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Short communication

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

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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,

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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.

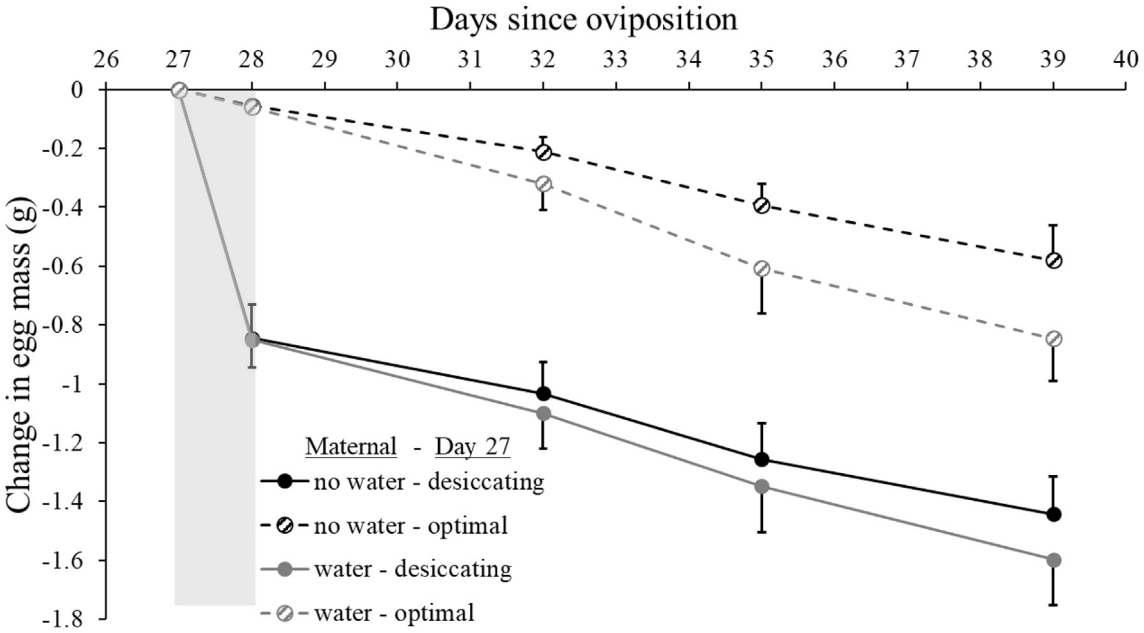

Fig. 1. Average mass change (g) of eggs from *Antaresia childreni* with water or no water from ovulation to oviposition. At Day 27 of incubation, eggs were held for 24 h (shaded grey area) in either optimal or desiccating incubation conditions. Error bars represent ± 1 SEM. Asterisk represents a significant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

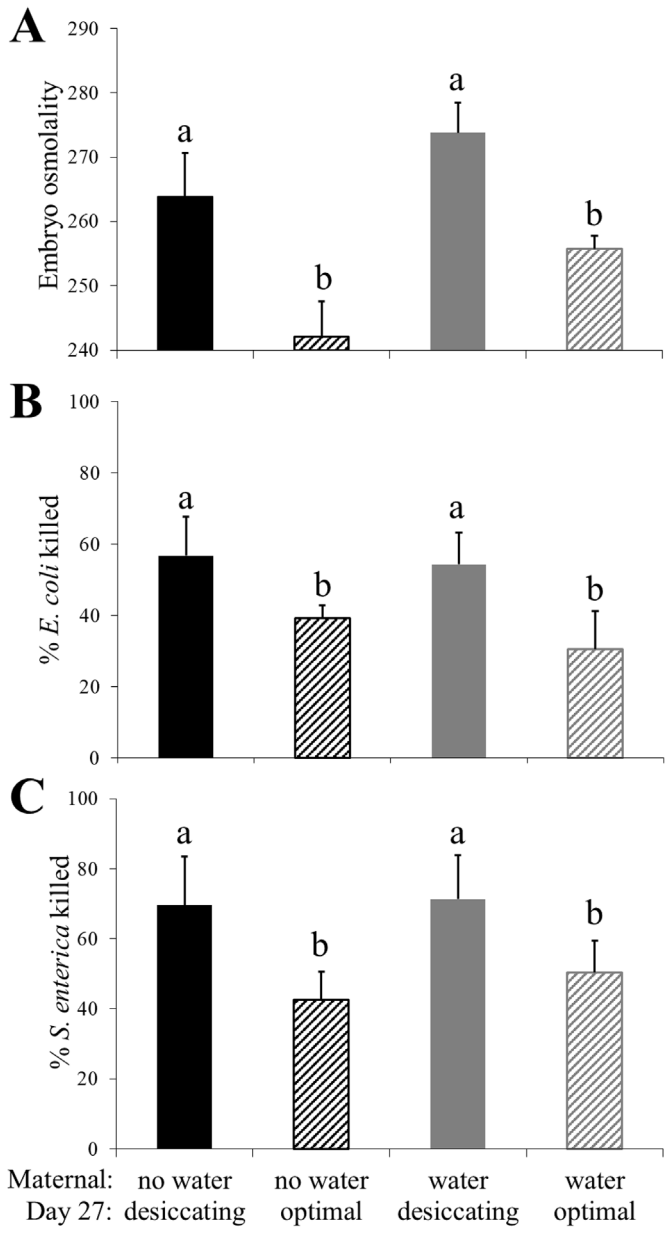

Fig. 2. Average embryonic fluid osmolality (A, mOsm/kg) and immune scores (B, *Escherichia coli* and C, *Salmonella enterica* bacterial-killing assay) measured in Day 39 embryos from *Antaresia childreni* with water or no water from ovulation to oviposition. At Day 27 of incubation, eggs were held for 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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