Too much of a good thing? Human disturbance linked to ecotourism has a "dose-dependent" impact on innate immunity and oxidative stress in marine iguanas, *Amblyrhynchus cristatus*

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ARTICLE INFO

ABSTRACT

Human population and thus anthropogenic disturbance of natural systems are increasing at staggering rates. However, the impact of disturbance on natural systems likely varies with the extent of disturbance. While a considerable amount of research has been conducted on high-impact disturbances (e.g., environmental contamination, urban development), much less is known about the effects of less obtrusive activities such as ecotourism and outdoor recreation. Past research has documented behavioral and hormonal changes to non-consumptive wildlife activities; however, these studies rarely provide direct health and survival consequences, or examine impacts over a gradient of disturbances. In the current study, we quantified endocrine and immune responses and reproductive investment in six Galápagos marine iguana (*Amblyrhynchus cristatus*) populations located across a gradient of human disturbance stemming from the ecotourism industry, including urban development, boat traffic, and ecotourist activities. We regressed fitness-related physiological metrics to the intensity of human disturbance across these populations. As predicted, immunological capabilities decreased and oxidative stress increased with increasing intensity of human disturbance and development. Sexes also differed in their endocrine responses to disturbance intensity, with only males showing elevated baseline corticosterone at sites impacted by tourism. Testosterone and estradiol were both positively related to disturbance intensity. There was also a significant overall negative effect of the presence of human disturbance, but not its intensity, on reproductive investment. These results are the first to demonstrate that measures of physiological performance are sensitive to the intensity, and not merely the presence/absence of human disturbance, and that immunological measures in addition to more traditional methods can serve as important indicators of stress and potentially compromised fitness in natural populations.

1. Introduction

Ecotourism is vital to conservation because it exposes the general public to the fascinations of nature and provides critical income to local efforts to protect wildlife (Reynolds and Braithwaite, 2001; Scheyvens, 1999). While widely considered to be less intrusive and thus less impactful on the environment than highly invasive human activities such as agriculture and urban development, ecotourism activities, which often appear to be completely non-invasive, may have indirect impacts related to the infrastructure required to support the ecotourism activity. However, little work has examined the relative impacts of varying levels of ecotourism-related anthropogenic disturbances on the environment. Since non-consumptive wildlife activities are projected to double over the next 50 years (Flather and Cordell, 1995), it is imperative that we understand the relationship between disturbance intensity and environmental impact. Integrating this understanding into future management practices is essential for modifying ecotourism approaches to maximize benefits and minimize detriment.

We and others have demonstrated that human activity associated with ecotourism can impact animals, sometimes adversely, by altering

behavior, stress physiology, immunology, and reproduction (Buckley, 2004; French et al., 2010; Knapp et al., 2013; Romero and Wikelski, 2002). For example, exposure to activities such as tourist boats alters behavior and thus energy expenditure in cetaceans (Blane and Jaakson, 1994; Williams et al., 2006), and pedestrian tourism impacts behavioral responses in various penguin (*Spheniscidae*) species (Burger and Gochfeld, 2007; Walker et al., 2005, 2006). In many species, behavioral changes include habituation where animals decrease their responsiveness and flight initiation distances in response to people (Hines, 2011; Walker et al., 2005, 2006). This type of behavioral modification may not seem overtly harmful, but physiological responses in addition to or as a direct result of the behavior can have detrimental consequences. This is evident in many vertebrate species that show altered hormonal responses to stress via the hypothalamic-pituitary-adrenal axis as measured via circulating glucocorticoids, a class of adrenal steroids, in tourist-exposed populations (Ellenberg et al., 2007; French et al., 2010; Müllner et al., 2004; Romero and Wikelski, 2002). However, endocrine responses in and of themselves do not directly inform the effects of tourism-related activities on the health or survival of populations.

Links between tourism and fitness-related measures have been documented in some species. Increased tourism exposure is associated with a decrease in growth rate and reproductive success in California sea lions (*Zalophus californianus*, French et al., 2011), and exposure to tourism leads to increased mortality in some avian species (Buckley, 2004). However, assessing fitness-related measures in this manner can take a prohibitively long time, and many studies do not integrate classic indicators of behavior and population dynamics with emerging physiological indicators (Wikelski and Cooke, 2006).

Within the more recent field of conservation physiology, this integration has typically entailed studying stress in wild organisms by using glucocorticoids as physiological indicators (Wikelski and Cooke, 2006). These hormones are advantageous to study in that they provide rapid assessment of population physiological state and potential overall health, which is something that more traditional methods, such as population monitoring, take years and even generations to assess. However, while these hormones can be indicative of stress, their primary role as energetic regulators and mobilizers complicates their use and interpretation in assessing stress, especially in natural systems. Furthermore, glucocorticoids do not necessarily inform the effects on health, survival, or reproduction in natural systems and are often context-dependent (Neuman-Lee and French, 2017). Thus, researchers have suggested a move toward additional indicators of population health (Breuner et al., 2013; Dickens and Romero, 2013).

Ecoimmunology, a burgeoning field, presents promising tools that are stress-sensitive and can rapidly and more directly assess the health of individuals within a population. These immunological tools inform functional pathways that provide researchers with an understanding of not simply whether a physiological effect has occurred, but also the downstream health implications for individuals. Yet only a few studies have employed ecoimmunological tools in a conservation setting, especially when determining the effects of tourism. For example, a study in southern stingrays (*Dasyatis americana*) documented that exposure to tourism resulted in reduced hematocrit, lymphocytes and heterophils, and elevated parasite load (Semeniuk et al., 2009). In Galápagos marine iguanas (*Amblyrhynchus cristatus*), exposure to tourism increased hormone responses to stress and generally suppressed immune responses, although these effects varied depending on breeding state of the animals (French et al., 2010). Similarly, rock iguanas (*Cyclura* spp.) exposed to tourism showed altered blood chemistry parameters and elevated parasite loads (Knapp et al., 2013).

While less common, oxidative stress indicators have been used to assess individual and population responses to long-term stressors (Constantini and Dell'Omo, 2006a; Constantini et al., 2014b; Lucas and French, 2012), and thus are useful additions to studies investigating anthropogenic disturbance. The accumulation of oxidative damage to cells and tissues via reactive oxygen metabolites can be elevated during stress and is related to senescence and death (Finkel and Holbrook, 2000; Lucas and French, 2012; Metcalfe and Alonso-Alvarez, 2010).

One current gap in the progression of the field of conservation biology is the failure to examine the extent and type of tourism exposure, with tourism instead being treated as a dichotomy – presence or absence. Quantifying tourism and linking the intensity of tourism with accompanying infrastructure development to physiological changes will lead to a greater understanding of how tourism affects the health and viability of populations. Few studies have taken this approach (Rehnus et al., 2013), likely due to a limitation in the availability of natural systems in which to study varying intensities of ecotourism.

Marine iguanas on the Galápagos Islands, Ecuador, are an ideal model for investigating the impacts of ecotourism and their underlying causes. Restrictions on tourist access and supporting development throughout the Galápagos Islands have created definable and stable spatial patterns of tourism-related impacts. That is, adjacent populations of iguanas are exposed to differing intensities of tourism disturbance that remain relatively consistent over time. The Galápagos Islands are a special place in which all human development on the islands is tied to the ecotourism industry; thus, any metric of human disturbance is tied to ecotourism, either directly or indirectly (Epler, 2007; Taylor et al., 2006). Marine iguana populations exist across the spectrum of disturbance intensities, but individual iguanas are territorial and show high site fidelity, thus linking them to the disturbance intensity specific to their locations. In addition, unlike other species of ecotourism interest, marine iguanas only feed on marine algae (Wikelski et al., 1997), with occasional records of other native plant ingestion such as succulent beach plants (Wikelski and Wrege, 2000). Thus, we can examine the effects of human presence and development without the confounding effects of dietary alterations. Moreover, ecotourism in the Galápagos Islands is a recent phenomenon relative to the lifespan and generation time of the marine iguana, making evolutionary changes unlikely in the present timeframe and allowing researchers to focus on individual responses to human disturbance. Finally, marine iguanas are currently at risk from the effects of changing climate patterns on food resources, introduced predators and disease, and the physiological impacts of ecotourism (French et al., 2010). Yet ecotourism is vital to the local and national economies, so sustained and even growing ecotourism will continue. The manner in which this industry continues in the future can be shaped by knowledge of the impacts on the iguanas and the extent to which disturbance intensity influences these impacts.

We measured body condition, endocrine responses (plasma corticosterone concentration) to a controlled stressor, oxidative stress (reactive oxygen metabolites and antioxidant concentrations in the plasma), ecoimmunological responses (bacterial killing ability, hemolytic complement activity, and wound healing), and reproductive investment (circulating sex steroids and clutch size) in four different populations of breeding Galápagos marine iguanas along the southern shore of Isla Santa Cruz and two populations along the southern shore of Isla Isabela in the Galápagos Islands. The populations on each island are spatially close enough that habitats are similar yet represent a gradient in ecotourism and infrastructure development intensity (quantified via remotely sensed imagery). We hypothesized that physiological and immunological responses to stress would correspond to the gradient of human disturbance derived from the ecotourist industry, with increasing amount and intensity of disturbance corresponding to elevated physiological impacts on iguanas. Similarly, if viewed in a dichotomous fashion (e.g., presence/absence), human disturbance should have significant impacts, such that animals inhabiting ecotourist sites should exhibit greater physiological impacts than iguanas in areas free of disturbances.

2. Materials and methods

2.1. Animals and study sites

We studied Galápagos marine iguanas in January 2013 during their breeding season. Animals were studied at four different sites on the island of Santa Cruz – at the Parque Nacional Galápagos park entrance (S 0° 44′ 29.4″ W 90° 18′ 24.9″), Estación Charles Darwin (CDF) (Santa Cruz; S 0° 44′ 38.3″ W 90° 18′ 10.3), a site 1.5 km away from CDF (SC1) (S 0° 44′ 43.4″ W 90° 17′ 46.6″), and a site isolated from the port city at Punta Núñez (S 0° 44′ 45.5″ W 90° 15′ 17.1″). The park entrance is a concrete dock with a near continuous presence of people. The CDF site is a natural lava rock beach where human presence is frequent (i.e., regular visits throughout the daylight hours). The other two sites are also natural lava beaches, with SC1 limited to rare visits (i.e., less than daily) by local residents and fishermen and the Punta Núñez site being undisturbed. We also studied two populations of iguanas on the island of Isabela– Playa del Amor (S 0° 57′ 41.2″ W 90° 59′ 20.4″) and within the town of Puerto Villamil (S 0° 57′ 22.7″ W 90° 58′ 17.4″). Both of these populations were exposed to human disturbance, although the disturbance within the town of Puerto Villamil was near continuous, with animals having to cross the main thoroughfare to feed on a daily basis. As described in the Introduction, although disturbance in Puerto Villamil is linked to normal civic activities, the town is established as a tourist center and port for Isabela and the surrounding islands, and thus is inherently tied to ecotourism. The Playa del Amor site was limited to intermittent daily tourist visitation. In addition, the habitat at Playa del Amor was natural habitat, whereas in Puerto Villamil the habitat was highly modified and disturbed by humans. Thus, the two Isabela island sites were similar in disturbance to the park entrance and SC1 sites on Santa Cruz.

We sampled both adult males and females at each of the 4 sites on Santa Cruz (Park Dock, high disturbance = 18 F, 13 M; Estación Charles Darwin (CDF), moderate disturbance = 20 F, 12 M; a site 1.5 km away from CDF and the main town (SC1), low disturbance = 20 F, 12 M; Punta Núñez, no disturbance = 20 F, 14 M) and only females (due to logistical constraints) at the two sites on Isabela (Puerto Villamil, high disturbance = 13 F; Playa del Amor, low disturbance = 15 F). Within the sexes, we selected sexually mature animals of a similar body size (male snout-vent length (SVL): 42.43 ± 0.72 cm; female SVL: 29.21 ± 0.31 cm; mean ± s.e.) and body mass (male: 3.96 ± 0.10 kg; female: 1.44 ± 0.05 kg; mean ± s.e.). Selection of adults was random within the given size class of animals being studied. Individuals were caught by hand and marked with non-toxic paint to allow for observation and avoid re-sampling of the same individuals. To assure identification at the time of recapture, individuals in this study were also marked using short strings of colored beads sutured to the dorsal spines. Paint and bead markings have not altered behavior in previous studies of marine iguanas (Audet and Wikelski, unpublished data). After processing (see below), all animals were released at the site of capture.

2.2. Blood samples, stress series, and size measures

Glucocorticoids (particularly corticosterone in reptiles) increase in response to capture in many species (Romero et al., 1997; Wingfield et al., 1995) including reptiles (Moore et al., 2000; Moore et al., 1991). Between 0800 h and 1300 h, blood samples (1 ml total from the caudal vein using a heparinized 1 ml syringe with a 25-gauge needle) were collected from each animal within 3 min (0.5 ml) of capture and again at either 15 or 30 minute post-capture (0.5 ml) to determine baseline and restraint stress-induced levels of corticosterone, respectively. The time of each blood sample collection was recorded to assess temporal variation in stress responses. Upon capture and after the initial blood sampling, we placed each iguana in an opaque cloth bag. We used restraint as a stressor because it is known to elicit large increases in circulating corticosterone levels in reptiles, including marine iguanas, and can be considered a standardized novel stressor (French et al., 2010; French et al., 2006; Romero and Wikelski, 2002). Most iguanas remained in cloth bags until the end of a 30-minute restraint period; however, half of the males from each site were tested at 15 min rather than 30 min at the request of Parque Nacional Galápagos. Samples were stored on ice until returning to the field camp (less than 6 h).

After collecting blood samples, ectoparasite load (number of ticks present on individual), was assessed for each animal. Ectoparasite counts were done by a careful visual scan (by the same observer), and total number of ectoparasites present on an animal at the time of capture was recorded. The only ectoparasites present were ticks of the *Amblyomma* group (Bequaert, 1932; Schatz, 1991; Wikelski, 1999). We did not discern between the two species (*Amblyomma darwini* and *Amblyomma williamsii*) due to limitations on holding time of the iguanas by the Parque Nacional Galápagos. Ticks of the *Ornithodoros* group only feed on marine iguanas as night, and so were not present during our ectoparasite counts (Wikelski, 1999).

Body mass was measured with a Pesola scale when iguanas were in cloth bags. A cloth measuring tape was used to measure SVL (ventral surface of the animal extending from the tip of the snout to the cloacal vent), tail length (ventral surface of the tail from vent to tip), and tail circumference (thickest part on the tail at the base near the vent). Tail volume was measured using water displacement whereby the tail was temporarily dipped to the vent into a water-filled cylinder, and then the cylinder was refilled with a measured amount of water. High resolution ultrasound (Sonosite MicroMaxx) was used to assess the number of developing follicles present in female marine iguanas. Because in most cases eggs were not ovulated and shelled, we did not measure egg size as this would be an inaccurate estimate of final egg size. Instead, we counted the number of developing eggs to estimate investment (Gilman and Wolf, 2007; Lucas and French, 2012).

2.3. Hormone assays

Plasma was separated from the cells via centrifugation and stored at − 20 °C until assayed. Plasma samples were analyzed using radio-immunoassay in three separate assays in duplicate for testosterone in males (Ab: Fitzgerald #WLI-T3003-01916), estradiol in females (Ab: Biogenesis # 7010-2650), and corticosterone in both sexes (Ab: MP Biomedicals # 07-120016) using a previously described and established protocol (French et al., 2010; Moore, 1986). For each sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction. These recoveries were used to adjust the final sample concentration values to account for any losses during these procedures. The intra-assay variation for the separate hormones was 7.2% for testosterone, 15.9% for estradiol, and 14.0% for corticosterone. Minimum detectable values were 0.4 ng/ml for testosterone, 0.4 ng/ml for estradiol, and 0.3 ng/ml for corticosterone. All assayed samples fell within the standard curve for the assay (i.e., were detectable values).

2.4. Cutaneous wound healing

After stress challenge, the 20 female iguanas from both the CDF and SC1 sites received a standardized 3.5 mm cutaneous biopsy to assess wound healing capability, a generalized measure of immune function and self-maintenance (French et al., 2006). Procedures followed French et al. (2010) with the only modifications being that animals were given a 0.05 ml subcutaneous injection of bupivacaine prior to biopsy, and digital images were captured using a Nikon d200 (10 megapixels, automatic shutter and focus, 28–560 mm zoom) camera. Wound size was determined using image analysis software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2009). Animals were photographed at the time of biopsy and again after recapture

10 days later, with percent change from initial wound area calculated to assess healing over time.

2.5. Bacterial killing assays

The bacterial killing assay characterizes a functionally relevant immune response that involves the action of phagocytes (macrophages, heterophils, and thrombocytes), opsonizing proteins (complement and acute phase proteins), and natural antibodies (predominantly IgM and IgA). Because this assay assesses the blood's ability to eliminate an actual pathogen, it provides an ecologically important assessment of host immune function (Tieleman et al., 2005). Working under a sterile laminar flow hood, we performed assays on both baseline and stressed plasma samples following French and Neuman-Lee (2012), using widely prevalent *E. coli* (E^{power}™ Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN) and a 1:5 dilution of plasma samples. Bactericidal capacity was calculated as the mean absorbance for each sample, which were run in duplicate, divided by the mean absorbance of positive controls (six wells containing only media and bacterial solution on each plate), and multiplied by 100 (i.e., % bacteria killed relative to the positive control). The coefficient of variation was 0.57%. Each plate also contained four wells that served as negative control (no bacteria added) to assure no background contamination was present.

2.6. Hemolytic complement activity assays

The hemolytic complement pathway, hereafter complement pathway or activity, is part of the innate immune response and consists of a series of proteins present in the plasma (Janeway et al., 2005). Activation of the complement system initiates an enzymatic cascade that leads to bacterial cell lysis, the formation of chemotactic peptides that attract immune cells, and an increase in phagocytic clearance of infected cells. This cascade acts as the major effector of the humoral immune response (Janeway et al., 2005; Mayer, 1948). A hemolytic complement activity assay (CH50), which evaluates the plasma response to introduced sheep red blood cells (SRBC), can be used to qualitatively measure overall pathway integrity, cell lysis, and functionality (Mayer, 1948).

In a previous study on marine iguanas, no difference in complement activity was detected between the baseline and 30 minute sampling times, and thus we only performed assays on baseline samples (French et al., 2010). Complement activity in plasma was measured based on methods previously described using duplicate 80 µl samples of both 1:5 and 1:10 dilutions of plasma (Freedberg et al., 2008; French et al., 2010; Greives et al., 2006; Sinclair and Lochmiller, 2000). Hemolytic complement activity was expressed as CH50 units/ml plasma, where 1 CH50 unit equals the reciprocal of the dilution of plasma required to lyse 50% of the SRBC in culture (Mayer, 1948). Because values violated the assumption of normality, all values were increased by two (so that all CH50 values were above one) and then normalized via a natural log transformation. The resulting values, ln(CH50 + 2), were then used in statistical analyses.

2.7. Oxidative stress assays

Oxidative stress was measured since the accumulation of oxidative damage to cells and tissues via reactive oxygen metabolites is elevated during stress and is related to senescence and death (Finkel and Holbrook, 2000; Lucas and French, 2012; Metcalfe and Alonso-Alvarez, 2010). To measure reactive oxygen metabolites (ROMs), we used a kit (Diacron, Grosseto, Italy) that detects the level of hydroperoxides, ROMs that signal lipid and protein oxidative damage (Costantini and Dell'Omo, 2006b; Finkel and Holbrook, 2000; Lucas and French, 2012; Metcalfe and Alonso-Alvarez, 2010; Van de Crommenacker et al., 2010). We diluted 5 µl of plasma into 100 µl of the provided acidic buffered solution and followed the 'end-point mode' manufacturer

instructions, with modifications for use on a 96-well microplate. Inter-plate variation was 0.41%, and intra-plate variations were 0.43% and 0.28% for the two plates.

Antioxidant capacity was measured using the OXY-Adsorbent test (Diacron, Grosseto, Italy), which measures the effectiveness of the blood antioxidant barrier by quantifying its ability to cope with the oxidant action of hypochlorous acid (HClO) (Vassalle, 2008; Vassalle et al., 2008). We diluted 2 µl of plasma in 100 µl of distilled water and followed manufacturer instructions with modifications for use on a microplate. We then mixed a 5 µl subsample of this diluted plasma with 100 µl of the HClO solution provided. Inter-plate variation was 0.43% and intra-plate variations were 0.14% and 0.44%.

2.8. Disturbance index

No consistent measures of tourism were available across the study areas, so we developed a spatially explicit disturbance index to quantify the potential human disturbance at each study site. In the Galápagos islands, any human development present at the study sites is either the direct or indirect result of the ecotourism industry, which comprises the majority of the resident and non-resident population activities in the Galápagos. These features provide an indirect estimate of human activity at the different sites. The index we developed combined the number of tourism-related infrastructure features including urban features, trails, and roads contained within a study site and the distance to the nearest urban feature, marina, trail, and road, the digitally quantified size of that feature in contact with the study site, and combined with a qualitative index of human activity of those different features. In developing the disturbance index, we assigned areas with high disturbance (based on the first author's assessment) or short distances to disturbance features a value of 10 (Table 1) to standardize and facilitate comparison across sites. A theoretical value of 110 indicated the maximum amount of potential human disturbance, a value of 0 indicated a minimum amount of disturbance, and a value of 55 indicated the study site was exposed to an intermediate amount of disturbance.

Study sites were represented as areas rather than individual points to account for the variable nature of the breeding congregations (i.e., leks). The boundaries of each study site (as determined by the first author – the boundary for which iguanas where physically caught and observed from the breeding lek) and urban structures were on-screen digitized while trails and roads were digitized from 2014 Google Earth imagery (Source: Isabela Island, May 5, 2013; Santa Cruz Island, March 19, 2014. Google Earth accessed May 13, 2014). Although iguanas could freely travel beyond these qualitative boundaries during the study, iguanas are highly territorial, especially during the breeding season when they are lekking (Wikelski et al., 1996). Marinas were

Table 1
Measures of rank used to develop a disturbance index for 6 marine iguana study sites in the Galápagos Islands. Higher values indicate higher potential human activity.

Rank measure	Low activity rank	High activity rank
Proportion urban features on site	Fewest features = 0	Most features = 10
Distance to nearest urban feature	Longest distance = 0	Shortest distance = 10
Distance to nearest marina	Longest distance = 0	Shortest distance = 10
Nearest marina activity	Lowest activity = 0	Highest activity = 10
Distance to nearest tourist beach	Longest distance = 0	Shortest distance = 10
Number of tourist beaches	Lowest number = 0	Highest number = 10
Nearest tourist beach activity	Lowest activity = 0	Highest activity = 10
Number of trails intersecting	Lowest number = 0	Highest number = 10
Length of trails intersecting	Shortest distance = 0	Longest distance = 10
Number of roads intersecting	Lowest number = 0	Highest number = 10
Length of roads intersecting	Shortest distance = 0	Longest distance = 10

identified as any water-based areas that consistently had boats of any size moored for > 30 min. Marinas (including docks and piers), urban features, and tourist beaches were subjectively assessed as having high, moderate, and low human levels of human activity, respectively, by the first author based on the observed human presence during sampling periods. (It should be noted that the overall disturbance rankings used in the analysis do not significantly change with exclusion of this qualitative human activity index). Identification of trails and roads and level of use (intensity) was based on a combination of the first author's field experience and visible paths in the remotely sensed imagery. Although we screen-digitized boats in each marina, each image represented a single "snap shot" in time that did not characterize activity during the sampling period. Thus, the number of boats in a marina was not included in the analysis; however, boat number corroborated the subjective human activity rankings. All digitized features were projected to Universal Transverse Mercator (UTM), WGS84 datum, and zone 15S to facilitate valid measures of areas and distances. All imagery processing and feature manipulations were conducted in ArcMap 10.0 (ESRI, Redlands, California, USA).

We quantified the number and size of urban features and the number and distance of trials and roads that intersected each study site to characterize the potential levels of human influence and disturbance. In general, we developed the disturbance index with the concept that areas ranked with a high disturbance index that were closer to a study site had the potential to influence iguanas more than the same type of area a greater distance away. Distances were measured between the study site and the nearest urban feature, the nearest marina, and the nearest tourist beach. Distances between study sites and polygonized features (i.e., urban features, marina, and tourist beaches) were measured between the two closest edges of each polygon. Distances of zero indicated the feature was within the site area. We examined the proportion of urban features present within each study site to evaluate the relative effect of urban features across all study sites. Sites included 4 populations on Santa Cruz (Park Dock, high disturbance = 18 F, 13 M; Estación Charles Darwin (CDF), moderate disturbance = 20 F, 12 M; a site 1.5 km away from CDF and the main town (SC1), low disturbance = 20 F, 12 M; Punta Núñez, no disturbance = 20 F, 14 M) and only females (due to logistical constraints) at the 2 sites on Isabela (Puerto Villamil, high disturbance = 13 F; Playa del Amor, low disturbance = 15 F). Sites were assigned the average measure of activity (Table 2). The overall score is a composite of all the features across all the sites ranking their overall size, intensity, and thus relative influence. While these scores may seem arbitrary in isolation, they quantify disturbance and allow for comparison across all study sites.

2.9. Statistical approaches

For all analyses we ran least squares regression for rank sum of human disturbance on morphological/physiological variables of inter-

est. For morphological and sex steroid analyses, the sexes were analyzed separately due to significant sex differences in body size (Wikelski, 2005) and testosterone (males) and estradiol (females) concentrations. Also, half of all males underwent a 15 min stress challenge instead of the usual documented 30 min challenge at the request of the Parque Nacional Galápagos, and thus these animals' stress induced samples had to be analyzed separately from males and females given the 30-minute stress challenge. For all other analyses, we included sex and interaction terms in all models to account for prevalent morphological and physiological differences between the sexes during breeding. When appropriate, Tukey posthoc honest significant difference (HSD) tests were performed to probe specific differences among groups. We performed a regression analysis of body mass against SVL to obtain residuals equivalent to "body condition". We also separately examined tail volume as a measure of resource stores and body condition. It should be noted that female condition (residual from the regression of body mass on SVL) but not tail volume may be largely confounded by clutch mass. Although islands differed in iguana body size (Isabela females being larger than Santa Cruz females), preliminary population-based analyses determined that size differences were not driving physiological variation across populations, and thus all models included disturbance ranks for all 6 populations (Isabela and Santa Cruz) for females and all 4 populations (Santa Cruz) for males that were sampled. Hormone data were log transformed to meet assumptions of normality. All analyses were completed in JMP version 12.1 (Cary, NC, USA). In all cases, differences between group means were considered statistically significant if $p \leq 0.05$ (García, 2004; Moran, 2003; Rice, 1989).

3. Results

3.1. Morphology

We observed significant negative relationships between the intensity of ecotourism disturbance measures and female body mass (Fig. 1a), SVL (Fig. 1c), and tail volume (Fig. 1f), but not body condition (Fig. 1e; Table 3.1). In males, we observed an opposite pattern, with significant positive relationships between disturbance intensity and body mass and body condition, but not SVL or tail volume (Fig. 1b, d, e, f; Table 3.1).

3.2. Reproductive investment

We found no significant effect of disturbance intensity on the number of developing follicles in females, but there was a significant difference in follicle number between sites with disturbance present versus sites with disturbance absent, with fewer follicles at disturbed sites (Fig. 2; Table 3.1). Estradiol was inversely related to follicle number ($F = 4.20$, $DF = 1, 88$, $p = 0.04$; $r^2 = 0.05$).

3.3. Endocrine effects

There were significant positive relationships between baseline estradiol (females) and disturbance intensity and between baseline testosterone (males) and disturbance intensity (Fig. 3a; Table 3.2). We found a significant positive relationship between disturbance and stress-induced testosterone in males undergoing a 30-min stress challenge, but not in males stressed for 15-min and not for delta testosterone response (delta = stress induced-baseline) in either 15 or 30-min group (Table 3.2). These results suggest that there is little difference in the magnitude of response of testosterone to stress as related to disturbance.

For baseline corticosterone, there was a significant interaction between sex and disturbance intensity, such that males showed a positive relationship between baseline corticosterone and disturbance and females showed a negative relationship (Fig. 3b; Table 3.3). The
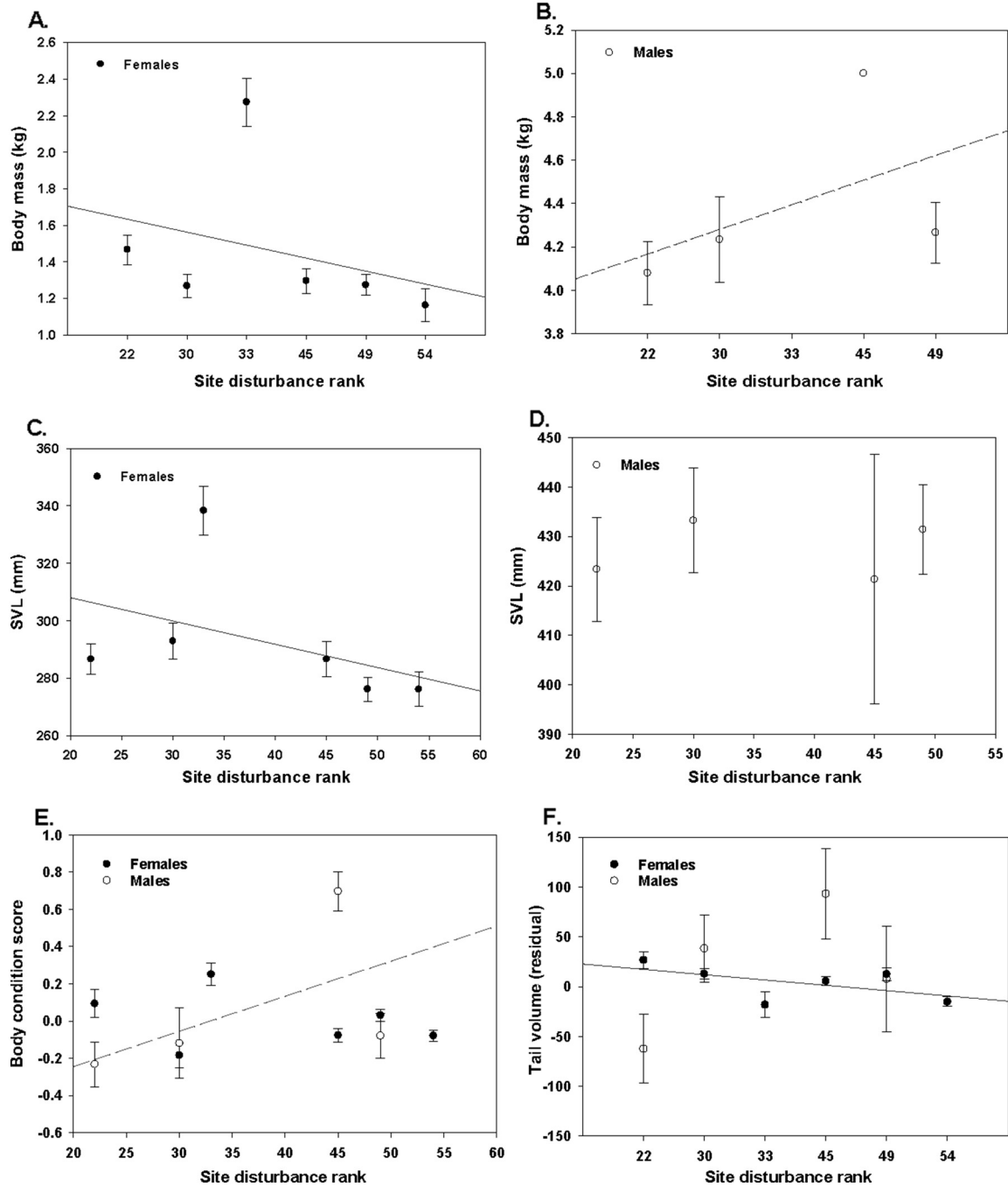
Table 2
Rank sum of a human disturbance index across 6 study sites in the Galápagos Islands. The index was developed with a maximum of 110 theoretically indicating high disturbance, a minimum of 0 indicating little to no disturbance, and disturbance values in between the maximum and minimum should be considered relative to the maximum and minimum values.

Island	Study area	Rank sum human disturbance	Potential human activity level
Isabela	Puerto Villamil	54	High
	Playa Del Amor	33	Low
Santa Cruz	Park Dock	49	High
	Charles Darwin Foundation	45	High
	SC1	30	Low
	Punta Núñez	22	Low

Fig. 1. Body size and condition scores across sites with varying amounts of disturbance. Mean (a) body mass in females p < 0.05*; (b) body mass in males p < 0.05*; (c) snout vent length (svl) in females p < 0.05*; (d) snout vent length (svl) in males p > 0.05; (e) body condition index in females p > 0.05, males p < 0.05*; and (f) tail volume in females p < 0.05*, males p > 0.05; in female (closed circles – solid line) and male (open circles – dashed line) marine iguanas from populations exposed to different amounts of human disturbance. Lines are only present for statistically significant relationships.

corticosterone response (delta = stress-baseline concentrations) to 30-min stress challenge was greater in females than males, and there was no effect of disturbance and no sex by disturbance interaction (Table 3.3). There was also no effect of disturbance intensity on corticosterone response to stress in males undergoing a 15-min stress challenge (Table 3.3).

3.4. Ectoparasites, immunity, and oxidative stress

We found that males had significantly more ticks than females, but there was no effect of disturbance intensity or sex by disturbance interaction on the number of ticks present (Table 3.4; Table 4). There

was a significant negative relationship among disturbance intensity and bacterial killing ability in both males and females, with females having lower overall bactericidal ability than males (Fig. 4a; Table 3.4). The sex by disturbance interaction was not significant for bactericidal ability. For stress-induced bactericidal response (Neuman-Lee and French, 2014), we found a significant effect of sex, with females have a greater stress-induced BKA response than males, a near significant effect of disturbance intensity such that responses are larger with greater disturbance, and no interaction between the two factors (Table 3.4). Additional analyses revealed a negative relationship between testosterone and bactericidal ability overall (F = 6.77, DF = 1, 49, p = 0.01; r^2 = 0.121), as well as between testosterone

Table 3

Statistics in female and male iguanas. Asterisks denotes statistically significant value p < 0.05. [†] denotes tests where independent variable is the presence of disturbance, for all other tests the independent variable is the rank sum of human disturbance unless otherwise stated.

	Parameter estimate	p value	F value	Df model	Df error
3.1 Morphology					
Female					
Mass	− 0.012	< 0.01*	8.2932	1	104
SVL	− 0.686	0.01*	6.48	1	104
Body condition	− 0.003	0.11	2.5439	1	104
Tail volume	− 0.694	0.01*	6.504	1	98
Egg number[†]	− 0.373	0.04*	4.3855	1	73
Male					
Mass	0.019	0.01*	6.568	1	49
SVL	0.062	0.93	0.0089	1	49
Body condition	0.018	0.01*	6.8273	1	49
Tail volume	3.297	0.09	2.1523	1	47
3.2 Sex steroids					
Female					
Baseline estradiol	0.013	0.04*	3.9827	1	88
Male					
Baseline testosterone	1.151	< 0.01*	12.2011	1	49
15 min stress testosterone	− 0.097	0.89	0.0195	1	22
15 min delta testosterone	− 0.753	0.20	1.7494	1	22
30 min stress testosterone	1.560	0.03*	5.306	1	24
30 min delta testosterone	− 0.008	0.99	0.003	1	24

|	Parameter estimate	p value	F value	F model	Df model	Df error
3.3 Corticosterone						
Baseline corticosterone						
Disturbance	0.010	0.15	2.1032	5.882	3	148
Sex	− 0.114	0.14	2.1874	5.882	3	148
Sex*disturbance	− 0.025	< 0.01*	16.0537	5.882	3	148
30 min delta corticosterone						
Disturbance	− 0.079	0.34	1.0923	3.8092	3	124
Sex	1.940	< 0.01*	10.2942	3.8092	3	124
Sex*disturbance	0.077	0.16	2.0427	3.8092	3	124
15 min delta corticosterone (males)	0.060	0.54		0.3948	1	22
3.4 Parasites, immunity, oxidative stress						
Parasites						
Disturbance	− 0.157	0.12	2.4019	3.1933	3	149
Sex	− 2.746	0.02*	5.9863	3.1933	3	149
Sex*disturbance	− 0.026	0.79	0.0678	3.1933	3	149
Wound healing (females)						
Disturbance	− 0.201	0.59	0.2964		1	29
Bactericidal ability						
Disturbance	− 1.322	< 0.01*	23.2814	13.9105	3	153
Sex	− 6.507	0.03*	4.491	13.9105	3	153
Sex*disturbance	− 0.436	0.11	2.5346	13.9105	3	153
30 min delta bactericidal response						
Disturbance	0.700	0.06	3.7078	5.0707	3	129
Sex	10.665	0.01*	6.766	5.0707	3	129
Sex*disturbance	0.146	0.69	0.162	5.0707	3	129
Hemolytic complement (CH50)						
Disturbance	− 0.002	0.58	0.3134	0.1479	3	151
Sex	− 0.012	0.76	0.0922	0.1479	3	151
Sex*disturbance	0.001	0.79	0.0693	0.1479	3	151
dROMS						
Disturbance	0.134	0.05*	3.8802	18.1268	3	122
Sex	5.211	< 0.01*	48.2813	18.1268	3	122
Sex*disturbance	0.069	0.31	0.3145	18.1268	3	122
OXY						
Disturbance	0.038	0.97	0.0018	1.5761	3	121
Sex	14.946	0.13	2.3197	1.5761	3	121
Sex*disturbance	− 1.352	0.13	2.3238	1.5761	3	121
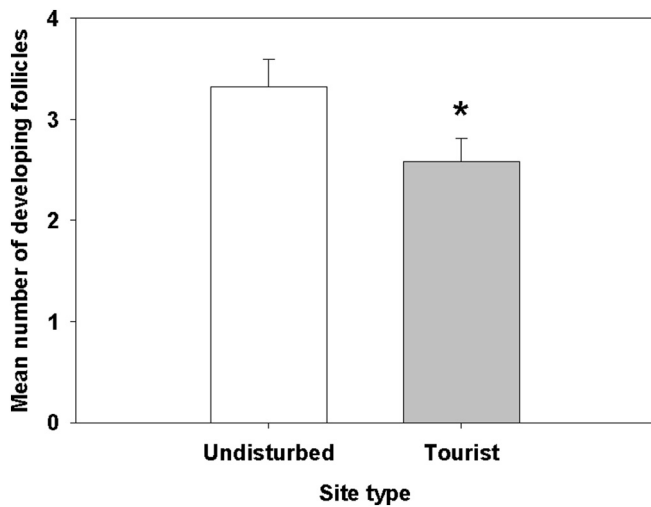
Fig. 2. Number of follicles. Mean number of developing follicles in females from sites exposed to human disturbance versus sites that are undisturbed (P = 0.04).
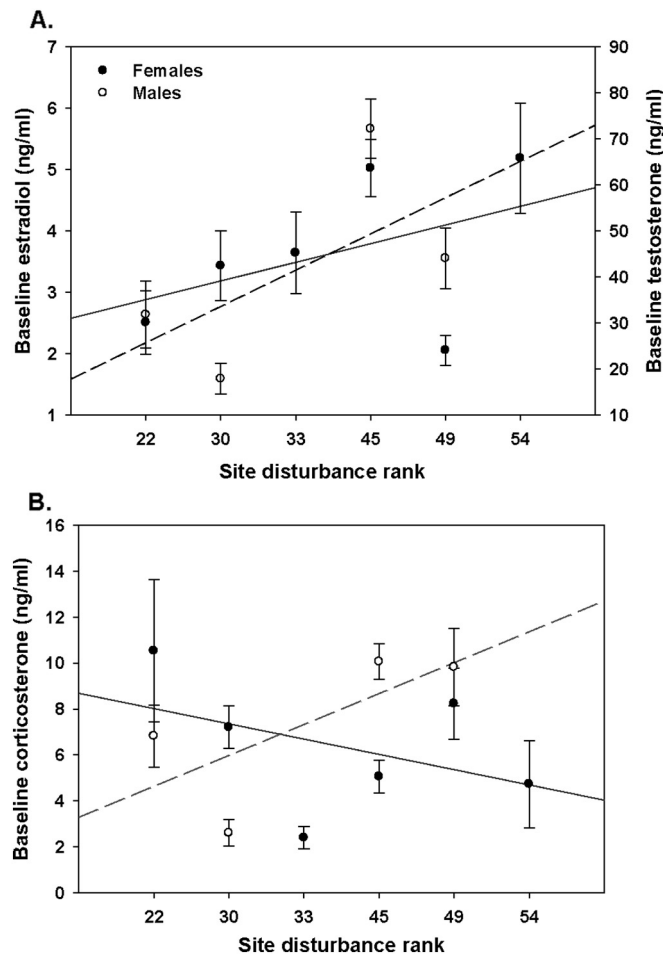

Fig. 3. Circulating hormone concentrations across sites with varying amounts of disturbance. Circulating baseline (a) corticosterone and (b) estradiol and testosterone concentrations in female (closed circles – solid line) and male (open circles – dashed line) marine iguanas from populations exposed to different amounts of human disturbance. Lines are only present for statistically significant relationships.

and parasite number at disturbed (F = 9.51, DF = 1, 23, p < 0.01; r^2 = 0.29) but not undisturbed (F = 0.03, DF = 1, 22, p = 0.86; r^2 = 0.00) sites.

We did not find a significant effect of disturbance intensity on

wound healing in females or total hemolytic complement activity in either sex (Table 3.4; Table 4). An additional regression revealed a negative relationship between complement activity and follicle number at disturbed sites (F = 9.24, DF = 1, 35, p < 0.01; r^2 = 0.21), but this relationship was absent at undisturbed sites (F = 0.49, DF = 1, 35, p = 0.49; r^2 = 0.01).

Reactive oxygen metabolites were higher with increasing levels of human disturbance in both sexes and were also significantly higher in females compared to males (Fig. 4b; Table 3.4). We obtained the same results when we analyzed oxidative stress (i.e., ratio of reactive oxygen metabolites/antioxidant barrier). We saw no significant effect of disturbance index, sex, or interaction between the two factors for antioxidant capacity alone (Table 3.4; Table 4).

4. Discussion

Overall, we found a significant relationship between the degree of human disturbance, which is inextricably tied with ecotourism in the Galápagos Islands, and indicators of endocrine, immunological, and oxidative stress, as well as condition and reproduction. While clutch size (i.e., number of developing follicles) did not directly relate to the intensity of tourism, there were still significant effects of tourism exposure (i.e., presence/absence) on reproduction irrespective of intensity, as females had more follicles in undisturbed sites than in disturbed sites.

4.1. Endocrine responses and morphology

Alterations in endocrine stress reactivity to human disturbance (i.e., tourism) have been repeatedly observed across taxa, including avian (Ellenberg et al., 2007; Müllner et al., 2004; Walker et al., 2005, 2006), mammalian (Rehnus et al., 2013), and reptilian species (French et al., 2010). Many studies find that tourist-exposed animals release more glucocorticoids (cortisol in mammals, corticosterone in reptiles and birds) in response to a stressor than animals not exposed to tourism. However, longer-term studies with repeated sampling show that these responses can be highly variable in both space and time. For example, in Magellanic penguins (*Spheniscus magellanicus*), offspring show elevated hormone responses at tourist sites while adults show reduced responses at these same sites (Walker et al., 2005, 2006). In non-breeding marine iguanas, Romero and Wikelski (2002) found a reduced corticosterone stress response in a tourist-exposed population in 1999, whereas in 2008 we found the opposite: an elevated corticosterone response at tourist-exposed (albeit different) populations of marine iguanas (French et al., 2010). However, in both 2008 and in the current study (2013) we found no significant effect of tourism on corticosterone stress reactivity during the marine iguana breeding season.

Independent of tourism, there are marked difference in immunological and endocrine responses between the sexes, and these responses were found to significantly covary with one another (Neuman-Lee and French, 2017). Consistent with the previous results, in the present study we found that the sexes differ in how their endocrine system covaries with human disturbance. For example, in the current study we found altered baseline levels of corticosterone in breeding season males and females in response to human disturbance; however, the responses were in opposing directions for the two sexes. Males had elevated corticosterone levels at sites with higher disturbance whereas females had lower levels at more disturbed sites. The disparate responses between the sexes are likely driven by intersexual differences in reproductive strategy and investment in marine iguanas (discussed below). Even though the patterns are not consistent, taken together these studies demonstrate a physiological effect of tourism with supplementary environmental factors that vary over time (e.g., El Niño) likely augmenting endocrine responses to stress.

Similarly, we found disparate morphological and condition metrics to disturbance, whereby males were larger and in better condition with

Table 4

Mean and standard error for parasite count, percent wound healed, total hemolytic complement activity, and total antioxidant barrier in females and male iguanas from populations exposed to different amounts of human disturbance.

| Disturbance rank	Sex	Parasites	SE	Wound healing	SE	Total hemolytic complement activity	SE	Total antioxidant barrier	SE
22	F	9.85	2.20	–	–	0.35	0.12	485.36	24.79
	M	32.23	22.38	–	–	1.89	1.78	409.62	20.04
30	F	5.70	0.84	42.45	4.69	0.27	0.16	447.72	24.97
	M	18.00	9.60	–	–	0.16	0.06	403.57	19.12
33	F	9.13	1.51	–	–	0.14	0.93	–	–
	M	–	–	–	–	–	–	–	–
45	F	11.50	1.78	39.44	3.21	0.12	0.04	442.00	23.49
	M	18.67	5.80	–	–	1.37	1.24	467.66	46.26
49	F	2.00	0.27	–	–	1.06	0.88	445.79	28.41
	M	3.92	1.50	–	–	0.11	0.01	421.43	21.72
54	F	1.62	0.49	–	–	0.65	0.63	–	–
	M	–	–	–	–	–	–	–	–
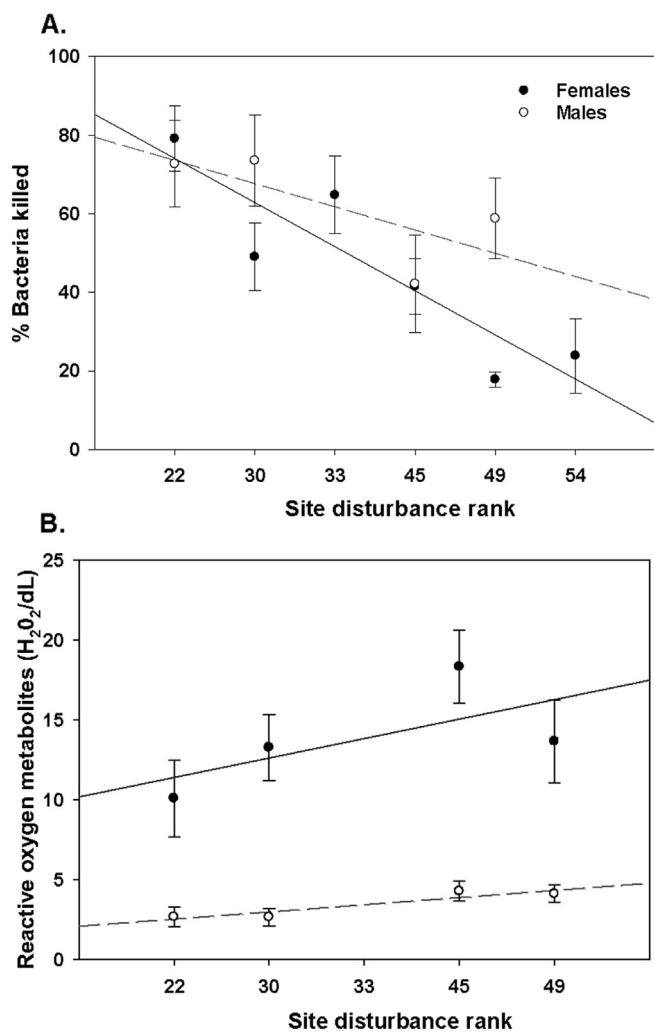
Fig. 4. Immunity and reactive oxygen metabolites. Mean (a) percent bacteria killed and (b) reactive oxygen metabolites in female (closed circles – solid line) and male (open circles – dashed line) marine iguanas from populations exposed to different amounts of human disturbance. Lines are only present for statistically significant relationships.

increasing intensity of disturbance while females were correspondingly smaller and had smaller fat stores in their tails. The male response is echoed by their testosterone levels, which also track disturbance intensity and corticosterone. This difference between the sexes may reflect different, sex-dependent intraspecific pressures during the breeding season, but the impact of morphology on reproductive success would need to be elucidated to further understand this result.

4.2. Immunity

Consistent responses were observed in immunological reactions to disturbance. We found that the ability of an animal's plasma to kill foreign bacteria was not only suppressed in populations exposed to disturbance, but that this reduction directly scaled in response to the intensity of tourism-related disturbance. This effect was observed in both male and female iguanas. This suggests that as human disturbance activities increase, the corresponding negative effects on the health of the iguana populations also increase in intensity. In males, baseline corticosterone levels are also inversely related to bacterial killing ability, suggesting that there are significant health consequences to elevated corticosterone levels, and they may act as a partial mediator of immunological changes.

As previously observed in marine iguanas, not all immune measure responded consistently to human disturbances. For example, in 2008, hemolytic complement activity (important innate immunological response found in all vertebrates) was significantly suppressed at tourist sites (French et al., 2010), but not in the current study. Instead, in the present study we found an apparent trade-off at tourist sites, where females producing more follicles had lower hemolytic complement activity, a relationship not present at undisturbed sites. There is a similar yearly discrepancy in the effects of tourism on wound healing ability, where there was a significant effect of tourism exposure on wound healing in 2008, but not in 2013. This incongruity may be due in part to a mild El Niño event in 2008, which is known to negatively affect iguana body condition through reduced food availability (Wikelski and Thom, 2000), perhaps exacerbating tourism-induced physiological effects. In support of this idea, iguana wound healing was faster and hemolytic complement activity were both greater in 2013 compared to 2008.

4.3. Oxidative stress

We found a significant response to disturbance intensity, whereby free radical expression was higher when exposed to greater levels of disturbance. Similarly, southern stingrays in the Cayman Islands exposed to tourism also experience elevated expression of free radicals (Semeniuk et al., 2009). We also found that these reactive oxygen metabolites were elevated in females relative to males, which is common in breeding animals and occurs as a response to extreme reproductive metabolic costs to females (Costantini et al., 2014a). We did not, however, find a difference in antioxidant levels across populations of marine iguanas, suggesting that tourist-exposed populations do not have any enhanced mechanism to deal with the increased free radicals and therefore are indeed experiencing greater oxidative stress.

4.4. Reproduction

Finally, and perhaps most significant for conservation purposes, we found that reproductive metrics for both males and females varied significantly according to tourism presence. Females had lower numbers of follicles at disturbed sites, which could ultimately lead to population declines in the long term. Smaller clutches and less energy mobilization may also be a contributing factor to lower circulating levels of baseline corticosterone present in females at disturbed sites. Similar reductions in reproductive success were present in yellow-eyed penguins (*Megadyptes antipodes*, Ellenberg et al., 2007) and California sea lions exposed to tourism (French et al., 2011). Juvenile California sea lions from tourist-exposed populations experienced reduced growth rates (French et al., 2011). We could not measure total clutch mass, because eggs were not fully shelled and, thus, there could still be compensatory investment in terms of egg volume in females with lower egg numbers, such that overall clutch investment in terms of energy would not differ between the populations.

In reptiles, estradiol stimulates vitellogenesis and is elevated during follicle production (Ho et al., 1982). However, we found elevated estradiol at the sites exposed to higher levels of disturbance despite these females at these sites having fewer follicles. This suggests that the endocrine signal for reproductive investment (i.e., increased estrogen) is positively responding but failing to induce normal reproductive output at disturbed locations. Although there could be differences in reproductive staging among the populations leading to the observed hormone differences, egg size measurements (unpublished data 2013, French), reproductive behaviors (personal observations, 2013), and the close proximity of the sites would suggest that the populations are fairly synchronous. Furthermore, the elevated oxidative stress levels in these populations may indicate that the elevation of the reproductive stimulus may impose increased costs to the female. Such an impact is further supported by the reduced body condition and fat stores in females at disturbed sites. The potential for a combination of greater female costs yet reduced reproductive output could be devastating to population stability and thus warrants further study to better understand the reproductive consequences of human disturbance and ways to best mitigate such effects.

Similarly, in males, baseline testosterone levels were altered such that they were significantly higher at sites with greater human disturbance, suggesting disturbance may be significantly augmenting reproductive suppression of immunity in this species. Testosterone levels were positively related to ectoparasite load and negatively related to innate immune response. Testosterone and estradiol are both known to suppress immunity in a variety of species (Al-Afaleq and Homeida, 1998; Fuxjager et al., 2011; Owen-Ashley et al., 2004a, 2004b), and the relationship between testosterone and parasite load has been demonstrated in other reptile species (Olsson et al., 2000; Pollock et al., 2012). Given that both testosterone and estradiol levels positively correlate with human disturbance, this could be a potential mechanism by which human disturbance negatively impacts immunity in breeding marine iguanas.

The downstream effects of increased testosterone and corticosterone (i.e., via increased disturbance and tourism exposure) may lead to altered behavior including territory guarding, a conspicuous behavior in this species, and thus have profound implications on reproductive behavior as a whole (DeNardo and Sinervo, 1994). For example, in marine iguanas, intense competition and energetically-expensive territory guarding during breeding can overwhelm and confound any additional outside factors (e.g., tourism or development) contributing to stress and immunosuppression (Wikelski et al., 1996). In fact, there are seasonal differences within a given year in immunological consequences of tourism depending on breeding state of the iguanas in question, such that breeding condition blurs the effects of human disturbance that are readily apparent when animals are not breeding (French et al., 2010).

At this point it is not clear what specific mechanisms mediate human disturbance-induced effects on reproduction in males versus females. However, it is well accepted that elevations in glucocorticoids in the long-term can lead to reduced reproduction (Wingfield and Kitaysky, 2002), and thus the more frequent elevations in corticosterone in response to tourist activities and development at tourist sites may lead to a decrease in clutch size in females or elevated testosterone levels in males over time.

4.5. Conclusions

This study illustrates the importance of quantifying not just the presence but the extent of tourism activities, such that all activities that provide the logistical support for tourism are included when analyzing the impacts on wild species. Together, the results demonstrate that human disturbance activities stemming from ecotourism have significant implications for the health of individual marine iguanas and possibly that of the populations. This study further indicates that both the presence and intensity of disturbance influences the resiliency of marine iguanas to human disturbance. Given that ecotourism and thus human disturbance will likely continue to increase in the Galápagos Islands, our research suggests that the amount of human disturbance at lek sites of marine iguanas should be closely monitored. Limiting the overall disturbance at lek sites year round (i.e., breeding and non-breeding seasons) could increase the overall health and fecundity of marine iguanas. Melding objectives of conservation with those of ecotourism are needed to maintain the Galápagos Islands' unique ecological systems and the economic benefits they provide. The ultimate goal of management should be to develop an effective strategy that mitigates the negative effects of human disturbance on animal health and physiology while preserving the rewards associated with ecotourism, both preservation of species and economic benefits to local populace.

References

Al-Afaleq, A.I., Homeida, A.M., 1998. Effects of low doses of oestradiol, testosterone and dihydrotestosterone on the immune response of broiler chicks. Immunopharmacol. Immunotoxicol. 20, 315–327.

Bequaert, J., 1932. On the ornate nymphs of the tick genus *Amblyomma* (Acarina: Ixodidae). Z. Parasitenk. 4, 776–783.

Blane, J.M., Jaakson, R., 1994. The impact of ecotourism boats on the St Lawrence beluga whales. Environ. Conserv. 21, 267–269.

Breuner, C.W., Delehanty, B., Boonstra, R., 2013. Evaluating stress in natural populations of vertebrates: total CORT is not good enough. Funct. Ecol. 27, 24–36.

Buckley, R., 2004. Impacts of ecotourism on birds. In: Environmental Impacts of Ecotourism. CAB International, Cambridge, pp. 187–209.

Burger, J., Gochfeld, M., 2007. Responses of emperor penguins (*Aptenodytes forsteri*) to

encounters with ecotourists while commuting to and from their breeding colony. Polar Biol. 30, 1303–1313.

Costantini, D., Casasole, G., Eens, M., 2014a. Does reproduction protect against oxidative stress? J. Exp. Biol. 217, 4237–4243.

Costantini, D., Dell'Omo, G., 2006a. Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 176, 575–579.

Costantini, D., Dell'Omo, G., 2006b. Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J. Comp. Physiol. B. 176, 575–579.

Costantini, D., Greives, T.J., Hau, M., Partecke, J., 2014b. Does urban life change blood oxidative status in birds? J. Exp. Biol. 217, 2994–2997.

DeNardo, D.F., Sinervo, B., 1994. Effects of steroid-hormone interaction on activity and home-range size of male lizards. Horm. Behav. 28, 273–287.

Dickens, M.J., Romero, L.M., 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. Gen. Comp. Endocrinol. 191, 177–189.

Ellenberg, U., Setiawan, A.N., Cree, A., Houston, D.M., Seddon, P.J., 2007. Elevated hormonal stress response and reduced reproductive output in yellow-eyed penguins exposed to unregulated tourism. Gen. Comp. Endocrinol. 152, 54–63.

Epler, B., 2007. Tourism, the Economy, Population Growth, and Conservation in Galapagos. Charles Darwin Foundation.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Flather, C.H., Cordell, H.K., 1995. Outdoor recreation: historical and anticipated trends. In: Knight, R.L., Gutzwiller, K.J. (Eds.), Wildlife and Recreationists: Coexistence Through Management and Research. Island Press, Washington DC, pp. 3–16.

Freedberg, S., Greives, T.J., Ewert, M.A., Demas, G.E., Beecher, N., Nelson, C.E., 2008. Incubation environment affects immune system development in a turtle with environmental sex determination. J. Herpetol. 42, 536–541.

French, S.S., DeNardo, D.F., Greives, T.J., Strand, C.R., Demas, G.E., 2010. Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). Horm. Behav. 58, 792–799.

French, S.S., González-Suárez, M., Young, J.A., Durham, S., Gerber, L.R., 2011. Human disturbance influences reproductive success and growth rate in California sea lions (*Zalophus californianus*). PLoS One 6, e17686.

French, S.S., Matt, K.S., Moore, M.C., 2006. The effects of stress on wound healing in male tree lizards (*Urosaurus ornatus*). Gen. Comp. Endocrinol. 145, 128–132.

French, S.S., Neuman-Lee, L.A., 2012. Improved ex vivo method for microbiocidal activity across vertebrate species. Biol. Open 1, 482–487.

Fuxjager, M.J., Foufopoulos, J., Diaz-Uriarte, R., Marler, C.A., 2011. Functionally opposing effects of testosterone on two different types of parasite: implications for the immunocompetence handicap hypothesis. Funct. Ecol. 25, 132–138.

García, L.V., 2004. Escaping the Bonferroni iron claw in ecological studies. Oikos 105, 657–663.

Gilman, C.A., Wolf, B.O., 2007. Use of portable ultrasonography as a nondestructive method for estimating reproductive effort in lizards. J. Exp. Biol. 210, 1859–1867.

Greives, T.J., McGlothlin, J.W., Jawor, J.M., Demas, G.E., Ketterson, E.D., 2006. Testosterone and innate immune function inversely covary in a wild population of breeding dark-eyed juncos (*Junco hyemalis*). Funct. Ecol. 20, 812–818.

Hines, K.N., 2011. Effects of ecotourism on endangered northern Bahamian Rock Iguanas (*Cyclura cychlura*). Herpetol. Conserv. Biol. 6, 250–259.

Ho, S.-M., Kleis, S., McPherson, R., Heisermann, G.J., Callard, I.P., 1982. Regulation of vitellogenesis in reptiles. Herpetologica 38, 40–50.

Tieleman, B. Irene, Williams, J.B., Ricklefs, R.E., Klasing, K.C., 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc. R. Soc. B Biol. Sci. 272, 1715–1720.

Janeway, C., Travers, P., Walport, M., Shlomchik, M., 2005. Immunobiology: The Immune System in Health and Disease, sixth ed. Garland Science, New York.

Knapp, C.R., Hines, K.N., Zachariah, T.T., Perez-Heydrich, C., Iverson, J.B., Buckner, S.D., Halach, S.C., Lattin, C.R., Romero, L.M., 2013. Physiological effects of tourism and associated food provisioning in an endangered iguana. Conserv. Physiol. 1, cot032.

Lucas, L.D., French, S.S., 2012. Stress-induced tradeoffs in a free-living lizard across a variable landscape: consequences for individuals and populations. PLoS One 7, e49895.

Mayer, M.M., 1948. Complement and complement fixation. In: Kabat, E.A., Mayer, M.M. (Eds.), Experimental Immunochemistry. Charles C. Thomas, Springfield, Ill, pp. 100–112.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24, 984–996.

Moore, I.T., Lemaster, M.P., Mason, R.T., 2000. Behavioural and hormonal responses to capture stress in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Anim. Behav. 59, 529–534.

Moore, M.C., 1986. Elevated testosterone levels during nonbreeding-season territoriality in a fall-breeding lizard, *Sceloporus jarrovi*. J. Comp. Physiol. A Sens. Neural Behav. Physiol. 158, 159–163.

Moore, M.C., Thompson, C.W., Marler, C.A., 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 81, 217–226.

Moran, M.D., 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos 100, 403–405.

Müllner, A., Eduard Linsenmair, K., Wikelski, M., 2004. Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). Biol. Conserv. 118, 549–558.

Neuman-Lee, L.A., French, S.S., 2014. Wound healing reduces stress-induced immune changes: Evidence for immune prioritization in the side-blotched lizard. J. Comp. Physiol. B. 184, 623–629.

Neuman-Lee, L.A., French, S.S., 2017. Endocrine-reproductive-immune interactions in female and male Galápagos marine iguanas. Horm. Behav. 88, 60–69.

Olsson, M., Wapstra, E., Madsen, T., Silverin, B., 2000. Testosterone, ticks and travels: a test of the immunocompetence-handicap hypothesis in free-ranging male sand lizards. Proc. R. Soc. Lond. B Biol. Sci. 267, 2339–2343.

Owen-Ashley, N.T., Hasselquist, D., Wingfield, J.C., 2004a. Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. Am. Nat. 164, 490–505.

Owen-Ashley, N.T., Hasselquist, D., Wingfield, J.C., 2004b. Examining the indirect pathways of testosterone-induced immunosuppression in male songbirds. I. Effects of corticosterone and estradiol on acquired immune function. Integr. Comp. Biol. 44, 616.

Pollock, N.B., Vredevoe, L.K., Taylor, E.N., 2012. The effect of exogenous testosterone on ectoparasite loads in free-ranging Western fence lizards. J. Exp. Zool. A Ecol. Genet. Physiol. 317, 447–454.

Rehnus, M., Wehrle, M., Palme, R., 2013. Mountain hares *Lepus timidus* and tourism: stress events and reactions. J. Appl. Ecol. 51, 6–12.

Reynolds, P.C., Braithwaite, D., 2001. Towards a conceptual framework for wildlife tourism. Tour. Manag. 22, 31–42.

Rice, W.R., 1989. Analyzing tables of statistical tests. Evolution 43, 223–225.

Romero, L.M., Ramenofsky, M., Wingfield, J.C., 1997. Season and migration alters the corticosterone response to capture and handling in an Arctic migrant, the white-crowned sparrow (*Zonotrichia leucophrys gambelii*). Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 116, 171–177.

Romero, L.M., Wikelski, M., 2002. Exposure to tourism reduces stress-induced corticosterone levels in Galapagos marine iguanas. Biol. Conserv. 108, 371–374.

Schatz, H., 1991. Catalogue of known species of Acari from the Galapagos Islands (Ecuador, Pacific ocean). Int. J. Acarol. 17, 213–225.

Scheyvens, R., 1999. Ecotourism and the empowerment of local communities. Tour. Manag. 20, 245–249.

Semeniuk, C.A.D., Bourgeon, S., Smith, S.L., Rothley, K.D., 2009. Hematological differences between stingrays at tourist and non-visited sites suggest physiological costs of wildlife tourism. Biol. Conserv. 142, 1818–1829.

Sinclair, J.A., Lochmiller, R.L., 2000. The winter immunoenhancement hypothesis: associations among immunity, density, and survival in prairie vole (*Microtus ochrogaster*) populations. Can. J. Zool. 78, 254–264.

Taylor, J.E., Hardner, J., Stewart, M., 2006. Ecotourism and Economic Growth in the Galapagos: An Island Economy-wide Analysis .

Van de Crommenacker, J., Horrocks, N.P.C., Versteegh, M.A., Komdeur, J., Tieleman, B.I., Matson, K.D., 2010. Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. J. Exp. Biol. 213, 3527–3535.

Vassalle, C., 2008. An easy and reliable automated method to estimate oxidative stress in the clinical setting. In: Armstrong, D. (Ed.), Advanced Protocols in Oxidative Stress I. Springer, New York, NY, pp. 31–39.

Vassalle, C., Pratali, L., Boni, C., Mercuri, A., Ndreu, R., 2008. An oxidative stress score as a combined measure of the pro-oxidant and anti-oxidant counterparts in patients with coronary artery disease. Clin. Biochem. 41, 1162–1167.

Walker, B.G., Boersma, P.D., Wingfield, J.C., 2005. Physiological and behavioral differences in Magellanic penguin chicks in undisturbed and tourist-visited locations of a colony. Conserv. Biol. 19, 1571–1577.

Walker, B.G., Boersma, P.D., Wingfield, J.C., 2006. Habituation of adult Magellanic penguins to human visitation as expressed through behavior and corticosterone secretion. Conserv. Biol. 20, 146–154.

Wikelski, M., 1999. Influences of parasites and thermoregulation on grouping tendencies in marine iguanas. Behav. Ecol. 10, 22–29.

Wikelski, M., 2005. Evolution of body size in Galapagos marine iguanas. Proc. R. Soc. B Biol. Sci. 272, 1985–1993.

Wikelski, M., Carbone, C., Trillmich, F., 1996. Lekking in marine iguanas: female grouping and male reproductive strategies. Anim. Behav. 52, 581–596.

Wikelski, M., Carrillo, V., Trillmich, F., 1997. Energy limits to body size in a grazing reptile, the Galapagos marine iguana. Ecology 78, 2204–2217.

Wikelski, M., Cooke, S.J., 2006. Conservation physiology. Trends Ecol. Evol. 21, 38–46.

Wikelski, M., Thom, C., 2000. Marine iguanas shrink to survive El Nino - changes in bone metabolism enable these adult lizards to reversibly alter their length. Nature 403, 37–38.

Wikelski, M., Wrege, P.H., 2000. Niche expansion, body size, and survival in Galápagos marine iguanas. Oecologia 124, 107–115.

Williams, R., Lusseau, D., Hammond, P.S., 2006. Estimating relative energetic costs of human disturbance to killer whales (*Orcinus orca*). Biol. Conserv. 133, 301–311.

Wingfield, J.C., Kitaysky, A.S., 2002. Endocrine responses to unpredictable environmental events: stress or anti-stress hormones? Integr. Comp. Biol. 42, 600–609.

Wingfield, J.C., O'Reilly, K.M., Astheimer, L.B., 1995. Modulation of the adrenocortical responses to acute stress in arctic birds: a possible ecological basis. Am. Zool. 35, 285–294.