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

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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.

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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 ($\pm 3$ 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 ($F_{1,58} =$

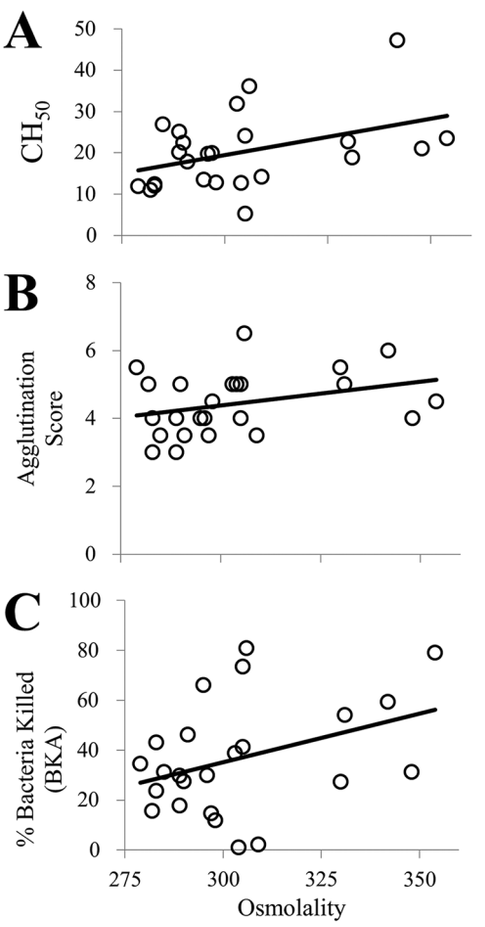


Figure 1. Positive relationship between osmolality (mOsm $kg^{-1}$) and lysis ($CH_{50}$; $P = 0.04$; *A*), agglutination ($P = 0.12$; *B*), and *Escherichia coli* bacterial-killing assay (BKA; $P = 0.07$; *C*) scores in wild-caught *Antaresia childreni* ($n = 24$). Circles represent individual animals.

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 ($P > 0.05$).

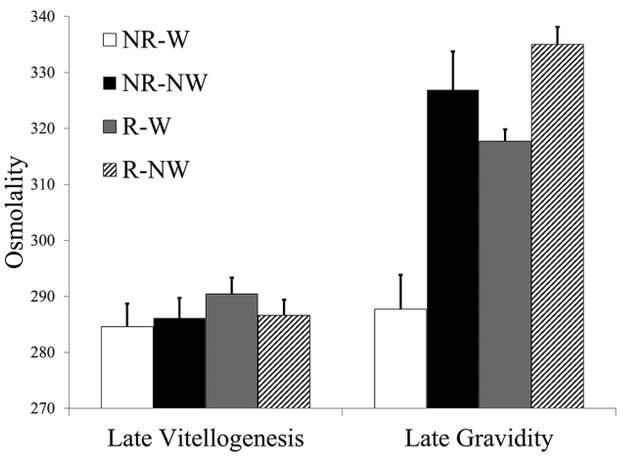


Figure 2. Average plasma osmolality (mOsm $kg^{-1}$) measured in nonreproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water during most of the duration of gravidity or its equivalent for nonreproductive yoked females. Plasma samples were collected 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.

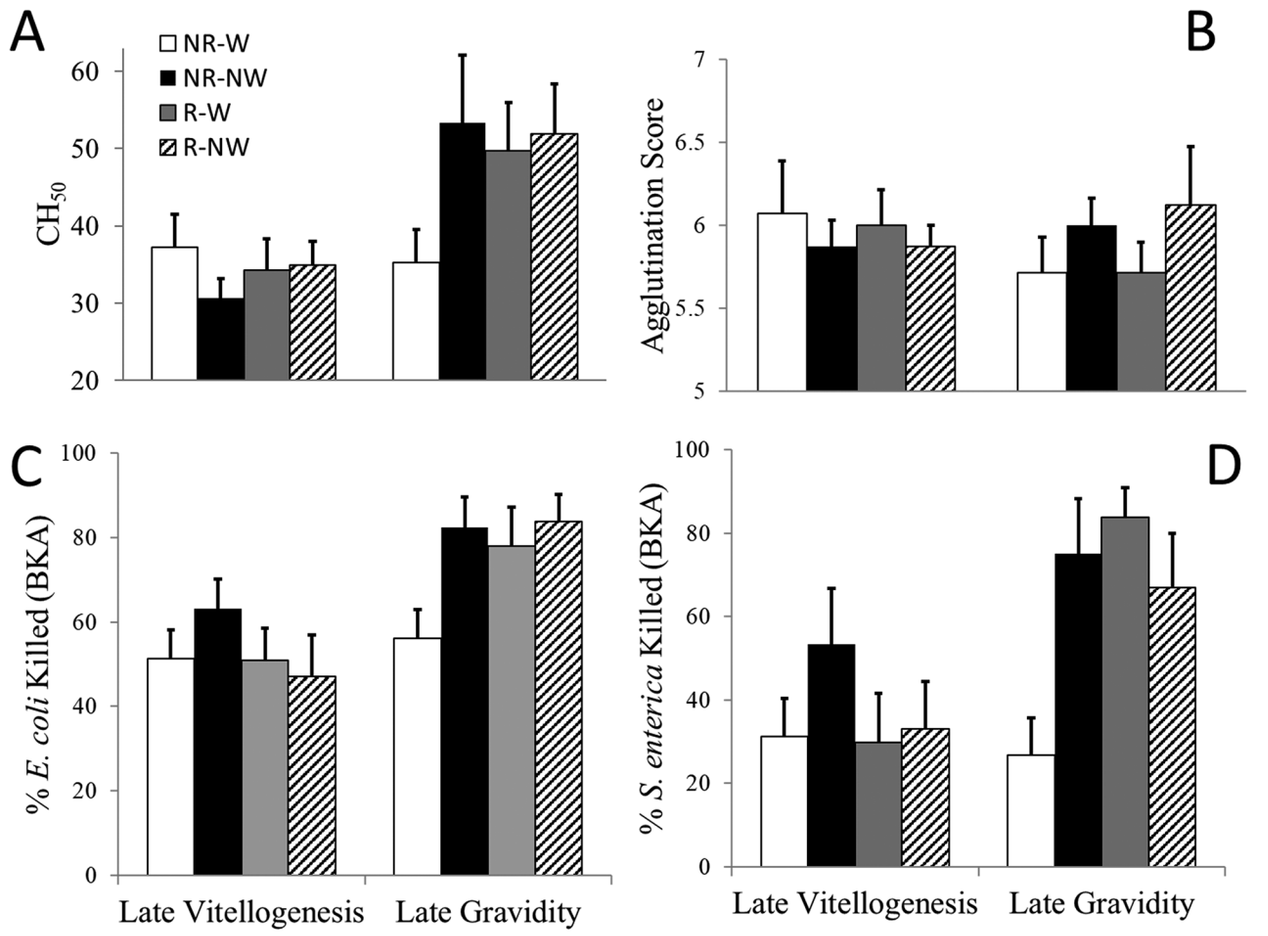


Figure 3. Average immune scores (*A*, lysis [$CH_{50}$]; *B*, agglutination; *C*, *Escherichia coli* bacterial-killing assay [BKA]; *D*, *Salmonella enterica* BKA) measured in nonreproductive (NR) and reproductive (R) female *Antaresia childreni* with (W) or without (NW) access to water during most of 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.

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