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IMPACTS OF TOURISM ON THE ECOPHYSIOLOGY OF THE ENDANGERED
NORTHERN BAHAMAIN ROCK IGUANA (*CYCLURA CYCHLURA*)

by

Alison C. Webb

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

of

DOCTOR OF PHILOSOPHY

in

Ecology

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2020

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ABSTRACT

Impacts of Tourism on the Ecophysiology of the Endangered Northern Bahamian Rock

Iguana (*Cyclura cyclura*)

by

Alison C. Webb, Doctor of Philosophy

Utah State University, 2020

Major Professor: Dr. Susannah S. French

Department: Biology

Increased interest in ecotourism over recent years has led to more direct human-animal interactions and a striking concomitant increase in the provisioning of non-natural food, that may have unintended consequences for the wildlife involved. The critically endangered Northern Bahamian Rock Iguana provides a valuable model to address the potential impact of food provisioning on health as there are populations that represent a graded variation in human presence, with sites experiencing high, low, or no tourism. To assess the potential impacts of tourism on iguana physiology I first reviewed the relevant literature on iguana physiology and then performed three investigations focusing on different facets of the relationships between human presence and iguana health. First, because physiological changes related to reproduction can mask changes caused by human disturbance, I examined the endocrine profiles of steroid hormones involved in reproduction to better define this natural variation and a “post reproduction” period. Next, I examined how specific reproductive stages were related to physiological measures and found that increased reproductive investment significantly altered both energetics metabolites and measures of oxidative stress. Finally, in my third investigation, I

examined the impact of tourism on iguana physiology during the post reproductive season and found that tourism and food supplementation significantly affected all measures of iguana physiology and body condition assessed.

(158 pages)

PUBLIC ABSTRACT

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Iguana (*Cyclura cyclura*)

Alison C. Webb

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measures of iguana physiology and body condition assessed.

ACKNOWLEDGMENTS

The research presented here would not have been possible without the help of many people. First, I would like to thank my advisor, Dr. Susannah French, for inviting me to participate in this exciting area of work. She has supported me with an enormous amount of her time and resources by writing grants, reviewing my work, and guiding my career with careful thought of my best interest. Above all, she led by example, never asking of me what she was not willing to do herself. I am so appreciative for the opportunity to have had her as a mentor.

Dr. John Iverson graciously welcomed me in to his research, allowing me to take ownership in a project that he has spent much of his life developing. His dedication to his work and contribution to science is inspiring. His priority has always been to ensure his students success and I consider myself extremely fortunate to have been one of his many mentees.

I would also like to thank each of my committee members for the time they have invested in my education. Dr. Karen Kapheim, Dr. Lise Aubry, and Dr. Scott Bernhardt spent many patient hours teaching me new skills, thoughtfully reviewing my work, and supporting me throughout the entire process, for which I am extremely thankful. I would also like thank all of my committee members for always treating me as a colleague, listening to and valuing my input.

Although not on my committee, Dr. Charles Knapp invested a great amount of his time and resources into my education, generously allowing me to participate in his long-term research. Dr. Dale Denardo provided thorough feedback on all of my manuscripts and participated in much of the field work, his participation has undoubtedly improved

the quality of each manuscript.

Each of my lab members were an essential part of my graduate school experience, they supported me academically and emotionally, and generally always made my day better by being around each of them. I wouldn't have wanted to do this without them.

Finally, I would like to thank my family for their participation in this journey. My daughter, Juniper, certainly made this work more challenging but also more meaningful. My husband, who quit his job and followed me across the country to go to grad school, supported me every step of the way. My parents, who never doubted my unusual interests and gave me every resource they could to ensure my success. To all of my family members who provided childcare so I could complete my field work and attend conferences. I could not have done this without my village.

For each chapter, I have specific individuals and funding sources I must thank for their participation in the project. They appear here as they appear in the articles.

Chapter 2 – Rock iguanas were handled and blood samples collected and exported with appropriate IACUC approval (#2530 USU), research permissions (The Bahamas Environment, Science & Technology Commission), and CITES export permits (Bahamas Department of Agriculture). Financial support was provided by Utah State University Research Catalyst Grant project (A35851 to SSF), Utah Agricultural Experiment Station project numbers 1104 & 1347 (SSF), Utah State University Ecology Center (ACW), Shedd Aquarium (CRK), the Earlham College Biology Department (to JBI), and the personal funds of most of the field assistants. Transportation was provided by the R/V Coral Reef II (Zoltan Bobick, Kip Mors, and Joseph Brozovich) and 7 C's Charters (Bruce Dunham, Sheila Young, Ron White, and crew). Field assistance from

Earlham College, Denison University, and Utah State University students and alumni, Bahamas National Trust and BREEF (Bahamas Reef Environmental Educational Foundation) interns, several citizen scientists, Jill Jollay, and Sandra Buckner are greatly appreciated.

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Alison C. Webb

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CHAPTER 1
INTRODUCTION
A REVIEW OF IGUANA PHYSIOLOGY

Iguanas

Currently, there are 44 extant species of iguana recognized in 8 genera (Buckley et al., 2016). Of the listed taxa, 82 % are in Threatened categories (Critically Endangered, Endangered, and Vulnerable) according to IUCN Red List of Threatened Species™ criteria, making this one of the most vulnerable vertebrate groups on the planet (Buckley et al., 2016). Developing a better understanding of iguana biology is essential to strengthening the conservation efforts aimed at protecting natural populations of iguanas. Additionally, iguanas are a valuable model for ecophysiology studies for several reasons, 1) iguanas maintain relatively small home ranges, allowing for them to be recaptured at multiple times throughout the year, 2) only some females reproduce in a given year, allowing for the direct comparison of reproductive and non-reproductive females, and 3) large blood samples can be collected with minimal invasion, allowing for a more comprehensive physiological examination. Further, the Northern Bahamian Rock Iguanas (*Cyclura cyclura*) are ideally suited for examining the impacts of tourism because they experience a gradient of tourism pressure, with some sites heavily visited while others are infrequently visited or not visited at all.

Physiological approaches

Assessing the physiological condition of individuals allows for a deeper understanding of the life-history and ecology of species. From a researcher's perspective, studying animal physiology allows for faster detection of potentially problematic situations. For instance, an animal being fed inappropriate food items may have changes in blood glucose and lipids before any changes in body mass or condition occur, measures which likely exhibit long term dietary changes. Measuring multiple physiological parameters provides an immediate, more comprehensive- measure of health that allows researchers to isolate individual-level differences in response to varying environmental conditions. Indeed, a single blood sample can yield measurements of baseline concentrations of energy metabolites and glucocorticoids, and assessments of immune function and oxidative stress (French et al., 2017). Integrating this physiological approach with long term population monitoring allows identification of the mechanisms by which anthropogenic disturbance can alter animal health, and can guide more directed management practices.

Iguana species studied

Iguanas have long been the subject of biological studies but the use of physiological techniques is still relatively new for this group of reptiles. The Galapagos Marine Iguana (*Amblyrhynchus cristatus*) and the Green Iguana (*Iguana iguana*) are the most common subject of physiological studies (Table 1), likely because of their unique feeding biology (Marine Iguana) and commonplace (Green Iguana). Other iguanas studied in physiological research include the Galapagos Land Iguana (*Conolophus marthae* and *C. subcristatus*), Rock Iguanas (*Cyclura cyclura* and *C. carinata*), Spiny

Tailed Iguanas (*Ctenosaurus ascanthura*, *C. bakeri*, and *C. similis*), Desert Iguanas (*Dipsosaurus dorsalis*), and Chuckwallas (*Sauromauls ssp.*). Several studies have examined iguana's thermal biology as a physiological measure, allow this is an essential component or reptile biology it is not included in this review.

Field vs. lab research

There are benefits and limitations for both field studies and lab-based experiments. Field studies are best suited for addressing questions of population demography and as they are focused on studying an animal in their natural environment, provide a level of biological relevance that cannot always be attained in a lab environment. The strength in lab-based experiments lies in the ability to manipulate certain aspects of animal's environment in a controlled manner. As many species of iguana are endangered, lab-based experiments are not always an option and sample collection instead must occur in the natural environment, minimizing disturbance to both the individual and population. By utilizing physiological assays, a single blood or tissue sample can be collected, and multiple physiological endpoints can be measured.

Hormones

Glucocorticoids are a group of steroid hormones that are largely involved in energy mobilization in response to changing environmental conditions (Angelier and Wingfield, 2013). Corticosterone is the main glucocorticoid hormone in reptiles (Norris and Carr, 2013) and is frequently measured in reptile physiology studies either to assess

baseline concentrations or reactivity to stressful stimuli. With such a broad function and the many possible changes to an animal's environment, both baseline and stress response levels of corticosterone are extremely context-dependent.

Baseline Corticosterone

Baseline concentrations of the reptilian glucocorticoid, corticosterone, can provide valuable information concerning an animal's current physiological state and energy balance. In iguanas, corticosterone has distinct changes associated with natural processes, such as reproduction (French et al., 2010) and changes in food availability (Romero and Wikelski, 2001), but also responds to human disturbance (French et al., 2017) and environmental disruption (Wikelski et al., 2001), making it a useful tool in assessing potential threats.

Circadian changes in corticosterone concentrations have been documented for some iguanas, but these patterns differ significantly among species, highlighting the importance of establishing daily patterns of this hormone when designing a study. Baseline CORT levels appear to be related to the tidal cycles for Galapagos Marine Iguanas, dropping when low tide occurred during the day (Woodley et al., 2003). However, baseline corticosterone levels in *Cyclura carinata carinata* were higher in mornings compared to afternoons (MacDonald et al., 2007) and in Desert Iguana (*Dipsosaurus dorsalis*) showed max levels at 1600 hours and min at 0400 hours (Chan and Callard, 1972). High levels of baseline corticosterone have been linked with low food availability/starvation (El Nino years for Galapagos Marine Iguanas) (Romero and

Wikelski, 2001) (Webb et al., in review) and likely play a role in both daily feeding strategies and the response to starvation. Although not extensively studied, temperature can also have affect corticosterone, with chronic suboptimal temperatures slightly, but not significantly, increasing baseline corticosterone levels (*Iguana iguana*) (Ávila-Mendoza et al., 2016).

Increases in baseline levels of corticosterone may act to facilitate breeding due in part to their role in mobilizing fat stores and converting glycogen to glucose for the energetically costly task of reproducing. Mating behaviors can also be inherently stressful events for both males and females, further elevating levels of this hormone in response to a stressful stimulus. The mating behavior of a species undoubtedly affects how stressful reproduction is, and males which rely on maintaining and defending a territory can have greater energetic demands and CORT, as observed in territorial lekking Galapagos Marine Iguana males (Berger et al., 2005). For female iguanas, reproduction brings about significant changes in behavior and physiology, starting with early investment into follicles (vitellogenesis) which is followed by copulation with potentially aggressive males, continued egg development, migration to nesting sites, nest construction, oviposition, and nest guarding. As each stage has unique demands, the role of CORT in facilitating and managing these must be dynamic. Female stage-specific changes in CORT have been observed in other iguana species, where CORT was highest during early nesting periods (Galapagos Marine Iguanas) compared to other stages (Rubenstein and Wikelski, 2005) and elevated in females carrying eggs compared to non-reproductive females sampled during the same period (Galapagos Land Iguanas, *Conolophus subcristatus*) (Onorati et al., 2017). However, depending on iguana species, not all

females reproduce each year, and comparing reproductive to non-reproductive females during the same sampling period can be helpful in determining the relationship between reproduction and iguana physiology. For example, receptive female Galapagos marine iguanas (*Amblyrhynchus cristatus*) had lower corticosterone than non-receptive females during late male assessment to post-breeding periods (Vitousek et al., 2010).

The relationship between human presence and baseline corticosterone is not consistent between the two species which have been studied, but could be due to differences in supplemental feeding and reproductive condition. Northern Bahamian Rock Iguanas (*Cyclura cychlura inornata* and *C.c. figginsi*) at low tourism sites, which have no supplemental feeding, had higher baseline corticosterone compared to those at high tourism and no tourism sites (Webb et al. in review) but this pattern was not observed during their reproductive season (Knapp et al., 2013). Whereas, for Galapagos Marine Iguanas, baseline and stress induced corticosterone were higher at tourist sites in the non-breeding season (French et al., 2010) and males but not females had elevated baseline CORT at tourist disturbed sites during the breeding season (French et al., 2017). Habitat perturbations can have a strong impact on baseline corticosterone for some iguana species, while others show little response (Northeastern Spiny-tailed iguana (*Ctenosaura acanthura*)) (Suárez-Domínguez et al., 2011). An oil spill in the Galapagos during a sampling period provided an ideal opportunity to assess physiological responses to such a catastrophe, and researchers found that iguanas elevated baseline and stress induced corticosterone compared with just 3 days before (Wikelski et al., 2001). The type and extent of environmental disruption likely affect the degree of response from an animal, making them difficult to compare across studies.

Stress Reactivity Corticosterone

Measuring stress reactivity of corticosterone can provide valuable insight into physiological responses to acute stressful stimuli, such as predation, approach from humans, aggressive conspecific interaction, and even sudden environmental changes.

In Galapagos Marine Iguanas, the introduction of new predators was associated with increased stress induced corticosterone levels, affecting juveniles baseline and stress response corticosterone significantly more than adults (Berger et al., 2007). Female Galapagos Marine Iguanas had higher corticosterone in response to stress than males, which was correlated with lower immune function (Neuman-Lee and French, 2017).

The degree to which corticosterone is elevated in response to a stressor can even alter an individual's chances of fitness and survival. Reproductive Galapagos Marine Iguanas females with lower corticosterone responses assessed more displaying males (Vitousek and Romero, 2013). Galapagos Marine Iguanas with high corticosterone release in response to a stressor, both experimental (Romero and Wikelski, 2010) and natural El Nino conditions (Romero and Wikelski, 2001), had reduced survival. Similar to baseline corticosterone, patterns in hormone release are not always consistent across individuals or species and the physiological and environmental context should be considered when interpreting this measure.

Estradiol and Progesterone

Estrogen (estradiol in reptiles) and progesterone are the primary gonadal hormones in female reptiles (Custodia-Lora and Callard, 2002; Norris and Carr, 2013). Elevated levels of these hormones stimulate receptive mating behaviors in females, elicit mating behaviors from males (Phillips et al., 1987), and facilitate resource deposition into offspring (Custodia-Lora and Callard, 2002). Estrogens stimulate the synthesis of vitellogenin proteins and increase circulating calcium for egg shell deposition and are also associated with secretions around the egg, such as albumin (Norris and Carr, 2013; Norris and Lopez, 2010). Estradiol is elevated during early stages of reproduction in both the Galapagos Marine Iguana (*Amblyrhynchus cristatus*) (Rubenstein and Wikelski, 2005) and Galapagos Land Iguana (*Conolophus marthae*) (Onorati et al., 2016) and peaks during follicle development in the Green Iguana (*Iguana iguana*), returning to baseline levels following post-reproductive follicular atrophy (Phillips et al., 1987). Progesterone increases the expression of proteins deposited into eggs and facilitates the formation of the eggshell (review of *Chelonia* species) (Custodia-Lora and Callard, 2002). Female Galapagos Marine Iguanas had elevated progesterone at the beginning of the mating period and again at arrival to nesting sites (Rubenstein and Wikelski, 2005), a trend also observed for female Galapagos Land Iguana (*Conolophus subcristatus*) which had elevated progesterone when gravid (Onorati et al., 2016). Non-receptive females had a peak in progesterone during follicle atresia and receptive females peaked after copulation (Vitousek et al., 2010). Progesterone may also have a role in modulating the immune system as levels were positively related with bacterial killing ability in female Galapagos Marine Iguana (Neuman-Lee and French, 2017).

Testosterone

While small amounts of estrogen and progesterone are produced in males, their primary sex steroid hormone is testosterone. Testosterone is typically higher in males than females during the breeding season (Galapagos Marine Iguanas) (French et al., 2010), is higher for breeding males compared to non-breeding (Galapagos Marine Iguanas) (French et al., 2010), and for at least one iguana species (Green Iguana), circulating levels increase with body mass (Pratt et al., 1994). Testosterone may even have important interaction with the immune system in iguanas, as levels in male Galapagos Marine Iguana were inversely related to a measure of innate immune performance, bacterial killing ability (Neuman-Lee and French, 2017). Testosterone is a potentially important steroid hormone in female reproduction as well, possibly due to it being a precursor in the synthesis of estrogens (Staub and De Beer, 1997), but also in facilitating behaviors that lead to successful reproduction such as copulation and nesting (Ketterson et al., 2005; Rubenstein and Wikelski, 2005; Vitousek et al., 2010). In female Galapagos Marine Iguanas, testosterone peaked during the height of copulation for receptive females and during follicle atresia for in non-receptive females (Vitousek et al., 2010). Another study found that female peaks in testosterone levels were associated with times of aggression, such as mating and nesting (Rubenstein and Wikelski, 2005). Testosterone is also positively related to bacterial killing ability in female Galapagos Marine Iguanas (Neuman-Lee and French, 2017) and positively related to tourism for (French et al., 2017). Together these studies suggest that testosterone likely has a significant role in female iguana reproduction, immune function, and maybe even behavior is sensitive to disruption from tourism.

Energy Metabolites and Oxidative Stress

By studying energy metabolites, we gain an understanding of current mobilization and utilization of energy within the body. Some of the energy metabolites that are often measured in animal physiology studies include glucose, triglycerides, and free-glycerol, however, few studies of iguanas have looked at these measures. Comprehensive hematological analyses have been performed for Green Iguanas, to establish normal values for veterinary care (Divers et al., 1996), and in Northern Bahamian Rock Iguanas to assess the impacts of food supplementation (Knapp et al., 2013). With little known about the role of these metabolites in iguanas, more research is greatly needed.

Reactive oxygen species (ROS) are primarily produced in the mitochondria of eukaryotic organisms as a by-product of cellular metabolism. Oxidative stress occurs when the production of reactive oxygen species exceeds the ability of antioxidant defenses to prevent damage (Lushchak, 2014; Monaghan et al., 2009; Sies, 1997; Vassalle et al., 2008). Changes in the production of ROS can occur during commonly encountered situations, such as increased exercise (Ji, 1999; Sies, 1997), and are not always an indicator of pathophysiological conditions. Antioxidant production can increase in response to increases in ROS production, but at times cannot adequately meet the demand, creating an imbalance between ROS and antioxidants. Researchers are now looking at oxidative physiology in the context of life-history traits and trade-offs, trying to identify which conditions are associated with oxidative stress and any resultant long-term impacts (Costantini et al., 2010). Very few studies have examined the context in which oxidative stress occurs in iguanas, but male Galapagos Marine Iguanas did have lower levels of oxidative stress compared to females and female reactive oxygen

metabolites varied significantly throughout the year (Costantini et al., 2009). Further work is needed to better understand when oxidative stress occurs and what the implications are for iguanas.

Immune function

Few studies have closely examined the immune system of iguanas, and there is still a significant amount of work to be done in describing immune function and relationships with other physiological processes. Despite little work in this area, researchers have established that immune challenges can alter iguana behavior and thermoregulation (Green Iguana) (Deen and Hutchison, 2001) and that several hormones appear to play a role in modulating the immune system (Galapagos Marine Iguanas) (Berger et al., 2005; French et al., 2017; Neuman-Lee and French, 2017). Even social rank can alter immune function, as territorial male Galapagos Marine Iguanas had lower swelling response to phytohemagglutinin than satellite males and corticosterone was found to suppress swelling response (Berger et al., 2005). Other interactions between reproduction and hormonal modulation of the immune system have also been described in Galapagos Marine Iguanas (French et al., 2010; French et al., 2017; Neuman-Lee and French, 2017), highlighting the importance of defining reproductive state when interpreting physiological data. Human disturbance was also found to alter immune function in Galapagos Marine Iguanas, with wound healing being slower (French et al., 2010) and bacterial killing ability decreased compared to undisturbed sites (French et al., 2017). Northern Bahamain Rock Iguanas are a valuable model for exploring the

interactions between reproduction and human disturbance on the reptile immune system, which is still greatly understudied.

Implications for conservation

Iguanas are one of the most endangered groups of vertebrates on the planet and conservation must be a priority if further decline is to be prevented. Tourism is an emerging threat to endangered populations of iguanas (French et al., 2017; Knapp et al., 2013; Romero and Wikelski, 2002) but also holds the potential to create positive change. Iguanas are not only an essential part of their ecosystem, with some species playing an important role in seed dispersal (Galapagos Land Iguanas) (Traveset et al., 2016), but are a large charismatic animal that people enjoy observing. By studying several aspects of iguana physiology, we can better inform conservation policy and public education, enabling people to make positive changes for iguana conservation.

For my dissertation, I have focused on several different aspects of animal health and fitness to better assess the impacts of human disturbance in the form of ecotourism. In working with an endangered species, all of this work has been minimally invasive, relying on data collected in the natural habitat of iguanas. I have conducted four studies to describe iguana physiology and the impact of ecotourism on iguanas.

Chapter II – Steroid hormone concentrations and reproductive condition in two subspecies of Northern Bahamian Rock Iguana (Cyclura cychlura inornata and C.c. figginsi)

The purpose of this study was to determine seasonal changes in steroid hormone concentrations associated with reproductive condition for two subspecies of endangered iguana, *Cyclura cyclura inornata* and *C.c. figginsi*. Sex steroid hormones have not yet been measured in this species and by assessing this aspect of physiology the impacts of disturbance on iguana reproduction can be better understood. To accomplish this, steroid hormones were measured in male and female iguanas at 3 distinct reproductive stages (early, late, and post reproduction) and an ultrasound examination was used to determine female reproductive condition. For both subspecies, progesterone was elevated during gravid states. Changes in estradiol were related to reproductive state for *C.c. inornata*, with vitellogenic females having the highest levels, and to season for *C.c. figginsi*, with all females having higher levels in late June. In males, testosterone peaked during early reproduction for *C.c. inornata*, with larger males having higher testosterone levels, while *C.c. figginsi* had a smaller peak during September. Not surprisingly, corticosterone levels varied significantly by sex, season, subspecies, and reproductive condition. During September, male *C.c. inornata* had low corticosterone = while male *C.c. figginsi* had high corticosterone. Reproductive female *C.c. inornata* had elevated corticosterone during late June but both reproductive and non-reproductive female *C.c. figginsi* had elevated corticosterone during the same time. Although reproduction is initiated at slightly different times of the year for the two subspecies, overall patterns of hormones associated with reproductive state were similar.

Chapter III – Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction

Oxidative stress is a potential cost of reproduction, but conclusive evidence for this relationship is lacking. The goal of this study was to serially assess across a seasonal gradient the relationship between reproduction, circulating plasma energy metabolites, and oxidative state. Here we examine a study animal ideally suited to test for the oxidative costs of reproduction: The Allen Cays Rock Iguana. Female rock iguanas reproduce at varying frequencies, often skipping years, allowing for a comparison between reproductive and non-reproductive females during the same narrow, annual breeding season. This feature of iguana life-history enabled us to address not just sex and seasonal differences in physiology, but also potential oxidative costs of reproduction in females. Male and female iguanas were sampled during the early (vitellogenic), late (gravid), and post-reproductive seasons. Ultrasound examinations were performed on females to quantify reproductive investment, and blood samples were collected for physiology assays, which included reactive oxygen metabolites (d-ROMs), antioxidants, triglycerides, free glycerol, and glucose. The early reproductive season was characterized by significant increases in reproductive female's triglycerides, free glycerol, and oxidative stress compared to late and post-reproductive periods and non-reproductive females and males during all sampling periods. Antioxidants were significantly elevated during the early reproductive season for reproductive females, non-reproductive females, and males when compared to late and post-season. Follicle number in early reproductive females was positively related to d-ROMs, triglycerides, and free glycerol, negatively related to antioxidants, and showed no relationship with glucose. Measures of oxidative stress, d-ROMs and oxidative index were positively correlated with circulating levels of the lipid metabolite free glycerol during the early reproductive period, but this

relationship weakened in the late season and disappeared in the post-season. Broadly, this study supports the hypothesis that the relationship between reproduction and oxidative stress is driven by energy investment, being greatest during early reproduction when vitellogenesis is occurring.

Chapter IV – Physiological consequences of ecotourism in an endangered species of iguana (Cyclura cychlura)

Increased interest in ecotourism over recent years has led to more direct human-animal interactions and a striking concomitant increase in the provisioning of non-natural food, that may have unintended consequences for the wildlife involved. The critically endangered Northern Bahamian Rock Iguana provides a valuable model to address the potential impact of food provisioning on health as there are populations that represent a graded variation in human presence, with sites experiencing high, low, or no tourism. We investigated the relationship between the provisioning of non-natural foodstuff and key physiological measures of health for two subspecies of Northern Bahamian Rock Iguanas, and found significant impacts on all measures. Iguanas experiencing the highest amount of food provisioning had elevated levels of energy metabolites, body condition, immune performance, and markers of oxidative stress but decreased circulating levels of the glucocorticoid hormone corticosterone compared to iguanas that were infrequently or not visited. Having sites that experience similar degrees of ecotourism and food supplementation for each subspecies enables us to understand more clearly the degree to which these activities impact physiology in a wild vertebrate. This model system could

have important conservation and management implications for not only this endangered iguana but other species as well.

Chapter VI – Conclusion

In this chapter I summarize the research conducted on the impacts of tourism on iguana health and physiology. I further discuss the implications of my finding and future directions that would benefit our understanding of human disturbance and the conservation of endangered species.

Table 1. Relevant literature on iguana physiology by species and physiological measures.

Iguana species	Physiology measure	Citation
Galapagos Marine Iguanas (<i>Amblyrhynchus cristatus</i>)		
	Baseline and acute stress, and maximal response of CORT (ACTH challenge)	Romero and Wikelski 2010
	Testosterone, estradiol, CORT, wound healing, bacterial killing ability, hemolytic complement activity, dROMS, antioxidants, and clutch size	French, Neuman-Lee et al. 2017
	Testosterone, CORT, wound healing, bacterial killing ability, and hemolytic complement activity	French, DeNardo et al. 2010
	Testosterone, estradiol, progesterone, CORT, wound healing, and bacterial killing ability	Neuman-Lee and French 2017)
	CORT response to tourism exposure	Romero and Wikelski 2002

	Performance and fitness	Wikelski and Romero 2003
	Baseline and stress response CORT	Wikelski, Romero et al. 2001
	Circadian rhythm of baseline CORT	Woodley, Painter et al. 2003
	Iguana survival after an oil spill	Romero and Wikelski 2002
	HPA axis function, ACTH and dexamethasone challenges	Romero and Wikelski 2006
	Ultrasound of follicle development, testosterone, progesterone, and CORT	Vitousek, Mitchell et al. 2010
	Baseline and stress response CORT	Vitousek and Romero 2013
	Heart rate, antipredator behavior (in response to Hawks), influence of CORT and epinephrine injections on escape behavior	Vitousek, Romero et al. 2010
	Baseline and CORT response	Romero and Wikelski 2001
	Changes in body length during El Nino years	Wikelski and Thom 2000
	PHA swelling response, CORT, and testosterone	Berger, Martin II et al. 2005
	Behavior and CORT response to predator introduction	Berger, Wikelski et al. 2007
	Steroid hormones (testosterone, estradiol, progesterone, and CORT) and aggression in female Marine Iguanas	Rubenstein and Wikelski 2005
Galapagos Land Iguanas (<i>Conolophus</i>)		
<i>Conolophus marthae</i> and <i>C. subcristatus</i>	Progesterone and estradiol	Onorati, Sancesario et al. 2016
<i>Conolophus marthae</i> and <i>C. subcristatus</i>	CORT, immune cell counts, reproductive status of females, and parasite counts	Onorati, Sancesario et al. 2017
<i>Conolophus subcristatus</i>	Oxidative stress: dROMS, antioxidants, and oxidative index	Costantini, Dell’Omo et al. 2009

<i>Conolophus subcristatus</i>	Salmonella infection	Franco, Hendriksen et al. 2011
<i>Conolophus subcristatus</i>		Constantini, Dell 'Omo et al. 2005
Rock Iguanas (<i>Cyclura</i>)		
Allens Cay Rock Iguanas (<i>Cyclura cychlura inornata</i>)	Baseline and stress response CORT, endoparasites, and nutritional parameters	Knapp, Hines et al. 2013
Allens Cay Rock Iguanas (<i>Cyclura cychlura inornata</i>)	Body size, growth, condition, and demography.	Smith and Iverson 2015
Turks and Caicos Iguanas (<i>Cyclura carinata carinata</i>)	Diurnal and seasonal patterns of CORT	MacDonald, Czekala et al. 2007
Spiny Tailed Iguanas (<i>Ctenosaura</i>)		
Northeastern Spiny-tailed Iguana (<i>Ctenosaura acanthura</i>)	Fecal CORT and daily activity patterns in response to habitat perturbation	Suárez-Domínguez, Morales-Mávila et al. 2011
Utila Spiny-Tailed Iguana (<i>Ctenosaura bakeri</i>)	Ectoparasites	Gutsche, Mutschmann et al. 2012
Black Spiny-Tailed Iguana (<i>Ctenosaura similis</i>)	CORT	Hanley and Stamps 2002
Green Iguanas (<i>Iguana iguana</i>)		
	Sleep and wakefulness	Ayala-Guerrero and Mexicano 2008
	Absorption of carotenoids	Raila, Schuhmacher et al. 2002
	Blood physiology	Divers, Redmayne et al. 1996
	LPS challenge and behavioral thermoregulation	Deen and Hutchison 2001

	Class 1 MHC gene characterization	Glaberman, Du Pasquier et al. 2008
	Intestinal microflora	Salb, Mitchell et al. 2007
	Pituitary growth hormone and receptors	Ávila-Mendoza, Carranza et al. 2014
	Changes in growth hormone, thyroid hormones, CORT, glucose, and fatty acids in response to acute and chronic sub optimal temperature	Ávila-Mendoza, Carranza et al. 2016
	Circulating testosterone, androstenedione, 17B-estrogen, and estrone levels	Judd, Laughlin et al. 1976
	Baseline and GnRh induced changes in progesterone and estradiol	Phillips, Frye et al. 1987
	Testosterone, estradiol, and iguana puberty	Pratt, Phillips et al. 1994
	Pineal gland function in temperature regulation and melatonin	Tosini and Menaker 1996

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CHAPTER II

STEROID HORMONE CONCENTRATIONS AND REPRODUCTIVE CONDITION IN TWO SUBSPECIES OF NORTHERN BAHAMIAN ROCK IGUANA (*CYCLURA* *CYCHLURA INORNATA* AND *C.C. FIGGINSI*)

The purpose of this study was to determine seasonal changes in steroid hormone concentrations associated with reproductive condition for two subspecies of endangered iguana, *Cyclura cychlura inornata* and *C.c. figginsi*. Sex steroid hormones have not yet been measured in this species and by assessing this aspect of physiology the impacts of disturbance on iguana reproduction can be better understood. To accomplish this, steroid hormones were measured in male and female iguanas at 3 distinct reproductive stages (early, late, and post reproduction) and an ultrasound examination was used to determine female reproductive condition. For both subspecies, progesterone was elevated during gravid states. Changes in estradiol were related to reproductive state for *C.c. inornata*, with vitellogenic females having the highest levels, and to season for *C.c. figginsi*, with all females having higher levels in late June. In males, testosterone peaked during early reproduction for *C.c. inornata*, with larger males having higher testosterone levels, while *C.c. figginsi* had a smaller peak during September. Not surprisingly, corticosterone levels varied significantly by sex, season, subspecies, and reproductive condition. During September, male *C.c. inornata* had low corticosterone = while male *C.c. figginsi* had high corticosterone. Reproductive female *C.c. inornata* had elevated corticosterone during late June but both reproductive and non-reproductive female *C.c. figginsi* had elevated corticosterone during the same time. Although reproduction is initiated at slightly different times of the year for the two subspecies, overall patterns of hormones associated with reproductive state were similar.

INTRODUCTION

While there are multiple naturally occurring steroid hormones in vertebrates, here we focus on the adrenocorticoid steroid- corticosterone, and the gonadal sex steroids progesterone, estrogen, and testosterone, because these hormones are critical both for reproduction and energy mobilization (Norris and Carr, 2013). Estrogen (estradiol in reptiles) and progesterone are the primary gonadal hormones in female reptiles (Custodia-Lora and Callard, 2002; Norris and Carr, 2013). Elevated levels of these hormones stimulate receptive mating behaviors in females, elicit mating behaviors from males (Phillips et al., 1987), and facilitate resource deposition into offspring (Custodia-Lora and Callard, 2002). Estrogens stimulate the synthesis of vitellogenin proteins and increase circulating calcium for egg shell deposition and are also associated with secretions around the egg, such as albumin (Norris and Carr, 2013; Norris and Lopez, 2010) Estradiol is elevated during early stages of reproduction in both the Galapagos Marine Iguana (*Amblyrhynchus cristatus*) (Rubenstein and Wikelski, 2005) and Galapagos Land Iguana (*Conolophus marthae*) (Onorati et al., 2016) and peaks during follicle development in the Green Iguana (*Iguana iguana*), returning to baseline levels following post-reproductive follicular atrophy (Phillips et al., 1987). Progesterone increases the expression of proteins deposited into eggs and facilitates the formation of the eggshell (review of *Chelonia* species) (Custodia-Lora and Callard, 2002). Female Galapagos Marine Iguanas had elevated progesterone at the beginning of the mating period and again at arrival to nesting sites (Rubenstein and Wikelski, 2005), a trend also

observed for female Galapagos Land Iguana (*Conolophus subcristatus*) which had elevated progesterone when gravid (Onorati et al., 2016).

While small amounts of estrogen and progesterone are produced in males, their primary sex steroid hormone is testosterone, which has been studied in other reptile species in relation with copulation and territory defense (Crews et al., 1978; Neuman-Lee et al., 2017), the development of secondary sexual traits (Pratt et al., 1994), and activity period (Klukowski et al., 2004). Male Galapagos Marine Iguanas have higher testosterone during the breeding season compared to non-breeding (French et al., 2010), and for at least one iguana species (Green Iguana), circulating levels increase with body mass (Pratt et al., 1994). Testosterone is a potentially important steroid hormone in female reproduction as well, possibly due to it being a precursor in the synthesis of estrogens (Staub and De Beer, 1997), but also in facilitating behaviors that lead to successful reproduction such as copulation and nesting (Ketterson et al., 2005; Rubenstein and Wikelski, 2005; Vitousek et al., 2010).

Corticosterone (CORT) is the main glucocorticoid hormone in reptiles (Norris and Carr, 2013) and has been measured in other physiology studies of iguanas either to assess baseline concentrations or reactivity to stressful stimuli (Neuman-Lee and French, 2017). Increases in baseline levels of corticosterone may act to facilitate breeding due in part to their role in mobilizing fat stores and converting glycogen to glucose for the energetically costly task of reproducing. Mating behaviors can also be inherently stressful events for both males and females, further elevating levels of this hormone in response to a stressful stimulus. The mating behavior of a species undoubtedly affects how stressful reproduction is, and males which rely on maintaining and defending a territory can have

greater energetic demands and CORT, as observed in territorial lekking Galapagos Marine Iguana males (Berger et al., 2005). For female iguanas, reproduction brings about significant changes in behavior and physiology, starting with early investment into follicles (vitellogenesis) which is followed by copulation with potentially aggressive males, continued egg development, migration to nesting sites, nest construction, oviposition, and nest guarding. As each stage has unique demands, the role of CORT in facilitating and managing these must be dynamic. Female stage-specific changes in CORT have been observed in other iguana species, where CORT was highest during early nesting periods (Galapagos Marine Iguanas) compared to other stages (Rubenstein and Wikelski, 2005) and elevated in females carrying eggs compared to non-reproductive females sampled during the same period (Galapagos Land Iguanas, *Conolophus subcristatus*) (Onorati et al., 2017).

Northern Bahamian Rock Iguanas, *Cyclura cychlura*, are distributed in isolated populations on 17 of the 365 cays of the Exuma island chain on the Great Bahama Bank (Malone 2003 & Hines 2017). These populations are separated into two geographically disjunct subspecies, *Cyclura cychlura inornata* and *Cyclura cychlura figginsi*, and are both listed as Critically Endangered according to IUCN Red List of Threatened Species™ criteria, respectively (Iverson, 2019; Knapp, 2004). Northern Bahamian Rock Iguanas begin mating in early to mid-May and migrate to nesting sites in mid to late June where females dig nests and defend them for at least 3-4 weeks after completion (Iverson et al., 2004). On average, one in three adult females nest each year with clutch sizes between 1-10 eggs, with frequency but not clutch size increasing with female size (Iverson et al., 2004). The populations of the subspecies *C.c. figginsi* included in this

study are located south of *C.c. inornata* and begin the breeding season approximately 2-3 weeks earlier. Some of the existing populations of *C. cythlura* have been studied for up to 40 years in an effort to assess changes in population demography, reproduction and physiology (Iverson et al., 2004; Knapp et al., 2013; Webb et al., 2018), and genetic structure (Aplasca et al., 2016; Malone et al., 2003; Malone et al., 2000). While the steroid hormone, corticosterone, has been assessed in relation to tourism pressure (Knapp et al., 2013), seasonal changes in corticosterone and the sex steroid hormones progesterone, estradiol, and testosterone have not yet been determined for this species.

Given the current body of knowledge on the timing of reproduction in *C. cythlura*, we predicted that there would be distinct seasonal changes in all steroid hormones associated with reproductive condition. Specifically, we hypothesized that **1)** estradiol would be elevated during early reproductive states compared to gravid states, **2)** progesterone would be elevated during gravid states, **3)** testosterone would be elevated for both males and females during early reproduction (copulation) and during nest guarding for reproductive females, **4)** corticosterone would be elevated for males during early reproduction and during gravid and nest guarding states for reproductive females, **5)** seasonal patterns would be similar between the two subspecies but would reflect the earlier initiation of breeding in *C.c. figginsi* relative to *C.c. inornata*, and finally, **6)** all three steroid sex hormones would be at baseline levels during September. As previously described (Webb et al., 2018), this study system is ideal for addressing such questions because individuals can be safely and reliably captured both during and after reproduction and because approximately one in three adult females nest each year

(Iverson et al., 2004), allowing for a direct comparison of reproductive and non-reproductive females during the sampling period.

MATERIALS AND METHODS

2.1 Study design and sample collection

Male ($n = 450$) and female ($n = 372$) Rock Iguanas were captured in May, June or September of 2016 and in June or September of 2018 from six different sites: 3 sites in the northern Exuma islands (*Cyclura cyclura inornata*) and 3 sites in the southern Exuma islands (*Cyclura cyclura figginsi*) in the Commonwealth of the Bahamas. These sampling months coincide with vitellogenic (May 2016 and early June 2018), gravid (Late June 2016) and post-reproductive (September 2016 and 2018) states for females and reproductive (May and June) and post-reproductive (September) states for males (Iverson et al., 2004) (Table 1). Rock Iguanas were captured and processed following a previously described protocol (Webb et al., 2018). To assess circulating levels of steroid hormones, a blood sample was collected from the caudal vein between 0800 and 1350 and the average time to bleed was 115.4 ± 3.05 seconds. There was no significant correlation between any hormone concentration and time to bleed or time of day ($r^2 < 0.05$).

2.2 Reproductive condition

Female reproductive states were determined in May and June by examination with a high-resolution ultrasound (Sonosite MicroMaxx). The exam allowed for the assessment of reproductive state and reproductive stage. Reproductive state was determined by the presences or absence of developed follicles or eggs during the reproductive months, females being either “reproductive” or “non-reproductive” for a given sampling period (Table 1). Reproductive stages were determined by size, shape, and position of follicles or eggs. Reproductive stages were either “vitellogenic” or “gravid”, with vitellogenic states having multiple small (diameter < 3.0 cm), round, centrally positioned follicles (Figure 1) and gravid states having large (diameter > 3.0 cm), oblong, eggs aligned in the oviducts.

Table 1. Reproductive stage and sample sizes by subspecies

Sampling period (day of year)	<i>C.c. inornata</i>	<i>C.c. figginsi</i>
May 2016 (138-144) June 2018 (152-159)	Vitellogenesis ($n = 52$) Non-reproductive ($n = 45$) Males ($n = 117$)	Gravid ($n = 33$) Non-reproductive ($n = 25$) Males ($n = 54$)
June 2016 (171-178)	Gravid ($n = 10$) Non-reproductive ($n = 25$) Males ($n = 51$)	Late gravid ($n = 10$) Non-reproductive ($n = 16$) Males ($n = 42$)
September 2016 & 2018 (245-255)	Females ($n = 62$) Males ($n = 84$)	Females ($n = 94$) Males ($n = 102$)



Figure 1. Ultrasound analysis of *Cyclura cyclura* illustrating small, spherical follicles during vitellogenesis.

2.3 Radioimmune assay of steroid hormones

Baseline circulating hormone concentrations of estradiol (Ab: Biogenesis #7010-2650), progesterone (Ab: Fitzgerald #WLI-P1604-S), testosterone (Ab: Fitzgerald #WLI-T3003-01916), and corticosterone (Ab: MP Biomedicals # 07-120016) were measured with radioimmune assays. Assays were performed following a previously described protocol (French et al., 2010; Moore, 1986; Moore et al., 1991). Samples were extracted with isooctane: ethyl acetate, dried down, and resuspended in a phosphate buffered saline (PBS). Each hormone was measured separately with a direct assay and all samples were assayed in duplicate and the mean value was used in analysis. The intra-assay coefficients

of variation were < 10 % for 3 estradiol assays, < 20 % for 3 progesterone assays, < 15% for 3 testosterone assays, and < 15 % for 5 corticosterone assays.

2.4 Statistical analysis

All statistical analyses were performed in JMP, version 14.0.0 (SAS Institute Inc., Cary, NC, USA). Parametric (ANOVA) and non-parametric (Kruskal-Wallis) tests were used to model circulating hormone concentrations as a function of reproductive condition, a variable which encompassed both season and presence or absence of follicles/eggs. An ANCOVA was used to model male hormone levels, testosterone and corticosterone, as a function of season and body mass. Body mass was not included in models of female hormones due to correlations with reproductive state. To compare concentrations of testosterone and corticosterone in males and females, the data was subset by sampling period and an ANOVA (May/early June and late June) or a two-tailed t-test (September) was used to compare females of each reproductive state to males. Each subspecies was modeled separately due to slight differences in the timing of reproduction. We did not include year as a factor, but an initial analysis with ANOVA showed no significant effect on physiological measures. Testosterone and corticosterone concentration values were \log_e transformed to normalize data for analysis. Progesterone and estradiol were not normalized with a \log_e transformation and were therefore modeled with a non-parametric test. *Post hoc* analyses were performed for significant interactions by Tukey's honest significant difference and Student's t-test for parametric tests, and Wilcoxon pairwise comparisons for non-parametric tests. Normality checks of residual distribution and Levene's tests were carried out and the assumptions were met.

RESULTS AND DISCUSSION

3.1 Estradiol

Estradiol was higher during vitellogenesis for *C.c. inornata* females compared to the non-reproductive females during the same time ($\chi^2(4) = 10.18, p = 0.037$) (Fig. 2). This trend appears to be common among reptiles, even across families, as estradiol was elevated during early reproduction for Galapagos Land Iguanas (*Conolophus marthae*) (Onorati et al., 2016), Galapagos Marine Iguanas (Rubenstein and Wikelski, 2005), and Green Sea Turtles (*Chelonia mydas*) (Licht et al., 1979). In addition, estradiol was also elevated during vitellogenesis in Asp Vipers (*Vipera aspis*) (Bonnet et al., 2001), Blue-Tongued Skinks (*Tiliqua nigrolutea*) (Edwards and Jones, 2001), and Western Diamond-backed Rattlesnakes (*Crotalus atrox*) (Taylor et al., 2004), all of which are viviparous. Reproduction alone did not appear to drive any substantial differences in estradiol concentrations for female *C.c. figginsi*, however, all females had significantly higher estradiol during late June compared to May/early June ($\chi^2(4) = 41.28, p = < 0.0001$) (Fig. 1). This could very likely be because the sampling periods occurred later than any potential peaks in estradiol for this subspecies.

Female *C.c. inornata* estradiol concentrations were lower in September than for breeding females in May/early June and non-breeding females in late June. September estradiol concentrations were most similar to early non-reproductive females. Female *C.c. figginsi* estradiol concentrations were still quite high in September, similar to concentrations observed for both breeding and non-breeding females in late June.

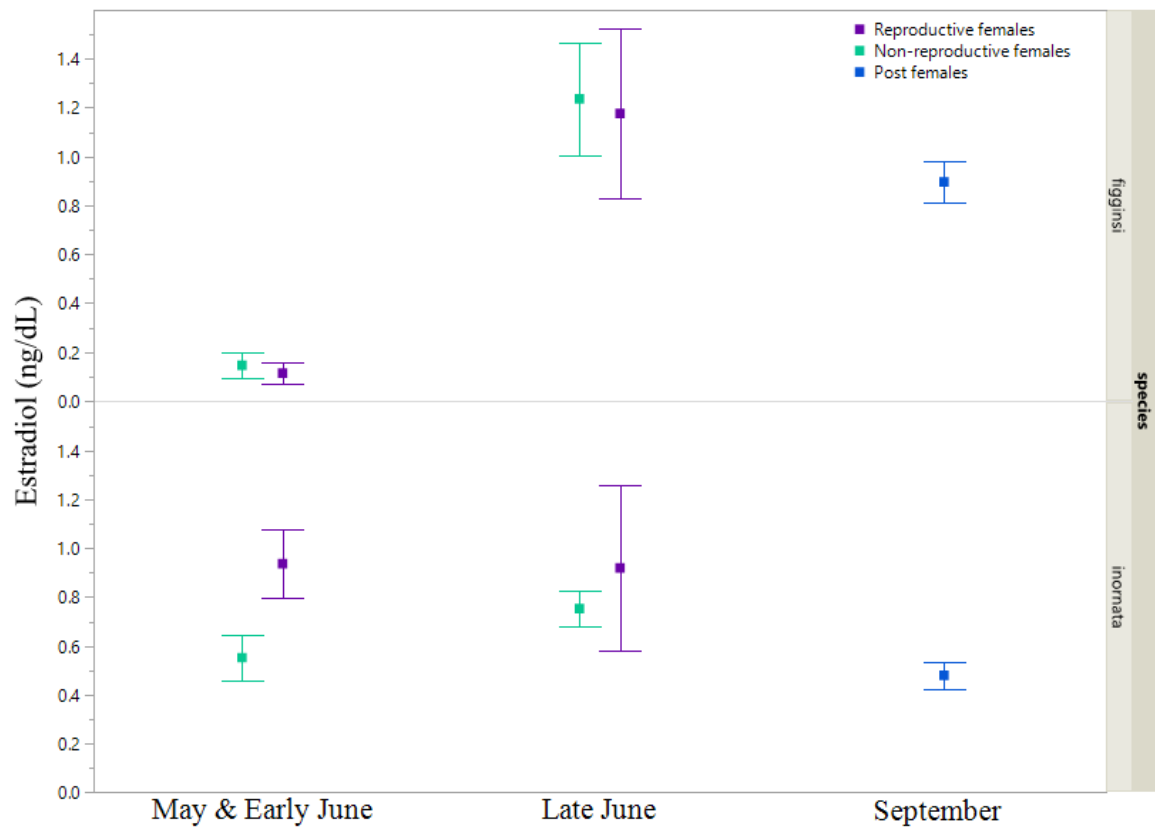


Figure 2. Seasonal changes in estradiol for female *Cyclura cychlura inornata* and *C.c. figginsi* by reproductive condition. Points display the mean \pm SE.

3.2 Progesterone

Reproductive female *C.c. inornata* had the highest levels of progesterone in late June compared to non-reproductive females and reproductive females sampled in May/early June ($\chi^2(4) = 1.47, p = < 0.0001$) (Fig. 3). Similarly, reproductive female *C.c. figginsi* had the highest levels of progesterone during gravid states, in May/early June, compared to non-reproductive females sampled during the same time and all females sampled in late June ($\chi^2(4) = 51.27, p = < 0.0001$). The peaks in progesterone observed during gravid states for Rock Iguanas (May/early June for *C.c. figginsi* and late June for *C.c. inornata*) were similar to those observed for Galapagos Marine Iguanas, which had elevated progesterone at the beginning of the mating period and again at arrival to nesting sites (Rubenstein and Wikelski, 2005), as well as several other species of reptiles (asp vipers, *Vipera aspis*, (Bonnet et al., 2001) and Blue-Tongued Skinks, *Tiliqua nigrolutea* (Edwards and Jones, 2001). A sharp decline in progesterone may be responsible for initiating laying, an idea that is also supported by an experiment that implanted progesterone in a pregnant viviparous lizard (*Sceloporus jarrovi*) and found it to delay parturition compared to control animals (Guillette Jr et al., 1991). It could be that the peak we observed corresponded to the beginning of the nesting periods as females were preparing to lay their eggs.

Though still significantly lower than reproductive females, non-reproductive female *C.c. inornata* had elevated levels of progesterone in late June compared to May/early June. Follicle atresia for non-reproductive females has been associated with progesterone peaks in non-receptive female Galápagos marine iguanas (*Amblyrhynchus cristatus*) (Vitousek et al., 2010), however, follicle atresia would have likely been

observed via ultrasound examination and probably doesn't explain the progesterone peak observed in Rock Iguanas. It is possible that some females laid early and were therefore mistakenly recorded as non-reproductive. Female *C.c. figginsi* and *C.c. inornata* progesterone concentrations in September were similar to non-breeding females in May/early June, which were both lower than all other sampling periods and reproductive states, likely representing baseline levels for this species. Similarly, non-reproductive Western Diamond-backed Rattlesnakes (*Crotalus atrox*) had consistently low levels of progesterone throughout the year (Taylor et al., 2004).

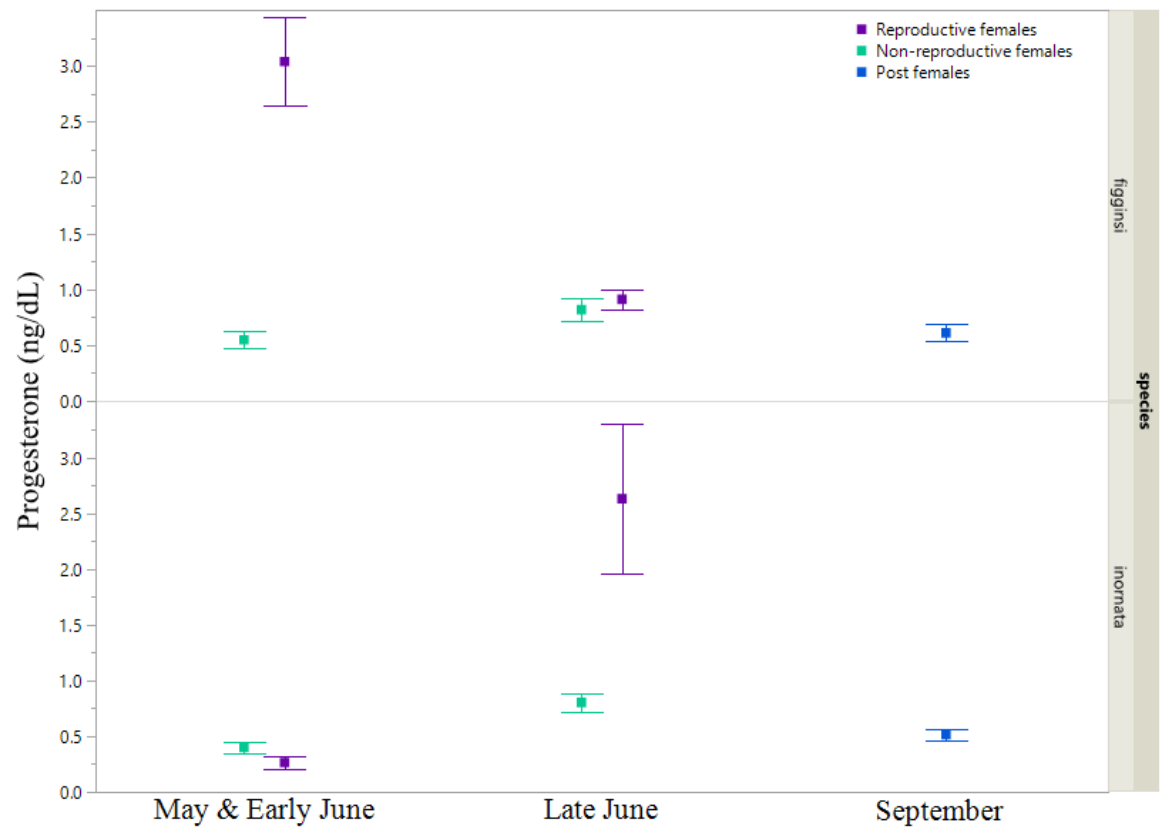


Figure 3. Seasonal changes in progesterone for female *Cyclura cyclura inornata* and *C.c. figginsi* by reproductive condition. Points display the mean \pm SE.

3.3 Testosterone

Testosterone was significantly elevated for reproductive female *C.c. figginsi* during early June, which corresponded with gravid states ($P < 0.0001$) (Fig. 4). Although not significant, testosterone tended to be higher in female *C.c. inornata* during May/early June, which also corresponded with gravid states ($P < 0.079$). Testosterone was higher in May/early June compared to late June for both reproductive and non-reproductive female *C.c. figginsi* ($P < 0.0001$). Female testosterone levels appear to be linked with reproduction in several taxa, although the stage of reproduction at which testosterone peaks does vary, possibly due to different reproductive strategies. Several species of bird had peak testosterone levels during the pre-laying or laying period, times that are associated with territory acquisition and nest defense (Ketterson et al., 2005). In a study of Western Diamond-backed Rattlesnakes (*Crotalus atrox*) reproductive females had elevated testosterone during vitellogenesis and non-reproductive females had consistently low levels of testosterone throughout the year (Taylor et al., 2004). In some mammals, female testosterone levels during pregnancy can be attributed to social rank as the most dominant meerkat females (*Suricata suricatta*) have higher testosterone than subordinate females (Clutton-Brock et al., 2006), and may be important for securing the limited nest sites on some of the islands.

Female *C.c. figginsi* testosterone in September was significantly lower than all other reproductive states and sampling periods except for breeding females in late June. Female *C.c. inornata* had no significant differences in testosterone attributed to season or reproductive condition. Individual variations in testosterone during reproduction and the

potential role in offspring behavior would be interesting aspects to consider in further interpreting the role of testosterone in female iguanas.

Male *C.c. inornata* had significantly higher concentration of testosterone in May/early June compared to the other sampling periods ($P < 0.0001$) (Fig. 4). This peak was not observed for male *C.c. figginsii* but may have been missed due to the earlier initiation of reproduction in this subspecies. Male Marine Iguanas also had higher testosterone during the breeding season compared to non-breeding season (French et al., 2010). Testosterone was significantly associated with aggressive behaviors in other iguana studies as well and testosterone increase preceded behaviors changes by approximately 1 month (Lock and Bennett, 2015). With mating occurring in mid-May for *C.c. inornata* (Iverson et al., 2004), and 2-3 weeks prior for *C.c. figginsii*, maximum levels of testosterone were possibly missed for both subspecies during our sampling.

Male *C.c. figginsii* had slightly higher testosterone in September compared to late June ($P < 0.0001$) and larger males had higher testosterone ($P = 0.002$), but unlike *C.c. inornata*, this relationship was independent of season. September peaks in testosterone could be related to spermatogenesis, which varies for reptiles by reproductive strategy (prenuptial, post nuptial, mixed, continuous). Temperate turtles and most temperate snakes are postnuptial in spermatogenesis patterns, starting after spring mating (reviewed in:) (Gribbins, 2011). Testosterone is necessary for several stages of spermatogenesis (Steinberger and Duckett, 1967; Walker, 2011) and it is not uncommon for reptiles to have bimodal peaks in testosterone which correspond with both mating behaviors and again with spermatogenesis (Mojave Rattlesnakes (*Crotalus scutulatus*) (Schuett et al., 2002), Karoo Girdled Lizard (*Cordylus polyzonus*) (Flemming, 1993), Stinkpot Turtles

(*Sternotherus odoratus*) (McPherson et al., 1982)). Although the timing of spermatogenesis in Rock Iguanas is not known, the bimodal peaks in testosterone provide good reason to investigate the occurrence of post nuptial spermatogenesis for this species. Testosterone in male Galapagos Marine Iguanas has also been shown to be sensitive to tourism disturbance (French et al., 2010), another possible explanation for the seasonal variation of testosterone in Rock Iguanas.

For *C.c. inornata*, there were no significant differences between males and females of either reproductive condition in testosterone levels during May/early June ($P = 0.37$) or between males and females in September ($t(115) = 1.04$, $P = 0.30$). However, in late June, reproductive females, but not non-reproductive females, had higher testosterone levels than males ($P = 0.003$). For *C.c. figginsi*, reproductive females, but not non-reproductive females had higher testosterone levels than males in May/early June ($P = < 0.0001$) but in late June, there were no significant differences between males and females of either reproductive condition ($P = 0.35$). In September, males had higher testosterone than females ($t(138) = 4.02$, $P = < 0.0001$).

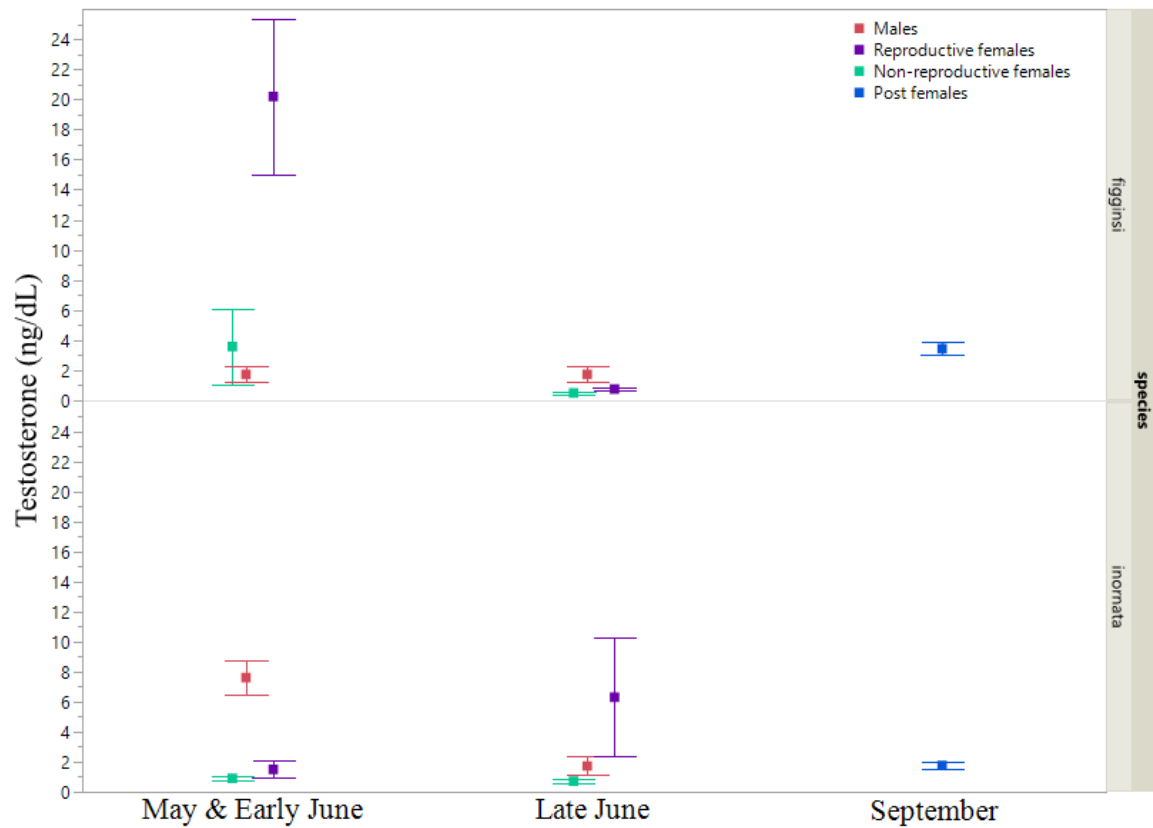


Figure 4. Seasonal changes in testosterone *Cyclura cychlura inornata* and *C.c. figginsi* by sex and reproductive condition. Points display the mean \pm SE.

3.4 Corticosterone

Corticosterone varied by season for males of both subspecies with concentrations highest in September for *C.c. figginsi* ($P = 0.0009$) and lowest in September for *C.c. inornata* ($P = 0.004$) (Fig. 5). Avian studies have found negative correlations between body mass and stress induced (but not baseline) corticosterone levels, and it has been suggested that individuals with lower energy stores, such as lipids, may have more need for the gluconeogenic activities of corticosterone (Schoech et al., 1997; Wingfield et al., 1994). Food restriction can lead to lower body mass and an increase in corticosterone (red-legged kittiwakes (*Rissa brevirostris*) (Kitaysky et al., 2001) and Northern Water Snakes (*Nerodia sipedon*) (Webb et al., 2017)) and differences in food availability or other environmental factors could explain the differences observed in corticosterone. Behavioral and social status differences have also been linked to baseline levels of corticosterone in male Galapagos Marine Iguanas, showing that territorial males had the highest corticosterone (Berger et al., 2007). Behavioral differences between the two subspecies could also be related to the different patterns in corticosterone.

Reproductive female *C.c. inornata* ($P = 0.046$) and *C.c. figginsi* ($P = < 0.0001$) (Fig. 5) had higher levels of corticosterone during late June compared to all females in September. Similarly, corticosterone was lower in post breeding female Eastern Fence Lizards (*Sceloporus undulatus*) compared to reproductive females (Phillips and Klukowski, 2008). Corticosterone was also elevated in egg carrying females for two species of Galapagos Land Iguana (Onorati et al., 2017) and highest during early nesting periods in Galapagos Marine Iguanas (Rubenstein and Wikelski, 2005). Receptive female Galapagos marine iguanas (*Amblyrhynchus cristatus*) had lower corticosterone than non-

receptive females during late male assessment to post-breeding periods (Vitousek et al., 2010). Maternal glucocorticoid levels can also alter offspring phenotype, in some cases even reducing offspring fitness (slowed growth in meerkats) and increasing parental fitness (increased cooperative behavior in offspring – pup rearing and feeding) (Dantzer et al., 2019).

Reproductive and non-reproductive females had higher corticosterone than males in May/early June for both subspecies (*C.c. inornata*, $P = 0.0006$; *C.c. figginsi*, $P = < 0.0001$). Reproductive females but not non-reproductive female *C.c. inornata* had higher corticosterone than males during late June ($P = 0.013$). Females also had higher corticosterone than males during September ($t(137) = -2.47$, $P = 0.015$). For *C.c. figginsi*, both reproductive and non-reproductive females had higher corticosterone than males during late June ($P = < 0.0001$). There were no significant differences between males and females during September ($t(190) = -1.97$, $P = < 0.05$).

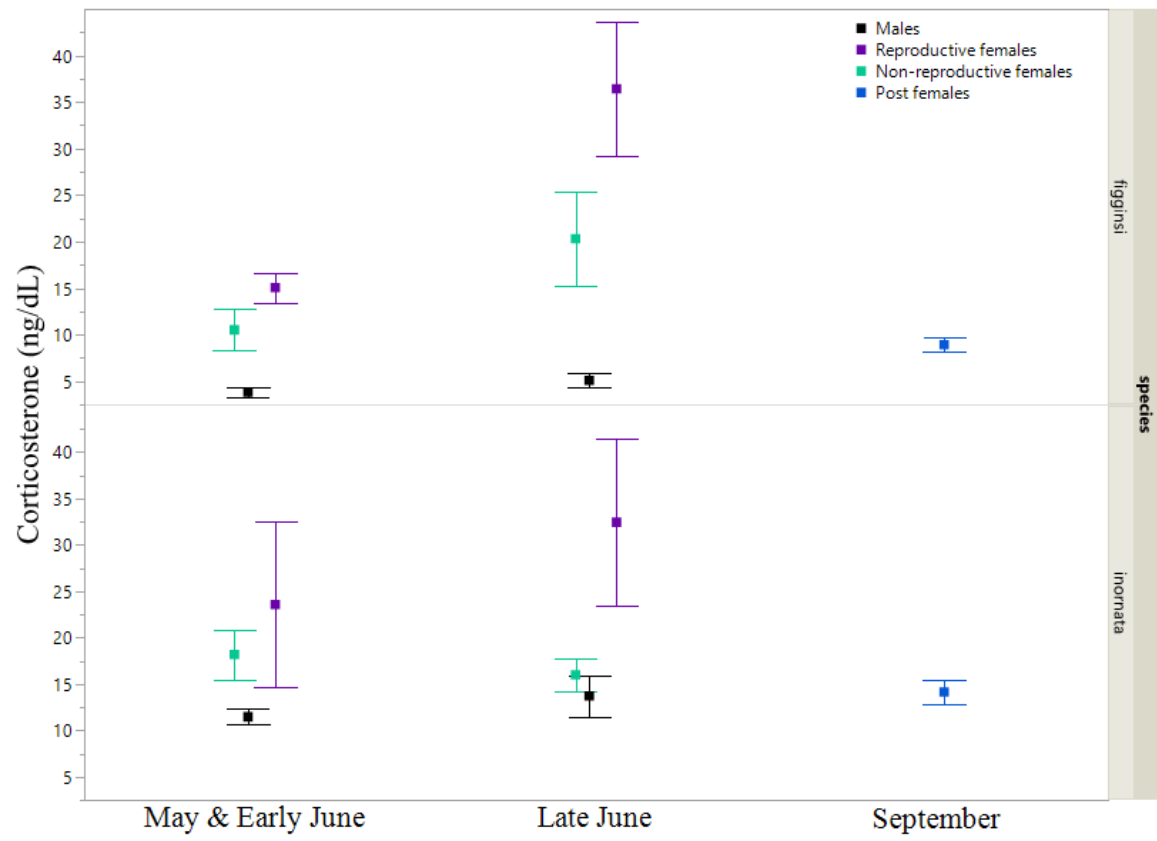


Figure 5. Seasonal changes in corticosterone for female *Cyclura cyclura inornata* and *C.c. figginsi* by sex and reproductive condition. Points display the mean \pm SE.

CONCLUSION

Reproduction is accompanied by distinct changes in steroid hormone concentrations which likely act to facilitate mating behaviors, receptivity, and offspring development and which are well documented for many vertebrate species (Norris and Carr, 2013; Norris and Lopez, 2010). As many species have differing life histories and environmental constraints, the endocrine profiles characterizing periods of reproduction are often distinct, but exact patterns are not well known for many species. Some but not all of our hypotheses concerning the seasonal patterns of endocrine profiles were supported. As we anticipated, estradiol was elevated during vitellogenesis, progesterone and corticosterone were elevated during gravid states, and testosterone was elevated during early reproduction for males and gravid states for females. The role of corticosterone in male reproduction was less clear than we initially hypothesized, likely due to high individual variation. While progesterone and testosterone appeared to be at baseline levels for females during September, estradiol and male hormones still showed significant variation that could also be driven by high individual variation. Overall, seasonal patterns in hormone concentrations were similar between the two subspecies, even though the timing of reproductive cycles differed by two to three weeks (*C.c. figginsi* earlier). Basic research on life history data for wild populations, as presented here, has been called for in an effort to better inform management strategies for these endangered iguanas (Alberts, 2004). By studying this aspect of the seasonal physiology of *Cyclura cychlura*, we gain a better understanding of their reproductive endocrinology

which can allow us the ability to assess the impact of current and future perturbations, such as anthropogenic disturbance and climate change.

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CHAPTER III

ENERGETIC INVESTMENT ASSOCIATED WITH VITELLOGENESIS INDUCES AN OXIDATIVE COST OF REPRODUCTION¹

Oxidative stress is a potential cost of reproduction, but conclusive evidence for this relationship is lacking. The goal of this study was to serially assess across a seasonal gradient the relationship between reproduction, circulating plasma energy metabolites, and oxidative state. Here we examine a study animal ideally suited to test for the oxidative costs of reproduction: The Allen Cays Rock Iguana. Female rock iguanas reproduce at varying frequencies, often skipping years, allowing for a comparison between reproductive and non-reproductive females during the same narrow, annual breeding season. This feature of iguana life-history enabled us to address not just sex and seasonal differences in physiology, but also potential oxidative costs of reproduction in females. Male and female iguanas were sampled during the early (vitellogenic), late (gravid), and post-reproductive seasons. Ultrasound examinations were performed on females to quantify reproductive investment, and blood samples were collected for physiology assays, which included reactive oxygen metabolites (d-ROMs), antioxidants, triglycerides, free glycerol, and glucose.

¹ Previously published in Webb, AC, JB Iverson, CR Knapp, DF DeNardo, and SS French. 2019. Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction. *Journal of Animal Ecology* 2019; 1-11.

The early reproductive season was characterized by significant increases in reproductive female's triglycerides, free glycerol, and oxidative stress compared to late and post-reproductive periods and non-reproductive females and males during all sampling periods. Antioxidants were significantly elevated during the early reproductive season for reproductive females, non-reproductive females, and males when compared to late and post-season. Follicle number in early reproductive females was positively related to d-ROMs, triglycerides, and free glycerol, negatively related to antioxidants, and showed no relationship with glucose. Measures of oxidative stress, d-ROMs and oxidative index were positively correlated with circulating levels of the lipid metabolite free glycerol during the early reproductive period, but this relationship weakened in the late season and disappeared in the post-season. Broadly, this study supports the hypothesis that the relationship between reproduction and oxidative stress is driven by energy investment, being greatest during early reproduction when vitellogenesis is occurring.

INTRODUCTION

Evolution has led to traits in populations that optimize reproductive success; however, at the same time, there are inherent costs of reproduction. Substantial nutritional resources must be acquired and allocated to offspring development, which can reduce the resources available for other functions such as growth and self-maintenance. The increased nutritional demands and metabolic activity occurring during reproduction

may also result in increased oxidative stress, possibly constraining this life-history trait (Metcalf & Alonso-Alvarez, 2010). While some researchers have found support for oxidative stress being a cost of reproduction, the relationships among specific reproduction stages, metabolic activity, and oxidative states are still not well understood.

Oxidative physiology

Reactive oxygen species (ROS) are by-products of cellular metabolism, which are primarily produced in the mitochondria of eukaryotic organisms. Most cellular processes result in the production of superoxides (O_2^-), which are quickly converted to a non-radical ROS that can cause damage to DNA, proteins, and other macromolecules (Ames, Shigenaga & Hagen 1993; Halliwell 1994; Monaghan, Metcalfe & Torres 2009). The antioxidant system protects an organism's cells and tissues from the damaging effects of ROS (Costantini *et al.* 2010). Some antioxidants come from dietary sources but many types are produced endogenously, all working together to provide protection (Lushchak 2014). Oxidative stress occurs when the production of reactive oxygen species exceeds the ability of antioxidant defences to prevent damage (Sies 1997; Vassalle *et al.* 2008; Monaghan, Metcalfe & Torres 2009; Lushchak 2014). Changes in the production of ROS can occur under pathophysiological conditions but also during commonly encountered situations, such as increased exercise (Sies 1997; Ji 1999). Antioxidant production can adapt to changes in ROS production, but at times cannot adequately meet the demand, creating an imbalance between ROS and antioxidants. Thus, oxidative stress occurs because of excessive production of ROS, decreased production or inactivation of antioxidants, or a combination of these (Monaghan, Metcalfe & Torres 2009; Lushchak

2014). Transient oxidative stress may occur during several life-history stages but even short periods of time in this state could be detrimental to the health and longevity of an animal (Finkel & Holbrook 2000), and oxidative stress occurring during reproduction can lead to abortion, maternal reproductive disorders, and even offspring defects (Agarwal, Gupta & Sharma 2005). Therefore, researchers are now looking at oxidative physiology in the context of life-history traits and trade-offs, trying to identify which conditions are associated with oxidative stress and any resultant long-term impacts (Costantini *et al.* 2010).

Reproduction and oxidative stress

A relationship between reproduction and oxidative stress has been proposed by several researchers (Metcalf & Monaghan 2013), but further research is needed to define and understand the differences among species and reproductive strategies. Reproduction is an energetically expensive process for females, increasing resource requirements, metabolism (Angilletta & Sears 2000), and potentially the production of ROS (Metcalf & Alonso-Alvarez 2010; Metcalf & Monaghan 2013; Costantini 2016). Oxidative costs of reproduction have been demonstrated in females of numerous species, including wild Song Sparrows (*Melospiza melodia*) (Travers *et al.* 2010), the viviparous Asp Viper (*Vipera aspis*) (Stier *et al.* 2017), Brown Boobies (*Sula leucogaster*) (Montoya *et al.* 2016), Zebra Finches (*Taeniopygia guttata*) (Noguera 2017), and White-Browed Sparrow Weavers (*Ploceus mahali*) (Cram, Blount & Young 2015). However, reproduction is a series of physiological events, and different stages have varying energetic demands and it is likely that oxidative stress also varies with reproductive stage. For instance, in the

Brown Booby, reactive oxygen species were highest during the early reproductive season for breeding pairs and decreased thereafter (Montoya *et al.* 2016). Song Sparrows also demonstrate an increase in oxidative stress during the laying period of reproduction as laying more eggs as a result of nest predation have higher oxidative stress (Travers *et al.* 2010). Thus, detailed studies are needed to reveal the key drivers behind the relationship between reproduction and oxidative stress.

Energy metabolites and reactive oxygen species

The high energetic demands of reproduction likely increase the metabolism of lipid stores. Lipolysis of stored lipids results in increased circulation of free-fatty acids and the glycerol backbone, which together form triglycerides (Lin 1977) and provides energetic resources needed for cellular metabolism and yolk production. Although metabolic rate and the production of ROS are not inherently linked (Metcalf & Alonso-Alvarez 2010; Costantini 2016), it does appear that certain physiological conditions that increase cellular metabolism, such as exercise, digestion, and reproduction, are followed by an increase in ROS (Finkel and Holbrook 2000; Butler *et al.* 2016; Ji 1999). Similarly, conditions that increase circulating levels of free-fatty acids may also increase ROS. One study found that mice with a mutation for Werner syndrome (premature ageing) also had increased triglycerides, free-fatty acids, and ROS but a direct correlation between energy metabolites and ROS was not observed (Massip *et al.* 2006). However, when explicitly investigating the relationship between plasma triglycerides and d-ROMS, a positive correlation was observed for several species of birds (Pérez-Rodríguez *et al.* 2015). Overall, few studies have attempted to link lipid metabolism, specifically free-glycerol,

during reproduction with oxidative stress despite the likely relationship between these variables.

Research and modelling goals

Given the current body of knowledge, we hypothesize that there is a relationship between reproduction and oxidative stress and that this relationship is driven by energy investment, which is often high during early reproductive stages. Specifically, we predict that (1) circulating energy metabolites will be highest for vitellogenic females, (2) elevated free-glycerol will be positively correlated with oxidative stress, and (3) that non-reproductive females will have physiological measures more similar to males than to reproductive females. To better understand oxidative stress in an ecological context, we investigated the relationship between specific reproductive stages and energy metabolites, reactive oxygen metabolites, and antioxidant defences in a free-living population of Allen Cays Rock Iguanas (*Cyclura cychlura inornata*). Secondly, we investigated the relationship between circulating energy metabolites and reactive oxygen metabolites as a potential mechanism linking reproduction and oxidative stress. This study system is ideal for addressing these relationships, since (1) individuals can be safely and reliably captured during and after reproduction, (2) nearly all adults are individually marked, and (3) approximately one in three adult females nest each year (Iverson, Hines & Valiulis 2004), providing an ideal comparison between reproductive and non-reproductive females during the same sampling period.

MATERIALS AND METHODS

Rock iguanas

The Allen Cays Rock Iguana is distributed among isolated populations on five of the 365 cays of the Exuma island chain on the Great Bahama Bank (Malone *et al.* 2003) and is considered Endangered according to International Union for Conservation of Nature (IUCN) Red List criteria. Long-term studies of population demography (Iverson & Mamula 1989; Iverson *et al.* 2006; Iverson 2007), genetic structure (Malone *et al.* 2003; Aplasca *et al.* 2016), evolution (Martins & Lamont 1998; Malone *et al.* 2000), reproduction (Iverson, Hines & Valiulis 2004; Knapp, Iverson & Owens 2006), and behaviour (Knapp 2000) of *C. cychlura* have provided an ideal foundation of information to augment physiological studies.

Study design

Male ($n = 166$) and female ($n = 133$) iguanas were captured in May, June, or September of 2016 from Allen's Leaf Cay, U Cay, and Flat Rock Reef Cay (Table 1), which are located in the Allen Cays (24° 45' N, 76° 50' W) of the northern Exuma islands in the Commonwealth of the Bahamas. These sampling months coincided with vitellogenic (May), gravid (June), and post-reproductive (September) states for females

and reproductive (May and June) and post-reproductive (September) states for males (Iverson, Hines & Valiulis 2004).

	May	June	September
Allen's Leaf Cay	Reproductive F = 19 Non-reproductive F = 9 Males = 37	Reproductive F = 3 Non-reproductive F = 13 Males = 21	Females = 16 Males = 22
U Cay	Reproductive F = 7 Non-reproductive F = 6 Males = 11	Reproductive F = 1 Non-reproductive F = 5 Males = 13	Females = 15 Males = 11
Flat Rock Reef Cay	Reproductive F = 4 Non-reproductive F = 9 Males = 16	Reproductive F = 5 Non-reproductive F = 7 Males = 14	Females = 9 Males = 12

Table 1. Sample sizes for Allen Cays Rock Iguanas (by sex, reproductive condition, site, and month).

Sample collection

Iguanas were captured by noose or net and a blood sample was collected from the caudal vein within 3 minutes to control for potential stress-induced changes in physiology. Blood samples did not exceed 0.5 ml and were collected with a 25-gauge heparinized 3 ml syringe (305270, BD Integra, New Jersey, USA). All blood samples were collected between 0800 and 1350 with an average time to bleed of 97 ± 3 (SE) seconds. There was no correlation between any physiological measure and time of day ($r^2 < 0.08$) or time to acquire blood sample ($r^2 < 0.001$). Iguanas were temporarily stored in cloth bags until processed to obtain morphometric data and determine sex via cloacal probing. Female reproductive state and follicle number were determined by ultrasound examination in May and June following a protocol previously described (French *et al.*

2017). Blood samples were stored on ice for up to 6 hours, after which a whole blood glucose test, which has been validated for use in vertebrate species (Stoot *et al.* 2014), was performed with a portable meter (Aviva AccuChek Aviva Plus, Roche Diagnostics, Indiana, USA), and plasma was separated by centrifugation at 6000 rpm (2000 x g) for 10 minutes. Plasma samples were frozen and transported back to the laboratory on dry ice where they were stored at -80°C until physiological assays were performed.

Reactive oxygen metabolites and antioxidant capacity

Oxidative physiology was assessed by measuring the derivatives of reactive oxygen metabolite species (d-ROMs) and the effectiveness of antioxidant defences. Circulating d-ROMs were quantified using an assay kit (MC435, Diacron International, Italy) which detects levels of hydroperoxides that oxidize an alkyl-substituted aromatic amine (A-NH₂). Plasma was diluted in the provided acidic buffered solution (5 µl : 100 µl) and samples were run in duplicate following the manufacturer instructions for ‘endpoint’ mode with modifications for use with a 96-well microplate (French *et al.* 2017). This reaction resulted in a colour change that was measured with a spectrophotometer at 505 nm (BioRad xMark). Values were calculated as absorbance change relative to the standard. The inter-assay coefficient of variation (CV) was 3.65 % and the intra-assay CV’s were < 5 % for each of the 10 plates. Antioxidant capacity was measured with the OXY-Absorbent kit (MC002 Diacron International, Italy) which quantifies the ability of plasma to prevent oxidative actions by hypochlorous acid (HClO). Plasma was diluted in distilled water (2 µl: 100 µl), and 5 µl of this solution were mixed with 100 µl of the provided HClO reagent. The inter-assay CV was 1.67 % and the

intra-assay CV's were < 3 % for each of the 10 plates. An oxidative index was then calculated by subtracting a standardized antioxidant value ((antioxidant value – mean antioxidants)/standard deviation) from a standardized d-ROMs value, representing the relative contribution of d-ROMs or antioxidant capacity, with greater values associated with greater levels of oxidative stress (Vassalle *et al.* 2008).

Energy metabolites

Two lipid metabolites (triglycerides and free glycerol) were measured via sequential enzymatic colour endpoint assays (F6428, T2449, and G7793, Sigma-Aldrich, Missouri, USA). The manufacturer's protocol and a dilution protocol (Guglielmo, O'Hara & Williams 2002) were followed to enable use with a 96-well microplate. The free glycerol reagent was added to 5µl of plasma, incubated for 5 minutes at 37°C, and the absorbance was measured at 505 nm (xMark, Bio-Rad, California, USA). This allowed for the measurement of free glycerol, which is indicative of endogenous triglyceride catabolism. Next, a lipase reagent was added to dissociate the fatty acids from the glycerol backbone of triglyceride molecules. The plate was again incubated for 5 minutes at 37°C, and absorbance was measured at 505 nm to estimate triglyceride concentration.

Statistical analysis

All statistical analyses were performed in R, version 3.3.2 (R Core Team 2016) using the 'car' (version 2.1-6, {Fox, 2011 #10}Fox and Weisberg, 2011), and 'lsmeans' packages (version 2.27-61, Length 2016). Samples were run in duplicate for each assay

and any samples with missing data were removed from analysis. There was no significant relationship between any physiological measures and body mass, snout-vent length (SVL), or body condition (calculated as mass/length and again as residuals from a mass/length regression) so these metrics were excluded from analysis. A two-way ANOVA was used to assess changes in physiology by reproductive state, site, and their interaction using type III tests to account for the unbalanced sample design. Reproductive state encompassed sex, presence/absence of follicles, and month (e.g. early reproductive females, early non-reproductive females, and early males) (Table 1). Adult females without enlarged vitellogenic follicles or oviducal eggs in May or June were considered non-reproductive for that year. All physiological variables were \log_e transformed to normalize data for analysis except for glucose, which was normally distributed without transformation. *Post hoc* analyses were performed for significant interactions using the least squares mean function of the 'lsmeans' package in R.

Linear models were used to assess physiological variables as a function of follicle number for vitellogenic females on Allen's Leaf Cay in May. Additional sites were not included in this analysis due to small sample sizes of follicle counts for females. Free glycerol was log-transformed prior to analysis, but all other variables were normally distributed for May females. Finally, we used an ANCOVA with type III contrasts to model dROMS and oxidative index as a function of free glycerol, reproductive condition, and site. Data for ANCOVA models were centered on zero prior to analysis by subtracting the mean from individual values. Free glycerol and triglycerides were moderately co-linear and we chose to include free glycerol but not triglycerides in our model because glycerol is indicative of the active breakdown of triglycerides. Annotated

models are described in Table 2. Normality checks of residual distribution and Levene's tests were carried out and the assumptions were met.

Table 2. Annotated models of physiological variables used for statistical analysis.

(a)	d-ROMs $\sim f(\text{reproductive condition} + \text{site} + \text{interaction})$
	Antioxidants $\sim f(\text{reproductive condition} + \text{site} + \text{interaction})$
	Oxidative index $\sim f(\text{reproductive condition} + \text{site} + \text{interaction})$
	Free-glycerol $\sim f(\text{reproductive condition} + \text{site} + \text{interaction})$
	Triglycerides $\sim f(\text{reproductive condition} + \text{site} + \text{interaction})$
(b)	d-ROMs $\sim f(\text{follicle number})$
	Antioxidants $\sim f(\text{follicle number})$
	Oxidative index $\sim f(\text{follicle number})$
	Free-glycerol $\sim f(\text{follicle number})$
	Triglycerides $\sim f(\text{follicle number})$
(c)	d-ROMs $\sim f(\text{free glycerol} + \text{reproductive condition} + \text{site} + \text{interaction})$
	Oxidative index $\sim f(\text{free glycerol} + \text{reproductive condition} + \text{site} + \text{interaction})$

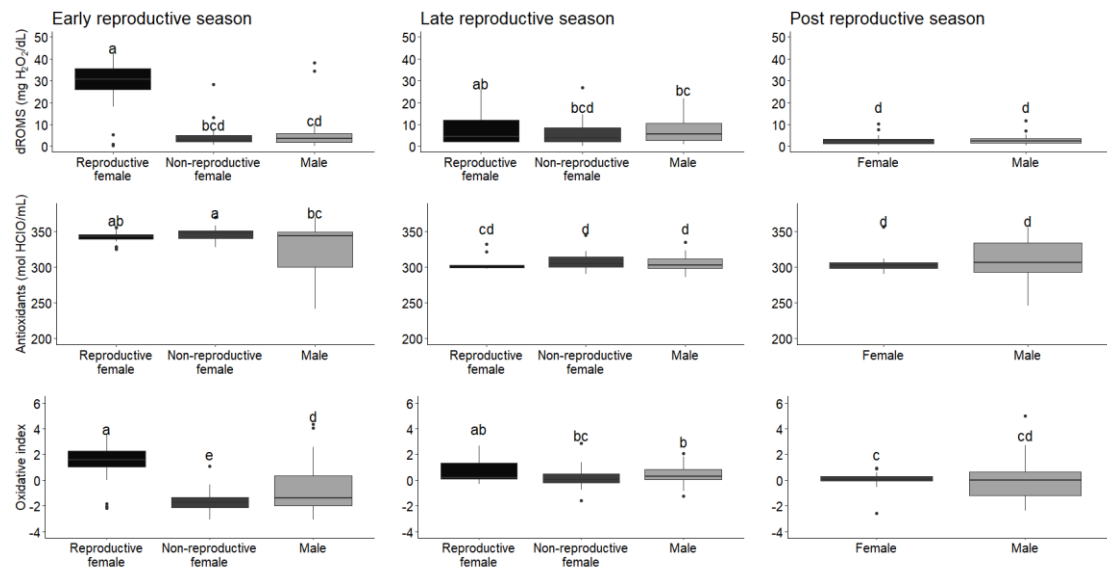
RESULTS

Influence of reproductive state on oxidative stress and energetic measures

There was a statistically significant interaction between the effect of reproductive state and site for d-ROMs, free glycerol, triglycerides, antioxidants, and oxidative index ($P < 0.05$), and a significant effect of reproductive state but not site on glucose levels (Table 3). Data trends were largely consistent across reproductive states at all sites but overall values differed, driving the observed interaction effect. Early season reproductive

females had significantly elevated d-ROMs, oxidative index, free glycerol, and triglycerides compared to all other reproductive states ($P < 0.05$, Fig. 1). Both male and female iguanas had elevated antioxidants during May with males showing the greatest variation across the seasons (Fig. 1 – A). Free glycerol and triglyceride levels were similar for non-reproductive females and males across the seasons (Fig 1 – B). Glucose levels did not vary greatly with reproductive condition in May and June but were significantly lower in September (Fig. 1 – B).

A



B

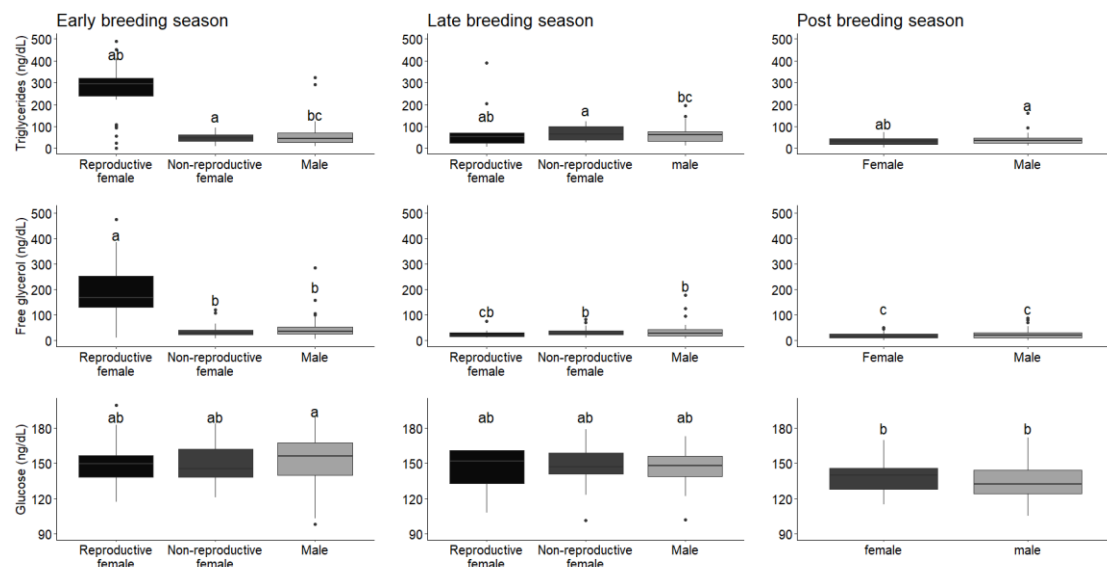


Figure 1. Seasonal changes in (A) d-ROMs, antioxidants, oxidative index, (B) triglycerides, free glycerol, and glucose by sex and reproductive condition. Different lower-case letters above samples for each parameter indicate statistically significant differences among groups across all seasons when analysing reproductive condition alone

($P < 0.05$, see Table 3). Boxplots display the median, upper and lower quartiles, two whiskers, and outlying points individually.

Table 3. ANOVA results for physiological variables as a function of reproductive state and site for Allen Cay Rock Iguanas. Significant P values are in bold.

	Sum of Squares	df	F	P
d-ROMs ~				
intercept	151.02	1	245	< 0.001
reproductive state	61.28	7	14.2	< 0.001
site	2.88	2	2.34	0.10
reproductive state: site	21.43	14	2.50	0.002
residuals	164.73	268		
Free glycerol ~				
intercept	488.48	1	1129	< 0.001
reproductive state	50.13	7	16.6	< 0.001
site	2.35	2	2.72	0.07
reproductive state: site	15.14	14	2.50	0.002
residuals	116.79	270		
Triglycerides ~				
intercept	564.48	1	1635	< 0.001
reproductive state	50.47	7	20.9	< 0.001
site	8.27	2	11.9	< 0.001
reproductive state: site	11.44	14	2.36	0.004
residuals	93.18	270		
Antioxidants ~				
intercept	647.22	1	10.0	< 0.001
reproductive state	0.46	7	6.06	< 0.001
site	0.00	2	3.47	0.88
reproductive state: site	0.35	14	19.1	< 0.001
residuals	0.92	261		

Oxidative index ~				
intercept	21.22	1	19.6	< 0.001
reproductive state	83.29	7	11.0	< 0.001
site	6.82	2	3.15	0.04
reproductive state: site	74.19	14	4.90	< 0.001
residuals	282.02	261		
Glucose ~				
intercept	437457	1	1406	< 0.001
reproductive state	5881	7	2.70	0.01
site	770	2	1.24	0.29
reproductive state: site	2661	14	0.61	0.86
residuals	83980	270		

Relationship between follicle number and oxidative stress and energy metabolites

Follicle number in early reproductive females on Allen's Leaf Cay was a significant factor for all models of physiology as a function of reproductive investment ($P < 0.008$) (Fig. 2). Follicle number was positively related to d-ROMS, free glycerol, oxidative index, and triglycerides and negatively related with antioxidants (Table 4). There was no relationship between follicle number and glucose. Follicle number was not significantly correlated with any physiological variables in June ($P > 0.8$).

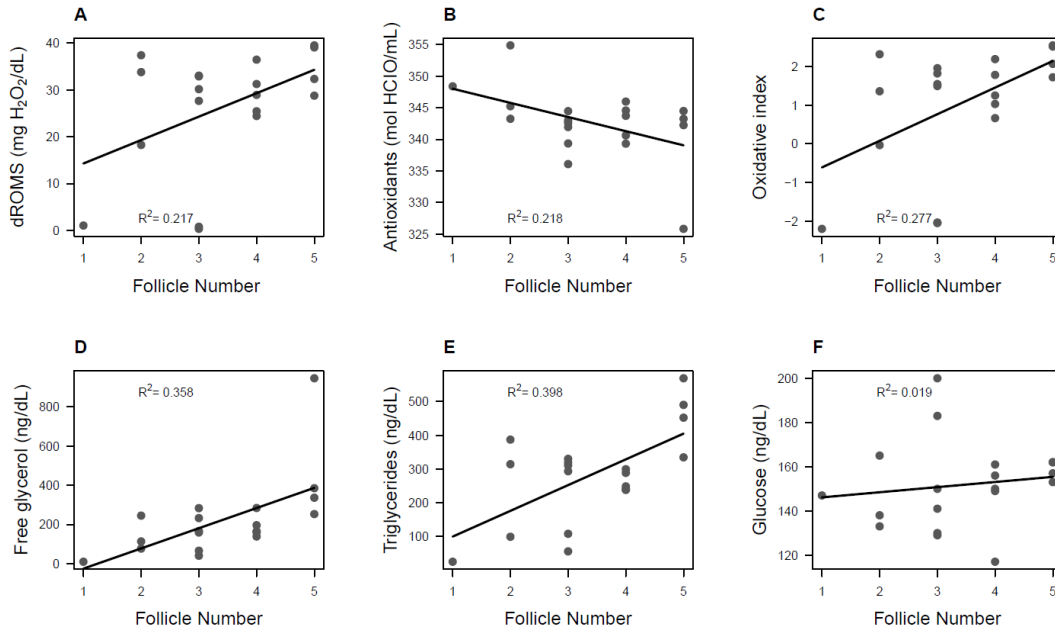


Figure 2. Physiological variables d-ROMs (A), antioxidants (B), oxidative index (C), free glycerol (D), triglycerides (E), and glucose (F), as a function of follicle number for May (early) reproductive female iguanas on Allen's Leaf Cay. The effect of follicle number was significant ($P < 0.05$, see Table 4) for all models except glucose.

Table 4. Linear model results of variables as a function of follicle number for Allen Cay Rock Iguanas. Significant *P* values are in bold.

	Estimate	Standard Error	<i>T</i>	<i>P</i>
d-ROMs ~				
intercept	9.29	8.29	1.22	0.28
follicle number	5.0	2.30	2.17	0.04
Antioxidants				
intercept	350.26	3.71	94.4	< 0.001
follicle number	-2.24	1.03	-2.18	0.04
Oxidative index				
intercept	-1.30	0.97	-1.34	0.20
follicle number	0.69	0.27	2.55	0.02
log(Free glycerol)				
intercept	3.00	0.50	5.95	< 0.001
follicle number	0.60	0.14	4.30	0.0005
Triglycerides				
intercept	23.62	81.98	0.29	0.77
follicle number	76.31	22.74	3.36	0.004
Glucose				
intercept	143.81	14.29	10.1	< 0.001
follicle number	2.32	3.96	0.58	0.57

Relationship between energy metabolism and oxidative stress

There was a significant positive relationship between d-ROMs and free glycerol, and between oxidative index and free glycerol (Table 5). The fixed effects of site, reproductive state, and the interaction of free glycerol and reproductive state were significant for both models, and the interaction between free glycerol and site was also

significant for the oxidative index model (Table 5). The relationship between d-ROMs and free glycerol was strongest in May for iguanas of all reproductive states and this relationship weakened in June and nearly disappeared in September (compare slopes in Fig. 3 – A). Similarly, the relationship between oxidative index and free glycerol was strong in May and June for all iguanas and disappeared in September (Fig. 3 – B). Model estimates and summaries are reported in (Table 5).

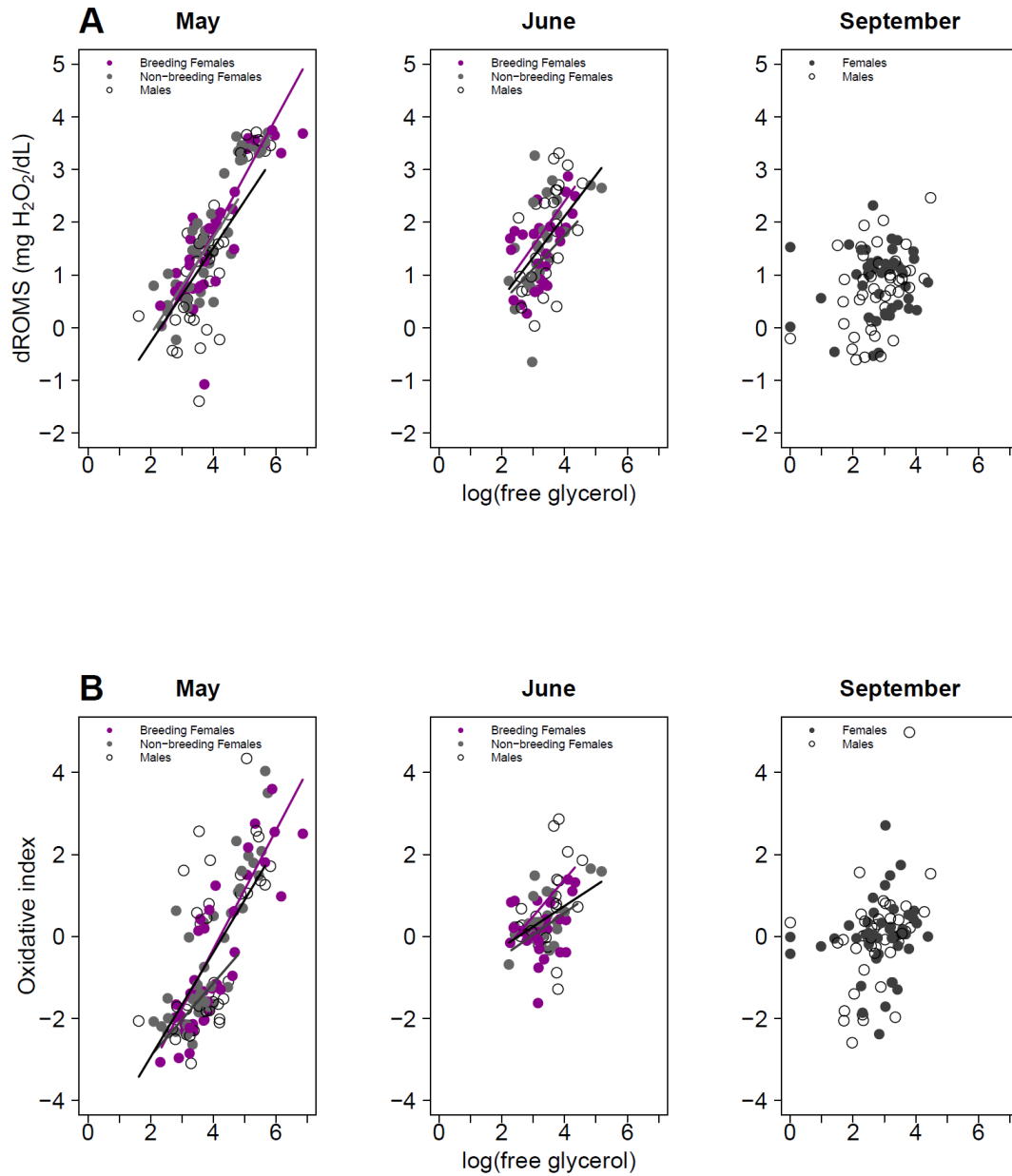


Figure 3. Oxidative stress measures, d-ROMs (A) and oxidative index (B), as a function of lipid catabolism into free glycerol for Allen Cay Rock Iguanas. Oxidative index is calculated by subtracting the standardized antioxidant value from the standardized d-ROMs value for each individual. Colour denotes reproductive condition. Lines represent predicted values plotted across the data range of each reproductive condition.

	Sum of Squares	df	<i>F</i>	<i>P</i>
d-ROMs ~				
intercept	0.06	1	0.13	0.71
free glycerol	22.09	1	43.4	< 0.001
site	9.73	2	9.56	< 0.001
reproductive state	24.46	7	6.86	< 0.001
free glycerol: site	0.61	2	0.60	0.55
free glycerol: reproductive state	17.51	7	4.91	< 0.001
residuals	134.93	265		
Oxidative index ~				
intercept	4.99	1	5.14	0.02
free glycerol	33.07	1	34.1	< 0.001
site	8.73	2	4.50	0.01
reproductive state	133.74	7	19.7	< 0.001
free glycerol: site	7.04	2	3.63	0.03
free glycerol: reproductive state	26.61	7	3.92	< 0.001
residuals	257.28	265		

Table 5. ANCOVA results for models of oxidative stress measures as a function of free glycerol, reproductive condition, and site for Allen Cay Rock Iguanas. Significant *P* values are in bold.

DISCUSSION

We investigated not only the relationship between specific reproductive states and oxidative stress, but also the extent to which circulating energy metabolite levels may drive this relationship. We have considered, independently, the influence of reproductive

state (e.g. season, sex, breeding vs. non-breeding), reproductive investment (number of developed follicles), and the impact of circulating energy metabolites on oxidative stress. This study was uniquely able to compare not just males to females in early, late, and post-reproductive states but non-breeding females to breeding females during the early and late reproductive season, providing a more in-depth assessment of how reproduction impacts physiological stress in females. Overall, we found a clear effect of reproduction on reactive oxygen species (ROS) and some evidence to support a relationship between reproductive investment and ROS. As predicted, we also observed a relationship between oxidative stress measures and circulating energy metabolites, demonstrating that the energetic costs of reproduction are likely driving the increase in ROS.

Influence of reproductive state on oxidative stress and energetic measures

Early reproductive activity in females was characterized by increased levels of both energy metabolites and oxidative stress. During the development of follicles early in reproduction, females are investing more nutritional resources into egg development than they are during later stages of reproduction. This likely explains why plasma triglycerides and free glycerol were so much greater for females in this state as some of these mobilized products are likely being deposited into the follicles. Accordingly, non-breeding adult females have lower circulating levels of these energy metabolites. These results, combined with increased levels of d-ROMs in vitellogenic but not gravid females point to a positive relationship between the energetic demands of reproduction and oxidative stress.

Antioxidants were significantly higher for iguanas of all reproductive states in May compared to the late and post-reproductive periods. This increase may be an oxidative shielding strategy where endogenous production of antioxidants is increased in anticipation of reproduction. In mammals, inadequate antioxidant defences in response to the bursts of ROS during early development can result in pregnancy loss (Myatt & Cui 2004). Some other organisms are thought to employ this shielding strategy as well to prevent damage during energetically expensive life phases, like reproduction or migration (Blount *et al.* 2016). However, this strategy does not appear to be ubiquitous as female Children's pythons (*Antaresia childreni*) in early reproductive stages had decreased antioxidants compared to post reproductive states (Stahlschmidt *et al.* 2013). Vitellogenin which is produced during follicle development for iguanas has also been identified as an antioxidant in honeybee workers (Seehuus *et al.* 2006). However, as antioxidants were elevated for non-reproductive females and male iguanas in May, it is unlikely that vitellogenin is solely responsible for seasonal differences in antioxidants. It is possible that iguanas are simply in better condition and have better access to nutritional resources during this time of the year.

Despite the elevated antioxidants in the early breeding season, reproductive females still had elevated oxidative index values, an indicator of oxidative stress. Consistent with our findings, female Galapagos Land Iguanas (*Conolophus subcristatus*) had higher d-ROM values and higher oxidative stress than males, although reproductive condition of the female land iguanas was not assessed precisely (Costantini *et al.* 2009). Similarly, female Galapagos Marine Iguanas (*Amblyrhynchus cristatus*) had significantly elevated levels of d-ROMs during the breeding season when compared to males, which

further points to an association between female reproduction and oxidative stress (French *et al.* 2017).

Oxidative stress has been associated with several energetically demanding activities, including sexual signalling (Alonso-Alvarez *et al.* 2007), exercise (Powers & Jackson 2008), growth (Rollo, Carlson & Sawada 1996), absorptive state of digestion (Ceriello *et al.* 1998; Butler *et al.* 2016), and reproduction (Alonso-Alvarez *et al.* 2004). Our study further supports this idea that transient levels of oxidative stress are a normal part of life, as we observed an increase in oxidative stress during reproduction and a return to lower levels post-reproduction. It is likely that evolution favours strategies that quickly re-establish oxidative balance and minimize or correct damage from short-term oxidative stress.

Relationship between follicle number and oxidative stress and energy metabolites

Within reproductive females, the number of developing follicles had a significant effect on several physiological measures. We observed a positive relationship between number of follicles in the early reproductive period and the amount of circulating d-ROMs, triglycerides, free glycerol, and oxidative index. The increase in d-ROMs that occurs during reproduction is likely a result of increased catabolism of fats into free glycerol and fatty acids that are needed for the production of follicles, but this has not been explicitly tested. A relationship between circulating levels of triglycerides and ROS has been demonstrated in other studies (Pérez-Rodríguez *et al.* 2015) but our study has

uniquely demonstrated a relationship between ROS and the catabolism of triglycerides into free-glycerol. Additionally, we did not observe an association between follicle number and elevated energy metabolites for late-reproductive females (i.e. gravid) likely because the majority of resource investment into the eggs had already occurred.

During May (early season) there was also a negative relationship between follicle number and antioxidants, and oxidative index increased, further suggesting an oxidative cost of energy-demanding reproductive activity. The decrease in antioxidants observed with increasing follicle number may be a result of investment into eggs, a depletion due to neutralizing ROS, or a decrease in production due to limited resources available for multiple physiological functions. Similar to our results, in a study of brood size manipulation in Zebra Finches, levels of the antioxidant superoxide dismutase were lower for females with a brood of 6 offspring compared to those with 2 offspring (Wiersma *et al.* 2004). However, unlike our results, viviparous, pregnant female Asp Vipers had higher antioxidants compared to non-pregnant females (Stier *et al.*, 2017). In another study, on vitellogenic Painted Dragons, body-length corrected clutch size was not significantly correlated with one measure of oxidative stress, superoxide production, but was negatively correlated with unspecified ROS (Olsson *et al.* 2009). Species differences likely reflect dissimilarities in the timing of resource acquisition and the extent of reproductive investments. However, further studies are needed to more explicitly understand the relationship between reproductive investment and oxidative stress.

Relationship between energy metabolism and oxidative stress

The relationship we observed between oxidative stress measures and circulating levels of free glycerol was strongest in May, regardless of reproductive condition or sex. It appears that this relationship is only present in our iguanas when lipid metabolism and ROS production is occurring at high rates. Currently, few studies that we are aware of have explicitly linked changes in lipid metabolites during reproduction with oxidative stress, and thus far the evidence is circumstantial, not experimental. For example, there are data in adult mammals to suggest that lipid metabolites and, in particular, glycerol levels tend to be elevated during times of energetic need such as starvation, exercise, and cold environments for adult mammals (Lin 1977). Another study found that high glucose and free-fatty acid exposure (palmitate) increased ROS production in specific tissues cultures (i.e., aortic smooth muscle cells of endothelial cells), supporting a link between these energetic metabolites and oxidative stress (Inoguchi *et al.* 2000). Despite a likely relationship between fatty acids and d-ROMS, only weak correlations have been observed (Constantini 2016).

The implications of oxidative stress in life-history biology is complex and current reviews have implored researchers to assess animals at multiple time points and life-stages (Isaksson, Sheldon & Uller 2011), which we have largely accomplished with this study. The elevated oxidative stress that accompanied follicle development in our lizards demonstrates an oxidative cost of reproduction. We have also addressed a current gap in literature by linking quantitative measures of circulating energy metabolites with reproduction and oxidative stress, specifically demonstrating that the energetic costs of reproduction are likely driving reproduction-associated increases in ROS. Moving

forward, we will investigate the role of diet in individual physiology and reproductive frequency. Additionally, it will also be important to examine the impacts of oxidative stress on DNA damage, survival, and longevity.

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CHAPTER IV

PHYSIOLOGICAL CONSEQUENCES OF ECOTOURISM IN AN ENDANGERED IGUANA (*CYCLURA CYCHLURA*): A NATURAL EXPERIMENT OF VISITATION AND FOOD SUPPLEMENTATION

Increased interest in ecotourism over recent years has led to more direct human-animal interactions and a striking concomitant increase in the provisioning of non-natural food, that may have unintended consequences for the wildlife involved. The critically endangered Northern Bahamian Rock Iguana provides a valuable model to address the potential impact of food provisioning on health as there are populations that represent a graded variation in human presence, with sites experiencing high, low, or no tourism. We investigated the relationship between the provisioning of non-natural foodstuff and key physiological measures of health for two subspecies of Northern Bahamian Rock Iguanas, and found significant associations on all measures. Iguanas experiencing the highest amount of food provisioning had elevated levels of energy metabolites, body condition, immune performance, and markers of oxidative stress but decreased circulating levels of the glucocorticoid hormone corticosterone compared to iguanas that were infrequently or not visited. Having sites that experience similar degrees of ecotourism and food supplementation for each subspecies enables us to understand more clearly the degree to which these activities impact physiology in a wild vertebrate. This model system could have important conservation and management implications for not only this endangered iguana but other species as well.

INTRODUCTION

Ecotourism is a growing industry that is touted for positively affecting human attitudes toward the conservation of nature and for supporting local economies [1, 2]. Increased interest in ecotourism over recent years has also led to more direct human-animal interactions through handling, observations, photography, and food provisioning, which may have unintended consequences for involved wildlife. Studies of such effects have been limited, but profound effects on animal behaviour, physiology, and even survival have been documented. In order to maintain a sustainable tourism industry and also protect wildlife, it is critical to understand the impacts of these interventions on wildlife health, especially when considering endangered species.

Tourism and food supplementation

The ecotourism industry involves many different wildlife species; however, only a small portion of these have been monitored consistently for possible impacts. The remoteness of many of these populations together with the unregulated nature of tourism in many locations make it difficult to ascertain specific impacts. Studies that have examined the influences of tourism on animals have found altered animal behaviour [3-5], modification of the hypothalamic pituitary adrenal axis release of hormones, such as corticosterone, in response to stressors [6-8], changes in body condition and physiology [9, 10], and changes in survival [11, 12].

As part of the human interactions with wildlife, many ecotourism excursions include feeding wildlife [13]. This activity can significantly alter natural behaviours and population densities while creating a dependency on non-natural food items and a habituation to humans [14]. Easy and concentrated access to this introduced food can lead to unnatural aggregation at feeding sites, which can lead to higher pathogen transmission (avian populations) [15], increased intra- and interspecific aggression (Sicklefin Lemon Sharks) [16], and even injury to tourists (several species of large mammals) [14]. Consuming unnatural food sources can also have negative impacts on animal health, causing injury and disease (several species of large mammals) [14], increased metabolic rate (Whitetip Reef Sharks) [17], and altered faeces consistency (Rock Iguanas) [9]. Supplementation with high calorie foods could also lead to elevated levels of blood glucose and lipids, which could alter metabolic and physiological processes dependent on nutritional resources, such as immune function. This access to more food can also alter reproductive rates and timing in females where food is often a limiting factor (Black Bears) [15, 18], and an increase in reproduction could further alter female health (Rock Iguanas) by increasing exposure to oxidative stress [19]. Given the demonstrated effects of food supplementation on animal behaviour and health, these, and other in-depth physiological assessments, should be considered more broadly across affected wildlife species.

Physiology as a metric of health

Measuring multiple physiological parameters provides an immediate and comprehensive- measure of health that allows researchers to isolate individual-level

differences in response to varying environmental conditions. Indeed, a single blood sample can yield measurements of baseline concentrations of energy metabolites and glucocorticoids, and assessments of immune function and oxidative stress [8]. As in other taxa, it has been demonstrated that food supplementation can alter iguana behaviour [20], but physiological measurements allow direct quantification of nutritional differences between visited and non-visited populations and a better understanding of how these relate to measures of health, such as immune function and oxidative stress. This integrative approach allows identification of the mechanisms by which anthropogenic disturbance can alter animal health, and can guide more directed management practices.

Northern Bahamian Rock Iguana ecotourism

Northern Bahamian Rock Iguanas, *Cyclura cyclura*, are distributed in isolated populations on at least 17 of the 365 cays of the Exuma island chain on the Great Bahama Bank [21, 22]. These populations are separated into two geographically disjunct subspecies, *Cyclura cyclura inornata* and *Cyclura cyclura figginsi*, and are listed as Endangered and Critically Endangered according to IUCN Red List of Threatened SpeciesTM criteria, respectively (IUCN Red List of Threatened Species 2014) [23, 24]. These Rock Iguanas face pressures from several anthropogenic disturbances such as poaching for food, introduction of mammalian competitors or predators [25], and more recently, ecotourism [9, 11, 20]. The emerging threat of ecotourism has prompted several studies on this species, including feeding behaviour [20], physiology [9], growth and body condition [10], and population demography [10, 11], which provides an ideal

foundation to expand upon and with which to integrate ecoimmunological and oxidative health indicators.

Tourist interactions vary in intensity across rock iguana populations, and while some populations are still found in relatively undisturbed settings, others are exposed to high levels of tourism. The most heavily visited cays have boats arriving daily (weather permitting) with multiple tour groups that can total hundreds of people offering food, observing, photographing, and sometimes physically harassing iguanas. Anticipation of daily feeding has drawn an unnaturally high density of iguanas to beaches where boats arrive, with the sound of boat engines often attracting iguanas to descend down beaches often to the waterline in anticipation of arriving tourists (Figure 1). In contrast, beaches infrequently visited by people have a strikingly different, more natural, dispersed distribution of individuals and iguanas are often difficult to locate. The rock iguanas exposed to constant tourism are less wary of humans and consume an atypical diet, primarily non-native fruits (e.g., grapes) with higher sugar and liquid content, left-over vegetables, and bread. Some physiological measures of dietary nutrition also differ significantly between populations of iguanas on tourist-visited islands versus non-visited islands [9] and survival probability has been found to be higher for populations experiencing less human visitation [11]. Island biogeography along with a graded variation in human visitation intensity and interactions with the Northern Bahamian Rock Iguanas provide a valuable model to study physiology associated with ecotourism and food provisioning. This model system could have important conservation and management implication for not only this endangered iguana but other species as well.



Figure 1. Northern Bahamian Rock Iguanas (*Cyclura cychlura*) gather on the beach in anticipation of food.

Research and modelling goals

The goal for this project was to assess the impact of ecotourism and food supplementation on iguana physiology at no tourism, low tourism, and high tourism sites for two subspecies of *C. cychlura* iguanas, with natural replicates across the geographic range of the species. To isolate the effects of tourism from changes related to reproductive physiology, we sampled animals in the non-reproductive season. Given the variation in tourism and feeding experienced at each site, we hypothesized that physiological profiles of iguanas at high tourism sites would differ in a graded manner

from iguanas at sites experiencing little or no tourism. Specifically, we predicted that increased feeding would be associated with **1)** an increase in the energy metabolites glucose, triglycerides, and free glycerol, **2)** either no differences in baseline corticosterone concentrations [9] or sex dependent relationships [8] as observed in other studies, **3)** increased immune performance due to both the aggregation of iguanas which may facilitate pathogen or parasite transfer and more nutritional resources available to allocate to the immune system, and finally **4)** increased markers of oxidative stress, which has been associated with increased feeding activity in other systems [26].

MATERIALS AND METHODS

Study design

Male ($n = 93$) and female ($n = 86$) rock iguanas were captured between September 3-9 in 2016 from six different sites (Table 1): a high, low, and no tourism site located in the northern Exuma Islands (*Cyclura cychlura inornata*) and a high, low, and no tourism site located in the southern Exuma Islands (*Cyclura cychlura figginsii*) in the Commonwealth of The Bahamas. This sampling period coincided with post-reproductive states for this species [27].

No tourism	<i>C.c. inornata</i>	Males: 13
		Females: 10
	<i>C.c. figginsi</i>	Males: 18
		Females: 10
Low tourism	<i>C.c. inornata</i>	Males: 13
		Females: 17
	<i>C.c. figginsi</i>	Males: 14
		Females: 15
High tourism	<i>C.c. inornata</i>	Males: 22
		Females: 17
	<i>C.c. figginsi</i>	Males: 13
		Females: 17

Table 1. Sample sizes by tourism exposure, subspecies, and sex.

Sample collection

Rock iguanas were captured and processed following the procedures described in Webb et al. [19]. A blood sample was collected from the caudal vein between 0800 and 1300 hrs with an average time to bleed of 110 ± 61 seconds (SD). There was no correlation between any physiological measure and time to acquire blood sample ($r^2 < 0.06$). Body condition was calculated as the residual from a snout-vent length-mass ordinary least squares regression [28]. Tail volume, as a proxy for energy stores, was measured by dipping the tail of an iguana up to the cloaca into a cylinder full of water,

and weighing the amount of water displaced. Tail volumes are reported as grams of water displaced and residuals from a snout-vent length-water mass regression were calculated.

Energy metabolites

Two lipid metabolites (triglycerides and free glycerol) were measured via sequential enzymatic colour endpoint assays (F6428, T2449, and G7793, Sigma-Aldrich, Missouri, USA). The manufacturer's protocol and a dilution protocol [29] were followed to enable use of a 96-well microplate with a 5-minute incubations at 37°C. Following the protocol described in Webb *et al.* [19], a 5µl sample from each individual was used to sequentially measure free glycerol and triglycerides.

Radioimmune assay of corticosterone

Baseline circulating hormone concentrations of corticosterone (Ab: MP Biomedicals # 07-120016) were measured using a radioimmune assay. Assays were performed following a protocol previously described by French *et. al* [30, 31]. Samples were extracted with isooctane: ethyl acetate, dried down, and resuspended in a phosphate-buffered saline (PBS). All samples were assayed in duplicate and the mean value was used in analysis. The intra-assay coefficient of variation (CV) was 13.2 %.

Bacterial killing assay

The bacterial killing assay involved the actions of phagocytes, opsonizing proteins, and natural antibodies to assess the ability of blood plasma to eliminate a pathogenic bacteria, *Escherichia coli*, providing a functionally relevant measure of host immune function [32, 33]. Following the protocol described in French and Neuman-Lee [32], plasma was diluted 1:12 with 0.9% PBS and combined with CO₂ – Independent Medium (Gibco, Grande Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), 10⁵ CPU (colony producing unit) *Escherichia coli* (EPower™ Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and tryptic soy broth on a 96-well microplate. Background absorbance was calculated using a BioRad xMark microplate reader. After a 12-h incubation at 37°C, absorbance was again read and bactericidal ability was calculated by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only medium and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative to the positive controls. Negative controls (containing only medium) were also run to ensure there was no background contamination. The inter-assay CV was 5.9% and the intra-assay CV was < 7% for each of 5 plates.

Reactive oxygen metabolites and antioxidant capacity

Oxidative physiology was assessed by measuring the derivatives of reactive oxygen metabolite species (d-ROMs) and the effectiveness of antioxidant defences. Following methods described in Webb et al. [19], circulating d-ROMs and antioxidant capacity were quantified using commercially available assay kits (MC435 and MC002, Diacron International, Italy). Plasma was diluted in the provided acidic buffered solution

(5 μ l : 100 μ l) for the d-ROMs assay and in distilled water (2 μ l: 100 μ l) for the antioxidant assay and all samples were run in duplicate. The d-ROMs inter-assay CV was 4.39% and the intra-assay CV was < 3% for each of 4 plates. The antioxidants inter-assay CV was 1.67% and the intra-assay CV was < 3% for each of 4 plates. An oxidative index was calculated by subtracting a standardized antioxidant value ((antioxidant value – mean antioxidants)/standard deviation) from a standardized d-ROMs value, representing the relative contribution of d-ROMs or antioxidant capacity- with greater values associated with greater levels of oxidative stress [34].

Tourism rankings

The sites studied in this project experienced a gradient of exposure to tourism and feeding. Sites were ranked as either “high-tourism” (average 131 tourist/day), “low-tourism” (average 18 tourist/day), or “no-tourism” (no tourists) based initially on our estimation of the intensity of human presence on each of the cays, and quantified during our visits in 2019. High-tourism sites were locations where multiple tour companies brought groups on a daily basis, weather allowing. Low-tourism sites were sites that allowed easy access, such as a landing beach, but were not generally visited by large tour groups though they were visited by small groups on private boats and charters. It should be noted that the *C.c. figginsi* low-tourism site was historically visited more heavily than it currently is, and for this reason it was considered low-tourism. Finally, no-tourism sites were ranked as such due to the extreme difficulty of access and the lack of any evidence of visitation by tourists. Each of these rankings was based on over 25 years of personal observations at each site.

Statistical analysis

All statistical analyses were performed in JMP, version 14.0.0 [35]. Samples were run in duplicate for each assay and any samples with missing data were removed from analysis. A three-way ANOVA was used to assess changes in physiology as a function of tourism ranking, sex, subspecies, and their interactions. All physiological variables were \log_e transformed to normalize data for analysis except for glucose and oxidative stress indices, which were normally distributed without transformation (Table 2). *Post hoc* analyses were performed for significant interactions by using a Student's T-test for the factors sex and subspecies and Tukey's honest significant difference test for tourism ranking and all interactions. Normality checks of residual distribution and Levene's tests were carried out and the assumptions were met.

RESULTS

Body condition and tail volume

There was a statistically significant effect of tourism rank ($P = 0.0007$) and the interaction between tourism rank and subspecies ($P = < 0.0001$) but no other variable, on iguana body condition (Table 2). On average, iguanas at high-tourism sites had higher body condition than those at low- and no-tourism sites for the *C.c figginsi* subspecies and iguanas at no-tourism sites had higher body condition than those at low- and high-tourism

sites for the *C.c. inornata* subspecies (Fig. 2). Tail volumes could not be measured for the no-tourism *C.c. figginsi* site and the subspecies were therefore modelled separately. There was a statistically significant effect of sex ($P < 0.005$; greater in males) and tourism rank ($P < 0.0001$; greater at high tourism site) for the *C.c. figginsi* subspecies but no differences were observed for *C.c. inornata* ($P > 0.05$) (Table 2, Fig. 6).

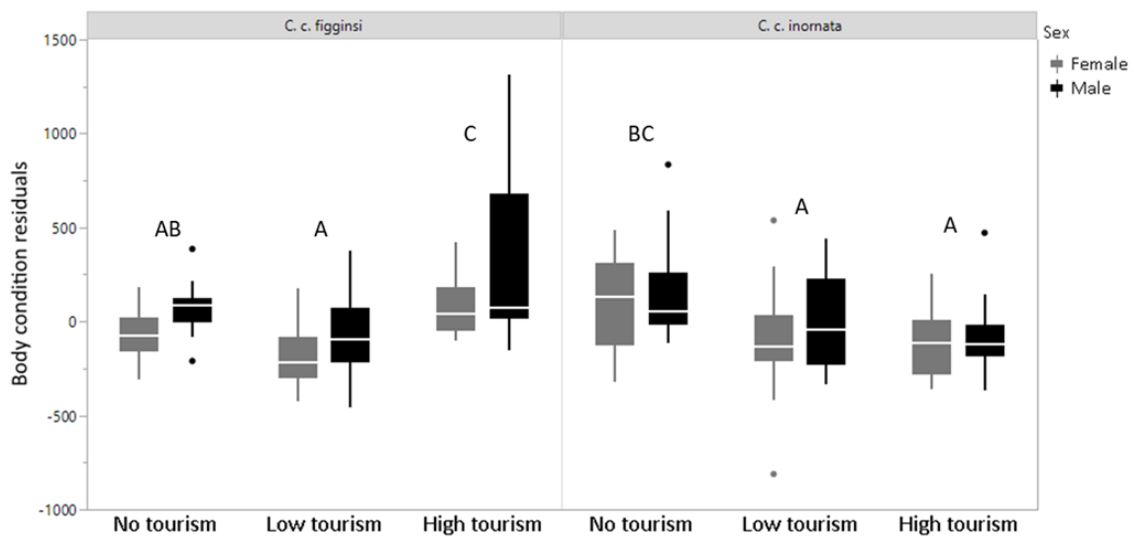


Figure 2. Site, subspecies (*Cyclura .c.inornata*, and *C.c. figginsi*), and sex differences in body condition. Different upper-case letters above samples for each parameter indicate statistically significant differences among groups when analysing the interaction between tourism rank and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

Energy metabolites

Whole blood glucose concentrations were significantly higher at high-tourism sites compared to low- and no- tourism sites ($P = 0.0001$) but no other factors were significant (Table 2, Fig.3). Plasma triglyceride concentrations were also significantly higher at high-tourism sites compared to the other sites ($P < 0.0001$) and there was a significant effect of subspecies ($P < 0.0001$) and the interaction of subspecies and tourism rank ($P = 0.0002$) where populations of *C.c. figginsi* had higher mean concentrations compared to *C.c. inornata* (Table 2, Fig. 4A). Free glycerol followed the same pattern, with higher concentrations observed at high-tourism sites compared to low- and no-tourism sites and significantly higher for *C.c. figginsi* compared to *C.c. inornata* (Table 2, Fig. 7).

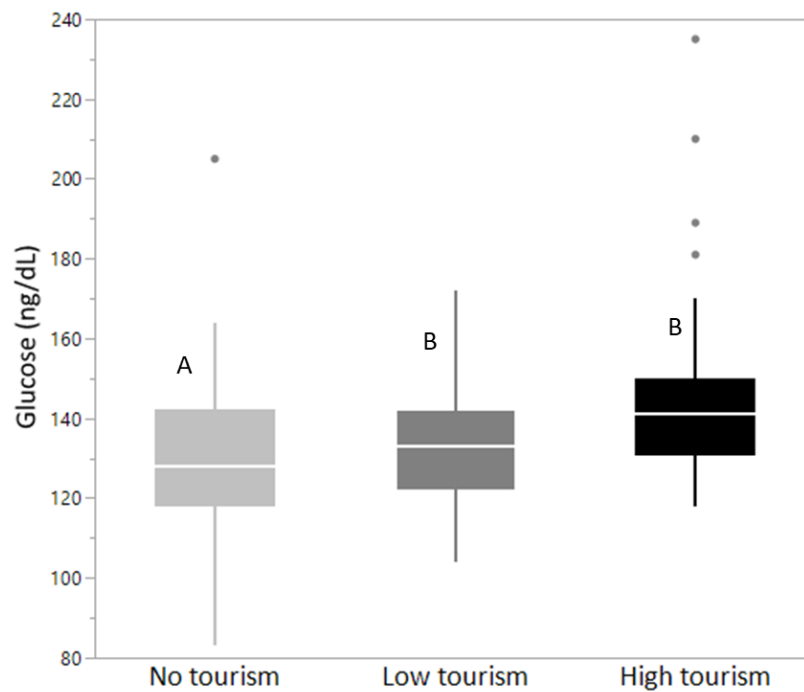
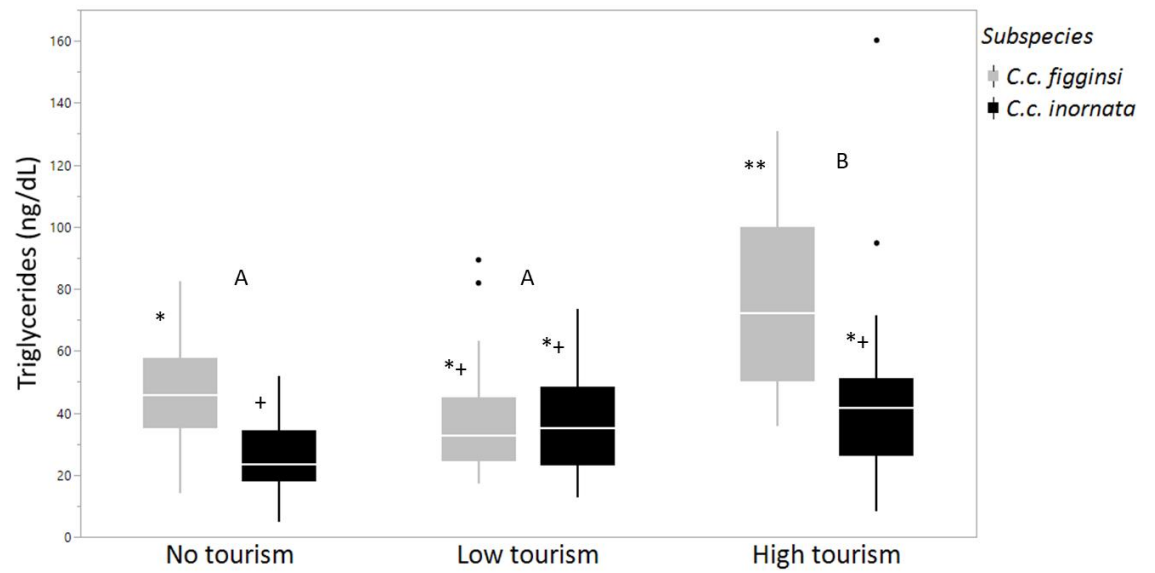
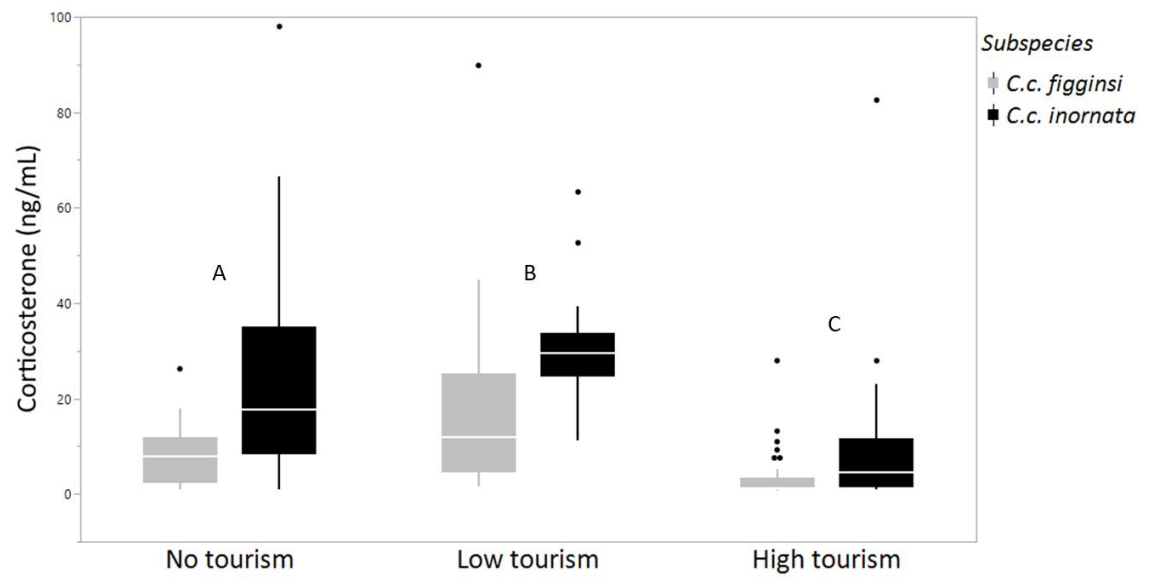


Figure 3. Glucose values for all rock iguanas grouped by exposure to tourism. Different upper-case letters above samples for each parameter indicate statistically significant differences among groups when analysing tourism rank differences alone ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

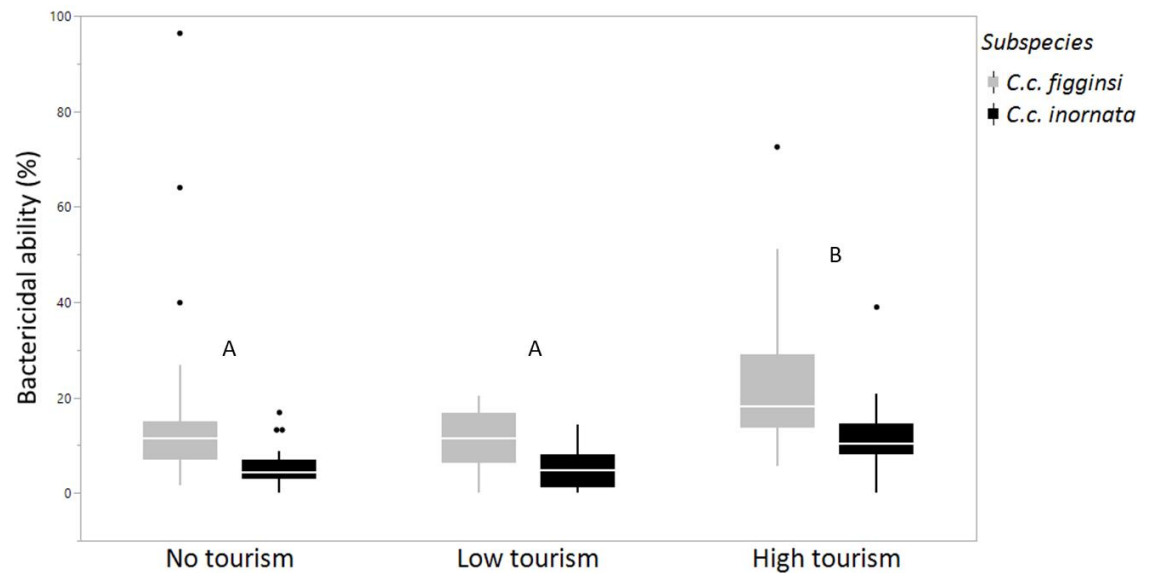
A



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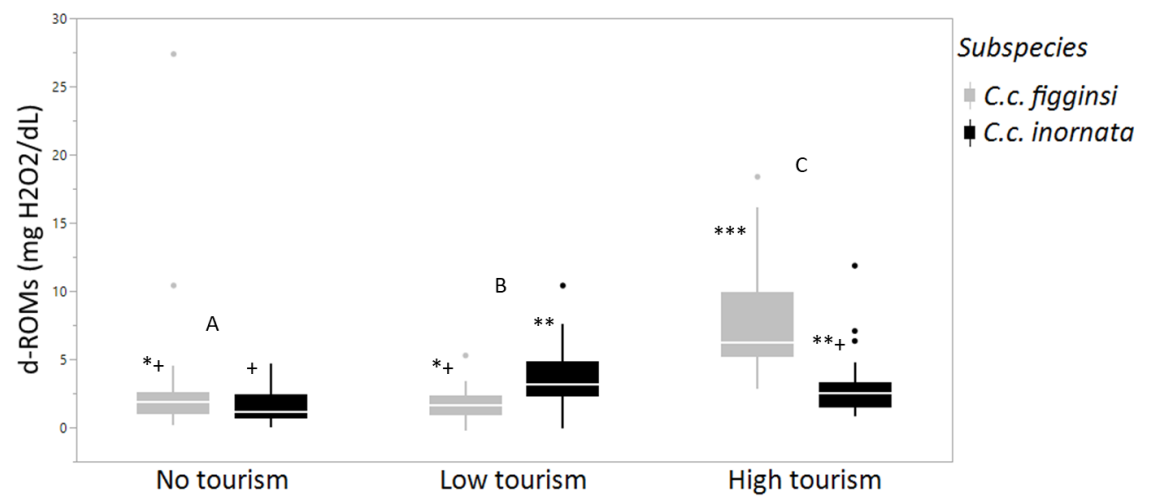


Figure 4. Site and subspecies (*Cyclura c. inornata*, and *C.c. figginsi*) differences in (A) triglycerides, (B) baseline corticosterone, (C) bacterial killing ability, and (D) d-ROMs. Different upper-case letters above samples for each parameter indicate statistically significant differences among groups when analysing tourism rank differences alone ($P < 0.05$). Different symbols above bars for each parameter indicate statistically significant differences among groups when analysing the two-way interaction between site and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

Baseline corticosterone concentrations and immune function

Corticosterone varied by tourism rank, being highest for rock iguanas at low-tourism sites and lowest for those at high-tourism sites for both subspecies ($P < 0.001$). Overall, corticosterone concentration was also lower for *C.c. figginsii* compared to *C.c. inornata* ($P < 0.001$) (Table 2, Fig 4B). Bacterial killing ability was higher at high-tourism sites compared to low- and no- tourism sites ($P < 0.001$) and was higher for *C.c. figginsii* than *C.c. inornata* ($P < 0.001$) (Table 2, Fig 4C).

Oxidative physiology

Rock iguana d-ROMs varied by tourism rank and were highest at high-tourism sites and lowest at no-tourism sites ($P < 0.001$). There was also a significant interaction of subspecies and tourism rank where *C.c. figginsii* at high-tourism sites had the highest observed values ($P < 0.001$) (Table 2, Fig 4D). Antioxidant capacity varied significantly by tourism rank, sex, subspecies, and their interactions (Table 2, Fig 8), but oxidative index was significantly elevated at high-tourism sites for both subspecies (Fig. 5).

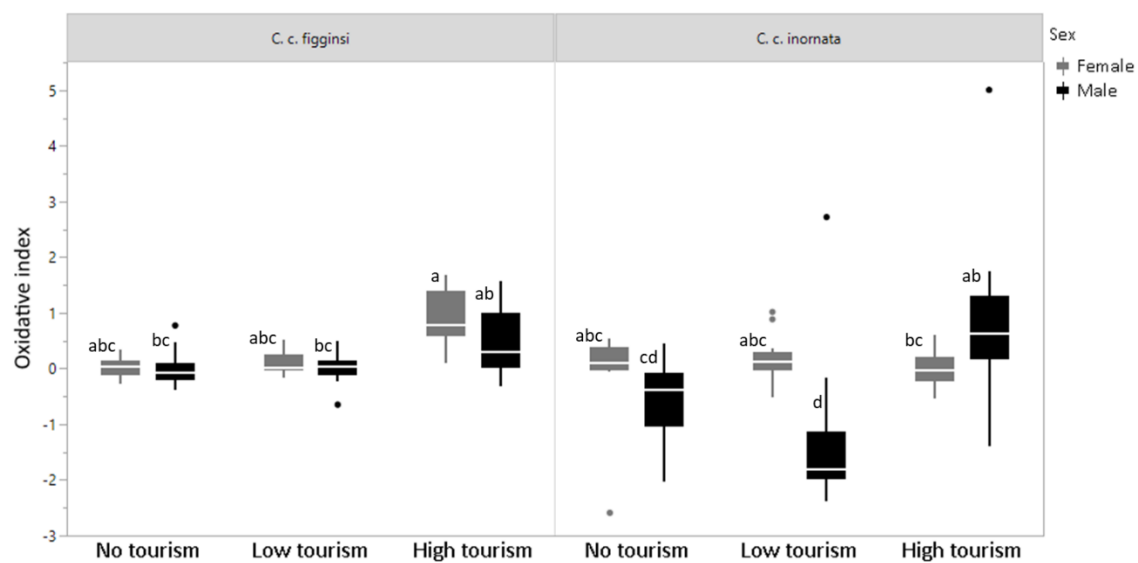


Figure 5. Site, subspecies (*Cyclura c. inornata*, and *C.c. figginsi*), and sex differences in oxidative index. Different lower-case letters above bars for each parameter indicate statistically significant differences among groups when analysing the three-way interaction among site, sex, and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

DISCUSSION

Tourism exposure and physiology

We investigated the relationship between exposure to ecotourism and food supplementation with non-natural foodstuff to key physiological measures of health for two subspecies of Northern Bahamian Rock Iguanas. Natural replicate populations during the non-reproductive season were used, and significant impacts of tourism activity on all measures were found. Iguanas at high-tourism sites had higher concentrations of all energy metabolite measures, immune function, and measures of oxidative stress as well as lower concentrations of baseline corticosterone. A previous study of these same populations during their reproductive season identified site differences in triglyceride concentrations but no difference in glucose, body condition, or corticosterone [9]. Obviously, reproduction significantly alters iguana physiology [19] and differing physiological states due to participation in reproduction that year could affect responses to tourism during the post breeding season. Not surprisingly, the higher values of all energy metabolites observed for rock iguanas at high-tourism sites is likely due to the feeding of non-natural foodstuffs such as grapes and bread.

Interestingly, corticosterone was highest for iguanas at low-tourism sites and lowest for those at high-tourism sites. Other studies that have specifically looked at baseline iguana corticosterone concentrations associated with tourism have found either no difference (rock iguanas and Galapagos Marine Iguanas) [6, 9], or sex-dependent

relationships (Galapagos Marine Iguanas) [8]. However, post-stress corticosterone levels were higher for no-tourism groups in one study [6] and higher for non-breeding tourist-exposed iguanas in another study [30], further demonstrating the importance of assessing reproductive condition when trying to understand these physiological relationships. Corticosterone, which plays a role in mobilizing energy stores, increases when some reptiles face periods of food deprivation compared to when recently fed [36]. In the current study, corticosterone may be down-regulated for rock iguanas experiencing heavy feeding simply because resources are in such abundance. An additional explanation could be that these iguanas experience such heavy exposure to tourists that they have habituated to humans, which continuously reinforces iguana aggregations on fed beaches. Given the relationships observed between human presence and baseline corticosterone concentrations for other iguanas species [6, 30], the former explanation is more strongly supported.

Bacterial killing ability was highest for iguanas at high-tourism sites, which is the opposite of what was observed for Galapagos Marine Iguanas exposed to tourism in the absence of food provisioning [8], possibly because rock iguanas acquire additional nutritional resources through feeding that can be allocated to the immune system. This hypothesis is further supported by the relatively better body condition of iguanas at tourist sites. Additionally, the unnaturally high densities of rock iguanas on feeding beaches could be causing an increased transmission of infection upregulation of immune activity. Fed iguanas at these sites have more faecal parasites [9], but a relationship between this and increased bacterial killing ability has not yet been established.

Iguana d-ROMs were highest for high-tourism sites and lowest for no-tourism sites, demonstrating a graded impact of tourism on this measure of oxidative stress. Galapagos Marine Iguanas also had higher d-ROMs at high-tourism sites [8], suggesting this relationship could be independent from the feeding experienced by rock iguanas. However, absorptive states of digestion are associated with increased d-ROMs in corn snakes (*Pantherophis guttatus*) [26], which could also explain, at least in part, the elevated levels for iguanas experiencing heavy food provisioning.

Antioxidants varied significantly by tourism rank, sex, subspecies, and their interactions, making associations with this measure difficult to interpret. However, the relationship between the relative measure of antioxidants to d-ROMs, oxidative index, and tourism were more distinct. Iguanas at high-tourism sites had higher oxidative indices suggesting that these iguanas may be experiencing a state of oxidative stress, such that their levels of antioxidants may not be sufficient to prevent harm from the reactive oxygen species being produced [34].

Sex and subspecies differences in physiology

Body condition was highest for iguanas at high tourism sites for *C.c. figginsii* but was highest for no-tourism sites for *C.c. inornata*. This may be due to inherent differences in demographics and habitat. Also, the northern islands have been historically fed at high rates for longer, and this we may be observing time-course differences in population density effects of tourism [10], whereby the more recent intense tourism effects in the southern islands could eventually be equivalent with the north.

While the blood samples were collected during the non-reproductive season, sex differences in antioxidants and oxidative index could be a residual effect of reproduction, where females previously experienced higher oxidative costs compared to males [19]. This possibility could be further explored by following females long-term and comparing results of females that recently reproduced to those that did not. With the exception of antioxidants and oxidative index, no other physiological sex differences were observed, which is not surprising since they were not in breeding condition. Similarly, differences in reproductive timing and clutch sizes could account for the subspecies differences that we observed.

The subspecies differences in body condition and physiology are likely a reflection of both genetic and site (i.e. environmental) differences between *C.c. inornata* and *C.c. figginsi* populations. The two subspecies begin their reproductive season a few weeks apart from each other [27], which could lead to the manifestation of minor seasonal differences in physiology or condition. There are also slight differences in tourism pressure and feeding frequency, with tourist-visited *C.c. inornata* populations experiencing a slightly higher rate of tourism than tourist-visited *C.c. figginsi* populations (average of 145 tourist/day versus 119 tourist/day) but *C.c. figginsi* experiences tourist visits for longer periods of the day. Although tourism pressure is slightly higher for the *C.c. inornata* population, supplemental feeding appears to be more intense for the *C.c. figginsi* population, which could explain the overall higher levels of triglycerides and free glycerol observed for this subspecies.

CONCLUSIONS

Basic research on life history data for wild populations has been sought in an effort to better inform management strategies for endangered species including these iguanas [37]. This and other studies have demonstrated the consequences of food provisioning on both behaviour [20] and health (higher endoparasitic infection rates) [9]. More comprehensive physiological assessments have demonstrated the importance of not assuming that all individuals throughout a species' range respond to the same stressors (tourism in this case), and that responses are not static as seasonal changes likely alter responses to tourism [19, 30]. It is also critical to place the importance of this work in its broader context of working to protect entire ecosystems. For example, iguanas of at least the genus *Cyclura* are thought to be important seed distributors, increasing germination rate after passage through their gut [38], likely impacting plant distribution on the islands, and current/future work should assess the impact of food provisioning on seed distribution in these ecosystems.

Finally, this work can help contribute to management practices for a wide variety of wildlife. For example, in an initial assessment of the impacts tourism and food provisioning on *Cyclura* populations, recommendations were made, which should be reiterated, for strategies to reduce the negative impacts of tourism on iguana health [9]. As previously stated, tourism is an essential part of local economies and suggestions to reduce or remove such activities would be impractical. Instead, amending current practises to protect animal well-being should be prioritized. These suggestions include limiting the number of sites that are visited, selecting more appropriate food items (i.e. iguana food pellets in this case), using a food delivery system that prevents sand from

being ingested, and continuing to endorse public education programs. For example, in The Bahamas, it should be noted that a previously recommended method of feeding iguanas by putting food items on the end of a stick or skewer appeared to be a positive solution that prevented sand from being ingested; however, this approach results in the littering of the beach with used skewers, posing an injury risk to tourist and, in some cases, the swallowing of part of a skewer by iguanas leading to gastrointestinal perforation [39], causing hazards to tourist and wildlife alike. It is imperative that all ecotourism practices be thoroughly scrutinized to optimize industry benefits without compromising the target species.

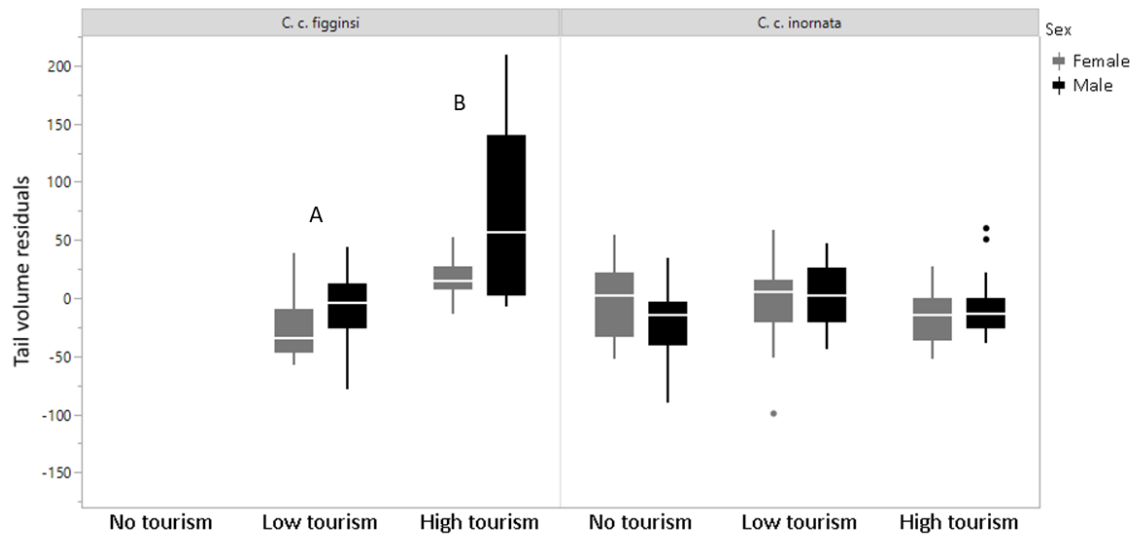


Figure 6. Site, subspecies (*Cyclura c. inornata*, and *C.c. figginsi*), and sex differences in tail volume. Different upper-case letters above samples for each parameter indicate statistically significant differences among groups when analyzing the interaction between tourism rank and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

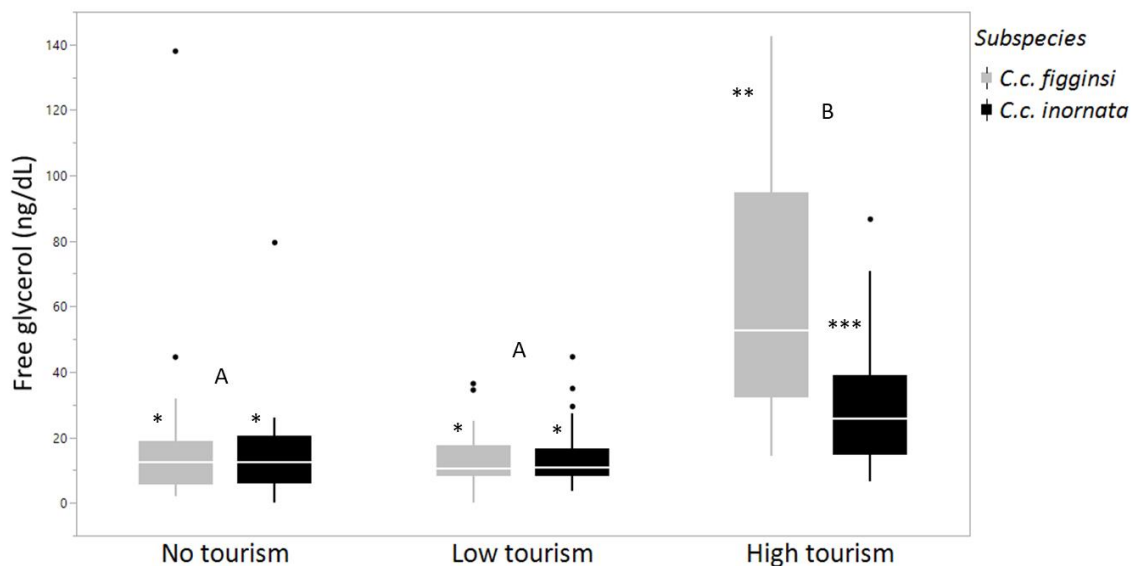


Figure 7. Site and subspecies (*Cyclura c. inornata*, and *C.c. figginsi*) differences in free glycerol. Different upper-case letters above samples for each parameter indicate statistically significant differences among groups when analyzing tourism rank differences alone ($P < 0.05$). Different symbols above bars for each parameter indicate statistically significant differences among groups when analyzing the two-way interaction between site and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

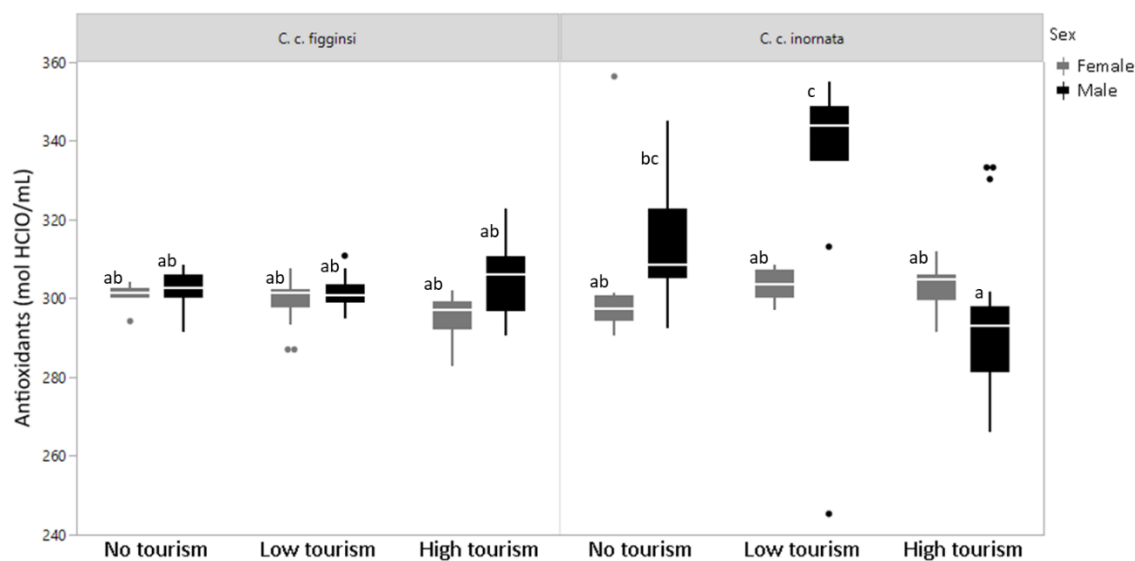


Figure 8. Site, subspecies (*Cyclura c. inornata*, and *C.c. figginsi*), and sex differences in antioxidants. Different lower-case letters above bars for each parameter indicate statistically significant differences among groups when analyzing the three-way interaction among site, sex, and subspecies ($P < 0.05$). Boxplots display the median, upper and lower quartiles, range as two whiskers, and outlying points.

Table 2. ANOVA results for physiological variables as a function of tourism rank, sex, and subspecies for the Northern Bahamian Rock Iguana (*Cyclura cychlura*). Significant *P* values are in bold.

	Sum of Squares	df	<i>F</i>	<i>P</i>
Body condition ~				
tourism rank	89629.2	2	7.66	0.0007
sex	635277.2	1	10.86	0.45
subspecies	33469.1	1	0.57	0.45
tourism rank: sex	18784.7	2	0.16	0.85
tourism rank: subspecies	1859898.1	2	15.90	< 0.0001
subspecies: sex	103500.4	1	1.77	0.19
tourism rank: sex: subspecies	125110.5	2	1.07	0.35
residuals	9648844	165		
Tail volume residuals (<i>C.c. inornata</i>) ~				
tourism rank	2097.70	2	1.14	0.33
sex	65.17	1	0.07	0.80
tourism rank: sex	2435.70	2	1.32	0.27
residuals	77373.85	84		
Tail volume residuals (<i>C.c. figginsi</i>) ~				
tourism rank	54364.05	1	31.56	< 0.0001
sex	15465.14	1	8.98	0.004
tourism rank: sex	4046.34	1	2.35	0.13
residuals	94745.51	55		
Glucose ~				
tourism rank	6219.60	2	9.70	0.0001
sex	16.45	1	0.05	0.82
subspecies	236.50	1	0.73	0.39
tourism rank: sex	168.30	2	0.26	0.77
tourism rank: subspecies	213.58	2	0.33	0.71
subspecies: sex	9.44	1	0.03	0.86
tourism rank: sex: subspecies	333.94	2	0.52	0.60
residuals	53562.80	167		

Log(Triglycerides) ~				
tourism rank	1.43	2	16.90	< 0.0001
sex	0.14	1	3.23	0.07
subspecies	1.42	1	33.54	< 0.0001
tourism rank: sex	0.15	2	1.80	0.17
tourism rank: subspecies	0.78	2	9.23	0.0002
subspecies: sex	0.04	1	1.06	0.31
tourism rank: sex: subspecies	0.22	2	2.63	0.08
residuals	6.98	165		
intercept				
tourism rank	9.92	2	54.60	< 0.0001
sex	0.08	1	0.90	0.35
subspecies	0.30	1	3.35	0.07
tourism rank: sex	0.31	2	1.73	0.18
tourism rank: subspecies	1.23	2	6.75	0.0015
subspecies: sex	0.00	1	0.004	0.95
tourism rank: sex: subspecies	0.27	2	1.48	0.23
residuals	14.63	161		
Log(Corticosterone) ~				
tourism rank	18.83	2	49.20	< 0.0001
sex	0.52	1	2.70	0.10
subspecies	7.90	1	41.26	< 0.0001
tourism rank: sex	0.64	2	1.68	0.19
tourism rank: subspecies	0.09	2	0.24	0.80
subspecies: sex	0.0002	1	0.001	0.97
tourism rank: sex: subspecies	0.58	2	1.50	0.23
residuals	31.39	164		
Log(Bacterial killing ability) ~				
tourism rank	2.97	2	15.39	< 0.0001
sex	0.05	1	0.50	0.48
subspecies	4.07	1	42.14	< 0.0001
tourism rank: sex	0.05	2	0.24	0.79
tourism rank: subspecies	0.03	2	0.15	0.86

subspecies: sex	0.01	1	0.14	0.70
tourism rank: sex: subspecies	0.06	2	0.31	0.73
residuals	15.06	156		
Log(d-ROMs) ~				
tourism rank	5.34	2	30.97	< 0.0001
sex	0.01	1	0.15	0.70
subspecies	0.25	1	2.86	0.09
tourism rank: sex	0.01	2	0.07	0.93
tourism rank: subspecies	4.95	2	28.7	< 0.0001
subspecies: sex	0.01	1	0.13	0.72
tourism rank: sex: subspecies	0.23	2	1.34	0.27
residuals	14.13	164		
Log(Antioxidants) ~				
tourism rank	0.007	2	6.30	0.002
sex	0.003	1	4.83	0.03
subspecies	0.003	1	4.90	0.03
tourism rank: sex	0.004	2	4.01	0.02
tourism rank: subspecies	0.007	2	6.34	0.002
subspecies: sex	0.0001	1	0.23	0.63
tourism rank: sex: subspecies	0.009	2	8.70	0.0003
residuals	0.09	157		
Oxidative index ~				
tourism rank	19.94	2	19.23	< 0.0001
sex	2.97	1	5.74	0.018
subspecies	6.69	1	12.90	0.0004
tourism rank: sex	5.56	2	5.36	0.006
tourism rank: subspecies	0.51	2	0.49	0.61
subspecies: sex	0.30	1	0.58	0.45
tourism rank: sex: subspecies	10.60	2	10.22	< 0.0001
residuals	81.38	157		

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CHAPTER VI

CONCLUSIONS

The goals of my research were to assess natural changes in iguana physiology related to reproduction and season and to then further determine the impact of ecotourism on these measures. Specifically, I aimed to **1)** define seasonal changes in steroid hormones, energy metabolites, body condition, and oxidative stress, **2)** determine the relationship between reproduction and physiology, and **3)** assess the impact of ecotourism and supplemental feeding on iguana physiology.

In chapter 1, I reviewed the current literature on iguana physiology and discussed the gaps in knowledge that each of my research chapters aimed to address. In chapter 2, I defined distinct reproductive states and their accompanying endocrine profiles for male and female iguanas by measuring the steroid hormones corticosterone, progesterone, estradiol, and testosterone for two subspecies of *Cyclura* iguanas across a seasonal gradient. This allowed me to describe physiological aspects of reproduction that have not been measured before in this species of iguana. Further, as reproduction has distinct physiological changes, this allowed me to choose a “non-reproductive” period for assessing the impacts of ecotourism outside of the reproductive season. In chapter 3, I further examined the relationship between iguana reproduction and physiology by assessing the physiological state of discrete reproductive stages. Here I found that early stages of reproduction for females have high energetic demands that are associated with oxidative costs. This study also addressed a current gap in literature by linking quantitative measures of circulating energy metabolites with reproduction and oxidative

stress. To separate the effect of reproduction and ecotourism on iguana physiology, Chapter 4 focused on the impacts of tourism and food supplementation on iguana physiology during the non-reproductive season. I found that tourism had distinct effects on several measures of physiology for both subspecies of *Cyclura* iguanas studied. By collecting data from sites that experience high, moderate, and no tourism, this study was able to assess not just the presence of humans but how animal responses are “dose-dependent.”

Basic research on life history data for wild populations, as presented here, has been called for in an effort to better inform management strategies for these endangered iguanas (Alberts, 2004). By examining several aspects of iguana physiology and fitness, I have provided a more comprehensive assessment of the impacts of ecotourism on the critically endangered Northern Bahamian Rock Iguana. My findings both significantly added to the field of research on reptilian physiology and ecology, and improved the understanding of the impact of human disturbance on an endangered species. Finally, this work can help contribute to management practices for a wide variety of wildlife. In several chapters, recommendations have been made for strategies that could improve the conservation efforts for these endangered iguanas.

APPENDICES

Appendix A: Coauthor Permission Letters

April 11, 2020

Ms. Alison C. Webb
Department of Biology
Utah State University, USA
84322-5305

Dear Alison,

As a coauthor on the following manuscript, I grant you permission to use this work in your dissertation.

Webb AW, Iverson J, Knapp C, DeNardo D, French SS. (2018) Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction. *Journal of Animal Ecology* <https://doi-org.dist.lib.usu.edu/10.1111/1365-2656.12936>

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Charles Knapp, Ph.D
Vice President, Conservation Research
Louis Family Conservation Chair
Daniel P Haerther Center for Conservation and Research
John G. Shedd Aquarium

April 11, 2020

Ms. Alison C. Webb
Department of Biology
Utah State University, USA
84322-5305

Dear Alison,

As a coauthor on the following manuscript, I grant you permission to use this work in your dissertation.

Webb AW, Iverson J, Knapp C, DeNardo D, French SS. (2018) Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction. *Journal of Animal Ecology* <https://doi-org.dist.lib.usu.edu/10.1111/1365-2656.12936>

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

John Iverson, Ph.D
Professor Emeritus
Department of Biology
Earlham College

April 11, 2020

Ms. Alison C. Webb
Department of Biology
Utah State University, USA
84322-5305

Dear Alison,

As a coauthor on the following manuscript, I grant you permission to use this work in your dissertation.

Webb AW, Iverson J, Knapp C, DeNardo D, French SS. (2018) Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction. *Journal of Animal Ecology* <https://doi-org.dist.lib.usu.edu/10.1111/1365-2656.12936>

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Dale Denardo, D.V.M., Ph.D.
Associate Professor
School of Life Sciences
Arizona State University

Appendix B: Publisher Permission Letters

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CURRICULUM VITAE

Alison Carey Webb

Education

Utah State University, Logan, UT 2019

PhD, Biology & Ecology

Dissertation: *Impacts of ecotourism on endangered Bahamian rock iguanas (Cyclura cyhlura figginsi and C.c. inornata)*

Advisor: Susannah French

Middle Tennessee State University, Murfreesboro, TN 2014

MS, Biology

Thesis: *Interactions between innate immunity, steroid hormones, and body condition in female fence lizards (Sceloporus undulates)*

Advisor: Matthew Klukowski

Middle Tennessee State University, Murfreesboro TN 2010

BS, Biology

Senior Thesis: *Effects of moderate food deprivation on plasma corticosterone and blood metabolites in common watersnakes (Nerodia sipedon)*

Advisor: Matthew Klukowski

Appointments

Utah State University

2016 – 2019 Research Assistant, Educational Outreach grant coordinator for Susannah French

2018 Research Assistant, USGS Polar Bear endocrinology
 2015 – 2019 Research Assistant (summer position), Immunology of *Uta stansburiana*
 2017 Instructor, Comparative Animal Physiology Lab
 2015 – 2017 Human Physiology Lab, Lead TA
 2014 Mammalogy Lab, Lead TA

Middle Tennessee State University

2013 General Biology Lab, TA
 2010 – 2012 National Science Foundation GK-12, Graduate Fellow
 2010 General Chemistry Lab, Undergraduate TA
 2009 – 2010 NSF STEP^{MT} Student Research Scholar

Publications

Webb AW, Iverson J, Knapp C, DeNardo D, French SS. (2018) Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction. *Journal of Animal Ecology* <https://doi-org.dist.lib.usu.edu/10.1111/1365-2656.12936>

French SS, **Webb AW**, Hudson S, Virgin EE. 2018. Town and country reptiles: A review of reptilian responses to urbanization. *Integrative and Comparative Biology*. <https://doi.org/10.1093/icb/icy052>

Stahlschmidt ZR, French SS, Ahn A, **Webb AC**, Butler MW. 2017. Simulated heat waves has diverse effects on immune function and oxidative physiology in the Corn Snake (*Pantherophis guttatus*). *Physiological and Biochemical Zoology* 90 (4), 434-444

Webb AC, Chick LD, Cobb VA, and Klukowski M. 2017. Effects of moderate food deprivation on plasma corticosterone and blood metabolites in common watersnakes (*Nerodia sipedon*). *Journal of Herpetology* (1) 134-141. <https://doi.org/10.1670/15-112>

Smith GD, Neuman-Lee LA, **Webb AC**, Angilletta MJ Jr., Denardo DF, French SS. 2017. Metabolic response to immune challenges: Reevaluating the energetics of immunity. *Journal of Comparative Physiology B* 187: 1173. <https://doi-org.dist.lib.usu.edu/10.1007/s00360-017-1095-4>

Anderson MA, Campbell JR, **Carey AN**, Dodge DR, Johnston RA, Mattison ER, Seddon RJ, Singer NL, and Miller BT. 2014. Population survey of the streamside salamander in the Nashville Basin of Tennessee. *Southeastern Naturalist* 13 (1): 101-107.

Notes and Species Distributions

Carey AN, Anderson MA. 2011. Geographic distribution *Lithobates palustris* (Pickerel frog). *Herpetological Review* 42 (3): 386.

Anderson MA, **Carey AN**. 2011. Geographic distribution *Lithobates clamitans* (Green frog). *Herpetological Review* 42 (2): 237.

Grants, Fellowships, and Awards

2019	SICB DEDE Best Student Poster Award
2018	IUCN Iguana Specialist Group Student Travel Award
2018	Graduate Enhancement Award, USU - for contribution to local and national communities and professional field
2018	Matt Del Grosso Endowed Graduate Research Award, USU
2016 – 2017	Graduate Student Travel Awards, USU
2017 – 2019	Charlotte Magnum Student Support Program, Society for Integrative and Comparative Biology
2015 – 2017	Ecology Center Graduate Student Research Award, USU
2010 – 2012	National Science Foundation GK-12 STEM travel awards, 2010 -2012
2010 – 2012	National Science Foundation GK-12 STEM Fellowship, 2010-2012
2012	Marion R. Wells Graduate Research Scholarship, MTSU
2012	Mitchell Magid Research Scholarship, MTSU
2012	John A. Patton Research Scholarship, MTSU
2011	International Travel Grant for NSF GK-12 research in Taiwan, MTSU
2011	Taiwan summer study scholarship, CYCU
2010 – 2011	Tennessee Academy of Science First Place in Science and Math Teaching Student Poster Presentations

- 2010 Sarah H. Swain Undergraduate Research Scholarship, MTSU
- 2010 Padget Kelly Research Scholarship, MTSU
- 2009 National Science Foundation - STEP^{MT} Research Scholar Award, MTSU

Conference Presentations

- 2019 **Webb AC**, Lilly N, Wood J, Warren C, and S.S. French. Behavioral thermoregulatory response to immune challenges in side blotched lizards (*Uta stansburiana*). Society for Integrative and Comparative Biology, Tampa, FL
- 2018 **Webb AC**, Kapheim K, Knapp C, Iverson J, DeNardo D, and French S. Impacts of site and diet on the gut microbiotic community of Bahamian rock iguanas, *Cyclura cychlura inornata* and *Cyclura cychlura figginsi*. IUCN Iguana Specialist Group Meeting, Fort Worth, TX
- 2018 French SS, **Webb AC**, Iverson J, DeNardo D, and Knapp C. Seasonal and reproductive variations in the physiology of the Bahamian Rock Iguana. Bahamas Natural History Conference, Nassau, Bahamas
- 2018 **Webb AC**, Knapp C, Iverson J, Denardo D, and French SS. The effects of tourism and food provisioning on the physiology of Exuma Rock Iguanas (*Cyclura cychlura*). Society of Integrative and Comparative Biology, San Francisco, CA
- 2017 **Webb AC**, Knapp C, Iverson J, Denardo D, and French SS. The effects of tourism and food supplementation on the physiology of Exuma Rock Iguanas (*Cyclura cychlura*) in the Bahamas. IUCN Iguana Specialist Group Meeting, Cuba
- 2017 **Webb AC**, Neuman-Lee LA, Watson E, French SS. Time course of the immune response in non-model organisms, *Thamnophis elegans*. Society of Integrative and Comparative Biology, New Orleans
- 2016 **Webb AC**, Knapp C, Denardo D, Iverson J, and French SS. Assessing the effects of tourism and food provisioning on Exuma Rock Iguanas (*Cyclura cychlura*) in The Bahamas. Iguana Specialist Group Meeting, Fiji
- 2016 Wood J, **Webb AC**, Neuman-Lee L, and French SS. The effects of wound healing on innate immune function in grass lizards (*Takydromus viridipunctatus*). Student Research Symposium, Utah State University

- 2016 Watson E, **Webb AC**, Neuman-Lee L, and French SS. Innate immune response to antigen exposure in the garter snake *Thamnophis elegans*. Student Research Symposium, Utah State University
- 2016 Smith GD, Neuman-Lee LA, **Webb AC**, Denardo DF, Angilletta MJ, and French SS. Lizards downregulate their metabolism in response to an immune challenge: implications for energy status. Society of Integrative and Comparative Biology, Portland, OR
- 2012 **Carey A**, Nichols C, Farone M, and Farone A. TRIAD: Teaching, research, and industry application to deepen scientific understanding in Middle Tennessee. National Science Foundation GK-12 Annual Meeting, Washington, DC
- 2012 **Carey A** and Klukowski M. Effects of reproductive status on innate immunity, hormones, and behavior in female fence lizards (*Sceloporus undulatus*). Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 **Carey A** and Anderson M. Herpetological surveying in Middle Tennessee. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 Shores J and **Carey A**. Growth trends of *Opuntia humifusa* in a Middle Tennessee cedar glade. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 Kennedy J and **Carey A**. Soil composition of a typical cedar glade habitat in Middle Tennessee. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 Hunt A and **Carey A**. Effects of *Cladonia ragnifera* and *Nostoc commune* on plant growth in cedar glade environments. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 Cooley V and **Carey A**. Plant and microbiological crust composition in a cedar glade community. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2012 **Carey A** and Anderson M. Herpetological surveying in Middle Tennessee. Middle Tennessee State University Scholars Week, Murfreesboro, TN
- 2011 Anderson MA, Campbell JR, **Carey A**, Dodge DR, Johnston RA, Mattison ER, Seddon RJ, Singer NL, and Miller BT. Population survey of the streamside salamander, *Ambystoma barbouri*, in the Central Tennessee Basin. Middle Tennessee State University Scholars Week, Murfreesboro, TN

- 2011 **Carey A**, Nichols C, Sadler K, Farone A, and Farone M. Teaching scientific research through cedar glade ecology in the NSF GK-12 program. Tennessee Academy of the Sciences, Jackson, TN
- 2011 **Carey A**, Nichols C, Farone M, and Farone A. Engaging students in original scientific research. National Science Foundation GK-12 Annual Meeting, Washington, DC
- 2011 **Carey A** and Klukowski M. Effects of food deprivation on plasma corticosterone and nutrient levels in *Nerodia sipedon*. Association of Southeastern Biologists, Huntsville, AL
- 2010 **Carey A** and Klukowski M. Home ranges of the Eastern fence lizard, *Sceloporus undulatus*. Association of Southeastern Biologists, Ashville, NC
- 2010 **Carey A**, Saul T, Vaden N, Lewis E, Sadler K, Farone A, and Farone M. Engaging high school ecology students in biodiversity lab activities in the TRIAD GK-12 program. Tennessee Academy of the Science, Cookeville, TN

Invited Talks

- 2019 Impacts of ecotourism on endangered Bahamian iguanas April 3, 2019 Arkansas State University

Teaching Experience

Utah State University

- | | |
|-------------|--------------------------------------------------------------|
| 2017 Fall | Comparative Animal Physiology Lab, Instructor (2 sections) |
| 2016 Spring | Human Physiology Lab, Course coordinator and TA (2 sections) |
| 2016 Spring | Human Physiology Lab, Course coordinator and TA (2 sections) |
| 2015 Fall | Human Physiology Lab, Course coordinator and TA (2 sections) |
| 2015 - 2017 | Human Physiology, Guest lecturer |
| 2015 Spring | Human Physiology Lab, TA (4 sections) |
| 2014 Fall | Mammalogy Lab, Lead TA (2 sections) |

Middle Tennessee State University

- | | |
|-----------|--------------------------------------|
| 2013 Fall | General Biology Lab, TA (3 sections) |
| 2013 Fall | Biology department tutor |

- 2010- 2012 National Science Foundation GK-12 Graduate Fellow, spent 10 hours a week in high school class rooms mentoring independent research projects and guest lecturing. Additional lectures and laboratory activities were designed prepared and led (8 lectures and 8 labs per year)
- 2010 General Chemistry, Undergraduate TA

Mentoring Experience

Utah State University

Mentored students

2014 – present

Nicholas Lilly - Behavioral thermoregulation of side-blotched lizards (*Uta stansburiana*) in response to immune challenges

Cheyne Warren – Behavioral thermoregulation of side-blotched lizards (*Uta stansburiana*) in response to immune challenges

Juliane Wood – Effects of wound healing on innate immune function in Taiwanese grass lizards (*Takydromus viridipunctatus*)

Eleanor Watson – Innate immune response to antigen exposure in the garter snake *Thamnophis elegans*.

Middle Tennessee State University

2010 – 2012 Mentored and coordinated 45 high school student independent research projects from project synthesis to conference presentation

Service to University and Professional Societies

Professional Societies

- 2018 Proceedings of the Royal Society B – refereed articles
- 2018 Journal of Animal Ecology – refereed articles

Utah State University

- 2018 – 2019 Co-chair Ecology Center Seminar Committee
- 2018 – 2019 Search Committee for Evolution/Developmental Biology Faculty Position
- 2016 – 2019 Ecology Center Seminar Committee
- 2016 Biology Graduate Student Association Officer, Graduate programs committee representative

Middle Tennessee State University

- 2012 Forensic Institute for Research and Education, Forensic Science Symposium Judge
- 2011 – 2012 Association for Pre-Professional Life Sciences (APLS), chapter president

Community Outreach and Education

-
- 2017 Early Education Center, Logan UT. Animal outreach program
- 2016 Stokes Nature Center, Logan UT. Outreach program volunteer
- 2014 Graduate Student Panel for Women in Science Class, Utah State University
- 2014 Science Unwrapped, Utah State University. Educational outreach volunteer
- 2013 – 2014 Tennessee Women in Science, Technology, Engineering, and Research (TWISTER), Adventure Science Museum, Nashville, TN. Program planning committee member and activity leader (Cookie-ases – Enzymes, genes, and how they make us who we are!)
- 2012 Nashville Zoo at Grassmere, Educational outreach program (Wildlife on Wheels) volunteer, Nashville, TN.
- 2012 Nature Research Center at North Carolina Museum of Natural Sciences, Raleigh. Educational outreach volunteer.
- 2011 Expanding your Horizons in Science and Mathematics Conference (EYH) activity instructor, Middle Tennessee State University. Instructed middle and high school girls in a hands-on lab activity.

2011 Parks and Recreation Reptile Camp, Murfreesboro, TN. Presented on herpetology research and assisted with field trips finding and identifying reptiles and amphibians with elementary school students.

Membership in Professional Societies

Society for Conservation Biology

Society for Integrative and Comparative Biology

American Society of Ichthyologist and Herpetologist

Tennessee Academy of Science

Graduate alliance for the Life Sciences

Sigma Alpha Lambda Honors Society

References

Susannah French, Assistant Professor, *PhD Advisor*

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Logan, UT 84322

Ph. 435.797.9175

Email: sfrench@biology.usu.edu

John Iverson, Professor Emeritus, *PhD Committee Member*

Earlham College

Department of Biology

Richmond, Indiana

Ph. 765.983.14.5

Email: johni@earlham.edu

Charles Knapp, Vice President, Conservation and Research

John G. Shedd Aquarium

1200 S Lake Shore Dr.
Chicago, IL 60605
Ph. 312.692.2735
Email: cknapp@sheddaquarium

Matthew Klukowski, Assistant Professor, *MS Advisor*
Middle Tennessee State University
Department of Biology
MTSU Box 60
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