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RESEARCH ARTICLE

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Energetic investment associated with vitellogenesis induces an oxidative cost of reproduction

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Abstract

1. 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.
2. 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.
3. 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.
4. 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.
5. 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.

KEYWORDS
antioxidants, free glycerol, reactive oxygen metabolites, reptile, triglycerides

1 | 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 (Metcalfe & 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.

1.1 | 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, Rowe, Butler, & McGraw, 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 ROS exceeds the ability of antioxidant defences to prevent damage (Lushchak, 2014; Monaghan et al., 2009; Sies, 1997; Vassalle, Pratali, Boni, Mercuri, & Ndreu, 2008). Changes in the production of ROS can occur under pathophysiological conditions but also during commonly encountered situations, such as increased exercise (Ji, 1999; Sies, 1997). 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 (Lushchak, 2014; Monaghan et al., 2009). 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).

1.2 | Reproduction and oxidative stress

A relationship between reproduction and oxidative stress has been proposed by several researchers (Metcalfe & 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 (Costantini, 2016a,b; Metcalfe & Alonso-Alvarez, 2010; Metcalfe & Monaghan, 2013). Oxidative costs of reproduction have been demonstrated in females of numerous species, including wild Song Sparrows (*Melospiza melodia*) (Travers, Clinchy, Zanette, Boonstra, & Williams, 2010), the viviparous Asp Viper (*Vipera aspis*) (Stier et al., 2017), Brown Boobies (*Sula leucogaster*) (Montoya, Valverde, Rojas, & Torres, 2016), Zebra Finches (*Taeniopygia guttata*) (Noguera, 2017) and White-Browed Sparrow-Weavers (*Plocepasse mahali*) (Cram, Blount, & Young, 2015). However, reproduction is a series of physiological events, and different stages have varying energetic demands; it is likely that oxidative stress also varies with reproductive stage. For instance, in the Brown Booby, ROS 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.

1.3 | Energy metabolites and ROS

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 (Costantini, 2016a,b; Metcalfe & Alonso-Alvarez, 2010), it does appear that certain physiological conditions that increase cellular metabolism, such as exercise, digestion and reproduction, are followed by an increase in ROS (Butler, Lutz, Fokidis, & Stahlschmidt, 2016; Finkel & Holbrook, 2000; 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.

1.4 | 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 (a) circulating energy metabolites will be highest for vitellogenic females, (b) elevated free glycerol will be positively correlated with oxidative stress, and (c) non-reproductive females will have physiological measures more similar to males than to reproductive females. To better understand oxidative stress in an ecological context, firstly, 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 (a) individuals can be safely and reliably captured during and after reproduction, (b) nearly all adults are individually marked, and (c) 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.

2 | MATERIALS AND METHODS

2.1 | 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, Knapp, Taylor, & Davis, 2003) and is considered Endangered according to International Union for Conservation of Nature (IUCN) Red List criteria. Long-term studies of population demography (Iverson, 2007; Iverson, Converse, Smith, & Valiulis, 2006; Iverson & Mamula, 1989), genetic structure (Aplasca, Iverson, Welch, Colosimo, & Hekkala, 2016; Malone et al., 2003), evolution (Malone, Wheeler, Taylor, & Davis, 2000; Martins & Lamont, 1998), reproduction (Iverson et al., 2004; Knapp, Iverson, & Owens, 2006) and behaviour (Knapp, 2000) of *C. cychlura* have provided an ideal foundation of information to augment physiological studies.

2.2 | Study design

Male (n = 166) and female (n = 133) iguanas were captured in May, June or September 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 et al., 2004).

2.3 | Sample collection

Iguanas were captured by noose or net, and a blood sample was collected from the caudal vein within 3 min 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 hr, 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 (AccuChek Aviva Plus; Roche Diagnostics, Indiana, USA), and plasma was separated by centrifugation at 6,000 rpm (2,000 × g) for 10 min. Plasma samples were frozen and

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

	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

transported back to the laboratory on dry ice where they were stored at −80°C until physiological assays were performed.

2.4 | 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-NH_2). Plasma was diluted in the provided acidic buffered solution (5 μl : 100 μl), and samples were run in duplicate following the manufacturer's 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 (xMark; Bio-Rad). Values were calculated as absorbance change relative to the standard. The inter-assay coefficient of variation (CV) was 3.65%, and the intra-assay CVs were <5% for each of the 10 plates. Antioxidant capacity was measured with the OXY-adsorbent 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 was mixed with 100 μl of the provided HClO reagent. The inter-assay CV was 1.67%, and the intra-assay CVs 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-ROM 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).

2.5 | 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 5 μl of plasma and incubated for 5 min 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 min at 37°C, and absorbance was measured at 505 nm to estimate triglyceride concentration.

2.6 | 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 and Weisberg, 2011) and LSMEANS packages (version 2.27-61, Lenth 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) and 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

TABLE 2 Annotated models of physiological variables used for statistical analysis

(a)	d-ROMs ~ *f* (reproductive condition + site + interaction)
	Antioxidants ~ *f* (reproductive condition + site + interaction)
	Oxidative index ~ *f* (reproductive condition + site + interaction)
	Free glycerol ~ *f* (reproductive condition + site + interaction)
	Triglycerides ~ *f* (reproductive condition + site + interaction)
(b)	d-ROMs ~ *f* (follicle number)
	Antioxidants ~ *f* (follicle number)
	Oxidative index ~ *f* (follicle number)
	Free glycerol ~ *f* (follicle number)
	Triglycerides ~ *f* (follicle number)
(c)	d-ROMs ~ *f* (free glycerol + reproductive condition + site + interaction)
	Oxidative index ~ *f* (free glycerol + reproductive condition + site + interaction)

an ANCOVA with type III contrasts to model d-ROMs and oxidative index as a function of free glycerol, reproductive condition and site. Data for ANCOVA models were centred 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.

3 | RESULTS

3.1 | 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 ($p < 0.05$, Figure 1). Both male and female iguanas had elevated antioxidants during May with males showing the greatest variation across the seasons (Figure 1a). Free glycerol and triglyceride levels were similar for non-reproductive females and males across the seasons (Figure 1b). Glucose levels did not vary greatly with reproductive condition in May and June but were significantly lower in September (Figure 1b).

3.2 | 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 ($p < 0.008$) (Figure 2). Follicle number was positively related to d-ROMS, free glycerol, oxidative index and triglycerides and negatively related to antioxidants (Table 4). There was no relationship between follicle number and glucose. Follicle number was not significantly correlated with any physiological variables in June ($p > 0.8$).

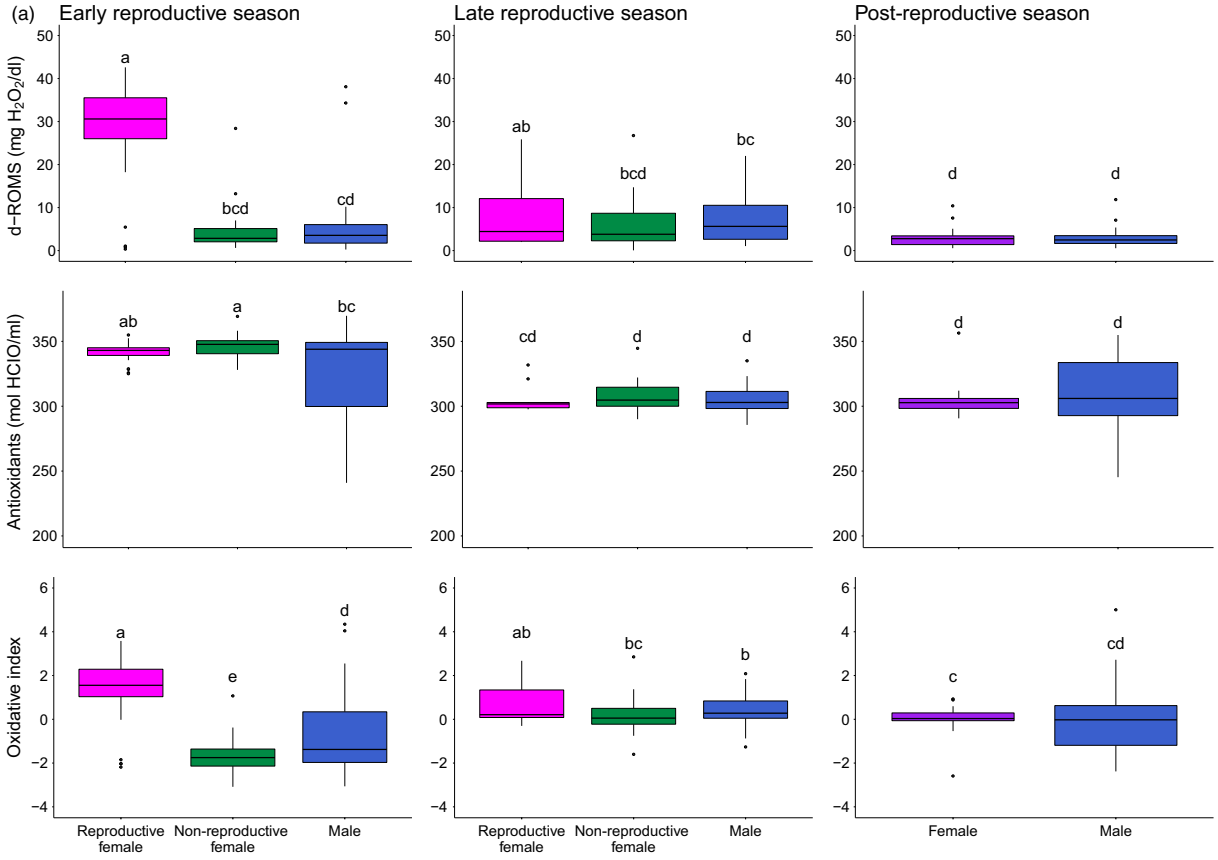
3.3 | Relationship between energy metabolism and oxidative stress

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 between 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 Figure 3a). Similarly, the relationship between oxidative index and free glycerol was strong in May and June for all iguanas and disappeared in September (Figure 3b). Model estimates and summaries are reported in Table 5.

TABLE 3 ANOVA results for physiological variables as a function of reproductive state and site for Allen Cay Rock Iguanas

	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	437,457	1	1406	**<0.001**
Reproductive state	5,881	7	2.70	**0.01**
Site	770	2	1.24	0.29
Reproductive state: site	2,661	14	0.61	0.86
Residuals	83,980	270		

Note. Significant *p* values are in bold.


(a)
Early reproductive season
Late reproductive season
Post-reproductive season
d-ROMS (mg H_2O_2/dl)
Antioxidants (mol HClO/ml)
Oxidative index
Reproductive female
Non-reproductive female
Male
Female

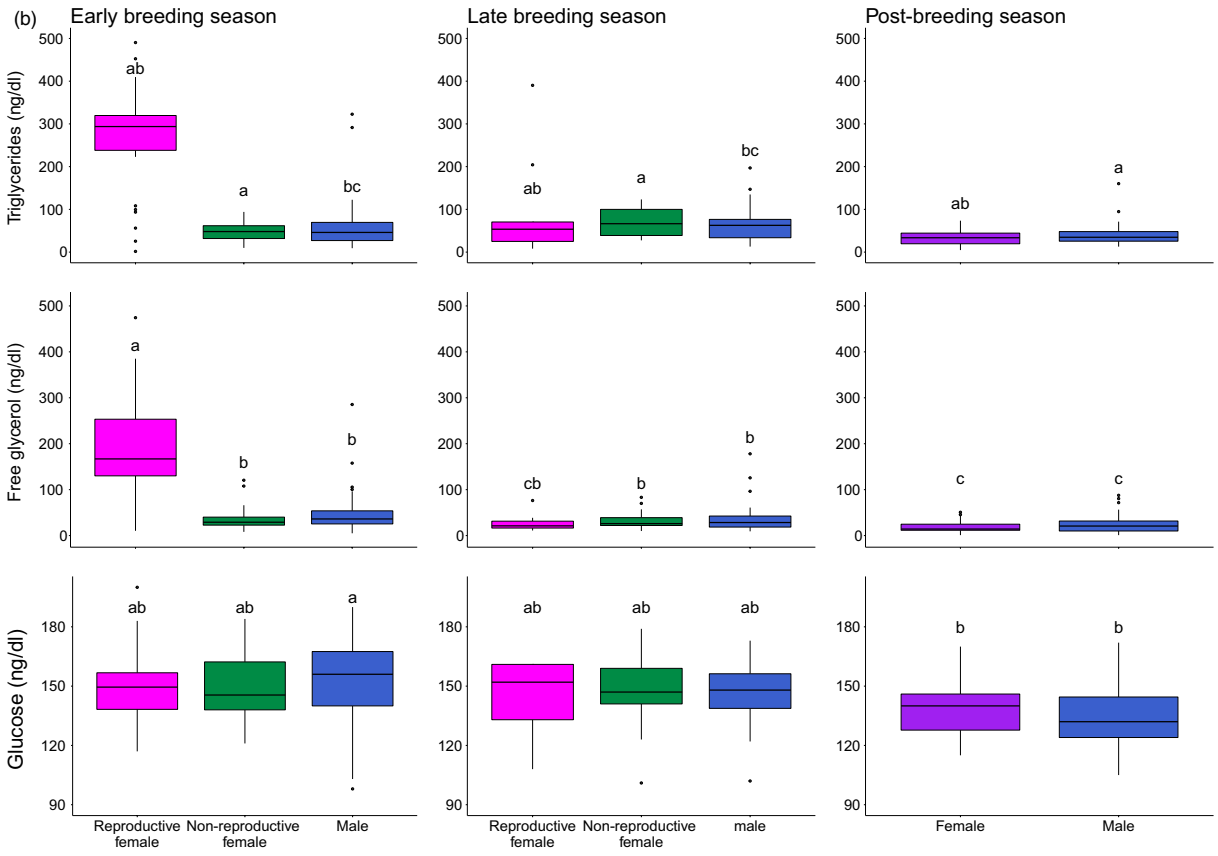

(b)
Early breeding season
Late breeding season
Post-breeding season
Triglycerides (ng/dl)
Free glycerol (ng/dl)
Glucose (ng/dl)
Reproductive female
Non-reproductive female
Male
male
Female

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

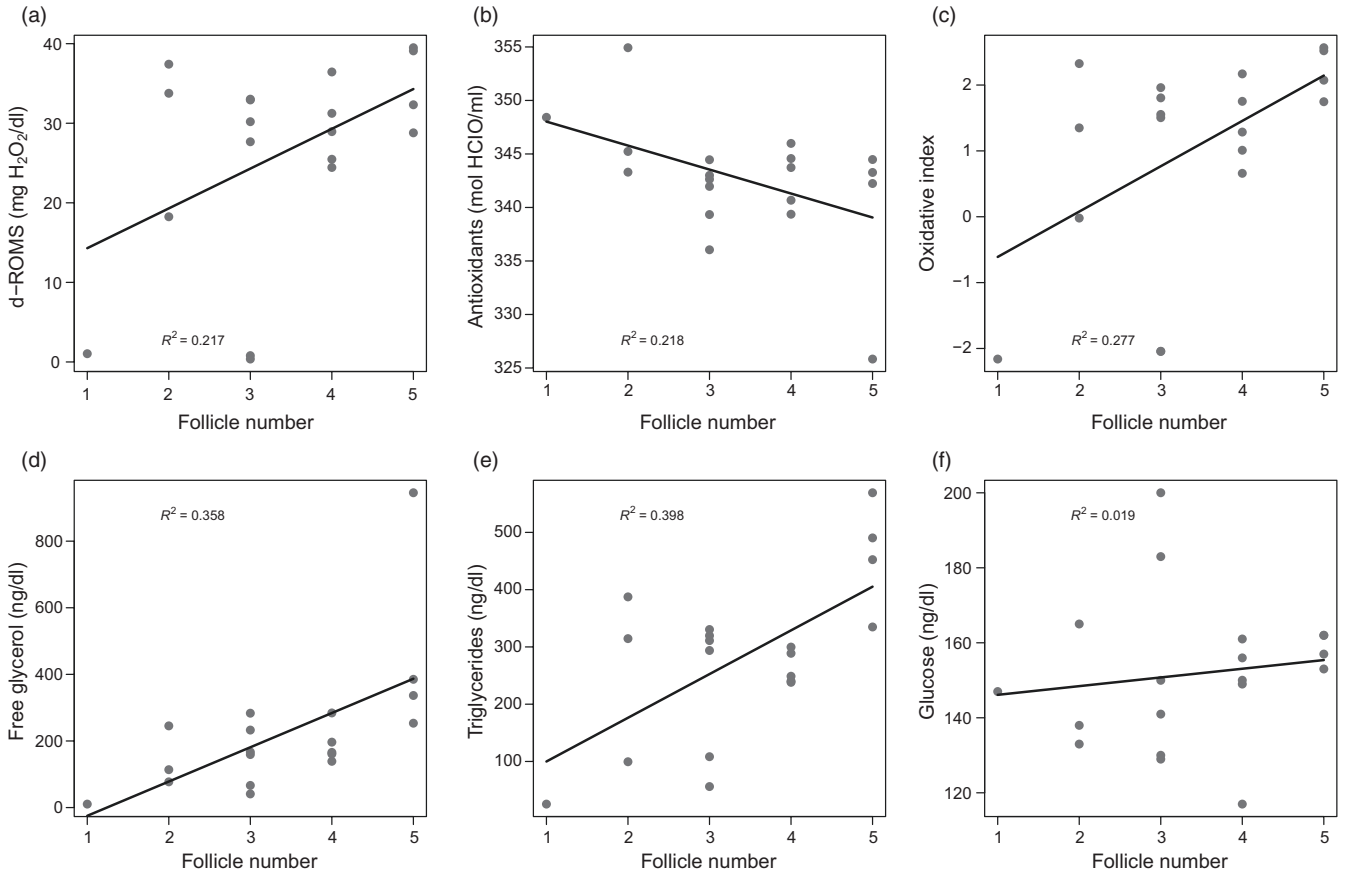


FIGURE 2 Physiological variables d-ROMs (a), antioxidants (b), oxidative index (c), free glycerol (d), triglycerides (e) and glucose (f), as a function of follicle number for May (early) reproductive female iguanas on Allen's Leaf Cay. The effect of follicle number was significant ($p < 0.05$, see Table 4) for all models except glucose

4 | 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 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.

4.1 | 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

TABLE 4 Linear model results of variables as a function of follicle number for Allen Cay Rock Iguanas

	Estimate	Standard error	*T*	*p*
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

Note. Significant *p* values are in bold.

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

	Sum of squares	*df*	*F*	*p*
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		

Note. Significant *p* values are in bold.

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, such as reproduction or migration (Blount, Vitikainen, Stott, & Cant, 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, Norberg, Gimsa, Krekling, & Amdam, 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, Bertrand, Faivre, Chastel, & Sorci, 2007), exercise (Powers & Jackson, 2008), growth (Rollo, Carlson, & Sawada, 1996), absorptive state of digestion (Butler et al., 2016; Ceriello et al., 1998) 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.

4.2 | 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

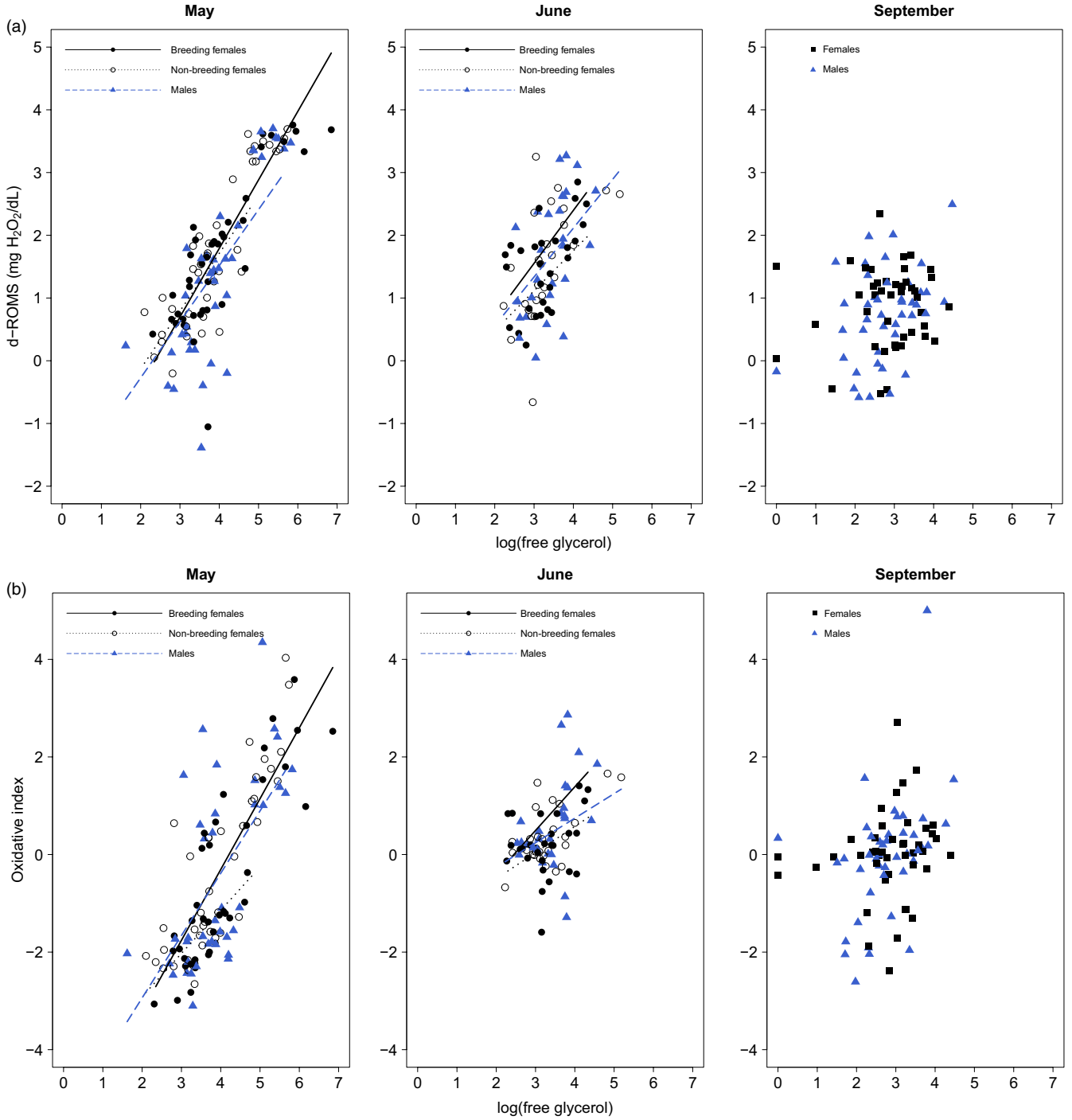


FIGURE 3 Oxidative stress measures, d-ROMs (a) and oxidative index (b), as a function of lipid catabolism into free glycerol for Allen Cay Rock Iguanas. Oxidative index is calculated by subtracting the standardized antioxidant value from the standardized d-ROM value for each individual. Colour denotes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

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 six offspring compared to those with two offspring (Wiersma, Selman, Speakman, & Verhulst, 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, Wilson, Uller, Mott, & Isaksson, 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.

4.3 | 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 tissue 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 (Costantini, 2016a,b).

The implications of oxidative stress in life-history biology are 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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AUTHORS' CONTRIBUTIONS

All authors involved in project conception, design and data collection; A.C.W. and S.S.F. led the laboratory assays, analyses of data and writing of the manuscript. All authors contributed critically to revisions of the manuscript and gave final approval for publication.

DATA ACCESSIBILITY

Data related to this article are available on the Dryad Digital Repository: https://doi.org/10.5061/dryad.3vm1h25 (Webb, Iverson, Knapp, DeNardo, & French, 2018).

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