Water as a Physiological Currency: Hydration State Impacts Immune Function, Metabolic Substrates, and Reproductive Investment

by

George Brusch IV

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Graduate Supervisory Committee:

Dale DeNardo, Chair
Joseph Blattman
Susannah French
John Sabo
Emily Taylor

ARIZONA STATE UNIVERSITY

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ABSTRACT

Environmental changes are occurring at an unprecedented rate, and these changes will undoubtedly lead to alterations in resource availability for many organisms. To effectively predict the implications of such changes, it is critical to better understand how organisms have adapted to coping with seasonally limited resources. The vast majority of previous work has focused on energy balance as the driver of changes in organismal physiology. While energy is clearly a vital currency, other resources can also be limited and impact physiological functions. Water is essential for life as it is the main constituent of cells, tissues, and organs. Yet, water has received little consideration for its role as a currency that impacts physiological functions. Given the importance of water to most major physiological systems, I investigated how water limitations interact with immune function, metabolism, and reproductive investment, an almost entirely unexplored area. Using multiple species and life stages, I demonstrated that dehydrated animals typically have enhanced innate immunity, regardless of whether the dehydration is a result of seasonal water constraints, water deprivation in the lab, or high physiological demand for water. My work contributed greatly to the understanding of immune function dynamics and lays a foundation for the study of hydration immunology as a component of the burgeoning field of ecoimmunology. While a large portion of my dissertation focused on the interaction between water balance and immune function, there are many other physiological processes that may be impacted by water restrictions. Accordingly, I recently expanded the understanding of how reproductive females can alter metabolic substrates to reallocate internal water during times of water scarcity, an important development in our knowledge of reproductive investments. Overall, by thoroughly

evaluating implications and responses to water limitations, my dissertation, when combined previous acquired knowledge on food limitation, will enable scientists to better predict the impacts of future climate change, where, in many regions, rainfall events are forecasted to be less reliable, resulting in more frequent drought.

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CHAPTER 1

INTRODUCTION

Water is a fundamental resource to sustain life, yet water availability can be limited. In many environments, the uneven distribution of precipitation throughout the year results in seasonal droughts: > 66% of the earth's land surface goes for 65 or more consecutive days per year without any measurable rainfall (Hao et al. 2018). An organism's ability to regulate its internal environment and maintain homeostasis is paramount to proper cell function. The importance of water balance to homeostasis in most vertebrates cannot be overstated; in many major taxa an absence of water can be lethal within days (Popkin et al. 2010). To cope with water scarcity, many organisms exhibit water-conserving behavioral, morphological, or physiological adaptations to maintain water balance (Takei 2000). Alternatively, other organisms tolerate dramatic fluctuations in plasma osmolality and endure bouts of dehydration (i.e., hyperosmolality) until water becomes available (Peterson 1996; Brusch and DeNardo 2017). Dehydration can impair activity (Davis and DeNardo 2009), thermoregulation (Montain et al. 1999), cognitive function (Wilson and Morley 2003), and damage to an organism at the membrane level (Prestrelski et al. 1993; Potts 1994). Osmoregulation, the process of regulating water and solutes, allows organisms to maintain the composition of their body fluids within an often-small osmotic range (Bradley 2009).

Many terrestrial mammals maintain a plasma osmolality of approximately 300 mOsm (Stockham and Scott 2008), and it rarely changes more than 5%, even during periods when they do not drink (Ramsay and Thrasher 1984). Juxtaposed against these norms are non-mammalian animals adapted to living in environments where water

availability is limited for portions of the year. These organisms can tolerate extreme variation in osmolality [toads: 250-370 mOsm (Johnson and Propper 2000), tortoises: 290-400 mOsm (Nagy and Medica 1986), lizards: 280-350 mOsm (Davis and DeNardo 2009), and birds: 325-425 mOsm (Williams et al. 1991)] and apparently function normally. The ability to dramatically fluctuate plasma osmolality makes these animals of particular interest when studying the relationship between hydration state and physiological performances. However, it is unknown to what extent non-lethal hyperosmotic states affect physiological functions including immune function, metabolism, and reproductive output.

Accordingly, the first chapter of my dissertation examined the relationship between immune function and hydration state using a species known to undergo substantial seasonal fluctuations in water availability, the western diamond-backed rattlesnake (*Crotalus atrox*). I tested the hypothesis that extended drought leads to measurable yet well-tolerated increases in osmolality in this species and that increased osmolality within this tolerated range results in improved immune function. To do this, I collected blood samples from free-ranging *C. atrox* in the Sonoran Desert to compare osmolality and immune function during the relatively cooler and moister spring, the hot and dry summer, and during the monsoons season when conditions are hot and wet. To remove potentially confounding factors from seasonal effects, I also examined dehydration and immune function using *C. atrox* held without food and water for 16 weeks in the laboratory. I collected blood samples from snakes as they dehydrated and subsequently provided them with *ad libitum* water before taking a final blood sample at the end of the experiment (Brusch and DeNardo 2017 [Appendix A])

Results from the first chapter of my dissertation indicated that, unlike what is seen with energy limitations, water limitations enhance immune function. However, this study did not examine the effects of dehydration on immunity when there was another concurrent, substantial physiological challenge. Thus, I examined the impact of reproduction and water-deprivation, individually and in combination, on immune performance in Children's pythons (*Antaresia childreni*). I collected blood samples from free-ranging *A. childreni* in Australia to evaluate osmolality and innate immune function during the Austral dry-season when water availability is limited, and this species is typically reproducing (Taylor and Tulloch 1985). To examine how reproduction and water-imbalance, both separately and combined, impact immune function in a controlled setting, I also included a laboratory-based 2 x 2 experiment (Brusch et al. 2017 [Appendix B]).

Interestingly, results from my second chapter revealed that reproductive and non-reproductive females held without water had similarly elevated osmolality values, suggesting that reproductive females are somehow able to limit dehydration-based osmolality increases when they are gravid. Accordingly, the third chapter of my dissertation investigated the interactive effects of reproductive investment and water deprivation on catabolism and reproductive output in female *A. childreni*. Using a similar 2 x 2 design, reproductive and non-reproductive females were either provided water *ad libitum* or were water-deprived for three weeks at the time when reproductive females were gravid. I measured a variety of physical and biochemical characteristics and compared between the four treatment groups to examine if reproductive females without

water were altering the type of internal resources they utilized and if dehydration affected their reproductive output (Brusch et al. 2018 [Appendix C]).

Results from my third chapter found no difference in the number of eggs hydrated and dehydrated females laid, however, individual eggs from dehydrated females tended to weigh less. This suggested a potential parent-offspring conflict for limited water resources during gravidity. I therefore tested the hypothesis that the mother-offspring conflict over limited water resources leads to fine-scale morphological and physiological impacts on the eggs in *A. childreni*. To do this, I explored how water deprivation during gravidity affects egg water content, yolk immune performance, and shell development. I examined a suite of physical and biochemical characteristics in eggs and yolks from reproductive females held with or without water throughout gravidity (Brusch et al. 2019 [Appendix D]).

The first two chapters of my dissertation and much of the current research into immune function dynamics focuses on newborn to adult life stages, despite previous studies documenting physiological responses in embryos to environmental stimuli such as temperature (Rafferty and Reina 2012) and oxygen (Stahlschmidt and DeNardo 2009). To explore the relationship between hydration and immune function in a critical but grossly understudied life-stage, I examined whether embryonic *A. childreni* show dehydration-enhanced immune function similar to that documented in adults of this species. I used a 2 x 2 experiment with hydrated or dehydrated mothers and hydrated or dehydrated mid-development embryos and collected embryonic fluid from late-stage embryos to measure osmolality and assess innate immune function (Brusch and DeNardo 2019 [Appendix E]).

CHAPTER 2

CONCLUSION

The vast majority of previous research examining the physiological consequences of resource limitations has focused on energy balance as the driver of changes. Physiological systems that are important to fitness such as immune performance, metabolism, and reproduction are often suppressed during periods of food scarcity or heavy energy investment (Lochmiller et al. 1993; Ardia et al. 2003; Uller et al. 2006). While energy is clearly a vital currency, other resources can also be limited and may impact physiological functions. Despite water being universally recognized as a fundamental resource, it has received limited consideration for its role as a physiological currency. Given the importance of water to most major physiological systems, it seems logical to expect that hydric state would have considerable impact on immune function, metabolism, and reproduction as well.

The immune system is a stimulated defense that destroys and removes particles recognized as foreign such as bacteria, parasites, fungi, and viruses (Murphy 2011). Evidence suggests immunocompetence, the ability of an organism to mount a normal response to an antigen, has energetic production costs (Klasing and Leshchinsky 1999), maintenance costs (Råberg et al. 1998), and substantial energetic costs associated with reacting to foreign pathogens (Klasing 2004). Much of the current focus on dynamic immune function has examined the theory that immune defenses compete with other physiological functions (e.g., growth and reproduction) for energetic resources. Stimulated immune responses have been shown to increase resting metabolic rates (Martin et al. 2003), decrease growth rates (Parmentier et al. 1996), lower reproductive

success (Bonneaud et al. 2003), and reduce parental care (Råberg et al. 2000). Immunocompetence can also be affected by non-energetic resources such as vitamins and carotenoids (Hartley and Kennedy 2004). However, there remain substantial gaps in our understanding of what mechanisms or resources directly modulate the immune system and the magnitude of immunity costs (Viney et al. 2005). To the best of my knowledge, prior to my dissertation work, there were only two studies that found evidence suggesting that water balance can influence immune function. Fruit flies (*Drosophila melanogaster*) with challenged immune systems did not survive desiccation for as long as flies with unchallenged immune systems (Hoang 2001). However, the flies were also subject to starvation so a direct link between immune performance and hydration was confounded by energy limitation. Conversely, Gila monsters (*Heloderma suspectum*) had elevated aspects of innate immune function when dehydrated (Moeller et al. 2013).

I therefore examined the relationship between immunocompetence and hydration using western diamond-backed rattlesnakes (*Crotalus atrox*) from the Sonoran Desert. These large-bodied snakes are naturally adapted to environments that experience substantial seasonal droughts when standing water and rainfall are typically non-existent. They are also binge-feeders; able to survive by consuming large, infrequent meals, and can go months without feeding, making them an ideal organism for studying the effects of hydration on immune function without results being confounded by concerns of an effect due to starvation.

Using a combination of field and laboratory-based experiments, I found that *C. atrox* in the wild experience considerable bouts of hyperosmolality during the seasonal drought. I also found that hyperosmolality, both in the wild and via manipulation in the

lab, led to enhanced innate immune performance. Specifically, animals held in the lab without food and water for 16-weeks showed gradual increases in innate immune performance. When animals were subsequently provided water towards the end of the experiment, innate immune metrics plummeted to baseline within 72-hours. These results suggest that whatever plasma proteins are responsible for my findings rapidly disassociate or become ineffective with rehydration. Additionally, I also found through a series of dilution experiments that elevated immune scores associated with hyperosmolality were not simply the result of increased concentration of immune factors within the plasma. Together, these findings suggest that plasma proteins associated with innate immunity are upregulated (in terms of number or activity) during periods of dehydration. I also found that rattlesnakes held in the lab without food, but who had *ad libitum* access to water, did not have altered immune performances. This validated that the study examined the relationship between hydration and immune function without limited energy confounding my results. My results (Brusch and DeNardo 2017 [Appendix A]), along with those of Moeller et al. (2013), suggest that, unlike what has been documented with energy limitations, water limitations enhance immune function.

While rattlesnakes and Gila monsters in these studies had relatively low energetic and water demands, other reptile species have enormous investment of both resources during reproduction. In many species inhabiting similar seasonal environments, offspring development occurs towards the end of the dry season so that they are born at the start of the wet season when food and water resources are plentiful (Bronson and Heideman 1994; Prendergast et al. 2001). While this strategy is widespread across diverse taxa, it may pose considerable physiological challenges to the female by requiring her to invest

substantial energetic and hydric resources during a time when these same resources are limited or non-existent in the environment. This temporal discrepancy between resource availability and investment has been thoroughly investigated from an energetic standpoint (reviewed in Bronson 2009) via the establishment of fat stores (i.e., capital breeding). However, water has been mostly ignored from consideration which is unfortunate, especially considering it is rarely stored in the body and thus reproductive females may not be well-buffered from environmental water limitations. Therefore, I found it important to examine the relationship between hydric state and immune function in an additional organism still capable of considerable fluctuations in osmolality, but under more rapid physiological water demands.

Children's pythons (*Antaresia childreni*) inhabit the wet-dry tropics of northern Australia (Wilson and Swan 2003) and experience substantial, natural fluctuations in available water resources throughout the year with free-standing water absent for 3-4 months at a time (Taylor and Tulloch 1985). Despite being characterized as a typical capital-breeder for energy resources (Stephens et al. 2009), breeding females face dramatic challenges to water balance during reproduction (Lourdais et al. 2013). Oviposition typically occurs towards the end of the dry season when females have had little access to free-standing water. Coupled with the relative lack of available water, developing embryos require greater amounts of water from the mother during later stages of gravidity (Deeming 2004; Lourdais et al. 2015) and female water balance is often compromised in favor of her developing embryos (Dupoué et al. 2015). Not surprisingly, Children's pythons in the weeks prior to oviposition can undergo significant osmolality

increases as females transfer substantial amounts of body water into their eggs (Stahlschmidt et al. 2011).

Again, using a combination of field and laboratory-based experiments, I found that *A. childreni* in the wild experience considerable bouts of hyperosmolality during Austral dry season when they are also reproductive. Hyperosmolality, in the wild or the lab, also resulted in enhanced innate immune performance as had been documented in other, non-reproductive reptiles (Moeller et al. 2013; Brusch and DeNardo 2017). I used a 2 x 2 experimental design for the laboratory portion (reproductive and non-reproductive females – with or without water) to investigate how reproduction and water-imbalance, both separately and combined, impact immune function. I predicted that non-reproductive females with water would be normosmic – they had access to a water source and relatively low hydric demands. Conversely, I predicted that reproductive females without water would have the highest levels of osmolality – they had a combined hydric cost of lacking drinking water and giving what little internal water they had to their developing eggs. I expected the remaining two treatment groups to have comparable osmolality values somewhere between the first two.

Surprisingly, I did not detect any additive effects of water deprivation and reproduction on osmolality. If the increased osmolality in non-reproductive females without water was due dehydration and similar increases in reproductive females with water was due to the hydric demands of reproduction, it was not clear why there was no additive osmolality increases in reproductive females without water. These results suggested that reproductive females faced with water deprivation were somehow buffered from overt osmolality increases (Brusch et al. 2017 [Appendix B]). Water sources

typically fall into three categories: free water (water from drinking), dietary water (that present in food), and metabolic water (as a byproduct of metabolism). Because I knew these reproductive females did not have access to water or food during the experiment, I examined the interactive effect of reproduction and water deprivation on metabolism in *A. childreni*.

As capital breeders, female *A. childreni* accumulate large fat reserves prior to reproducing which sustains their energic requirements from vitellogenesis through egg brooding (Stephens et al. 2009). However, despite water being crucial for reproduction (Kleiner 1999), it was unknown whether a capital breeding strategy extends to water reserves prior to my dissertation. It was also unclear how females without water were seemingly buffered from hyperosmolality during reproduction (Brusch et al. 2017). Previous research in non-reproductive mammals and birds found that increased muscle catabolism can maintain water balance during periods of hydric imbalance (Bintz and Mackin 1980; Gerson and Guglielmo 2011). Gram for gram, protein catabolism only provides half the energy compared to fat. However, protein, especially skeletal muscle, is stored in >70% bound-water compared to fat which only is only composed of ~10% bound-water (Candlish 1981). Mobilizing wet protein yields over five times more water than fat when accounting for both metabolic and bound water (Jenni and Jenni-Eiermann 1998). I accordingly examined the interactive effects of reproduction and water deprivation on muscle catabolism. Using a 2 x 2 experimental design (reproductive and non-reproductive females – with or without water), I measured physical (body mass and epaxial muscle width) and biochemical (osmolality, glucose, triglycerides, total proteins, ketones, and uric acid) characteristics to compare between the four treatment groups. I

also measured clutch mass and number of eggs between the two reproductive treatment groups.

I found that water deprivation increased protein catabolism and greatly reduced body mass in reproductive females. This suggested, because protein is a much lower energetic source compared to fat, that the catabolism of proteins was mostly driven by water needs. This study was the first to link muscle atrophy during reproduction to water allocation requirements of developing offspring. Water deprivation had no impact on the number of eggs per clutch. However, females without water produced lighter clutch masses compared to females with water. This impact on reproductive output suggests that muscle catabolism was not sufficient to completely satisfy the water requirements of the developing embryos during gravidity. This also implied that there is a water-based parent-offspring conflict for finite hydric resources. Similar transgenerational conflicts have been previously demonstrated in other species of snake (Dupoué et al. 2015, 2018) and the egg mass difference I found was potentially due to reduced water allocation (Brusch et al. 2018 [Appendix C]).

Maternal hydration has been explored after gestation (Hanson et al. 1994) and as a potential cue for reproductive timing (Nelson et al. 1989). However, the direct consequences of maternal hydration during gestation is far less understood. Thus far, my dissertation work showed that, when water is limited, reproductive snakes have elevated plasma osmolality (Brusch et al. 2017) and catabolize greater amounts of muscle mass relative to fat to free up bound water (Brusch et al. 2018). Loss of muscle, not surprisingly, has been associated with reduced performance by the post-reproductive females (Lourdais et al. 2013). As a result, it was assumed that the hydric aspects of the

parent-offspring conflict are often compromised in favor of developing embryos (Dupoué et al. 2015). However, these assumptions relied on relatively broad, easily assessable metrics of reproductive output such as egg mass or volume to examine potential conflicts for hydric resources (Dupoué et al. 2015; Brusch et al. 2018), and a more detailed examination of egg quality was needed to reveal more cryptic impacts of the parent-offspring conflict over water resources.

Using reproductive *A. childreni* with or without water during gravidity, I next examined the impact of maternal hydration on reproductive output. Specifically, whether female dehydration altered investment into eggs and subsequently affected egg water content and shell development. I also explored whether dehydrated eggs had elevated immune performances like what had been documented in dehydrated adults (Brusch et al. 2017). To do this, I measured a variety of physical (egg mass, water content, water loss rate, and shell thickness) and biochemical (yolk osmolality and innate immune performance) characteristics in freshly oviposited eggs. As with my previous study (Brusch et al. 2018), I found that water-deprived females laid lighter eggs. I also found that eggs from water-deprived females were dehydrated as indicated by reduced percent water and greater yolk osmolality compared to eggs from females that received *ad libitum* water. Additionally, eggs from water-deprived mothers had thinner shells and higher water-loss rates. The impacts were not entirely negative though, as dehydrated eggs had higher antimicrobial capabilities. Thinner and more permeability eggshells may also allow for elevated rates of rehydration from nest substrate. These results provided the first evidence of a water-based mother-offspring intergenerational trade-off in an oviparous vertebrate. By examining an array of egg traits, I also demonstrated that

dehydration of gravid females impacts the eggs, not just the females as had been previously reported (Brusch et al. 2019 [Appendix D]).

There remained several important, unanswered questions. It was not clear if enhanced immunity in eggs was due to an embryonic response to dehydration or because of vertical transmission from mothers. It was also uncertain if thinner shelled eggs could, in fact, absorb environmental moisture. In oviparous species, the embryonic experience as it develops outside the mother is strongly influenced by the environment (Furness et al. 2015). Factors such as light (Archer et al. 2009), oxygen (Stahlschmidt and DeNardo 2009), and temperature (Bull 1980) have been shown to directly affect the physiology of the embryo. However, prior to my dissertation, none had examined the impact of environmental water availability on the embryo directly. I therefore examined potential trans-generational immune effects of female dehydration as well as the effects of egg desiccation on embryonic hydration and innate immunity using *A. childreni*. I used a 2 x 2 experimental design with water-provided or water-deprived mothers and eggs either incubated under continuous optimal conditions or experiencing desiccating conditions for 24 hours. I found that, regardless of maternal hydration, environmental conditions had the only significant impact on embryonic hydration. I also found that embryonic dehydration resulted in aspects of innate immunity being elevated. These results demonstrated that, similar to adults, embryos independently enhance some metrics of innate immunity when they are dehydrated and that their hydration is dynamic and dependent on environmental conditions (Brusch and DeNardo 2019 [Appendix E]).

While much of the previous research in environmental physiology has explored limited energetic resources, I took a novel approach for my dissertation and explored how

water (a fundamental, non-energetic resource) interacts with numerous physiological functions. Through my experiments on multiple species and life-stages I have demonstrated the profound effect that hydration can have on multiple physiological systems and life-stages (Appendices A-E). My research has greatly contributed to our understanding of physiological dynamics and has laid a foundation for the study of hydration immunology as a component of the burgeoning field of environmental immunology. Additionally, by expanding our understanding of how reproductive females can alter metabolic substrates to reallocate internal water sources during times of water scarcity, I have provided an important development in our knowledge of reproductive phenology. I have also applied my research questions to include cane toads in Australia, which provided the ideal backdrop to effectively infuse my physiological research into a critical conservation effort and contributed to our limited understanding of the role of rapid adaptation in the range expansion of an invasive species across the globe (Brusch et al. *In Press*).

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APPENDIX A

WHEN LESS MEANS MORE: DEHYDRATION IMPROVES INNATE IMMUNITY IN RATTLESNAKES

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RESEARCH ARTICLE

When less means more: dehydration improves innate immunity in rattlesnakes

George A. Brusch, IV* and Dale F. DeNardo

ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

Arizona State University, School of Life Sciences, 417 East Tyler Mall, Tempe, AZ 85281, USA.

*Author for correspondence (bruschg@gmail.com)

G.A.B., 0000-0001-7740-6066

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

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

MATERIALS AND METHODS

Study animals

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

Field-based experiment

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

Laboratory-based experiment

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

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

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

Sample collection

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

Immune function assays

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

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

Plasma dilution experiment

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

Plasma degradation experiment

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

Statistical analysis

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

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

Ethical statement

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

RESULTS

Field-based experiment

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

Laboratory-based experiment

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

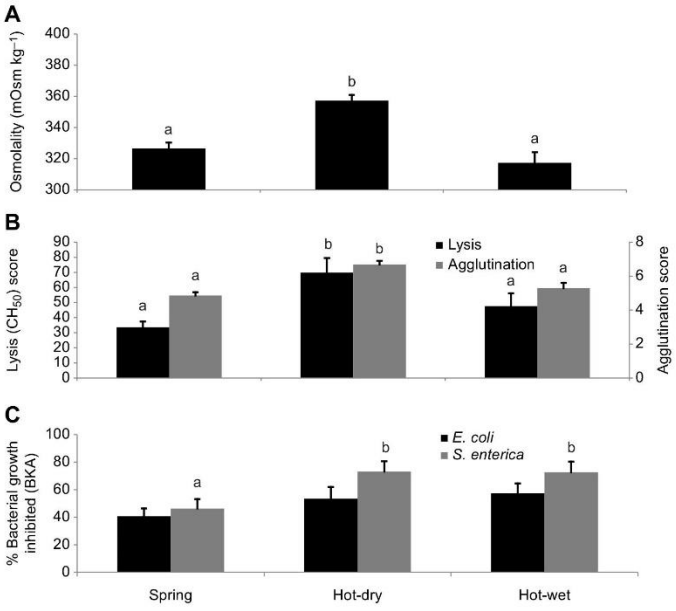

Fig. 1. Seasonal fluctuations in osmolality and immune performance in free-ranging rattlesnakes. Average osmolality (A) and immune scores [B: lysis and agglutination; C: *Escherichia coli* and *Salmonella enterica* bacterial killing ability (BKA)] from free-ranging *Crotalus atrox* during the spring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

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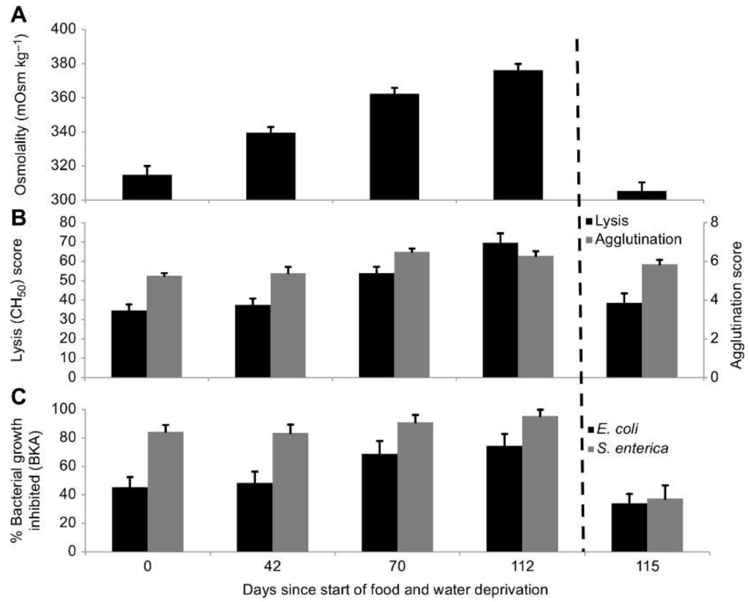

Fig. 2. Fluctuations in osmolality and immune performance in captive rattlesnakes held without food and water. Average osmolality (A) and immune scores (B: lysis and agglutination; C: *E. coli* and *S. enterica* BKA) in captive *Crotalus atrox* (n=14) during a 16-week period without water and 72 h after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

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

Plasma dilution experiment

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

Plasma degradation experiment

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

DISCUSSION

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

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

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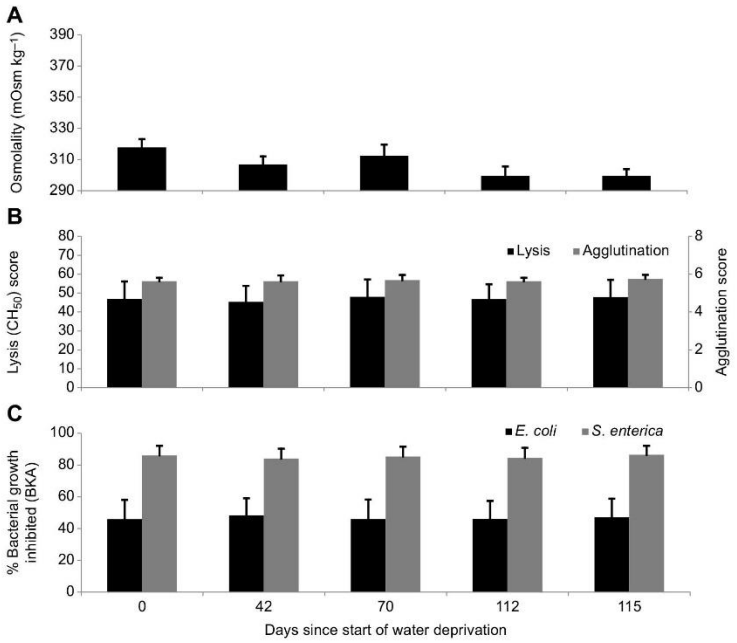

Fig. 3. Fluctuations in osmolality and immune performance in captive rattlesnakes held without food. Average osmolality (A) and immune scores (B: lysis and agglutination; C: *E. coli* and *S. enterica* BKA) in captive *Crotalus atrox* (n=8) during a 16-week period with no food and *ad libitum* access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

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

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

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

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

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

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

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

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Competing interests
The authors declare no competing or financial interests.

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

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

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APPENDIX B

REPRODUCTION ALTERS HYDRATION STATE BUT DOES NOT IMPACT THE POSITIVE EFFECTS OF DEHYDRATION ON INNATE IMMUNE FUNCTION IN CHILDREN'S PYTHONS (*ANTARESIA CHILDRENI*)

Reproduction Alters Hydration State but Does Not Impact the Positive Effects of Dehydration on Innate Immune Function in Children's Pythons (*Antaresia childreni*)

George A. Brusch IV[1,*]
Gopal Billy[2]
Joseph N. Blattman[1]
Dale F. DeNardo[1]
[1]School of Life Sciences, Arizona State University, 427 East Tyler Mall, Tempe, Arizona 85281; [2]Université Claude Bernard Lyon 1, 43 Boulevard du 11 Novembre 1918, 69100 Villeurbanne, France

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ABSTRACT

Resource availability can impact immune function, with the majority of studies of such influences focusing on the allocation of energy investment into immune versus other physiological functions. When energy is a limited resource, performance trade-offs can result, compromising immunity. Dehydration is also considered a physiological challenge resulting from the limitation of a vital resource, yet previous research has found a positive relationship between dehydration and innate immune performance. However, these studies did not examine the effects of dehydration on immunity when there was another concurrent, substantial physiological challenge. Thus, we examined the impact of reproduction and water deprivation, individually and in combination, on immune performance in Children's pythons (*Antaresia childreni*). We collected blood samples from free-ranging *A. childreni* to evaluate osmolality and innate immune function (lysis, agglutination, bacterial growth inhibition) during the austral dry season, when water availability is limited and this species is typically reproducing. To examine how reproduction and water imbalance, both separately and combined, impact immune function, we used a laboratory-based 2 × 2 experiment. Our results demonstrate that *A. childreni* experience significant dehydration during the dry season and that, overall, osmolality, regardless of the underlying cause (seasonal rainfall, water deprivation, or reproduction), is positively correlated with increased innate immune performance.

*Corresponding author; e-mail: bruschg@gmail.com.

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Introduction

The immune system is a remarkably complex network involved in host defense, repair, and maintenance, and its overall effectiveness can vary based on life stage (Schwanz et al. 2011), season (Buehler et al. 2008), or an animal's ecology (French et al. 2009). Resource availability is also of extreme importance to immune function, with most studies focusing on the allocation of energy investment into immune versus other physiological functions (Toomey et al. 2010; Nebel et al. 2012) or the importance of nutrients to immune function (Wintergerst et al. 2007; Cotter et al. 2011).

Fluctuations in energy availability can create life-history trade-offs (Uller et al. 2006). For example, during periods of low resource availability, the high energetic demands of reproduction can limit energy resources available for other physiological processes such as immune function (Martin et al. 2008). This is especially true in income breeders, which rely on concomitant energy intake and reproductive investment (French et al. 2007; Ruiz et al. 2011). When greater amounts of food are available, females can simultaneously invest in reproductive efforts and immune function without compromising the performance of either (French et al. 2007; Ruiz et al. 2011).

While energy is clearly a vital currency that influences immune function, other resources can also be limited and potentially compromise performance and lead to conflicts between physiological functions. As with energy, water limitations and resulting imbalances (i.e., hyperosmolality) can also alter various physiological processes (e.g., protein production [Burg et al. 2007], cellular composition [Reinehr and Häussinger 2006], organismal development [Wilson and Morley 2003]). Furthermore, water is not readily stored in the body, and, thus, as with income breeders and energy resources, animals are not well buffered from environmental water limitations. Interestingly, while dehydration is considered a physiological challenge (El Fazaa et al. 2000; Tsuchida et al. 2004), unlike negative energy balance, dehydration has been shown to enhance certain aspects of immunity in insect, lizard, and snake species (Hoang 2001; Moeller et al. 2013; Brusch and DeNardo 2017).

Despite recent attention, substantial gaps remain in our understanding of what mechanisms or resources directly modulate the immune system and the magnitude of immunity costs (Viney et al. 2005). Additionally, our knowledge of immune function

dynamics is limited in that most studies to date have focused on single physiological challenges, with limited insight into how concurrent challenges, as are often experienced in nature, impact immune function. In many species, offspring development within the female occurs toward the end of the dry season so that offspring are born at the beginning of the wet season, when food and water resources are plentiful for the offspring (Bronson and Heideman 1994; Prendergast et al. 2001). While this strategy is widespread across diverse taxa because of the benefits it provides the offspring (reviewed in Bronson 2009), it may pose considerable physiological challenges to the female by requiring substantial energy and water investment during a time when these resources are limited in the environment. While such a discrepancy can be managed from an energy standpoint through a dissociation between energy intake and expenditure via the establishment of fat stores (i.e., capital breeding), water is rarely stored in the body and thus typically cannot be stockpiled for future use. Therefore, reproductive females may face a water-based trade-off between immune function and reproduction similar to the energy-based trade-off documented in income-breeding species.

To investigate such a possibility, we examined the impact of reproduction and water deprivation, individually and in combination, on immune performance in Children's pythons (*Antaresia childreni*). This species develops its eggs during the end of the austral dry season and therefore may face considerable natural osmotic challenges from a lack of free-standing water (Taylor and Tulloch 1985). Reproductive females face the added burden of transferring significant amounts of water to developing eggs just before oviposition (Stahlschmidt et al. 2011). Coupled with these hydric challenges, Children's pythons typically do not eat when reproductively active, and previous studies have shown that females face considerable physiological and performance costs associated with the energetic demands of reproduction (Lourdais et al. 2013).

We sampled free-ranging Children's pythons to ascertain whether they are dehydrated during the dry season and, if so, what influence this has on multiple assessments of immune function. Furthermore, we used a laboratory-based 2 × 2 experimental design to examine more precisely how reproduction and water imbalance, both separately and combined, impact immune function. We tested the hypothesis that immune function is dynamic and limited resources during high demand for those resources impose a physiological imbalance that suppresses immune function. We predicted that (1) dehydration naturally occurs in free-ranging Children's pythons during the dry season; (2) reproduction, likely because of its high energy demands during a period of inappetence, will suppress immune function; (3) because of high water demands associated with reproduction, water deprivation during gravidity will suppress immune function; and (4) the combined physiological challenges of reproduction and dehydration will have an additive inhibitory effect on immune function.

Methods

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

Study Species

Children's pythons (*Antaresia childreni*) inhabit the wet-dry tropics of northern Australia (Wilson and Swan 2003) and experience substantial natural fluctuations in available water resources throughout the year, with free-standing water mostly absent for 3–4 mo at a time, typically between May and August (Taylor and Tulloch 1985). Females accumulate large lipid reserves before reproducing that sustain their energy requirements from vitellogenesis through egg brooding. Despite being characterized as a typical capital breeder for energy resources (Stephens et al. 2009), breeding females face dramatic challenges to water balance during reproduction (Lourdais et al. 2013). Egg development and oviposition typically occur toward the end of the dry season, when females have had little access to free-standing water. Coupled with the relative lack of available water, developing embryos require greater amounts of water from the mother during later stages of gravidity (Deeming 2004; Lourdais et al. 2015) and female water balance is often compromised in favor of her developing embryos (Dupoué et al. 2015). Not surprisingly, Children's pythons in the weeks before oviposition can face significant hydric challenges as females transfer substantial amounts of body water into their eggs (Stahlschmidt et al. 2011).

Field Study

To determine whether these natural fluctuations in water availability during the austral dry season lead to dehydration and whether increased osmolality is correlated with immune function, we collected blood samples from 24 individual Children's pythons during the mid to late dry season (June and July 2016) at Beatrice Hill Farm, Northern Territory, Australia. Upon capture, we determined mass (by placing the snake in a tared bag and hanging it from a spring scale [±2 g; model 40300, Pesola, Schindellegi, Switzerland]), sex (by probing the cloaca caudally), and snout-vent length (SVL; using a cloth tape). We also collected a blood sample and marked each snake on its back with a permanent marker to avoid duplicative sampling. We temporarily stored the collected blood in a cooler with ice until return to the lab. We then used the samples to determine plasma osmolality and in immune function assays.

Lab Study

To control for potential variation associated with seasonal effects, sex, and reproductive status, we used 30 captive-born adult Children's pythons that were housed individually in 91 × 71 × 46-cm

cages (Freedom Breeder, Turlock, CA) and part of a long-term colony at Arizona State University. Before the start of our study, we cooled the snakes for ~2 mo (early December to early February) in a temperature-controlled room with a 6L:18D/20°:15°C light and temperature regimen. During the 20°C portion of the cycle, we provided the snakes with a subsurface heating element (Flexwatt, Maryville, TN) below one end of each cage to provide a thermal gradient. In mid-February, we switched the light cycle to 12L:12D and held the room temperature constant at 25°C. We also provided subsurface heating 24 h d^{-1}, creating a continuous thermal gradient (25°–35°C).

We assigned snakes to two groups of similar mean body mass: reproductive and nonreproductive. Reproductive females ($n = 15$) had individual male snakes rotated through their cage, resulting in each reproductive female being housed with six to eight individuals during this period. Nonreproductive females ($n = 15$) had no access to males and remained housed individually throughout the study. All females, regardless of reproductive assignment, had ad lib. access to water during this time and were not fed during the duration of the study.

We used ultrasonography (Sonosite MicroMaxx, Bothell, WA), as described by Stahlschmidt et al. (2011), to monitor follicular growth in all females once per week. Once follicles reached 15 mm in diameter, we ultrasounded the females once every 2 d. At this time, we assigned yoked pairs made up of one reproductive female and one nonreproductive female of similar size. The water treatment and blood collection schedule for each nonreproductive female followed that of the reproductive female to which she was yoked. Once reproductive female follicles reached 20 mm in diameter, we collected a 0.8-mL blood sample from her and her yoked nonreproductive female. Females typically ovulate when follicle diameters reach 20–25 mm; therefore, this first bleed occurred during late vitellogenesis when nutrient deposition into developing follicles was considerable and nearing completion (Stahlschmidt et al. 2011).

We used periovulation ecdysis as an indicator of ovulation and the onset of gravidity, which typically occurs 21–25 d before oviposition (Lourdais et al. 2008). Once a reproductive female shed, we assigned her and her yoked nonreproductive female to one of two water treatment groups: ad lib. access to water or water deprived (hereby referred to as water or no water, respectively). Females in the water treatment group (reproductive with water, $n = 7$; nonreproductive with water, $n = 7$) had ad lib. access to water throughout the study, while females in the no water treatment group (reproductive with no water, $n = 8$; nonreproductive with no water, $n = 8$) were completely water deprived from periovulation ecdysis to oviposition. Twenty days after the periovulation ecdysis (and 1–5 d before oviposition), we collected a second 0.8-mL blood sample from the reproductive female and her yoked nonreproductive female, representing a late-gravidity state for the reproductive female. Thereafter, we inspected reproductive females daily for oviposition. After oviposition, we removed females from the study and provided them with ad lib. access to water. As with the field study, we used the blood samples to determine plasma osmolality and conduct immune function assays.

Blood Sample Collection

We used heparinized 1-mL syringes with a 25-gauge × 1.6-cm (5/8-in) needle to collect a 0.8-mL blood sample via cardiocentesis. After blood collection, we either returned the snake to its cage (lab) or marked and released it at its capture site (field). Total time for capture, restraint, and collection was typically less than 4 min and did not exceed 9 min for both lab and field portions. We immediately centrifuged the blood samples from captive snakes at 3,000 rpm for 3 min to separate plasma from blood cells. We aliquoted plasma (~50 μL) into separate vials and froze at −80°C until we used them within 40 d to measure plasma osmolality and evaluate immune function. Similarly, we separated, aliquoted, and stored (−20°C) blood samples from the field study until we used them within 21 d for the same assessments.

Blood Osmolality Determination

We determined plasma osmolality for all samples using a vapor pressure osmometer (±3 mOsm kg^{-1}; model 5600 for lab, model 5100C for field; Wescor, Logan, UT). We ran samples in triplicate as described in Davis and DeNardo (2009).

Immune Function Assays

We used several assays to assess innate immune function and examine the relationship among immunocompetence, hydration state, and reproductive status. We used agglutination and lysis assays to evaluate the involvement of natural antibodies and complement, respectively, in reacting to a novel antigen, sheep red blood cells (SRBC; SBH050, Hemostat Laboratories, Dixon, CA, for lab; SB050, Thermo Fisher Scientific, Scoresby, Victoria, Australia, for field) and thus serve as a measure of innate immunity (Matson et al. 2005). In brief, we serially diluted 20 μL of each plasma sample from 1:2 to 1:2,048 with phosphate-buffered saline (PBS) along a row of a 96-well plate. We then added 20 μL 1% SRBC to each well. We did not add plasma to the final column; the first four wells contained only 20 μL PBS and 20 μL 1% SRBC (negative control, 0% lysis), and the bottom four wells contained 20 μL ammonium-chloride-potassium lysing buffer (Lonza, Basel, Switzerland) and 20 μL 1% SRBC (positive control, 100% lysis). We incubated the plates at 30.5°C, representing the preferred temperature of gravid females (Lourdais et al. 2008), for 90 min and then placed them at room temperature, ~25°C, for 20 min, after which point we scanned them at 600 dots per inch using a flatbed scanner (Hewlett-Packard, ScanJet 3670) for agglutination images. Plates remained at room temperature for an additional 70 min, and we then centrifuged them for 5 min (500 rpm; Sorvall, Newtown, CT), after which we aspirated the supernatant into a clean 96-well plate. We then measured absorbance using a microplate spectrophotometer (405 nm; BioTek Instruments, Winooski, VT, for lab; Bio-Rad, Hercules, CA, for field) to calculate lysis scores. Hemolytic-complement activity was expressed in CH_{50} units $(mL\ plasma)^{-1}$, where 1 CH_{50} unit equals the reciprocal of the dilution of plasma required to lyse 50% of the SRBC.

We also conducted bacterial-killing assays (BKA) outlined in French and Neuman-Lee (2012) to assess the ability of Children's pythons to inhibit the growth of infectious microorganisms. For the laboratory-collected samples, we used two different species of gram-negative bacteria, *Escherichia coli* and *Salmonella enterica*, while for the field-collected samples we used only *E. coli*. Briefly, we combined 1 : 4 plasma dilution with CO_2-independent media plus 4 nM L-glutamine, 10^6 colony-producing units of *E. coli* (lot 483-478-1 for lab; lot 483-306-1 for field; ATCC 8739, MicroBioLogics, St. Cloud, MN) or 10^6 colony-producing units of *S. enterica* (lot 501-13-1; ATCC 51741, MicroBioLogics), and agar broth on a 96-well microplate. We calculated absorbance using a microplate reader (300 nm; BioTek Instruments for lab; Bio-Rad for field) at 0 h and after 12 h of incubation at 37°C. We calculated percent bacterial growth inhibited as the mean number of colonies for each sample, which we ran in triplicate, divided by the mean number of colonies for the positive control (triplicate wells containing only media and bacteria), multiplied by 100.

Statistical Analysis

We performed all statistical analyses in R, version 3.3.2 (R Development Core Team 2016). We checked to ensure the data met the assumptions for parametric testing and used transformations where necessary. First, we examined the effect of reproductive status and water treatment on osmolality and immune scores in laboratory-collected samples using repeated-measures analysis of variance (rmANOVA). We tested for three-way interactions and used treatment (water or no water), status (reproductive or nonreproductive), and time (late-vitellogenesis and late-gravidity bleeds) as fixed effects and individual as a random effect. We included parameters addressing potential size using a body condition index (standardized residuals from a linear regression using mass and SVL) and seasonal (i.e., date of blood collection) effects. However, these two variables were removed from the final model as a result of stepwise removal using the change in Akaike's information criterion (ΔAIC) and model weights (Arnold 2010; Zuur et al. 2010).

After comparing the combined effects of time, treatment, and status, we used variance-partitioning methods described by Anderson and Gribble (1998) to decompose our full response into orthogonal subsets to examine how treatment (water or no water in nonreproductive snakes) or status (reproductive or nonreproductive snakes with access to water) affected immune performance using separate rmANOVAs. We used a post hoc Tukey's honest significant difference test on our orthogonal subsets to determine which of the groups was significantly different. For field-collected samples, we first determined whether there was a sex effect on osmolality or immune scores using Student's *t*-tests. We then used linear regressions comparing the profiles between individuals to explore the relationship between osmolality and immune scores. We performed similar linear regressions using laboratory data, independent of treatment regimen.

We used the packages "nlme" and "multcomp" (Hothorn et al. 2008; Pinheiro et al. 2016) for rmANOVAs, "CAR" (Fox and Weisberg 2011) for linear regressions, and "agricolae" (Mendiburu 2014) for post hoc tests. Significance was set at $\alpha = 0.05$.

Results

Field-Collected Data

Wild-caught Children's pythons ($n = 24$, male = 11, female = 13) plasma osmolality ranged from 279 to 354 mOsm kg^{-1}. Osmolality and immune scores were not significantly different ($P > 0.05$) between sexes. We found a significant positive relationship between osmolality and SRBC lysis ($F_{1,22} = 4.622$, $P = 0.043$, $R^2_{adj} = 0.136$) in our field samples. There were nonsignificant relationships between osmolality and agglutination ($F_{1,22} = 2.61$, $P = 0.121$) and between osmolality and *Escherichia coli* BKA scores ($F_{1,22} = 3.5$, $P = 0.074$, $R^2_{adj} = 0.098$; fig. 1).

Lab-Collected Data

In our laboratory samples, we found a significant positive relationship between osmolality and SRBC lysis ($F_{1,58} = 14.092$, $P < 0.001$, $R^2_{adj} = 0.182$), *E. coli* BKA ($F_{1,58} = 13.386$, $P < 0.001$, $R^2_{adj} = 0.174$), and *Salmonella enterica* BKA ($F_{1,58} =$

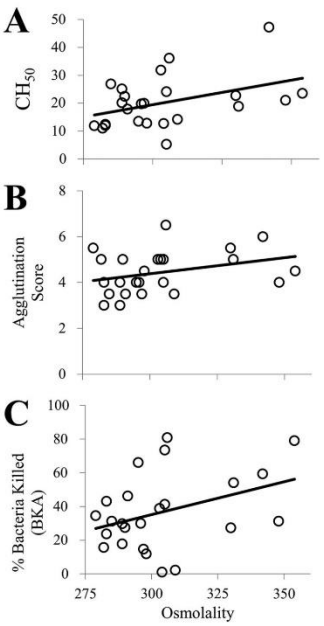

Figure 1. Positive relationship between osmolality (mOsm kg^{-1}) and lysis (CH_{50}; $P = 0.04$; *A*), agglutination ($P = 0.12$; *B*), and *Escherichia coli* bacterial-killing assay (BKA; $P = 0.07$; *C*) scores in wild-caught *Antaresia childreni* ($n = 24$). Circles represent individual animals.

13.642, $P < 0.001$, $R^2_{adj} = 0.176$). There was a nonsignificant relationship between osmolality and agglutination ($F_{1,58} = 0.003$, $P > 0.05$). We found significant time–by–reproductive status ($F_{1,26} = 5.544$, $P = 0.026$) and time–by–water treatment ($F_{1,26} = 20.20$, $P = 0.0001$) interactions in osmolality levels (fig. 2). There was also a significant time-by-treatment interaction in SRBC lysis scores ($F_{1,26} = 5.217$, $P = 0.032$). We found a significant three-way interaction between time, water treatment, and reproductive status in *S. enterica* BKA scores ($F_{1,26} = 5.912$, $P = 0.0222$) and a significant time–by–reproductive status interaction in *E. coli* BKA scores ($F_{1,26} = 7.443$, $P = 0.014$). We did not detect any significant interactions or main effects in agglutination scores ($P > 0.05$; fig. 3).

We used orthogonal subset analyses to independently assess the effect of treatment (±water) and the effect of status (±reproductive activity). Evaluating the effects of treatment (water [$n = 7$] vs. no water [$n = 8$] in nonreproductive females) revealed a significant time-by-treatment interaction on osmolality ($F_{1,13} = 10.53$, $P = 0.006$), lysis ($F_{1,13} = 7.62$, $P = 0.016$), *E. coli* BKA ($F_{1,13} = 5.56$, $P = 0.035$), and *S. enterica* BKA ($F_{1,13} = 5.62$, $P = 0.034$) values. Nonreproductive females with no access to water had significantly higher ($P < 0.05$) osmolality (fig. 2), lysis, *E. coli* BKA, and *S. enterica* BKA (fig. 3) means compared to nonreproductive females with access to water. We did not find any significant interaction or main affects in agglutination scores ($P > 0.05$). When evaluating the effect of reproductive status (water-provided reproductive [$n = 7$] vs. water-provided nonreproductive [$n = 7$] females), we found a significant time-by-status interaction on osmolality ($F_{1,12} = 8.40$, $P = 0.013$), lysis ($F_{1,12} = 9.53$, $P = 0.009$), *E. coli* BKA ($F_{1,12} = 5.70$, $P = 0.034$), and *S. enterica* BKA ($F_{1,12} = 19.24$, $P < 0.001$) values. Reproductive females with access to water had significantly higher ($P < 0.05$) osmolality (fig. 2), lysis, *E. coli* BKA, and *S. enterica* BKA (fig. 3) means compared to nonreproductive females with access to water. We did not find any significant interaction or main affects in agglutination scores ($P > 0.05$).

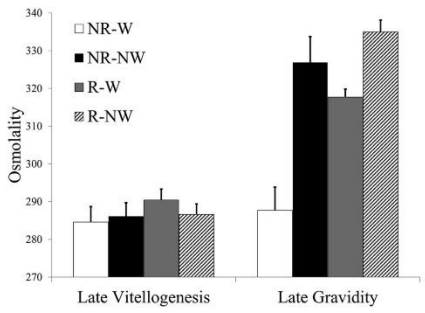

Figure 2. Average plasma osmolality (mOsm kg^{-1}) measured in nonreproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water during most of the duration of gravidity or its equivalent for nonreproductive yoked females. Plasma samples were collected when reproductive females were in late vitellogenesis and late gravidity. There was a significant ($P < 0.05$) time-by-treatment (W or NW) and time-by-status (R or NR) interaction. Error bars represent ±1 SEM.

Discussion

Many organisms have reproductive cycles that have been modified to suit annual changes in their local environments. These seasonal breeders reproduce during specific times of the year so that offspring are delivered under favorable climatic conditions, typically when food and water are readily available (Norris and Lopez 2010). Photoperiod, temperature, food availability, and rainfall (Brown and Sexton 1973; Ballinger 1977; Visser et al. 2009; Nishiwaki-Ohkawa and Yoshimura 2016) have all been shown to influence the length of gestation or incubation in seasonal breeders (Lincoln and Short 1980). Furthermore, limited energetic resources create trade-offs between growth, self-maintenance, and reproduction (Stearns 1989) and frequently result in females reproducing biannually or triannually so that energetic stores used for the previous litter can be replenished (Fitch 1960). Previous research suggests that Children's pythons require relatively stable temperature regimes during gravidity (Lourdais et al. 2008) and brooding (Stahlschmidt and DeNardo 2009). Accordingly, annual temperature variations in their native range are relatively limited, and water availability has been suggested as the most important proxy for reproductive timing (Shine and Brown 2008). Our field data demonstrate that Children's pythons can be dehydrated during the dry season (May through August) when they are also reproductive, with the considerable variation in their osmolality (279–354 mOsm kg^{-1}), likely reflecting inconsistent water availability among individuals across the site where we captured the snakes. These results demonstrate the relevant ecological context of our laboratory study where average osmolality values ranged from 289–347 mOsm kg^{-1} depending on water availability and reproductive status.

We found that, regardless of whether osmolality increases were due to natural fluctuations or manipulation in the lab, aspects of innate immune function were enhanced in dehydrated animals. Despite our reproductive animals having greater water demands, the results were consistent with previous findings in other nonreproductive reptiles (Moeller et al. 2013; Brusch and DeNardo 2017)—we found a positive relationship between SRBC lysis scores and increased osmolality. We did not detect a significant relationship between bacterial-killing ability and osmolality in wild Children's pythons (fig. 1), but we did in the laboratory portion of our study (fig. 2). However, the trend in *E. coli* BKA scores from wild pythons prevents us from ruling out the possibility of a positive relationship between bacterial killing and increased osmolality and emphasizes the value of laboratory studies where variables such as sex, reproductive status, and seasonal affects can be controlled.

Unlike bacterial killing and hemolytic activity, we did not detect any significant relationships between agglutination scores and osmolality in both the field and laboratory portions of our study.

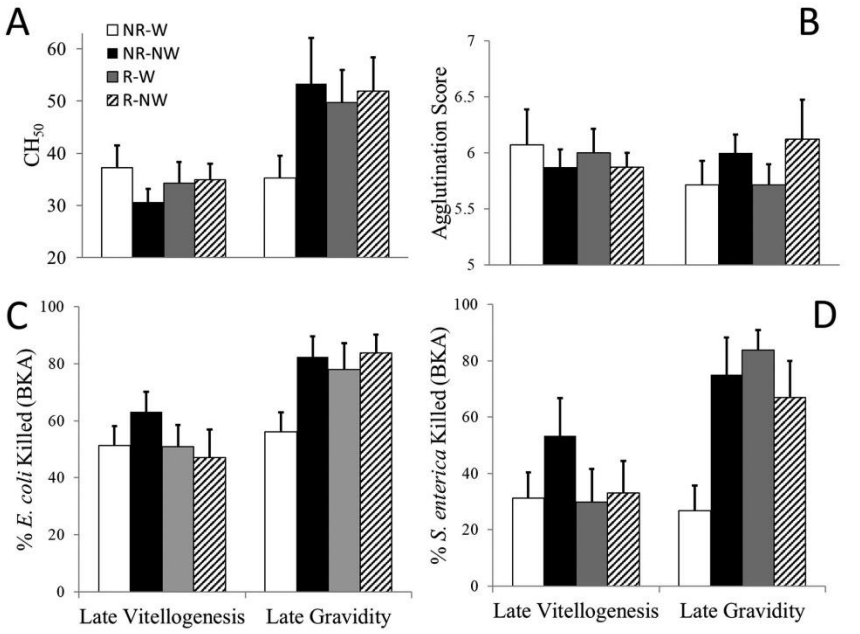

Figure 3. Average immune scores (*A*, lysis [CH_{50}]; *B*, agglutination; *C*, *Escherichia coli* bacterial-killing assay [BKA]; *D*, *Salmonella enterica* BKA) measured in nonreproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water during most of the duration of gravidity or its equivalent for nonreproductive yoked females. Plasma samples were collected when reproductive females were in late vitellogenesis and late gravidity. There was a significant ($P < 0.05$) time-by-treatment (W or NW) interaction in lysis scores; a significant time-by-status (R or NR) interaction in *E. coli* BKA scores; and a significant three-way interaction between time, treatment, and status in *S. enterica* BKA scores. There were no significant interactions or main effects detected in agglutination scores. Error bars represent ±1 SEM.

Immunoglobulin M (IgM), frequently called naturally occurring antibodies, is typically the key isotype for mediating agglutination (Ehrenstein et al. 2010) because it is polyreactive and can bind to conserved structures on invading microorganisms without the need for prior exposure (Briles et al. 1981). The binding of IgM (or IgG following a previous immune response) to an antigen allows for initiation of the classical complement cascade and eventual cell lysis by the membrane attack complex or phagocytosis by immune cells (Murphy 2011). Previous research has shown that IgM, IgD, and IgY (roughly synonymous with IgG) are the primary isotypes found in reptiles (Portis and Coe 1975; Wei et al. 2009). However, there appear to be species-specific differences in IgY structure that influence its relationship with activating the complement cascade (Sekizawa et al. 1984; Lundqvist et al. 2006). Without previous exposure to an antigen, as is presumably the case with SRBC, IgM would be vital to agglutination and initiation of the classical complement cascade in Children's pythons. IgM concentrations may not be upregulated in dehydrated animals, which may explain why we did not detect any significant changes in agglutination scores. These results, coupled with our findings that lysis scores did significantly increase, suggest that another complement pathway that requires preexisting antibodies (i.e., lectin or alternative) is responsible for agglutination.

The low metabolic demands of nonreproductive females in different treatment groups (water or no water) enabled us to examine how a physiological challenge (dehydration) affects immune performance independent of high energetic demands (as in reproductive females). Nonreproductive females without water showed significant increases in osmolality (fig. 2) and most measures of innate immune function (fig. 3), further validating the link between increasing osmolality and enhanced immune performance. Interestingly, our exploration of increased energetic demands independent of hydric challenge (i.e., the comparison of reproductive and nonreproductive females with access to water) produced results opposite of what we initially predicted. As capital breeders, Children's pythons accumulate large amounts of fat reserves be-

fore reproducing to sustain the energetic demands of gravidity. Despite these reserves, females have been shown to incur dramatic expenditures throughout reproduction in the form of significant resource and performance costs (Bonnet et al. 1998; Lourdais et al. 2013; Stahlschmidt et al. 2013). We found that reproductive females had significantly higher immune scores compared to their yoked nonreproductive females (fig. 3) in the late stages of gravidity, when we assumed they would be in an energetic deficit.

Coupled with this, we also found that reproductive females with access to water throughout the experiment had significantly higher osmolality values (fig. 2). This may be due to the increased hydric demands females face toward the end of gravidity. Previous research has shown that females deposit a large amount of water into developing eggs before oviposition (Stahlschmidt et al. 2011). An alternative explanation is that the reproductive females were behaviorally thermoregulating to sustain an elevated preferred body temperature near the heat source in their cage (which was at the end opposite their water). We did not record body temperature in our study, and previous work has found that gravid Children's pythons sustain higher and more stable body temperatures (Lourdais et al. 2008). It may be that there are trade-offs between drinking water at the cooler end of the cage and dehydrating while maintaining an ideal temperature for developing eggs. Future research should either record internal body temperatures or maintain a stable thermal environment to remove any potential thermal influence.

Surprisingly, we did not detect any additive effects of water deprivation and reproduction on osmolality or immune performance. If the osmolality increases that we found in nonreproductive females without water can be attributed to dehydration (i.e., no water for ~3 wk) and the osmolality increases in reproductive females with water (assuming they drank throughout gravidity) are due to the hydric demands of reproduction, why is there not an additive osmolality increase in reproductive females without water? Again, the water-restricted group may have been behaviorally thermoregulating and opted to remain at a cooler temperature to avoid increased evaporative water loss from elevated body temperatures. Another possibility is our measurement of hydration, osmolality, as the total number of solutes (dissolved particles) in a solution (Sweeney and Beuchat 1993). In an organismal context, increased osmolality can be due to decreased water volume in the plasma, increased solutes in the plasma, or both. Typically, the major extracellular solutes that contribute to osmolality are plasma proteins (albumin) and ions (mainly sodium and chloride and to a lesser extent magnesium and calcium; Burg and Ferraris 2008). The maturation of parchment-shelled eggs in the oviducts requires the female to invest considerable amounts of structural (eggshell) and functional (extraembryonic fluids) proteins (White 1991; Blackburn 1998), which might also be contributing to the elevated osmolality we found in reproductive females with access to water. We propose that reproductive females without access to water are faced with a twofold hydric challenge: decreased water volume in their plasma and increased solute concentrations for egg maturation. These females might be hitting an "osmotic ceiling" where their plasma osmolality approaches a physiologically dangerous level and they are forced to mobilize fewer solutes for their developing eggs. In support of this, the maximum osmolality detected in our field study (354 mOsm kg^{-1}) was comparable to the maximum value we found in laboratory animals (357 mOsm kg^{-1}). Future work should examine egg composition in females with and without water to examine whether there is a trade-off between egg quality and maintaining tolerable plasma osmolality.

We found that, overall, osmolality, regardless of the underlying reason for it (seasonal rainfall, water deprivation, or reproduction), was positively correlated with increased immune performance. Dehydration is considered by some to be a physiological stressor (El Fazaa et al. 2000; Tsuchida et al. 2004), and stress typically suppresses immune function via increases in immune inhibitory hormones (Maule et al. 1989; Morici et al. 1997). While we did not measure stress hormones in this study, previous research has shown that, during periods of dehydration, Children's pythons do not exhibit an increase in plasma glucocorticoid concentrations, which is indicative of a stress response (Dupoué et al. 2014). While these results may seem initially surprising, recent work has shown that deviations from homeostatic balance illicit inflammatory responses through the activation of the NLRP3 inflammasome by a wide array of stimuli, including transient receptor potential channels (Han and Yi 2014; Kotas and Medzhitov 2015), which can respond to osmotic stimuli (Liedtke 2006). Most females in our study were faced with one or more physiological challenges (i.e., dehydration, energetic demands), which may have disrupted homeostasis and stimulated such an inflammatory response. It would be valuable to explore the mechanism by which dehydration triggers an upregulation of the innate immunity, as little is known about the regulation of immune activity in squamate reptiles. Furthermore, it is also of great interest to determine whether these physiological challenges imposed on mothers affect offspring quality. Reproduction is a crucial life-history stage that ensures the survival of a species yet can jeopardize individual future reproductive effort and even survival, especially when undertaken during times of resource limitation. Given the limitations of current knowledge and the expected changes in resource availability that will result from climate change, further work is needed to understand the interactions between critical resource limitations, reproduction, and immune function.

Acknowledgments

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APPENDIX C

MUSCLES PROVIDE AN INTERNAL WATER RESERVE FOR REPRODUCTION

PROCEEDINGS B

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Research

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Author for correspondence:
George A. Brusch IV
e-mail: bruschg@gmail.com

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Muscles provide an internal water reserve for reproduction

George A. Brusch IV[1], Olivier Lourdais[2], Brittany Kaminsky[1] and Dale F. DeNardo[1]

[1]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA
[2]Centre d'Etudes Biologiques de Chizé, CNRS, 79360 Villiers en Bois, France

GAB, 0000-0001-7740-6066; DFD, 0000-0003-3145-8367

The use of fat to support the energy needs of reproduction (i.e. capital breeding) has been studied in a diversity of taxa. However, despite reproductive output (i.e. young or eggs) being approximately 70% water, little is known about the availability of internal resources to accommodate the hydric demands of reproduction. Recent research suggests that dehydration increases the catabolism of muscle as a means of maintaining water balance. Accordingly, we investigated the interactive effects of reproductive investment and water deprivation on catabolism and reproductive output in female Children's pythons (*Antaresia childreni*). Both reproductive and non-reproductive females were either provided water ad libitum or were water-deprived for three weeks at the time when reproductive females were gravid. We found that water-deprived reproductive females had, in general, greater body mass loss, epaxial muscle loss, plasma osmolality and plasma uric acid concentrations relative to the other groups. Furthermore, water-deprived females had similar clutch sizes compared with females with access to water, but produced lighter eggs and lower total clutch masses. Our results provide the first evidence that selective protein catabolism can be used to support water demands during reproduction, and, as a result, these findings extend the capital breeding concept to non-energetic resources.

1. Introduction

Optimizing reproductive phenology is essential to fitness, with offspring typically being born when trophic resources and abiotic conditions maximize offspring survival [1,2]. However, producing offspring at such a time may require reproductive activities (i.e. migration, mate acquisition and energy allocation) to occur when resources are limited [3]. Multiple strategies exist to cope with this possible temporal conflict. For example, females can unlink resource acquisition from allocation by accumulating resources when available and then relying on internal stores to invest in subsequent reproduction (i.e. capital breeding [4]). To date, such breeding strategies have solely been approached from an energetic viewpoint, where fat stores are primarily used to fuel reproductive efforts [5–7]. However, offspring production requires other nutrients that cannot be supplied by the catabolism of fat alone. For example, if not adequately obtained from feeding, amino acids needed for offspring production must come from the catabolism of muscle mass [8,9].

Water is another high-demand resource required for reproduction and notably embryonic development [10]. While in many environments water is readily available when energy resources are not, this is not the case in arid ecosystems, where access to drinking water may be extremely limited for extended periods. In these ecological contexts, the appearance of offspring coincides with the rainy season to take advantage of this period of high productivity. Such temporal optimization may require females to start investing in reproduction during times of concomitantly limited water and food resources. Capital breeding provides a robust way to support the energy demands of reproduction,

especially in seasonal environments [11,12]. Despite the crucial importance of water for reproduction [13], to date it is unknown whether such a capital breeding tactic also pertains to water. We posit that mobilizing stored water (i.e. 'capital') should provide benefits when environmental water resources (i.e. 'income') are limiting at the time of reproductive effort.

A water-based capital breeding strategy is challenged by the facts that water imbalance can have debilitating effects on physical, cognitive and physiological functions [14], and most species do not possess distinct water stores. However, bound water is a significant component of all body tissues, typically representing approximately 70% of the wet mass of vertebrates. Thus, internal tissues could provide a water source for reallocation to reproductive needs. While fat is an exceptional source of energy, providing twice the energy per gram as does protein, it is a less effective source for the reallocation of bound water. Bound water represents only approximately 10% of the wet mass of fat, while it represents approximately 76% of skeletal muscle [15]. Overall, wet protein mobilization yields more than five times the total (metabolic and bound) water (0.155 g water kJ^{-1}) than does wet lipids (0.029 g water kJ^{-1}) [16]. Therefore, muscle protein may serve as an important water depot when environmental water resources are not available. In fact, mammals and birds enhance the catabolism of muscle relative to that of fat to maintain water balance [17,18]. However, no previous efforts have examined whether selective protein catabolism (beyond that required for the reallocation of amino acids) can be used to meet the water demands of reproduction.

Children's pythons (*Antaresia childreni*) reside in the wet–dry forest of northern Australia, and their eggs hatch shortly after the onset of the rainy season. Consequently, reproduction occurs during the latter parts of the dry season when both food and water are extremely limited. In fact, plasma osmolality of Children's pythons increases as the dry season progresses [19]. Children's pythons are typical capital breeders, using energy stores to support reproduction and notably yolk deposition during vitellogenesis. The ensuing period of gravidity is characterized by a shift in maternal thermoregulation, development of the eggshell and the investment of a significant amount of water into developing eggs [20,21]. While reproductive female Children's pythons provided with ad libitum water but no food catabolize muscle to provide energy and amino acids for egg production [9], it is unknown whether additional muscle catabolism can support the investment of water into the eggs when this resource is limited.

We examined the interactive effects of reproduction and water deprivation on plasma osmolality, muscle catabolism and reproductive output in female Children's pythons. We hypothesized that structural proteins serve as a valuable source of water for developing eggs when environmental water availability is insufficient. We tested the following predictions:

(1) Females deprived of water dehydrate (i.e. increase plasma osmolality) more than females with continuous access to water, and the extent of this constraint is greater in gravid relative to non-reproductive females due to the water demand associated with gravidity.
(2) To support embryonic water demands, water-deprived females catabolize more protein than females with ad libitum access to water, as evidenced by a greater loss of muscle and an increased plasma concentration of uric acid, a by-product of protein catabolism.
(3) Protein catabolism is insufficient in covering all water demands associated with gravidity, so water-deprived females will have lower reproductive output than do females that have access to water.

2. Methods

(a) Experimental design

To stimulate the reproductive cycle, snakes were overwintered (6 L : 18 D cycle and a daily temperature cycle of 20°C : 15°C) from 6 December 2016 to 7 February 2017. Snakes were deprived of food from the onset of overwintering to the end of the experiment (approx. five months). Upon emergence, the room temperature was increased to a constant 31.5°C, which is the preferred temperature of gravid Children's pythons [20]. Females were held at a constant temperature without opportunity to thermoregulate to prevent a temperature-based difference in water loss as a result of differential thermoregulation between reproductive and non-reproductive females.

Females were randomly assigned to either the reproductive or non-reproductive groups, with reproductive females, but not non-reproductive ones, being exposed to a different male every 2–3 days, resulting in each reproductive female being sequentially housed with six to eight males during this 18-day breeding period. Each non-reproductive female was yoked to a reproductive female so that the timing of its treatments, measurements and sampling coincided with that of the reproductive female to which it was yoked. At ovulation (based on a peri-ovulation shed [20]), half of the reproductive females and their yoked partners, were deprived of water until the reproductive females laid her eggs (21–23 days). Treatment groups were: gravid with water provided ad libitum ($n = 9$), non-reproductive with water provided ad libitum ($n = 8$), gravid with no water provided ($n = 10$) and non-reproductive with no water provided ($n = 8$).

Assessments focused on changes that occurred predominantly over the vast majority of the gravidity period. Body mass, snout–vent length (SVL) and epaxial muscle width (at both 50% and 75% of each snake's SVL) were measured at ovulation and again during late gravidity (21 days post-ovulation). Epaxial muscle widths were measured following previously described methods [9,22]. In brief, there is a palpable demarcation where the longissimus dorsi muscle rests against the iliocostalis muscle, so the distance between the lateral edges of the two longissimus dorsi muscles can be measured with digital calipers [22]. Four measurements were taken each at 50% and 75% of the distance from the head to the vent, and an overall average was used for statistical analyses. Blood samples were collected from females once the reproductive female of the yoked partners reached late vitellogenesis and again when it reached late gravidity. From the collected blood samples, plasma osmolality values (vapour pressure osmometer, #5600, Wescor Inc.) and circulating concentrations of nutrients were measured (glucose [blood glucose meter, #EG220546, Medline Industries], triglycerides [assay kit #TR0100, Sigma-Aldrich], total proteins [assay kit #23236, Thermo Scientific]) and their catabolic by-products (ketones [blood ketone meter, # OS020102A, Nova Biomedical] and uric acid [assay kit #A22181, Life Technologies], for fatty acid and protein catabolism, respectively). At oviposition, female body mass, clutch size and clutch mass were measured.

(b) Statistical analyses

One-factor analyses of variance (ANOVAs) were performed to compare mass and epaxial muscle changes over the course of the experimental treatment (21 days) among the four treatment groups. Two-factor ANOVAs were also performed to examine

the influences of water treatment (water or no water), reproductive status (reproductive or non-reproductive), and their interaction on epaxial muscle and mass changes.

Repeated-measures analysis of variance (rmANOVA) was used to examine the effect of status (reproductive or non-reproductive) and treatment (water or no water) on biochemical assessments over the two time periods (late vitellogenesis and late gravidity). Three-way interactions using treatment, status and time period as fixed effects and individual identity as a random effect were also tested.

The influence of treatment on clutch size (i.e. number of eggs per clutch) was tested using a linear model with treatment (water or no water) as a fixed effect and the SVL as a covariate. As clutch size and body size are closely related in snakes, size-adjusted fecundity was calculated by extracting residuals of the linear relationship between clutch size and the SVL. The effect of treatment on clutch mass was then tested using a linear model with treatment as a fixed effect and the SVL and size-adjusted fecundity as covariates. The data were checked to ensure they met the assumptions for parametric testing, and transformations were used where necessary. All statistical analyses were performed in R v. 3.3.2 [23] with the packages 'nlme' and 'multcomp' [24,25] for rmANOVAs and 'agricolae' [26] for post hoc tests. Significance was set at $\alpha = 0.05$.

3. Results

(a) Morphological changes during treatment

Females at the onset of the experiment had similar SVL ($F_{1,34} = 1.32$, $p = 0.28$) and body mass ($F_{1,34} = 1.17$, $p = 0.33$). We found significant differences in mass loss during the three-week experimental period among the four groups ($F_{3,34} = 8.05$, $p = 0.004$) with higher values in water-deprived reproductive females when compared with water-provided reproductive females (figure 1*a*). Using a two-factor ANOVA, we found that mass loss was influenced by treatment, being significantly higher in water-deprived females than water-provided females (respectively, -85.46 ± 9.45 g versus -25.19 ± 9.68 g, $F_{1,34} = 19.81$, $p < 0.001$). We found no effect of reproductive status ($F_{1,34} = 2.18$, $p = 0.14$) and no interaction between treatment and status ($F_{1,34} = 0.87$, $p = 0.35$).

The mean epaxial muscle width was not related to body size ($F_{1,34} = 2.35$, $p = 0.13$) and there was no initial difference in muscle width among experimental groups ($F_{1,34} = 1.35$, $p = 0.27$). Comparing the four experimental groups revealed that epaxial muscle loss was greatest in water-deprived reproductive females compared to the other groups ($F_{3,34} = 11.38$, $p = 0.0001$; figure 1*b*). A two-factor ANOVA revealed that muscle loss was influenced by treatment with higher values in water-deprived females relative to water-provided females (-2.75 ± 0.18 versus -1.81 ± 0.2 mm, $F_{1,34} = 10.96$, $p = 0.002$). We also found a significant influence of status with higher values in reproductive females relative to non-reproductive females (-2.94 ± 0.19 and -1.62 ± 0.2 mm, $F_{1,34} = 21.8$, $p < 0.0001$). No interaction was found between status and treatment ($F_{1,34} = 0.14$, $p = 0.71$). Epaxial muscle loss and mass loss were correlated ($F_{1,34} = 11.44$, $p = 0.002$), emphasizing that muscle catabolism was associated with mass decreases.

(b) Influence of treatment on blood parameters

Plasma osmolality prior to the onset of water treatment (late vitellogenesis) was similar among groups ($F_{1,31} = 0.49$, $p = 0.42$). By contrast, at the end of treatment (late gravidity), osmolality was higher in water-deprived females when compared with water-provided ones. We found significant time by water treatment ($F_{1,31} = 56.90$, $p < 0.001$) and time by reproductive status ($F_{1,31} = 34.72$, $p < 0.001$) interactions, but no three-way interaction ($F_{1,31} = 1.00$, $p = 0.34$). When considering osmolality change (i.e. final osmolality minus initial osmolality), we found a significant influence of both treatment ($F_{1,31} = 55.39$, $p < 0.0001$) and reproductive status ($F_{1,31} = 34.97$, $p < 0.0001$), but there was no interaction ($F_{1,31} = 2.13$, $p = 0.16$). An HSD post hoc test revealed that osmolality increases were highest in water-deprived reproductive females

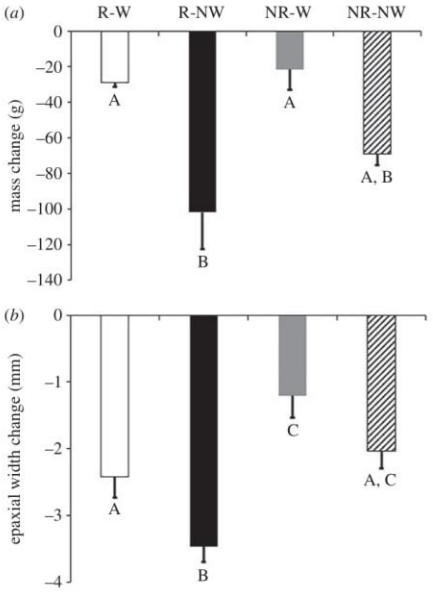

Figure 1. (*a*) Average mass change (grams) and (*b*) average epaxial muscle width change (millimetres) measured in non-reproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water from ovulation to oviposition or its equivalent duration for yoked, non-reproductive females. Error bars represent ± 1 s.e.m. Different letters indicate significant differences among groups (HSD post hoc test).

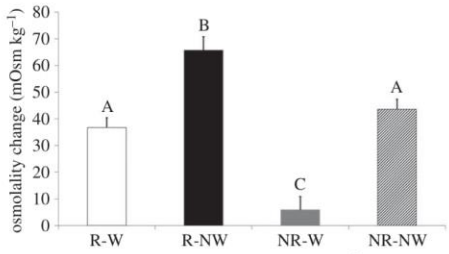

Figure 2. Average plasma osmolality change (mOsm kg^{-1}) measured in non-reproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water from ovulation to oviposition or its equivalent duration for yoked, non-reproductive females. Error bars represent ± 1 s.e.m. Different letters indicate significant differences among groups (HSD post hoc test).

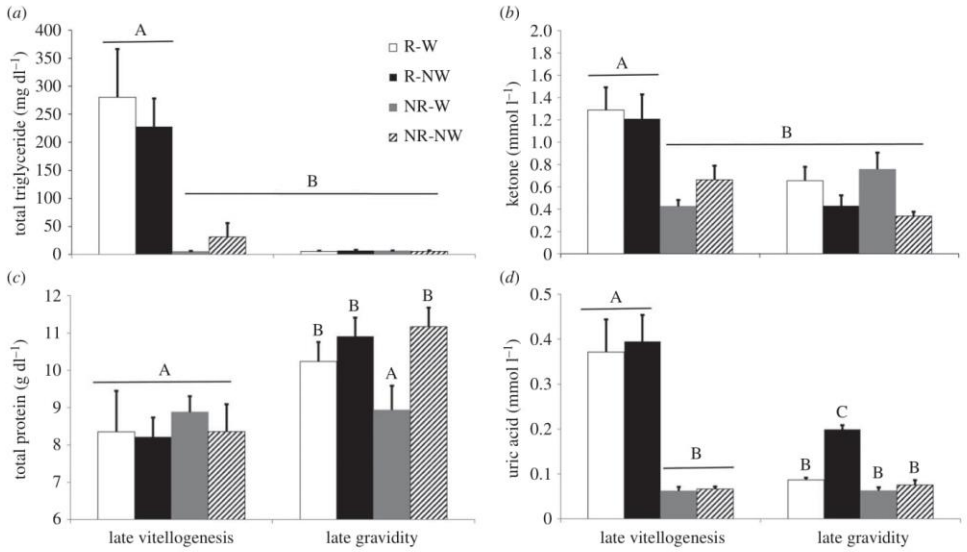

Figure 3. Mean plasma concentrations of (*a*) total triglycerides, (*b*) ketones, (*c*) total protein and (*d*) uric acid measured during late vitellogenesis and late gravidity in non-reproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water from ovulation to oviposition, or its equivalent duration for yoked, non-reproductive females. Error bars represent ± 1 s.e.m. Different letters indicate significant differences among groups (HSD post hoc test).

(65.70 ± 4.17 mOsm kg^{-1}, all $p < 0.05$). Osmolality increase was not different between water-deprived non-reproductive females (43.62 ± 4.66 mOsm kg^{-1}) and water-provided reproductive females (36.77 ± 4.39 mOsm kg^{-1}). Finally, the lowest osmolality changes were found in water-provided non-reproductive females (5.87 ± 4.66 mOsm kg^{-1}, HSD post hoc tests, all $p < 0.05$; figure 2).

Regarding the biochemical assays, we found a significant time by reproductive status interaction in plasma concentrations of total triglycerides ($F_{1,31} = 18.06$, $p < 0.001$), ketones ($F_{1,31} = 10.72$, $p = 0.003$) and uric acid ($F_{1,31} = 23.51$, $p < 0.001$; figure 3*a*,*b*,*d*) where late vitellogenic females, regardless of water treatment, had higher levels than did non-reproductive females. During late gravidity, water-restricted gravid females had higher uric acid levels compared to the other three groups (all $p < 0.05$). For total protein, we did not detect any significant interactions (all $p > 0.05$); however, there was a significant main effect of time where total proteins increased from late vitellogenesis to late gravidity ($F_{1,31} = 16.80$, $p < 0.005$; figure 3*c*). We did not detect any significant interactions or main effects in plasma glucose concentration (all $p > 0.05$).

(c) Influence of experimental treatment on reproductive output

Clutch size was influenced by SVL ($F_{1,18} = 6.77$, $p = 0.02$), but not by treatment (12.5 ± 0.7 versus 12.2 ± 0.7 eggs for water-deprived and water-provided females, respectively; $F_{1,18} = 0.09$, $p = 0.76$) or the interaction term ($F_{1,18} = 0.55$, $p = 0.46$).

Clutch mass was positively influenced by SVL ($F_{1,15} = 22.86$, $p < 0.001$). We found an influence of size-adjusted fecundity, with relatively more fecund females having heavier clutches ($F_{1,15} = 17.40$, $p = 0.001$). After accounting for these two independent covariates, we detected a significant effect of treatment with water-deprived females producing slightly lighter clutch masses than water-provided females (figure 4; $F_{1,15} = 7.62$, $p = 0.014$). Therefore, water-deprived females produced 16% lighter eggs than did water-provided females (mean egg mass $= 9.48 \pm 0.36$ versus 11.38 ± 0.62 g, respectively, $F_{1,17} = 6.74$, $p = 0.02$). Finally, we found that average epaxial muscle loss was negatively related to clutch mass ($F_{1,17} = 5.65$, $p = 0.03$) and average egg mass ($F_{1,17} = 3.04$, $p = 0.007$). When considering each group separately, the negative relation between epaxial muscle loss and clutch mass was marginal in water-provided ones ($F_{1,8} = 1.31$, $p = 0.28$).

4. Discussion

Resource-based trade-offs have shaped many of our current concepts in evolutionary ecology, and energy is usually the currency being balanced. For example, most of our understanding of the gradient between income and capital breeding strategies has focused solely on energetic requirements and cost/benefit balances associated with storage [6,7,27]. Our results provide the first evidence that protein catabolism can be used to support water demands during reproduction, and these findings extend the capital breeding concept to non-energetic resources.

Vitellogenic females had higher plasma concentrations of total triglycerides, ketones and uric acid than did non-reproductive females (figure 3*a*,*b*,*d*), reflecting the energy mobilization required for yolk production into developing follicles of lecithotropic species [28–30]. All plasma osmolalities measured in this study were within the osmolality range of free-ranging *A. childreni* (279–354 mOsm kg^{-1}) [19], suggesting ecological relevance despite being a laboratory

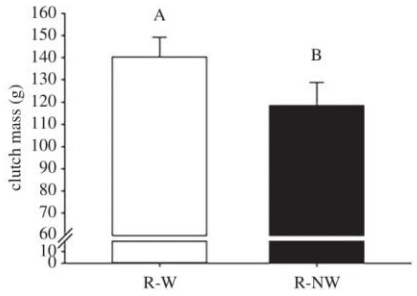

Figure 4. Influence of treatment on clutch mass (grams) measured in reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water from ovulation to oviposition. Plotted values are LS means adjusted for maternal size (SVL) and relative clutch size (see text for statistics). Error bars represent ± 1 s.e.m. Different letters indicate significant differences among groups (HSD post hoc test).

study. Initially, osmolality was similar among all four female groups but, after the water treatment, it was strongly influenced by both treatment and reproductive status. Water-deprived, non-reproductive females had a higher plasma osmolality than did those provided with water. Additionally, females during late gravidity had a higher plasma osmolality than did non-reproductive females, even when both groups had ad libitum access to water (figure 2). These results suggest distinct water imbalances associated with water deprivation and reproduction. Plasma osmolality in females facing both of these challenges simultaneously (i.e. gravid females deprived of water) was significantly different from non-reproductive females deprived of water. Furthermore, the change in osmolality was greater in reproductive, water-deprived females when compared with the three other groups, which underlines the cumulative constraints imposed by reproduction and concurrent water restrictions.

Water deprivation strongly impacted body mass loss and increased protein catabolism in reproductive females (as evidenced by greater loss of epaxial musculature and higher plasma uric acid concentrations; figures 1*b*, 3*d*). Because proteins are a much lower energetic source than are lipids (5.3 kJ g^{-1} and 37.6 kJ g^{-1}, respectively), this suggests that the oxidation of proteins was mostly driven by water needs, as protein supplies five times more total water than do lipids [16]. Muscle loss during reproduction has been documented in insects [31,32], fish [33], reptiles [34], birds [35,36] and mammals [37,38]. Protein mobilization is typically associated with extended fasting and is thought to cover energy and amino acid requirements when lipid reserves and food intake are insufficient [9,39]. Alternatively, protein mobilization can serve as a water resource during times of water restriction or increased water demands [40]. Our study is the first to link muscle atrophy during reproduction to water allocation requirements of developing offspring. Muscle loss associated with fasting and reproduction is known to alter locomotor performance [34,41,42] and induce predation costs [43]. Therefore, using muscle as a water depot under water-limited circumstances leads to an important water-based trade-off between reproduction and performance that is similar to that commonly demonstrated with energy as the currency.

Water deprivation had no impact on clutch size, probably because treatment started at ovulation, after clutch size determination and energy investment is mostly completed [44]. Importantly, water-deprived females produced lighter clutch masses when compared to water-provided females. This impact on reproductive output suggests that maternal catabolism of muscle, while greater in water-deprived females, was not to the extent needed to completely satisfy the water requirements of the developing embryos during gravidity. This implies that there is a trade-off that creates a parent–offspring conflict [45–47]. Evidence for a water-based transgenerational conflict has been previously demonstrated in two viviparous squamates [47,48]. Our study clearly supports the existence of a trade-off between female condition (e.g. muscle mass and osmolality) and reproductive output (e.g. egg size). For the female, loss of epaxial muscle width has been previously correlated with reductions in whole-body performances [49,50] and increases in plasma osmolality alter physiological performances [19,51,52]. From the perspective of the offspring, egg mass in squamates is typically positively correlated with offspring size [53,54]. Additionally, the egg mass difference we report probably reflects reduced water allocation, and lower egg water-content reduces embryonic yolk absorption, resulting in smaller size and reduced offspring performance [55,56].

Our experimental water restriction was limited to the time when females were gravid, which lasted for a rather brief period (three weeks). An even more significant conflict may take place if water deprivation occurs over a longer period and encompasses additional reproductive stages (e.g. vitellogenesis). Furthermore, examining only simple quantitative metrics of reproductive output (e.g. clutch size and egg mass) may miss more cryptic effects, so future research on water-based trade-offs should examine compositional components of eggs, offspring phenotype and offspring performance.

Uricotelic species (i.e. reptiles and birds) produce a highly concentrated, and frequently solid, form of nitrogenous waste. Therefore, the elimination of the nitrogen freed during muscle catabolism requires little water, enabling water acquired from muscle catabolism to be reallocated to internal needs. By contrast, it has been proposed that ureotelic animals do not rely on protein catabolism for water [57], mostly as a result of the nitrogenous wastes requiring a considerable increase in urine production. However, there are examples of ureotelic animals facing times without any external water sources [58–62]. Given the consistent demands for water during reproduction and results presented herein, muscle catabolism to fulfil both energy and water requirements may be a widespread phenomenon deserving of further attention across taxa. This is especially relevant given that current changes in rainfall patterns are challenging reproductive strategies [1,63,64] and can have negative impacts on entire ecosystems [65]. Further alteration in water availability is predicted widely across the globe, and increased muscle mobilization during reproduction may result in higher survival or performance costs of reproduction.

Ethics. All work was conducted under the oversight of the Arizona State University Institutional Animal Care and Use Committee (protocol no. 17-1532R).

Data accessibility. The datasets supporting this article can be accessed at https://doi.org/10.6084/m9.figshare.6075614.

Authors' contributions. G.A.B., O.L. and D.F.D. designed the study and wrote the manuscript. G.A.B., B.K. and D.F.D. collected the samples. G.A.B. conducted all assays. G.A.B. and O.L. performed the statistical analyses.

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APPENDIX D

DEHYDRATION DURING EGG PRODUCTION ALTERS EGG COMPOSITION AND YOLK IMMUNE FUNCTION

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Dehydration during egg production alters egg composition and yolk immune function

George A. Brusch IV[a,*], Benoit Heulin[b], Dale F. DeNardo[a]

[a] School of Life Sciences, Arizona State University, 427 East Tyler Mall, Tempe, AZ 85281, USA
[b] Paimpont Biological Station, CNRS UMR6553, University of Rennes 1, 35380 Paimpont, France

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ABSTRACT

Parent-offspring conflicts occur when resources are limited for allocation, and, historically, energy has been the primary currency of focus when examining these trade-offs. Water is a fundamental resource that has received far less consideration for parent-offspring conflicts. Previous research suggests that, when water is limited, reproductive females are compromised in favor of developing embryos. However, these studies limited their assessments to standard metrics such as clutch size and mass. We tested the hypothesis that the mother-offspring conflict over limited water resources leads to finer scale morphological and physiological impacts on the eggs in Children's pythons (*Antaresia childreni*). We predicted that water deprivation during gravidity alters female investment into her eggs, impacting egg water content and shell development. Additionally, we predicted that the yolk in these dehydrated eggs would have enhanced immune performance metrics, as has been documented in dehydrated adults. We found that eggs from water-deprived females were dehydrated as indicated by reduced percent water and greater yolk osmolality compared to eggs from females that received ad libitum water. We also found that eggs from dehydrated mothers had thinner shells and higher water loss rates. The impacts were not entirely negative as dehydrated eggs had higher antimicrobial capabilities. Also, thinner and more permeability eggshells might allow for elevated rates of rehydration from nest substrate. Overall, by examining an array of egg traits, we demonstrated that dehydration of gravid females impacts the eggs, not just the females as previously reported. As a result, the mother-offspring conflicts are indeed two-sided.

1. Introduction

Reproduction requires a considerable amount of resource investment. When resources are limited, this can lead to a resource trade-off between the parent and offspring (Trivers, 1974). Historically, the currency of interest in studies of parent-offspring conflicts has been energy (Calow, 1979; Doughty and Shine, 1997; Reznick et al., 2000; Kotiaho, 2001), and the outcomes determined by examining the post-reproductive female body condition (Rutherford and Tardif, 2008; Fowden and Moore, 2012) along with the quantity and quality of her resulting young (Denardo et al., 2012; Schwarzkopf and Andrews, 2012; Shine, 2012). However, such approaches ignore other currencies as well as more cryptic developmental metrics that might be influenced by such conflicts. In oviparous species, the egg provides the early developmental environment for the embryo, which can have profound impacts on fitness (Lindström, 1999; Monaghan, 2008). Therefore, consideration of egg metrics can provide a more complete understanding of the repercussions of parent-offspring conflicts.

Water is a fundamental resource that has received far less consideration as a potential currency to mediate maternal-offspring trade-offs. The impacts of maternal hydration have been explored after gestation (Hanson et al., 1994) and as a potential proximate cue for reproductive timing (Nelson et al., 1989; Gesquiere et al., 2008; Bukovetzky et al., 2012). However, there is far less known about the direct consequences of maternal hydration during gestation. Recent studies show that, when water is limited, reproductive female snakes face significant increases in plasma osmolality (Brusch et al., 2017; Dupoué et al., 2018) and may catabolize greater amounts of muscle mass relative to fat in order to free up bound water (Brusch et al., 2018). Loss of muscle mass not surprisingly has been associated with reduced performance by the post-reproductive female (Lourdais et al., 2013). As a result, it is assumed that the water aspects of the parent-offspring conflict are often compromised in favor of the developing embryos (Dupoué et al., 2015). However, maternal hyperosmolality can also enhance maternal immune performance (Brusch et al., 2017) and reduce egg size (Brusch et al., 2018), indicating a much more complex

* Corresponding author.
E-mail address: gbrusch@asu.edu (G.A. Brusch).

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relationship between the mother and her offspring. This newly appreciated complexity deserves further study, especially considering that past studies have relied on relatively broad, easily assessable metrics of reproductive output to examine potential conflicts for hydric resources (e.g., egg mass or volume, Dupoué et al., 2015, Brusch et al., 2018). More detailed examinations of egg quality may reveal relatively cryptic impacts of the parent-offspring conflict over water resources.

Accordingly, we examined the impact of maternal dehydration on reproductive output in the Children's python (*Antaresia childreni*), which is a capital-breeding, oviparous squamate that has proven to be a valuable study system for examining parent-embryo interactions (e.g., Stahlschmidt and Denardo, 2008, 2009a, 2009b, 2010; Lorioux et al., 2012; Lourdais et al., 2013). Regarding water limitations, female Children's pythons deprived of water during gravidity are hyperosmotic, experience greater muscle catabolism, and produce lighter eggs (Brusch et al., 2017, 2018). We furthered our understanding of water-based parent-offspring trade-offs by testing the hypothesis that the mother-offspring conflict over limited water resources leads to morphological and physiological impacts on the eggs. We predicted that water deprivation during gravidity alters female investment into her eggs and thus impacts egg water content and shell development, two critical investments into the egg that occur in the oviduct. Furthermore, we predicted that the yolk in these dehydrated eggs would have enhanced immune performance metrics, as has been documented in dehydrated adults (Brusch et al., 2017) and dehydrated late-stage embryos (Brusch and Denardo, 2019).

2. Material and methods

2.1. Study Species

Children's pythons (*Antaresia childreni*) inhabit the wet-dry tropics of northern Australia, where eggs are laid towards the end of the dry season so that offspring hatch at the start of the wet season (Wilson and Swan, 2013). Thus, reproductive females naturally face dramatic challenges to water balance during the period when egg development occurs (Brusch et al., 2017). Additionally, female Children's pythons brood their eggs and, in doing so, the eggs have minimal contact with substrate for water absorption but also have reduced evaporative water loss (Stahlschmidt et al., 2008).

2.2. Experimental design

All work was conducted under the oversight of the Arizona State University Institutional Animal Care and Use Committee (protocol # 17-1532R). Snakes used for this study were part of a long-term colony at Arizona State University, AZ, USA, and were housed individually in 91 × 71 × 46 cm cages (Freedom Breeder, Turlock, CA, USA). Snakes were deprived of food from the onset of overwintering until oviposition (~5 months) since they typically will not eat when reproductively active. To stimulate the reproductive cycle, snakes were over-wintered for six weeks (mid-December through January) by providing them a light-dark cycle of 6:18 h. During the dark cycle, room temperature was 15 °C, while during the light cycle room temperature was 20 °C and supplemental sub-surface heat was provided under one end of each cage to allow for thermoregulation. After overwintering, room temperature was increased to a constant 31.5 °C, which is the preferred temperature of gravid Children's pythons (Lourdais et al., 2008), with a 12 L:12D light cycle (see Brusch et al., 2017 for breeding protocol). Males were rotated through the female cages so that all females were with multiple males with each visit separated by 2–3 days. Once females underwent their peri-ovulatory shed (which approximates the time of ovulation; Lourdais et al., 2008), each snake was alternately assigned to one of two maternal treatment groups of similar mean body mass: water (number of reproductive females and mean mass ± SEM: n = 9, 542 ± 33 g) or no water (n = 10, 507 ± 27 g) provided for the duration of gravidity (i.e., ovulation to oviposition; 22 ± 1 d). Deprivation of water during this time leads to ecologically relevant levels of dehydration (control females: 297 ± 13 mOsm kg^{-1}, deprived females: 354 ± 12 mOsm kg^{-1}; Brusch et al., 2018).

At oviposition of the fertile eggs, clutch size (number of eggs) and clutch mass were measured, and the clutch was moved to a 1.0 L plastic container filled with ~0.5 L of moistened perlite substrate (1:3 by weight) and stored in an environmental chamber at 31.5 °C. Concurrently, an egg from each clutch was placed on a scale at room temperature (~25 °C) and weighed (± 0.01 g) every 10 min for 1 h before being returned to the clutch. A separate egg was selected and weighed, after which a 1 cm^2 eggshell section was removed and stored in 95% ethanol. These cross-sections were air-dried at room temperature and mounted with double-sided transparent tape onto brass stubs. They were then coated with gold-palladium using a sputtering fine coater (JEOL JFC 1000) for structural analyses using scanning electron microscopy ([SEM], JEOL JSM 6301F SEM. Photographs (x600) of cross sections from each eggshell were used to take serial measurements (n = 20 for each eggshell) of the fibrous layer, calcareous crust, and total thickness (fibrous + calcareous). From this same egg, a 1 mL yolk sample was collected (see details below) and the remaining egg contents were moved to a pre-weighed, flat aluminum dish, re-weighed, and placed in an isothermal chamber at 60 °C. The mass of the dish and egg contents were re-weighed every 24 h until mass stabilized. Percent water of the egg contents were calculated as:

$$\%\text{water} = 100 \times [(\text{initial mass of dish with egg contents}) - (\text{final mass of dish with egg contents}) (\text{initial mass of dish with egg contents}) - (\text{mass of dish})]$$

2.3. Yolk preparation

Using slight modifications to the water dilution methods described in Akita and Nakai (1993) and Kim and Nakai (1996), yolk plasma proteins were separated from the granules and lipids. Briefly, 1 mL of egg yolk was diluted with 9 mL Nanopure water (pH of 5, acidified with 0.1 N HCl). Samples were then kept at 4 °C for 2 h, after which they were centrifuged at 10,000 *g* for 2 h at 4 °C. Aliquots of the fluid (50 μL) were separated into vials and frozen at −80 °C to be used within 30 d to measure osmolality and evaluate immune function. Yolk osmolality was determined using a vapor pressure osmometer (± 3 mOsm kg^{-1}; model 5600; Wescor Inc., Logan, Utah, USA) with samples run in triplicate as described in Brusch et al. (2017).

2.4. Immune assays

To examine immunocompetence, we performed several plasma-based innate immune function assays. To evaluate the involvement of complement (C′) and natural antibodies (NAbs) in reacting to a novel, eukaryotic antigen, we used sheep red blood cells (sRBC, SBH050, Hemostat Laboratories, Dixon, CA, USA) to quantify agglutination and lysis, which are standard measures of soluble constitutive immunity (Matson et al., 2005). Briefly, 20 μL of egg yolk plasma were serially diluted with phosphate buffered saline (PBS) along a row of a 96-well plate, after which 20 μL of 1% sRBC were added to each well. Plates were incubated at 31.5 °C for 90 min and then placed at room temperature (~25 °C) for 20 min after which point they were scanned at 600 dots per inch (Hewlett-Packard Co., ScanJet 3670) for agglutination images. After an additional 70 min, plates were centrifuged for 5 min (500 rpm, Sorvall, Newtown, CT, USA) and the supernatant was dispensed into a clean 96-well plate. Absorbance values were measured (405 nm, Bio-Rad, Hercules, CA, USA) to calculate lysis scores. Hemolytic-complement activity was expressed in CH_{50} units mL $plasma^{-1}$, where 1 CH_{50} unit equals the reciprocal of the dilution of plasma required to lyse 50% of the sRBC.

Bactericidal activity was also assessed to determine the ability of egg yolk plasma proteins to kill a prokaryotic microorganism (French and Neuman-Lee, 2012). We used two species of gram-negative bacteria, *Escherichia coli* and *Salmonella enterica*. In brief, we combined 1:1 egg yolk dilution with CO_2-independent media plus 4 nm L-glutamine, agar broth, and either 10^5 colony-producing units of *E. coli* (Lot#483-478-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA) or 10^4 colony-producing units of *S. enterica* (Lot#501-13-1, ATCC 51741, MicroBioLogics, St. Cloud, MN, USA) on a 96-well microplate. Absorbance values were measured (300 nm, Bio-Rad, Hercules, CA, USA) immediately and again after 12 h of incubation at 37 °C. Bactericidal ability percentages were calculated as one minus the mean absorbance for each sample, run in triplicate, divided by the mean absorbance for the positive control, and then multiplied by 100.

2.5. Statistical analysis

We used linear mixed-effect models to examine the effect of maternal treatment (water or no water) on egg (mass, percent water, and mass loss after an hour) and yolk (osmolality, agglutination and lysis scores, and bacterial killing ability of *E. coli* and *S. enterica*) metrics. We used maternal treatment as a fixed effect and maternal plasma osmolality nested within maternal ID as a random effect. Within maternal ID, we included parameters to address potential size effects (using a body condition index – standardized residuals from a linear regression using mass and SVL). To test the effect of maternal treatment on eggshell thickness measurements (calcareous, fibrous, and total) we used a repeated measures analysis of variance (rmANOVA) with maternal treatment and measurement (to account for variation in thickness measurements) as fixed effects, and individual egg as a random effect. We tested for compound symmetry to ensure linearity of repeated thickness measurements within eggshells and a homogenous relationship between samples. We checked to ensure the data met the assumptions for parametric testing and used transformations where necessary. We performed all statistical analyses in R, version 3.3.2 (R Development Core Team 2016) with the packages *lme4* (Bates et al., 2015) and *car* (Fox and Weisberg, 2002). Significance was set at $\alpha = 0.05$.

3. Results

When comparing the masses of the eggs randomly selected to be sacrificed for yolk and eggshell samples we found that eggs from water-deprived females were significantly lighter that those from females with water ($F_{1,17} = 5.35$, $p = 0.034$; Fig. 1A). The eggs from the water-deprived females also had significantly lower percent water ($F_{1,17} = 4.65$, $p = 0.041$; Fig. 1B) and higher yolk osmolality ($F_{1,17} = 8.59$, $p = 0.001$; Fig. 1C) compared to eggs from the water-provided females. Additionally, eggs from water-deprived females had higher percentages of *E. coli* ($F_{1,17} = 7.77$, $p = 0.010$; Fig. 2A) and *S. enterica* ($F_{1,17} = 5.31$, $p = 0.016$; Fig. 2B) killed. However, we did not detect any significant differences between treatment groups for agglutination ($F_{1,17} = 0.27$, $p = 0.612$; Fig. 2C) and lysis ($F_{1,17} = 0.01$, $p = 0.900$) values (Fig. 2D).

Eggs from water-deprived females had greater mass loss after 1 h in a dehydrating lab environment ($F_{1,17} = 4.62$, $p = 0.046$; Fig. 3). Additionally, eggshells from water-deprived females had thinner fibrous layers ($F_{1,17} = 4.88$, $p = 0.044$) and thinner total thickness ($F_{1,17} = 4.44$, $p = 0.047$; Fig. 4), but there was no significant treatment-based difference in calcareous crust thickness ($F_{1,17} = 0.01$, $p = 0.909$; Table 1). We did not detect any significant interactions between mass loss under dehydrating conditions and eggshell thickness measurements (all $p > 0.05$).

4. Discussion

When considering the relative physiological costs of reproduction, there is frequently an emphasis on energy balance and how limited energetic resources can create maternal-offspring conflicts and life-history trade-offs (Stearns, 1992; Walker et al., 2008; Bleu et al., 2012). In these energetic contexts, limited intake or finite internal reserves results in associated costs to mothers (e.g., reduced self-maintenance, Lochmiller and Deerenberg, 2000, Martin et al., 2008) or offspring (e.g., reduced size, Stearns, 1989, Reznick et al., 2000). While energy is clearly an important resource for reproductive investment, our results provide the first evidence that water balance also poses maternal-offspring conflicts in oviparous vertebrates. Additionally, our study emphasizes the importance of including effects that are more cryptic than the number of eggs and egg mass when exploring consequences of mother-offspring conflicts.

We found that, similar to previous findings (Brusch et al., 2018), maternal dehydration (i.e., hyperosmolality), caused by water restriction during gravidity, led to the production of lighter eggs (Fig. 1A). As the energy content of the eggs was determined during vitellogenesis, which occurred prior to our water manipulation, the lighter mass of the

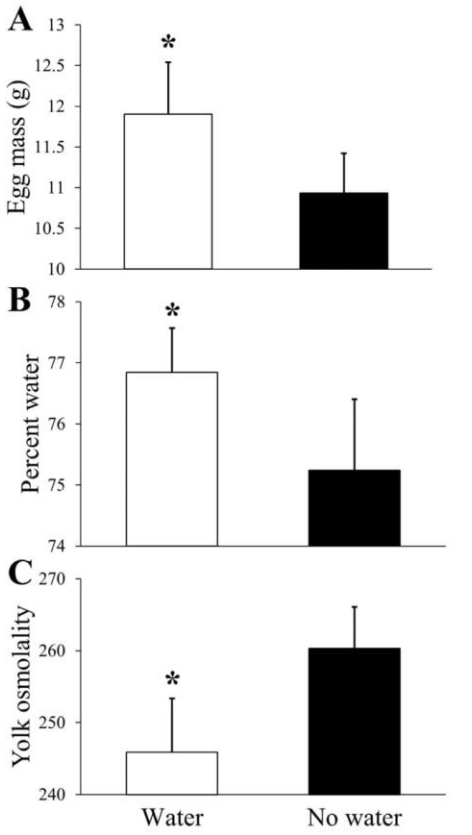

Fig. 1. Average (A, egg mass; B, percent water; C, yolk osmolality [mOsm kg^{-1}]) measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access to water throughout gravidity. Error bars represent ± 1 SEM. Asterisk represents a significant difference between treatment groups.

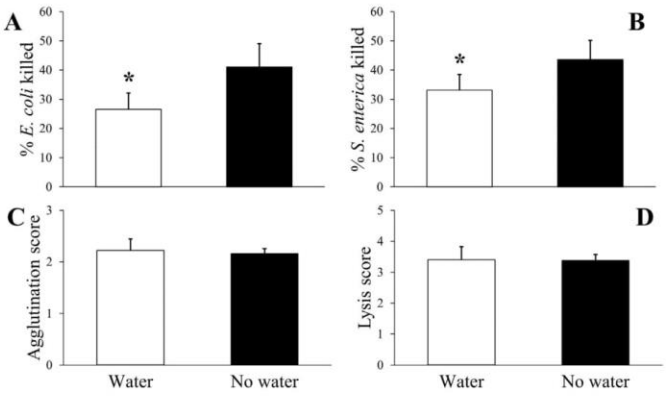

Fig. 2. Average yolk immune scores (A, *Escherichia coli* bacterial-killing; B, *Salmonella enterica* bacterial-killing; C, agglutination; D, lysis) measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access to water throughout gravidity. Error bars represent ± 1 SEM. Asterisk represents a significant difference between treatment groups.

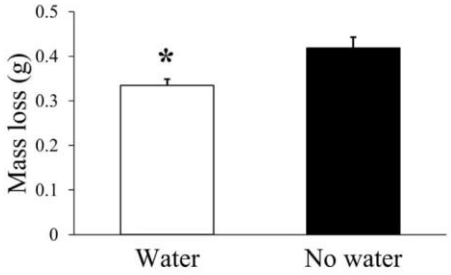

Fig. 3. Average egg mass loss after 1 h in desiccating conditions measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access to water throughout gravidity. Error bars represent ± 1 SEM. Asterisk represents a significant difference between treatment groups.

eggs produced by water-deprived females is likely attributable to reduced water investment during gravidity, a time when females typically invest considerable amounts of water into their eggs (Stahlschmidt et al., 2011a). This presumption is supported by the findings that eggs from water-deprived females had less percent water and higher yolk osmolality compared to eggs from females that received ad libitum water throughout reproduction (Fig. 1B–C).

Previous studies examining trade-offs between maternal and embryonic hydration during water deprivation concluded that maternal hydration is compromised in favor of embryonic hydration (Dupoué et al., 2015; Sparkman et al., 2018). However, these studies relied on egg volume and mass as their lone egg metrics. Our study, which used additional, more cryptic egg quality metrics (e.g., percent water and yolk osmolality) suggests that the effects of maternal dehydration during gravidity are also present in the oviposited eggs. These previously undetected effects add to a growing body of knowledge regarding water flux from mothers to their developing offspring throughout gravidity (Brown and Shine, 2009; Dupoué et al., 2015; Lourdais et al., 2015; Bonnet et al., 2017).

While water balance is critical for embryonic survival (Cagle et al., 1993; Warner and Andrews, 2002), water imbalance is not purely detrimental as dehydration-based immune enhancement has been documented in multiple taxa (insects, Hoang, 2001; lizards, Moeller et al., 2013; snakes, Brusch and Denardo, 2017) including adult and

Table 1
Eggshell component thickness measurements of Children's python (*Antaresia childreni*) eggs at oviposition from females with or without water throughout gravidity (mean ± SEM).

Maternal treatment	Calcareous crust (μm)	Fibrous layer (μm)*	Total thickness (μm)*
Water	6.8 ± 0.2	94.8 ± 3.3	101.6 ± 3.6
No water	6.5 ± 0.7	85.2 ± 2.8	91.8 ± 3.5

* Significantly different between treatment groups.

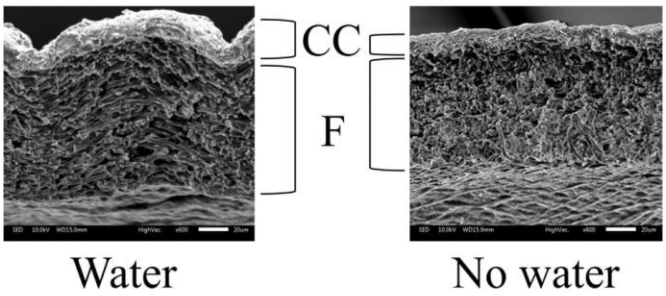

Fig. 4. Scanning electron microgram image of Children's python (*Antaresia childreni*) eggshells at oviposition from females with or without water throughout gravidity. Cross sections were used to measure calcareous crust (CC), fibrous layer (F), and total thickness (CC + F) over 20 measurements.

embryonic Children's pythons (Brusch et al., 2017; Brusch and Denardo, 2019). This suggests that there is a complex interplay between osmolality and physiological functions essential for survival. Our results expand the newly appreciated relationship between hydric state and immune function to include freshly oviposited eggs as another life-stage that exhibits a similar pattern. We found that dehydrated eggs had greater antimicrobial capabilities compared to hydrated eggs (Fig. 2A–B). Microbial infection of eggs can drastically reduce clutch viability and, once pathogens pass through the membrane and infect the developing embryo, there is typically a low chance of survival (Kiesecker and Blaustein, 1997; Adamo, 1999; Cook et al., 2003; Brandl et al., 2014). A robust antimicrobial defense is therefore fundamental to the health of developing embryos (Shawkey et al., 2008; Horrocks et al., 2014). Python embryos at oviposition are approximately one-third through development (Lourdais et al., 2008) and have fully functional livers, which is primary source of synthesis for antimicrobial peptides (van Hoek, 2014). Thus, it is uncertain as to whether the peptides responsible for the bacterial killing ability of the egg are products of the mother or the embryo. Regardless, while reduced water content can impact egg survival (Aubret et al., 2003; Lourdais et al., 2007), this may be offset by increased ability to ward off potentially lethal pathogens. To better understand the benefits of an enhanced immune system when dehydrated, future studies should examine survival rates between dehydrated and hydrated eggs experimentally exposed to microorganisms, as well as the source of the antimicrobial agents. Additionally, the cellular mechanisms responsible for dehydration-based innate immune enhancement at any life stage remain unknown and are in need of further study.

In contrast to the positive effects of dehydration on egg bacterial killing ability, agglutination and lysis were not impacted by dehydration (Fig. 2C-D). These results are in accordance with previous findings that found embryonic dehydration later in development (just prior to hatching), resulted in increased bactericidal ability but not enhanced agglutination and lysis (Brusch and Denardo, 2019). After recognition of foreign pathogens, agglutination is often the next step in an immune response, whereby cells and microorganisms are clumped together (typically in the presence of complement and/or antibodies) and destroyed via phagocytosis or direct membrane disruption (i.e., lysis, Medzhitov and Janeway, 2000). Adult Children's pythons have elevated agglutination and lytic abilities when dehydrated (Brusch et al., 2017), which suggests that complement and antibodies are neither invested into eggs by the female nor effectively produced by the embryos. As it is often presumed that maternal antibodies are passed to the offspring through vertical transmission to prime the immunological defenses of their naive offspring (Grindstaff et al., 2003; Boulinier and Staszewski, 2008), future studies should focus on the ontogeny of humoral immunity to help understand the discrepancies between adult and embryonic immune responses to dehydration.

A potential explanation for our immune results is that dehydration leads to a concentration of innate immune molecules. Previous work to address this possibility showed that diluting plasma samples from dehydrated squamate reptiles to adjust for the decreased volume, still resulted in higher immune metrics compared to plasma samples from hydrated animals (Brusch and Denardo, 2017; Moeller et al., 2013). This suggests that dehydration leads to an upregulation (either in total number or activity) of innate molecules. Although we did not conduct a similar experiment for our study, it is reasonable to suspect a similar upregulation occurs in dehydrated eggs.

We also found that the eggs from dehydrated mothers had higher water loss rates (Fig. 3, which may be a result of their thinner shells, specifically thinner fibrous layers (Fig. 4; Table 1). Our results are consistent with previous research that found that the calcareous crust of Children's python eggs does not change from oviposition to hatching, while the fibrous layer gradually thins (Stahlschmidt et al., 2010). This change is believed to support the increased oxygen demands of the growing embryos whose metabolism dramatically increases just prior to hatching. This shell thinning, however, comes with the cost of higher water loss rates (Stahlschmidt et al., 2010). For our study, eggs from dehydrated mothers started with less water and had the potential to lose water faster. However, trans-shell water movement has a bidirectional potential. Eggs with thinner shells may lose water faster but they may also be able to absorb environmental water faster as has been previously documented in other oviparous species (Tracy and Snell, 1985; Ji and Braña, 1999; Verdú-Ricoy et al., 2014). Nest site selection is extremely important for Children's pythons (Stahlschmidt et al., 2011b) and dehydrated mothers, who cannot allocate enough water to their eggs without jeopardizing their own hydric needs, may select moister nest sites so that their eggs could absorb environmental moisture. Alternatively, thinner eggs shells may simply reflect a reduced ability of dehydrated female to mobilize and deposit egg shell components during gravidity.

Our results provide the first evidence of a water-based mother-offspring intergenerational trade-off in an oviparous vertebrate. While it was previously assumed that dehydrated mothers were burdened with the majority of the water imbalance in the form of increased plasma osmolality (Dupoué et al., 2015, 2018) and increased muscle catabolism (Brusch et al., 2018), our exploration of cryptic traits found that eggs are also significantly impacted. It is not clear, however, if these impacts are entirely negative, as dehydrated eggs had higher antimicrobial capabilities and thinner shells which might allow for elevated rates of rehydration. We suspect that these results are not limited to squamates, but may be applicable to many other oviparous organisms. By and large, the vast majority of previous research has focused on the importance of energy allocation during reproduction (Gittleman and Thompson, 1988; Lambert and Dutil, 2000; Dugas et al., 2015). Water is an essential resource that has received far less consideration, and we strongly encourage future work to focus on the role hydration plays on both reproductive mothers and their offspring using a variety of metrics that may help elucidate underappreciated trade-offs.

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Competing interests

No competing interests declared.

Author contributions

GABIV and DFD designed the study and collected the samples. BH performed the SEM analyses. GABIV conducted all assays, performed the statistical analyses, and led the writing of the manuscript. DD and BH contributed to revisions and gave final approval for publication.

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Data availability

The datasets supporting this article can be accessed at https://doi.org/10.6084/m9.figshare.6846845.v1

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APPENDIX E

EGG DESICCATION LEADS TO DEHYDRATION AND ENHANCES INNATE IMMUNITY IN PYTHON EMBRYOS

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Short communication

Egg desiccation leads to dehydration and enhanced innate immunity in python embryos

George A. Brusch IV*, Dale F. DeNardo

Arizona State University, School of Life Sciences, 427 East Tyler Mall, Tempe, AZ, 85281, USA

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ABSTRACT

The immune system is essential for survival and its performance can vary depending on the physiological state of the organism. Much of the current research into immune function dynamics has examined newborn to adult life stages, despite previous studies documenting physiological responses in embryos to environmental stimuli. While energy balance has been the predominant focus as the driver of changes in immune function, recent research has found a positive relationship between dehydration and innate immune performance in adult reptiles. We expanded the understanding of this relationship by examining trans-generational immune effects of female dehydration as well as the effects of egg desiccation on embryonic hydration state and innate immunity using Children's pythons, *Antaresia childreni*. We used a 2 × 2 experiment with hydrated or dehydrated mothers and eggs either incubated under continuous optimal conditions or experiencing desiccating conditions for 24 h. Our results demonstrate that, similar to adults, embryos enhance some metrics of innate immunity when they are dehydrated.

1. Introduction

The immune system is vital for the recognition and elimination of foreign pathogens (Murphy and Weaver, 2017). Yet, immune performance is dynamic, being influenced by environmental conditions and organism state. Energy limitations, either associated with limited intake or high metabolic demand, can compromise immune function (Martin et al., 2008; Ritz and Gardner, 2006; Scrimshaw and SanGiovanni, 1997). In contrast, recent studies have demonstrated that water limitation, leading to subsequent hyperosmolality (i.e., dehydration), enhances multiple measures of innate immune performance (Brusch and DeNardo, 2017; Moeller et al., 2013).

To date, most studies have examined immune function dynamics in the free-living life stage, particularly during adulthood. However, the embryonic life stage must also maintain a functional immune system to protect itself from pathogens, and this is especially true for oviparous species since eggs are directly exposed to environmental conditions. Protection of the developing embryo is provided by numerous egg components including albumen, protease inhibitors, nutrient sequesters, a lytic enzyme, and antibodies (Stevens, 1996). Yet, the sensitivity of embryonic immune function to environmental conditions has received little attention. Developmental temperature has been shown to influence hatchling immunocompetence (Ardia et al., 2010; Martin et al., 2011), but there is no information on the immediate effects of temperature on immune function of the developing embryo or on how other environmental factors influence embryonic immune function.

To expand knowledge of embryonic immune function dynamics, we investigated whether embryonic Children's pythons (*Antaresia childreni*) show dehydration-enhanced immune function similar to that documented in adults of this species. Children's pythons inhabit the wet-dry forest of northern Australia, and females lay eggs during the dry season so that their eggs hatch shortly after the start of the wet season (Wilson and Swan, 2013). As a result, females can be significantly dehydrated at the time of egg production (Brusch et al., 2017), and eggs from dehydrated females weigh less than those from hydrated females (Brusch et al., 2018). Thus, embryos may face hydric challenges as a result of female dehydration during egg production and as a result of dry incubation conditions leading to egg water loss.

Because of the ecological importance of egg water balance and the vital role of embryonic immune function, we tested the hypothesis that water restrictions would lead to embryonic dehydration and enhanced innate immunity. We predicted that late embryos from females deprived of water throughout gravidity (i.e., the first third of embryonic development; Lourdais et al., 2007) would have elevated osmolality as would embryos exposed to desiccating conditions during egg incubation. We further predicted that such increases in embryonic osmolality,

* Corresponding author.
E-mail address: gbrusch@asu.edu (G.A. Brusch).

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regardless of what developmental stage the osmotic challenge occurred, would result in elevated innate immune function.

2. Materials and methods

2.1. Reproductive females

Snakes used for this study were part of a long-term colony at Arizona State University, Tempe, AZ, USA, and were housed individually in 91 × 71 × 46 cm cages (Freedom Breeder, Turlock, CA, USA). Snakes were provided a winter cooling period to stimulate reproduction and then held at 31.5 °C (the preferred body temperature of gravid *A. childreni*; Lourdais et al., 2008) with a 12L:12D light cycle. Snakes were not fed throughout the study but had access to *ad libitum* water from overwintering through to ovulation (see Brusch et al., 2017 for breeding protocol). At the time of ovulation, each snake was alternately assigned to one of two maternal treatment groups – water (n = 9) or no water (n = 10). Females in the water treatment group were provided water *ad libitum* throughout gravidity (i.e., ovulation to oviposition), while females in the no water group had no access to water during this stage (22 ± 1 d).

2.2. Offspring

At day 20 post-ovulation (1–4 d prior to oviposition), the female was moved to an oviposition chamber that had limited air flow that provided sufficient oxygen but reduced the possibility of egg desiccation once laid. Females were monitored daily for oviposition. At oviposition, the female and her egg clutch were weighed, the number of eggs counted, and the clutch then moved to a 1.0 L plastic container filled with ~0.5 L of moistened perlite substrate (1:3 by weight) and stored in an environmental chamber at 31.5 °C. Twenty-seven days after oviposition, two eggs from each clutch were randomly selected and assigned a 24 h treatment – control (incubation continued unchanged) or desiccating conditions (alone in a 120 ml container with no substrate and a single 5 mm diameter hole in the lid). Eggs were weighed (± 0.01 g) immediately prior to and after the 24 h treatment. After the 24 h treatment, the eggs were returned to their clutch's original incubation container for the remainder of incubation. The mass of each experimental egg was again measured on days 32, 35, and 39 post-oviposition (incubation duration for eggs allowed to hatch was 45–48 d). After weighing the egg on day 39, the embryo was removed, and a 0.8 ml sample of embryonic fluid was collected. Briefly, the shell was incised with surgical scissors and the embryo removed with forceps. The embryo was then blotted dry, suspended head-down, and decapitated with clean surgical scissors. Embryonic fluid was palped from the body cavity into a 1.5 ml collecting tube, and then immediately centrifuged at 4000 × *g* for 3 min to separate blood cells from the fluid. Sub-samples of the fluid were aliquoted (~20 µl) into separate vials and frozen at −80 °C to be used within 12 d to measure osmolality and evaluate immune function. Osmolality was determined using a vapor pressure osmometer (± 3 mOsm/kg; model 5600; Wescor Inc., Logan, Utah, USA) with samples run in triplicate as described in Brusch et al. (2017).

2.3. Immune assays

Several assays were used to assess innate immune function and examine the relationship between embryonic hydration state and immunocompetence. Agglutination and lysis assays were used to evaluate the involvement of natural antibodies and complement in reacting to a novel antigen, sheep red blood cells (sRBC, SBH050, Hemostat Laboratories, Dixon, CA, USA), and thus serve as standard measures of innate immunity (Matson et al., 2005). Briefly, 20 µl of each embryonic fluid sample were serially diluted (1:2–1:2048) with phosphate-buffered saline (PBS) along a row of a round bottom, 96-well microplate. To each well we also added 20 µl 1% sRBC. To separate rows we added 20 µL PBS with 20 µL 1% sRBC to serve as a negative control (0% lysis) and 20 µl 1% sRBC with 20 µL ACK lysing buffer (Lonza, Basel, Switzerland) as a positive control (100% lysis). We incubated plates at 29 °C for 90 min and then placed them at room temperature (~25 °C) for 20 min after which point they were scanned at 600 dots per inch using a flat-bed scanner (Hewlett-Packard Co., ScanJet 3670) for agglutination images. Plates remained at room temperature for an additional 70 min and were then centrifuged for 5 min (700 × *g*, Sorvall, Newtown, CT, USA). We next transferred the supernatant to a clean 96-well plate and measured absorbance using a microplate reader (405 nm, BioTek Instruments, Winooski, VT, USA) to calculate lysis scores. Hemolytic-complement activity was expressed in CH_{50} units/ml plasma, where one CH_{50} unit equals the reciprocal of the dilution of plasma required to lyse 50% of the sRBC.

We also conducted bacterial killing assays (BKA) as outlined in French and Neuman-Lee (2012) to assess the ability of Children's python embryos to inhibit the growth of infectious microorganisms. We used two different species of gram-negative bacteria, *Escherichia coli* and *Salmonella enterica*, that are known to be pathogenic to snakes (Jacobson, 2007). Briefly, we combined 1:4 embryonic fluid dilution with CO_2-independent media plus 4 nm L-glutamine, agar broth, and either 10^4 colony-producing units of *E. coli* (Lot#483-478-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA) or 10^4 colony-producing units of *S. enterica* (Lot#501-13-1, ATCC 51741, MicroBioLogics, St. Cloud, MN, USA) onto a round bottom, 96-well microplate. We calculated absorbance using a microplate reader (300 nm, BioTek Instruments, Winooski, VT, USA) initially and after 12 h of incubation at 37 °C. Percent bacteria killed was calculated as the mean absorbance for each of the samples, which were run in triplicate, divided by the mean absorbance for the positive control (triplicate wells containing only media and bacteria), and multiplied by 100.

2.4. Statistical analysis

We first used a repeated measures analysis of variance (rmANOVA) to examine the effect of maternal treatment (access to water during gravidity or not) and incubation condition (optimal or desiccating) on egg mass change over the five time periods (i.e., mass changes between days 27, 28, 32, 35, and 39 post-oviposition). We ensured our data met the assumptions for parametric testing and had non-significant sphericity. We tested for three-way interactions using treatment, condition, and time as fixed effects and individual identity as a random effect.

To test the effect of maternal treatment, incubation condition, and their interaction on immune function, we next ran a two-factor analysis of variance (ANOVA) on parametric embryonic immune scores. We used maternal treatment and incubation condition as fixed effects and individual embryo nested within maternal clutch as a random effect. A Tukey's honest significant difference (HSD) post hoc test was used when interaction terms were insignificant to determine which of the groups were significantly different. All statistical analyses were completed in R with the packages "nlme" and "multcomp" (Hothorn et al., 2008; Pinheiro et al., 2018; R Core Team, 2018). Significance was set at $\alpha = 0.05$.

3. Results and discussion

In oviparous species, the experience of the embryo as it develops outside the mother is strongly influenced by environmental factors (Deeming, 2004; Furness et al., 2015). Stimuli such as light (Archer et al., 2009), oxygen (Stahlschmidt and DeNardo, 2009), and temperature (Bull, 1980) have been shown to have direct physiological effects on the embryonic life-stage. Other key factors, such as water balance, have been explored (Li et al., 2017; Robbins and Warner, 2010); however, ours is the first study to examine the impact water balance, as a result of environmental conditions, has on the embryo directly.

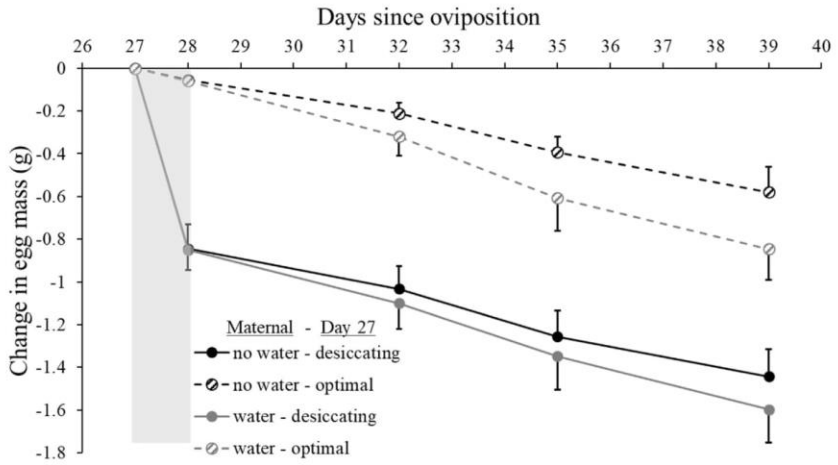

Fig. 1. Average mass change (g) of eggs from *Antaresia childreni* with water or no water from ovulation to oviposition. At Day 27 of incubation, eggs were held for 24 h (shaded grey area) in either optimal or desiccating incubation conditions. Error bars represent ± 1 SEM. Asterisk represents a significant difference in mass loss from Day 27 to Day 28 between eggs held under optimal conditions vs. those held under desiccating conditions ($P < 0.05$).

3.1. Egg mass and yolk osmolality

At oviposition, we found that, on average, water-deprived females produced lighter eggs (total clutch mass divided by number of eggs) than did water-provided females (9.5 ± 0.4 versus 11.4 ± 0.6 g, respectively, $F_{(1,16)} = 6.74$, $P = 0.02$). However, at Day 27 maternal water treatment had no effect on the mass of eggs that were used in this study ($F_{(1,16)} = 0.23$, $P = 0.22$). Instead, short-term exposure to desiccating incubation conditions had a strong influence on egg mass (Fig. 1), and over the 24 h desiccation period, mass loss was significantly greater in the desiccation condition eggs ($F_{1,16} = 111.51$, $P < 0.01$). For the remainder of the experiment (Days 28–39 post-oviposition), eggs, regardless of maternal treatment or incubation condition, had similar mass loss rates (all $P > 0.05$; Fig. 1), thus maintaining the mass difference established during the 24 h treatment period.

Contrary to what we predicted, Day 39 embryos from mothers deprived of water throughout gravidity did not have elevated plasma osmolality values relative to embryos from mothers with *ad libitum* access to water during the same period (Fig. 2A). We did not detect a significant treatment-condition interaction ($P > 0.05$) or a main effect of maternal treatment ($F_{(1,16)} = 0.25$, $P = 0.62$); however, there was a significant main effect of incubation condition ($F_{(1,16)} = 28.13$, $P < 0.01$). An HSD post hoc test revealed that osmolality was higher in eggs that were held under desiccating conditions on Day 27 ($P < 0.05$; Fig. 2). As with egg mass, this suggests that short-term exposure to desiccating conditions not only reduced egg mass but chronically elevated embryonic osmolality. Interestingly, while water-deprived females produced lighter eggs at oviposition, it appears that eggs from dehydrated mothers corrected any hydric imbalance by taking up environmental moisture after oviposition, as has been documented in other, relatively thin-shelled reptile eggs (Booth and Yu, 2009; Tang et al., 2018; Zhao et al., 2013). In contrast, eggs that lost mass due to the desiccating conditions imposed on them at Day 27 failed to regain mass after returning to optimal conditions.

3.2. Yolk immune metrics

We found that some features of innate immunity were enhanced in dehydrated embryos. These immune-enhancing responses to dehydration are similar to previous studies in adult Children's pythons (Brusch et al., 2017) and other reptiles (Brusch and DeNardo, 2017; Moeller et al., 2013). Immune function in newborns and juveniles has conventionally been thought to be underdeveloped (Apanius, 1998). However, recent evidence suggests that adaptive immune responses are critical to newborn survival (Arriero et al., 2013; King et al., 2010; Palacios et al., 2009). Our results add to a growing body of evidence that immune function develops early in ontogeny and includes both innate and adaptive responses.

When evaluating the effect of maternal treatment and incubation condition on immune scores of Day 39 embryos we did not find any significant main effect of maternal treatment or interaction (all $P > 0.05$). However, there was a significant main effect of condition where *E. coli* killing ($F_{(1,16)} = 10.13$, $P = 0.01$; Fig. 2B) and *S. enterica* killing ($F_{(1,16)} = 10.53$, $P = 0.01$; Fig. 2C) were significantly greater in embryos with higher plasma osmolality due to being held in desiccating conditions for 24 h on Day 27. However, we did not detect significant interaction or main effects to suggest a similar relationship between osmolality and either lysis or agglutination scores ($P > 0.05$; Supplementary Fig. 1). The lysis and agglutination assays are used to evaluate the efficacy of natural antibodies (NAbs) and complement proteins (Matson et al., 2005), while the BKA measures multiple effector molecules such as complement, acute phase proteins, and antimicrobial peptides (French and Neuman-Lee, 2012). Adult Children's pythons have elevated agglutination and lytic abilities when dehydrated (Brusch et al., 2017). However, our results suggest that, while present in embryos, complement and NAbs are not affected by increased osmolality in embryos. Future studies should explore if maternal antibodies are passively transferred to eggs which may help explain some of the discrepancies between adult and embryonic immune responses to dehydration.

Previous studies have shown that increased plasma concentrations of immune molecules are not responsible for dehydration-based immune enhancement, suggesting an upregulation by some unknown mechanism is responsible (Brusch et al., 2017; Moeller et al., 2013). Because our BKA scores closely resembled results from adults of multiple squamate species facing similar osmotic challenges (Brusch and DeNardo, 2017; Brusch et al., 2017; Moeller et al., 2013) it is clear that

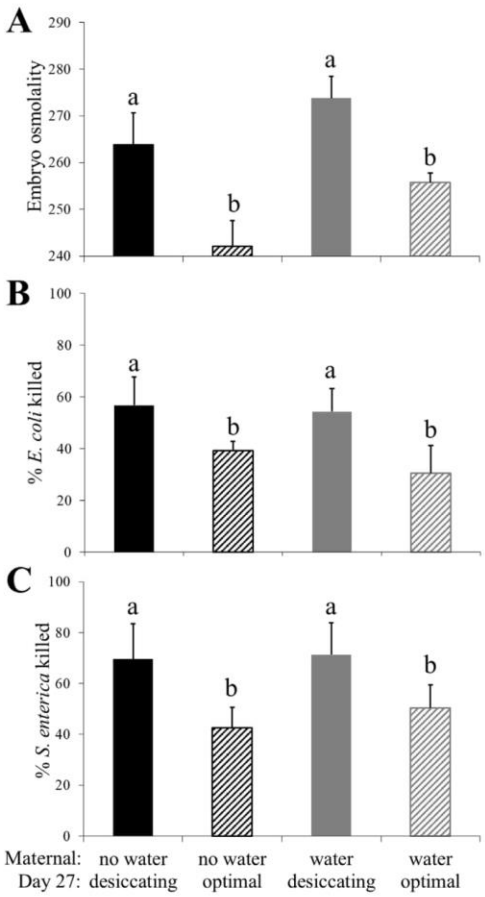

Fig. 2. Average embryonic fluid osmolality (A, mOsm/kg) and immune scores (B, *Escherichia coli* and C, *Salmonella enterica* bacterial-killing assay) measured in Day 39 embryos from *Antaresia childreni* with water or no water from ovulation to oviposition. At Day 27 of incubation, eggs were held for 24 h in either optimal or desiccating incubation conditions. There were no significant effect of maternal treatment or interactions ($P > 0.05$) detected on osmolality; however, there was a significant ($P < 0.05$) main effect of incubation condition on osmolality, *E. coli*, and *S. enterica* scores. Error bars represent ± 1 SEM. Groups that share the same letter did not have statistically significant differences in their means (HSD post hoc test).

python embryos are capable of elevating production of at least some proteins associated with innate immunity. Human fetal livers begin to synthesize innate immune proteins as early as 8 weeks after conception (Colten, 1972); however, much less is known about immune development in oviparous species, and we suggest future research explore the ability of hepatic cells to synthesize innate effector molecules throughout egg incubation. We exposed embryos to desiccating conditions during late-stage development, so it is unclear as to whether this immune response to desiccation occurs throughout most of development. Future research should investigate changes over developmental time in both the ability to correct for water imbalances and the ability to mount an immune response to water loss.

4. Conclusions

Our results provide the first evidence that embryos respond to dehydration in a manner similar to what has been documented in adults. That is, at least some metrics of innate immunity are enhanced when the organism is dehydrated. It is important to understand how organisms cope with scarce resources throughout multiple life-stages, including the embryonic stage. Recognizing how animals cope with resource restrictions, especially water, will enable us to better predict how they might be affected by anticipated climate change scenarios, where, in many xeric environments, rainfall events are predicted to be less reliable, resulting in extended and perhaps more severe droughts.

Data accessibility

The datasets supporting this article can be accessed at https://doi.org/10.6084/m9.figshare.6847550.v1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2018.09.013.

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APPENDIX F

PERMISSIONS

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