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Dehydration during egg production alters egg composition and yolk immune function

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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.

Keywords: Hydration**;** Immunocompetence; Innate immunity; Maternal-offspring conflict; Osmotic challenge

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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 Tadif, 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

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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 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,b, 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).

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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 x 71 x 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,

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which is the preferred temperature of gravid Children's pythons (Lourdais et al., 2008), with a 12L: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 ºC. Concurrently, an egg from each clutch was placed on a scale at room temperature (~25 ºC) 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

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dish, re-weighed, and placed in an isothermal chamber at 60 ºC. 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:

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

2.3 Yolk Preparation

Using slight modifications to the water dilution methods described in Akita & Nakai (1993) and Kim & 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 ºC for 2 h, after which they were centrifuged at 10,000 g for 2 h at 4 ºC. 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

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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 ºC 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 ºC. 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

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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* ($F_{1,17}$

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= 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).

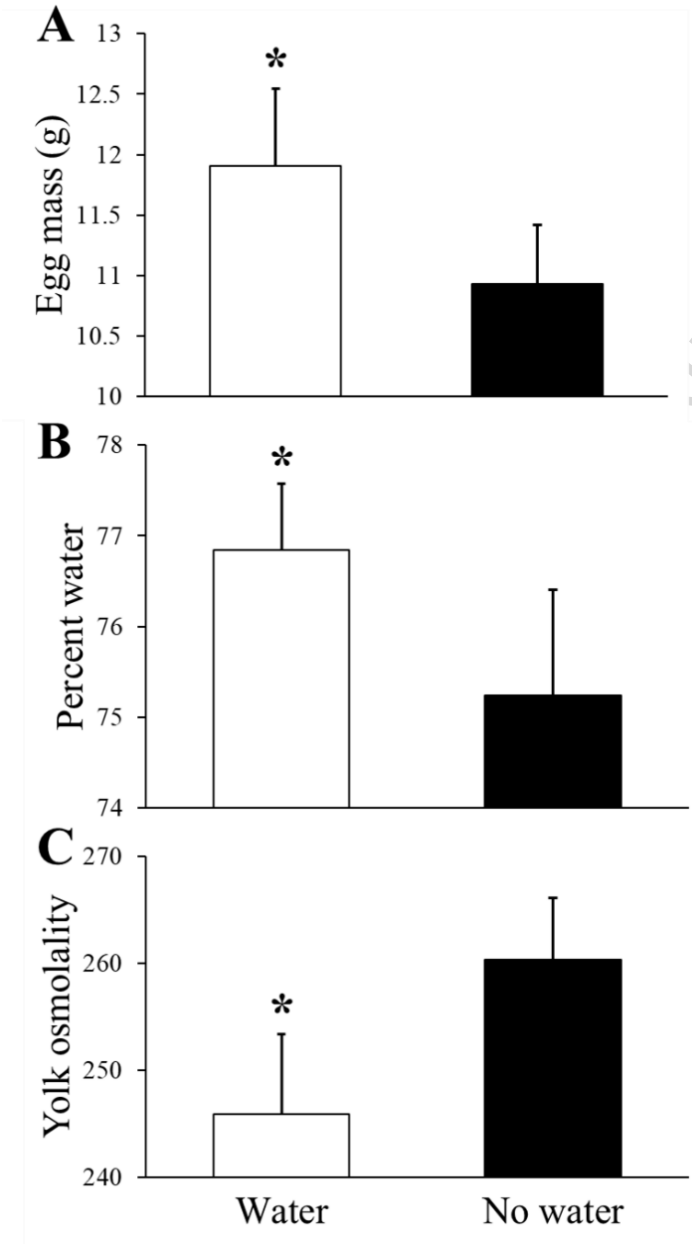


Fig. 1. Average (A, egg mass; B, percent water; C, yolk osmolality [mOsm kg^{-1}]) measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access

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to water throughout gravidity. Error bars represent ±1 SEM. Asterisk represents a significant difference between treatment groups.

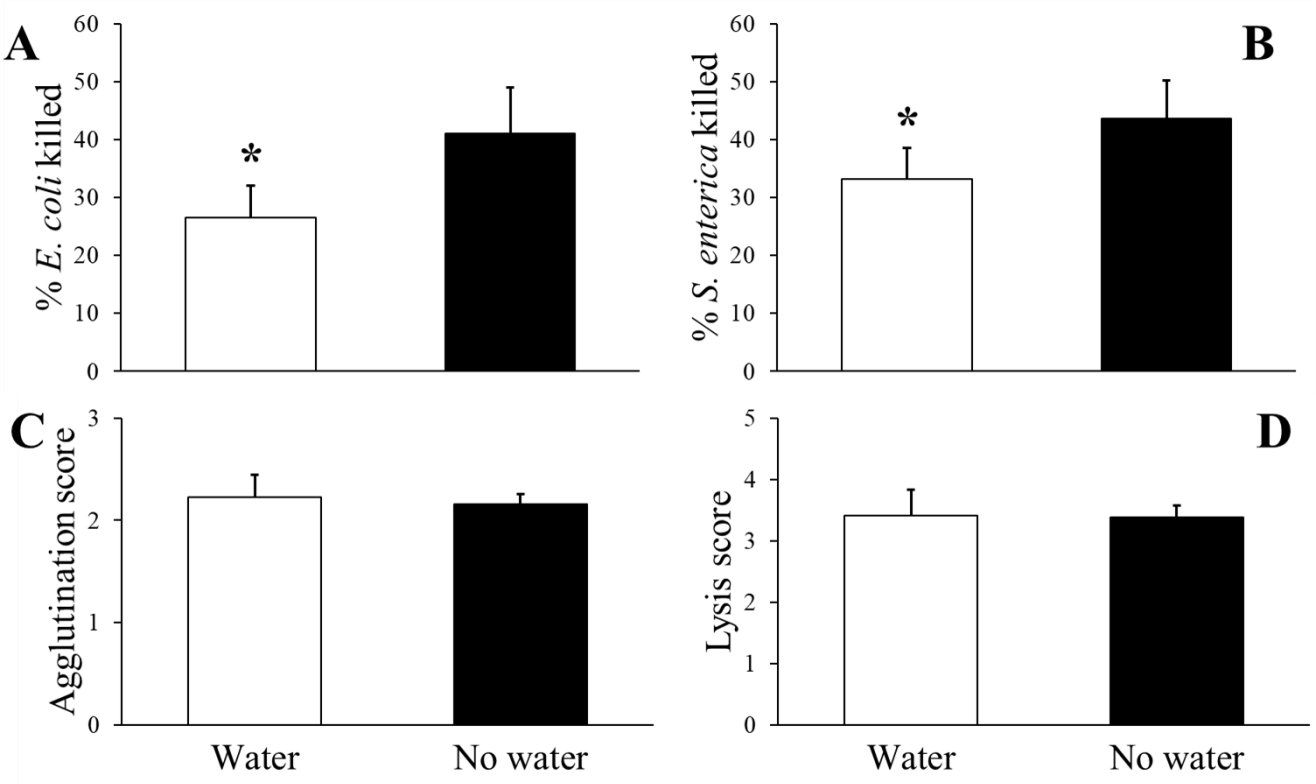


Fig. 2. Average yolk immune scores (A, *Escherichia coli* bacterial-killing; B, *Salmonella enterica* bacterial-killing; C, agglutination; D, lysis) measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access to water throughout gravidity. Error bars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

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

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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 $p > 0.05$).

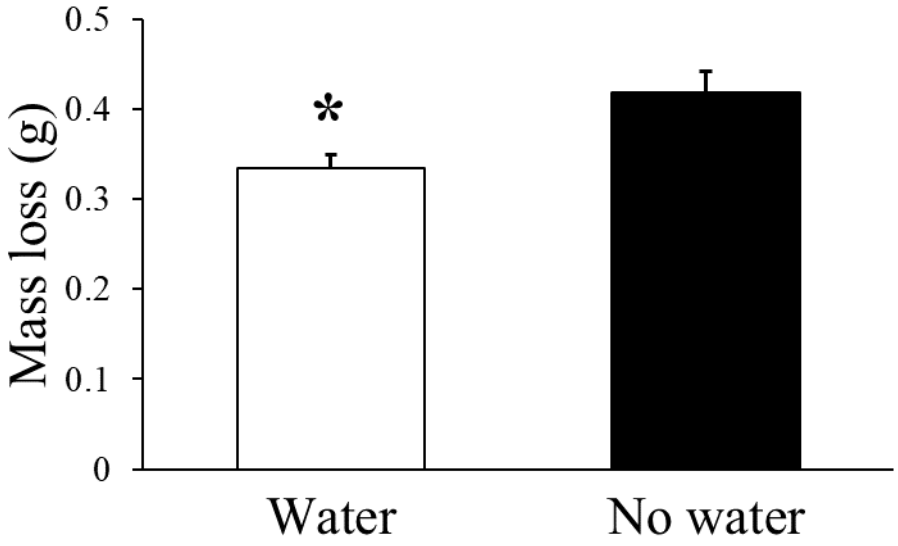


Fig. 3. Average egg mass loss after 1 h in desiccating conditions measured in Children's python (*Antaresia childreni*) eggs at oviposition from females with or without access to water throughout gravidity. Error bars represent ±1 SEM. Asterisk represents a significant difference between treatment groups.

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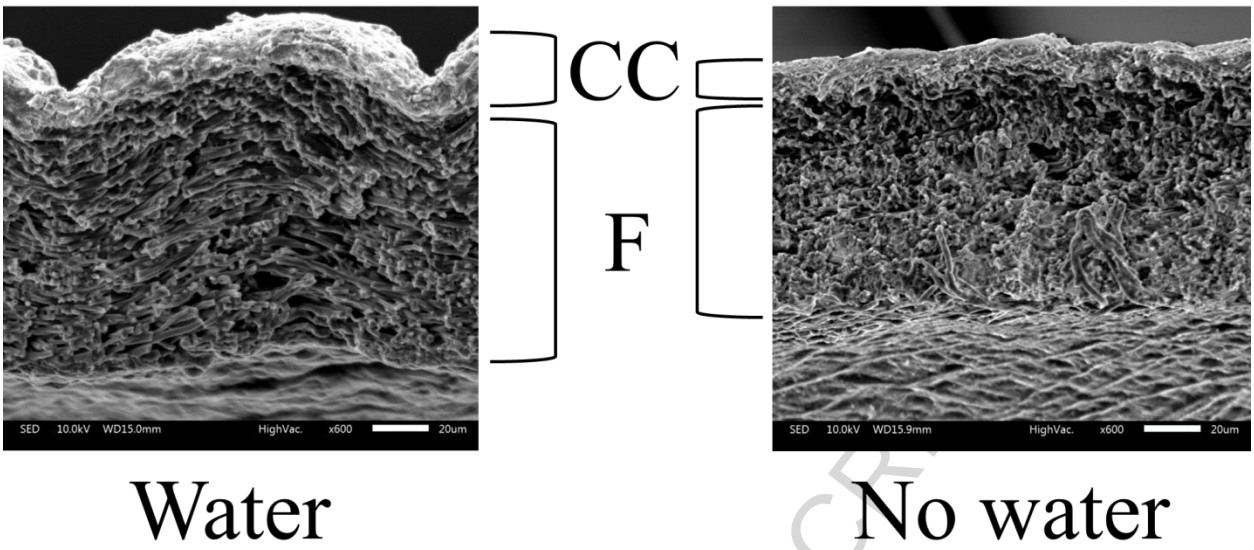


Fig. 4. Scanning electron micrograph image of Children's python (*Antaresia childreni*) eggshells at oviposition from females with or without water throughout gravidity. Cross sections were used to measure calcareous crust (CC), fibrous layer (F), and total thickness (CC + F) over 20 measurements.

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

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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 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

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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 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

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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.

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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.

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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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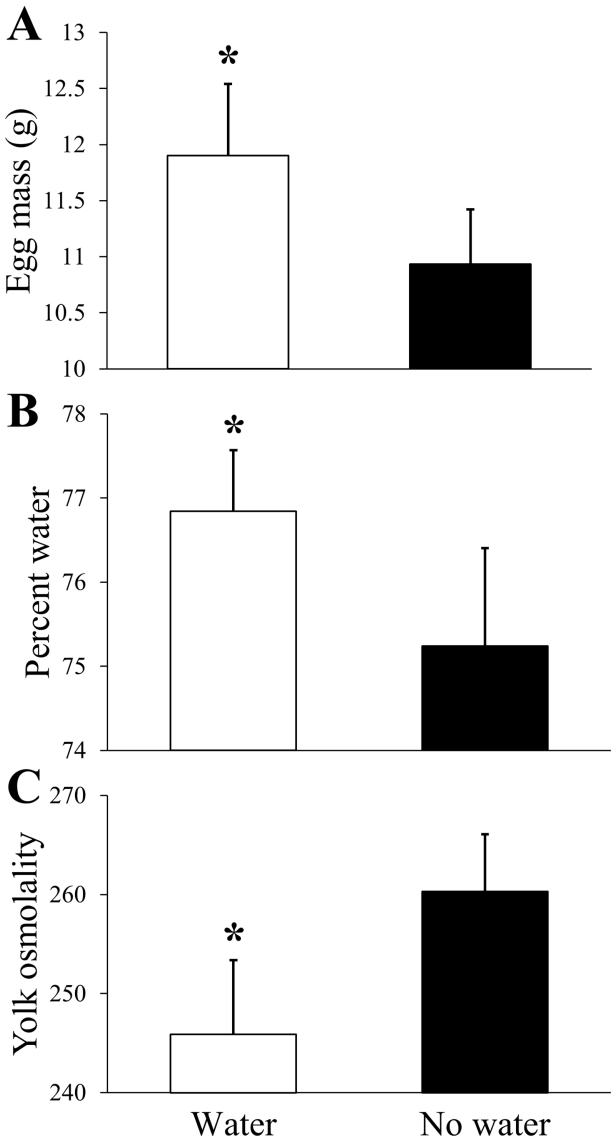

A
Egg mass (g)
13
12.5
12
11.5
11
10.5
10
*
B
Percent water
78
77
76
75
74
*
C
Yolk osmolality
270
260
250
240
*
Water
No water

Figure 1

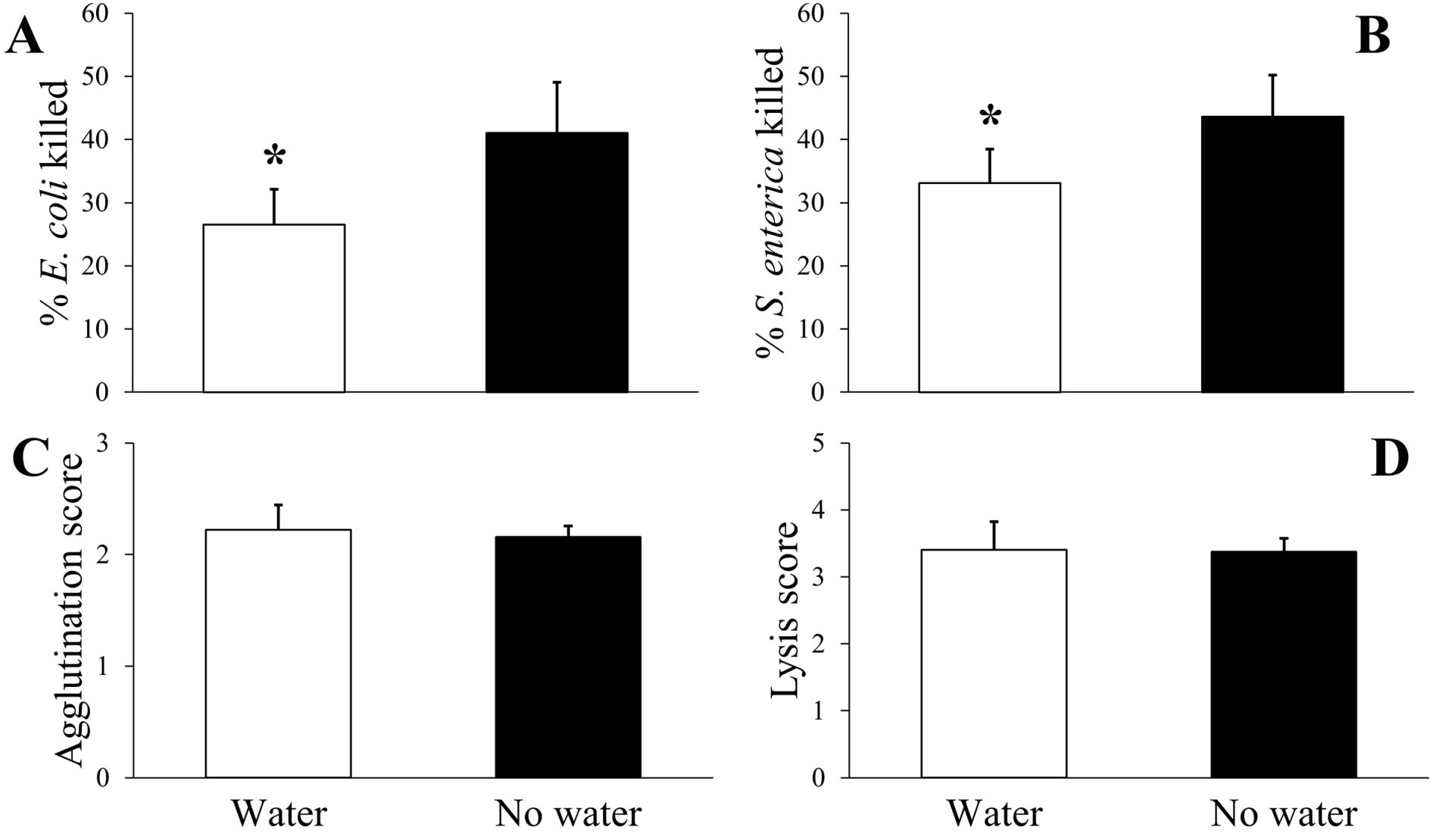

A
% E. coli killed
60
50
40
30
20
10
0
*
B
% S. enterica killed
60
50
40
30
20
10
0
*
C
Agglutination score
3
2
1
0
Water
No water
D
Lysis score
5
4
3
2
1
0
Water
No water

Figure 2

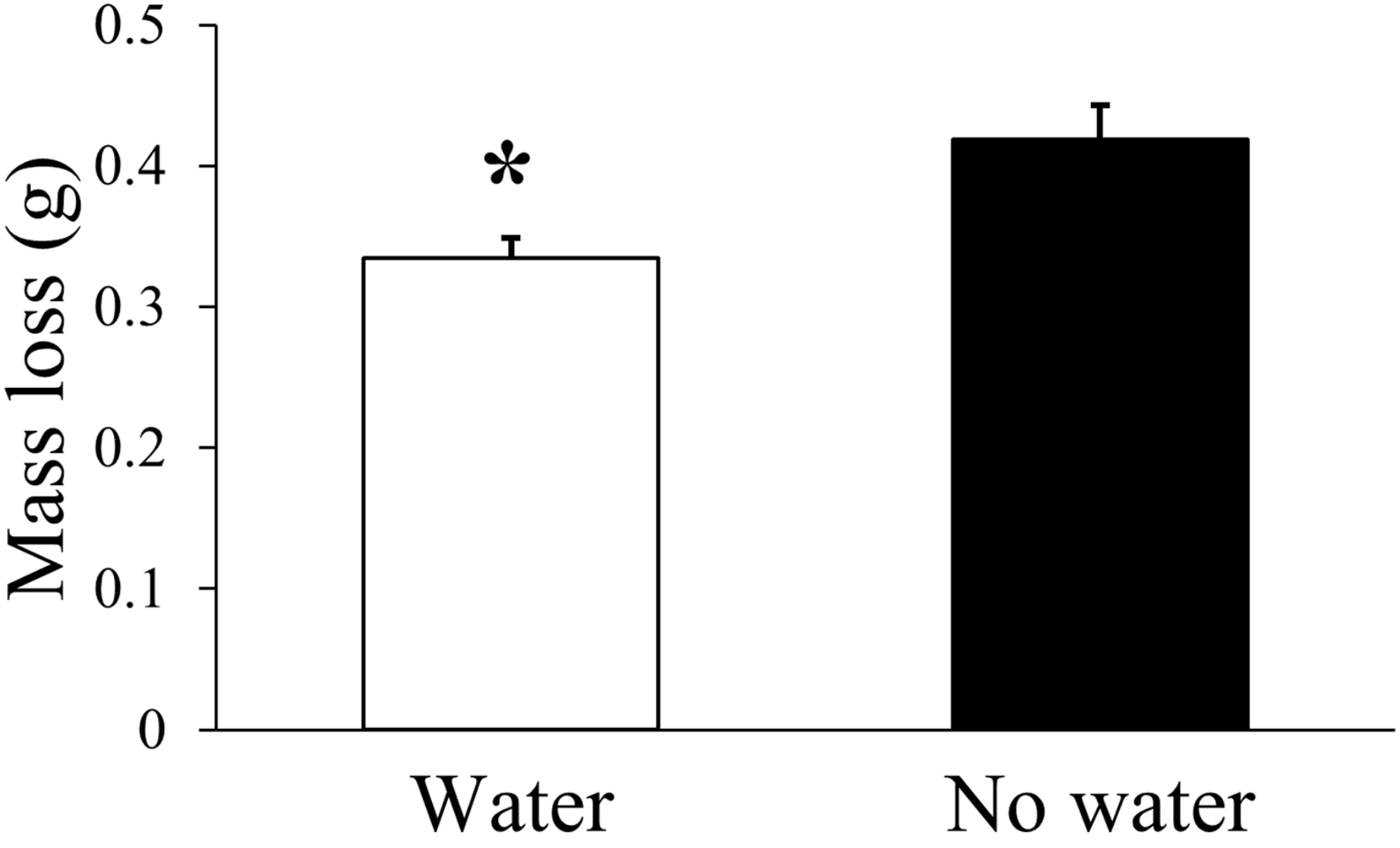


Figure 3

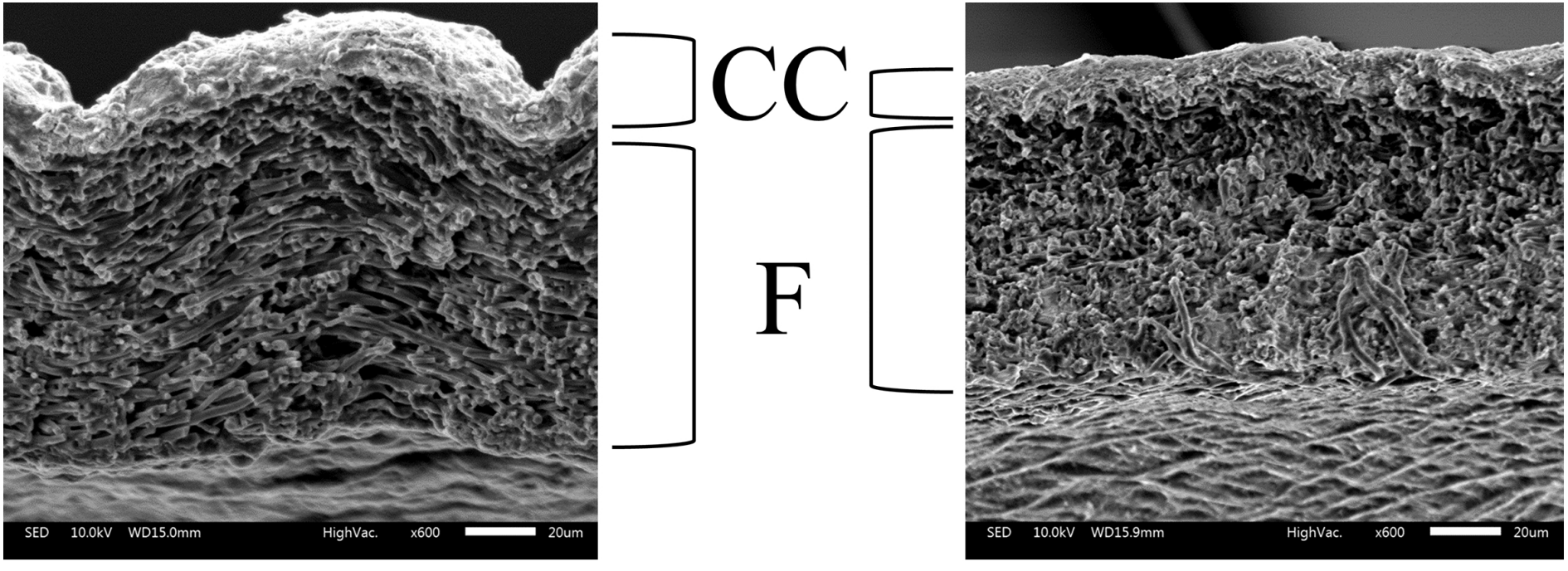

CC
F
SED 10.0kV WD15.0mm HighVac. x600 20um
SED 10.0kV WD15.9mm HighVac. x600 20um
Water
No water

Figure 4