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Immunological Tradeoffs and the Impacts of Urbanization on the Reproductive Ecology and Physiology of the Side-Blotched Lizard (*Uta stansburiana*)

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IMMUNOLOGICAL TRADEOFFS AND THE IMPACTS OF URBANIZATION ON THE REPRODUCTIVE ECOLOGY AND PHYSIOLOGY OF THE SIDE-BLOTCHED LIZARD (UTA STANSBURIANA)

by

Emily E. Virgin

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology and Ecology

Approved:

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ABSTRACT

Immunological tradeoffs and the impacts of urbanization on the reproductive ecology and physiology of the Side-blotched Lizard (*Uta stansburiana*)

by

Emily E. Virgin, Doctor of Philosophy

Utah State University, 2022

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Department: Biology

Life history theory posits that organisms have optimized energetic allocation strategies into different life history components to maximize reproductive fitness. However, anthropogenic disturbance can alter life history strategies by exerting different selective pressures on a species. Urban and rural Side blotched lizard (*Uta stansburiana*) populations in southern Utah exhibit diverging life history strategies, such that urban lizards have lower survival rates and maximize reproductive investment relative to their rural counterparts; but it is unclear whether these changes in egg and clutch size translate to differences in yolk physiological composition. To assess the physiological costs of urbanization and its effects on maternal investment, I performed three distinct research studies. First, I characterized baseline differences in female immune function and oxidative stress and related these metrics to clutch size, total clutch mass, and egg yolk immune function and oxidative stress. Immune function and oxidative stress were higher in females with larger clutches, and increased as overall clutch mass increased, suggesting physiological costs of reproduction can vary depending on clutch size. In my second research chapter, I investigated the metabolic, immune, and oxidative costs of reproduction prior to and following an immune challenge in females at different stages in vitellogenesis. I found metabolic rate to be highest at the onset of vitellogenesis (peak yolk investment) and lowest just prior to ovulation. Early- and mid-stage females mounted significantly higher immune and metabolic responses and lower oxidative responses than control females. However, there were no significant differences in immune, metabolic, or oxidative responses in late-stage females, suggesting they were energetically constrained. In my final chapter, I combine research approaches from previous chapters and investigate the impacts of urbanization on female immunity and oxidative stress with respect to reproductive investment and egg yolk physiology. I also tested whether simulating infection alters what physiological factors are invested in the egg yolk. Female immunity increased and egg yolk immunity decreased with maternal ectoparasite burden, but only at rural sites. At urban sites, female and egg yolk immune function did not covary with ectoparasite load, suggesting maternal investment is consistent despite a significant immunological burden. Finally, a simulated infection altered egg yolk oxidative damage, and urban lizards laid a significantly higher proportion of unfertilized eggs, highlighting a potential cost of urbanization.

(173 pages)

PUBLIC ABSTRACT

Immunological tradeoffs and the impacts of urbanization on the reproductive ecology and physiology of the Side-blotched Lizard (*Uta stansburiana*)

Emily E. Virgin

Investing resources into reproduction can limit energy available to other competing demands, such as fighting off an infection; yet, both processes are necessary for organisms to survive and pass on their genes to the next generation. These strategies often follow patterns associated with lifespan, such that shorter-lived animals are more likely to invest more resources into reproduction over survival, and vice versa in long-lived animals. However, environmental change caused by urbanization can disrupt these relationships, and the within- and transgenerational costs of urbanization on females and offspring are unknown. I address these uncertainties in three research chapters to better understand the effects of urbanization on reproductive investment in female Side-blotched Lizards (*Uta stansburiana*), a small and abundant species of reptile found throughout the western United States. In my second chapter, I examine general variation in female immunity and stress and how it relates to egg number, egg mass, and egg yolk immunity and stress. I found oxidative stress and immunity vary in females depending on how many eggs they produced, and that larger eggs had lower yolk stress levels. I built upon this by examining how metabolism differs across the reproductive cycle and tested whether simulating an infection in females affected immunity, stress, and metabolism. Metabolism was higher at the onset of reproduction and decreased until ovulation, and

females differentially responded to infection depending on their stage in the reproductive cycle, which may suggest limited resources underly these findings. In my final chapter, I investigated the impacts of urbanization on female and egg yolk physiology and tested whether simulating infection altered female investment into egg yolk. I found immunity and stress in females and eggs were only apparent in rural females with ectoparasites, but not in urban females and eggs. Fertilization rates were lower in urban populations, which also influenced egg yolk physiology. Differential physiological investment can drastically alter offspring traits; therefore, it is imperative to develop a better understanding of the transgenerational costs of inhabiting an urban environment.

For Zoey and Klaus

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CHAPTER 1 – INTRODUCTION

Tradeoffs between Reproduction and Immunity

A central tenet in life history theory is that organisms must invest finite energetic resources into multiple competing life history traits, which may result in a negative association between two or more components if resources are limited (Stearns 1992). Therefore, optimal allocation of resources to life history traits, such as reproduction or maintenance, have likely evolved to follow patterns associated with lifespan to maximize reproductive fitness (Williams, 1966). For example, long-lived species are predicted to invest more resources into survival and maintenance than reproductive output within a given breeding event due to future reproductive opportunities (Stearns, 1989; Williams, 1966), whereas short-lived species are predicted to maximize investment into reproduction over maintenance and survival. These strategies, however, are at the extremities of the life history continuum, while in reality organisms' investment strategies can fall anywhere in between these extreme examples (Cucherousset et al., 2005).

Experimental studies have demonstrated that energetic constraints often underly tradeoffs between different life history components (Cox et al., 2022; French et al., 2007a; French et al., 2007b) and share the same resource pools (Durso and French, 2018). For example, ovariectomized female brown anole lizards (*Anolis sagrei*) were in better body condition, had more fat stores, and had higher survival prospects than females given a sham surgery (Cox et al., 2010). Immunocompetence, or the maintenance of a well-functioning immune system, has emerged as a potential life history trait constraining reproduction (Nordling et al., 1998; Sheldon and Verhulst, 1996). Mounting an immune response is energetically costly, and as a result, can reduce resources allocated toward concurrent life history demands (Demas et al., 2012; Lochmiller and Deerenberg, 2000). For example, experimental elevation of reproductive investment negatively influenced immune function in Ornate tree lizards (*Urosaurus ornatus*) and vice versa, demonstrating a bidirectionality of this relationship in resource tradeoffs (French et al., 2007a; French et al., 2007b). Although tradeoffs associated with reproductive investment and immune function are well-documented, resource constraints may also vary within a reproductive event and subsequently cause variation in physiological tradeoffs.

Vitellogenesis, or the hepatic synthesis of yolk lipids and proteins to be deposited into developing follicles, incurs a significant energetic burden during reproduction in reptiles; however, energy expenditure is significantly reduced after vitellogenesis ends and ovulation begins (Van Dyke and Beaupre, 2011). A decrease in energy expenditure following vitellogenesis either suggests that the energetic requirements of ovulation and oviposition are lower than vitellogenesis, or that resource constraints following yolk investment may result in an attenuated response. Pregnant pygmy rattlesnakes (*Sistrurus miliarius*) exhibited a hindered immune response relative to non-pregnant females and males (Lind et al., 2020), highlighting a tradeoff between reproduction and immunocompetence across different reproductive stages. However, this was not explicitly tested across vitellogenesis, and therefore represents an interesting continuation of my dissertation research.

Maternal Effects

A parental effect is defined as the phenotypic adjustment of offspring traits through non-genetic mechanisms, which can involve epigenetic inheritance (Crean and Immler, 2021; Weaver et al., 2004), changes in maternal physiology and investment during prenatal development (Schwabl, 1996; Sinervo, 1990), and/or postnatal offspring investment via parental care and provisioning (Mousseau and Fox, 1998; Weber et al., 2018). Parental effects were previously considered inconvenient variation in scientific studies; however, environmentally mediated changes in maternal physiology during reproduction can significantly affect offspring phenotypes (Eising et al., 2006). Differential allocation of hormones, immune factors, and nutrients via the placenta or yolk can cause long-standing changes in offspring growth and size (Hutchings, 1991; Schwabl, 1996), stress-reactivity (Ensminger et al., 2018; Haussmann et al., 2012), immune function (Bowers et al., 2015; Henriksen et al., 2013), and subsequent survival (Hao et al., 2021; Khan et al., 2016). Therefore, physiological maternal effects have received considerable attention in life history evolution and ecological research due to the potential to influence population dynamics and evolutionary outcomes (Bian et al., 2015; Mousseau et al., 2009; Wolf and Wade, 2009).

Urbanization and Maternal Effects

In a rapidly changing world, organisms are currently, and will continue to be, tasked with surviving and reproducing under novel environmental conditions (Angelier and Wingfield, 2013). For instance, urbanization can alter resource use and availability

(Larson et al., 2020; Seress and Liker, 2015; Williams et al., 2006), predation risk (Fischer et al., 2012), wildlife disease dynamics and health (Bradley and Altizer, 2007; Cohen et al., 2022), and species richness and composition (Fischer et al., 2012; McKinney, 2006; McKinney, 2008). While urbanization is inherently harmful for many species (French et al., 2018; Kaiser et al., 2015), urbanization can act as an environmental buffer and extend the length of breeding seasons for some species (Leveau, 2018; Leveau and Leveau, 2016). As a result, urbanization has altered life-history tradeoffs (Partecke and Gwinner, 2007), such as survival probability (Ibáñez-Álamo and Soler, 2010; Lucas and French, 2012; Sepp et al., 2018) and reproductive investment (Lucas and French, 2012; Sepp et al., 2018; Seress et al., 2020).

Maternal physiological state is highly sensitive to environmental perturbations and the resulting change in physiological exposure or investment can modify offspring phenotypes (Meylan et al., 2012). In the context of anthropogenic change, maternal effects may confer an advantage to offspring in fluctuating environments (Lind et al., 2020; Marshall and Uller, 2007; Meylan et al., 2012; Shama et al., 2014; Sinervo et al., 2018). For example, early seasonal warming has altered breeding phenology and lay dates of blue tits (*Cyanistes caeruleus*) to mirror caterpillar emergence for feeding offspring (Visser et al., 2006). Some species of lizards may be able to adequately respond to climate change and warming by altering thermoregulatory behavior and corticosterone deposition during vitellogenesis (Hao et al., 2021; Paranjpe et al., 2013). Differences in precipitation may impact gas exchange and water use efficiency in progeny of *Eucalyptus* trees (Vivas et al., 2019). Hence, both maternal effects and transgenerational plasticity may represent key mechanisms allowing for the persistence of species despite anthropogenic change.

The Side-blotched Lizard as a model

Understanding the physiological costs of urbanization and its effects on maternal investment is crucial in predicting the persistence of urban dwelling species. The Side-blotched Lizard (*Uta stansburiana*) is a small, abundant, phrynosomatid lizard species commonly found in the western United States (Tinkle, 1967). Females can lay between one to three clutches a year, although this can vary considerably depending on latitude and annual rainfall (Smith et al., 2019; Tinkle, 1967). Side-blotched Lizard populations occupying urban and rural areas in southern Utah (Washington County, UT, USA) exhibit differences in life history strategy, such that urban lizards in proximity to St. George, Utah, have lower survival rates and maximize reproductive investment via larger eggs and overall clutch sizes (Lucas and French, 2012). In contrast, rural populations approximately twenty miles outside of St. George have higher survival prospects and exhibit lower reproductive investment (Lucas and French, 2012). Urbanization is evidently altering reproductive investment strategies in Side-blotched Lizard populations, but it is unclear whether these changes in egg and clutch size translate to differences in yolk physiological composition.

Documented differences in life history strategies combined with a distribution across an urban landscape, makes urban and rural Side-blotched Lizard populations an excellent study system to investigate (1) changes in physiology and egg yolk investment following experimental manipulation of the reproductive and immune systems and (2) urban and rural differences in reproductive investment, egg yolk physiology, and its covariation with maternal physiology. Because Side-blotched Lizards are oviparous, they are ideal models to study the effects of urbanization on life history tradeoffs and maternal investment. Deposition of resources into the egg occurs in a short, discrete period and is easily measured via egg contents (Warner and Lovern, 2014), although recent work has demonstrated that steroidal hormones and toxins pass across the eggshell within the oviduct (Ensminger et al., 2018; Johnston, 2009). Moreover, in comparison to avian species, we are able to quantify and assess prenatal investment in oviparous lizards without having to account for variation via postnatal parental investment.

Objectives

In three research chapters, I address this gap in knowledge of the impacts of urbanization on reproductive and immune system tradeoffs and the transgenerational impacts on egg quality. I utilize both observational and experimental laboratory studies to gain a better understanding of (1) the physiological costs of reproduction in reptiles within and across vitellogenesis, (2) the relationship between maternal and egg yolk physiology, and (3) how inhabiting an urban environment impacts these relationships.

Chapter 2 – Immunological and oxidative costs of reproduction and associations between maternal and egg yolk physiology in a reptile

In this chapter, I assessed existing variation in both female Side-blotched Lizard and egg yolk physiology. I examined how reproductive investment (clutch size and mass) influenced maternal physiology, whether these metrics covaried with egg yolk physiology, and determined factors influencing variation in egg yolk physiology within and across clutches. I found that females with smaller clutches had higher immune function and oxidative status than females with larger clutch sizes, and that maternal immune and oxidative status increased as clutch mass increased, regardless of clutch size. Finally, there was no relationship between maternal and egg yolk physiology, but yolk oxidative status decreased as egg mass increased.

Chapter 3 – Baseline differences in metabolic rate and the physiological responses to an immune challenge vary by vitellogenic stage in an oviparous reptile

In my third chapter, I assessed baseline differences in metabolic rate across different stages of vitellogenesis in female Side-blotched Lizards and tested whether the physiological costs of an immune response differ across vitellogenic stages following an immune challenge. I found that baseline metabolic rate was highest in early-stage vitellogenesis and lowest in late-stages vitellogenesis. Following an immune challenge, I found that early- and mid-stage females overall mounted significantly higher immune and metabolic responses and lower oxidative responses than control females. However, there were no significant differences in immune, metabolic, or oxidative responses in late-stage females, suggesting they were constrained and unable to mount an immune response.

Chapter 4 – Immunological tradeoffs, ectoparasites, and the impacts of urbanization on yolk corticosterone, immune function, oxidative stress, and fertilization in Side-blotched Lizard eggs

In my fourth chapter, I combined research approaches from my first two chapters and conducted two separate experiments. First, I compared urban and rural differences in maternal and egg yolk physiology and their interactions with egg mass, viability, and maternal parasite load. Second, I tested the effects of an immune challenge on maternal physiology and maternal investment of egg yolk physiology in females from urban populations. I found that urban female Side-blotched Lizards had higher ectoparasite loads than rural females, but only correlated with egg yolk immunity from rural females. Interestingly, urban females laid a higher proportion of unfertilized eggs, which had higher yolk immune function, corticosterone, and triglyceride levels than fertilized eggs. Finally, I found that immune challenged females had higher immune function and laid eggs with lower yolk oxidative damage markers, which supported previous findings in these populations. These results help us better understand potential downstream impacts of urbanization on offspring survival, fitness, and overall population health.

Chapter 5 – Conclusions

In the concluding chapter, I discuss the potential implications of my research findings. Because urban lizards exhibit higher reproductive investment than rural lizards, I investigated whether documented differences in life-history strategies impacted tradeoffs between reproduction and immunity and extended to differences in egg yolk physiological investment. In my first chapter, I determined that Side-blotched Lizard egg yolk physiology varied considerably within and across clutches, and with respect to maternal physiology and investment. In my second chapter, I determined that energetic constraints associated with ovulation and oviposition may impact the ability of female

Side-blotched Lizards to physiologically respond to an immune challenge. Finally, in my final chapter, observed differences in life-history strategies and environmental stressors between urban and rural Side-blotched Lizards may extend to egg quality and egg yolk physiology. Overall, these findings expand our understanding of the physiological costs of reproduction in reptiles and lay a foundation for future research investigating the transgenerational costs of urbanization.

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CHAPTER 2 – IMMUNOLOGICAL AND OXIDATIVE COSTS OF REPRODUCTION AND ASSOCIATIONS BETWEEN MATERNAL AND EGG YOLK PHYSIOLOGY IN A REPTILE [1]

Abstract

Energetic constraints caused by reproductive investment may incur immunological and oxidative costs to a female and potentially to her developing offspring. Oviparous reptiles can alter offspring phenotype through differential allocation of physiological factors during vitellogenesis (yolk formation), depending upon the external environment and the physiological state of the female. Despite its importance in protecting developing offspring against infection, maternal transfer of immune factors has rarely been studied in reptiles. Furthermore, the extent to which offspring may be impacted by the inherent oxidative costs of vitellogenesis is not well understood. Using the Side-blotched Lizard (*Uta stansburiana*) as a model, we examined how reproductive investment (clutch size and mass) influenced maternal immunological (BKA) and oxidative state (ROMs) and whether these metrics covaried with egg yolk immunity and oxidative state. We assessed factors influencing variation in egg yolk BKA and ROMs within and across clutches from all females and found females with smaller clutches had higher BKA and ROMs than females with larger clutches. There was also an overall positive relationship between maternal BKA and ROMs and clutch mass, regardless of clutch size. We did not find maternal and egg yolk BKA and ROMs to be directly related, but yolk ROMs decreased as egg mass increased. Within-clutch variation in yolk BKA

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and ROMs was not related to maternal investment, but variation among clutches was explained by maternal identity. Because egg yolk physiology can impose long-lasting or permanent changes to offspring, these results create a foundation for future work investigating factors contributing to yolk physiology and highlight the necessity of considering multiple physiological and ecological variables when conducting eco-immunological research.

Introduction

The maternal phenotype and environment are both known to have profound effects on offspring development. Such effects may extend into adulthood and impact subsequent generations. Maternal traits, such as body size, age, (Paitz et al., 2007; Valenzuela, 2001), and behavior (Hepp et al., 2006; Schwabl, 1996) can result in lasting effects on offspring survival and fitness. These maternal effects are potential mechanisms by which mothers can modulate the phenotype of offspring through non-genetic means (Bernardo, 1996a; Mousseau and Fox, 1998). In oviparous species, traditional studies have used egg size and egg number as a proxy for maternal effects or maternal investment (Bernardo, 1996b). However, egg size and number do not quantify egg quality. For example, maternal physiological state during gestation, lactation or egg/yolk formation can influence offspring phenotypes by altering the allocation of hormones (Gasparini et al., 2007; Groothuis and Schwabl, 2008), immune factors (Brusch et al., 2019; Gasparini et al., 2007; Merrill and Grindstaff, 2014), and nutrients (Warner and Lovern, 2014). Because maternal physiology during reproduction can variably affect offspring of other oviparous taxa, the extent egg physiology is impacted by maternal condition in reptiles should be explored.

Among the traits of potential importance for females and developing eggs is oxidative status, or the balance between reactive oxygen species and antioxidants (Sies, 1997). Work in reptiles have shown that oxidative status and its relationship with other physiological metrics differ between breeding and non-breeding females (Webb et al., 2019; French et al., 2021). While transient changes in oxidative status are generally innocuous, maternal oxidative damage markers were associated with clutch size (Webb et al., 2019; Lister et al., 2016) and offspring oxidative status (López-Arrabé et al., 2018; Ruuskanen et al., 2020; Giordano et al., 2015). However, some studies have found no relationship between maternal and offspring oxidative status, whatsoever (Taylor et al., 2015). Nonetheless, an association between maternal and offspring oxidative status could be mediated via egg yolk provisioning. Indeed, developing embryos within an egg rely on lipids from egg yolk for growth and nutrition. However, these biomolecules are susceptible to oxidation (Speake et al., 1998) and can affect the viability and performance of the resulting offspring (Rosa et al., 2017). Because studies comparing maternal oxidative state and egg quality yield mixed results, it is unclear how maternal oxidative status might influence egg yolk oxidative status.

Maternal transfer of immunological history via antibodies, immune proteins, and epigenetic modification has recently been recognized as an important mediator of offspring protection (Grindstaff, 2008; Hasselquist and Nilsson, 2009; Merrill and Grindstaff, 2014; Roth et al., 2018). Extensive research in avian species has demonstrated maternal transfer of immunological protection via a relationship between maternal antibodies in circulation and antibody content in egg yolk (Gasparini et al., 2002; Ramos et al., 2014) and resulting offspring (Ramos et al., 2014). However, immunological provisioning to the egg and/or offspring has not been studied as extensively in reptiles (Roth et al., 2018). Similar to birds, reptiles can provision IgY to egg yolk (Hassl, 2005; Warr et al., 1995) and pathogen-specific antibodies to confer protection during growth and development (Schumacher et al., 1999). Further, it appears innate immune components are also present in reptile egg yolk and can be influenced by maternal physiological state. Children's pythons (*Antaresia childreni*) undergoing a dehydration treatment produced eggs with higher innate immune function (Brusch et al., 2019). Although similar elevated immune responses are found in dehydrated, reproductive females (Brusch et al., 2017), it is not clear whether these changes are passed directly to the egg.

While it is clear that both the environment and physiological state of a mother impact the allocation of physiological factors to egg yolks and thus, offspring traits, direct comparisons of maternal immunological and oxidative state to egg yolk characteristics are lacking. In the current study, we investigated how reproductive investment influenced maternal immune and oxidative state, how it covaried with egg yolk immune and oxidative state, and whether maternal factors influenced variation in the aforementioned yolk metrics within and across clutches. Using the Side-blotched Lizard (*Uta stansburiana*) as a model, we (1) determined maternal characteristics (clutch size and mass) significantly contributing to maternal immune and oxidative state; (2) tested the relationship between maternal and individual egg yolk immune and oxidative state; and (3) tested the impact of maternal identification and/or maternal characteristics (mentioned above) on variation in egg and clutch-level immune and oxidative state. We hypothesized maternal immune and oxidative state would be influenced by maternal investment in the form of clutch size and clutch mass. As a cost of increased reproductive investment, we predicted females with larger clutches would have lower immune function and higher markers of oxidative stress. Alternatively, females with larger clutches may have higher immune function and lower oxidative stress if they optimized reproductive investment to minimize the potential physiological costs of egg production. As a function of passive transfer, we hypothesized maternal immune and oxidative states would covary directly with egg yolk innate immunity and oxidative state. We also hypothesized maternal identity would significantly explain variation in egg yolk and clutch physiology. This three-pronged approach furthers our understanding of maternal/eco-immune interactions in an understudied taxon.

Materials and methods

Field Sampling

The Side-blotched Lizard (*Uta stansburiana*) is a small, desert-dwelling lizard found commonly throughout the western and southwestern United States (Tinkle, 1967). Vitellogenic females can be found from March to August in populations of *Uta stansburiana* in southwestern Utah (pers. obs). Although clutch sizes vary considerably across latitudes (Smith et al., 2019), female *U. stansburiana* in southwestern Utah have clutches ranging from 2 to 6 eggs (pers. obs), with an average clutch size between 2.56 (± 0.154 SEM) and 4.82 (± 0.134 SEM) depending on drought conditions (Smith et al., 2019). We collected female Side-blotched Lizards (n=13) between middle to late-stage vitellogenesis via lassoing in Washington County, Utah, USA, during peak breeding season between May 8-10, 2017. Given females in middle- and late-stage vitellogenesis are actively investing resources into their eggs (Durso and French, 2018; Pettit et al., 2019), we determined obtaining blood samples immediately upon capture would best reflect a female's current physiological status, her eggs, and would not be influenced by handling and laboratory housing. We collected blood within three minutes to control for stress-related physiological changes. Blood samples were stored on ice until plasma separation. At the end of each sampling day, we measured mass (2.33 g ± 0.56 SEM), snout-vent-length (SVL) (45.15 mm ± 0.317 SEM), extracted blood plasma, and determined vitellogenic stage via an ultrasound of follicles or eggs (clutch size: 3.75 ± 0.074). Upon completion of sampling and data collection, we kept the captured females in cloth bags in a cool, shaded area and stored plasma samples in a -20 °C freezer until they were transported to Utah State University (USU).

Laboratory housing

We housed females in climate-controlled environmental chambers at 60% relative humidity and a 12L:12D photoperiod at 36 ± 1°C for the light period and 20 ± 1°C for the dark period at USU from May 14-29, 2017. Females were housed individually in plastic tubs (48 x 18 x 23 cm) on racks containing a hide, a water dish, and an inch of moist vermiculite for substrate. We fed females between 0.10 and 0.15 grams of small (1/4"), calcium-dusted crickets (Fluker Farms) and recorded the amount (g) consumed every other day. After females laid their eggs, they were fed ad libitum and their resource intake was no longer recorded. All procedures described below were permitted under Utah State Department of Wildlife Resources (COR #1COLL8382) and were approved by the USU Institutional Animal Care and Use Committee (protocol #2068). At the end of the study, we released the females back at their locations of capture.

Egg collection and extraction

Oviposition occurred between May 18-29, 2017, during which we checked for eggs twice a day and immediately extracted them upon discovery. We could not determine laying order due to logistical constraints and concern associated with adding unneeded stress during oviposition. Once collected, we measured length (11.47 mm ± 0.23 SE), width (6.13 mm ± 0.14 SEM), and mass (0.25 g ± 0.01 SEM) of eggs using calipers and an electronic scale. Eggs were then rinsed in deionized (DI) water, 1% bleach solution, and then rinsed again in DI water in a sterile weigh-boat to reduce chances of bacterial contamination of the egg sample for later bactericidal assay (see below). Eggs were poked with a sterile 30G PrecisionGlide™ needle and the contents of the eggs were squeezed out into a sterile, 2.0 mL Eppendorf tube with a pre-recorded mass. We determined the amount of yolk extracted by taking the difference between total mass and Eppendorf tube mass. To account for differences in yolk volumes, we suspended egg yolk samples in phosphate buffered saline (PBS) at a 2:5 dilution factor to control for differences in extracted yolk volume within and across clutches. This dilution factor was validated for bactericidal and oxidative stress assays prior to this study. After extraction and dilution, we stored egg samples in a -80°C freezer until later use in oxidative stress and bactericidal assays.

Bactericidal assay for plasma and egg samples

Following validated protocol for this species (French and Neuman-Lee, 2012; Lucas and French, 2012), we performed a bactericidal assay using *Escherichia coli* (ATCC #8739; Microbiologics, St. Cloud, MN) on female plasma samples and on eggs collected from those females. Immune defense toward this strain involves phagocytes, opsonizing proteins, and natural antibodies (French and Neuman-Lee, 2012). For female plasma samples, we added 6 ul of plasma to 14 ul of CO_2-dependent media. We plated 20 ul and 24 ul of CO_2-independent media (PBS) to the positive and negative controls, respectively, and then added 4 ul of 10^4 working *E. coli* solution to the positive controls and the plasma samples. After vortexing for one minute, we incubated the plate at 37°C for 30 minutes. Following, we added 125 ul tryptic agar soy broth in each well, vortexed for 1 minute, then measured background absorbance at 300 nm using a microplate reader (BioRad xMark spectrophotometer). We incubated the plate at 37°C for 12 hours and again measured the absorbance at 300 nm. To calculate microbicidal capacity, we subtracted the background absorbance from post-incubation absorbance, and calculated % of bacteria killed by subtracting net absorbance from 1 and dividing sample absorbance by absorbance of the positive control. We ran each female's plasma sample in duplicate and took the average of both samples to calculate % bacteria killed. In one instance, we removed a value because it was the same value as the negative control, leading us to believe *E. coli* was not added. All female samples were assayed on one 96-well plate.

We modified the assay for egg samples by using PBS instead of CO_2-dependent media and pipetting 8 ul of yolk sample per well. We validated this volume and media via a serial dilution (in triplicate) of a pooled yolk sample on a round-bottom microplate. We determined the volume at which 50% killing was achieved to capture potential variation in egg yolk bactericidal capacity within and across clutches. Because of the high volume of egg samples, we ran this assay in triplicate for each sample, and triplicates were averaged for final calculation. We subtracted the background absorbance from the final absorbance to account for sample “cloudiness” due to the high concentration of lipids in yolk samples. If one replicate significantly differed from the other two it was excluded from the average. Intra-assay CV of the positive controls for female BKA was 5.28%, and between 5.51% and 6.93% for egg BKA. Inter-assay CV for egg BKA was 11.02%.

d-ROMS Assay for plasma and egg samples

To detect markers of oxidative damage, we measured reactive oxygen metabolites (ROMs) using the d-ROMS kit (Diacron, Grosseto, Italy), a colorimetric assay which indirectly measures organic hydroperoxides in serum or plasma. The presence of organic hydroperoxides indicates lipid or protein molecules were damaged by free radicals (Costantini, 2016). We measured ROMs in egg yolk because it is primarily comprised of lipids and proteins, which are highly susceptible to oxidation (Speake et al., 1998). We performed a validation via serially diluting yolk samples to assure the assay was quantifying ROMs in a linear fashion. We quantified ROMs of female *U. stansburiana* plasma following validated protocol (Lucas and French, 2012). The associated absorbance of the volume we validated fell within the standard curve of the assay. We followed the “End-point Mode” mode protocol under the manufacturer’s instructions but modified it to work on a 96-well plate. Briefly, we pipetted 5 microliters of maternal plasma or diluted egg sample singly into a 96-well plate along with 100 ul of the provided acidic buffered solution. After a 90-minute incubation at 37°C, we read the plate at 505 nm and calculated the amount of hydroperoxides in CARR U (Carretelli Units) by dividing the sample absorbance by the calibrator absorbance and multiplying by the calibrator concentration. We converted CARR U to mg H_2O_2/dL by multiplying CARR U by 0.08. Intra-assay variation based on the calibration standard was 1.16%.

Statistical treatment of data

We constructed linear mixed models for each of four response variables: (i) maternal BKA, (ii) maternal ROMs, (iii) egg BKA, and (iv) egg ROMs. Prior to model construction, we Log_e-transformed values for BKA and ROMs to analyze all response variables under the assumption model error terms and random effects were normally distributed. For models of maternal physiology, we included the number of eggs per clutch (i.e., clutch size) and total clutch mass as fixed effects with individual identity as a random intercept. For models of egg physiology, we included clutch size, egg mass, and maternal BKA or ROMs as fixed effects with clutch identity as a random intercept. We did not include lay date as a fixed effect because (1) it was not significant in our models,

(2) females were in similar reproductive stages, and (3) we did not want to over-parameterize our statistical models given our small sample sizes. With the uncertainty of continuous fixed effects being strongly correlated, we conducted model diagnostics to ensure multicollinearity, and inflation in the variances of parameter estimates were not detected (Tolerance > 0.1, VIF < 3). We also visually inspected residual plots for each model and did not find any marked deviations from homoscedasticity or normality. We performed Type III Sums of Squares tests to determine the significance of fixed-effect parameters in each model. Model summary outputs provided us with estimates of beta coefficients with 95% confidence intervals (CI) and p-values for each fixed effect as well as the variance (σ^2) with 95% CI for the random intercept. If fixed effects were significant within a model, we acquired estimated marginal means and trends to compare level differences.

We used log likelihood ratio tests (LRT) based on 100,000 simulated values to assess inter-clutch variance in models of egg BKA and ROMs. Here, we determined whether the variance attributed to the random intercept of clutch identity deviated from zero while controlling for clutch size, egg mass, and maternal BKA or ROMs. We also quantified intra-clutch variation using the variation coefficients associated with each clutch. Variation coefficients for egg BKA and ROMs were calculated as: (standard deviation within clutch / mean within clutch) x 100. This standardized measure of dispersion represents overall variation irrespective of egg characteristics (e.g., laying sequence, relative size). We note that coefficients for a subset of clutches (n = 4) were underrepresented, since we were unable to include 1-2 eggs in sampling. To analyze factors influencing intra-clutch variation, we used general linear models with relative laying date and clutch size as explanatory variables and variation coefficients as response variables.

For all data analysis and visualization, we used R statistical software (version 3.5.1, R Core Team 2018 with the following packages: ‘reshape2’ (version 1.4.3, Wickham 2007, ‘car’ (version 3.0.2, Fox and Weisberg 2019, ‘nlme’ (version 3.1.142, Pinheiro et al. 2007), ‘emmeans’ (version 1.4.7, Lenth et al., 018), ‘RLRsim’ (version 2.0.5; Scheipl and Bolker 010), ‘ggplot2’ (version 3.1.0, Wickham2016), and ‘cowplot’ (version 0.9.3, Wilke 2016.

Results

Summary Statistics

We collected morphometric and physiological data for 44 eggs from 12 females. Means, ranges, and standard errors are reported on the raw values of egg and female characteristics in Table 1.

Table 1. Summary statistics for morphometric and physiological parameters in female Side-blotched Lizards (*Uta stansburiana*) and their eggs.

Variable	**N**	**Mean ± SEM**	**Range**
Maternal SVL (mm)	12	45.25 ± 0.166	43.0 - 47.0
Maternal BKA (%)	12	22.9 ± 0.012	15.05 - 41.93
Maternal ROMs (mg H_2O_2/dL)	8	8.80 ± 0.965	1.87 - 18.27
Clutch Size	44	3.75 ± 0.074	2 - 4
Egg Mass (g)	43	0.248 ± 0.009	0.12 - 0.43
Egg BKA (%)	43	41.3 ± 0.020	18.1 - 68.9
Egg ROMs (mg H_2O_2/dL)	26	12.92 ± 0.41	7.14 - 17.35

Reproductive implications for maternal BKA and ROMs

Lizards with smaller clutch size ($p = 0.0156$; Fig. 1A), but larger clutch mass ($p = 0.0012$; Fig. 1B), exhibited greater BKA. Lizards with smaller clutch sizes ($p = 0.044$; Table 1-2; Fig. 2A), but larger clutch masses ($p = 0.0004$; Fig. 2B), had higher ROMs.

Table 2. Summary table of linear mixed models for physiological responses to clutch size and total clutch mass in female Side-blotched Lizards (*Uta stansburiana*).

Model Term **Fixed Effect**	**Log$_e$ BKA** **β Estimate (95% CI)**	**df**	**F**	**p**	**Log$_e$ ROMs** **β Estimate (95% CI)**	**df**	**F**	**p**
				Maternal Physiology				
Clutch Size	0.49 (0.117 – 0.861)	1,9	8.86	0.0156	0.71 (0.029 – 1.393)	1,5	7.18	0.0439
Total Clutch Mass	1.16 (2.27 – 3.37)	1,9	21.60	0.0012	3.48 (2.44 -4.54)	1,5	73.23	0.0004
Random Effect	σ^2 (95% CI)				σ^2 (95% CI)			
Maternal ID	0.482 (0.245 – 0.945)				0.648 (0.364 – 1.154)			
				Individual Egg Physiology				
Egg Mass	-1.56 (-3.68 - 0.55)	1,29	2.25	0.144	-1.17 (-2.30 -0.04)	1,17	4.76	0.043
Clutch Size	0.013 (-0.37 - 0.41)	1,9	0.005	0.944	-0.071 (-0.18 - 0.33)	1,5	0.52	0.505
Maternal BKA	-0.11 (-1.11 - 0.93)	1,9	0.055	0.819	-	-	-	-
Maternal ROMs	-	-	-	-	-0.073 (-0.40 - 0.26)	1,5	0.32	0.594
Random Effect	σ^2 (95% CI)				σ^2 (95% CI)			
Clutch ID	0.256 (0.140 - 0.470)				0.155 (0.072 - 0.332)			

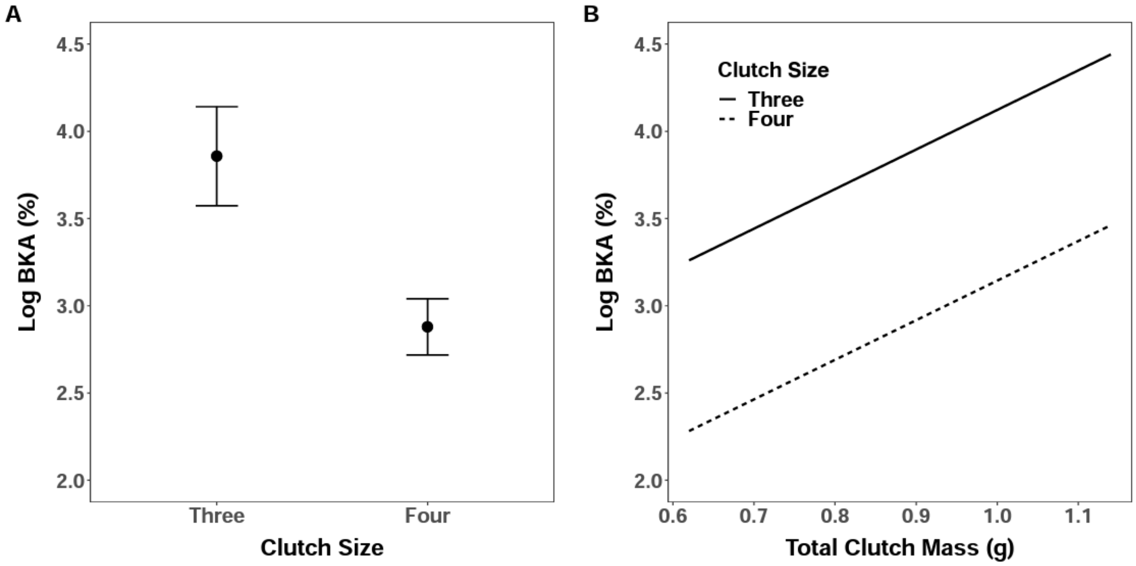

Figure 1. Predicted BKA (%) for female Side-blotched Lizards (*Uta stansburiana*) with respect to their clutch size and total clutch mass.

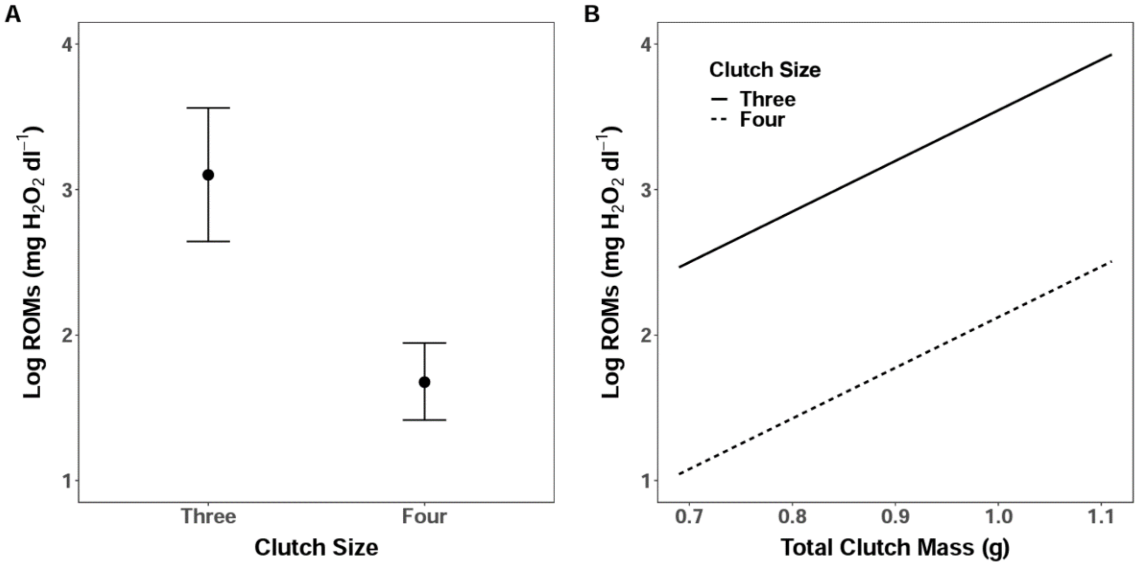

Figure 2. Predicted Log ROMs (mg H2O2/dL) for female Side-blotched Lizards (*Uta stansburiana*) with respect to their clutch size and total clutch mass.

Individual egg BKA and ROMs

We found no relationships between individual egg BKA and maternal BKA ($p = 0.819$) while accounting for egg mass ($p = 0.144$) and clutch size ($p = 0.944$; Table 2). We found individual egg ROMs negatively corresponded with egg mass ($p = 0.042$; Fig. 3), irrespective of clutch size ($p = 0.505$) and maternal ROMs ($p = 0.594$).

Intra-clutch variation in egg BKA and ROMs

We found the variation coefficients for both egg BKA and ROMs to be wide-ranging (Table 3). We did not find intra-clutch variation in egg BKA to be related to total clutch mass ($F_{1,9} = 2.221$, $p = 0.175$), nor to clutch size ($F_{1,8} = 2.374$, $p = 0.162$). Similarly, we found intra-clutch variation in egg ROMs was not related to total clutch mass ($F_{1,8} = 2.854$, $p = 0.135$) nor clutch size ($F_{1,7} = 0.029$, $p = 0.870$). We did not find intra-clutch variation in egg mass to be related to total clutch mass ($F_{1,9} = 3.998$, $p = 0.081$) nor clutch size ($F_{1,8} = 0.368$, $p = 0.561$).

Inter-clutch variation in egg physiology

While accounting for clutch size, egg mass, and maternal BKA, we found inter-clutch variance in egg BKA to be significantly greater than zero ($n_{females} = 12$, $n_{eggs} = 43$, LRT $\chi^2_{0:1} = 7.284$, $p = 0.0006$). Similarly, we found inter-clutch variance for ROMs to be significantly greater than zero ($n_{females} = 8$, $n_{eggs} = 26$, LRT $\chi^2_{0:1} = 5.262$, $p = 0.0014$) when accounting for clutch size, egg mass, and maternal ROMs.

Table 3. Summary statistics for intra-clutch variation coefficients of egg mass, BKA, and ROMs for female Side-blotched Lizards (*Uta stansburiana*).

Variation Coefficient Parameter	**Mean ± SEM**	**Range**
Egg Mass (g)	14.01 ± 2.41	3.226 - 31.353
Egg BKA (%)	22.13 ± 3.13	4.076 - 40.768
Egg ROMs (mg H_2O_2/dL)	13.27 ± 2.22	2.180 - 27.466

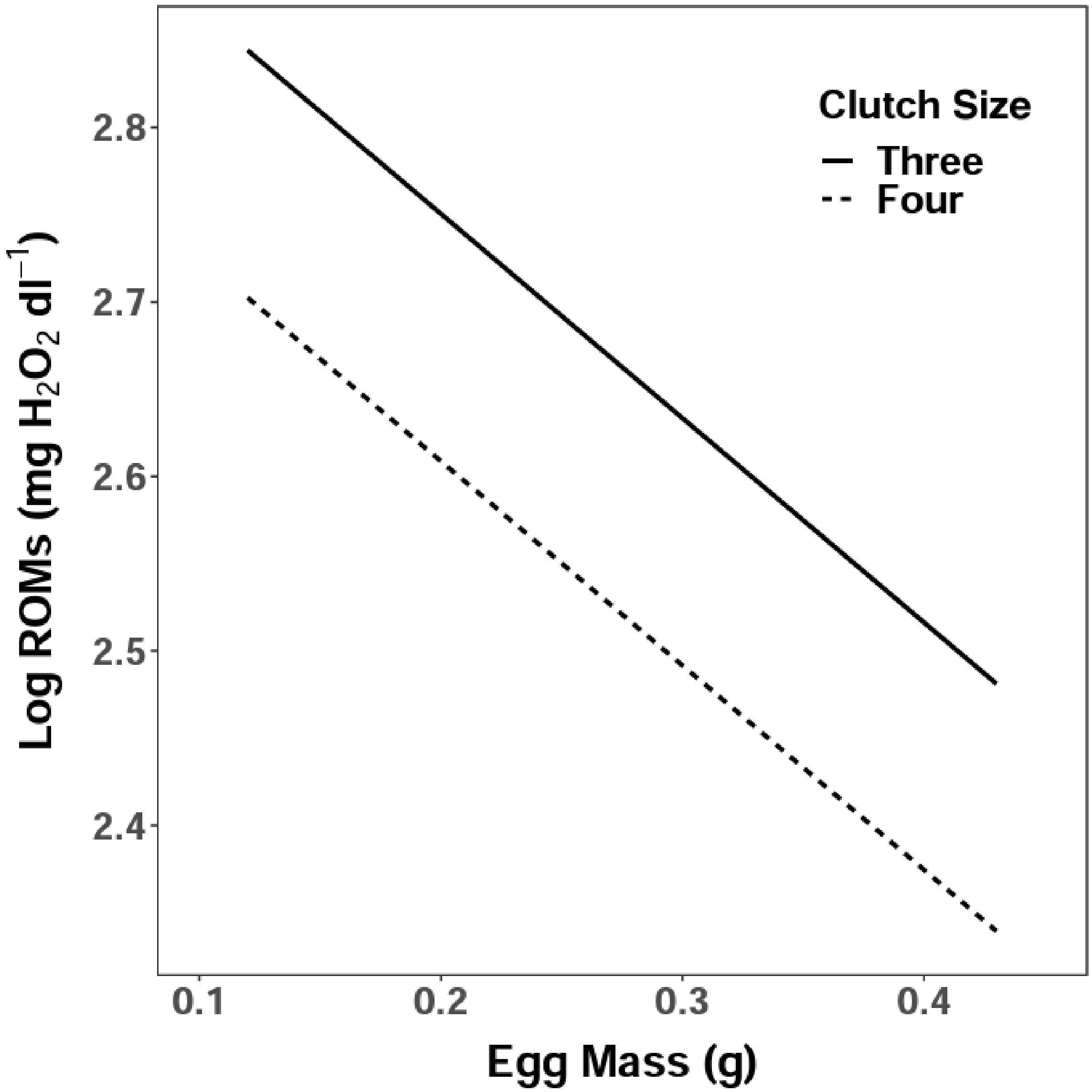

Figure 3. Predicted Log ROMs (mg H2O2/dL) in the egg yolk of female Side-blotched Lizards (*Uta stansburiana*) with respect to egg mass.

Discussion

Summary

To the best of our knowledge, this is the first study in lizards that measures immunological and oxidative indices in egg yolks and compares them to maternal physiological metrics. In doing so, we demonstrated how immunity and oxidative status for vitellogenic lizards can vary in accordance with multiple aspects of reproductive investment (clutch size and mass) prior to oviposition. By considering maternal condition (BKA, ROMs, and SVL), we also reveal the degree of inter-clutch variation that remains for egg physiological measures of BKA and ROMs.

Reproductive implications for maternal BKA and ROMs

We found an effect of reproductive investment (i.e., clutch size and mass) on maternal innate immunity despite a relatively low sample size. While clutch sizes can vary across the reproductive season and with female size (Tinkle, 1967), we do not expect either factor to influence our results given females were of similar size, were caught within a short period of time, and had similar clutch sizes. Females with smaller clutches had higher bactericidal capacity than females with larger clutches. Bactericidal capacity increased as clutch mass increased for all lizards but was lower in females with larger clutch sizes. Reproduction can impose deficits by reducing energy available for other physiological processes, such as maintenance of the immune system, and may incur costs if an individual is resource-limited (French and Moore, 2008). In situations where resources are readily available, tradeoffs are not likely to manifest. Our results demonstrate this tradeoff when comparing maternal immune responses between clutch sizes, such that females with a larger clutch size exhibited lower immune function. However, increases in bactericidal capacity as clutch mass increases among these females is contradictory. Instead of this pattern indicating better immunocompetence, higher immune function may instead indicate a current infection (Hawley and Altizer, 2011), which could suggest larger clutch masses ensue subsequent costs, such as increased susceptibility to infection. If higher immunity suggests infection, then females with larger clutch masses may be in better condition to respond to infection since they were able to produce a larger clutch to begin with. Future research on this subject should account for current infection status by simultaneously measuring endo- and ectoparasites.

Females with smaller clutch sizes had higher ROMs, but ROMs increased as clutch mass increased in both females with small and large clutches. This finding partially supported our initial hypothesis that increased reproductive investment (clutch mass) would incur oxidative costs via an increase in reactive oxygen metabolites. Costantini et al. (2010) found similar results in adult female Starlings (*Sturnus vulgaris*), such that females with larger broods, but smaller eggs, had higher ROMs. Manipulation of reproductive effort has uncovered the relationship between reproduction and oxidative stress in animals (Blount et al., 2016; Metcalfe and Monaghan, 2013). For example, larger clutch sizes were associated with higher oxidative damage markers in two different iguana species (Webb et al. 2019; French et al. *in press*), which directly opposes our findings. Breeding and brood-manipulated canaries in captivity experienced lower markers of oxidative damage relative to non-breeding individuals (Costantini et al., 2014). Similarly, breeding bank voles in captivity experienced lower oxidative damage in tissues compared to non-breeding individuals (Oldakowski et al., 2012). It is possible an unrestricted diet during captivity may have masked the oxidative costs of reproductive effort via clutch size. However, we argue in this case, clutch mass represents reproductive effort better than clutch size. Larger clutch sizes do not necessarily equate to greater reproductive investment, because larger clutches often include smaller eggs by mass (Ford and Seigel, 1989; Herman and Bout, 1998; Sinervo and Licht, 1991). Despite the limited variation in clutch size of the females within this study, our results trend toward this relationship, although a larger sample size is needed to resolve this question. Nonetheless, larger eggs result in larger offspring that are more likely to survive (Hutchings, 1991; Krist, 2011; Sinervo and Doughty, 1996), and this relationship may be the result of the oxidative cost of producing larger, higher quality eggs. Recent work has shown a yolk protein, vitellogenin, may offset potential oxidative costs relating to reproduction in lizards (Lindsay et al., 2020); given that vitellogenin is directly deposited into follicles, this yolk protein may provide antioxidant effects to offspring. Future research pertaining to this system should examine how antioxidant capacity and vitellogenin concentrations change with reproductive investment.

Individual egg BKA and ROMs

Because the embryo is present at oviposition, yolk physiology could be altered by embryonic physiological processes. However, we are confident the physiological attributes of the egg are maternal and not embryonic. At oviposition, *U. stansburiana* embryos have undergone ⅓ of their development (Andrews and Mathies, 2000). Embryo mass is marginal relative to the amount of egg yolk allocated to an egg. Therefore, any physiological contribution of the embryo, or yolk utilization by the embryo, is assumed to be minimal.

Contrary to our hypotheses and predictions, maternal innate immunity did not covary with egg yolk innate immunity. Maternal antibody titers correspond to egg yolk antibodies during yolk formation in avian species (Gasparini et al., 2002; Saino et al., 2002; Sun et al., 2013), and these antibodies provide immediate protection to offspring early in development when their immune systems are underdeveloped. This phenomenon was also documented in desert tortoises (*Gopherus agassizii*), in which females allocated antibodies against *Mycoplasma agassizii* to their offspring (Schumacher et al., 1999). Previous studies have shown birds and reptiles can provision antimicrobial or innate immune components to eggs, which correlate with maternal antimicrobial factors (Saino et al., 2002). However, a later finding in Saino et al. (2007) suggested antimicrobial components in avian egg albumen are not derived via circulation in the blood stream, which could explain why components of the innate immune system found in the yolk may not correlate with maternal innate immunity during vitellogenesis. It is also important to note that defense against the microbe used in our immune assay measures a functional response by innate immune components and natural antibodies (French and Neuman-Lee, 2012). Natural antibodies are present in the egg yolk of *U. stansburiana*, as indicated by agglutination against sheep red blood cells (Virgin and French, unpublished data). Thus, it is possible yolk natural antibodies were utilized in this assay and might be directly related to maternal natural antibodies. Further studies should seek to quantify specific immune system components in reptilian eggs.

We did not find a relationship between maternal ROMs and egg yolk ROMs, rejecting our hypothesis that maternal oxidative status would influence egg yolk oxidative status. Embryonic development is a metabolically intense process which may produce reactive oxygen species and result in oxidative damage to developing tissues (Blount et al., 2000). Embryos utilize yolk for growth and development; therefore, it is likely beneficial for a yolk to have lower oxidative damage markers to minimize potential oxidative stress from development. Reduction of yolk oxidative damage markers, such as organic hydroperoxides, is a signal for higher nutritive content in egg yolk (Faitarone et al., 2016; Vieira et al., 2017), and is often achieved through maternal dietary addition of antioxidants (Akdemir et al., 2012). Hence, the measurement of maternal antioxidant capacity may have better predicted egg yolk ROMs.

Despite a lack of association between maternal ROMs and egg yolk ROMs, we found ROMs decreased as egg mass increased, regardless of clutch size. Decreased ROMs in larger eggs could be due to differences in water content (Brusch et al., 2019; Ferguson and Deeming, 1991), such that larger eggs might be more hydrated which could displace/dilute the total amount of yolk and yolk components within an egg. However, we did not find this relationship with egg mass and BKA, so it is unlikely that egg hydration state was the primary factor influencing this. A more likely explanation is elevated ROMs in females acted as a reproductive constraint (Stier et al., 2012; Lister et al., 2016). Work in other systems have shown females under oxidative stress produced smaller clutch or litter sizes (Costantini et al., 2016; Stier et al., 2012), but Viblanc et al. (2018) found females under higher oxidative stress had larger litter masses, further stressing the importance of context and species.

Intra- and inter-clutch variation in egg physiology

Because differential allocation of egg yolk components can lead to different offspring phenotypes (Muller and Groothuis, 2013; Reed and Vleck, 2001), high variation in egg yolk physiology among eggs in a given clutch may be adaptive in unpredictable environments (Love et al., 2008). Conversely, low variation in egg yolk traits may be adaptive in more stable environments. We found there was a wide range of variation in egg yolk BKA, ROMs, and egg mass within and across clutches (See Table 3). We found neither clutch size, nor mass, predicted the level of variation within clutches. Overall, these results suggest variation in physiology among eggs, and thus phenotype, is not determined by the metrics of reproductive investment characterized in this study and is instead a likely combination of intrinsic (Pilz et al., 2003) and extrinsic factors (Hargitai et al., 2009; Postma et al., 2014).

Maternal traits can significantly impact egg characteristics. This study demonstrated significant inter-clutch variation when we controlled for clutch size, egg mass, and maternal physiology, suggesting the variation described could be attributed to other aspects of maternal identity. Because egg yolk content is directly provisioned by the mother, it is not surprising our results support this. Maternal identity has also previously explained variation in egg yolk immunoglobulins (Counihan et al., 2015), nestling cell-mediated immunity (Westneat et al., 2004) and ROMs (Costantini and Dell'Omo, 2006). Whether the maternal influence on egg yolk characteristics is related more to genetics (Rubolini et al., 2006; Ruuskanen et al., 2016) or to maternal condition during vitellogenesis may depend on the physiological factor measured (Ruuskanen et al., 2016). Our results suggest while maternal identity contributed to observed egg yolk BKA and ROMs, maternal physiology did not. There was no effect of capture/lay date, total food intake, or female body size (SVL) on any of the egg yolk metrics measured. However, we were unable to account for environmental factors that might have occurred prior to capture (e.g., paternity, mate quality). Incorporation of more environmental and maternal physiological factors may better capture the relationship between maternal and egg physiological status, especially in a wild-caught species.

Conclusion

In conclusion, the present study provides new insights regarding the relationship between reproductive investment and maternal physiology. Specifically, much of the variation in maternal innate immunity and reactive oxygen species is due to reproductive investment, including both clutch size and mass. Further, the significant proportion of inter-clutch variation in egg innate immunity and egg reactive oxygen species is due to factors not identified in this study. Although we rejected our hypothesis that variation in maternal immune and oxidative physiology would directly correspond with egg yolk physiology, the knowledge that immune factors exist and vary substantially both within and across clutches, thus conferring variable protection to eggs, is a valuable insight. Given egg yolk physiologies vary within and across clutches, researchers should exercise caution with the assumption that one egg is representative of an entire clutch. Our findings build upon previous work identifying the physiological costs of reproduction in female Side-blotched Lizards and introduce creative methods to measure immune function and oxidative state in yolks.

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CHAPTER 3 – BASELINE DIFFERENCES IN METABOLIC RATE AND THE PHYSIOLOGICAL RESPONSES TO AN IMMUNE CHALLENGE VARY BY VITELLOGENIC STAGE IN AN OVIPAROUS REPTILE [1]

Abstract

The energetic requirements associated with reproduction may limit resources available for co-occurring physiological processes, such as immune system maintenance. In oviparous reptiles, egg yolk investment during vitellogenesis precedes ovulation and eggshell development and requires substantial resources; however, it is unclear whether the energetic demands of egg yolk investment vary across vitellogenesis (early-, middle-, and late-stage), or whether tradeoffs between reproductive investment and immune function are dependent on vitellogenic stage. Using female Side-blotched Lizards (*Uta stansburiana*) as a model species, we addressed these research gaps *ex situ* by testing whether energy metabolism (respirometry) differs across the reproductive cycle and by examining the metabolic, immune, and oxidative costs of an immune challenge in female lizards varying in vitellogenic stage. Metabolism was significantly higher at the onset of vitellogenesis (early-stage) and lowest just prior to ovulation (late-stage). Furthermore, females differentially responded to an immune challenge depending on vitellogenic stage, which may suggest limited resources underly these findings.

Introduction

Reproduction is a costly physiological process which requires an influx of energetic resources to be used toward offspring development, growth, and survival. Life history theory posits that investment of resources into reproduction necessitates a deficit in another life history trait (Reznick, 1985; Stearns, 1989), such as future reproduction (Hanssen et al., 2005), longevity (Helle and Lummaa, 2013), or self-maintenance (French et al., 2007; French and Moore, 2008). However, recent work has demonstrated tradeoffs are more nuanced, such that costs from investment into different traits may vary both within and across life-history stages (Hegemann et al., 2013). Reproduction can incur direct energetic costs via additional energetic expenditure. For example, gravid *Sceloporus undulatus* experienced a 122% increase in metabolic rate compared to non-gravid conspecifics (Angilletta and Sears, 2000), and some viviparous snake species experience a 30% increase in metabolism during vitellogenesis (Van Dyke and Beaupre, 2011). Pregnant pygmy rattlesnakes (*Sistrurus miliarius*) exhibited hindered metabolic and immune responses relative to non-pregnant females and males (Lind et al., 2020), highlighting a tradeoff between reproduction and immunocompetence. In addition to an increase in energy expenditure, the extra weight from carrying a clutch of eggs or a litter of pups may alter an animal's ability to interact with their environment. Studies assessing the impacts of gravidity/pregnancy on endurance and locomotor performance have shown a considerable cost via reduced endurance capacity (Miles et al., 2000; Zani et al., 2008) and locomotor performance (Shine, 2003; Winne and Hopkins, 2006), traits inherently linked to survival and fitness (Miles et al., 2000).

The energetic requirements of reproduction may also reduce energy available for other physiological systems, such as the immune system. Maintaining a well-functioning immune system is energetically costly, but necessary for survival (Lochmiller and Deerenberg, 2000; Nordling et al., 1998; Sheldon and Verhulst, 1996). Studies have demonstrated tradeoffs between the reproductive and immune systems in the field (Ardia, 2005), and experimentally (Deerenberg et al., 1997; Fedorka et al., 2004; French et al., 2007; McCallum and Trauth, 2007), whereby manipulating one system induces a tradeoff in the other. For example, female Texas field crickets (*Gryllus texensis*) had reduced fecundity (oviposition rate) when given a bacterial challenge (Stahlschmidt et al., 2013). Furthermore, reproductive investment may elicit detrimental effects related to oxidative stress (French et al., 2021; Webb et al., 2019). For example, female Alpine Swifts (*Apus melba*) with lower oxidative stress resistance laid fewer and lower quality eggs than females with higher resistance (Bize et al., 2008), and female Bahamian Rock Iguanas with larger clutches had higher markers of oxidative damage (Webb et al., 2019). Despite growing evidence linking reproduction and oxidative stress, it is still largely debated (Costantini et al., 2014; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014); to complicate things, immune system elevation may incur costs in the form of oxidative damage, such as an increase in ROS via oxidative burst (Sorci and Faivre, 2009) or indirectly through a change in metabolic rate, albeit this relationship is equivocal (Barja, 2007; Fletcher et al., 2013; Mangel and Munch, 2005; Speakman et al., 2004). Hence, it is unclear how interactions between the immune and reproductive system may influence oxidative physiology.

Although it is evident the energetic demands of reproduction induce tradeoffs in other physiological systems, the costs of reproduction and thus the tradeoffs also change across the reproductive cycle. In the Colorado checkered Whiptail (*Aspidoscelis neotesselata*), a parthenogenetic lizard, energy metabolites (triglycerides and free glycerol) were highest early in vitellogenesis and lowest at gravidity (Hudson et al., 2020). Manipulation of the immune system has elicited tradeoffs across reproduction as well (Caracalas et al. 2021). Female Side-blotched Lizards (*Uta stansburiana*) alter investment in wound-healing depending on how far they have progressed in vitellogenesis (Durso and French, 2018; Pettit et al., 2019). Similarly, vitellogenic female Ornate Tree Lizards (*Urosaurus ornatus*) exhibited the poorest wound healing ability relative to pre-reproductive, gravid, and post-reproductive females (French and Moore, 2008), further supporting the idea that energetics vary across reproduction, and thus, physiological tradeoffs. However, little has been done to directly test how simulated infection influences the metabolic, immunological, and oxidative costs of reproduction across vitellogenesis.

In this experiment, we tested whether **(1)** resting metabolic rates (RMR) differed in female side-blotched lizards across vitellogenic stages, **(2)** whether an immune challenge via LPS injection (lipopolysaccharide, LPS) induces metabolic and oxidative responses and **(3)** whether these responses vary depending on vitellogenic stage. We hypothesized resting metabolic rate and the immune and oxidative response to LPS would differ across vitellogenic stages. Because females assumedly have more energetic reserves available at the onset of reproduction (Hudson et al., 2020), we predicted females in early and mid-stage vitellogenesis treated with LPS would mount significantly different responses than control females. Conversely, we predicted late-stage females treated with LPS would respond similar to the control females injected with PBS.

Methods

Model organism

The Side-blotched Lizard (*Uta stansburiana*) is a desert-dwelling, phrynosomatid lizard species which lays multiple clutches each breeding season and occupy a wide-range of habitats (Parker and Pianka, 1975). Reproduction represents a significant energetic cost to female Side-blotched Lizards. Clutches can contribute as much as 30% to the total mass of a gravid female in our specific populations (Virgin, unpublished data). Furthermore, reproductive investment (follicle length and clutch size) can be assessed across the entire reproductive cycle of female Side-blotched Lizards via ultrasonography (Lucas and French, 2012). Hence, Side-blotched Lizards are an ideal model to examine the energetic costs of reproduction across vitellogenesis and reveal tradeoffs that may be dependent on the stage of the reproductive cycle.

Animal capture and housing

We collected 77 female Side-blotched Lizards using lassos in Washington County, Utah, USA. Females were captured during the peak breeding season (French, pers. obs.) in two iterations between mid-April 2018 and mid-May 2018. Upon capture, we determined vitellogenic stage and clutch size via palpation of the body cavity. We included females in our study as long as they were not gravid (i.e., possessing fully shelled eggs within the oviduct). We transported females in individual containers to Utah State University where we took morphometric measurements (mass, SVL) and validated vitellogenic stage, follicle size, and clutch size via ultrasonography (SonoSite, Inc., Bothell, Washington, USA).

We housed females in Percival environmental chambers (DR-36VL, Percival Scientific) at 36 ±1°C, 60% RH, for 72 hours prior to experiment to allow for environmental acclimation. Females were housed in plastic containers (48×18×23 cm) with a two-inch bed of moist vermiculite, a plastic hide made of PVC pipe, and a water dish. Females were fed 0.10g-0.15g of calcium-dusted crickets every 48 hours unless undergoing metabolic trials the next day.

Determination of vitellogenic stage

We collected 39 females in early-vitellogenesis (SVL: 45.461 ± 0.454 SE; clutch size: 1.230 ± 0.253 SE), 28 females in mid-vitellogenesis (SVL: 46.214 ± 0.367 SE; clutch size: 3.678 ± 0.145 SE), and 9 late-vitellogenesis females (SVL: 46.600 ± 0.622 SE; clutch size: 3.200 ± 0.200 SE). We separated females into three distinct stages of vitellogenesis based on follicle length and shape. Females in early-stage vitellogenesis possessed round follicles with a size of up to 0.35 mm. Females in mid-stage vitellogenesis possessed round follicles ranging between 0.36 to 0.59 mm. Females in late-stage vitellogenesis possessed unshelled, oval-shaped follicles ranging from 0.60 to 0.85 mm. Moving forward, we will refer to each vitellogenic stage as early-, mid-, and late-stage.

Experimental design and treatment

Table 4. Timeline of daily procedures for both iterations of experiments.

Day of Experiment	Task(s)
0	Morphometrics and Ultrasonography
	Housing and feeding
	Assigned treatment and group
3	Pre-treatment metabolic measurements
	LPS and PBS injections
6	Post-treatment metabolic measurements
	Blood sample

We captured animals within two discrete time periods (April and May) and therefore conducted this experiment in two iterations, adhering to the same design and schedule (see Table 4). We used an interspersion design to ensure an equal number of females pertaining to a certain vitellogenic stage, clutch size, and SVL were distributed between treatments. Because we could only measure resting metabolic rate in seven lizards at a time, we distributed lizards of different treatments and vitellogenic stages into different "groups", in which the timing of metabolic measurements, treatment injections, and blood collection was kept consistent for each female.

To determine whether metabolic rate varied among females in different stages of vitellogenesis, we measured resting metabolic rate (RMR) prior to treatment injections after allowing the lizards to acclimate for 72 hours. To test whether sickness induces physiological responses dependent on vitellogenic stage, we used lipopolysaccharide (LPS) to simulate an immune response. LPS is a component of gram-negative bacterial cell walls which mimics a bacterial infection without pathogenic effects and is frequently used in eco-immunology research (Demas et al. 2011). Females were given either a mass-dosed injection (20 µl/1g body mass) of LPS (10 µg lyophilized LPS/20 µl PBS); serotype 0127:B8 (Sigma-Aldrich, St Louis, MO, USA) or an equivalent volume of PBS (0 µg lyophilized LPS/20 µl PBS). Furthermore, this dilution of LPS and dose was previously validated in Side-blotched Lizards via an increase in plasma bactericidal ability (Hudson et al. 2021) and altered thermoregulatory behavior. Immediately following the pre-treatment metabolic trial, we administered injections into the body cavity (intraperitoneally) to minimize handling stress and returned lizards to their respective terraria.

To test the metabolic and physiological effects of an immune challenge on lizards in different vitellogenic stages, we measured RMR 72 hours post-injection of LPS or PBS. Immediately following each post-treatment metabolic trial, we collected a blood sample from each lizard via the retro-orbital sinus within 3 minutes of handling and returned them to their respective terraria. All procedures described below were permitted under Utah State Department of Wildlife Resources (COR #1COLL8382) and were approved by the USU Institutional Animal Care and Use Committee (protocol #2068). At the end of the study, we released the females at their locations of capture.

Metabolic measurements and analysis

Resting metabolic rates among lizards were measured through an eight-channel closed-flow respirometry system (Sable Systems, Las Vegas, Nevada, USA) between 1000 – 1500 hours on Day 3 and Day 6. Pre- and post-measures were included to assess (1) reproductive stage-dependent differences in resting metabolic rate, and (2) the overall impact of immune investment on mass-adjusted O_2 consumption and CO_2 production during the sensitive period (Smith and French, 2017). Since females were actively progressing through reproduction over the course of the study, we did not directly compare pre and post metabolic rates. Instead, we compared pre-treatment metabolic rates among different stage females and compared post-treatment RMR to that of the control females. Food was restricted 24 h prior to metabolic trials to limit potential interactive effects of digestion. Lizards were then transferred from their terraria to assigned glass metabolic chambers (500 mL) located in a dark incubator maintained at 36 °C, a temperature within the optimal thermal range for this species (Goller et al., 2014; Waldschmidt and Tracy, 1983). Metabolic chambers were slowly flushed with dry, CO_2-free air (i.e., air scrubbed by Drierite® and Ascarite®) before beginning an automated sampling program adapted from Kolbe et al. (2014). During this time, lizards were allotted 1 h within the chambers to acclimate and achieve resting metabolic state. After the 1-h flush period, scrubbed air was pushed through the chambers by a mass-flow regulator (Mass Flow System, Sable Systems, Las Vegas, Nevada, USA), which then passed through a Drierite® column for subsequent sampling by a carbon dioxide analyzer (CA-10, Sable Systems, Las Vegas, Nevada, USA). Air then flowed through a column of Drierite® and Ascarite® before being sampled by an oxygen analyzer (Oxzilla, Sable Systems, Las Vegas, Nevada, USA). Metabolic rate was measured in milliliters of oxygen hr−1g−1 and carbon dioxide hr−1g−1. We mass-adjusted resting metabolic rates because (1) LPS doses were mass-adjusted, and (2) none of the females were gravid, and therefore we did not need to consider the metabolic rates of each embryo within an egg (Angilletta and Sears, 2000).

Plasma physiological assays

BKA

We measured plasma bactericidal capacity (BKA) to validate the effectiveness of our immune challenge and to assess its overall effects on lizards varying in vitellogenic stage. Following French and Neuman-Lee (2012), we added 6 µl of plasma to 14 µl of CO_2-dependent media. We plated 20 µl and 24 µl of CO_2-independent media to the positive and negative controls, respectively, and then added 4 µl of 10^4 working *Escherichia coli* (ATCC#8739) solution to the positive controls and the plasma samples. After vortexing for one minute, we incubated the plate at 37C for 30 minutes. Following, we added 125 µl tryptic agar soy broth in each well, vortexed for 1 minute, then measured background absorbance at 300 nm using a microplate reader (BioRad xMark). We incubated the plate for 12 hours and again measured the absorbance at 300 nm. To calculate microbicidal capacity, we subtracted the background absorbance from post-incubation absorbance, and calculated % bacteria killed by subtracting net absorbance from 1 and dividing sample absorbance by absorbance of the positive control. We ran each female's plasma sample in duplicate, so we took the average of both samples to calculate % bacteria killed. Due to low sample volume, we ran 14 samples singly. Intra-assay CVs were between 2.23% and 5.21%, Inter-assay variation of the positive controls was 3.34%.

d-ROMs Kit and OXY-Adsorbent Test

To determine whether an immune challenge alters oxidative status within and across stages of vitellogenesis, we measured reactive oxygen metabolites (d-ROMs) and antioxidant capacity (OXY) using the d-ROMs test (Diacron, Grosseto, Italy) and OXY-Adsorbent Test (Diacron, Grosseto, Italy), respectively. For both assays, we followed “endpoint mode” protocol validated in Side-blotched Lizards with modifications for use on a microplate (Lucas and French, 2012).

The d-ROMs test indirectly measures organic hydroperoxides in biological samples, which may signal lipid oxidative damage (Costantini, 2016). Briefly, we pipetted 5 µl of plasma in (singlicate) in 100 µl of the provided acidic buffer solution (1:100 R1/R2 mixture) and vortexed the microplate on a plate shaker for 30 seconds. We incubated assay plates at 37°C for 90 minutes, and immediately read each plate at 505 nm on a spectrophotometer (xMark, BioRad). Absorbance values were converted into units of mg H_2O_2/dL by dividing CARR Us by 0.08 mg H_2O_2/dL. Intra-assay CVs of the calibrator (i.e., standard) were between 2.55% and 4.35%, and inter-assay variation was 2.91%. The OXY-Adsorbent Test quantifies the ability of a sample to withstand oxidation from hypochlorous acid (HClO). We diluted 2 µl of plasma into 100 µl of nanopure water in which 5 µl of diluted sample was then pipetted onto a 96-well plate along with 100 µl of provided HClO Reagent (R_1). Each plate was gently vortexed for 30s, and then incubated at 37°C for 10 minutes. We measured sample absorbance at 505 nm post-incubation and measured sample absorbance by row after adding 5 µl of R_2 to each well. Absorbance values were converted into µmol of HClO/mL following kit instructions.

Statistical analysis

All statistical analyses were performed in R CRAN Statistical Software and RStudio (R Core Team, 2022; RStudio Team, 2022). When needed, we log-transformed metabolic and physiology data to fit assumptions of residual normality and improve model fit. To validate the efficacy of the LPS treatment, we compared differences in bactericidal capacity within vitellogenic stages by performing a non-parametric one-way Wilcoxon test using the *rstatix* package (Kassambara, 2021). To assess baseline metabolic differences across vitellogenesis, we performed a 1-way ANCOVA with RMR metrics as our dependent variable, vitellogenic stage as our predictor, and group as our blocking factor. Group, or the assigned time in which lizards underwent metabolic trials, treatment injections, and blood sampling, was included as a blocking factor to control for circadian changes in physiology. Using the *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) packages, we ran linear mixed effects models to assess **(1)** baseline resting metabolic rate and **(2)** the metabolic and oxidative responses to LPS, and **(3)** the interaction between vitellogenic stage and physiology following an immune challenge. We included group as a random effect of intercept to account for circadian differences in physiology. All post-hoc analyses were performed using the *emmeans* package (Lenth, 2022), in which pairwise comparisons were performed to assess treatment effects on physiology within vitellogenic stages. We assessed model fit through visual inspection of the model residuals. Graphs to depict results from linear mixed effects models are reflected through the estimated marginal means calculated from the *emmeans* package. All graphics used were created in RStudio (RStudio Team, 2022) using ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020).

Results

Summary statistics

Females across vitellogenic stages did not differ in SVL ($F = 1.2638$; $p = 0.2886$) but differed in clutch size ($F = 34.036$; $p < 0.0001$) because over half of the females in early-vitellogenesis were classified as having undetectable follicles. However, snout-vent-length and clutch size did not differ across treatments (SVL: $F = 0.4888$; $p = 0.4866$; clutch size: $F = 0.0947$; $p = 0.7591$), suggesting females across vitellogenic stages were evenly dispersed across treatments, as desired.

Metabolic differences across vitellogenic stages

Prior to treatment injections, females varied in CO_2 production by vitellogenic stage (One-Way ANCOVA; $F = 3.272$; df = 2; $p = 0.044$; Table 5). Early-stage females had significantly higher CO_2 production than late-stage females (t = 2.521; p = 0.0366; Fig. 4), and mid-stage females had marginally higher CO_2 production than late-stage females (t = 2.255; p = 0.0689). However, there were no significant differences between early- and mid-stages (t =0.463; p = 0.888). Similarly, vitellogenic stage was marginally related to O_2 consumption (One-Way ANOVA; $F = 2.317$; df = 2; $p = 0.106$). When comparing across stages, early-stage females had marginally higher O_2 consumption than late-stage females (t =2.151; p = 0.087) but there was no difference between early- and mid-stage females (t =1.049; p = 0.549). Finally, O_2 consumption was not significantly different between mid- and late-stage females (t =1.338; p = 0.379).

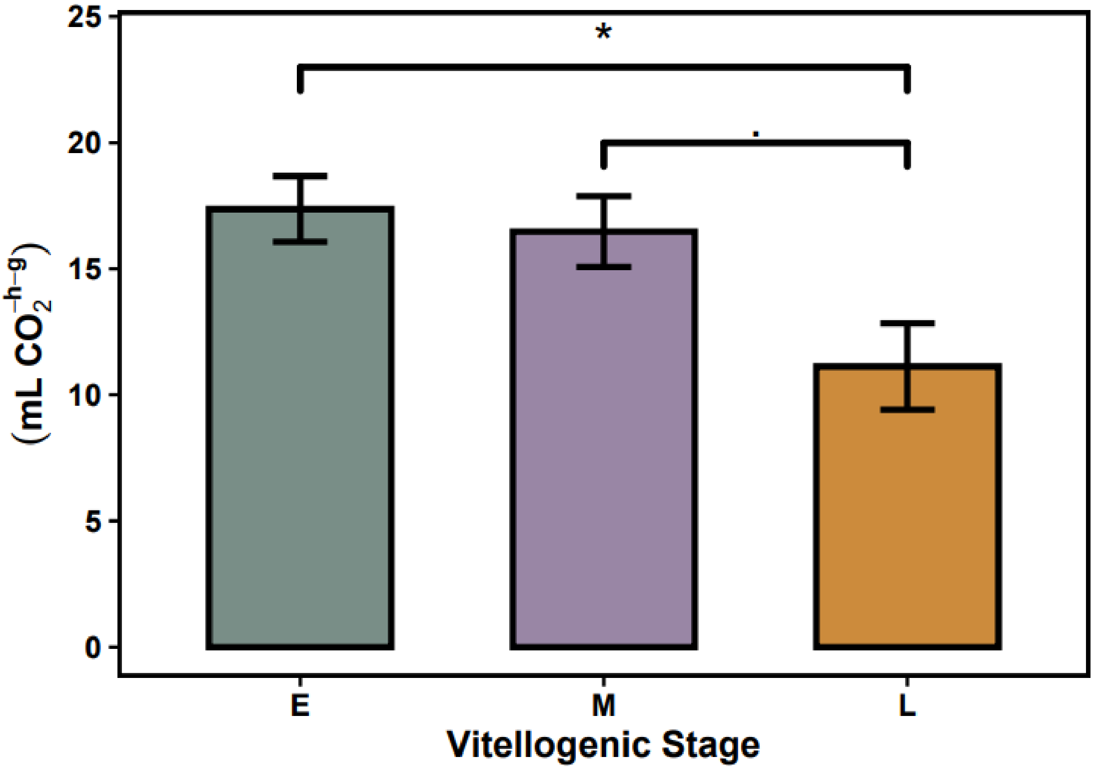

Figure 4. Baseline CO_2 Production (ml CO_2-h-g) of early-, mid-, and late-stage female Side-blotched Lizards (*Uta stansburiana*) prior to treatment. An asterisk (*) over the bracket indicates a significance level less than 0.05.

Table 5. Summarized ANCOVA results for assessing the effect of vitellogenic stage on CO_2 production and O_2 consumption in females Side-blotched Lizards (*Uta stansburiana*).

Resting Metabolic Rate by Vitellogenic Stage (Pre-Treatment)	**CO_2 Production (ml $CO_{2\text{-h-g}}$)**				**O_2 Consumption (ml $O_{2\text{-h-g}}$)**			
Predictor Variables	*SS*	*df*	*F*	*p*	*SS*	*df*	*F*	*p*
Vitellogenic Stage	0.2495	2	3.272	**0.044**	0.1214	2	2.317	0.106
Group (Block)	0.0004	1	0.011	0.918	0.0464	1	1.772	0.187
Residuals	2.7447	72			1.8873	72		

Physiological and metabolic responses to an immune challenge across vitellogenic stages

Metabolic rate

Model summaries are reported in Table 6. In the overall model, females treated with LPS had significantly different CO_2 production (*F* = 9.50; p = 0.003) than females treated with PBS (Fig. 5). The effect of vitellogenic stage on CO_2 production was marginally significant (*F* = 2.53; p = 0.087). However, its interaction with treatment did not influence post-injection CO_2 production (*F* = 0.49; p = 0.616). Within vitellogenic stages, early-stage (t = -2.140; p = 0.0365) and mid-stage (t = -2.433; p = 0.0180) females had significantly higher CO_2 production rates than their respective control treatments, but treatment did not influence CO_2 production in late-stage females (t = -0.359; p = 0.7211). Similarly, O_2 consumption was significantly different in females depending on treatment (*F* = 8.59; p = 0.005) but not vitellogenic stage or their interaction (*F* < 1.912; p > 0.156). Finally, early-stage and mid-stage females treated with LPS had higher O_2 consumption than females treated with PBS within the same stage (early: t = -2.140; p = 0.0365;

middle: t = -2.292; p = 0.0255). Late-stage females exhibited similar responses between treatments (t = -362; p = 0.719).

Table 6. Type-II ANOVA results summarizing the effects of treatment, vitellogenic stage, and their interaction on female Side-blotched Lizard (*Uta stansburiana*) physiology.

Physiological Responses to Treatment	**Reactive Oxygen Metabolites (mg H_2O_2/dL)**			**CO_2 Production (ml $CO_{2\text{-h-g}}$)**			**O_2 Consumption (ml $O_{2\text{-h-g}}$)**		
Predictor Variables	*F*	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
Treatment	**7.05**	**1,56.4**	**0.01**	**9.50**	**1,59.5**	**0.003**	**8.59**	**1,59.6**	**0.005**
Vitellogenic Stage	1.07	2,60.9	0.35	2.53	2,64.4	0.087	1.91	2,64.6	0.156
Treatment* Vitellogenic Stage	0.44	2,54.6	0.65	0.49	2,57.9	0.616	0.56	2,57.9	0.573
Random Effect	σ^2	σ		σ^2	σ		σ^2	σ	
Group (Intercept)	4.38	2.09		0.008	0.088		0.005	0.073	
Residual	15.98	4.00		0.039	0.197		0.029	0.170	

BKA

Within vitellogenic stages, BKA was higher in early-stage females treated with LPS (W = 32; p = 0.009) but was not significantly different in mid- (W = 40; p = 0.5612) or late-stage females (W = 14; p = 0.4127; see Fig. 6).

Reactive oxygen metabolites (d-ROMS) *and antioxidant capacity* (OXY)

Treatment significantly influenced d-ROMs (t = 2.212; p = 0.0311; Fig. 7) but not vitellogenic stage or the interaction between treatment and vitellogenic stage (t <1.415; p > 0.1622). When comparing within vitellogenic stage, plasma d-ROMs were significantly lower in mid-stage females treated with LPS (t = 2.35; p = 0.0223), but not in early- or late-stage females (t <1.475; p > 0.1458). There was no effect of treatment, vitellogenic stage, nor their interaction on plasma OXY levels (*F* < 0.484; p > 0.504).

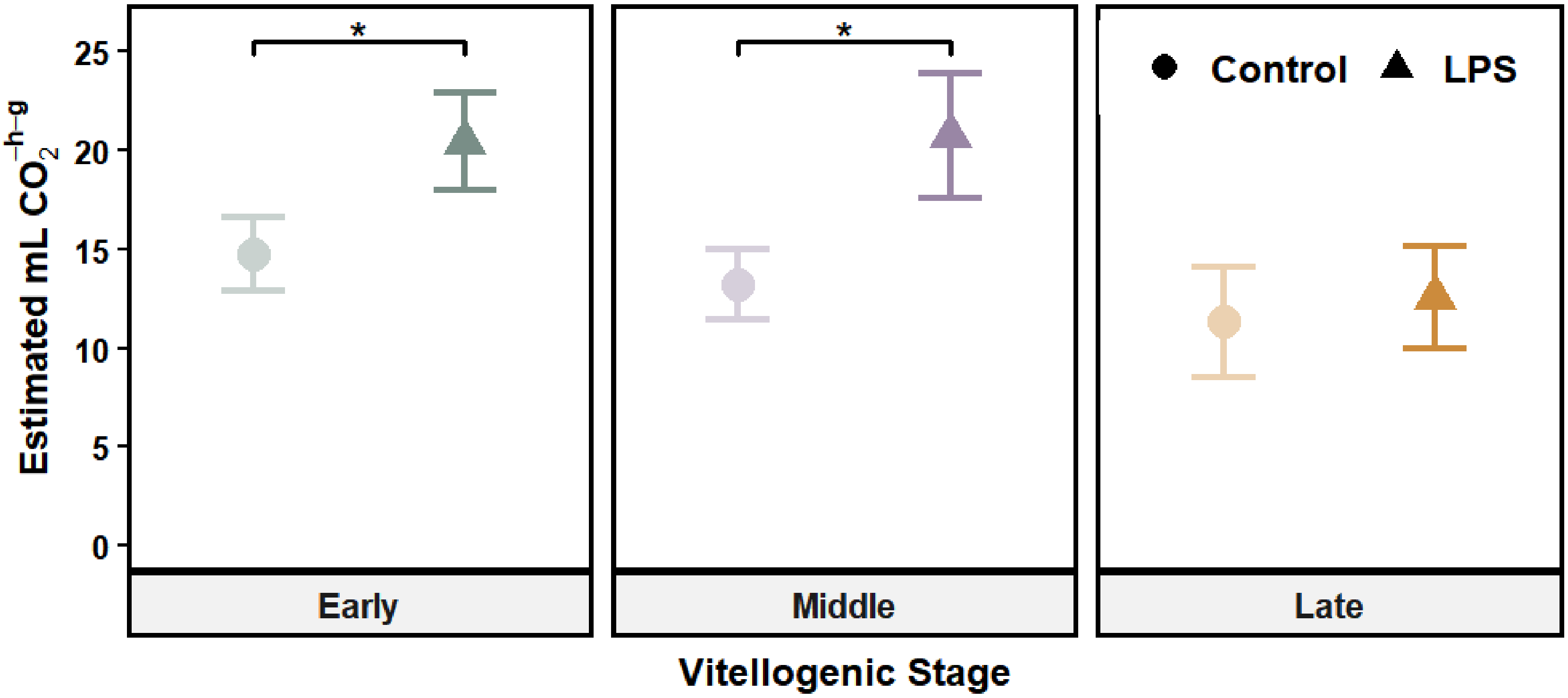

Figure 5. Effect of PBS and LPS on CO2 Production (ml CO2-h-g) in vitellogenic female Side-blotched Lizards (*Uta stansburiana*).

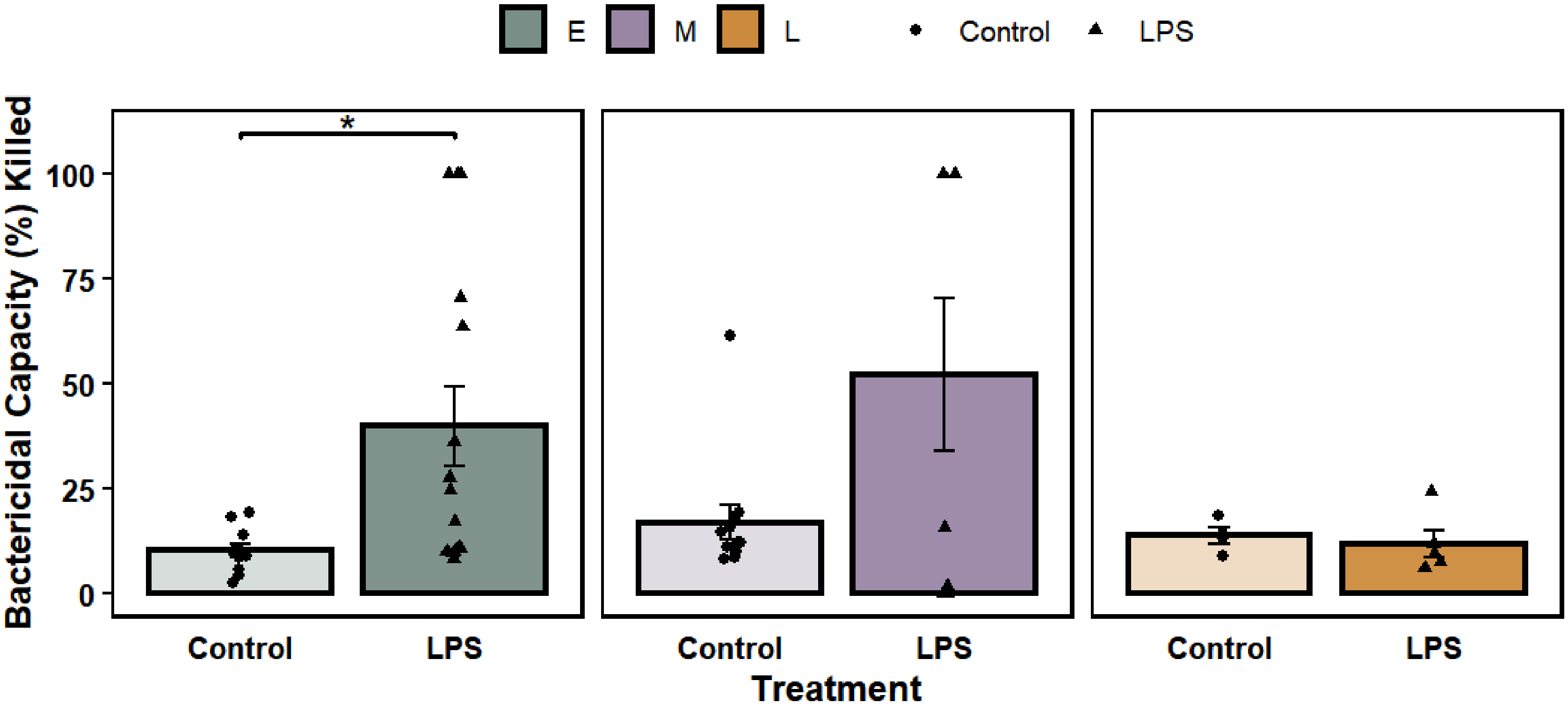

Figure 6. Effect of LPS and PBS on bactericidal capacity (BKA) of female Side-blotched Lizards (*Uta stansburiana*).

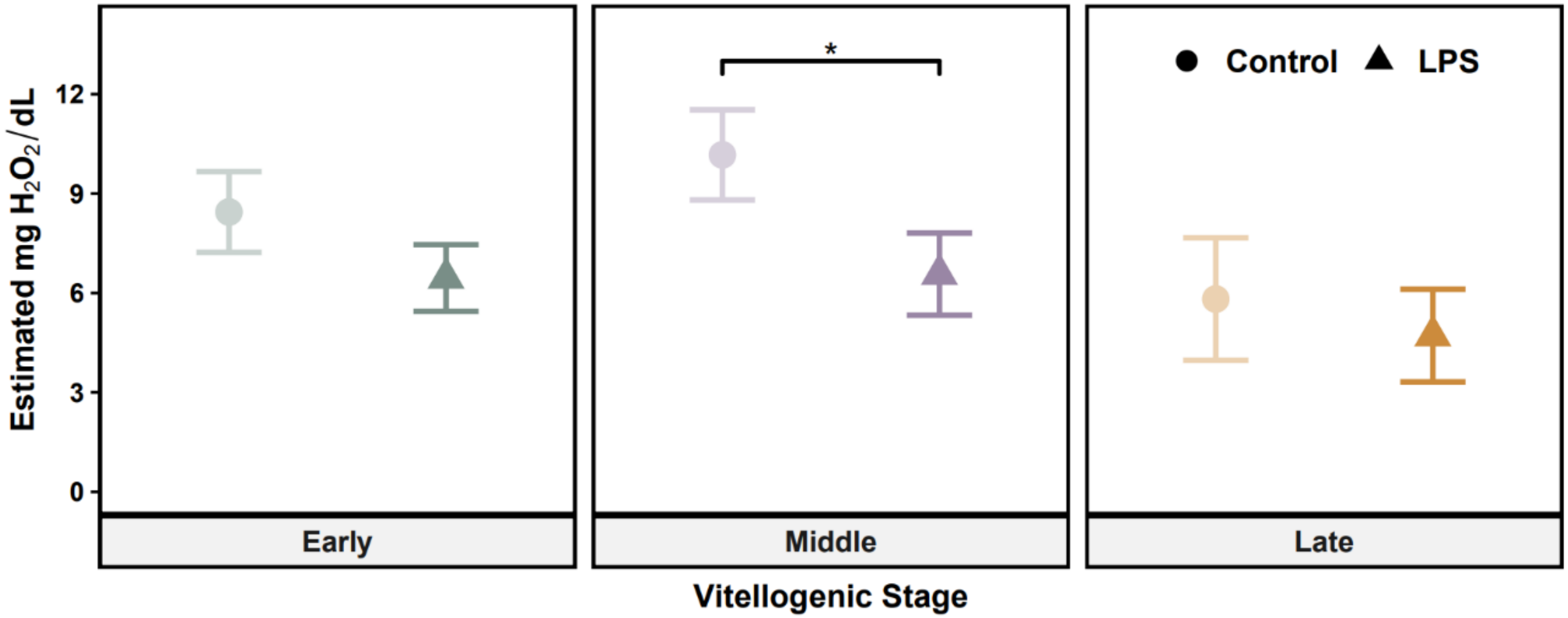

Figure 7. Effect of PBS and LPS on Reactive Oxygen Metabolites (mg H2O2/dL) in vitellogenic female Side-blotched Lizards (*Uta stansburiana*).

Discussion

To our knowledge, this is the first study that aimed both (1) to test whether there were baseline metabolic differences across different stages of vitellogenesis and (2) to document treatment differences in metabolic rate, oxidative damage markers, and bactericidal capacity following a simulated infection, both within and across vitellogenic stages. Prior to treatments, CO_2 production and O_2 consumption varied across vitellogenesis, such that females in late-stage had significantly lower metabolic rates than early-stage females. Moreover, we found early-stage female side-blotched lizards mounted significantly different immunological and metabolic responses following simulated infection. In mid-stage females, immune function, oxidative damage markers, and metabolic rate differed depending on treatment as well. However, females in late-stage exhibited no differences in physiological metrics between treatment groups. While our respirometry measurements represent a proxy for metabolism and energetic expenditure, we also argue the observed stage-dependent responses between treatments are potentially related to the costs of reproduction and its interaction with energetic deficits.

Baseline metabolic differences across reproduction

The energetic costs associated with reproduction are a well-studied area of research due to its implications in life history tradeoffs and evolution (Reznick, 1985; Stearns, 1989). Indeed, both the quantification and manipulation of energetics during reproduction has highlighted its direct influence on life history traits, such as future reproductive events (Aragón et al., 2009; Miles et al., 2000; Tallamy and Denno, 1982) and offspring size, number, and performance (Sinervo, 1990). In this study, we found female side-blotched lizards exhibit differences in resting metabolic rate depending on how far they have progressed into vitellogenesis. Females at the onset of reproduction (early) had higher levels of CO_2 production and O_2 consumption compared to females which had progressed to at least halfway through vitellogenesis (middle and late). These results corroborate previous findings in other reptile species, in which plasma energy metabolites (Hudson et al., 2020) and vitellogenin (an egg yolk precursor) were highest in early vitellogenesis females and decreased throughout until ovulation (Lindsay et al., 2020). Studies quantifying the metabolic demands of reproduction in viviparous snakes have similarly found resting metabolic rate was highest during vitellogenesis and steadily decreased until yolk deposition ceased (i.e., ovulation) (Van Dyke and Beaupre, 2011). We similarly considered female side-blotched lizards to be in late-stage vitellogenesis just prior to ovulation (Tinkle, 1967), which suggests peak RMR during vitellogenesis and its linear decrease until ovulation is evident across multiple taxonomic groups. However, it is important to note some studies have found metabolic rates in reptiles were highest at ovulation (Foucart et al., 2014; Lima-Santos et al., 2021), and during gestation and pregnancy (Foucart et al., 2014), warranting future research. While our metabolic measurements represent an excellent proxy for energy use, lower RMR doesn’t always correspond to lower energetic consumption; for example, females may also mediate their energetics through reducing activity or via behavioral thermoregulation, which were not assessed in the current study. Nonetheless, our results following treatment with LPS suggest a lack of energy available may mediate responses to simulated sickness.

Sickness-induced physiological differences within and across vitellogenic stages

Metabolic Rate

When resources are scarce, the energetic demands of reproduction often incur compensatory costs through the inability to respond to internal and external stressors (Sinervo et al., 1991). Maintenance of a well-functioning immune system is a costly process, but necessary for survival (Lochmiller and Deerenberg, 2000; Ots et al., 2001). Consequently, organisms are required to balance energetic investment into competing life history traits (e.g., reproduction vs. self-maintenance) (Durso and French, 2018; French et al., 2007). Following an immune challenge, early- and middle-stage females treated with LPS had significantly higher CO_2 production than their respective control groups, whereas late-stage females exhibited no difference in CO_2 production regardless of treatment.

One potential explanation for these findings is injection with LPS incurs additional costs via higher metabolic rates in early and mid-stage females. In pygmy rattlesnakes, reproductive females exhibited significantly higher resting metabolic rates prior to and after administration of LPS compared to non-reproductive females and males (Lind et al., 2020), suggesting pregnant females were still able to appropriately respond to an immune challenge, albeit at a cost of increased energy expenditure. Female side-blotched lizards in early- and mid-stage are actively investing yolk lipids and proteins into their follicles during this point in vitellogenesis (Durso and French, 2018). In contrast, females in late-stage vitellogenesis are assumedly expending less energy because yolk deposition is no longer necessary post-ovulation (Lindsay et al., 2020).

Previous studies have demonstrated direct competition for resources between the reproductive and immune systems and these expected relationships change depending on how far an animal has progressed in vitellogenesis (Durso and French, 2018; French and Moore, 2008). For example, when administered a wound, female side-blotched lizards in early-stage invested more protein and lipids into healing than their eggs, whereas late-stage females exhibited the exact opposite relationship (Durso and French, 2018; Pettit et al., 2019). While it could be considered an additional metabolic cost in early- and mid-stage females, if this were the case, we would expect late-stage females injected with LPS to have a significantly different metabolic response than late-stage females injected with PBS. Therefore, it is more likely elevated responses to LPS in early- and mid-stage females—and the lack of a response from late-stage females—is mediated through variation in available energy across vitellogenesis. However, it is important to note impaired stress and the associated physiological responses in reproductive females may benefit survival prospects and fitness by reducing the risks of becoming egg-bound (Woodley and Moore, 2002), or to prevent nest abandonment and/or conserve resources (Kitaysky et al., 1999). While not mutually exclusive, it is imperative future studies incorporate field-based research to better understand downstream effects on survival and reproductive output within a given breeding season and across lifetimes.

BKA

Female side-blotched lizards in early-stage vitellogenesis exhibited significantly elevated bactericidal capacity if injected with LPS, whereas mid- and late-stage females were not significantly different from within-stage females injected with PBS. Simulating an infection through using heat-killed microbes or cell membrane ligands of pathogens recognized by the immune system is a common tool to assess the eco-immunological costs of mounting an immune response (Demas et al., 2011). Within this study, these differences demonstrate LPS is a useful tool in (1) elevating an integrated immune response (French and Neuman-Lee, 2012) and (2) the experimental assessment of trade-offs between the reproductive and immune systems.

Female side-blotched lizards and other reptiles have previously exhibited stage-dependent responses following an immune challenge via wound administration (Pettit et al., 2019) and through exposure to a mitogen (Lind et al., 2020). As our results demonstrate, these responses can differ depending on progression within a specific reproductive process (i.e., vitellogenesis) and when comparing across different reproductive processes (i.e., vitellogenesis vs. ovulation).

Immune function can be highly variable depending on the season measured (Hudson et al., 2020; Palacios and Bronikowski, 2017), immune factor measured (Madelaire et al., 2021; Tylan and Langkilde, 2017), and organismal condition (Downs et al., 2014), among many other factors. Therefore, we are not surprised in finding substantial variation in bactericidal capacity of early- and mid-stage females treated with LPS. Because of this variation and due to poor model fit, we were unable to run parametric statistics to compare the interaction between treatment and vitellogenic state. Provided we were able to increase sample sizes within mid-stage females, we expect to observe substantial differences between treatments, similar to what was observed in early-stage females. In contrast, the lack of variation in response to LPS in late-stage females further supports these relationships are mediated by energetics, and that late-stage females are energetically constrained.

d-ROMs

We observed an overall effect of treatment on reactive oxygen metabolites (d-ROMs), such that females treated with LPS exhibited lower d-ROMs, irrespective of vitellogenic stage. These findings contradict the traditional idea that elevation of the immune system will elicit an increase in markers associated with oxidative damage through its importance in an organism's innate immune system (Sorci and Faivre, 2009). While the effects of LPS on oxidative stress are well-documented in human health and animal science research (De Filippis et al., 2007; Giri et al., 2020; Noworyta-Sokołowska et al., 2013)) in the context of ecoimmunological research, this relationship is less clear, especially in reptiles (Costantini, 2022). For example, Egyptian fruit bats (*Rousettus aegyptiacus*) injected with LPS elicited an inflammatory response without an effect on d-ROMS (Costantini et al., 2022). Marri and Richner (2015) similarly found no difference in d-ROMs after great tit nestlings were treated with LPS. In contrast, LPS elicited an increase in d-ROMs even at extremely low doses (0.01 mg LPS /in tree swallow nestlings (*Tachycineta bicolor*) (Butler et al., 2021) and in northern bobwhite quail (*Colinus virginianus*) (Armour et al., 2020). Despite the current uncertainty in this research area, there are a few potential co-occurring processes which may explain our results. First, an elevation of antioxidants in response to an immune challenge (Marri and Richner, 2015), or dietary intake of antioxidants (Schneeberger et al., 2014) may alleviate the deleterious effects of ROS. We found no significant effect of vitellogenic stage, treatment, nor their interaction, on plasma OXY levels, suggesting plasma antioxidant capacity did not impact d-ROMS. However, we cannot rule out other potential antioxidants not measured in this study. For example, vitellogenin, a yolk precursor, may offset the oxidative costs of vitellogenesis through functioning as an antioxidant post-ovulation (Lindsay et al., 2020). Because vitellogenin is directly linked to reproductive investment and antioxidant protection, incorporation of this measurement could further our understanding of the oxidative costs of reproduction.

Finally, an increase in metabolic rate may explain our findings in which d-ROMs were significantly lower overall in LPS treated females. While is commonly thought an increase in metabolic rate is directly related to an increase in oxidative stress (Harman, 1956), increases in metabolic rate downregulate the production of free radicals (Barja, 2007; reviewed in Speakman and Garratt, 2014; Speakman et al., 2004). Indeed, high metabolic rates were accompanied by low d-ROMs levels in grass snakes (*Natrix natrix*) acclimated to warm temperatures for six months (Bury et al., 2018). When comparing within vitellogenic stage, mid-stage females injected with LPS exhibited significantly lower d-ROMs, while d-ROMs from early- and late-stage females did not differ by treatment. However, the effect of treatment within early-stage females was marginally significant, which may suggest a similar trend in both early- and mid-stage females. A corresponding increase in CO_2 production further supports that lower d-ROMs may be directly related to higher metabolic rates from an immune challenge within early- and mid-stage females. Alternatively, LPS-treated females may be experiencing a suppression in reproductive investment. The oxidative costs of reproduction are well-documented across taxonomic groups, often resulting in an increase in oxidative damage markers (Bergeron et al., 2011; French et al., 2021; Pap et al., 2018; Webb et al., 2019). Simulating an infection in a reproductive female may temporarily halt reproductive investment and the inherent oxidative costs associated with it.

Conclusions

We believe these results are the first to demonstrate the energetic demands of reproduction can vary across vitellogenesis, and, when challenged with an immune-stimulant, exhibit stage-dependent physiological responses. A considerable amount of variation existed in immunological, metabolic, and oxidative responses to LPS in both early- and mid-stage females. Given this variation was absent in the respective PBS controls, inter-individual variation likely contributed to this; indeed, variation in body condition, fat stores, age, and previous reproductive investment are all important factors which influence tradeoffs between the reproductive and immune systems (Abrams and Miller, 2011; Downs et al., 2014; Durso and French, 2018; French et al., 2007; French et al., 2009; Gustafsson et al., 1994). While we were able to control for these factors through our experimental design, variation attributed to aspects of female quality should not be ignored, especially in the context of life-history theory (Hamel et al., 2009). Finally, the lack of a difference in physiological responses to PBS and LPS in late-stage females suggests late-stage females are energetically constrained, especially when considering their significantly lower metabolic rates at baseline. This work not only builds on the continual effort to better understand reproductive and immune tradeoffs in reptiles, but it also highlights the importance of accounting for reproductive stage when designing experiments within this research realm.

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CHAPTER 4 - EGG VIABILITY AND EGG MASS UNDERLY IMMUNE TRADEOFFS AND DIFFERENCES BETWEEN URBAN AND RURAL LIZARD EGG YOLK PHYSIOLOGY [1]

Abstract

Urbanization can cause innumerable abiotic and biotic changes that have the potential to influence the ecology, behavior, and physiology of native resident organisms. Relative to their rural conspecifics, urban Side-blotched Lizard (*Uta stansburiana*) populations in southern Utah have lower survival prospects and maximize reproductive investment via producing larger eggs and larger clutch sizes. While egg size is an important predictor of offspring quality, physiological factors within the egg yolk are reflective of the maternal environment and can alter offspring traits, especially during energetically costly processes, such as reproduction or immunity. Therefore, maternal effects may represent an adaptive mechanism by which urban-dwelling species can persist within a variable landscape. In this study, we assess urban and rural differences in egg yolk bacterial killing ability (BKA), corticosterone (CORT), oxidative status (d-ROMs), and energy metabolites (free glycerol and triglycerides), and their association with female immune status and egg quality. Within a laboratory setting, we immune challenged urban lizards via lipopolysaccharide injection (LPS) to test whether physiological changes associated with immune system activity had an impact on egg yolk investment. We found urban females had higher ectoparasite loads than rural females, however ectoparasite status was related to yolk BKA in rural but not urban eggs. While

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yolk BKA differed between urban and rural sites, egg mass and egg viability (fertilized vs. unfertilized) were strong predictors of yolk physiology and may imply tradeoffs between maintenance and reproduction. LPS treatment caused a decrease in egg yolk d-ROMs relative to the control treatments, supporting results from previous research. Finally, urban lizards laid a higher proportion of unfertilized eggs, which differed in egg yolk BKA, CORT, and triglycerides in comparison to fertilized eggs. Because rural lizards laid only viable eggs during this study, these results suggest that reduced egg viability is a potential cost of living in an urban environment. Furthermore, these results help us better understand potential downstream impacts of urbanization on offspring survival, fitness, and overall population health.

Introduction

Life history theory suggests that organisms must invest their finite energetic resources into multiple competing life history traits and will result in a negative association between two or more components if resources are limited (Stearns, 1989). As a consequence, optimal allocation of resources to life history traits, such as reproduction or maintenance, have likely evolved to follow patterns associated with lifespan to maximize reproductive fitness (Williams, 1966). Resources allocated to reproduction and maintenance come from the same pool of reserves (Durso and French, 2018; Pettit et al., 2019) and, as a result, can cause energetic deficits in one or both systems (French and Moore, 2008; Hegemann et al., 2013). Elevated production of immune system components in response to parasitism or infection is not only energetically costly but can impact offspring provisioning and quality (Criscuolo et al., 2020). For example, maternal transfer of pathogen-specific antibodies may alleviate the energetic costs of mounting an immune response in some species (Grindstaff, 2008), but can be dependent on the immune factor (Fassbinder-Orth et al., 2019; Madelaire et al., 2021), life-history strategy (Fassbinder-Orth et al., 2019; Garnier et al., 2013), or species (Garnier et al., 2013). Consequently, balance in energetic investment into current stressors while allocating resources toward reproduction is crucial to maximize current survival prospects as well as that of developing offspring.

Maternal physiological condition during pre-natal offspring development can have profound influences on offspring traits (Grindstaff et al., 2003; Hasselquist and Nilsson, 2009; Moore et al., 2019; Mousseau and Fox, 1998; Parolini et al., 2019) and has therefore received considerable attention in life history evolution and ecological research. Changes in maternal glucocorticoids (Miltiadous and Buchanan, 2021; Owen et al., 2018; Weber et al., 2018), immune function (Gomez-Chamorro et al., 2019; Grindstaff, 2008), and oxidative status (Parolini et al., 2019; Possenti et al., 2018), directly impact factors invested into egg yolk (Gomez-Chamorro et al., 2019; Miltiadous and Buchanan, 2021) and influence post-natal offspring physiology and development (Parolini et al., 2019; Possenti et al., 2018). Consequently, altered maternal physiological state in response to environmental cues during reproduction may affect a multitude of traits associated with survival and fitness (Haussmann et al., 2012; Liu et al., 2020; Owen et al., 2018; Sparks et al., 2020; Weber et al., 2018), and influence population trends and dynamics (Bernardo, 1996; Marshall and Uller, 2007; Mousseau and Fox, 1998).

The novel environmental conditions caused by unpredictable climate fluctuations and rapid expansion of anthropogenic landscapes pose challenges to maternal survival and reproduction (Angelier and Wingfield, 2013). Unpredictable fluctuations in climate, coupled with simultaneous rapid expansion of an anthropogenic landscape, are altering community composition, introducing non-native predators and diseases, and causing changes in resource availability (Bradley and Altizer, 2007). In response to these challenges and everyday challenges, vertebrates activate the stress response and the subsequent downstream release of glucocorticoids (Angelier and Wingfield, 2013). Glucocorticoids are a class of steroid hormones which aid in the mobilization of stored energy in response to external stressors or demands (Sapolsky et al., 2000). Though this cascade of physiological changes is crucial for survival, it is not without costs. In oviparous animals, maternal transfer of corticosterone (CORT) to the yolk can directly affect offspring body condition (Owen et al., 2018), growth (Hayward and Wingfield, 2004), and physiology (Haussmann et al., 2012), both positively and negatively.

Maternal physiological state is highly sensitive to environmental perturbations and the resulting change in physiological exposure or investment can modify offspring phenotypes (Meylan et al., 2012). However, maternal physiological effects may mitigate the detrimental effects of climate change and/or anthropogenic disturbance. For example, experimental warming during offspring development conferred positive effects on hatchling growth of female Jacky Dragons (Eremias multiocellata) reared in the same warming treatment (Hao et al., 2021). In a different study, offspring from food-limited female brown anoles (Anolis sagrei) that also developed in food-limited environments had higher survival (Warner et al., 2015). Maternal effects via “environmental matching” may represent a key mechanism allowing for the persistence of species in the face of anthropogenic change (Breuner, 2008). To better understand the costs of urbanization on egg and offspring quality, multivariate assessments of egg morphometry and physiology are crucial in predicting the persistence of increasingly urbanized species.

We employed a multi-tiered approach to assess the effects of urbanization and an immune challenge on female and egg yolk physiology in different populations of an oviparous species of reptile. The Side-blotched Lizard (*Uta stansburiana*) is an abundant species of phrynosomatid lizard occupying wide-ranging environments in the western United States (Tinkle, 1967). Side-blotched Lizard populations occupying urban and rural areas in southwest Utah exhibit differences in life-history strategies; compared to their rural counterparts, urban lizards maximize reproductive investment at a cost of reduced survival and immune function (Lucas and French, 2012), alter reproductive investment when immune challenged (Durso and French, 2018), and have lower survival estimates (Lucas and French, 2012). First, we determined whether urbanization was associated with female condition and reproductive investment by comparing the effect of “site type” on female ectoparasite number, baseline physiology, and clutch size and egg quality (i.e., egg mass & egg viability). Second, we tested whether urbanization, mite counts, and egg quality impacted egg yolk physiology across urban and rural populations. Finally, we tested the effect of maternal immune challenge on egg yolk physiology. As an extension of findings from Lucas and French (2012), we hypothesized that egg yolk physiological metrics would differ between urban and rural Side-blotched Lizard eggs and urban females would produce larger clutches and eggs. We also predicted yolk CORT and oxidative index would be higher in urban eggs because of higher CORT responsiveness in urban lizards and its marked effects on oxidative indices (Lucas and French, 2012). We hypothesized that changes in egg yolk physiological metrics following an immune challenge would depend on female treatment. Immune factors, glucocorticoids, and energetics can elevate in response to LPS (Gardner et al., 2020; Xu et al., 2020) and prior work in this model species may suggest decreases in oxidative status following an immune challenge (Virgin, in prep). We predicted yolk BKA, CORT, and energy metabolites would elevate in response to an immune challenge, and oxidative indices would be lower in females given LPS injections during vitellogenesis.

Methods

Field collection

Using snare poles, we collected a total of twenty-eight (N = 28) female *Uta stansburiana* from two urban sites (n = 18) and one rural site (n =10) in Washington County, Utah, USA, between 11 May 2019 and 15 June 2019, during peak breeding season. All collected individuals were considered reproductive – in which they were either vitellogenic (yolked follicles) or gravid (shelled eggs) – and later confirmed via palpation of the abdomen. Upon capture, we assigned each lizard a unique ID using a non-toxic paint marker and stored individuals in opaque and breathable cloth bags made of nylon. We transported females to Utah State University (USU) within 72 hours of capture for subsequent processing and to await oviposition. Upon completion of this experiment, all individuals were returned to their location of capture. All methods previously described for capture and sample collection were permitted under Utah State Department of Wildlife Resources (COR #1COLL8382), and field sites used for individual collection were previously established within this lab (Durso and French, 2018; Hudson et al., 2021; Lucas and French, 2012; Pettit et al., 2019; Smith and French, 2017; Smith et al., 2019).

Laboratory housing

Female Side-blotched Lizards were housed in cage racks within the Laboratory Animal Research (LARC) facility at Utah State University. Upon arrival, we measured mass (mean s.e.m.; 4.02 g ± 0.12 g), snout-vent-length (SVL; 45.95 mm ± 0.30 mm) and confirmed reproductive status of each female using an ultrasound, in which we measured clutch size (4.29 eggs ± 0.13) and of each follicle or egg (largest follicle; 0.80 mm ± 0.02 mm). Due to differences in ectoparasite loads between sites and their previously established effect on immune function, we also quantified the number of scale mites (*Geckobiella texana*) on each lizard using a hand lens (Spence et al., 2017). Scale mites were commonly found in folds around the neck, legs, and tail, but can also be found in between the digits of Side-blotched Lizards. Following morphometric and reproductive data collection, we assigned females from urban sites into three different treatment groups: Lipopolysaccharide (LPS) injection, control (Phosphate buffered saline; PBS) injection, or no injection (NI). We assigned females to their respective treatment based on SVL, clutch size, and largest follicle length (mm) to minimize variation from female size, age and vitellogenic stage.

Each female was housed individually in a plastic terrarium (30 x 45 x 15 cm), containing a shelter made from PVC pipe, a water dish, heat tape, and a UV bulb operating on a 14L:10D photoperiod. Mean room temperature was 23°C, which is commonly experienced by *U. stansburiana* within the breeding season (Goller et al., 2014), apart from a more pronounced decrease in ambient temperature at night. This set temperature is below their optimal temperature of 37°C (Goller et al., 2014), which allows animals to thermoregulate within their cages using their heat lamp and heat strip. To prevent egg desiccation following oviposition, we added 2 inches of a 1:1 moist sand/peat moss mixture to the bottom of each container, while maintaining a dry substrate on the opposite side of the cage. Lizards were fed three, calcium-dusted crickets (ranging between 0.15 – 0.20 g in total mass) (3/4 in, Fluker Farms, Port Allen, Louisiana, USA) every 72 hours. We did not record food intake due to complications in detecting crickets within the substrate mixture.

Following oviposition, females were offered food and water *ad libitum* until returned to their site of capture. If individuals failed to progress in vitellogenesis within two weeks of initial housing, we returned them to their site of capture with no further data collection. All procedures were approved prior to this study by the USU Institutional Animal Care and Use Committee (protocol #2068).

Administration of LPS and PBS to Urban Female Side-blotched Lizards

Females were administered their assigned treatments 48 hours after they were housed in the LARC. To simulate an innate immune response, we injected females with reconstituted lipopolysaccharide (LPS; serotype 0127:B8, Sigma-Aldrich, St Louis, MO, USA). LPS is a cell wall component of gram-negative bacteria and elicits a strong immune response (Demas et al., 2011). Females were given either a mass-dosed injection (20µl /1g body mass) of LPS (15µg lyophilized LPS/20µl PBS); or an equivalent volume of phosphate buffered saline (PBS tablet; Sigma). This dosing protocol was previously validated in Side-blotched Lizards and was found to increase in plasma bactericidal ability (Hudson et al., 2021). Females assigned to the no injection (NI) group were handled for the same duration and promptly returned to their assigned terraria.

Egg monitoring, collection, and extraction

Because we were unable to regulate relative humidity within the facility, we checked for viable eggs every 90 to 120 min between 0800 and 1900 h to minimize risk of egg desiccation (which can occur within an hour or two of oviposition). Egg monitoring consisted of briefly glancing within each cage (without disturbing females) for eggs or signs of oviposition, such as a female exhibiting digging behaviors, or a mound of overturned substrate. Upon discovery, eggs were collected and prepared for yolk sample collection according to protocols developed in Virgin et al. (2022).

We collected morphometric data and yolk samples from sixty eggs (n = 60) in total, in which we randomly selected two to three viable eggs within a given clutch (n = 51), and any unfertilized eggs (n = 9), while the remaining eggs were retained for use in additional laboratory studies. Because a significant proportion of clutches had one or more unfertilized eggs (lacking a circular aggregate of blood cells, and possessing an underdeveloped shell), we noted which eggs were unfertilized for later use in statistical analyses. Before collecting egg yolk samples, we measured egg mass (0.22 g ± 0.04 g), length (10.97 mm ± 0.70 mm), and width (6.30 mm ± 0.43 mm), using a digital scale (± 0.2 g, model HH120D, Ohaus, FL, USA) and digital calipers (± 0.01 mm, model #501601, World Precision Instruments, NJ, USA). To minimize bacterial contamination, we rinsed eggs with autoclaved DI water, a 10% bleach solution, and then rinsed with DI water a second time before collecting yolk samples.

Because eggs from this research project were used in multiple studies, we did not collect the entire yolk contents. We extracted egg yolk samples by making a small incision with sterile surgical scissors into the eggshell. With a sterile, 1000μL pipette and tip, we pipetted yolk samples into sterile Eppendorf tubes with pre-recorded masses and suspended egg yolk samples in PBS at a 2:5 dilution to control for differences in collection volumes. After processing, we stored egg samples in a -80°C freezer until later use in physiology assays.

Physiological Assays

Bactericidal capacity (BKA)

We compared differences in egg yolk innate immune function by performing a bactericidal assay (BKA), which tests the ability of immune factors within a biological sample to restrict growth of an ecologically relevant microbe (French and Neuman-Lee, 2012). In summary, we pipetted 6μL of sample, 14μL of CO_2-dependent media, and 4μL of 10^4 working *E. coli* solution (ATCC #8739; Microbiologics, St. Cloud, MN) in duplicate onto a 96-well plate and incubated for 30 minutes. Following, we added 125μL tryptic agar soy broth and measured the change in absorbance at 300nm on a spectrophotometer (xMark, Bio-Rad, California, USA) before and after a 12-hour incubation as a proxy for bacterial killing. We calculated percent (%) killing by subtracting the average sample absorbance from 1 and dividing sample absorbance by absorbance of the positive control (0% killing). Intra-assay coefficient of variation (CV) of the positive controls was between 5.7% and 12.7%. Inter-assay CV was 11.1%.

Radioimmunoassay and validation for corticosterone (CORT)

Following established protocol (French et al., 2010; Moore, 1986), we quantified egg yolk CORT levels using an in-house radioimmunoassay (RIA). RIAs indirectly quantify hormone levels through competitive binding of endogenous hormone and a known amount of radiolabeled hormone (CORT; Perkin Elmer, LOT#2178418) to an anti-CORT antibody (MP Biomedicals., LOT#3R3-PB17). Briefly, we extracted hormone from 40µL of diluted egg yolk samples (in duplicate) and let sit overnight at 4°C. For lipid separation, we snap froze samples after adding 4mL of a 30% ethyl acetate:isooctane solution and dried down the supernatant in a separate glass tube using N_2. Samples were resuspended in 500µL of 1X PBSg, vortexed, and left overnight again. The next day, 200µL of sample was then assayed in duplicate with individual recoveries using 100µL anti-corticosterone antibody (MP Biomedicals 07120016) and 100µL corticosterone label (Corticosterone, [1,2,6,7-3H(N)]-, 250µCi (9.25MBq), MP Biomedicals NET399250UC). To remove unbound CORT, we added 500µL of half strength charcoal solution, consisting of PBS (gelatin-free), charcoal and dextran (Sigma-Aldrich D8821), before centrifuging at 2200 RPM at 4°C. Finally, we decanted the supernatant into scintillation vials, then added 3.5mL of scintillation fluid to all samples, recoveries, standard curve, and controls and then loaded sample vials into a scintillation counter. Data from duplicates were averaged and then adjusted based on yolk volume, controls, standards, and individual recoveries for the final concentration of corticosterone in yolk samples. The minimum detectability of this assay was 0.15 ng/mL and fell within the standard curve of this assay.

We validated measuring CORT in Side-blotched Lizard egg yolks prior to running a complete assay, via assay spikes, recovery tests, parallelism curves, and a serial dilution of egg yolk volumes at 40µL, 60µL, 80µL and 100µL, in which assay linearity was high (R^2=0.96). To test for binding interference, we spiked samples with low and high CORT standard concentrations from our standard curve and tested expected versus actual resulting values to calculate percent recovery. The average percent recovery of the validation was 81%.

Reactive oxygen metabolites (d-ROMs)

To detect markers of oxidative stress, we measured reactive oxygen metabolites in egg yolk samples using the d-ROMs kit (Diacron, Grosseto, Italy) following validated protocol for Side-blotched Lizard plasma (Lucas and French, 2012) and egg yolk samples (Virgin et al., 2022). This assay kit indirectly measures the presence of organic hydroperoxides, which can indicate oxidative damage to lipid or protein molecules (Costantini, 2016). Following “end-point mode” protocol in the kit instructions, we pipetted 5µL of egg yolk sample and 100µL of the provided acidic buffer (1:10 R_1/R_2) solution in duplicate onto a 96-well plate. Plates were incubated for 90 minutes at 37°C, gently vortexed, and read at 505 nm on a spectrophotometer. CARR Us (Carretelli Units) were calculated by multiplying the change in sample absorbance relative to the standard by the calibrator concentration. We converted these values into mg H_2O_2/dL by multiplying CARR U by 0.08. The intra-assay CV of the calibrator was between 1.8 and 3.6%, and 3.3% across two microplates and the inter-assay CV was 1.6%.

Energy metabolites

We quantified triglyceride and free glycerol concentrations in egg yolk samples using a colorimetric endpoint assay (F6428; T2449; G7793; Sigma-Aldrich, Missouri, USA). We reconstituted reagents following the manufacturer's instructions and modified the protocol for use on a microplate (Webb et al., 2019). Briefly, we pipetted 5µL of diluted egg yolk sample in duplicate into a 96-well plate with 240µL prewarmed free glycerol reagent and incubated plates at 37°C for 5 minutes. After incubation, we gently vortexed each plate for 60 seconds and measured the absorbance at 540 nm to quantify free glycerol. To quantify triglycerides, we added 60µL of prewarmed triglyceride reagent to each well and incubated plates at 37°C for 5 minutes. After a 60 second vortex, we read the plate a second time at 540 nm.

Data curation and analysis

All statistical analyses were performed in R CRAN Statistical Software (R Core Team, 2022). We log-transformed CORT data to fit assumptions of normality and improve model fit. When performing linear mixed effects models, we used lme4 and LmerTest packages (Bates et al., 2015; Kuznetsova et al., 2017). We conducted post-hoc analyses using the emmeans package (Lenth, 2022). All graphics used were created in R using ggplot2 (Wickham, 2016), and ggpubr (Kassambara, 2020). We assessed model fit through visual inspection of the model residuals and by comparing marginal and conditional R^2 values of each model using sjPlot packages (Lüdecke, 2022). For all analyses, we set an alpha-level of $P = 0.05$.

Statistical analysis: urban and rural differences in female condition and reproduction

We performed separate one-way ANOVAs to determine the effect of site type on female condition, immune status, and reproduction. To test whether the proportion of fertilized vs. unfertilized eggs was significantly different between urban and rural sites, we performed a Fisher's Exact Test, which is a commonly employed method when testing proportions from small sample sizes.

Statistical analysis: urban and rural differences in egg yolk physiology and viability

When making comparisons between urban and rural eggs, we omitted eggs from females treated with LPS and unfertilized eggs to control for potential confounding effects of treatment and egg viability. We included eggs from urban females injected with PBS because egg yolk physiology was not significantly different from individuals receiving no injection.

We performed linear mixed effects models to assess the effect of urbanization and egg mass on egg yolk physiology and included Maternal ID as a random intercept. Under a model selection framework, we compared AICc values to determine whether the individual, additive, and interactive models of site type and egg mass better predicted egg yolk physiology than the null model. Apart from including site type, we included egg mass as a fixed effect because it is an important predictor of egg yolk BKA and d-ROMs in Side-blotched Lizard eggs (Virgin et al., 2022). We considered models to be significantly different from the null model if the AICc was more than two values different from the null model. Once determining the best model fit, we performed post-hoc comparisons to assess individual importance of each fixed effect. Due to the absence of unfertilized eggs from rural sites, we ran separate analyses using eggs from urban sites to determine the effect egg viability on egg yolk physiology while controlling for individual egg mass.

Statistical analysis: ectoparasites, immune challenge and egg yolk physiology

We performed linear mixed effects models to assess the effect of female ectoparasites or treatment on egg yolk physiology after controlling for egg mass and used an identical random effects structure. We ran separate analyses within each site type to determine the effect of female ectoparasites on egg yolk physiology while controlling for individual egg mass. When assessing the effect of treatment on urban lizard egg yolk physiology, we omitted eggs originating from our rural sites since rural females were not part of this experimental study. To validate the efficacy of the LPS injection, we performed a one-way ANOVA comparing the effect of treatment on female BKA.

Results

Urban and rural differences in female condition and reproduction

We found that female snout-vent-length (SVL), and clutch size were not significantly different between urban and rural sites (ANOVA; $p > 0.21$). Urban females had significantly higher ectoparasite loads than rural females (ANOVA; $F = 8.7976$; $p = 0.0079$; Fig. 8), but mites were not associated with female BKA or site type ($t < 0.616$; p

> 0.545). Finally, we found that the proportion of fertilized and unfertilized eggs was significantly different between urban and rural sites (Fisher's Exact Test; n = 93; p = 0.0193), such that urban lizards laid a higher proportion of unfertilized eggs than rural sites (See Fig. 9).

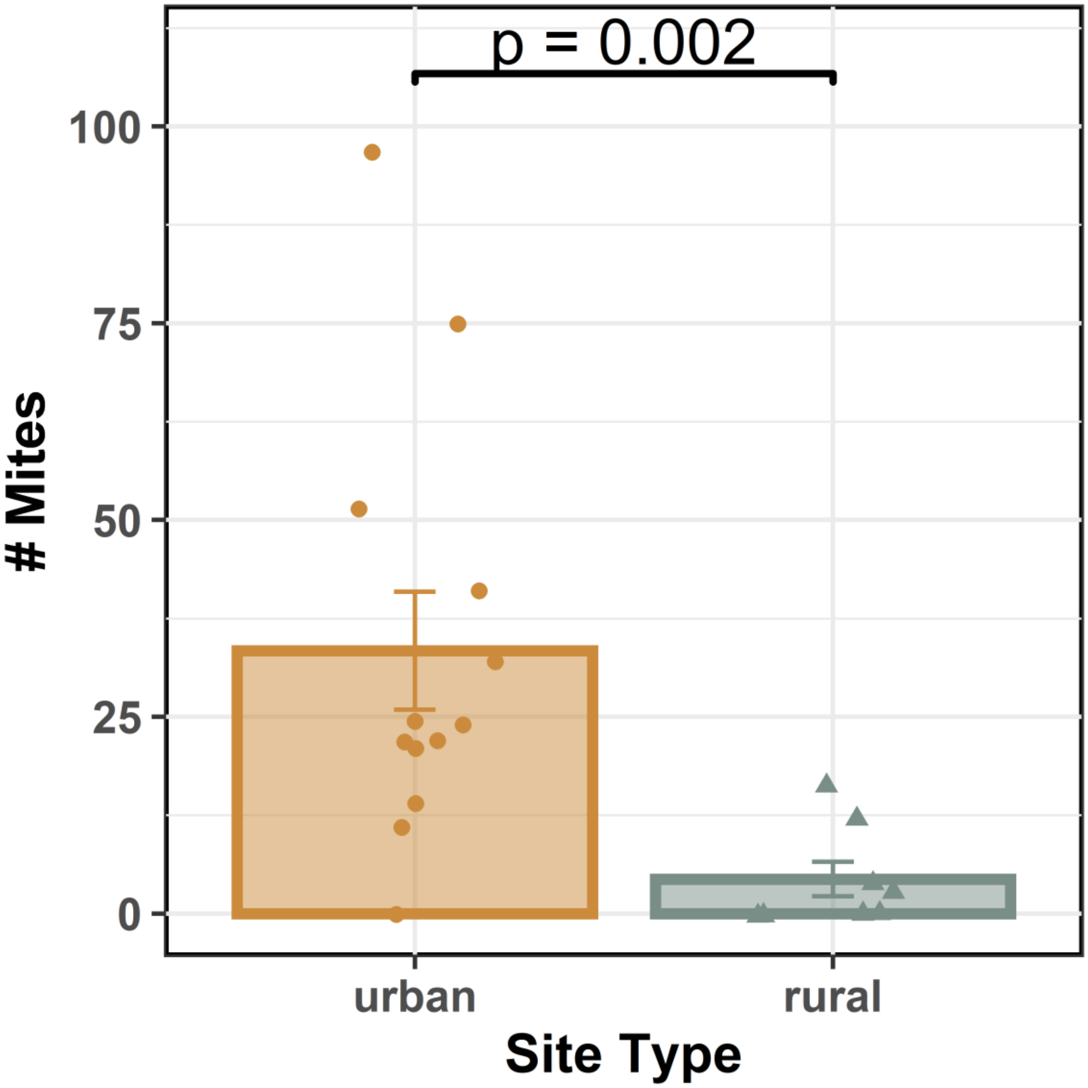

Figure 8. Urban and rural differences in the number of mites (*Geckobiella texana*) on female Side-blotched Lizards.

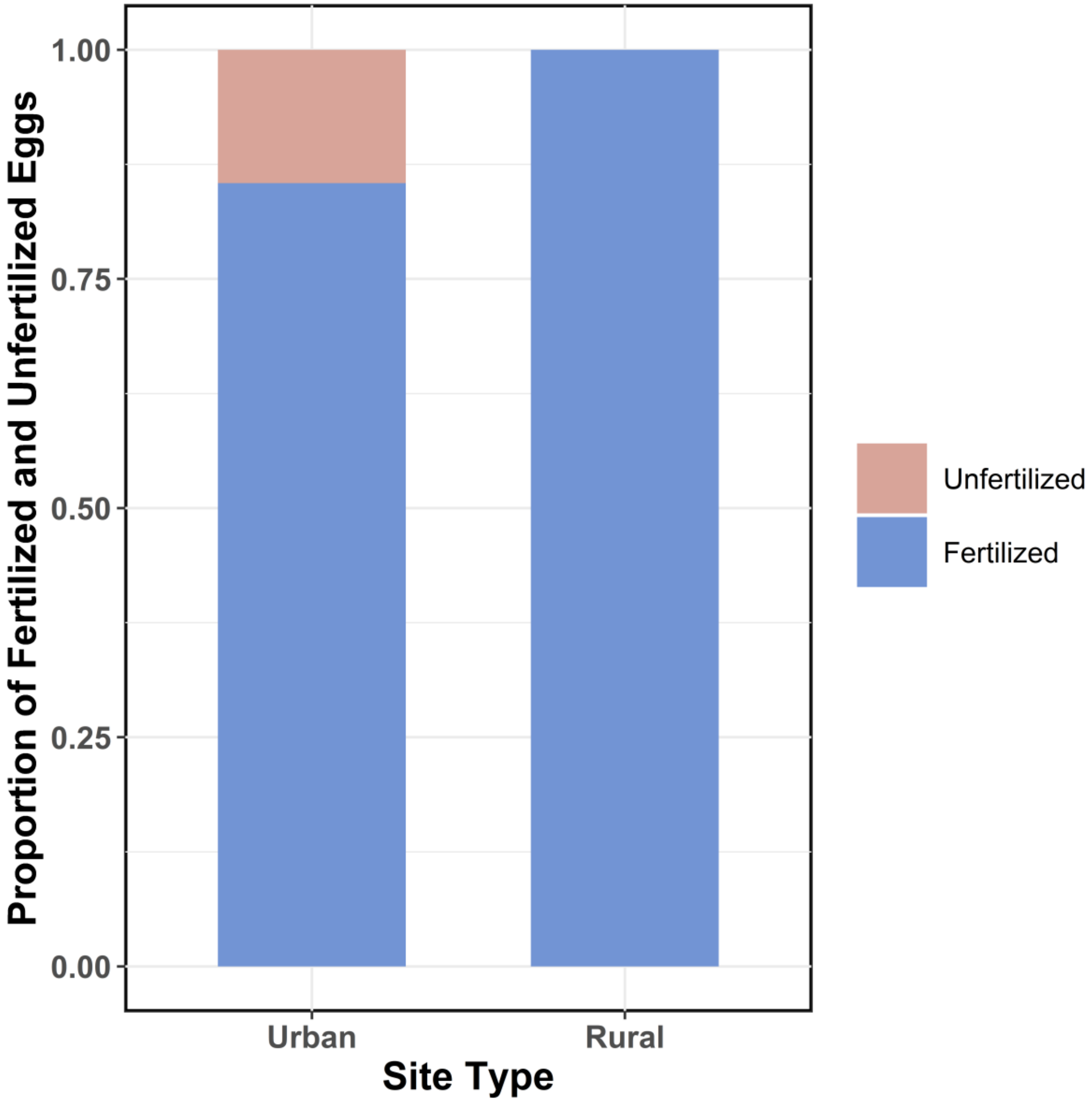

Figure 9. Urban and rural differences in the proportion of unfertilized eggs.

Urban and rural differences in egg yolk physiology and viability

Candidate models and reported AICc and log-likelihood estimates are reported in Table 7. A summary of the model parameters and regression coefficients for each candidate model variable is described in Table 8. Site type and egg mass were both important predictors for egg yolk BKA and had the lowest AICc (Table 7). When performing post-hoc comparisons, there were marginal differences in immune function between urban and rural eggs (t = 1.99; p = 0.062; Fig. 10) and a slight negative relationship with egg mass (t = -1.61; p = 0.12). Egg mass was an important predictor of egg yolk d-ROMs (t = -3.397; p = 0.00375; Fig. 11), free glycerol (t = -2.77; p = 0.011; Fig. 11), and true triglycerides (t = -3.029; p = 0.00616). While the candidate model for CORT included egg mass, there was a marginal negative association between egg mass and yolk CORT (t = -1.649; p = 0.11; Fig. 11). When controlling for egg mass, unfertilized eggs had higher BKA (t = 2.13; p = 0.0436; Fig. 12), CORT (t = 4.71; p = 0.0001; Fig. 12) and free glycerol (t = 2.45; p = 0.022; Fig. 12) than fertilized eggs within urban sites. Egg yolk d-ROMs and triglycerides were not significantly different between unfertilized and fertilized eggs (t > -1.642; p > 0.113).

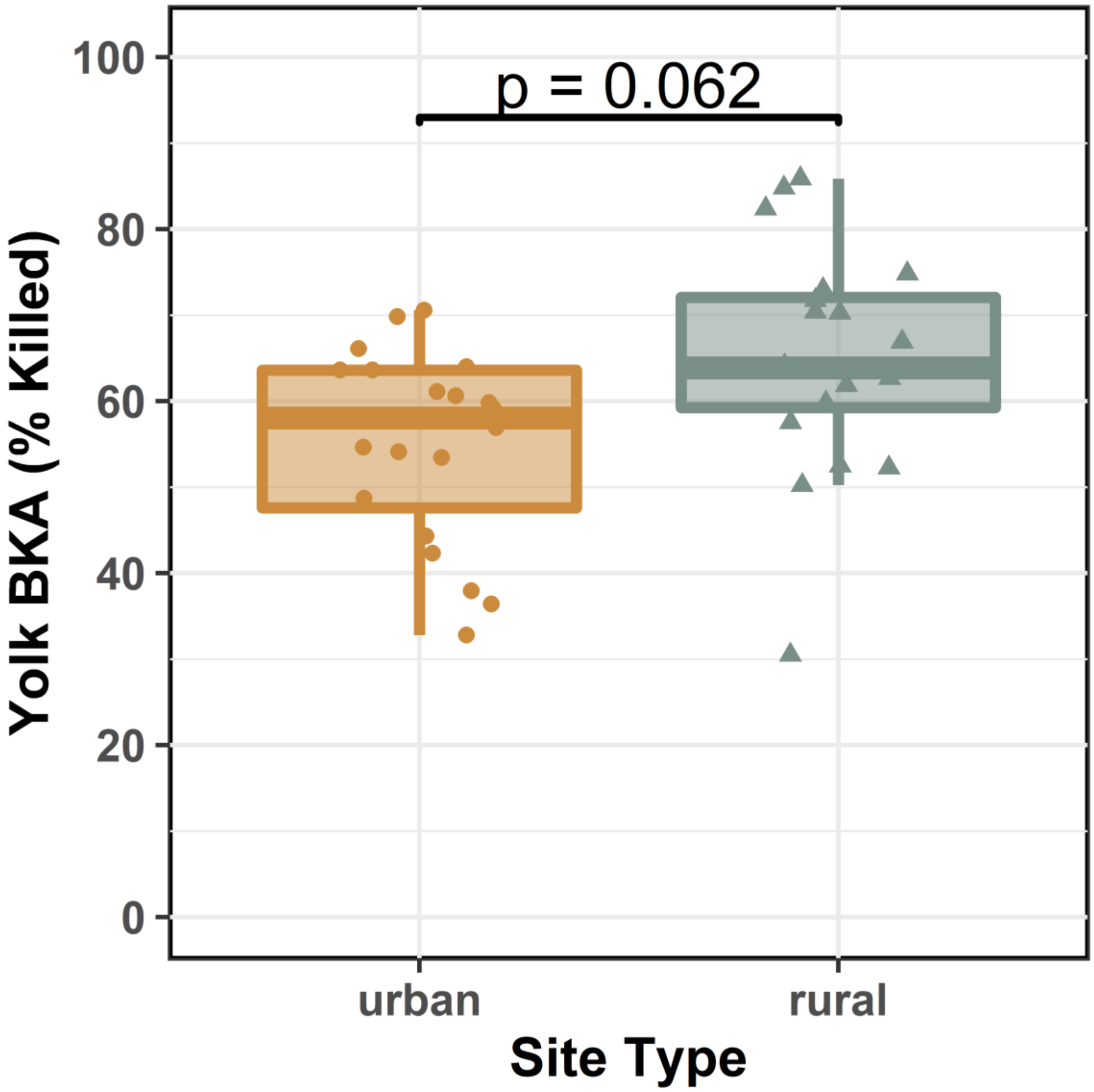

Figure 10. Urban and rural differences in the Side-blotched Lizard egg yolk bacterial killing ability (BKA).

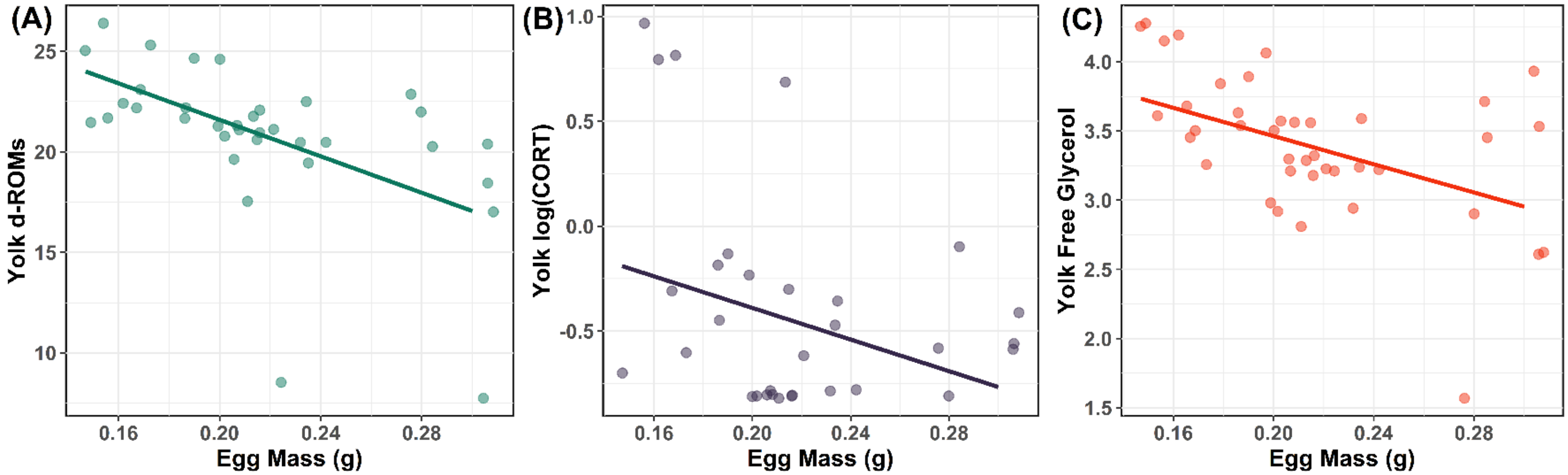

Figure 11. The relationship between egg mass and egg yolk (A) d-ROMs, (B) CORT, and (C) free glycerol of fertilized eggs from both urban and rural sites included.

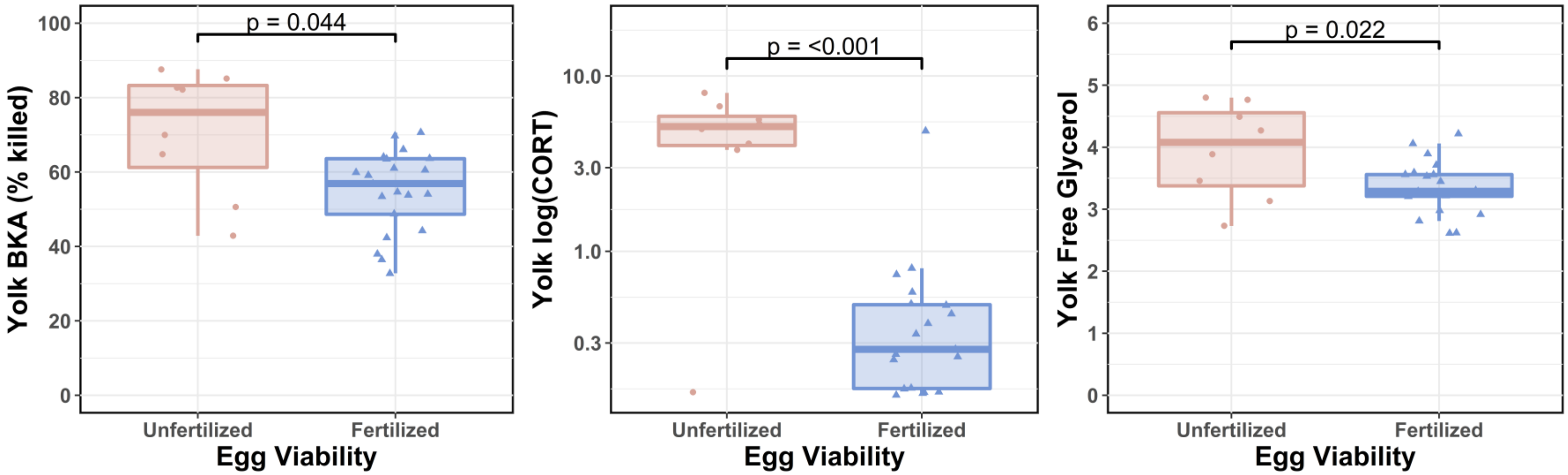

Figure 12. The effect of egg viability (unfertilized vs. fertilized) on egg yolk physiology.

There was a marginal, negative relationship between egg yolk BKA and female ectoparasite load in rural eggs ($t = -2.074$; $p = 0.0807$; Fig. 13). Bactericidal Capacity in females was significantly influenced by treatment (One-Way ANOVA; $F = 5.7405$; $p = 0.0091$), and females treated with LPS had significantly higher BKA than PBS and NI treatment groups. When controlling for egg mass, BKA was not significantly different between treatments ($t = 1.635$, $p = 0.122$). However, egg yolk d-ROMs were significantly different between treatments ($t = 3.477$, $p = 0.0035$; Fig. 14), such that eggs from LPS-treated females were significantly lower than eggs from control females. Free glycerol and triglycerides were not significantly different between treatments ($t < 1.094$; $p > 0.28$).

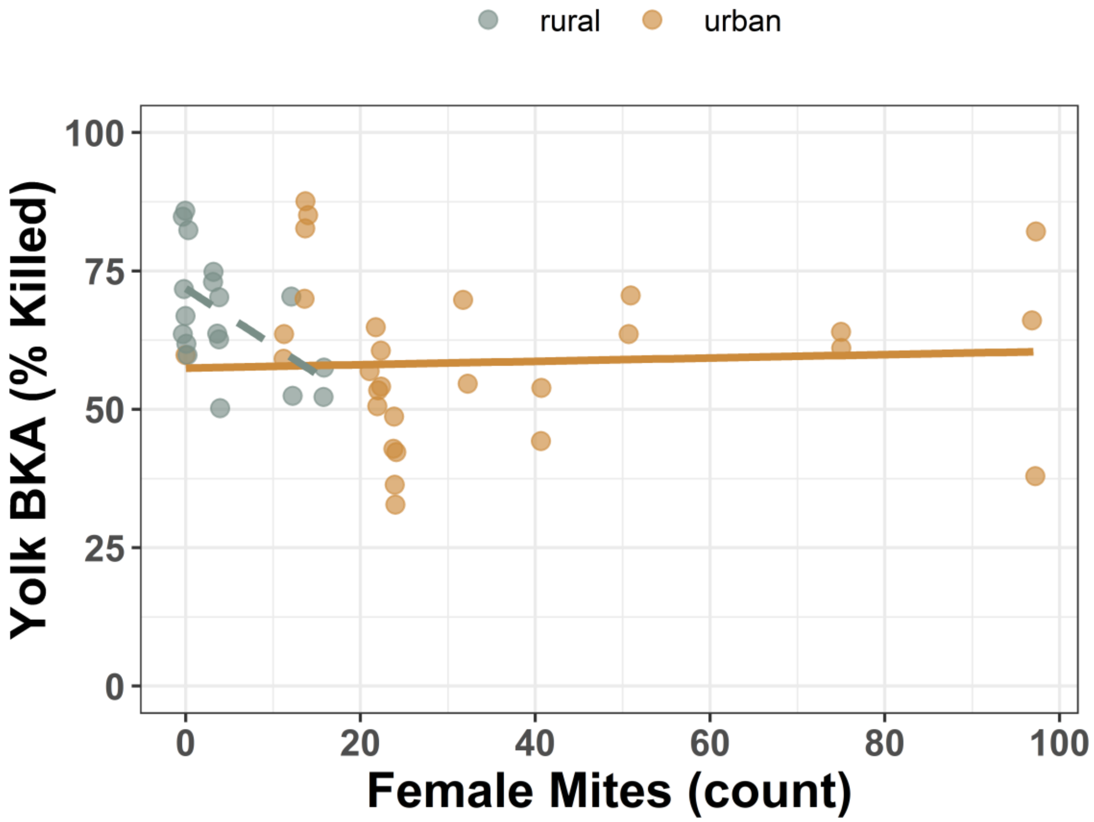

Figure 13. The relationship between female mites and bacterial killing ability of yolk from urban and rural eggs.

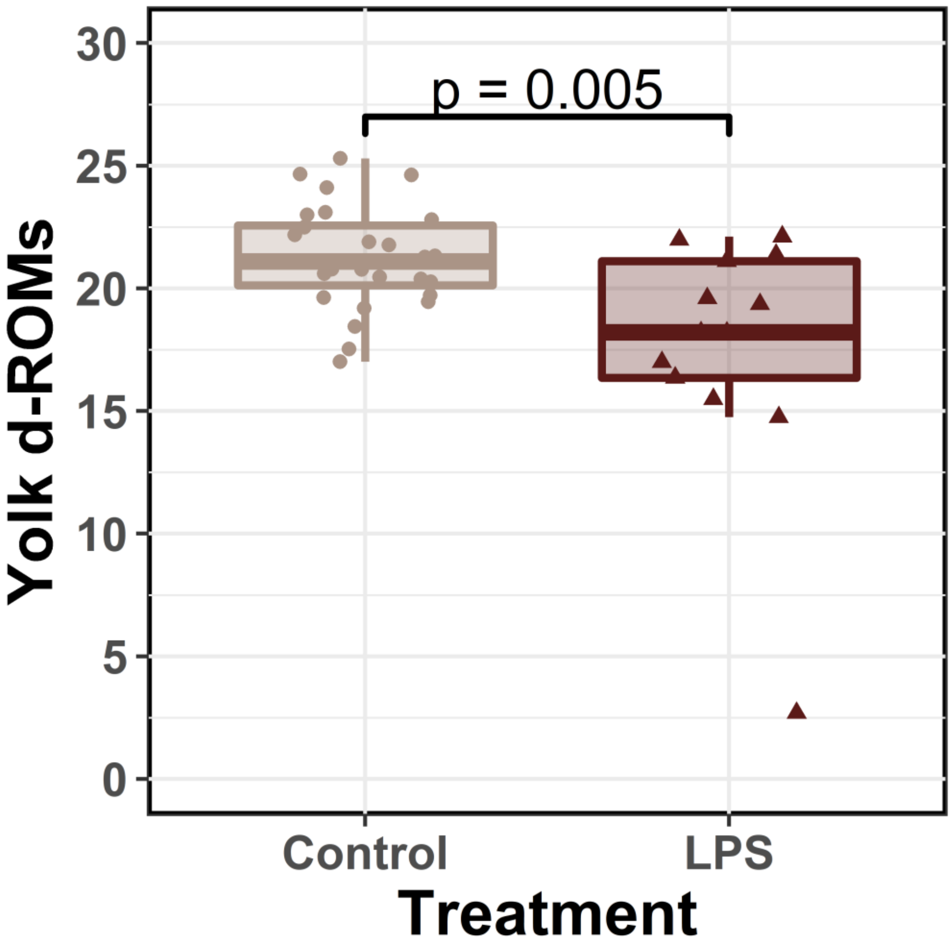

Figure 14. The effect of control or lipopolysaccharide (LPS) treatment on Side-blotched Lizard egg yolk d-ROMs.

Table 7. Results of Model Selection Procedure used to determine model fit of site type, egg mass, and the additive and interaction model.

		BKA				**d-ROMs**				**log(CORT)**				**Free Glycerol**				**Triglycerides**			
	K	**AICc**	**$_{\Delta}$AICc**	**$_{w}$AICc**	**LL**	**AICc**	**$_{\Delta}$AICc**	**$_{w}$AICc**	**LL**	**AICc**	**$_{\Delta}$AICc**	**$_{w}$AICc**	**LL**	**AICc**	**$_{\Delta}$AICc**	**$_{w}$AICc**	**LL**	**AICc**	**$_{\Delta}$AICc**	**$_{w}$AICc**	**LL**
Null Model	3	319.59	2.73	0.09	-156.46	197.32	8.01	0.01	-95.27	47.29	0.07	0.36	-20.20	55.23	4.52	0.06	-24.28	42.38	5.84	0.03	-17.86
Site Type	4	317.18	0.32	0.30	-154.02	199.80	10.49	0.00	-95.23	49.89	2.67	0.10	-20.18	56.85	6.13	0.03	-23.85	42.96	6.42	0.02	-16.91
Egg Mass	4	318.43	1.57	0.16	-154.64	**189.31**	**0.00**	**0.60**	**-89.99**	47.22	0.00	0.38	-18.84	**50.71**	**0.00**	**0.60**	**-20.78**	**36.54**	**0.00**	**0.52**	**-13.70**
Site Type + Egg Mass	5	**316.86**	**0.00**	**0.35**	**-152.55**	191.05	1.75	0.25	-89.49	50.05	2.84	0.09	-18.83	52.95	2.24	0.20	-20.59	38.02	1.48	0.25	-13.13
Site Type * Egg Mass	6	319.48	2.62	0.10	-152.47	192.40	3.09	0.13	-88.70	50.50	3.29	0.07	-17.50	54.19	3.48	0.11	-19.82	38.70	2.16	0.18	-12.08

Table 8. Summary table of candidate linear mixed effect models used for model selection of Side-blotched Lizard egg yolk physiological parameters.

	Null Model				Site Type				Egg Mass				Additive				Interaction			
BKA	**B**	**S.E.**	***t***	***p***	**B**	**S.E.**	***t***	***p***	**B**	**S.E.**	***t***	***p***	**B**	**S.E.**	***t***	***p***	**B**	**S.E.**	***t***	***p***
Intercept	59.57	2.52	23.64	<0.001	65.18	3.41	19.14	<0.001	80.20	10.99	7.30	<0.001	81.49	10.40	7.83	<0.001	78.27	14.03	5.58	<0.001
Site Type					-10.32	4.64	-2.23	0.032					-8.95	4.49	-2.00	0.054	-1.63	21.62	-0.08	0.940
Egg Mass									-95.08	49.62	-1.92	0.063	-78.77	47.71	-1.65	0.108	-63.17	65.80	-0.96	0.344
Site Type * Egg Mass																	-33.89	97.59	-0.35	0.730
Residual σ^2	87.91				86.85				90.50				87.73				88.62			
Female ID σ^2	84.18				63.91				64.35				53.15				56.71			
Marginal R^2 /Conditional R^2	0.000 / 0.489				0.153 / 0.512				0.113 / 0.482				0.229 / 0.520				0.225 / 0.528			
d-ROMs																				
Intercept	20.78	0.75	27.58	**<0.001**	20.46	1.22	16.73	**<0.001**	30.67	2.98	10.30	**<0.001**	30.59	3.04	10.07	**<0.001**	34.11	4.16	8.20	**<0.001**
Site Type					0.54	1.61	0.34	0.740					1.27	1.32	0.96	0.345	-6.11	6.20	-0.99	0.332
Egg Mass									-45.38	13.36	-3.40	**0.002**	-48.36	13.83	-3.50	**0.001**	-65.34	19.43	-3.36	**0.002**
Site Type * Egg Mass																	34.11	27.91	1.22	0.231
Residual σ^2	9.39				8.85				7.97				7.68				7.27			
Female ID σ^2	5.57				7.08				3.09				3.56				3.99			
Marginal R^2 /Conditional R^2	0.000 / 0.372				0.005 / 0.447				0.294 / 0.491				0.303 / 0.523				0.333 / 0.569			
CORT																				
Intercept	-0.45	0.10	-4.44	**<0.001**	-0.43	0.18	-2.43	**0.022**	0.36	0.51	0.72	0.480	0.37	0.52	0.71	0.484	1.28	0.80	1.60	0.122
Site Type					-0.04	0.22	-0.18	0.856					0.04	0.22	0.20	0.843	-1.49	1.03	-1.44	0.162
Egg Mass									-3.77	2.29	-1.65	0.111	-3.95	2.40	-1.64	0.112	-8.31	3.81	-2.18	**0.039**
Site Type * Egg Mass																	7.15	4.76	1.50	0.146
Residual σ^2	0.14				0.15				0.13				0.13				0.14			
Female ID σ^2	0.10				0.10				0.10				0.11				0.09			
Marginal R^2 /Conditional R^2	0.000 / 0.403				0.002 / 0.414				0.108 / 0.498				0.107 / 0.520				0.185 / 0.505			
Free Glycerol																				
Intercept	3.38	0.10	33.06	**<0.001**	3.48	0.15	22.68	**<0.001**	4.48	0.41	10.99	**<0.001**	4.50	0.42	10.81	**<0.001**	4.91	0.55	9.00	**<0.001**
Site Type					-0.18	0.21	-0.89	0.379					-0.11	0.18	-0.59	0.560	-1.03	0.83	-1.24	0.223
Egg Mass									-5.10	1.84	-2.77	**0.009**	-4.89	1.90	-2.58	**0.014**	-6.89	2.55	-2.70	**0.011**
Site Type * Egg Mass																	4.28	3.76	1.14	0.263
Residual σ^2	0.10				0.10				0.10				0.10				0.10			
Female ID σ^2	0.16				0.17				0.11				0.11				0.11			
Marginal R^2 /Conditional R^2	0.000 / 0.633				0.032 / 0.647				0.217 / 0.629				0.224 / 0.645				0.262 / 0.649			
Intercept	1.97	0.08	25.13	**<0.001**	2.09	0.11	18.33	**<0.001**	2.91	0.32	9.23	**<0.001**	2.92	0.32	9.24	**<0.001**	3.30	0.42	7.93	**<0.001**
Site Type					-0.21	0.15	-1.34	0.189					-0.14	0.14	-1.02	0.317	-1.01	0.64	-1.57	0.126
Egg Mass									-4.31	1.42	-3.03	**0.005**	-4.04	1.45	-2.78	**0.009**	-5.85	1.95	-3.00	**0.005**
Site Type * Egg Mass																	4.00	2.90	1.38	0.176
Residual σ^2	0.09				0.09				0.08				0.08				0.08			
Female ID σ^2	0.08				0.08				0.05				0.05				0.05			
Marginal R^2 /Conditional R^2	0.000 / 0.484				0.063 / 0.502				0.236 / 0.508				0.262 / 0.530				0.305 / 0.564			

Discussion

Summary

To our knowledge, this is the first study to assess differences in egg yolk physiology **(1)** across urban and rural sites and with respect to female condition, **(2)** following a maternal immune challenge, and **(3)** in relation to egg viability (i.e., whether an egg was fertilized at oviposition) and egg mass. Urban lizards laid more unfertilized eggs than rural lizards, suggesting there may be a reproductive cost associated with inhabiting an urban environment. Across studies, egg viability and mass were important determinants of egg physiology, and ectoparasites were marginally related to BKA in rural eggs. Parasite loads were higher in urban females and eggs of these females had higher BKAs than the eggs of rural females. Lastly, eggs d-ROMs were lower from females treated with LPS. This study stresses the importance of measuring multiple variables when attempting to assess the effects of anthropogenic change and life-history constraints on egg yolk physiology and concomitant offspring viability.

Urban and rural egg yolk physiology, egg mass, and viability

Maternal provisioning of hormones, immune factors, and nutrients are sensitive to environmental stressors and can drastically alter offspring phenotypes (Hao et al., 2021; Haussmann et al., 2012; Johnson-Dahl et al., 2017; Warner et al., 2015). Whether these phenotypic changes result from proximate mechanisms (i.e., physiological, or epigenetic) remains unclear (Hao et al., 2021). In the present study, we found a marginal difference in BKA between eggs from urban and rural sites but found no significant differences in d-ROMS, CORT, or energy metabolites. Seasonal differences in maternal investment are widely documented in the literature (Höbel et al., 2021; Mitchell et al., 2018; Warne et al., 2012) and in the context of yolk investment (Counihan et al., 2015; Partecke et al., 2020). Therefore, it is possible that maternal investment in our study populations varies throughout the breeding season. Maternal deposition of immune factors is not always linear and there may be a high degree of variation between females or among eggs in a given clutch (Coakley et al., 2014; Pihlaja et al., 2006; Virgin et al., 2022). Given the prior arguments, we find it unlikely that urban and rural variability in passive transfer is the mechanism underlying these differences.

The local disease environment is an important mediator of maternal investment in the context of immune factor modulation (Boonyarittichaikij et al., 2018; Grindstaff, 2008; Grindstaff et al., 2006; Müller et al., 2004). In the context of anthropogenic disturbance, differences in microclimate, food availability, overall population densities, and community composition are just a few of the many influences on parasitism and disease dynamics (Bradley and Altizer, 2007; Cohen et al., 2022; Fischer et al., 2012). Female Side-blotched Lizards from urban populations had higher ectoparasite loads than females from rural sites. Urban Side-blotched Lizard populations in southern Utah occur in higher densities which may foster increased disease and parasite transmission between individuals. In lizards and eggs from urban sites, the apparent lack of association between maternal BKA, ectoparasite load, and egg yolk BKA suggest that differences in maternal condition and available resources may facilitate responses to reproduction and immunity (French et al., 2007). Alternatively, as survival estimates are much lower in urban populations, it is possible that attenuated immune function in response to ectoparasites is a consequence of maximized reproductive investment (Lucas and French, 2012).

Likewise, egg yolk immune function was not related to maternal ectoparasite burdens. Urbanized areas tend to have less spatiotemporal variability in microclimates and more consistent access to food and water (Leveau, 2018). Low environmental variability in desert urban sprawls coupled with more consistent exposure to ectoparasites could explain the consistency in egg yolk immune function, regardless of ectoparasite load. In eggs from rural sites, there was a negative, but marginal relationship between maternal ectoparasite load and egg yolk BKA. Rural Side-blotched Lizards have higher survival estimates and longer lifespans than urban lizards (Lucas and French, 2012) and may favor survival over reproductive investment when overburdened by ectoparasites. However, higher mite loads in rural European blackbirds did not translate to higher levels of antibody deposition in the yolk (Partecke et al., 2020), suggesting that these relationships are context dependent.

Exposure to CORT during development can have long-lasting and/or permanent effects on offspring physiology, survival, and fitness, and is hypothesized to be a main driver of species persistence in a changing world (Angelier and Wingfield, 2013). However, egg yolk CORT did not differ between eggs from urban and rural sites despite previous studies showing elevated baseline CORT in urban House Wren nestlings (Ouyang et al., 2019). In this study, egg yolk CORT did not differ between eggs from urban and rural sites, suggesting maternal deposition of CORT may not differ between urban and rural females. These findings are supported by recent work demonstrating the effect of urbanization on glucocorticoids is mixed (Injaian et al., 2020). Meta-analyses of vertebrate taxa have shown no difference in baseline and stress-induced CORT between urban and rural populations (Iglesias-Carrasco et al., 2020; Injaian et al., 2020).

Therefore, it is possible that yolk CORT from urban and rural eggs does not differ significantly because urban females are not exhibiting stress via an elevated glucocorticoid response.

There were no apparent differences between urban and rural females in d-ROMS nor energy metabolites in the present study. Dietary-derived antioxidants can act as a buffer against the oxidative costs of energetically intense life history stages, such as reproduction or embryonic development (Giordano et al., 2015; Watson et al., 2018). A recent study focusing on Side-blotched Lizards living near windfarms found lower ectoparasite loads, but a similar positive trend in ectoparasite burden and oxidative stress (Alaasam et al., 2021). They found no difference in ectoparasite burden or an association with oxidative status in undisturbed populations, suggesting that anthropogenic disturbance measures cannot be generalized across populations. Previous work found differences in stable isotope ratios among urban and rural Side-blotched Lizards, suggesting dietary distinctions presumably due to differences in arthropod diversity and geographical variation (Durso et al., 2020). However, free glycerol and true triglycerides, our selected proxies for energetic state, were not significantly different between urban and rural lizards. Maternally derived yolk fatty acids and proteins are important predictors of offspring phenotype and can influence offspring oxidative status (Lindsay et al., 2020; Mentesana et al., 2021). Because egg yolk free glycerol and triglycerides were similar across urban and rural populations, it is likely any potential differences in diet and oxidative status (mediated by parasites) are not reflected within the parameters that we measured. Recent work has shown the majority of lipids in lizard egg yolks are derived from stored energy (i.e., fat bodies), but egg yolk protein comes from income resources

(Warne et al., 2012). Vitellogenin, a yolk precursor protein synthesized in the liver, may provide antioxidant benefits individual lizards post-ovulation (Li and Zhang, 2017; Lindsay et al., 2020) and exhibit antimicrobial properties (Li and Zhang, 2017). Phosvitin, a yolk protein derived from the cleavage of vitellogenin, can bind to and neutralize endotoxins (e.g., LPS) from microbes, such as *E. coli.* Measuring yolk precursor proteins in accordance with immune metrics and oxidative status in future studies will aid in our understanding of the complex relationships among urbanization, female condition, and physiological investment.

Perhaps the most noteworthy finding from this study is the marked difference in fertilization rates between urban and rural eggs. Approximately 15% of eggs from urban females were unfertilized, compared to rural females whose clutches did not have any unfertilized eggs. Genetic crosses between isolated populations of Side-blotched Lizards resulted in higher frequencies of unfertilized eggs and was dependent on male color morph, suggesting fertilization probability is dependent on multiple processes, such as reproductive isolation between populations, post-copulatory cryptic female choice, and gamete incompatibilities (Corl et al., 2012). Alternatively, manipulation of female densities can cause reptiles to lay higher proportions of unfertilized eggs potentially due to stress from crowding, or a reduction in copulatory frequency (Talent and Talent, 2013). However, other studies have found no difference in the proportion of unfertilized eggs and mating frequency, so this relationship remains unclear (Olsson and Madsen, 2001). Pregnancy termination (via extrusion from the oviduct) may confer survival advantages in response to stress or captivity (Blackburn et al., 1998) and neutralize the physiological costs on viable eggs by limiting investment to unfertilized eggs (Reinke et al., 2018). The latter claim, however, has never been explicitly tested, and other studies have found conflicting results (Weiss et al., 2011). It is likely that a combination of site type, population density, maternal stress, and gamete quality are factors influencing whether an egg is fertilized or unfertilized. Because fertilization probabilities are influenced by both male and female condition, future work should incorporate within-season comparisons of sperm quality and the proportion of viable eggs to determine whether these relationships are mediated by male quality.

Effect of egg mass and egg viability on yolk physiology

Across both studies presented in this manuscript, egg mass was a significant predictor of egg yolk physiology, despite controlling for differences in egg yolk volumes within assays. When egg mass was significant in the statistical models, there was a consistent negative relationship between egg mass and egg yolk physiology across all measured parameters. Recent work within this study system found a negative correlation between egg yolk d-ROMs and egg mass, and eggs from smaller clutches tended to have higher d-ROMs (Virgin et al., 2022). The negative relationship between egg mass and d-ROMs may reflect a reproductive constraint given that oxidative stress may reduce clutch sizes (Lister et al., 2016; Stier et al., 2012). A consistent negative relationship between egg mass and egg yolk physiology (e.g., BKA and free glycerol) across multiple variables may also suggest there is a limit to what females can invest (Weiss et al., 2011). A similar study in striped plateau lizards showed that offspring from larger clutches had lower yolk antioxidants compared to eggs from smaller clutches, indicating possible constraints in the bioavailability of yolk components; or competing needs between reproduction and maintenance (Weiss et al., 2011). Nonetheless, egg mass is a well-known proxy for maternal investment and offspring quality (Krist, 2011; Sinervo, 1990) and these results suggest a nuanced relationship between egg mass and quality.

Egg viability was also an important predictor of egg yolk physiology. Fertilized and unfertilized eggs have distinctive differences in appearance (Blackburn et al., 1998; Corl et al., 2012). Unfertilized eggs do not possess an embryonic disc, differ in yolk consistency, and often lack a well-developed shell (Blackburn et al., 1998; Corl et al., 2012; Reinke et al., 2018). Despite these differences, few studies have examined physiological differences between fertilized and unfertilized eggs. Egg yolk BKA, CORT, and free glycerol were higher in unfertilized eggs than in fertilized eggs. In another study, carotenoid supplemented female *Anolis* lizards differentially allocated pro-oxidants between unfertilized and fertilized eggs to limit the potential oxidative costs on viable eggs (Reinke et al., 2018). However, other studies in lizards and caimans have found no differences in the chemical composition or antioxidant capacity of fertilized and unfertilized eggs (Leiva et al., 2018; Weiss et al., 2011). It is important to note that vitellogenesis in oviparous reptiles occurs prior to ovulation (Ho et al., 1982; Miles et al., 2000), therefore It is conceivable that differences in yolk physiology may impact whether or not an egg is fertilized. However, in reptiles one-third of embryonic development occurs prior to oviposition (Andrews and Mathies, 2000), and it is possible that lower egg yolk components in fertilized eggs is due to yolk assimilation by the embryo. The inherent costs and/or benefits to investing in unfertilized eggs remains unknown and should be examined. Regardless of potential physiological differences, it is surprising to witness the presence of unfertilized eggs only in urban sites. While our results suggest only rural lizards are exhibiting tradeoffs in allocation to reproduction vs. maintenance, it is possible the costs of urbanization are instead manifesting via reduced egg viability.

Egg yolk physiology following maternal immune challenge

Maternal condition is a leading driver in resource provisioning to offspring (Coakley et al., 2014; Gasparini et al., 2007; Hargitai et al., 2009), and individual immune responses (Durso and French, 2018; Sheldon and Verhulst, 1996); as a result, energetic constraints from mounting an inflammatory response can alter investment of immune factors (Coakley et al., 2014). LPS-treated females had higher BKA relative to both controls, validating the efficacy of our immune challenge. Females treated with LPS had eggs with lower d-ROM values, suggesting that a mounted maternal immune response may impact markers of oxidative damage in the yolk.

Elevated production of immune system components can indirectly lead to oxidative stress by initiating an oxidative burst during phagocytosis and producing reactive oxygen species (Sorci and Faivre, 2009). An increase in oxidative damage markers in the yolk may represent an indirect cost of elevated maternal immune function. Resulting changes in oxidative status can have direct effects on offspring quality and fitness (Lister et al., 2016; Romero-Haro and Alonso-Alvarez, 2020). While d-ROMs were slightly lower in females treated with LPS, previous work within this study system has shown that LPS injection in reproductive females significantly reduced plasma d-ROMs (Virgin et al., *in prep*), potentially linking LPS treatment to both maternal and egg yolk oxidative damage. The immune challenge did not have a significant effect on the other egg yolk parameters measured in this study. It is possible that females had progressed far enough in vitellogenesis for LPS to have no influence on energy metabolites or CORT. Females within this study were in middle to late-stage vitellogenesis and had already invested a significant amount of yolk by this point, minimizing any effects on energetic investment. Maternal CORT can be transferred to eggs at any point within vitellogenesis, even after the eggs are shelled (Ensminger et al., 2018; Johnston, 2009). Alternatively, the overall effects of LPS on egg yolk CORT may be minimal relative to total yolk investment across the reproductive cycle. Chronic stress manipulations may be necessary to elicit substantial changes in egg yolk hormone deposition (Ensminger et al., 2018).

Conclusions

In summary, the present study provides new insights into the complexities of anthropogenic disturbance via physiological maternal effects, parasitism, and life-history tradeoffs. Maternal investment strategies appear to differ between urban and rural females and depend on ectoparasite load. Simulating an acute infection in reproductive females may alter egg oxidative status and immune function, akin to the impact of ectoparasites within this study. While urbanization was not the main driver, egg mass and egg viability were important predictors of egg physiology and may impact interpretation of traditional proxies of maternal investment. Finally, egg viability was lower in urban sites, suggesting a potential cost of urbanization. Our findings highlight the potential impacts of urbanization on embryonic viability and the importance of physiological context (e.g., parasitism) when assessing the effect of urbanization on maternal physiological investment. Future studies should address long-term effects, including survival and fitness of the resulting offspring.

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CHAPTER 5 – CONCLUSIONS

The main objectives of my research were to assess the impacts of urbanization on tradeoffs between the reproductive and immune systems in female Side-blotched Lizards and determine whether an increase in reproductive investment corresponded to differences in egg yolk physiology. Specifically, I tested **(1)** whether female physiology and egg yolk physiology correspond with metrics of reproductive investment, **(2)** if the physiological costs of reproductive investment differ across the reproductive cycle, and **(3)** if life history differences in response to urbanization correspond with maternal investment and egg yolk physiology.

In my second chapter, I compared female immune function and oxidative status and their relationship with reproductive investment and egg yolk immune function and oxidative status. By collecting information on female Side-blotched Lizards in an unmanipulated state, I gained a better understanding of natural variation in female physiology and egg yolk physiology to compare as a baseline for subsequent chapters in where I experimentally manipulated female physiological status. In my third chapter, I examined whether metabolic rate was significantly different across the reproductive cycle, and I simulated an infection in female Side-blotched Lizards at different points in vitellogenesis to test whether variation in total reproductive investment can alter metabolic, immunological, and oxidative responses to sickness. Females had the highest metabolic rates in earlier stages and lowest in later stages, suggesting a significant energetic cost associated with vitellogenesis. Because females earlier in reproduction showed significantly different responses to a simulated infection and females later in reproduction did not, energetic constraints associated with vitellogenesis may underly attenuated responses in later stages of reproduction. In my fourth chapter, I examined natural variation in female physiological condition and egg yolk physiology between urban and rural populations and tested whether simulation of an infection in urban females altered egg yolk provisioning. I found that urban and rural differences in female parasite load were associated with egg yolk immune function and oxidative status at rural sites and not at urban sites. Females at urban sites laid a significantly greater number of unfertilized eggs than rural females, and there were differences in egg yolk physiology between fertilized and unfertilized eggs. Egg yolk physiology was negatively related to egg mass as well, suggesting a potential limit in yolk investment or quality between smaller and larger eggs. Finally, simulating an infection in females was associated with lower egg yolk oxidative status, but not with egg yolk immune function, stress, or energetics. By comparing the effects of urbanization on maternal physiological condition and the influence on egg yolk physiology, I gained a better understanding of reproductive investment strategies between urban and rural females when burdened by ectoparasites or a simulated infection. Lastly, manifestation of reproductive costs may differ between urban and rural populations; although rural females exhibited significant physiological costs from having higher ectoparasite burdens, which manifested via differences in yolk physiology, the costs of reproduction in an urban setting may only result in a higher proportion of unfertilized eggs.

The findings from my dissertation research build upon existing work aimed at understanding physiological tradeoffs and complex interactions across different reproductive, immunological, and environmental contexts. Through physiological comparisons between females and egg yolks, it is abundantly clear that comparisons of multiple physiological variables in females and egg yolks are needed to understand the interactive, synergistic, and additive effects of egg yolk provisioning on offspring quality. Finally, this work expands our understanding of the potential impacts of urbanization in an understudied taxonomic group and sets a foundation for future work investigating the longitudinal effects of urbanization on offspring quality and fitness.

APPENDICES

Appendix A: Publisher Permission and Copyright Guidelines

Instructions to Authors — Ichthyology & Herpetology (ichthyologyandherpetology.org)

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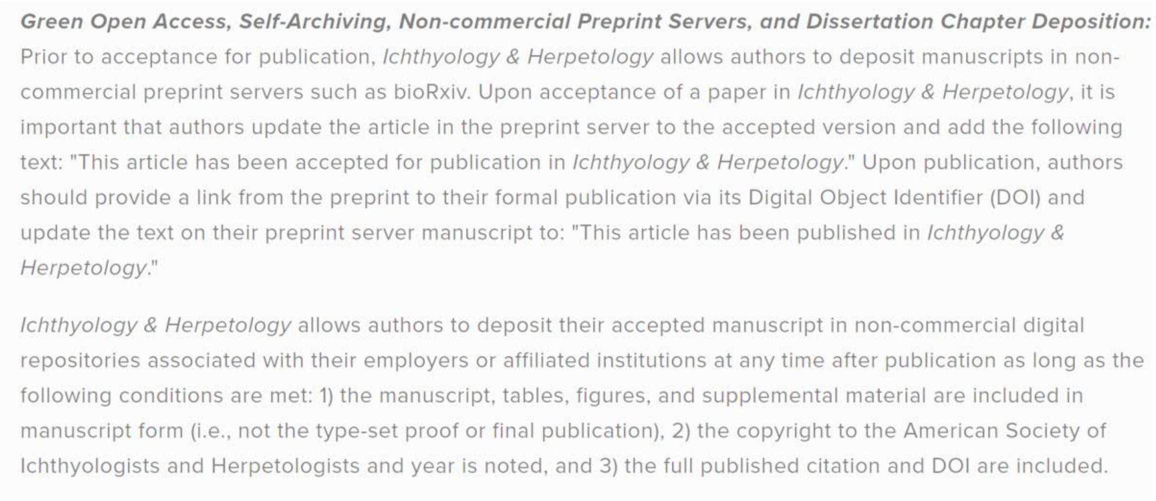

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Appendix B: Coauthor Permission Letters

November 10th, 2022

Emily E. Virgin
Department of Biology
Utah State University, USA
84322-5305

Dear Emily,

As a coauthor on this manuscript, I grant you permission to use this published manuscript in your dissertation:

Virgin, E.E., Hudson, S.B., Webb, A.C., French, S.S. 2022. The immunological and oxidative costs of reproduction and associations between maternal and egg yolk physiology in a reptile. *Ichthyology and Herpetology.* https://doi.org/10.1643/h2021040

You were the primary contributor to this research, which involved independent work in synthesis, experimental design, implementation, and manuscript preparation.

Sincerely,

Alison C. Webb, PhD
Program Director, Pacific Northwest Biology
Maple Valley, Washington, USA, 98038

November 10th, 2022

Emily E. Virgin
Department of Biology
Utah State University, USA
84322-5305

Dear Emily,

As a coauthor on this manuscript, I grant you permission to use this published manuscript in your dissertation:

Virgin, E.E., Hudson, S.B., Webb, A.C., French, S.S. 2022. The immunological and oxidative costs of reproduction and associations between maternal and egg yolk physiology in a reptile. *Ichthyology and Herpetology.* https://doi.org/10.1643/h2021040

You were the primary contributor to this research, which involved independent work in synthesis, experimental design, implementation, and manuscript preparation.

Sincerely,

Spencer B. Hudson, PhD Candidate
Department of Biology
Utah State University, USA
84322-5305

CURRICULUM VITAE

Emily E. Virgin
Ph.D. Candidate at Utah State University
Dept. of Biology and The Ecology Center,
5305 Old Main Hill, Utah State University, Logan, UT 84322-5305

EDUCATION

06/2022 – Ph.D. in Biology, Ecology at Utah State University (Logan, Utah)
Advisor: Dr. Susannah French

2016 – B.S. in Biology, Ecology at Northern Illinois University (Dekalb, Illinois)
Advisor: Dr. Richard King

RESEARCH/TEACHING APPOINTMENTS

Utah State University
2022 – Organismal Biology Lab, Teaching Assistant (Spring)
2021 – Human Physiology Lab, Teaching Assistant (Fall)
– Organismal Biology Lab, Teaching Assistant (Spring)
– Research Assistant, Effects of Ecotourism on Rock Iguana Physiology
2020 – Microbiology Lab, Prep Teaching Assistant (Spring)
– Presidential Doctoral Research Fellowship (Summer)
– Human Physiology Lab, Teaching Assistant (Fall)
2019 – Presidential Doctoral Research Fellowship (Spring/Fall)
– Research Assistant, Effects of Urbanization on Side-blotched Lizards
2018 – Presidential Doctoral Research Fellowship (Spring/Summer)
– Comparative Animal Physiology Lab, Instructor (Fall, 2 sections)
2017 – Presidential Doctoral Research Fellowship (Spring/Summer/Fall)
2016 – Presidential Doctoral Research Fellowship (Spring)

RESEARCH PUBLICATIONS

1. Smith, G.D., Wilcoxen, T., Hudson, S. B., **Virgin, E.E.**, Durso, A.M., Van der Walt, M., Spence, A., Neuman-Lee, L., Webb, A.C., Terletzky, P., French, S.S. *in review*. Anthropogenic and climatic factors interact to influence reproductive timing and effort.

2. French, S.S., Webb, A.C., Knapp, C.R., **Virgin, E.E.**, Smith, G.D., Lewis, E.L., Iverson, J.B., DeNardo, D.F. *in press.* Glucose tolerance of iguanas is affected by high sugar diets in the lab and supplemental feeding by ecotourists in the wild. *Journal of Experimental Biology.*
3. **Virgin, E.E**., Hudson, S.B., Webb, A.C., French, S.S. *in press.* The immunological and oxidative costs of reproduction and associations between maternal and egg yolk physiology in a reptile. *Ichthyology and Herpetology.*
4. Hudson, S.B., **Virgin, E.E**., Kepas, M.E., & French, S.S. 2021. Energy expenditure across immune challenge severities in a reptile: consequences for innate immunity, locomotor performance, and oxidative status. *Journal of Experimental Biology*.
5. French, S.S., **Virgin, E.E.**, Ki, K.C., Maryon, D.F., Goode, A.B., & Pasachnik, S.A. 2021. Reproductive stage and clutch size incur energetic and oxidative costs in an endangered iguana, *Ctenosaura oedirhina*. *Journal of Herpetology*.
6. Hudson, S.B. **Virgin, E.E**., Kepas, M.E., & French, S.S. 2021. Recovery from discrete wound severities in side-blotched lizards (*Uta stansburiana*): implications for energy budget, locomotor performance, and oxidative stress. *Journal of Comparative Physiology B.*
7. **Virgin, E.E.**, & King, R.B. 2019**.** What does the snake eat? Breadth, overlap and non-native prey in the diet of three sympatric natricines. *Herpetological conservation and biology.*
8. **Virgin, E. E.**, & Rosvall, K. A. 2018. Endocrine-immune signaling as a predictor of survival: A prospective study in developing songbird chicks. *General and comparative endocrinology*.
9. French, S. S., Webb, A. C., Hudson, S. B., & **Virgin, E. E.** 2018. Town and country reptiles: A review of reptilian responses to urbanization. *Integrative and comparative biology*.

INTERDISCIPLINARY/MISCELLANEOUS RESEARCH EXPERIENCE

2021	– Elevational differences in thermal physiology of coqui frogs – Hawaii – Shedd Aquarium Bahamian Rock Iguana Research Trip – Exumas, Bahamas
2019	– Iguana Conservation Workshop – Roatán, Honduras
2018	– Shedd Aquarium Bahamian Rock Iguana Research Trip – Exumas, Bahamas

SELECT PROFESSIONAL PRESENTATIONS

2020 – Virgin, E.E., Hudson, S.B., Kepas, M.E., and French, S.S. “Comparisons of Egg Yolk Physiology between Urban and Rural Side-blotched Lizards.” (SICB) ***Featured in *Life in the City* blog*** **click here to access blog post**

2019 – Virgin, E.E., French, S.S. “Physiological responses to an immune challenge vary by reproductive stage in female Side-blotched Lizards (*Uta stansburiana*).” (JMIH)

2018 – Virgin, E.E., Webb, A.C., Hudson, S.B., and French, S.S. “Inter and intra-clutch variation in egg immunity in Side-blotched Lizards (*Uta stansburiana*).” (SICB)

2016 – Virgin, E.E. and Rosvall, K.A. “Interleukin-6, Parasites, and Death: Why are big chicks better than small chicks?” (SICB)

2016 – Virgin, E.E. and King, R.B. “What does the snake eat? Dietary overlap in three sympatric grassland species.” (MEEC)

GRANTS, AWARDS AND FELLOWSHIPS

Utah State University
2020 – Ecology Center Graduate Research Award
– **Best Student Poster for Division of Ecoimmunology and Disease Ecology (DEDE)**
2019 – ASIH Diversity and Inclusion Award
– Charlotte Magnum Student Support Program, Society for Integrative and Comparative Biology (SICB)
2018 – Ecology Center Graduate Research Award
– Matt Del Grosso Endowed Graduate Research Award
– Charlotte Magnum Student Support Program, Society for Integrative and Comparative Biology (SICB)
2017 – Charlotte Magnum Student Support Program, Society for Integrative and Comparative Biology (SICB)
2016 – Presidential Doctoral Research Fellowship (4 years)

Northern Illinois University
2016 – Winifred Halsey Scholarship
2015 – NSF BIO REU Travel Award
– Student Engagement Fund Research Award
– August M. Gorenz Award
2014 – Student Engagement Fund Research Award
– Research and Apprenticeship Program

LEADERSHIP AND SERVICE

2020 – Reviewer for *Urban Ecosystems*
2019 – USU Ecology Center Seminar Committee

2018 – Graduate Student Representative – Evo/Devo Candidate Search Committee
– USU Ecology Center Seminar Committee
2017 – USU Ecology Center Seminar Committee
– Outreach Chair in USU Biology Graduate Student Association
2016 – Outreach Chair in USU Biology Graduate Student Association

COMMUNITY OUTREACH

2021 – Utah State University – Uinta Basin – Research Presentation
2019 – Mountain Crest High School – Research Presentation and Outreach
– Wildlife Society Outreach – Lab/Research tour
– Utah State University – Uinta Basin – Research Presentation
2018 – Science Unwrapped Volunteer at USU
– Science Education trip with Utah Natural History Museum Y2Y
– Mountain Crest Middle School Science Presentations
– Wildlife Society Outreach – Reptile Collection
2017 – Science Unwrapped Volunteer at USU
– Animal Outreach at Venture Academy Elementary School
– Animal Outreach at Edith Bowen Elementary School
2016 – Science Unwrapped Volunteer at USU

UNDERGRADUATE MENTORING EXPERIENCE

Utah State University
2016 – present

- Kelsi Chugg– Effect of an Immune Challenge on Egg Yolk Physiology in Side-blotched Lizards (*Uta stansburiana*) – **Undergraduate Research Presentation**
- Audrey Lidgard– Standard laboratory procedures, sample collection and physiology assays
- Jack Marchetti– Standard laboratory procedures and physiology assays
- Jordan DuPaul– Field work, data management and sample collection
- Elizabeth Graves– Field work, data management and sample collection
- Nicholas Sauer– Standard laboratory procedures and animal husbandry
- Brandon Pace– Standard laboratory procedures and animal husbandry
- Justine Lamm– Standard laboratory procedures and animal husbandry
- Eleanor Watson– Standard laboratory procedures and animal husbandry
- Holly Flann– DNA Extractions
- Mikell Goldsberry– DNA Extractions

RELEVANT COURSEWORK

Physiological Ecology of Vertebrates
Genes and Behavior
Biometry (R-based)
Mixed Effects Models in Ecology (R-based)
Communicating Science

MEMBERSHIP IN PROFESSIONAL SOCIETIES

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American Society of Ichthyologists and Herpetologists
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