Blood chemistry and biliverdin differ according to reproduction and tourism in a free-living lizard

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Abstract

Changes in the physiological health of species are an essential indicator of changing conditions and environmental challenges. Reponses to environmental challenges can often induce stress, influence physiology, and change metabolism in organisms. Here we tested blood chemistry parameters indicative of stress and metabolic activity using an i-STAT point-of-care blood analyzer in seven populations of free-ranging rock iguanas exposed to varying levels of tourism and supplemental feeding. We found significant differences in blood chemistry (glucose, oxygen, carbon dioxide, hematocrit, hemoglobin, calcium, potassium, and biliverdin levels) among populations exposed to varying levels of tourism, and some variation between sexes and reproductive states. However, different variables are not directly related to one another, suggesting that the causal physiological pathways driving tourism-induced differences are influenced by mechanisms that are not detected by common analyses of blood chemistry. Future work should investigate upstream regulators of these factors affected by tourism. Regardless, these blood metrics are known to be both stress-sensitive and related to metabolic activity, suggesting exposure to tourism and associated supplemental feeding by tourists are generally driven by stress-related changes in blood chemistry, biliverdin, and metabolism.

Keywords: *Cyclura*, Iguana, Metabolism, Stress, Tourism

Statements and Declarations: The authors have no competing interests to declare.

Acknowledgments: We thank Sandra Buckner, Jill Jollay, Spencer B. Hudson, Geoffrey R. Smith, Lynne Pieper, Emily N. Taylor, Frank Oliaro, Sabrina Bainbridge, Andy McCall, Earlham College undergraduate students, Bahamas National Trust interns (Christopher Fox and Melissa Elaine) and several citizen scientists for their assistance with field work. Transportation was provided by Zoltan Bobick on the R/V *Coral Reef II* and by Bruce Dunham, Sheila Young and Ron White on the *Beacon Won*. We also thank several Utah State University students for their assistance with lab work, including Emily Virgin, Jack Marchetti, Audrey Lidgard, Megen Kepas and Bailey Crowley. We also thank Lafayette College students Bridget Corpus and Bridget McNish, and Lafayette College professor Justin Hines for assistance with reagent preparation. All handling and blood sample collection was done under appropriate Institutional Animal Care and Use Commissions approval (#2530 Utah State University) and Research permission was granted through The Bahamas Environment, Science and Technology Commission, with CITES sample export permission granted through the Bahamas Department of Agriculture. This work was supported by National Science Foundation [grant number (IOS)-1752908 to S.S.F.]; Utah Agricultural Experiment Station [project numbers [1347 to S.S.F.]; Disney Conservation Fund via the Association of Zoos and Aquariums (AZA) Conservation Grants Fund.

Author Contributions: Susannah French and Charles Knapp conceived the ideas and designed methodology; Susannah French, Alison Webb, Charles Knapp, Erin Lewis, John Iverson, Zachary Cullen, Michael Butler collected the data; Kwanho Ki analyzed the data; Susannah

French, Erin Lewis, Kwanho Ki, and Michael Butler led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Availability: Data related to this article is available on USU Digital Commons: https://doi.org/10.26078/7tjp-yp51.

Introduction

Animals respond to environmental change through a suite of physiological reactions with the hallmark including the vertebrate stress response. Upon activation of the stress response both the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis are activated resulting in release of catecholamines (within seconds) and glucocorticoids (minutes) (Sapolsky et al. 2002). These hormones allow animals to cope with changing conditions that often cause stress (Romero and Butler 2007). While much work has focused on HPA activation and resulting glucocorticoid hormones, less is known about other related physiological changes that occur in response to environmental challenges, especially from non-model organisms in the wild.

Blood chemistry can provide valuable information regarding the health of animals in the wild. However most detailed chemical analyses in wild animals have thus far been limited to veterinary and medical studies, much of which has been performed in mammalian species, with a few exceptions in free-living reptiles (Knapp et al. 2013; e.g., James et al. 2006; Lewbart et al. 2015; Muñoz-Pérez et al. 2017). Markers commonly used include partial pressures of carbon dioxide (CO_2), oxygen (O_2), and blood pH levels, which help inform the metabolic demands of an animal (Severinghaus 1966). These measures may also serve as indicators of stress because stress often induces metabolic adjustments (e.g., increased O_2 consumption, increased CO_2 production, and decreased pH) (DuRant et al. 2008; Haase et al. 2016; Rabasa and Dickson 2016). For example, treatment with the exogenous stress-related hormone corticosterone increases metabolic rate in Western Fence Lizards (*Sceloporus occidentalis*) (DuRant et al. 2008). Similarly, blood chemistry variables, hematocrit and hemoglobin (Hb), may alter according to stress and accompanying metabolic adjustments to compensate for changing gas concentrations and demands (Biron and Benfey 1994).

More recently, additional stressor-induced factors have been investigated, including heme oxygenase (HO) expression and biliverdin concentrations (which can serve as a proxy for HO expression) in tissues (Butler et al. 2020). Heme oxygenase is the enzyme that converts heme to the pigment biliverdin, and multiple cellular stressors and disease states increase expression of HO (Otterbein and Choi 2000). Such changes to the heme oxygenase-biliverdin pathway could have important physiological consequences, including changes to metabolic pathways. In mammals, HO expression decreases in response to high-triglyceride diets (Varga et al. 2018) and there are negative correlations between HO expression and circulating triglyceride levels (Cimini et al. 2021; Palipoch et al. 2016). Heme oxygenase may also play a critical role in diabetes (Tiwari and Fomusi Ndisang 2014) and metabolic syndrome (Barbagallo et al. 2014; Vanella et al. 2014). However, mammals readily convert biliverdin to bilirubin (McDonagh 2001), making it difficult to link biliverdin directly to physiological processes. Emerging evidence in non-mammalian systems has demonstrated the potential informative value of exploring metabolic consequences of the heme oxygenase-biliverdin pathway, including identifying links between circulating biliverdin levels and body mass maintenance in quail (Baylor and Butler 2019). The heme oxygenase-biliverdin pathway may interact with additional systems, including spleen size in Veiled Chameleons (*Chamaeleo calyptratus*) (Butler and Ligon 2015), degree of immune response in Mallard Ducks (*Anas platyrhynchos*) (Butler and McGraw 2013), and modulation of oxidative stress in Northern Bobwhite Quail (*Colinus virginianus*) (Baylor and Butler 2019), but see Armour et al., (2020).

Ions such as calcium (Ca^{2+}), sodium (Na^{+}) and potassium (K^{+}) can also serve as stress indicators, at least in mammals (Harshfield et al. 2009; Light et al. 1983). Stress increases blood Ca^{2+} via a variety of mechanisms (Joëls et al. 2003). Moreover, oxidative stress induces Ca^{2+} transport to the cytoplasm of cells, where Ca^{2+} can directly act on mitochondria to increase and disrupt normal metabolism (Ermak and Davies 2002). Sodium and K^{+} can also respond to stress but not in the same direction as Ca^{2+} (Harshfield et al. 2009; Light et al. 1983). Notably, most previous work focuses on mammals so it is critical to expand our understanding to other systems such as ectotherms. Studies in non-mammalian species reveal similar responses of ion levels to stressors. For example, Northern Bahamian Rock Iguanas (*Cyclura cychlura*) exposed to tourism have lower levels of Na^{+} and K^{+} (Knapp et al. 2013). Similarly, broiler chickens exposed to heat stress exhibit reductions in both Na^{+} and K^{+} levels (Borges et al. 2004).

In addition to stressor-induced changes in physiology, seasonality and life-history states can impact blood chemistry in ways that align with short-term stressors. For example, reproduction, a key life-history event, is known to induce increased markers of stress across both endothermic and ectothermic species (Webb et al. 2019; Wingfield and Sapolsky 2003), and likewise can alter physiological responses to challenges in the environment (Romero et al. 1998).

The challenges associated with reproduction can also differ between the sexes, and lead to sex differences in physiological responses to stress (Grassman and Hess 1992; Tilbrook et al. 2002). Thus, sex and reproductive status need to be accounted for when studying physiological responses to stress.

Northern Bahamian Rock Iguanas (*Cyclura cychlura*) are found on a small number of cays in The Bahamas and are listed as Critically Endangered according to IUCN Red List of Threatened Species^{TM} criteria (Knapp and Buckner 2004; Iverson et al. 2019). While habitat and vegetation are fairly similar across the inhabited cays, the degree of tourism exposure (including supplemental feeding by tourists) varies considerably, as does the physiological state of the iguanas (French et al. 2022b). We have previously shown significant differences in energy metabolites, including glucose, free-glycerol, and triglycerides, whereby tourist-fed populations show elevated energy markers and glucose intolerance (French et al. 2022b; French et al. 2022a). There are also stress markers in the blood, including reduced baseline corticosterone concentrations, but elevated reactive oxygen metabolites indicative of oxidative stress (French et al. 2022b). Webb et al. (2019) also demonstrated significant changes in energy metabolites and oxidative stress according to reproductive state. Given these known physiological differences related to tourism in this well-studied species, investigating additional less-studied chemical parameters in the blood may provide a more thorough understanding of related physiological stress changes in free-living ectothermic vertebrates.

To assess health in free-living rock iguanas, we investigated a set of blood chemistry parameters more commonly used in veterinary practice. Because heme concentration is one of these metrics, we complemented this analysis by looking at the downstream product, biliverdin. Accordingly, we measured blood chemistry and circulating biliverdin levels across seven different islands with varying levels of tourism exposure. We predicted that these parameters would vary according to tourism exposure, expecting blood markers associated with stress to increase in proportion to increasing levels of tourism. We also expected significant effects of sex and reproductive status, and controlled for those effects in our statistical models.

Materials and Methods

Study design

Northern Bahamian Rock Iguanas, *Cyclura cychlura,* are distributed in isolated populations across The Bahamas (Malone et al. 2003; Hines 2017). The current study populations are found within the Exuma Island Chain and the populations are separated into two geographically separate (by 80 km) subspecies, *C. c. inornata* and *C. c. figginsi.* The subspecies also have slightly different phenologies, where the southernmost populations of *C. c. figginsi* included in this study begin the breeding season approximately 1–2 weeks earlier (JBI, CRK, SSF, unpublished).

For each subspecies, we studied a cluster of adjacent cays (3 in the north and 4 in the south), whereby all cays have similar habitats but experience significant differences in tourist visitation and supplemental feeding. Disturbance is quantified via the number of boats and people that visit these uninhabited cays (Knapp 2004; Iverson et al. 2006). We counted the total number of visitors each day over the sampling period and calculated the mean per day per island to generate ranks. Sites were ranked as either “high tourism” (average 131 tourists/day), “moderate tourism” (average 18 tourists/day), or “no tourism” (no tourists), which was quantified during our visits in 2019 as the number of visitors per day averaged over the sampling time frame. High tourism sites experience consistent daily visits from multiple tour companies, weather permitting. Moderate tourism sites are visited intermittently throughout the day by people from private yachts or smaller, personalized tour groups. To our knowledge, sites identified as no tourism experience no visitors due to the absence of landing beaches and very difficult accessibility. These rankings are further supported by over 25 years of annual personal observations at each site.

We captured adult rock iguanas (i.e., snout-vent length [SVL] over 24 cm) within the reproductive season from 16 May – 9 June (Male n = 84, Female n = 90) from high, moderate, and no tourism sites located in the both northern (*C. c. inornata*) and southern Exuma Islands (*C. c. figginsi*). A total of 102 iguanas were caught at our high tourism sites (Allens Leaf Cay n = 65 and Leaf Cay n = 37), 104 at our moderate tourism sites (Gaulin Cay n = 24, U Cay n = 41, White Bay Cay n = 38) and 65 at our no tourism sites (Flat Rock Reef Cay n = 17, North Adderly n = 20, Noddy Cay n = 28). We measured iguana body mass (Pesola scale, +/- 5 g) and snout to vent length (SVL, +/- 1 mm) and estimated body condition as mass divided by SVL (Schulte-Hostedde et al. 2005).

We sexed animals using a cloacal probe, unless a hemipenis was confirmed visually. To account for reproductive state, we used high-resolution ultrasonography (Sonosite iViz, Bothell, WA, USA) to quantify total clutch volume in females (number and size of both follicles and eggs).

Physiological metrics

Sample collection and i-STAT analyses

We collected blood samples following the procedures described in Webb et al. (2019). Our capture and collection methods allowed for true baseline sampling of individual physiology, independent of handling and capture stress (Delehanty and Boonstra 2012). We collected blood samples using 1-inch 22G syringes (BD Integra 305271) from the caudal vein between 0800 and 1300 hrs to reduce daily fluctuations in measures, with an average time to bleed of 115 ± 85 seconds (SD). Neither time of day nor time to capture and sample significantly altered blood measures (a prior model selection analysis demonstrated that time was not among the top models for any of our blood parameters). Whole blood was then stored on ice until processing later the same afternoon. We analyzed a 0.1 ml subsample of blood using a VetScan i-STAT blood gas analyzer (Abaxis, Union City, CA, USA) with CG8+ cartridges to record values for 13 variables (Table 1), including Glu (glucose), Na^+ (sodium), K^+ (potassium), Ca^{2+} (ionized calcium), pO2 (partial pressure of oxygen in blood), sO2 (oxygen saturation), pCO2 (partial pressure of carbon dioxide in blood), tCO2 (total carbon dioxide), pH, Hct (hematocrit), Hb (hemoglobin) Beecf (metabolic acid-base disturbance), and HCO_3 (bicarbonate).

Biliverdin analysis

We quantified biliverdin similarly to Butler et al. (2017) using a fluorophore developed by Berlec and Štrukelj (2014) that fluoresces in the presence of biliverdin. Biliverdin can serve as a proxy for heme oxygenase expression. First, we combined 20 µl of plasma with 20µl ddH_2O to achieve a volume of 40 µl of aqueous solution. To this, we added 60 µl dimethyl sulfoxide (DMSO) to generate 100 µl of a solution that was 40:60 ddH_2O:DMSO. This solution was briefly vortexed, and then centrifuged at 12,000 X *g* for 4 minutes at 4ºC. We then added 40µl of this solution, in duplicate, to a black 96-well plate. We also added a standard curve using a known concentration of biliverdin (Frontier Scientific, Inc., Logan, Utah) in 40:60 ddH$_2$O:DMSO. To each well, we added 65 µl of biliverdin determination reagent (Berlec and Štrukelj, 2014; Butler et al., 2017), which contains a fluorescent protein that binds with high affinity to biliverdin molecules, allowing for the quantification of biliverdin in each sample. After a 75-minute incubation at room temperature in the dark, we measured fluorescence (excitation: 680 nm; emission: 714 nm) using an Infinite M200Pro. We then calculated biliverdin concentration in each sample using the standard curve and correcting for initial volume of plasma. For a subset of samples ($n = 9$), we calculated recovery by performing the same procedure as above but added a further step. For these samples, we divided the vortexed 40:60 ddH$_2$O:DMSO solution into two aliquots of 40 µl. To each aliquot, we added 60 µl of either 0.25 µM biliverdin in 40:60 ddH$_2$O:DMSO, or just 40:60 ddH$_2$O:DMSO. We used the difference in biliverdin concentration between the two aliquots to calculate recovery for each sample, with a mean recovery of 95.6% (standard deviation: 5.3%; range: 87.1% to 102.7%).

Table 1. Physiological blood parameters evaluated in this study using an i-STAT handheld blood analyzer with exception of Biliverdin, which was analyzed in the laboratory. Significant main effects among groups denoted with a "+" and marginal differences denoted with a "*" ($p < 0.05$).

Measure	Units	Definition	Tourism	Reproduction	Subspecies
Glu	mmol/L	Glucose levels present in the blood	+	+	
Na^+	mmol/L	Na (sodium) levels present in the blood	+		+
K^+	mmol/L	K (potassium) levels present in the blood		+	+
Ca^{2+}	mmol/L	iCa (ionized calcium) levels present in the blood	+	+	+
pO2	mmHg	pO_2 is the partial pressure of oxygen gas in the blood (i.e., dissolved oxygen in the blood)	+		+

sO2	%	sO2 is the percent oxygen saturation (i.e., the amount of oxyhemoglobin as a fraction of the total hemoglobin that is able to bind oxygen_	+		+
pCO2	mmHg	PCO2 is the partial pressure of carbon dioxide gas in the blood (i.e., dissolved carbon dioxide in the blood)		+	+
tCO2	mmol/L	tCO2 (total carbon dioxide) is the measure of total carbon dioxide. This includes bicarbonate (HCO3) and dissolved CO2. While HCO3 and tCO2 levels are generally similar (Chittamma and Vanavanan 2008)			+
pH		Measure of acidity or basicity of blood		*	+
Hct	% PCU	Hct (hematocrit) is the proportion of red blood cells present in the blood	+		+
Hb	g/dL	Hb (hemoglobin) is the presence of oxygen transport metalloprotein in the red blood cells	+		+
Beecf	mmol/L	Beecf is the measurement of base excess of			

		extracellular fluid and is used to measure the effects of acid-base disturbance			
HCO3	mmol/L	HCO$_3$ (bicarbonate) is a blood buffer that is a byproduct of metabolism that regulates acidity within the blood			+
Biliverdin	umol/L	Biliverdin is a blue/green bile pigment, and the product of heme catabolism	+		+

Statistical Analyses

We assessed the differences in i-STAT variables and biliverdin of iguanas found on islands with different levels of tourism using statistical analyses in R, version 4.1.1 (R Core Team 2021), with the "effectsize" package (version 0.5, (Ben-Shachar et al. 2020; Champely 2020; Ogle et al. 2021; Mangiafico 2022; Cribari-Neto and Zeileis 2010), and "AICcmodavg" package (2.3-1) (Mazerolle 2020). To incorporate reproductive state and sex in the model within our power constraints, we created a combined variable "rep/sex" which had three levels (reproductive females, non-reproductive females, and males). The tourism index had three levels (no tourism, moderate tourism, high tourism) and subspecies had two levels (*figginsi* and *inornata)*. We ran an AIC model selection for each ISTAT variable to ensure that body size and mass did not influence our models. A body mass index (BMI) was created by dividing mass by SVL which was used as a covariate. 14 models were created with the ISTAT variable as the dependent variable and a combination of BMI, reproductive/sex, tourism, and sub-species as the explanatory covariates. BMI also did not significantly differ according to tourism level or subspecies (all $F < 1.80$, all $p > 0.17$), but did differ among the sexes as expected (female 29.15 ± 0.52; male 39.98 ± 1.43).

Two-way ANOVAs were then used to assess differences in blood chemistry variables (biliverdin, pH, pCO2, pO2, beecf, HCO3, tCO2, Na^+, iCa^{2+}, and glucose) among tourism levels and subspecies. A separate two-way ANOVA was used to assess difference in blood chemistry variables between tourism index and rep/sex. Post hoc analyses were completed using a Tukey test and Cohen's f for effect size. A three-way ANOVA with tourism index, subspecies, and rep/sex was not used because we did not have enough power to run the analysis. This constraint was due to limited sample size which ranged from n=2 to n=20 and the high variability among the 18 groups generated from this ANOVA design. While the 3-way ANOVA still provided significant results (consistent with 2-way ANOVA results reported here), the power generated by a 3-way ANOVA was ~ 60% which is much lower than the standard 80% power needed to minimize chances of a Type I error (Cohen 1992). With the two-way ANOVA, we achieved power higher than 85% (depending on the model), which was calculated using the pwr.f2.test in the 'pwr' package where we used Cohen's f calculated from the ANOVA model and a significance level of 0.05 (f= 0.10 small effect, f=0.25 moderate effect, f=0.40 large effect). The variables biliverdin and pCO2 were log-transformed to achieve normality.

The i-STAT variables potassium, hematocrit, and hemoglobin were unable to meet assumptions of normality despite attempts at transformation and were analyzed using the Kruskal Wallis test for sex effects, subspecies effects, and then tourism effects, all separately. Significant results were followed by a Dunn test for pairwise comparisons using the "FSA" package. Using the "rcompanion" package, epsilon square was also analyzed for the effect size under which a value less than 0.01 was a very small effect, 0.01-0.06 was small, 0.16-0.14 was moderate, and more than 0.14 was large. Finally, pO2 was analyzed with a beta regression using the "betareg" package due to the percent nature of the data. The data were changed from percent to a decimal by dividing by 100 and then, as suggested by the package, transformed so that all data points were between 0 and 1 but not 0 and 1 using: (y * (n-1) + 0.5) / n where n is the sample size. Two separate regressions were done for rep/sex and tourism index, and then subspecies and tourism index.

Results

i-STAT Parameters

Glucose

Blood glucose levels did not differ between subspecies, and reproductive females had higher glucose levels than males and non-reproductive females (Table 2, S.4). Iguanas on high-tourist islands had higher blood glucose levels than those on no-tourist islands, and moderate tourist islands were intermediate (Fig. 1, Table 3, S.4).

Na^+ (sodium), K^+ (potassium), and Ca^{2+} (calcium)

Sodium levels in the blood were higher in *C. c. figginsi* than in *C. c. inornata* (Table 3, 4), but there was no effect of reproductive state (hereafter "rep/sex" (Table 2). However, iguanas on the high-tourist islands had less Na^+ in their blood than those on the moderate and no-tourist islands (Fig. 1, Table 3, S.4).

Potassium levels were lower in *figginsi* than in *inornata*, but there was no statistical effect of reproductive condition or level of tourism (Table 4). However, there was tendency for K^+ to be higher in males than females, and to decrease with increasing tourism (Table S.4).

Calcium levels were higher in *figginsi* than in *inornata* (Table 3, S.4). Reproductive females had higher Ca^{2+} than both males and non-reproductive females (Table 2, 3). Within the *figginsi* subspecies, iguanas on no and moderate tourist islands had less blood Ca^{2+} than iguanas on high-tourist islands (Fig.1, Table S.4). For the *inornata* subspecies, iguanas on no-tourist islands had lower Ca^{2+} than either those on high or moderate tourist islands (Fig. 1, Table S.4).

pO2 (partial pressure of oxygen in blood), sO2 (oxygen saturation), pCO2 (partial pressure of carbon dioxide in blood), tCO2 (total carbon dioxide), and pH

Oxygen partial pressures were higher in *inornata* than *figginsi*, and values were lowest in reproductive females, though not significantly so (Table 2, 3, S.4). Iguanas on the no-tourist islands have higher pO2 than iguanas on the moderate and high tourist islands (Fig. 2, Table 3, S.4). Patterns in sO2 were identical to those for pO2 (Fig. 2, Table 5, S.4).

Carbon dioxide partial pressure and total carbon dioxide were both generally higher in *figginsi* than *inornata* (Table 2, S.4). There was no main effect of rep/sex or tourism, although there was a pattern for higher pCO2 with tourism (Table 2, S.4). Finally, pH was higher in *inornata* than *figginsi*, but there was no effect of either rep/sex or tourism (Table 2, 3, S.4).

Hematocrit and Hb (Hemoglobin)

The subspecies *figginsi* had higher hematocrit values than *inornata* (Table 4, S.4), but there was no rep/sex effect (Table 4). There was also an effect of tourism, whereby iguanas on high and moderate tourist islands had a higher hematocrit than those on no-tourist islands (Fig. 2, Table 4, S.4). The same subspecies and tourism effects were also apparent in Hb (Fig. 2).

Beecf (metabolic acid-base disturbance) and HCO3 (bicarbonate)

There were no effects of subspecies, sex, or tourism on beecf (Table 2, 3). For HCO3, *figginsi* had higher HCO3 than *inornata*, but there were no effects of rep/sex or tourism (Table 3, S.4).

Biliverdin

The subspecies *figginsi* had higher biliverdin levels than *inornata* (Table 3, S.4), but there was no difference in biliverdin among rep/sex groups (Table 2). However, there was a significant effect of tourism, where iguanas on high tourism islands had lower biliverdin than iguanas on either moderate and no tourism islands (Fig. 3, Table 2, 3, S.4).

Table 2. Results of 2-way ANOVA analyzing the effects of tourism and reproductive state (rep/sex) on physiological parameters in *Cyclura cychlura*.

Dependent Var.	Independent Var.	df	F	p	Cohen's f
Glucose	**Tourism**	**2**	**14.327**	**<0.001**	**0.42**
	Rep/sex	**2**	**12.44**	**<0.001**	**0.39**
	Tourism*Rep/sex	4	1.149	0.335	0.17
Na^+	**Tourism**	**2**	**4.526**	**0.012**	**0.23**
	Rep/sex	2	0.994	0.372	0.11
	Tourism*Rep/sex	4	0.801	0.526	0.14
Ca^{2+}	**Tourism**	**2**	**8.041**	**<0.001**	**0.31**

	Rep/sex	**2**	**6.355**	**0.002**	**0.28**
	Tourism*Rep/sex	4	0.66	0.621	0.13
pO2	**Tourism**	**2**	**14.566**	**<0.001**	**0.42**
	Rep/sex	2	0.422	0.656	0.07
	Tourism*Rep/sex	4	0.929	0.448	0.15
pCO2	Tourism	2	0.728	0.485	0.09
	Rep/sex	2	2.171	0.117	0.16
	Tourism*Rep/sex	4	0.402	0.807	0.1
tCO2	Tourism	2	1.63	0.199	0.14
	Rep/sex	2	1.059	0.349	0.11
	Tourism*Rep/sex	4	0.87	0.484	0.15
pH	Tourism	2	1.614	0.202	0.14
	Rep/sex	2	1.592	0.207	0.14
	Tourism*Rep/sex	4	0.313	0.869	0.09
beecf	Tourism	2	2.253	0.108	0.17
	Rep/sex	2	0.84	0.434	0.1
	Tourism*Rep/sex	4	0.642	0.633	0.13
HCO3	Tourism	2	1.863	0.158	0.15
	Rep/sex	2	0.986	0.375	0.11
	Tourism*Rep/sex	4	0.948	0.438	0.15
Biliverdin	**Tourism**	**2**	**35.556**	**<0.001**	**0.52**
	Rep/sex	2	2.093	0.125	0.13

	Tourism*Rep/sex	**4**	**4.385**	**0.002**	**0.26**

Table 3. Results of 2-way ANOVA analyzing the effects of tourism and subspecies (Spcc) on physiological parameters in *Cyclura cychlura*.

Dependent Var.	**Independent Var.**	**df**	**F**	**p**	**Cohen's f**
Glucose	**Tourism**	**2**	**15.419**	**<0.001**	**0.5**
	Subspecies	1	0.592	0.443	0.07
	Tourism*Spcc	2	0.606	0.547	0.1
Na^+	**Tourism**	**2**	**9.482**	**<0.001**	**0.33**
	Subspecies	**1**	**182.153**	**<0.001**	**1.04**
	Tourism*Spcc	**2**	**4.301**	**0.015**	**0.22**
Ca^{2+}	**Tourism**	**2**	**10.16**	**<0.001**	**0.4**
	Subspecies	**1**	**15.19**	**<0.001**	**0.35**
	Tourism*Spcc	**2**	**13.06**	**<0.001**	**0.46**
pO2	**Tourism**	**2**	**16.398**	**<0.001**	**0.44**
	Subspecies	**1**	**14.83**	**<0.001**	**0.3**
	Tourism*Spcc	**2**	**4.154**	**0.017**	**0.22**
pCO2	Tourism	2	0.984	0.376	0.11
	Subspecies	**1**	**37.344**	**<0.001**	**0.47**
	Tourism*Spcc	**2**	**12.739**	**<0.001**	**0.39**
tCO2	Tourism	2	1.856	0.159	0.15
	Subspecies	**1**	**18.998**	**<0.001**	**0.34**

	Tourism*Spcc	**2**	**3.595**	**0.030**	**0.21**
pH	Tourism	2	1.952	0.145	0.15
	Subspecies	**1**	**6.307**	**0.013**	**0.19**
	Tourism*Spcc	**2**	**6.779**	**<0.001**	**0.28**
Beecf	Tourism	2	2.327	0.101	0.17
	Subspecies	1	2.828	0.093	0.13
	Tourism*Spcc	2	1.95	0.145	0.15
HCO3	Tourism	2	2.052	0.132	0.16
	Subspecies	**1**	**14.372**	**<0.001**	**0.29**
	Tourism*Spcc	2	2.784	0.064	0.18
Biliverdin	**Tourism**	**2**	**35.935**	**<0.001**	**0.52**
	Subspecies	**1**	**16.559**	**<0.001**	**0.25**
	Tourism*Spcc	2	2.586	0.077	0.14

Table 4. Statistical results on physiological parameters in *Cyclura cychlura*. (HCT uses Kruskal Wallis).

Dependent Var.	Independent Var.	Chi-squared	df	p-value	Epsilon Square
K^+	Rep/sex	5.781	2	0.06	0.033
	Subspecies	**41.465**	**1**	**<0.001**	**0.240**
	Tourism	2.605	2	0.271	0.015
HCT	Rep/sex	1.234	2	0.539	0.007

	Subspecies	**44.581**	**1**	**<0.001**	**0.258**
	Tourism	**13.612**	**2**	**<0.001**	**0.079**
Hb	Rep/sex	0.411	2	0.814	0.003
	Subspecies	**46.032**	**1**	**<0.001**	**0.282**
	Tourism	**11.52**	**2**	**0.003**	**0.071**

Table 5. Statistical results of sO_2 run with a beta regression for tourism and reproductive (rep) state/sex and subspecies (Spcc) in *Cyclura cychlura*.

Dependent Var.	Independent Var.	df	F. ratio	P
Tourism*Rep/sex	**Tourism**	**2**	**9.084**	**<0.001**
	Rep/sex	1	0.209	0.648
	Tourism*Rep/sex	2	1.896	0.150
Tourism*Spcc	**Tourism**	**2**	**14.954**	**<0.001**
	Subspecies	**1**	**24.594**	**<0.001**
	Tourism*Spcc	**2**	**8.847**	**0.001**

Discussion

Overall, we found significant effects of sex, reproduction, tourism, and subspecies for many of the blood parameters. As expected, sex and reproduction both influenced blood chemistry, particularly those measures associated with energy expenditure. Reproduction is energetically costly in all animals, but in particular for females in most species (Shine and Schwarzkopf 1992). Because rock iguanas are biennial breeders, not all animals are breeding in a given year and thus the physiology of females with differing breeding status may be highly variable as a result. Specifically, we found that reproductive females have significantly elevated blood glucose relative to non-breeding females and males, suggesting that they are mobilizing energy for reproduction. These results support previous work in the same species that found elevated glucose, free glycerol, and triglycerides in the blood of female iguanas during vitellogenesis (Webb et al. 2019). To further support this idea, we also found that reproductive females have elevated PCO_2 and marginally reduced (more acidic) pH relative to non-breeding females and males, indicative of increased metabolism. Finally, reproducing females had elevated blood Ca^{2+} and K^+ important for egg production, eventual shelling, developing offspring, but also potentially markers of the stress of reproduction (Packard and DeMARCO 1991).

Although, we found variation in blood chemistry among populations due to sex, reproductive status and subspecies (see below), the degree of tourism consistently resulted in blood chemistry differences among populations. For example, blood glucose was affected by tourism intensity in a stepwise manner resulting in the highest glucose levels at high tourism sites, intermediate at moderate tourism sites and lowest at no tourism sites (Fig. 1). These results are consistent with previous work on these study populations (French et al. 2022b; Knapp et al. 2013), and are likely the result of supplemental feeding by tourists that has been steadily increasing over time. We have documented that it takes longer for iguanas at tourist sites to clear glucose and that these animals have elevated levels of energy metabolites, including free glycerol and triglycerides (French et al. 2022b). While elevated levels of glucose can ensure that iguanas on high tourism cays are unlikely to be calorically challenged, the quality of the food items may affect other nutrient levels that are less beneficial to iguanas (Knapp et al. 2013; French et al. 2022a). Moreover, elevated glucose, triglycerides, and/or cholesterol have been associated with anthropogenic disturbance such as tourism or urbanization and feeding on non-natural foodstuffs in other reptiles and birds (Townsend et al. 2019; Monzón-Argüello et al. 2018; French et al. 2022b). This is significant, because chronic elevations in glucose, triglycerides and cholesterol are generally linked to health problems in humans, although the implications for wildlife health are still unclear. However, it is important to note that stress can also influence these energetic markers in the blood (Remage-Healey and Romero 2001). For example, catecholamines released almost immediately at the onset of a stressor stimulate gluconeogenesis and result in increased circulating glucose levels (Nonogaki 2000). Glucocorticoids can also affect blood glucose levels via altering transcription (Sapolsky et al. 2000), although the effects occur later, are longer lasting, and complex (Romero and Beattie 2022).

In the present study, we found that blood Ca^{2+} was higher and Na^{+} was lower in iguanas of both sexes on tourist islands, corroborating previous work on these populations (Knapp et al. 2013), and confirming that the effects of tourism on specific ion concentrations can persist over time. Transport of ions is known to change in response to different stressors (Ermak and Davies 2002; Malinovska et al. 1991; Teng et al. 2008), and therefore differences in blood ion concentrations are less likely to be the direct result of supplemental feeding alone. The effects of stress on blood Ca^{2+} are seemingly mediated via augmenting ligand and potential-dependent Ca^{2+} channels (Liashenko and Lukashov 2003), with evidence for glucocorticoids affecting the transcriptional regulation of Ca^{2+} channels (Joëls et al. 2003). Similarly, glucocorticoids modulate Na+/K+-ATPase activity and thus the movement of Na^{+} in the blood, but also kidney retention of Na^{+} in the body (Rayson and Gupta 1985). Consistent with a previous study in chickens that demonstrated stress-induced reductions in Na^{+} (Borges et al. 2004), we also found lower levels of blood Na^{+} in iguanas at tourist-visited sites. The opposite might be expected if ion changes were due to supplemental feeding (e.g., increased sodium intake via non-natural [i.e., human] food at tourist sites), suggesting this difference in Na+ among tourist-visited and unvisited animals may be stress driven (i.e., without a food effect). Alternatively, iguanas from tourist islands that are fed grapes regularly, might eat fewer native leaves and fruits, which have different nutritional profiles than grapes (Knapp and Alvarez-Clare 2016). Moreover, native fruits and leaves may be encrusted with sea salt, resulting in higher Na^{+} intake in iguanas primarily eating a natural diet.

Elevated Ca^{2+} levels may also be the result of tourist-induced differences in stress physiology beyond their diet. We previously documented that animals at tourist sites have lower circulating baseline corticosteroid levels (French et al. 2022b). Corticosteroids are energy-mobilizing hormones that also increase intracellular Ca^{2+} levels in part by the uptake of extracellular (blood) Ca^{2+} (Das et al. 2021; Johnstone III et al. 2019), and so relatively lower corticosterone levels in tourist animals may thus be allowing for elevated blood Ca^{2+} levels. Moreover, during chronic stress in some species, bones are depleted of Ca^{2+}, which is moved to muscle tissues, suggesting an additional consequence of tourism, where animals have elevated markers of oxidative stress (French et al. 2022b). This stress effect on Ca^{2+} depletion can be exacerbated with high fat diets, and so something similar may be occurring in iguanas that were supplementally fed by tourists (Malinovska et al. 1991), whereby not only stress but also supplemental feeding were affecting Ca^{2+} levels. Another potential mechanism affecting Ca^{2+} levels in the body is oxidative stress, where elevated oxidative stress can increase intracellular Ca^{2+}, which in turn disrupts mitochondrial function and alters metabolism (Ermak and Davies 2002). Iguanas at tourist sites had elevated oxidative stress markers (French et al. 2022b), which may have additional impacts on the Ca^{2+} levels as well as the metabolic indicators we measured. Finally, in accordance with our results, elevated CO_2 and reduced pH are known to lead to Ca^{2+} effusion from the bones resulting in elevated blood Ca^{2+} levels (Wang et al. 2002); however, no direct relationships among these variables was detected.

Stress generally results in metabolic increases (DuRant et al. 2008; Haase et al. 2016), and our data were consistent with this pattern. Dissolved gas concentrations, and Hb and HCT levels, differed relative to tourism, suggesting metabolic effects. Specifically, both blood oxygen concentration and saturation were lower in animals that experienced high levels of tourism, whereas Hb and HCT were elevated. This corroborates experimental work demonstrating that stress directly reduced pO^2 in humans (Hildesheimer et al. 1985), and that lower levels of blood oxygen are likely due to elevated metabolic demands during stress (Haase et al. 2016). In Zebra Finches (*Taeniopygia guttata*), changes in metabolism and corticosterone are directly related, but not only in response to stress (Jimeno et al. 2018). Alternatively, these metabolic markers may be the result of differing digestive costs. Supplemental feeding at tourist sites likely leads to higher food intake and digestion of unnatural foodstuff, which may in turn lead to elevated metabolism in tourist animals via postprandial responses (i.e., specific dynamic action) (Secor 2009). Moreover, there was a trend toward elevated blood CO_2 in tourist-exposed animals, which would further support higher metabolic demands. Finally, in relation to blood oxygen levels, tourist-exposed animals also had elevated blood Hb and HCT relative to animals not exposed to tourism, and these increases may serve as compensatory responses to the relatively lower levels of blood oxygen.

Because Hb contains heme, and heme is directly converted to biliverdin, differences in Hb or HCT could have a direct relationship to circulating biliverdin concentration. However, we recorded contradictory patterns among these metrics. We found the lowest levels of Hb and HCT from iguanas inhabiting islands with low tourism but the lowest levels of biliverdin were from iguanas exposed to high levels of tourism. Because concentrations of substrate (heme, in the form of Hb) and product (biliverdin) show differing patterns with respect to tourism, it is probable that HO expression or activity differs with respect to tourism levels, reducing the ability of high tourism individuals to produce biliverdin from heme. Because increased expression of HO in mammalian systems is associated with antioxidant (Consoli et al. 2021) and anti-inflammatory (Mölzer et al. 2017) pathways, iguanas exposed to high levels of tourism may experience oxidative stress or pro-inflammatory conditions at higher rates. In a previous study we have documented elevated markers of oxidative stress in the populations of rock iguanas exposed to tourism (French et al. 2022b). While we did not measure adiposity or circulating lipid levels in these individuals, the high circulating levels of glucose and low levels of biliverdin in high tourism individuals is consistent with protective effects of HO and bilirubin in mammals with diabetes (Cimini et al. 2021), insulin sensitivity (Liu et al. 2015), and metabolic syndrome (Cho et al. 2016; Li et al. 2017). Therefore, while tourism may allow iguanas to avoid starvation, it may still constitute a stressor due to its effects on other physiological metrics.

Finally, there were significant differences in blood chemistry between the subspecies. In general, the southern *figginsi* subspecies had higher CO2 levels, HCO3 levels, lower O2 levels and lower pH than the northern subspecies *inornata*. All of these suggest relatively higher metabolic activity in *figginsi*. We also saw elevated Hb and HCT in *figginsi*, which may be compensatory. Moreover, Na^+ and Ca^{2+} were both higher in *figginsi*. This is the same overall pattern found with increasing tourism, except that biliverdin was higher in *figginsi.* Genetic differences between subspecies may underly some of these observed differences (Colosimo et al. 2021), however, there are also likely environmental and human-induced factors that vary among the northern and southern Exuma islands that the different subspecies inhabit (e.g., observed differences in phenology), which may also lead to the observed differences.

Conclusions

Overall, we found higher blood Ca^{2+}, Hb, HCT, and lower blood Na^+, O2, and biliverdin in tourist-exposed animals, which are consistent with elevated metabolism and stress in iguanas exposed to tourism. These markers may not be directly correlated (i.e., causally affecting one another) and may all still be responding to the same stimulus or signal. These results further suggest that additional markers, other than glucocorticoids, are useful in measuring stress in wildlife. Moreover, biliverdin concentration may complement traditional blood chemistry analyses, particularly those used in experiments investigating stress or nutrient status in free-living animals. Thus, these markers may prove useful indicators for wildlife health, including the critically endangered iguanas.

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Figure Legends

Figure 1. Tourism-related differences in glucose and ions **in *Cyclura cychlura***. Mean values (± 1 standard error of the mean) in populations of iguanas exposed to high, moderate, or no tourism in blood **a)** glucose, **b)** sodium, and **c)** calcium levels. Identical small letters (a or b) indicate no significant difference among samples; different letters indicate significant difference (α = 0.05). These data were gathered using individuals as replicates in a field setting.

Figure 2. Tourism-related differences in oxygen, and hemoglobin **in *Cyclura cychlura***. Mean values (± 1 standard error of the mean) in populations of iguanas exposed to high, moderate, or no tourism in blood **a)** partial pressure of oxygen, **b)** oxygen saturation, **c)** hemoglobin and **d)** hematocrit. Identical small letters (a or b) indicate no significant difference among samples; different letters indicate significant difference (α = 0.05). These data were gathered using individuals as replicates in a field setting.

Figure 3. Tourism-related differences in biliverdin **in *Cyclura cychlura***. Mean values (± 1 standard error of the mean) in populations of iguanas exposed to high, moderate, or no tourism in blood biliverdin concentrations. Identical small letters (a or b) indicate no significant difference among samples; different letters indicate significant difference (α = 0.05). These data were gathered using individuals as replicates in a field setting.