

Dehydration enhances multiple physiological defense mechanisms in a desert lizard, *Heloderma suspectum*

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Summary statement: We investigated how dehydration affected immunity and plasma corticosterone concentrations in Gila monsters. Dehydration did not elevate corticosterone, but enhanced the physiological defense mechanisms of stress reactivity and innate immunity.

## Abstract

The physiological challenges associated with dehydration can induce an increase in plasma glucocorticoid concentrations, a response thought to provide the mechanism for dehydration suppressing immune function. However, a comprehensive examination of the interrelationship of dehydration, stress, and immune function has not been conducted within a single species. We previously demonstrated that Gila monsters (*Heloderma suspectum*), which inhabit a xeric environment with a predictable seasonal drought, have enhanced measures of innate immunity when dehydrated. These results suggest that, in this species, dehydration may not induce a glucocorticoid response, but, instead, enhances physiological defense mechanisms. To explore this possibility, we examined multiple measures of innate immunity as well as initial and reactive plasma concentrations of glucocorticoids in captive and free-ranging Gila monsters at various hydration states. Our results show that, in this species, dehydration alone does not cause a substantial increase in plasma glucocorticoids, and we provide broader evidence that dehydration enhances defensive mechanisms including stress reactivity and various measures of innate immune function. These findings suggest that physiological responses to dehydration may 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 water-limited environments 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). Furthermore, as both survival and reproduction rely on the availability of sufficient water resources, water imbalance can reduce animal fitness.

Dehydration can often be viewed as a physiological stressor as endocrine responses may be an integral part of an organism's response to dehydration (Sebaai et al., 2002; Maresh et al., 2004). In particular, dehydration increases plasma glucocorticoids, the primary steroid hormones involved in the stress response, in mice (*Mus musculus*; Tsuchida et al., 2004; Bekkevold et al., 2013), rats (*Rattus norvegicus*; Sebaai et al., 2002; Arnhold et al., 2007), rabbits (*Oryctolagus cuniculus*; Kallaras, 2004), quail (*Colinus virginianus*; Cain and Lien, 1985), chickens (*Gallus gallus domesticus*; Klandorf, 1984), and cattle (*Bos indicus*; Parker et al., 2004). In general, elevated glucocorticoids have initial enhancing effects on immunity, but, if prolonged, they have more depressive effects, though response may also depend on stress hormone concentration (Sapolsky et al., 2000).

Glucocorticoids affect many physiological processes that are involved in coping with challenges (Busch and Hayward, 2009). For example, immune function is affected by glucocorticoids in various ways (Webster Marketon and Glaser, 2008; Martin, 2009; Stier et al., 2009).

Glucocorticoids can have a depressive effect on the synthesis or release of immune-promoting molecules and can stimulate or depress the proliferation of B and T cells, depending on physiological conditions (Sapolsky et al., 2000).

Because dehydration-induced elevation in plasma glucocorticoids is well documented, as are the negative effects of prolonged elevation of glucocorticoids on immune function, a linkage or cascade among them is somewhat assumed—namely, dehydration increases plasma glucocorticoid concentrations, which, if sustained, decreases immune function. However, some species tolerate dehydration for days, weeks, or months (e.g., Arabian camel (*Camelus dromedarius*), Schmidt-Nielsen et al., 1956; desert tortoise (*Gopherus agassizii*), Peterson, 1996; various dormant insects, Danks, 2000). Adjustments employed during times of dehydration may slow water loss (e.g., through modified activity patterns; Davis and DeNardo, 2010), alter growth patterns (Lorenzon et al., 1999; Dođramacı 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).

Despite these adjustments, dehydration should pose similar physiological challenges whether or not a species tolerates dehydration, especially if the dehydration-glucocorticoid-immunity cascade is universal. Interestingly, however, a glucocorticoid response to dehydration is lacking in Children's pythons (*Antaresia childreni*; Dupoué et al., 2014), Awassi sheep (*Ovis aries*; Jaber et al., 2004; Hamadeh et al., 2006; but see Ghanem et al., 2008), and humans (*Homo sapiens*; Malarkey et al., 1993; Hoffman et al., 1994; but see Maresh et al., 2006). Similarly, the negative relationship between dehydration and immune function is also not without exception, as we have demonstrated that Gila monsters, *Heloderma suspectum*, have improved innate immune performances when dehydrated (Moeller et al., 2013). Unfortunately, the interplay of the three components of this relationship (dehydration, the stress response, and immunity) has not been studied in a single species.

Accordingly, we explored the interrelationship of hydric state, glucocorticoids, and innate immunity in Gila monsters. We examined these relationships in captive animals under controlled environmental conditions as well as in free-ranging Gila monsters under varying seasonal conditions. We have previously shown that Gila monsters regularly experience prolonged seasonal dehydration (Davis and DeNardo, 2010) that suppresses foraging behaviors and can limit food acquisition (Davis and DeNardo, 2009). We collected blood samples from captive and free-ranging Gila monsters in hydrated and dehydrated states to: 1) test the repeatability of our previous findings of increased innate immunity (i.e., hemolysis and hemagglutination tests; Moeller et al., 2013) during moderate dehydration, 2) examine additional measures of innate immunity including bacterial killing ability and white blood cell differential, and 3) determine plasma corticosterone (CORT, the natural glucocorticoid of reptiles) concentrations prior to and after a stress perturbation in multiple hydric states. Comparing measures of initial CORT during the hydrated and dehydrated states will inform us as to whether dehydration can be viewed as a direct stressor, while the post-perturbation measure of CORT tests whether dehydrated animals exhibit elevated stress responsiveness to their surroundings. Unlike persistently elevated initial CORT levels, an acute CORT response to a stressor is generally classified as an adaptive physiological defense mechanism.

Together, this compilation of assessments will provide a simultaneous look at stress and defense responses stimulated by species-typical dehydration during seasonal drought. Specifically, we

hypothesized that, in species that cope with drought in part by tolerating dehydration (Stahlschmidt et al., 2011), moderate dehydration does not induce a stress response but stimulates physiological defense mechanisms. Thus, we predicted that dehydration in Gila monsters does not induce an increase in plasma CORT concentrations, which could explain the lack of an immunosuppressive response to dehydration. We further predict, in line with our previous findings, that dehydration in Gila monsters enhances physiological defense mechanisms including enhanced metrics of innate immunity and stress responsiveness.

## Methods

### *Study Species and Overview*

The Gila monster is a large-bodied lizard that is found in southwestern desert scrub habitats of North America that receive rain during the winter, early spring, and late summer monsoon period. Between early spring and the monsoon, there is typically a rain-free 60- to 80-day 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, we 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 spring and early summer, respectively; Davis and DeNardo, 2010). All procedures were reviewed and approved by Arizona State University's Animal Care and Use Committee (protocol 13-1299R).

### *Captive Gila Monster Experiment*

We used 8 captive adult Gila monsters (3 females, 5 males) obtained from the Arizona Game and Fish Department (permits #SP577864, SP684760) to examine the effects of hydration state on innate immunity 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).

The three different hydration states were reached by regulating access to water and monitoring blood plasma osmolality. Animals were provided water *ad libitum* for two weeks as they acclimated to their experimental environment. We then collected an initial 0.7 ml blood sample from each Gila monster 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 perturbed by repeated gentle prodding of its head, back, rear feet, and 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 perturbation. Thirty minutes is an adequate duration 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). We 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, ultrasonography was used to ensure that all bladder contents had been removed. The animal was then denied access to water to begin the dehydration process.

During dehydration, animals were checked daily and weighed every two to seven days. Blood samples (0.06 ml, taken as described above) for monitoring hydration state were collected during the second week, and once a week thereafter. When each animal approached a state of moderate

dehydration (~330 mOsm; often the extent of dehydration in the wild), a dehydrated state blood sample set (0.7 ml initial, 0.1 ml post-stress-treatment) was collected at least 4 full days after the previous monitoring bleed to avoid any potential effects of acute stress from the bleeding process. All samples were collected within three minutes of the start of handling to avoid a handling-associated elevation of plasma corticosterone (Romero and Reed, 2005). 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*. After having seven days of free access to water, we 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 Preparation and Assays below).

### *Free-Ranging Gila Monster Study*

To provide ecological relevance to our findings in captive animals, we collected initial and post-perturbation 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. We 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 innate immune function and plasma corticosterone concentrations varied with hydration state. 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 and all were compared to ensure individuals were unique. Within 12 hours of collection, blood samples were processed and stored for later assays.

### *Sample Processing and Assays*

All pre-perturbation 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 initial plasma CORT concentration. The post-perturbation blood samples were used only to determine plasma CORT concentration (i.e., CORT responsiveness). To process each pre-perturbation 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  $\mu$ l aliquots. All aliquots were stored at  $-80^{\circ}\text{C}$  for later assays. Post-perturbation blood samples were centrifuged and the plasma separated and frozen at  $-80^{\circ}\text{C}$  for later assays.

### *Assays: Determination of Hydration State*

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

To measure hematocrit, which often changes with hydration state, two capillary tubes were partially filled 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.

### *Assays: Agglutination and Lysis*

We 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, we serially diluted 20  $\mu$ l 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  $\mu\text{l}$  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). We 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. We 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.

#### *Assays: Bacterial Killing Ability*

Bacterial killing ability (BKA) assays were conducted using plasma from the pre-perturbation samples, following methods in French and Neuman-Lee (2012). Briefly, using autoclaved instruments in a sterilized laminar flow hood, frozen initial plasma samples were thawed and 2  $\mu\text{l}$  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  $\mu\text{l}$  phosphate-buffered saline and 18  $\mu\text{l}$  CO<sub>2</sub>-independent media plus 4 mM L-glutamine (no bacteria). Positive-control wells were made with 6  $\mu\text{l}$  of working bacteria solution, which consisted of 10<sup>4</sup> colony-forming units of *Escherichia coli* (NO. 8739, ATCC, Manassas, VA), along with 18  $\mu\text{l}$  of media. Sample wells also contained 6  $\mu\text{l}$  working bacteria solution and 16  $\mu\text{l}$  media, so that all wells had a final volume of 24  $\mu\text{l}$ . 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  $\mu\text{l}$  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 xMark™ Microplate Absorbance Spectrophotometer) at 300 nm. Following the reading, the plates were incubated at 37°C for 12 hrs, gently vortexed for 1 minute, and then 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.

### *Assays: Corticosterone*

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.

We diluted 25  $\mu$ l of plasma with assay buffer containing steroid displacement reagent before assaying. The average intra-assay coefficient of variation (CV) was 19.5% and the average inter-assay CV was 2.3%. The high intra-assay CV is possibly due to the low levels of CORT in many of the samples, as small changes in low values can lead to higher CVs, despite the difference in hormone levels still being small. The average assay sensitivity was 6.4 pg/ml.

### *Assays: Blood Cell Differentials*

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, we used repeated-measures analyses of variances (rmANOVAs) to validate differences in osmolality among hydric states 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 among hydric states. 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 we used Welch's t-tests to assess differences between hydration states 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 we still used a Welch's test as it is robust to unequal variances. Bacterial killing ability was problematic with 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 differences were non-significant. We also used a Kendall's rank correlation to test for a relationship between BKA and both agglutination and lysis. The rank correlation was only completed on free-ranging animals, in which different individuals were at different levels of hydration, so a correlation could be appropriately tested. Post-perturbation CORT and delta CORT (the difference between initial and post-perturbation 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 initial and post-perturbation levels, to assess stress reactivity. All plots were made using Graph Pad Prism (GraphPad Software, Inc., La Jolla, CA).

## Results

### *Plasma Osmolality*

All captive animal hydric states had significantly different plasma osmolality (Figure 1; Table 1). Captive animals in a dehydrated state had a significantly higher osmolality than when they were hydrated and rehydrated ( $F_{2,12} = 300.24$ ;  $P < 0.001$ ). Pre-catheterized, water-deprived Gila

monsters reached a moderate state of dehydration in  $28 \pm 9$  days, a duration that is comparable to a similar increase in osmolality reported in free-ranging animals during the dry season (1 month, Davis and DeNardo, 2009). 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*

Agglutination was highest during dehydration in both captive ( $F_{2,14} = 12.19$ ;  $P = 0.0016$ ; Figure 2; Table 1) and free-ranging animals ( $t(9) = -2.86$ ;  $P = 0.021$ ; Figure 3; Table 1). Lysis also increased during dehydration in both captive ( $F_{2,12} = 31.48$ ;  $P < 0.001$ ; Figure 2; Table 1) and free-ranging animals ( $t(9) = -2.80$ ;  $P = 0.021$ ; Figure 3; Table 1).

### *Bacterial Killing Ability*

Bacterial killing ability was highest in the dehydrated state for captive animals ( $F_{2,14} = 7.84$ ;  $P = 0.0052$ ; Figure 2; Table 1), but there was no difference among states in free-ranging animals (however, see Methods for difficulties with statistical analyses of these data;  $t(2) = -1.45$ ;  $P = 0.28$ ; Figure 3; Table 1). Among free-ranging animals, BKA was correlated with both agglutination ( $\tau_b = 0.680$ ;  $p = 0.013$ ) and with lysis ( $\tau_b = 0.640$ ;  $p = 0.018$ ).

### *Corticosterone*

In captive animals, baseline CORT did not vary among hydric states, though there was a near-significant difference, with the highest mean CORT in the dehydrated state ( $F_{2,14} = 2.99$ ;  $P = 0.083$ ; Figure 2; Table 1). However, post-perturbation CORT levels were significantly different among hydration states ( $F_{2,14} = 35.14$ ;  $P < 0.001$ ), with considerably higher post-perturbation CORT in dehydrated animals (Figure 2; Table 1). The hydrated and rehydrated states did not have significantly different post-perturbation CORT levels ( $P = 0.41$ ; Figure 2; Table 1). Delta CORT, a direct measure of CORT reactivity, showed similar results, and was significantly higher during dehydration than during the other two hydration states ( $F_{2,14} = 13.01$ ;  $P < 0.001$ ). Initial and post-perturbation samples did not differ when animals were hydrated ( $P = 0.13$ ), but post-perturbation CORT was significantly higher than initial levels when the animals were 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 2; Table 1). These treatment effects were detectable, despite the high intra-assay CV.

Results for free-ranging Gila monsters were very similar to those of captive animals. Baseline CORT did not vary between hydric states, though there was a near-significant difference, with the dehydrated state having a higher mean CORT level ( $t(9) = -1.94$ ;  $P = 0.089$ ; Figure 3; Table 1). Post-perturbation CORT levels were considerably higher in dehydrated animals ( $t(9) = -3.78$ ;  $P < 0.005$ ; Figure 3; Table 1). Post-perturbation samples had higher CORT than initial samples 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 when animals were dehydrated ( $F_{2,14} = 16.34$ ;  $P < 0.001$ ; Figure 2; Table 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; Table 1).

### *White Blood Cell Differentials*

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 2; Table 1). Monocyte percentages were also not affected by hydration ( $P = 0.17$ ), but basophils were more common when hydrated compared to the other two hydration states ( $F_{2,14} = 6.94$ ;  $P < 0.001$ ). In free-ranging animals, there were no significant differences between hydration states 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; Table 1).

### Discussion

Our results from both free-ranging and experimentally dehydrated Gila monsters 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 significant change in initial levels of glucocorticoids, though it does increase CORT responsiveness to a behavioral stressor.

Thus, dehydration does not induce a substantial hormonal stress response, but it does enhance certain physiological defense mechanisms, including some measures of innate immunity and stress reactivity.

### *Corticosterone*

Most studies of the effects of hydric state on stress show that CORT increases with dehydration in vertebrates. 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 likely 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 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 prolonged exposure to 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 our previous result of enhanced innate immune function in dehydrated Gila monsters (Moeller et al., 2013), we predicted that initial levels of CORT would not be affected by hydric state in the Gila monster. Though based on a limited number of animals (see Methods), our data show no significant effect of dehydration on initial CORT levels. As heterophil to lymphocyte ratio, another assessment of chronic stress, was also not different among hydration states, the combined results suggest that dehydration to naturally experienced levels is not a highly stressful event for Gila monsters.

Interestingly, some other species that experience seasonal water limitations also lack a hormonal stress response as a result of dehydration, with no dehydration-induced increase in initial 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 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 could be

beneficial as CORT release can increase energy expenditure and water loss (Parker et al., 2003), which would have the potential to worsen resource imbalances. While the trend across taxa is interesting, currently there are insufficient data that test whether the environmental conditions of a species' natural habitat has an effect on endocrine responses to dehydration.

The mechanism behind an absent or minimal increase in initial CORT 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 water-limited 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). Though such studies focus on acute responses, they may provide insight into compensations for physiological stressors in general. 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 perturbation ( $P = 0.114$ ), yet the same perturbation was stressful among dehydrated and rehydrated captive *Gila* monsters, as well as in hydrated and dehydrated animals that were free-ranging. 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.

Even when the perturbation induced a response in hydrated animals, the response was greater when the animals were dehydrated. This finding indicates that although dehydration may not be stressful in itself, it may enhance responsiveness to other stressors during dehydration, in a sense upregulating a physiological defense mechanism. We only found one other study that examined stress-induced plasma CORT concentrations in dehydrated animals: Dupoué and colleagues (2014) presented data from Children's pythons that were similar to ours. 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*

Our immune response results verify our previous findings that dehydration enhances agglutination and lysis in the Gila monster (Moeller et al., 2013). Additionally, using captive animals, we show that another estimate of innate immune response, bacterial killing ability, also increases with dehydration in this species. We suggest caution with interpretation of the insignificant enhancement in bacterial killing ability in samples from free-ranging animals because of weaker statistical tests due to the small sample size (see Methods) or the lower average osmolality of the dehydrated free-ranging animals, suggesting a lesser state of dehydration relative to the captive dehydrated animals.

Agglutination and lysis assays measure levels of natural antibodies and complement activity, respectively (Demas et al., 2011). While bacterial killing ability also addresses these innate 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 innate immune function of dehydrated Gila monsters that could be beneficial in an ecologically relevant context. Additionally, our captive animal experiment removes any effect of seasonality, and thus demonstrates that the results are related to hydration state.

Increased immunity during dehydration may boost an organism's defenses against pathogens during times when other physiological systems could be experiencing 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 innate 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). We posit that this response of the immune and endocrine systems to dehydration,



which goes against the general expected pattern, may be an adaptation of species that regularly experience bouts of dehydration. Comparing the relationship between hydration state and immune function in additional 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 critical as we try to understand potential impacts of anticipated changes in temperature and rainfall patterns due to climate change.

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### Competing Interests

No competing interests declared.

### Author Contributions

KTM and DFD conceived of the study, KTM and GD completed all sample collection, KTM completed immune assays, KTM and SD completed hormone assays, KTM drafted the first version of the manuscript, and KTM, GD, SD, and DFD edited the manuscript.

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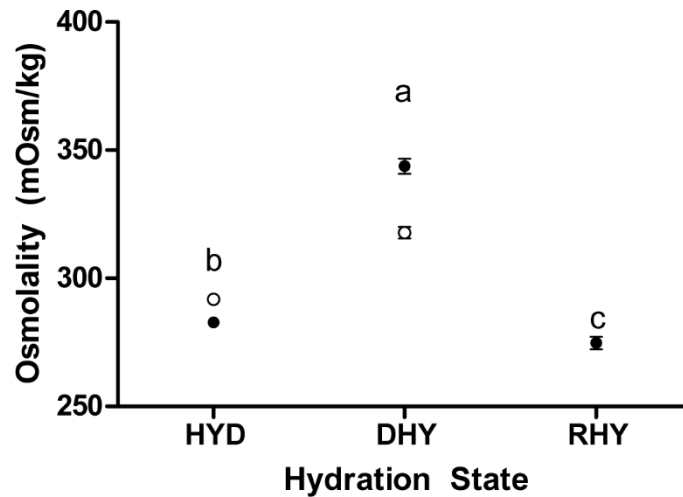
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**Table 1. Mean ( $\pm$  SEM) values for all physiological assessments made on captive and free-ranging Gila monsters at different hydration states.** HYD: hydrated, DHY: dehydrated, and RHY: rehydrated. Significant differences among hydration states within captive animals and within free-ranging animals are shown using superscript letters, in descending order of value (i.e., "a" is highest).

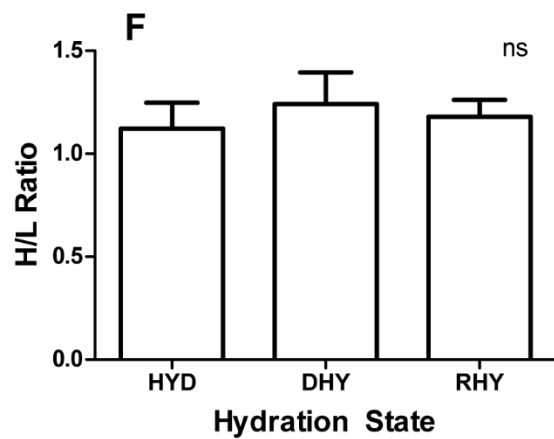
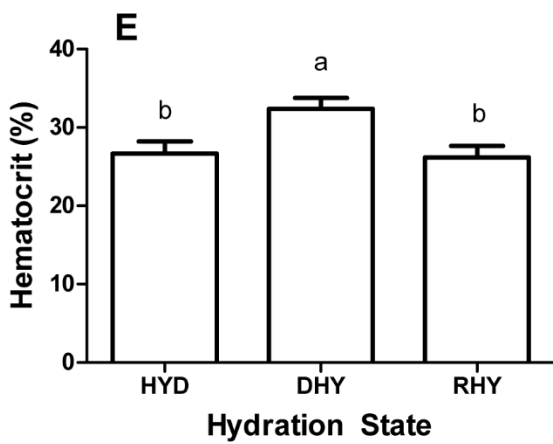
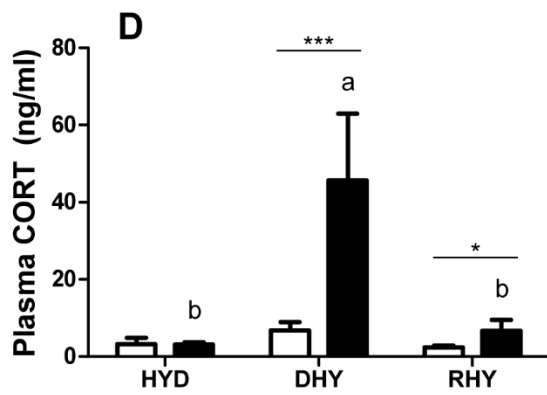
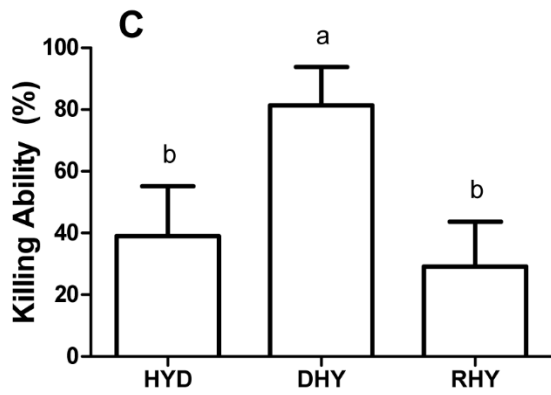
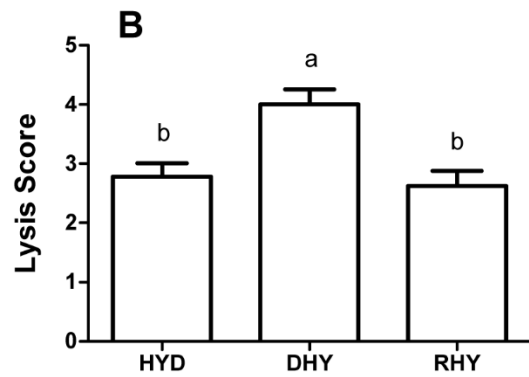
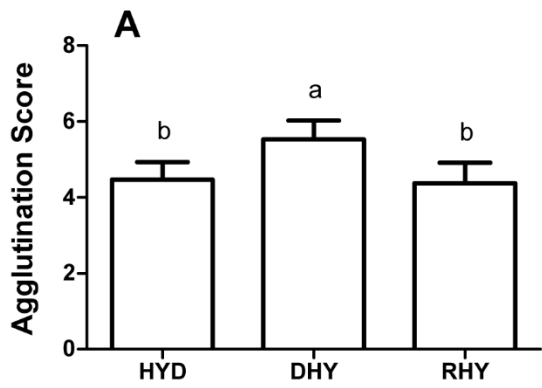
	Captive Gila Monsters			Free-ranging Gila Monsters	
	HYD	DHY	RHY	HYD	DHY
Osmolality (mOsm)	282.8 <sup>b</sup> $\pm$ 1.6	343.8 <sup>a</sup> $\pm$ 3.0	274.8 <sup>c</sup> $\pm$ 2.4	292.0 <sup>b</sup> $\pm$ 1.5	317.8 <sup>a</sup> $\pm$ 2.3
Agglutination Score	4.47 <sup>b</sup> $\pm$ 0.47	5.53 <sup>a</sup>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

## Figures



**Figure 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;  $n = 8$ ;  $P < 0.001$ ) and free-ranging (open circles; HYD  $n = 5$ ; DHY  $n = 6$ ;  $P < 0.001$ ) animals. Error bars represent standard error and letters depict significant differences among hydration states.





**Figure 2. Plasma immune performance and plasma corticosterone concentrations for captive Gila monsters across hydric states.** Repeated measures ANOVAs showed dehydrated (DHY) captive Gila monsters ( $n = 8$ ) had more robust agglutination (A;  $P = 0.0016$ ), lysis (B;  $P < 0.001$ ), and bacterial killing ability (C;  $P = 0.0052$ ), as well as higher plasma corticosterone (CORT) concentration after perturbation (D, solid bars;  $P < 0.001$ ) and hematocrit (E;  $P < 0.001$ ) 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-perturbation ( $* = P < 0.05$ ;  $*** = P < 0.001$ ). ns: no significant differences.

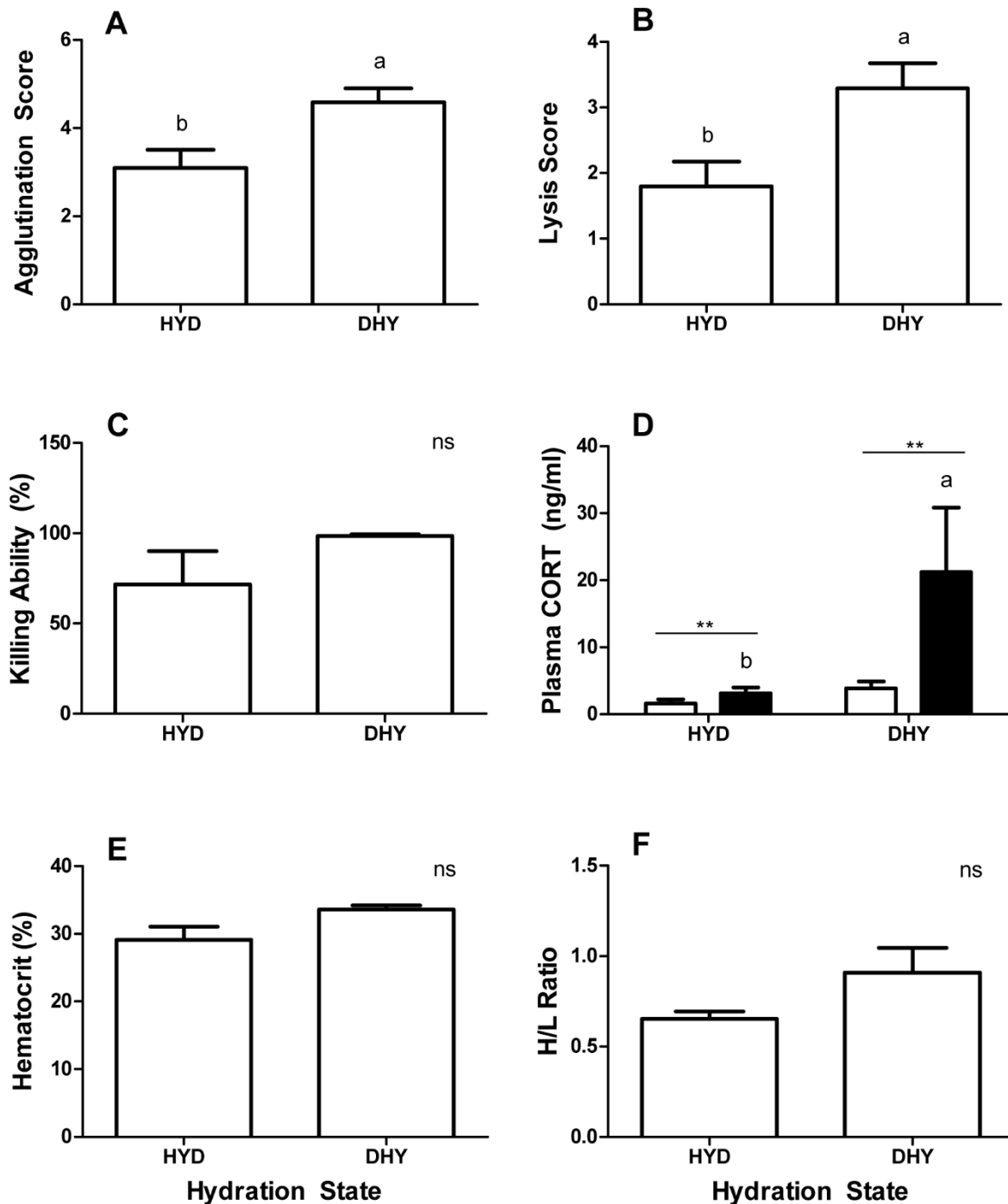


Figure 3. **Plasma immune performance and plasma corticosterone concentrations for free-ranging Gila monsters across hydric states.** Paired t-tests showed dehydrated (DHY; n = 6) free-ranging Gila monsters had more robust agglutination (A;  $P = 0.021$ ) and lysis (B;  $P = 0.021$ ), and higher plasma corticosterone (CORT) concentrations after perturbation (D, solid bars;  $P < 0.005$ ) compared to hydrated animals (HYD; n = 5). 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-perturbation (\*\* =  $P < 0.01$ ). ns: no significant difference.