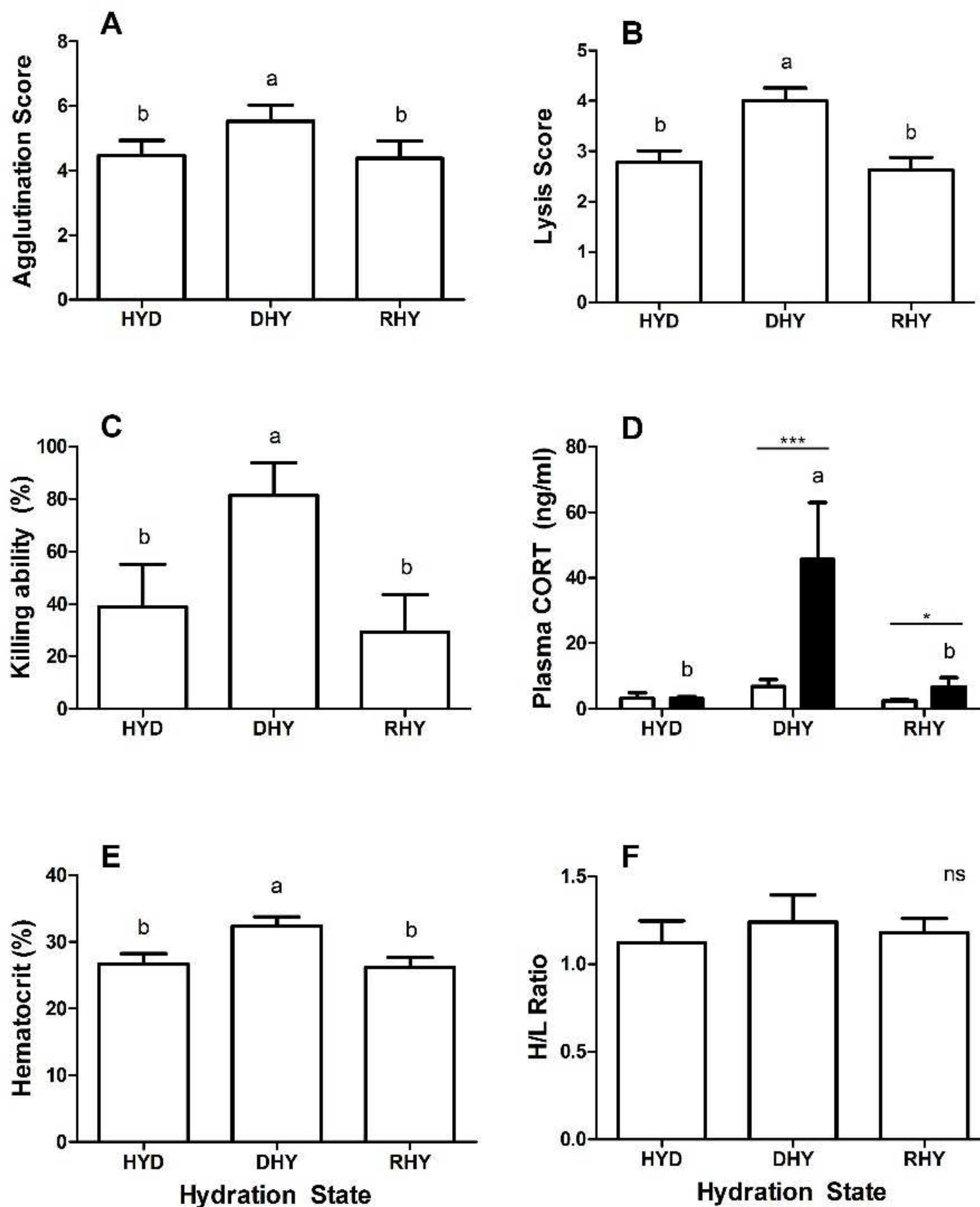
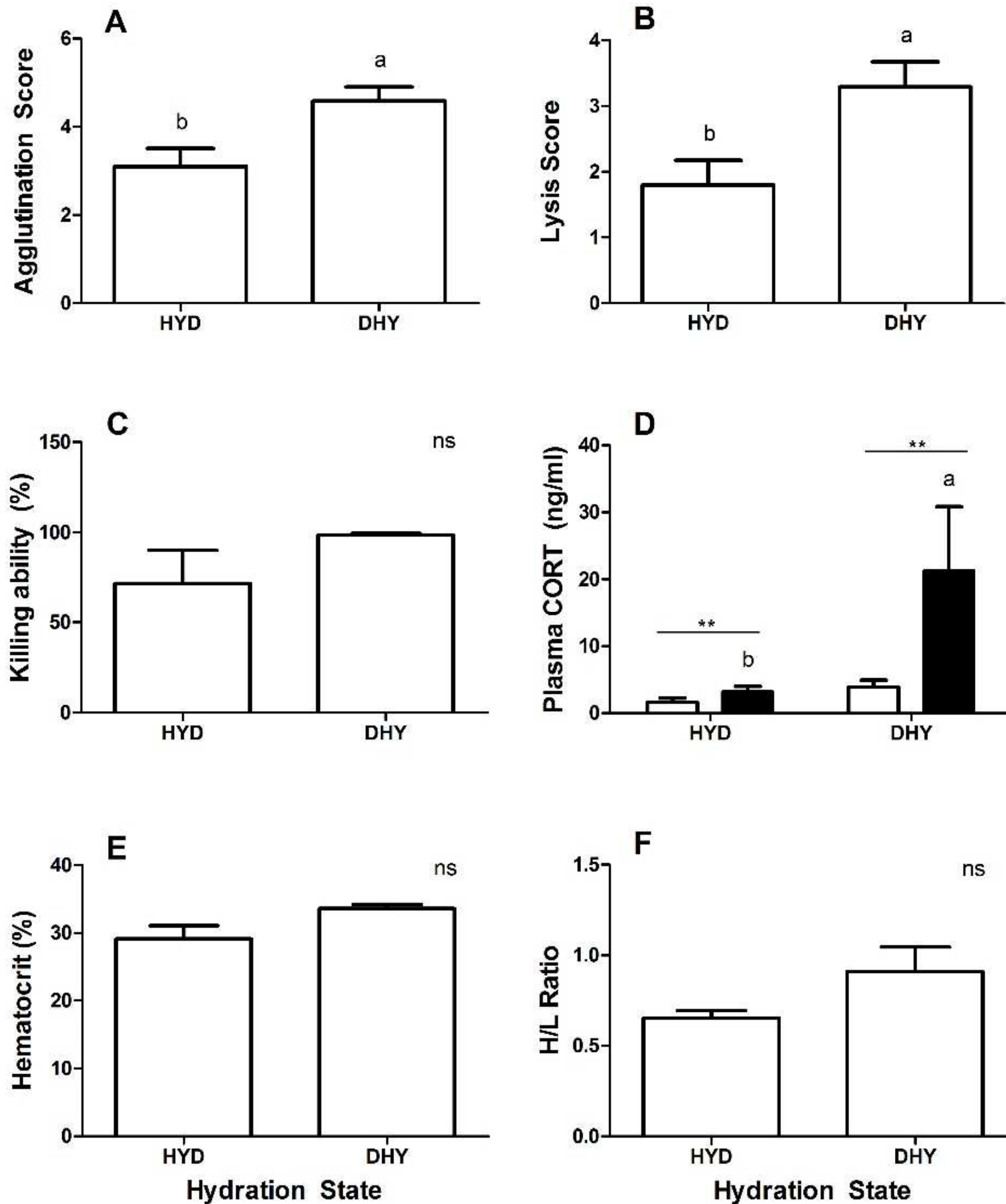


Figure 3.3. Immune and stress hormone levels for free-ranging Gila monsters across hydric states. Dehydrated (DHY) free-ranging Gila monsters had more robust agglutination (A) and lysis (B), and higher plasma corticosterone (CORT) concentrations after stress treatment (D, solid bars) compared to hydrated animals (HYD). In contrast, bacterial killing ability (C), hematocrit (E), and heterophil to lymphocyte (H/L) ratios (F) did not differ significantly between hydric states. Letters above bars represent significant differences between hydration states, while asterisks depict a significant difference in CORT pre- and post-stress (** = P <0.01). ns: no significant difference.



CHAPTER FOUR:

Dealing with Dehydration: Species-Specific Stress Responses to Dehydration and a Decoupling of the Chronic Stress-Immunity Response in Reptiles.

Introduction

In all natural environments, the availability of certain resources can be unpredictable or seasonally lacking. However, the challenge of survival demands that species adapt to such conditions. For example, in xeric environments, water is often a limiting resource, but organisms that inhabit xeric habitats have various adaptations that enable them to survive despite these limits. Plants in xeric habitats may maintain water balance by reducing water loss by having a waxy cuticle and respiring at night, or they may maintain access to water with long tap roots that can reach ground water or by having the capacity for water storage (Hadley 1980; Chaves et al. 2002; Herrera 2009; though these traits are not restricted to plants found in xeric environments; Haworth and McElwain 2008). Animals in xeric regions may maintain water balance by being able to reduce the water expelled with waste, adjusting active periods to avoid the hottest and most desiccating times of the day or year, obtaining the water they need from high-water-content food, or using specialized organs for water storage (McClanahan Jr. 1967; Kotler et al. 2006; Davis and DeNardo 2007; Davis and DeNardo 2010).

Alternatively to maintaining water balance, some xeric species tolerate dehydration by withstanding increasing levels of solutes in the body or within the egg or seed (Davis and

DeNardo 2009; Peterson 1996; Crowe et al. 1992). Even if an organism can tolerate dehydration to some extent, this condition can still have lasting and varied effects on physiological functions. Dehydration may affect development (Lorenzon et al. 1999), locomotion (Moore and Gatten 1989), reproduction (Lourdais et al. 2015), and immune function (Moeller et al. 2013; King and Bradshaw 2010). In many species, dehydration is a stressful condition that causes increased plasma glucocorticoids (Tsuchida et al. 2004; Maresh et al. 2006). However, the vast majority of studies that support this idea have been conducted in animals that do not regularly experience natural periods of dehydration (in lab mice, Bekkevold et al. 2013; Tsuchida et al. 2004; in lab rats, Sebaai et al. 2002; Arnhold et al. 2007; in quail, Cain and Lien 1985; in cattle, Parker et al. 2004; Hogan et al. 2007). The calibration of an individual's stress response likely depends on the environment to which it is adapted (Wingfield et al. 1995) or in which it has developed (Ellis et al. 2006). Due to the predictable seasonal nature of water limitation in many xeric environments, xeric-adapted animals may cope with dehydration without a stress response and the associated increase in circulating glucocorticoids. The avoidance of an increase in glucocorticoid production would likely be physiologically beneficial in drought-adapted animals, as this could enable the avoidance of the immunosuppressive effects of an increase in corticosterone (CORT) production over longer time scales (Sapolsky et al. 2000); such decreases in immune function are connected to dehydration through the vertebrate dehydration cascade. Increased CORT circulation also has the potential to increase rates of water loss (Parker et al. 2003), an effect that would worsen dehydration.

According to Boonstra (2005), stress responses must generally be adaptive, beneficial responses to stressful situations. While Boonstra proposed this idea mainly in a context of stress as a result of predation risk, this tenet also applies to the potential stress response during dehydration. In some animals, a CORT response to dehydration may be adaptive, enabling mobilization of energy (Romero 2002) and giving the animal a better chance to survive by finding water or escaping dehydrating conditions. For example, in the spinifex hopping mouse (*Notomys alexis*), which depends partly on metabolic water, water deprivation causes an increase in CORT, increasing food intake and the resulting production of metabolic water to maintain water balance (Takei et al. 2012). However, for many animals in xeric environments, the effects of dehydration over an extended period may have negative effects on fitness if CORT is released and immunosuppression and increased water loss result. Based on these ideas, I hypothesized that the presence or absence of a glucocorticoid response to dehydration is not a consistent trait among vertebrates. Specifically, the effect of dehydration on the release of stress hormones and immune function likely varies among species and may depend on the species' environment and tolerance mechanisms it employs (Stahlschmidt et al. 2011).

Various studies tentatively support this hypothesis. Dehydration is often found to cause release of glucocorticoids in mice (Tsuchida et al. 2004), rats (Sebaai et al. 2002), rabbits (Kallaras 2004), quail (Cain and Lien 1985), chicken (Klandorf 1984) and cattle (Parker et al. 2004). These subjects and the subjects of many similar studies are often domesticated animals that do not experience water limitations. However, many animals that experience seasonal drought or desert environments do not tend to exhibit the same dehydration-induced glucocorticoid response, such as Gila monsters (Moeller,

unpublished data; chapter 3), Children's pythons (Dupoué et al. 2014), and Awassi sheep (Jaber 2004; Hamadeh 2006; but also see Ghanem 2008), though studies of dehydration in such species are limited in number.

Though these studies show an interesting trend, no study has directly compared hormone and immune responses to dehydration among species that inhabit environments of varying water availability. Thus, to further investigate variation in the vertebrate response to dehydration and to examine whether the ecology of a species influences the physiological responses to dehydration, I tested the effect of dehydration on glucocorticoid release and various measures of innate immune function in four phylogenetic pairs of squamate species; one of each pair inhabits mainly xeric environments that experience annual drought, while the other of the pair inhabits environments where water is rarely limited. In each species, I tested the effects of moderate dehydration on levels of corticosterone (CORT, baseline and reactivity response) and on various measures of innate immunity (agglutination, lysis, bacterial killing abilities, and white blood cell differentials).

Methods

Overview

To test the effects of hydration state on innate immune function and glucocorticoid response, I collected blood samples from serially hydrated, dehydrated, and rehydrated individuals from captive populations of the following squamate species: Gila monster

(*Heloderma suspectum*), beaded lizard (*Heloderma horridum*), Children's python (*Antaresia childreni*), ball python (*Python regius*), gopher snake (*Pituophis catenifer*), diamondback water snake (*Nerodia rhombifer*), western diamond-backed rattlesnake (*Crotalus atrox*), and water moccasin (*Agkistrodon piscivorus*). These species represent confamilial pairs (within Helodermatidae, Pythonidae, Colubridae, and Viperidae) that differ in the extent to which the environments they inhabit experience seasonal water limitations. To validate differences in their adaptations to water limitation, we also measured the rate of cutaneous evaporative water loss for each species (as described below) and compared these metrics between xeric and mesic species groups.

Helodermatidae: Gila Monster and Beaded Lizard

Gila monsters are large lizards that inhabit desert scrub habitats in southwestern North America. In the Sonoran Desert, which encompasses the majority of the species' range, Gila monsters experience an extended hot, dry season prior to the monsoon season in late summer. During the dry season, Gila monsters experience a 60 to 80-day drought, during which there typically is no rainfall and standing water is unavailable.

Beaded lizards are large-bodied lizards that are found in Mexico and northern Central America, mainly in tropical deciduous forests. Overall, these areas get much more rainfall and are more humid that those where the Gila monster lives, however beaded lizards do experience periods with little rain that last at least four months (Murphy and Lugo 1986). During this dry season, standing water is restricted.

I used eight adult Gila monsters received from the Arizona Game and Fish Department (AZGFD) and seven captive-bred beaded lizards of a size similar to the Gila monsters. Both of these species were held under an AZGFD Holding Permit (#SP598954; #SP666234).

Pythonidae: Children's Python and Ball Python

Children's pythons are relatively small, thin pythons that live in the wet-dry tropical rainforests of northern Australia. The dry season in these areas lasts for several months between May and October, potentially causing extended periods during which these snakes likely do not have access to water.

Ball pythons are heavier bodied, medium-sized pythons that live in tropical grasslands and rainforests of sub-Saharan Africa. These areas have a cool, dry season, when rain is infrequent (from November to May), but there are no extended periods without rain. I used eight captive-bred adult Children's pythons and eight captive-born adult ball pythons in this experiment.

Colubridae: Gopher Snake and Diamondback Water Snake

Gopher snakes are medium-sized snakes that inhabit a wide variety of habitats across western North America, including the Mojave and Sonoran deserts, which both have extended hot, dry periods with no rainfall.

Diamondback water snakes are small to medium-sized snakes that are restricted to marshes, ponds, or other habitats along bodies of water in the middle southern United

States. They primarily eat fish and amphibians, and thus rely heavily on constant access to water. I used eight wild-caught gopher snakes from the Sonoran Desert in southern Arizona (AZGFD permit #SP609640) and eight wild-caught diamondback water snakes obtained by S. Secor in Itta Bena, Mississippi (Mississippi Department of Wildlife, Fisheries, and Parks collecting permit #1127121).

Viperidae: Western Diamond-backed Rattlesnake and Water Moccasin

Western diamond-backed rattlesnakes are medium to large venomous snakes that are found in the southwestern United States and central and northern Mexico. They are widely distributed across a variety of habitat types, including the Mojave, Sonoran, and Chihuahuan deserts, where they withstand extended hot, dry seasons with no access to water.

Water moccasins are medium to large venomous snakes that live in the southeastern United States. They are found in marshes, swamps, and other slow-moving water habitats. Like the diamondback water snakes, they eat fish and amphibians, but they will also eat more terrestrial prey, including mammals and birds. These snakes are heavily dependent on fairly constant access to water. I used eight wild-caught western diamond-backed rattlesnakes obtained as nuisance animals and eight wild-caught water moccasins obtained from a colleague.

All venomous species were held under AZGFD permits #SP598954 and #SP666234.

Dehydration Experiment

I used the above described individuals to serially examine the effects of hydration state on immune and stress responses. For Gila monsters, beaded lizards, and both python species, blood samples were collected from all subjects in hydrated (~290 – 305 mOsm/kg; HYD), moderately dehydrated (~315 – 360 mOsm/kg; DHY), and rehydrated (~265 – 300 mOsm/kg; RHY) states. In the other species, due to logistical limitations only two samples were collected from each snake – one when hydrated (either initial or rehydrated) and the other when dehydrated, but the osmolality range for each hydration state was the same in all species.

To begin the experiment, animals in a post-absorptive state (i.e., fasted for at least two weeks) were placed in individual cages (75 X 35 X 13 cm; Freedom Breeder, Turlock, CA, USA) in an environmental chamber at 30°C. They were then given two weeks to acclimate, during which time they were provided water *ad libitum*. I then collected an initial 0.7 ml blood sample from each individual from the caudal vein or by cardiocentesis using a heparinized 1 ml syringe with a 25g X 1.59 cm (5/8 inch) needle. Following each animal's initial sample, I administered a 30-minute stress treatment, which is sufficient time for a reptile to elicit a glucocorticoid response to stress (Langkilde and Shine 2006). Stress was induced by restricting the animal's movement and prodding it gently with a stick for 10 out of every 30 seconds of the treatment. Immediately after the stress treatment, I collected a 0.1 ml blood sample.

I adjusted animal hydration state by regulating water availability and monitoring mass and plasma osmolality. After the initial set of blood samples were collected, water was

removed from the animal's cage. For the lizard species, all fluid was also removed from the urinary bladder, since some lizards, including Gila monsters, are able to use the urinary bladder as a water reservoir (Davis and DeNardo 2007). Removing urinary fluid thus increased the rate of dehydration in the lizards (Gila monsters and Mexican beaded lizards). Bladder fluid was removed using transurethral catheterization. Briefly, after lizards were anesthetized using isoflurane, a Foley catheter was inserted through the cloaca, into the colon, and inflated. Gently pulling on the inflated catheter enabled partial eversion of the cloaca, revealing the urethral opening. A smaller Foley catheter was then inserted up the urethra so that bladder contents could be removed. The lack of bladder fluid post-procedure was confirmed using ultrasonography. As snakes lack a urinary bladder, only lizards were catheterized.

Once deprived of water, animals were visually checked daily and weighed every two to seven days to provide an estimate of water loss and thus dehydration. To monitor plasma osmolality, small blood samples (0.06 ml) were collected after two weeks of dehydration, and then once a week thereafter. When plasma osmolality indicated an animal was close to moderate dehydration, I scheduled a blood sample four days later and the animal was not disturbed until then to reduce the chance of stress affecting sample CORT concentration. After the four days without disturbance, the dehydrated blood sample set (0.7 ml initial, 0.1 ml post-stress-treatment) was collected and the animal was then given water *ad libitum*.

Seven days after the dehydrated blood sample set was collected, the rehydrated set was taken (0.7 ml initial and 0.1 ml post-stress-treatment sample), after which the animal was

removed from the experiment. All samples were collected within three minutes of initial disturbance to avoid elevation of plasma corticosterone associated with handling (Romero and Reed 2005). Samples were immediately refrigerated until they could be processed, aliquoted, and frozen (all within six hours of sampling) for later assays.

If the effort to collect a blood sample failed, the animal was thoroughly evaluated and then left for four days of no disturbance before another attempt at sampling was made. Any time three attempts for a sample were made without success, the sample was skipped and the animal was moved to the next experiment phase. Animals with faster dehydration rates (i.e., water snakes and water moccasins) were given controlled amounts of water (amount of water given was roughly equivalent to 50% of the mass lost in the previous four days) to slightly rehydrate them, so they would still safely reach moderate dehydration four days later. In these cases, amount of water given was equivalent to 50% of mass lost over the previous four days.

Sample Preparation and Assays

Plasma from each baseline blood sample (0.7 ml) was assayed to determine animal hydration state (osmolality, hematocrit), immune function (lysis, agglutination, bacterial killing ability (BKA), and white blood cell differential), and baseline plasma corticosterone concentration. Post-stress-treatment plasma samples were only used to determine corticosterone concentrations as a measure of stress reactivity.

After collecting the initial sample, I used two drops of whole blood to create blood smears for analysis of white blood cell counts (described below). I also used whole blood

to fill two capillary tubes to measure hematocrit. The remaining blood preparation process was the same for the rest of the initial sample and the post-stress-treatment sample: plasma was then separated from the remaining blood via centrifugation, and the plasma then divided into 50 ul aliquots. The separated blood cells and plasma were stored at -80°C until later analysis.

Sample Processing: Determination of Hydration State

Plasma osmolality was analyzed for each sample using vapor pressure osmometry (±6 mOsm/kg; model 5100C; Wescor, Inc., Logan, Utah, USA) as described in Moeller (chapter 3).

To measure hematocrit, I quantified packed cell volume in capillary tubes that had been filled three-quarters full with whole blood, sealed with clay, and centrifuged at 4000 rpm for 90 seconds.

Sample Processing: Agglutination and Lysis Assays

I assayed agglutination and lysis abilities of all initial samples following Moeller et al.'s (2013) modified protocol, originally from Matson et al. (2005). Briefly, I serially diluted plasma from 1:2 to 1:2048 in a 96-well plate and added diluted heparinized sheep blood (SBH050, HemoStat Laboratories, Dixon, California, USA) to each well. After incubation at 29°C (the active season mean diurnal body temperature of Gila monsters (Davis and DeNardo 2010) and an average active temperature for many of the species tested), I assayed for agglutination, incubated the plates again, then assayed for lysis.

Sample Processing: Bacterial Killing Ability

Bacterial killing assays were conducted using plasma following French and Neuman-Lee (2012). Briefly, initial plasma samples were thawed and 2 µl were pipetted in duplicate onto 96-well round-bottom microplates. Negative-control wells were made with 6 µl phosphate-buffered saline and 18 µl CO_2-independent media plus 4 mM L-glutamine and no bacteria, to ensure contamination was avoided. Positive-control wells had 6 µl working bacteria solution, which consisted of 10^4 colony-forming units of *Escherichia coli* (ATCC NO. 8739), along with 18 µl media. Samples were also given 6 µl working bacteria solution, and 16 µl media, so that all wells had a final volume of 24 µl. All wells were thoroughly mixed. The microplates were vortexed gently for 1 min, then incubated at 37°C for 30 min for bacterial killing to occur.

After incubation, the plates were vortexed gently for another minute, then 125 µl sterile tryptic soy broth (Sigma-Aldrich NO. T8907;15 g broth/500 ml nanopure water) was added to each well. The plates were gently vortexed for an additional minute, and their absorbance read at 300 nm (BioRad xMarkTM Microplate Absorbance Spectrophotometer). The plates were incubated at 37°C for 12 hours, then gently vortexed for one minute and then read again. Sample absorbance before and after the 12 hours of bacterial growth were compared to the positive controls (0% bacterial killing) and all absorbance differences were converted to percentage values.

Sample Processing: Corticosterone Assays

Plasma samples were assayed for CORT in duplicate using enzyme-linked immunoassay kits following the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA). To validate this assay for use in the eight species included in this study, a pilot assay determined that the curves generated by serially diluted (5× to 80×) pooled samples for each species were parallel with that of the standard curve. Based on this pilot assay, the plasma samples from all of the species were diluted 40× with assay buffer before being assayed. Ten assay plates were used in total, with a standard curve on each plate, and run over three days. All of the samples from a species were assayed on the same day and samples were randomly assigned to an assay plate on given day. The average inter- and intra-assay coefficients of variation were 7.1% and 13.2%, respectively. The average assay sensitivity was 6.34 pg/ml.

Sample Processing: Blood Cell Profiles

I created and analyzed duplicate blood smears from each initial sample. Briefly, one drop of blood was smeared on a slide using a cover slip, after which slides were dried and stored in a desiccator until preserved with methanol and stored again for up to six months. I then stained slides with Giemsa-Wright stain (1:15 dilution for Gila monsters and beaded lizards; 1:18 dilution for all other species) for 60 minutes and then rinsed slides in two nanopure water baths for five minutes each. Heterophils, lymphocytes, monocytes, and basophils were counted on each slide under a light microscope (BX60, Olympus Optical Co., Tokyo, Japan) at 400x magnification, counting the number of each blood cell type (identified according to Cooper-Bailey et al, 2011) until the first 100

white blood cells were counted. Eosinophils and azurophils were not found in the majority of samples and so were left out of analysis.

Cutaneous Evaporative Water Loss Measurements

Cutaneous evaporative water loss (CEWL) rate was measured under standard room conditions (25.8 ± 0.1 °C, 23.3 ± 0.1 % relative humidity) for eight animals of each species except *H. horridum*, for which 7 animals were assessed. CEWL was measured using an AquaFlux AF200 (Biox Systems Ltd., London, England, UK), which derives water vapor flux from humidity gradient measurements using a condenser within a closed chamber. AquaFlux version 6.2 software was used to calibrate the unit prior to each use and to record CEWL rate ($g \cdot m^{-2} \cdot h^{-1}$) in real-time. To maintain tight contact between the standard measurement cap (orifice diameter of 7 mm) and an animal's dorsolateral skin at mid-body, the measurement cap was fitted with a donut-shaped piece of closed-cell foam with the hole in the center of the foam being of the same size as and lined up with the orifice of the measuring cap. Contact between the unit and the animal's skin was continued until CEWL rates stabilized (+/- 0.02 $g \cdot m^{-2} \cdot h^{-1}$ for 10 sec). Measurements were run in duplicate to check for repeatability.

Dehydration Tolerance

I also tested gopher snakes (5), diamondback water snakes (7), western diamond-backed rattlesnakes (6), and water moccasins (5) for the extent of dehydration they could tolerate, the days until they reached that level of dehydration, and the body mass they lost over that time. The experiment was conducted similarly to the Dehydration Experiment

above, but rather than stopping the trial once moderate dehydration was reached, I continued withholding food and water until the animals showed the first symptoms of clinical dehydration (e.g., lethargy, loss of righting response, reduced responsiveness to stimulation). Animals were checked daily initially, but then two to four times daily after they reached moderate dehydration. Monitoring and sampling of animals was similar to the above Dehydration Experiment protocol, but animals did not experience additional stress treatments and blood samples were 0.1 to 0.2 ml and were collected a maximum of 10 times over the course of the trial. These data were used to determine osmolality at which clinical signs first appeared and days to clinical dehydration. Once clinical signs appeared, a final blood sample (0.1 ml) was collected to test osmolality and the animal was provided with access to water *ad libitum*. Two days after animals were rehydrated, the animal was removed from the study and fed.

Statistical Analyses

All statistical analyses were performed using R (3.0.2; R Core Team 2013). Repeated-measures analyses of variances (rmANOVAs) were used to validate the effect of hydration state on osmolality, hematocrit, innate immune function, and glucocorticoid levels using the *nlme* R package (Pinheiro et al. 2013) and conducted post-hoc tests to assess differences between hydration groups using the *multcomp* R package (Hothorn et al. 2008). To compare select assays between xeric and mesic groups or subgroups across hydration states, the *nlme* R package (Pinheiro et al. 2013) was used to run two-factor rmANOVA. Paired student’s t-tests validated effectiveness of stress treatments, comparing baseline and post-stress CORT within each hydric state. All data sets were

tested for normality by visually assessing qq-plots and sphericity was validated when possible using a Mauchly's test from the *car* R package (Fox and Weisberg 2011). If normality assumptions were not met, log or square root transformations were used to achieve normality. If sphericity was not met, Greenhouse-Geisser corrections were used on the F-value using the *car* R package (Fox and Weisberg 2011). If an animal's osmolality from a specific treatment did not match the target range, their data were excluded from all analyses for that treatment. Because of this, one water moccasin was removed from the RHY treatment analysis and six gopher snakes were removed from treatments (two from HYD, four from RHY). One children's python was excluded from CORT analysis, as it had levels of CORT that were outside of the standard curve in all treatment groups. Some individuals did not have data for various physiological measures and so those individuals were excluded from those analyses. Finally, the *ggplots* R package (Wickham 2009) was used to create the plots.

Results

Hydration: Water Loss, Osmolality, and Hematocrit

Cutaneous evaporative water loss (CEWL) rates varied among species. When all xeric species were compared to all mesic species, CEWL was greater among mesic species than xeric species ($F_{1, 61} = 84.38$; $P < 0.001$). Ball pythons had the lowest CEWL of all species, while beaded lizards had the highest. Species-specific averages for CEWL are listed in Table 4.1.

In all species, the DHY animals had significantly higher osmolality than when they were HYD or RHY (Figure 4.1; Table 4.1). Osmolality was highest in DHY and lowest in RHY, with all hydration states significantly different in Gila monsters ($F_{2, 14}$ = 300.24; P < 0.001) and Children's pythons ($F_{2, 14}$ = 191.17; P < 0.001). For all remaining species, DHY was higher than HYD and RHY, which were not different. This pattern held for the additional xeric-dwelling species (western diamond-backed rattlesnakes $F_{2, 14}$ =147.45, P < 0.001; gopher snakes $F_{2, 14}$ = 81.95, P < 0.001), as well as for beaded lizards ($F_{2,12}$ = 128.38; P < 0.001), ball pythons ($F_{2, 14}$ = 201.62; P < 0.001), diamondback water snakes ($F_{2,6}$ = 18.75; P = 0.0026), and water moccasins ($F_{2, 14}$ = 81.76; P < 0.001).

Hematocrit levels did not have a consistent pattern across hydration states in different species (Figure 4.2; Table 4.1). Hematocrit was highest in DHY in Gila monsters ($F_{2, 14}$ = 16.34; P < 0.001). In several of the species, hematocrit was decreased in RHY, but similar in HYD and RHY (Children's pythons $F_{2, 14}$ = 11.06, P = 0.0013; western diamond-backed rattlesnakes $F_{2, 14}$ = 8.83; P = 0.0033; diamondback water snakes $F_{2, 14}$ = 14.41, P = 0.0051; and water moccasins $F_{2, 14}$ = 6.52, P = 0.015). In gopher snakes, beaded lizards, and ball pythons, hematocrit was unchanged by hydration state (P = 0.77; P = 0.30; P = 0.16).

Innate Immunity: Plasma

Agglutination was highest during DHY for several species (Figure 4.3; Table 4.2), including Gila monsters ($F_{2, 14}$ = 12.19; P = 0.0016), gopher snakes ($F_{2, 14}$ = 6.60; P = 0.020), and beaded lizards ($F_{2, 14}$ = 4.99; P = 0.027). Agglutination was higher during DHY than RHY (but similar to HYD) in diamondback water snakes ($F_{2, 14}$ = 8.53; P =

0.018) and water moccasins ($F_{2, 14} = 10.43$; $P = 0.0045$). Hydric state did not affect agglutination in Children's pythons ($P = 0.13$), western diamond-backed rattlesnakes ($P = 0.41$), or ball pythons ($P = 0.10$).

Lysis was highest during DHY in three species (Figure 4.4; Table 4.2): Gila monsters ($F_{2, 14} = 31.48$; $P < 0.001$), beaded lizards ($F_{2, 14} = 5.43$; $P = 0.021$), and ball pythons ($F_{2, 14} = 6.31$; $P = 0.011$). Lysis was higher during DHY than RHY (but similar to HYD) in water moccasins ($F_{2, 14} = 9.69$; $P = 0.0057$). A near-significant effect of hydric state was found in lysis among Children's pythons ($P = 0.059$), with highest levels during DHY. There was no effect of hydric state on lysis in gopher snakes ($P = 0.29$), western diamond-backed rattlesnakes ($P = 0.28$), or diamondback water snakes ($P = 0.33$).

Bacterial killing ability was significantly higher during DHY than other treatments (Figure 4.5; Table 4.2) in Gila monsters ($F_{2, 14} = 7.84$; $P = 0.0052$) and western diamond-backed rattlesnakes ($F_{2, 14} = 6.63$; $P = 0.0094$). BKA was higher in DHY than RHY in Children's pythons ($F_{2, 14} = 6.41$; $P = 0.011$). Three species had near-significant increases in BKA during DHY, including gopher snakes ($F_{2, 14} = 3.44$; $P = 0.083$), ball pythons ($F_{2, 14} = 3.12$; $P = 0.076$), and diamondback water snakes ($F_{2, 14} = 4.01$; $P = 0.078$). The BKA assay results could not be properly tested in beaded lizards and water moccasins, as at least one hydric state was entirely maxed out, with killing ability at 100%; in both species, DHY was maxed out and in beaded lizards, HYD was also maxed out.

Hydric state had no effect on heterophil percentages for the majority of species (Table 4.3), including Gila monsters ($P = 0.33$), Children's pythons ($P = 0.16$), gopher snakes ($P = 0.86$), ball pythons ($P = 0.22$), and diamondback water snakes ($P = 0.11$). Heterophil percentages were higher during RHY in western diamond-backed rattlesnakes ($F_{2, 14} = 12.67$; $P = 0.0018$) and water moccasins ($F_{2, 14} = 12.41$; $P = 0.026$). In beaded lizards, heterophil percentages were lower during HYD than the other two treatments ($F_{2, 14} = 18.35$; $P < 0.001$).

Hydric state also had no effect on percentages of lymphocytes for the majority of species (Table 4.3), including Gila monsters ($P = 0.91$), gopher snakes ($P = 0.71$), ball pythons ($P = 0.52$), diamondback water snakes ($P = 0.11$), and water moccasins ($P = 0.78$). In beaded lizards, all hydric states had different lymphocyte percentages ($F_{2, 14} = 29.99$; $P < 0.001$), with the highest in DHY and the lowest in HYD. Lymphocytes showed the opposite pattern in Children's pythons ($F_{2, 14} = 8.45$; $P = 0.039$), with the lowest in DHY and the highest in HYD. In western diamond-backed rattlesnakes, lymphocytes were lower in RHY than in the other two treatments ($F_{2, 14} = 6.87$; $P = 0.013$).

The ratios of heterophils to lymphocytes (H/L ratio) were not affected by hydric state in most species (Figure 4.6; Table 4.3), including Gila monsters ($P = 0.70$), Children's pythons ($P = 0.15$), gopher snakes ($P = 0.53$), ball pythons ($P = 0.93$), diamondback water snakes ($P = 0.094$), and water moccasins ($P = 0.12$). The H/L ratio was different across hydric states in beaded lizards ($F_{2, 14} = 15.51$; $P < 0.001$), being highest in DHY and

lowest in HYD. In rattlesnakes, H/L was higher in RHY than in the other two treatments ($F_{2, 14} = 12.79$; $P = 0.0018$).

Monocyte percentages were not affected by hydric state (Table 4.3) in Gila monsters ($P = 0.17$), gopher snakes ($P = 0.42$), or beaded lizards ($P = 0.99$). They were also not affected, but near-significant in diamondback water snakes ($P = 0.078$) and water moccasins ($P = 0.057$). In western diamond-backed rattlesnakes ($F_{2, 14} = 13.13$; $P = 0.0016$), and ball pythons ($F_{2, 14} = 4.40$; $P = 0.033$), monocyte percentages increased during DHY. In Children's pythons, monocyte percentages were lower during HYD than in the other two treatments ($F_{2, 14} = 8.15$; $P = 0.0045$).

Basophil percentages were not affected by hydric state (Table 4.3) in gopher snakes ($P = 0.57$), western diamond-backed rattlesnakes ($P = 0.28$), beaded lizards ($P = 0.98$), ball pythons (though near-significant; $P = 0.067$), diamondback water snakes ($P = 0.37$), or water moccasins ($P = 0.10$). Basophil percentages were higher in HYD than in the other two treatments in Gila monsters ($F_{2, 14} = 6.94$; $P < 0.001$) and Children's pythons ($F_{2, 14} = 7.51$; $P = 0.0061$).

Corticosterone

Baseline CORT increased during DHY in half of the tested species (Figure 4.7; Table 4.4), including Children's pythons ($F_{2, 12} = 12.26$; $P = 0.0013$), beaded lizards ($F_{2,12} = 6.57$; $P = 0.002$), diamondback water snakes ($F_{2,6} = 22.88$; $P = 0.0016$), and water moccasins ($F_{2,5} = 8.96$; $P = 0.022$). A similar trend of increase during DHY was observed in Gila monsters, though the finding was only near-significant ($F_{2, 14} = 2.99$; $P = 0.083$).

Hydric state had no effect on baseline CORT in gopher snakes ($P = 0.95$) and western diamond-backed rattlesnakes ($P = 0.34$). In ball pythons, CORT decreased during RHY ($F_{2,14} = 7.00$; $P = 0.0078$).

Stress-induced CORT (absolute level) was not affected by hydric state in most species (Figure 4.7; Table 4.4), including Children's pythons ($P = 0.11$), gopher snakes (absolute $P = 0.76$), western diamond-backed rattlesnakes ($P = 0.17$), ball pythons (absolute $P = 0.16$), and water moccasins ($P = 0.23$). In Gila monsters ($F_{2,14} = 35.14$; $P < 0.001$) and beaded lizards ($F_{2,12} = 4.25$; $P = 0.027$), stress-induced CORT was significantly higher during DHY than the other treatments. In diamondback water snakes, stress-induced CORT differed with hydric state ($F_{2,6} = 7.78$; $P = 0.022$) and was higher during DHY than RHY, but both of these treatments were similar to HYD levels.

Delta CORT was not affected by hydric state in most species (Table 4.4), including Children's pythons ($P = 0.37$), gopher snakes ($P = 0.76$), ball pythons ($P = 0.52$), diamondback water snakes ($P = 0.24$), and water moccasins ($P = 0.26$). Delta CORT was higher during DHY in both Gila monsters ($F_{2,14} = 13.01$; $P < 0.001$) and beaded lizards ($F_{2,12} = 5.36$; $P = 0.022$), and was higher during RHY than the other treatments in western diamond-backed rattlesnakes ($F_{2,6} = 6.19$; $P = 0.035$).

Stress treatments successfully elevated CORT levels in most species across most hydric states (Figure 4.7). Thirty-minute perturbations increased CORT in all treatments for Children's pythons (HYD $t_6 = 8.74$, $P < 0.001$; DHY $t_6 = -5.54$, $P = 0.0015$; RHY $t_6 = -7.61$, $P < 0.001$), beaded lizards (HYD $t_6 = 7.32$, $P <0.001$; DHY $t_6 = -2.48$, $P = 0.048$; RHY $t_6 = -3.05$, $P = 0.023$), ball pythons (HYD $t_7 = 6.60$, $P < 0.001$; DHY $t_7 = -5.35$, $P =$

0.0011; RHY $t_7 = -7.71$, $P < 0.001$), and water moccasins (HYD t3 = -14.02, $P < 0.001$; DHY t7 = -7.29, $P < 0.001$; RHY t2 = -5.42, $P = 0.032$). The RHY stress treatment did not change CORT levels in gopher snakes (HYD $t_3 = -8.83$; $P = 0.0031$; DHY ($t_7 = -3.60$; $P = 0.0087$; RHY $t_2 = -2.20$; $P = 0.16$), western diamond-backed rattlesnakes (HYD $t_3 = -9.00$, $P = 0.0029$; DHY $t_7 = -10.82$, $P < 0.001$; RHY $t_3 = -2.57$, $P = 0.082$), or diamond-backed water snakes (HYD $t_3 = -9.00$, $P = 0.0029$; DHY $t_7 = -10.82$, $P < 0.001$; RHY $t_3 = -2.32$, $P = 0.10$). The HYD stress treatment did not change CORT levels in Gila monsters (HYD, $P = 0.13$; DHY, $t_7 = -4.21$, $P = 0.004$; RHY, $t_7 = -2.47$; $P = 0.043$).

Species Comparisons: Xeric vs. Mesic

Across all species, innate immunity heavily depended on hydration state, but did not differ between xeric and mesic species groups. Agglutination ($F_{2,110} = 26.08$; $P < 0.001$) and lysis ($F_{2,110} = 25.15$; $P < 0.001$) varied with hydration, but not with group ($P = 0.85$; $P = 0.45$), and there was no interaction of these two factors ($P = 0.84$; $P = 0.52$). BKA varied with hydration state ($F_{2,110} = 27.42$; $P < 0.001$), but not with group ($P = 0.81$), though there was an interaction between factors ($F_{2,110} = 4.38$; $P = 0.015$). Percentages of heterophils ($F_{2,110} = 13.30$; $P < 0.001$), lymphocytes ($F_{2,110} = 14.97$; $P < 0.001$), and basophils ($F_{2,110} = 8.96$; $P < 0.001$) were significantly affected by hydration state, as were H/L ratios ($F_{2,110} = 12.16$; $P < 0.001$), but only lymphocytes ($F_{1,61} = 17.74$; $P < 0.001$) and H/L ratios ($F_{1,61} = 12.16$; $P < 0.001$) were affected by group, and neither showed an interaction of factors ($P = 0.32$; $P = 0.44$). Hydric state affected CORT during baseline ($F_{2,88} = 28.15$; $P < 0.001$) and post-stress ($F_{2,88} = 22.44$, $P < 0.001$), as well as delta

CORT ($F_{2,88} = 3.86$, $P = 0.025$), but CORT was not affected by species groups (though there was an interaction of these factors for baseline CORT: $F_{2,88} = 3.51$; $P = 0.034$).

Species Comparisons: Xeric vs. Semi-Aquatic

Across semi-aquatic species and their paired confamilial species, agglutination and lysis also depended on hydration state, but not species groups. The immune assays which did rely on species group were BKA ($F_{1, 30} = 16.54$; $P < 0.001$), which was also affected by hydration state ($F_{2, 48} = 10.08$; $P < 0.001$), though there was no interaction between factors ($P = 0.32$); lymphocyte percentage ($F_{1,30} = 26.82$; $P < 0.001$), which was also affected by hydration state ($F_{2,48} = 6.14$; $P = 0.0042$), with no interaction ($P = 0.27$); and H/L ratio ($F_{1,30} = 12.92$; $P = 0.0011$), which was also affected by hydration state ($F_{2,48} = 12.09$; $P < 0.001$), with no interaction ($P = 0.52$). Hydric state affected baseline CORT ($F_{2,28} = 10.08$; $P < 0.001$), but baseline CORT did not vary with species group ($P = 0.75$). Post-stress CORT varied with hydration state ($F_{2,28} = 9.12$; $P < 0.001$), but not species group ($P = 0.50$). Delta CORT did not vary with hydration state ($P = 0.13$) or species group ($P = 0.30$).

Dehydration tolerance (osmolality of plasma when an animal showed clinical signs of dehydration) was similar between groups ($P = 0.61$; Table 4.5). Body mass loss also did not differ between groups ($P = 0.13$), but the days that it took for animals to reach clinical dehydration was significantly higher in the xeric group ($F_{1,18} = 117.31$; $P < 0.001$).

Discussion

This comparative study explored whether the response to dehydration in terms of CORT production and innate immunity (together, termed the vertebrate dehydration cascade) varies among squamate species. Additionally, these data address whether habitat type, particularly in terms of the extent of naturally occurring water limtiations, affects whether dehydration induces a stress response or influences innate immune function among squamates. Species were extremely variable in their CORT and immune responses to dehydration, and there were no consistent differences between species more adapted to seasonal water limitation and those that have more reliable water availabilty. However, hydric state affected, at least in some species, all dehydration, CORT, and immune metrics except for the percentage of monocytes. The only general response across species and within each habitat group was that innate immunity, regardless of how evaluated, did not decrease with dehydration, even in species that showed an increase in circulating CORT with dehydration. These data provide the first comprehensive set of results looking at dehydration and resulting effects on the production of glucocorticoids and the innate immune system across several ectothermic vertebrate species; with these data, we can begin to deconstruct the generally accepted vertebrate dehydration cascade. In place of this dehydration cascade, we instead posit that the effects of dehydration on CORT and the resulting long-term effects on immune function are quite varied among reptiles, even in closely related species, regardless of whether the species naturally experience water limitations.

Hydration

Osmolality results demonstrate the effectiveness of my hydration state manipulations, as all species showed similar initial hydrated osmolalities and increased osmolalities at the dehydrated sampling point (Figure 4.1). In all species, RHY greatly reduced osmolality, but in two species (Gila monsters and Children's pythons), the RHY osmolality was lower than it was when hydrated. While these two species were considered seasonally water-limited, I did not see a similar effect in the other two seasonally water-limited species and thus cannot conclude whether this is an adaptive change to this condition. Hematocrit did not increase during DHY for any species except Gila monsters (Figure 4.2). Generally, an increase of hematocrit during DHY is expected, due to decreased plasma in relation to packed cell volume with water loss. However, if an animal regulates blood volume loss by recruiting fluids from interstitial spaces, such a change may be avoided. In such a situation, it would also be likely that rehydration may decrease packed cell volume (as was seen in several species) if excess water taken in was temporarily not voided.

One metric that suggested an adaptive response to water limitation was CEWL, as CEWL in the more mesic species was 1.5 to 2.8 times the values for the confamilial species from a water-limited environment. Among the snakes, the two semi-aquatic species had the highest CEWL rates. Interestingly, the Helodermatid lizards had higher CEWL than the other species within their habitat groups. This result is in line with previous results showing total evaporative water loss of Gila monsters as higher than other xeric-adapted lizard species (DeNardo et al. 2004). Other studies have provided evidence that CEWL

may be influenced heavily by aridity of specific habitats (in kangaroo rats; Tracy and Walsberg 2001), but perhaps future studies can examine whether there are familial influences on evaporative water loss rates among squamates.

Innate Immune Function

The immune results confirm that dehydration increases multiple measures of innate immune function in several squamate species. Agglutination, lysis, and BKA had increased performance during dehydration in roughly half of the species tested. For agglutination, three of the four significant increases were in species categorized as mesic (beaded lizards, diamondback water snakes, and water moccasins), though differences between the species groups were not significant. Those same three species had increased lysis. Bacterial killing ability was significantly increased in Gila monsters, western diamond-backed rattlesnakes, and Children's pythons, all xeric-adapted species. All other species either had near-significant increases in BKA during dehydration, or their BKA was maxed out in the DHY treatment group (and possibly other treatment groups as well), so the change could not be properly analyzed. So, while I did not find a consistent difference in the effect of dehydration on immune function based on habitat type, I did find numerous examples of elevated immune performance during dehydration and no instances of dehydration reducing these immune performances across eight species. There seems to be a dogma regarding the impact of dehydration on the release of glucocorticoids, namely that it acts as a stressor (across a variety of taxa: quail, Cain and Lein 1985; pigeons, De and Ghosh 1993; rabbits, Kallaras 2004; rats, Sebaai et al. 2002; mice, Tsuchida et al. 2004) and experienced chronically, would thereby reduce immune

function (though the interplay between glucocorticoids and the immune system is complex; Sapolsky 2000) and have various other potentially negative physiological effects (at least in mammals; Brocker et al. 2012) through this vertebrate dehydration cascade. However, my results show that in species which dehydrate over the course of days to months and experience increases in CORT during dehydration, these measures of innate immunity are not reduced. My results also show that enhancement of immune function during dehydration is not consistent across all species; there is not yet a clear understanding of what might be influencing these different effects.

Though more literature is available on leukocyte differentials than the other immune measures I used, data is just as varied among the few published studies which do not confound dehydration with another immune driver such as food restriction or heat treatment (e.g., Ohira 1981). Neutrophils (the mammalian equivalent of heterophils) can increase after dehydration (though this was during exercise; Penkman et al. 2008) or granulocytes (neutrophils, eosinophils, and basophils) more generally may not change in number in response to dehydration, but may become activated, producing more toxins intended to kill microorgansims (Tsuchida et al. 2004). Lymphocytes within various organ tissues may decrease due to dehydration (in mice, Tsuchida et al. 2004; in rats, Guseinov and Guseinova 2008), or may not change in number in circulation (in humans, Mitchell et al. 2002; Laing et al. 2008). I found that white blood cell differential responses were extremely variable across hydric states among species, with no real patterns. Lymphocytes, if any differences were present, were most common during HYD or DHY, an odd finding as they are often thought to decrease in relative number in circulation during stress (Hanssen et al. 2003; Davis et al. 2008). Heterophils, H/L ratios,

and monocytes, if any differences were present, showed the highest percentages during DHY or RHY. Heterophils are usually associated with inflammation but can increase during stress (neutrophils, Baker et al. 1998) and are often used as stress indicators (Maxwell and Robertson 1998), though stress does not always elicit changes in heterophil percentage or H/L ratios (Clinchy et al. 2004; Mueller et al. 2011). Basophils, if any differences were present, were highest during HYD. Perhaps these vasodilators may not be helpful to tissues during times when blood volume may be decreased during DHY. More in-depth study of leukocyte production and release would be needed to fully understand the physiological triggers of the immune cell redistributions observed, as well as potential impacts on animal ecology.

Corticosterone

Among the CORT findings, many of the same species that had increased innate immunity during dehydration also had an increase in baseline CORT during DHY, but DHY treatment did not increase CORT in all species. This variation validates that dehydration could be stressful for some species (Breuner et al. 2013). However, these findings show that an increase in baseline CORT does not necessitate a decrease in innate immune function in these species, as is often assumed (Sapolsky et al. 2000). The decoupling of stress and physiological or behavioral responses can occur in species depending on life history (Hau et al. 2010). Most often, decoupling seems to occur in species that experience unpredictable or harsh conditions annually or seasonally or during specific developmental or reproductive periods when stress effects would create additional challenges (Wingfield et al. 1995; Wingfield and Hunt 2002; Wada et al. 2007). In these

cases, stress affecting physiological functions such as reproduction would have direct negative effects on fitness. As part of this decoupling, the CORT response can either be attenuated in general (e.g., Wingfield and Hunt 2002), or the effects of increased CORT can be dampened. As an example of the latter, during a test of the immunocompetence handicap hypothesis (that there is a trade-off between sexual signal development and immune function), Roberts and colleagues (2007) showed that increased CORT is not necessarily immunosuppressive in zebra finches. As CORT is a broad physiological driver, its production is also under constant selection and thus often the CORT response aligns with species-specific needs based on social, developmental, or environmental conditions (Wingfield and Sapolsky 2003; Boonstra 2005; Bókony et al. 2009). Thus, in cases where a CORT response would have negative fitness effects, the response may be dampened or decoupled from some facets of the traditional response. My data suggest a similar phenomenon. Agglutination, lysis, and BKA did not decrease in any species during DHY, including those experiencing significantly increased levels of CORT. Further testing would be required to see if increased CORT levels are uncoupled from additional physiological responses.

CORT reactivity to a stressor (delta CORT) increased during DHY in two species, Gila monsters and beaded lizards. I did not see an increase in CORT reactivity among Children's pythons, though Dupoué et al. (2014) observed increased reactivity (and no change in baseline CORT) with dehydration. The Children's pythons in our study were water restricted for roughly two and a half weeks longer (Dupoué et al.: 52 days; here a 69-day average), and, in the previous experiment, animals were allowed access to a thermal gradient down to 25˚C, whereas my animals were held at 30˚C constantly;

pythons observed by Dupoué et al. lost an average of 10.1 – 12.7% of body mass, while my snakes lost an average of 17.2%, suggesting a greater loss of water because metabolic rate is quite low and would not solely account for this difference. The greater levels of dehydration in my subjects could explain the difference in results for baseline CORT, as it has been shown previously that immune response to water restriction may vary with duration and severity of dehydration (Guseinov and Gusinova 2008). Additionally, increased baseline levels may limit the degree to which CORT levels can increase during a stressful event, as there are physiological maximums for CORT production (Jefcoate et al. 1987); this may potentially explain the lack of a significant difference in reactivity (delta CORT) among already-stressed DHY Children's pythons, as well as in water snakes and water moccasins.

Conclusions

Overall, the findings in this study suggest that variation exists among species, but it does not show that this variation is due to the environmental conditions of species' habitats. However, the data show that 1) dehydration, often thought to be a chronic stressor, did not increase baseline CORT significantly in half of the squamate species tested, and that 2) when CORT did increase, it did not result in a corresponding decrease in innate immune function for those species. Taking these data into account, we see that both levels of the vertebrate dehydration cascade (i.e., dehydration causes increased CORT and that increased CORT is immunosuppressive) fail to incorporate this variation, so the model begins to break down. Additionally, my data suggest that a decoupling of CORT levels and immune responses is occurring in these species. Whether the decoupling

mechanism involves the binding of CORT in plasma (to CORT-binding globulin; Breuner et al. 2013) or modification of response within target tissues remains to be tested. However, it appears that both the stress response and immune function are dynamic and responsive on a fine, physiological scale, and they may also be context-dependent within a broader evolutionary scale.

Table 4.1. Hydration measures for all species across hydration states and cutaneous evaporative water loss. Osmolality, hematocrit, and cutaneous evaporative water loss (CEWL) for each species (mean ± standard error of the mean). All species had a significantly higher osmolality during DHY.

Species	**Habitat**	**Osmolality**			**Hematocrit**			**CEWL**
		HYD	DHY	RHY	HYD	DHY	RHY	HYD
Gila monster	Xeric	283 ± 1	344 ± 3	275 ± 2	27.6 ± 1.5	32.9 ± 1.4	27.6 ± 1.3	6.41 ± 0.25
Children's python	Xeric	286 ± 3	339 ± 2	272 ± 2	32.9 ± 1.6	32.1 ± 1.4	26.9 ± 1.1	3.46 ± 0.14
Gopher snake	Xeric	300 ± 2	341 ± 3	296 ± 2	28.0 ± 2.1	26.6 ± 2.5	31.5 ± 1.1	3.65 ± 0.18
W. diamond-backed rattlesnake	Xeric	293 ± 3	333 ± 3	290 ± 2	22.2 ± 1.4	21.8 ± 2.5	18.4 ± 2.1	3.44 ± 0.08
Beaded lizard	Mesic	288 ± 2	357 ± 5	285 ± 2	27.8 ± 2.0	30.1 ± 1.3	27.1 ± 1.1	12.09 ± 0.89
Ball python	Mesic	282 ± 2	345 ± 2	280 ± 2	19.8 ± 0.7	19.4 ± 0.8	22.4 ± 2.0	5.35 ± 0.22
Diamondback water snake	Semi-aquatic	292 ± 3	339 ± 7	291 ± 2	35.6 ± 3.3	34.9 ± 1.4	20.4 ± 2.2	10.37 ± 0.68
Water moccasin	Semi-aquatic	295 ± 2	356 ± 7	287 ± 2	26.4 ± 0.8	25.5 ± 1.5	22.7 ± 1.8	8.29 ± 0.48

Table 4.2. Innate immune function for all species across hydration states. Agglutination, lysis, and bacterial killing ability (BKA) of plasma for each species as mean ± standard error of the mean. Gopher snakes, beaded lizards, water snakes, and water moccasins all had increased agglutination during DHY. Beaded lizards, ball pythons, and water moccasins had increased lysis during DHY. Western diamond-backed rattlesnakes and Children's pythons had increased BKA during DHY, and all other species had similar trends or the assay maxed out on DHY samples.

Species	Habitat	Agglutination			Lysis			BKA		
		HYD	DHY	RHY	HYD	DHY	RHY	HYD	DHY	RHY
Gila monster	Xeric	4.47 ± 0.47	5.53 ± 0.50	4.38 ± 0.54	2.78 ± 0.23	4.00 ± 0.25	2.63 ± 0.25	38.96 ± 16.26	81.33 ± 12.44	29.08 ± 14.54
Children's python	Xeric	6.47 ± 0.33	7.00 ± 0.25	6.19 ± 0.24	5.38 ± 0.32	5.88 ± 0.30	4.88 ± 0.23	51.98 ± 18.17	84.85 ± 11.51	40.94 ± 17.42
Gopher snake	Xeric	6.42 ± 0.37	7.22 ± 0.34	7.13 ± 0.41	4.75 ± 0.20	5.06 ± 0.19	5.06 ± 0.06	24.63 ± 13.16	55.56 ± 14.75	7.98 ± 2.68
W. diamond-backed rattlesnake	Xeric	7.25 ± 0.44	7.72 ± 0.54	7.44 ± 0.64	4.47 ± 0.44	5.03 ± 0.46	4.78 ± 0.48	12.50 ± 12.50	55.56 ± 16.51	0 ± 0
Beaded lizard	Mesic	4.29 ± 0.14	4.61 ± 0.46	4.14 ± 0.07	3.14 ± 0.14	3.50 ± 0.45	2.89 ± 0.07	100 ± 0	100 ± 0	85.20 ± 12.25
Ball python	Mesic	6.19 ± 0.34	6.75 ± 0.41	6.19 ± 0.32	4.50 ± 0.33	5.34 ± 0.31	4.63 ± 0.27	0.43 ± 0.34	18.51 ± 11.49	4.24 ± 1.28
Diamondback water snake	Semi-aquatic	6.00 ± 0	6.34 ± 0.16	5.69 ± 0.19	4.50 ± 0.29	4.69 ± 0.21	4.25 ± 0.25	44.21 ± 24.27	64.18 ± 16.27	3.37 ± 1.70
Water moccasin	Semi-aquatic	7.61 ± 0.43	9.31 ± 0.44	8.30 ± 0.59	5.68 ± 0.49	7.19 ± 0.32	6.60 ± 0.40	87.50 ± 12.50	100 ± 0	97.90 ± 2.10

Table 4.3. Leukocyte differential for all species across hydration states. Proportion of heterophils, lymphocytes, monocytes, and basophils, as well as heterophil/lymphocyte ratios for each species as mean ± standard error of the mean.

Species	Habitat	Heterophils			Lymphocytes			H/L ratio			Monocytes			Basophils		
		HYD	DHY	RHY	HYD	DHY	RHY	HYD	DHY	RHY	HYD	DHY	RHY	HYD	DHY	RHY
Gila monster	Xeric	38 ± 3	41 ± 2	43 ± 2	37 ± 3	36 ± 3	37 ± 1	1.12 ± 0.13	1.24 ± 0.15	1.18 ± 0.08	13 ± 2	16 ± 2	14 ± 1	12 ± 2	7 ± 2	7 ± 2
Children's python	Xeric	9 ± 2	20 ± 7	13 ± 2	51 ± 3	33 ± 5	37 ± 2	0.19 ± 0.04	0.86 ± 0.42	0.37 ± 0.07	23 ± 2	37 ± 4	40 ± 3	17 ± 3	9 ± 2	11 ± 3
Gopher snake	Xeric	34 ± 4	35 ± 6	39 ± 9	36 ± 3	33 ± 7	30 ± 1	1.01 ± 0.21	1.36 ± 0.27	1.29 ± 0.3	23 ± 3	20 ± 4	15 ± 4	2 ± 0	2 ± 1	1 ± 1
W. diamond-backed rattlesnake	Xeric	26 ± 1	26 ± 4	55 ± 6	13 ± 2	16 ± 3	8 ± 2	2.10 ± 0.30	1.89 ± 0.39	10.29 ± 3.31	4 ± 1	11 ± 3	1 ± 0	3 ± 1	5 ± 2	4 ± 1
Beaded lizard	Mesic	28 ± 2	51 ± 4	44 ± 3	53 ± 2	31 ± 3	39 ± 3	0.53 ± 0.05	1.82 ± 0.29	1.22 ± 0.17	11 ± 2	11 ± 2	11 ± 2	6 ± 2	6 ± 2	6 ± 1
Ball python	Mesic	33 ± 2	29 ± 2	30 ± 3	16 ± 1	14 ± 1	16 ± 2	2.13 ± 0.21	2.22 ± 0.40	2.19 ± 0.40	44 ± 2	52 ± 3	50 ± 3	7 ± 1	6 ± 1	4 ± 0
Diamondback water snake	Semi-aquatic	22 ± 3	27 ± 4	39 ± 11	13 ± 2	10 ± 1	10 ± 2	1.96 ± 0.46	3.06 ± 0.54	5.57 ± 2.27	12 ± 2	17 ± 2	12 ± 5	6 ± 2	2 ± 1	8 ± 7
Water moccasin	Semi-aquatic	32 ± 8	35 ± 5	52 ± 7	5 ± 1	5 ± 1	4 ± 0	7.30 ± 2.10	10.16 ± 3.39	13.54 ± 2.27	8 ± 3	3 ± 1	3 ± 1	7 ± 2	4 ± 1	3 ± 1

Table 4.4. Plasma corticosterone activity for all species across hydration states. Baseline CORT, post-stress CORT, and delta CORT for each species as mean ± standard error of the mean. Beaded lizards, Children's pythons, diamondback water snakes, and water moccasins had increased baseline CORT during DHY. Gila monsters, beaded lizards, and western diamond-backed rattlesnakes had increased post-stressed CORT during DHY.

Species	Habitat	Baseline CORT			Post-stress CORT			Delta CORT		
		HYD	DHY	RHY	HYD	DHY	RHY	HYD	DHY	RHY
Gila monster	Xeric	3.25 ± 1.57	6.75 ± 2.21	2.37 ± 0.55	3.10 ± 0.58	45.64 ± 17.35	6.67 ± 2.84	-0.15 ± 1.57	38.89 ± 15.76	4.30 ± 3.10
Children's python	Xeric	44.11 ± 13.91	95.21 ± 33.15	39.39 ± 10.62	128.66 ± 43.86	210.07 ± 37.84	134.70 ± 38.82	84.54 ± 30.95	114.86 ± 20.74	95.31 ± 29.74
Gopher snake	Xeric	18.25 ± 8.21	40.83 ± 19.09	32.26 ± 14.71	93.05 ± 14.20	99.49 ± 14.25	80.9 ± 12.90	74.80 ± 8.47	60.00 ± 17.52	48.64 ± 2.37
W. diamond-backed rattlesnake	Xeric	52.87 ± 15.46	74.12 ± 19.63	104.26 ± 60.80	223.67 ± 98.42	178.77 ± 42.55	128.89 ± 53.53	170.80 ± 83.48	104.65 ± 24.36	24.64 ± 9.58
Beaded lizard	Mesic	4.06 ± 0.33	21.25 ± 10.39	3.22 ± 0.38	7.86 ± 1.47	105.87 ± 69.82	6.73 ± 1.24	3.80 ± 1.46	84.62 ± 69.94	3.50 ± 1.15
Ball python	Mesic	86.00 ± 12.30	99.24 ± 20.24	52.84 ± 13.59	362. 34 ± 85.92	350.68 ± 60.49	263.72 ± 35.17	276.34 ± 81.41	251.44 ± 46.97	210.88 ± 27.34
Diamondback water snake	Semi-aquatic	31.31 ± 13.42	107.04 ± 26.75	24.49 ± 9.41	119.30 ± 33.20	206.06 ± 59.46	95.34 ± 54.07	88.00 ± 23.26	99.02 ± 49.51	70.85 ± 45.29
Water moccasin	Semi-aquatic	16.83 ± 2.35	29.63 ± 3.40	16.38 ± 2.40	63.22 ± 8.66	82.38 ± 10.13	40.84 ± 9.10	46.39 ± 7.11	52.75 ± 7.89	24.46 ± 7.46

Table 4.5. Dehydration tolerance of select species. Maximum osmolality at first sign of clinical dehydration, time to maximum osmolality, and body mass loss during that time for semi-aquatic species and confamilial xeric species as mean ± standard error of the mean. Maximum osmolality and body mass loss did not differ between groups, but xeric species took significantly longer to reach clinical dehydration.

Species	Habitat	Max osmolality (mOsm/kg)	Time to max osm (days)	Body mass loss (%)
Gopher snake	Xeric	464 ± 17	56 ± 6	34 ± 2
W. diamond-backed rattlesnake	Xeric	382 ± 24	99 ± 21	25 ± 5
Water snake	Semi-aquatic	417 ± 12	7 ± 1	25 ± 1
Water moccasin	Semi-aquatic	450 ± 32	13 ± 0	22 ± 1

Figure 4.1. Osmolality across hydric states for all species. Dehydration created a similar significant increase in osmolality across all species. In Gila monsters and Children's pythons, the rehydrated state had a significantly lower osmolality than did the hydrated state. Points are means and error bars are ± standard error of the mean, though points were often so precise that the error bars are within the marks for the mean.

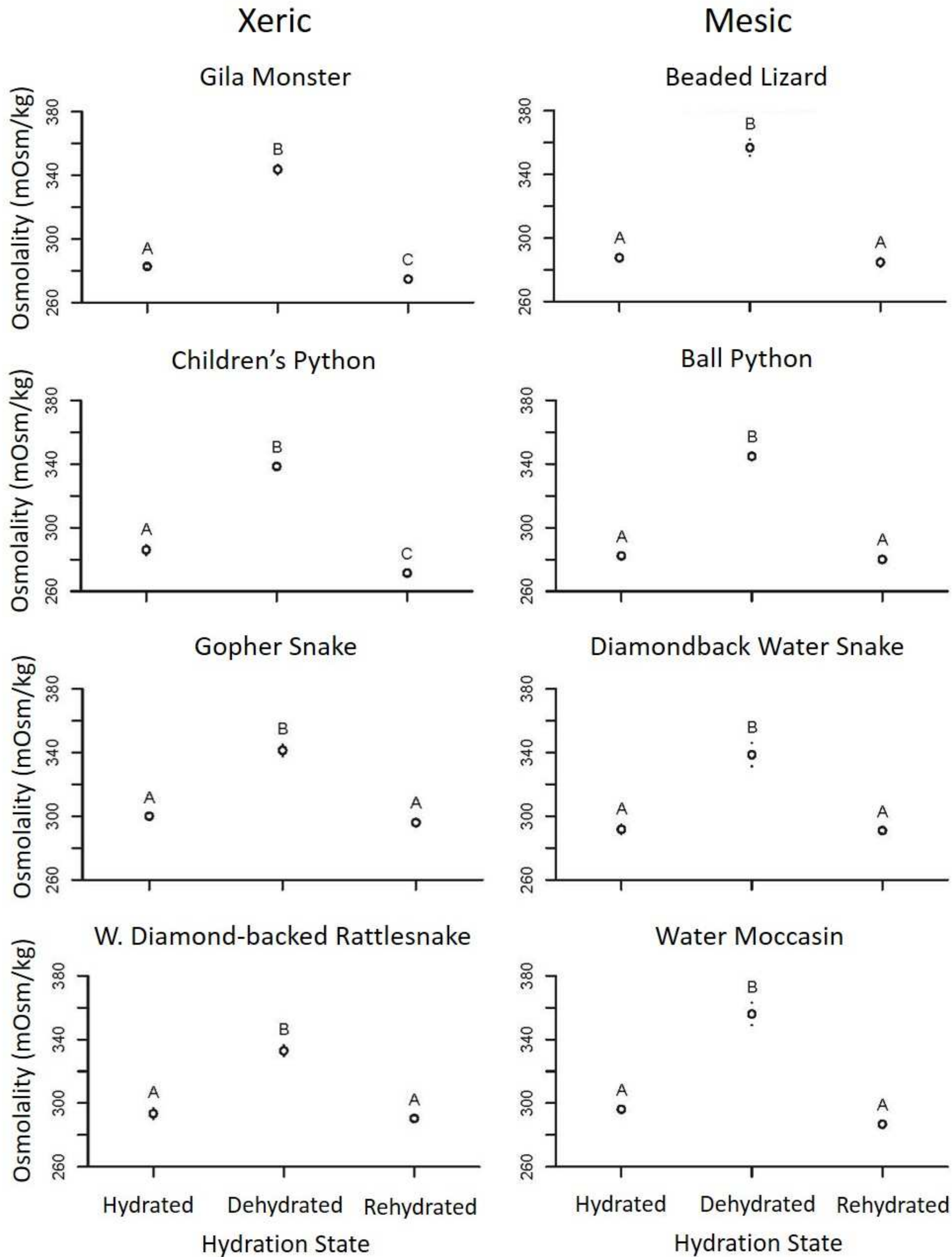


Figure 4.2. Hematocrit across hydric state treatments for all species. Most species showed a decrease in hematocrit when rehydrated. Gila monsters had increased hematocrit during dehydration and beaded lizards, gopher snakes, and diamondback water snakes showed no differece in hematocrit across hydration states. Bars show means and error bars show ± standard error of the means.

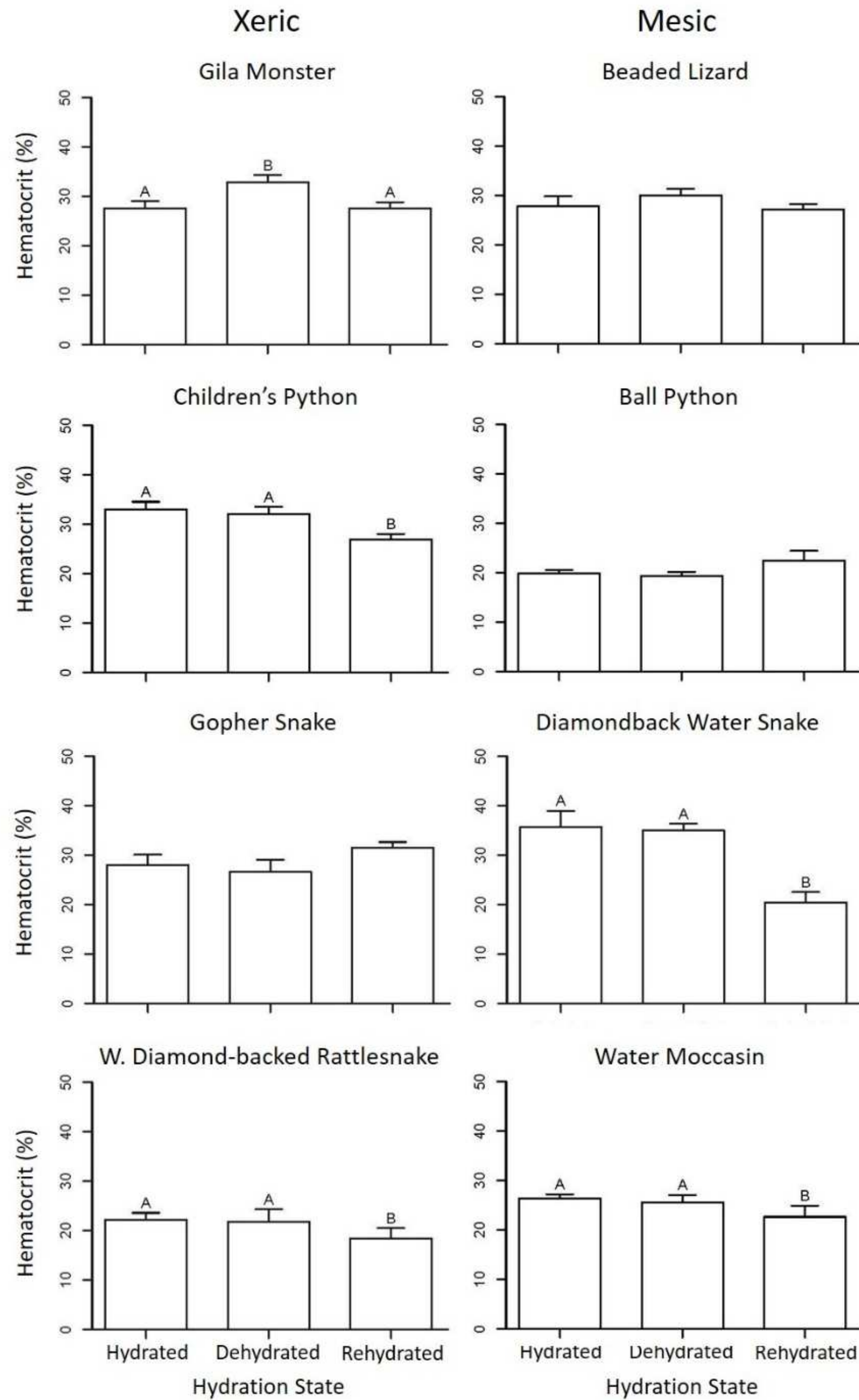


Figure 4.3. Agglutination across hydric state treatments for all species. Agglutination was increased during dehydration in five species. The remaining three, Children's pythons, ball pythons, and western diamond-backed rattlesnakes, showed no change in agglutination across hydration states. Bars show means and error bars show ± standard error of the means.

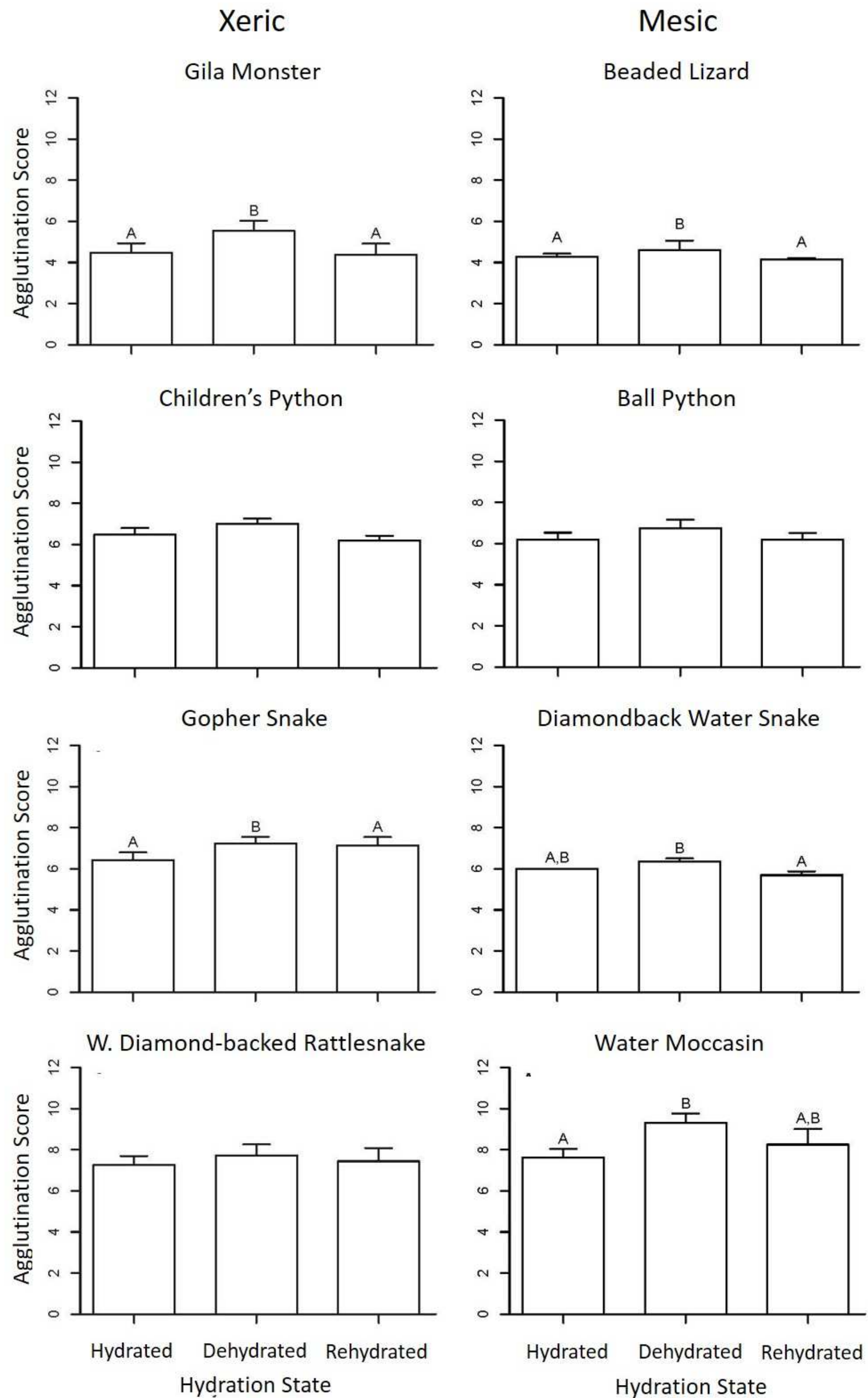


Figure 4.4. Lysis scores across hydric state treatments for all species. Lysis was increased during dehydration in four species, including Gila monsters, beaded lizards, ball pythons, and water moccasins. The remaining four species showed no change in lysis across hydration states. Bars show means and error bars show ± standard error of the means.

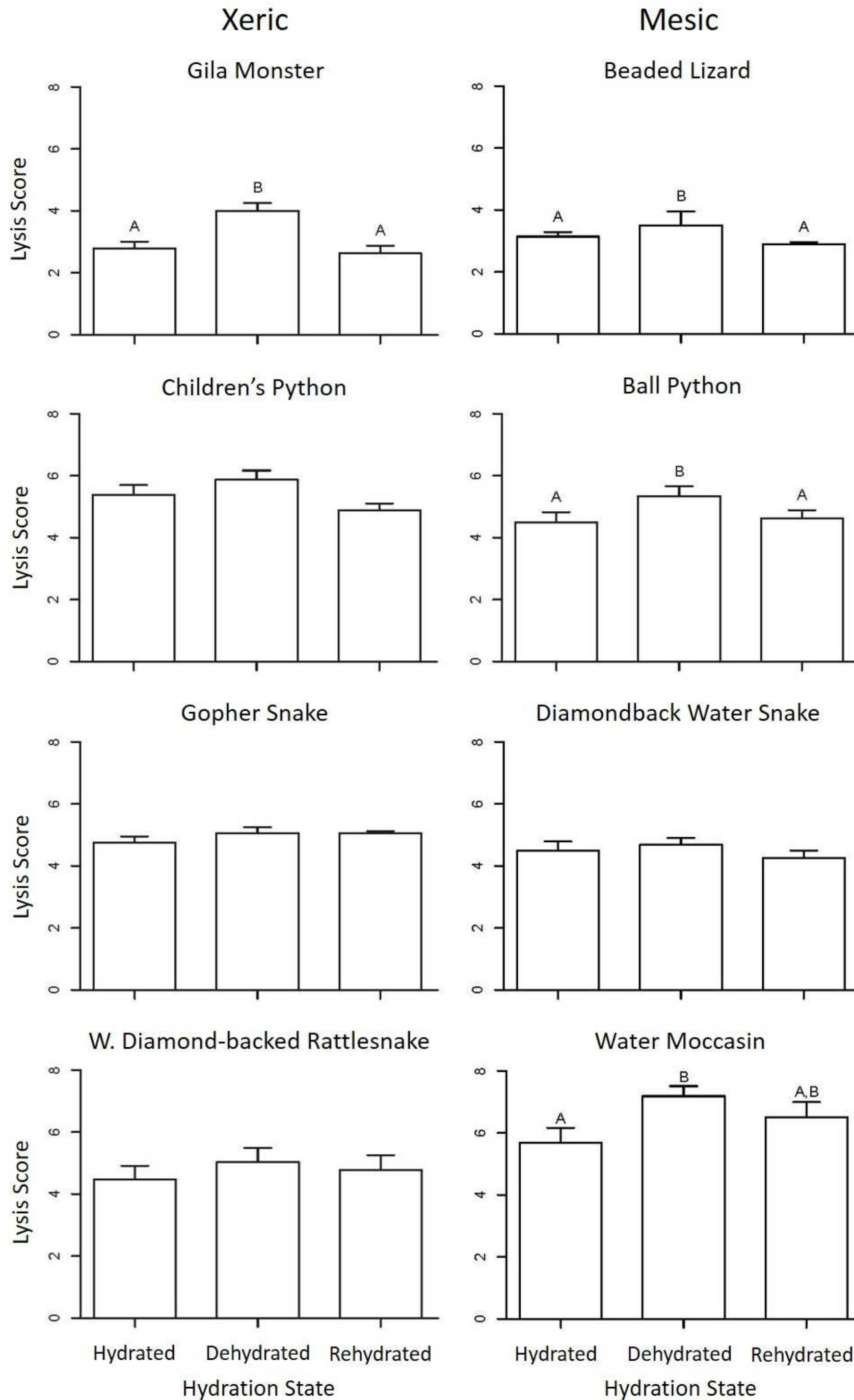


Figure 4.5. BKA scores across hydric state treatments for all species. BKA was significantly higher during dehydration in three xeric species, including Gila monsters, Children's pythons, and western diamond-backed rattlesnakes. BKA tended to be higher, with near significance, in gopher snakes, ball pythons, and diamondback water snakes. In beaded lizards and water moccasins, BKA was maxed out during dehydration and during hydration in beaded lizards. Bars show means and error bars show ± standard error of the means.

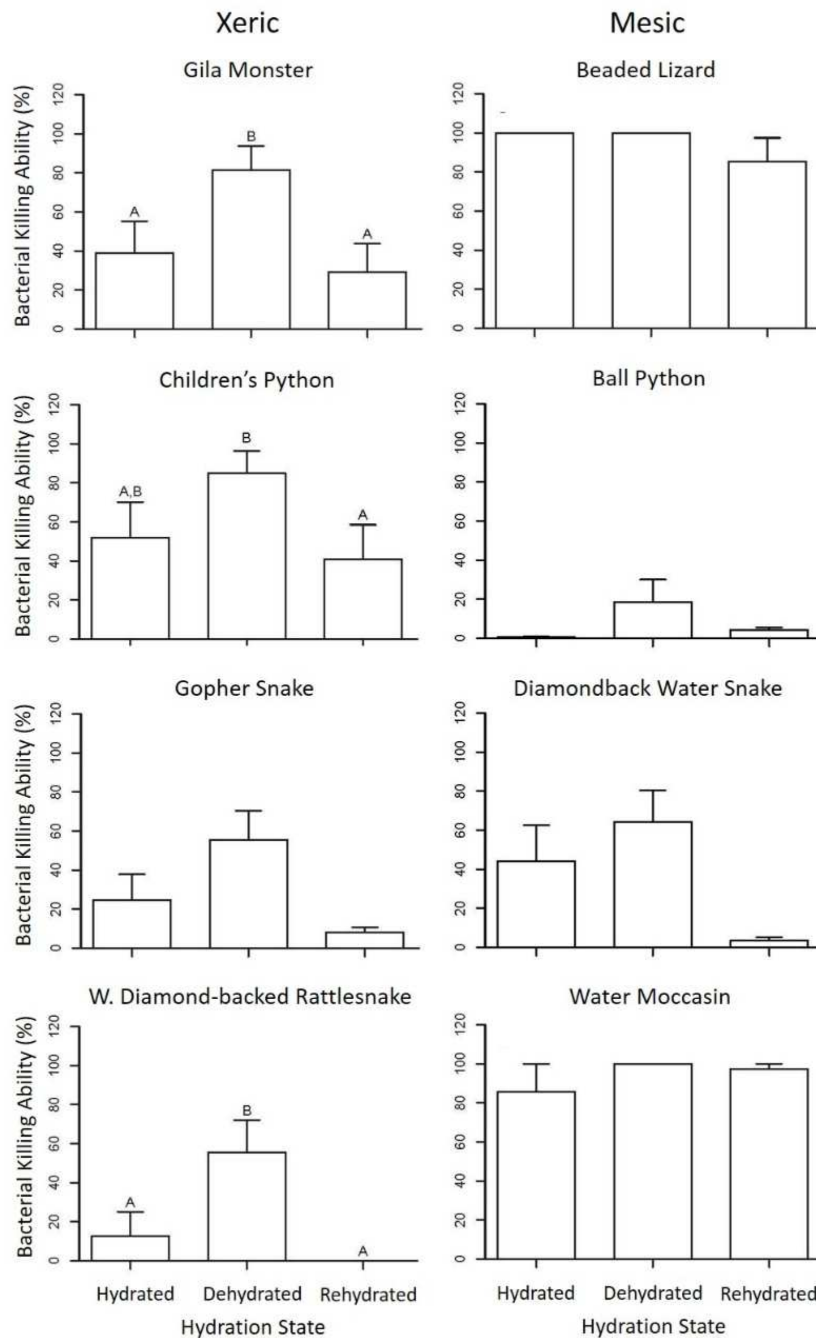


Figure 4.6. Ratios of heterophils to lymphocytes (H/L ratios) across hydric state treatments for all species. Most species showed no change in H/L ratio across hydration states. In western diamond-backed rattlesnakes, H/L ratios were highest during rehydration and in beaded lizards, they were highest during dehydration. The scale is 0 – 8 for most species, but 0 – 20 for the Viperids. Bars show means and error bars show ± standard error of the means.

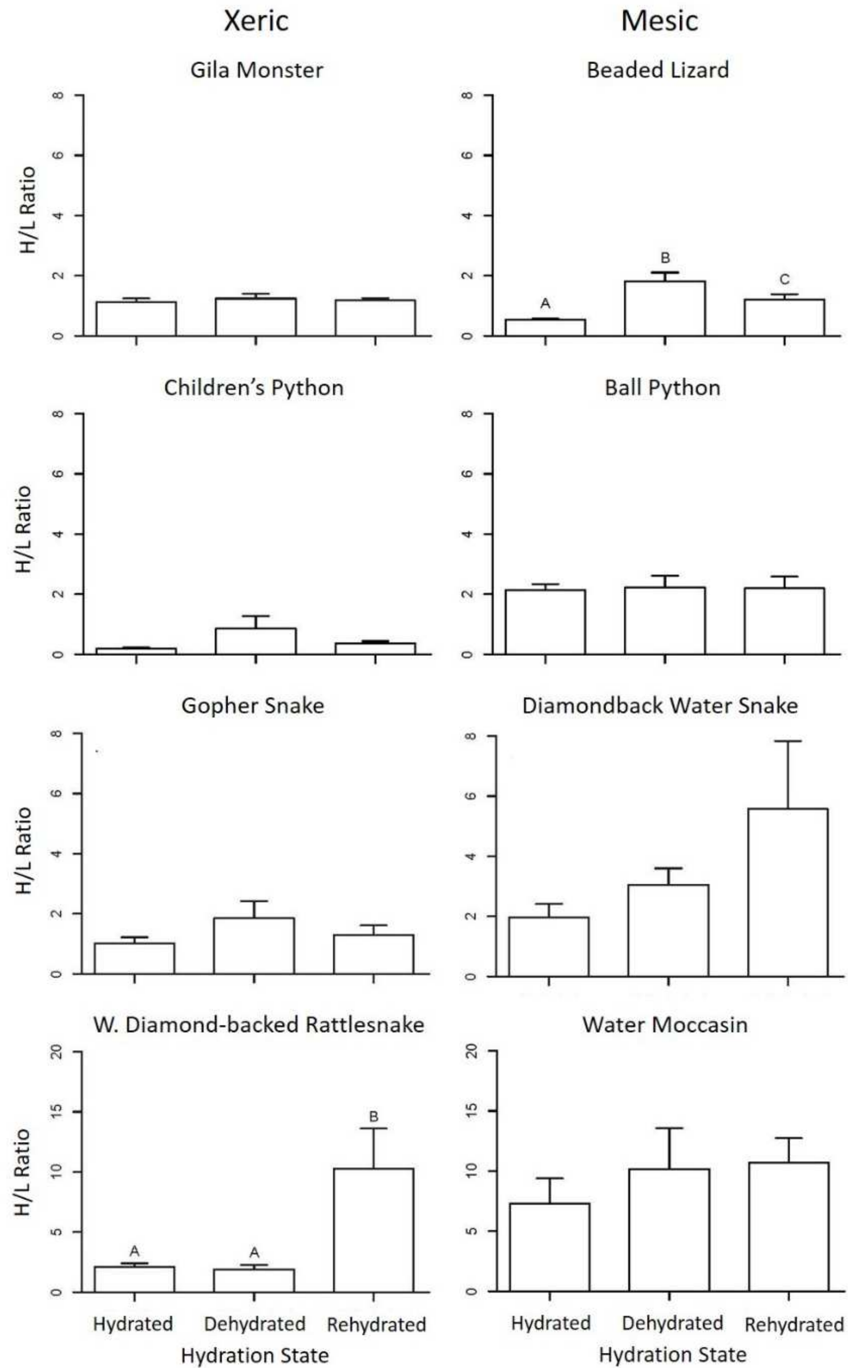
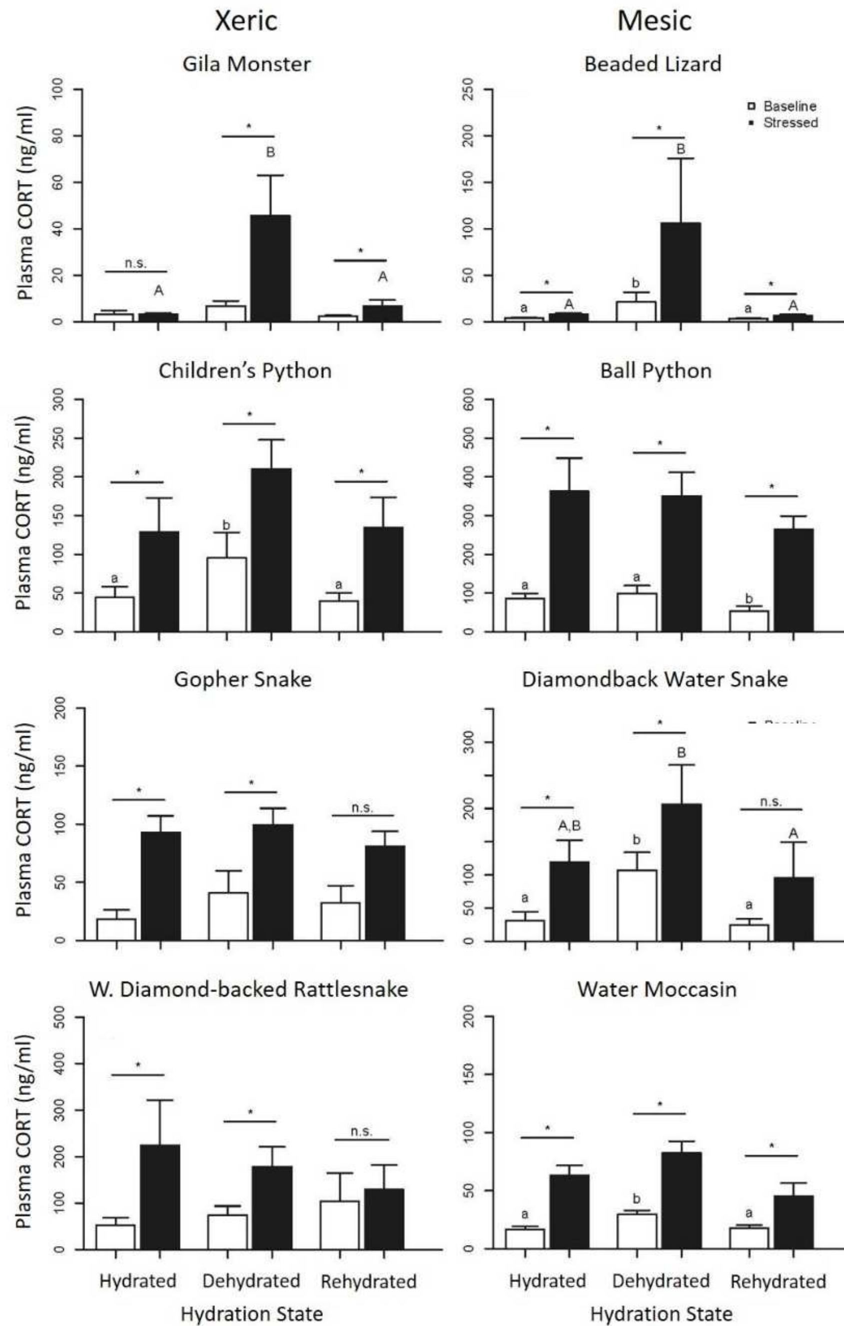


Figure 4.7. Baseline and post-stress-treatment CORT across hydric states for all species. Half of the species showed an increase in baseline CORT during dehydration, including Children's pythons, and three of the mesic species—beaded lizards, diamondback water snakes, and water moccasins. Gila monsters showed a near-significant increase in baseline CORT during dehydration. Gila monsters, beaded lizards, and diamondback water snakes also showed an increase in post-stress-treatment CORT during dehydration. Bars show means and error bars show ± standard error of the means.



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APPENDIX A

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APPENDIX B

COAUTHOR APPROVAL

All coauthors of published chapters granted permission to use those chapters in this dissertation.