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High human presence is correlated with lower faecal glucocorticoid metabolite levels in an urban bird population

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ABSTRACT

Urban wildlife must cope with diverse challenges and stressors, including human presence. However, in addition to being a disturbance, humans can provide energy-rich food and protection from predators. We evaluated the impact of human presence on red-winged starlings (*Onychognathus morio*) in a highly urbanised environment using faecal glucocorticoid metabolite (fGCM) concentrations as a stress-related biomarker. We performed an adrenocorticotropic hormone (ACTH) challenge to validate a non-invasive method for quantifying glucocorticoids in red-winged starlings. Using this method, we quantified fGCMs in excreta collected from free-living starlings during weekdays (high human presence) and weekends (low human presence) to determine the birds' responses to fluctuating human numbers. Following the ACTH challenge, starlings' circulating glucocorticoid (GC) concentrations increased by 127 % within 30 min and the corresponding fGCM concentrations increased within 1 h of injection. Of the four enzyme immunoassays (EIA) tested, an 11-oxoaetiocholanolone EIA, performed best, detecting a 310 % increase in fGCM concentrations post-ACTH challenge and suggested a 1-h lag between injection and peak fGCM excretion in this species. Human foot-traffic was significantly higher on weekdays compared to weekends, yet free-living red-winged starlings showed overall 30.4 % lower fGCM concentrations on weekdays compared to weekends. Red-winged starlings consume a higher proportion of anthropogenic food on weekdays than weekends and we cannot rule out the possibility that diet-related alteration in gut passage time affect fGCM concentrations. However, the correlation between fGCMs and human foot traffic may also suggest urban red-winged starlings benefit from human presence. Our results raise the possibility that, under certain conditions, the benefits associated with human presence outweigh potential negative effects associated with human activity, at least during the non-breeding season.

Summary statement

Validation and application of an enzyme immunoassay shows that high human foot traffic is associated with lower faecal glucocorticoid metabolite (fGCM) concentrations in a free-ranging urban bird: the red-winged starling.

1. Introduction

Urbanization is one of the main drivers of global anthropogenic environmental change (Rebolo-Ifrán et al., 2015; Parris, 2016). Given that 68 % of the world's human population is expected to be living in urban areas by the year 2050 (United Nations, 2018), it is important to understand how increasing urbanization will affect wildlife. Whereas urbanization is known to affect the behavior, physiology, and health of wildlife (Meillère et al., 2015; Murray et al., 2019; Sumasgutner et al., 2023), the mechanisms underlying these responses remain largely unknown (Bonier, 2012; Meillère et al., 2015). One of the novel factors wildlife must deal with in urban habitats is high human presence (Rebolo-Ifrán et al., 2015). While humans may be considered a predator

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and therefore their presence a stressor by a wide range of species (Rebolo-Ifrán et al., 2015; Scheun et al., 2015; Majelantle et al., 2020), not all human activities might be challenging to wildlife. For example, humans are often associated with reliable resources, such as food and water that may improve body condition, reduce predation risk associated with the search for natural food items and lower baseline glucocorticoid levels due to this high resource availability (Møller, 2012; Murray et al., 2019). High human presence may also deter natural predators, creating a 'safer' environment (e.g. Shannon et al., 2014). As patterns of wildlife responses to urbanization remain unclear (Bonier, 2012, 2023), it is important to understand how individuals perceive their environment to assess the extent to which urbanization acts as a challenge or an opportunity (Rebolo-Ifrán et al., 2015).

The hypothalamic-pituitary-adrenal (HPA) axis moderates, among others, behavioural and physiological responses to environmental change (Ricklefs and Wikelski, 2002; Deviche et al., 2023). Therefore, the endocrine mechanisms associated with the HPA axis are highly relevant to whether species are likely to persist in urban environments (Deviche et al., 2023). When faced with stressful stimuli, vertebrates mount a neuro-endocrine response that often culminates in, inter alia, increased release of glucocorticoids (GCs) to cope with the challenge (Wingfield et al., 1998; Touma and Palme, 2005; Palme, 2019). Baseline concentrations of GCs are associated with a wide range of physiological, developmental and behavioural activities in vertebrates (Wingfield and Kitaysky, 2002; MacDougall-Shackleton et al., 2019). These concentrations are known to fluctuate with season and life history stage based on the energetic requirements of the individual (Romero, 2002). Whereas elevated GCs may result in phenotypic changes that enable individuals to survive better in their environment (Wingfield, 2003; Badyaev, 2005), long-term stress-induced elevations may have detrimental effects on immune function, reproduction, and growth (Wingfield et al., 1998; Romero, 2004). Thus, quantifying GCs can be useful for monitoring the fitness and health status of individuals and populations (Romero, 2004; Wikelski and Cooke, 2006). However, traditional methods of quantifying GCs using blood sampling are invasive and can cause stress in the focal animal (Goymann, 2005; Touma and Palme, 2005; Palme, 2019). The metabolism of circulating GCs in the liver to be excreted as part of faeces and urine (Touma and Palme, 2005; Sheriff et al., 2011) makes it possible to use minimally invasive methods that do not require the handling of sampled individuals. The quantification of faecal glucocorticoid metabolites (fGCMs) as a biomarker for neuro-endocrine activity has become a popular approach in recent years (Touma and Palme, 2005; Palme, 2019).

The metabolism, conjugation and excretion of GCs can vary both intra- and interspecifically (Goymann, 2005; Touma and Palme, 2005), resulting in differences in the compositions and proportions of GC metabolites (Goymann, 2005; Touma and Palme, 2005). Additionally, fGCM concentrations represent an accumulation of metabolized GCs over a species-specific lag period, influenced in part by gut passage time (Möstl and Palme, 2002; Touma and Palme, 2005; Palme, 2019). Thus, the methods for quantifying fGCMs require a reliable validation prior to first use for each species (Möstl et al., 2005; Jepsen et al., 2019; Palme, 2019). These validation methods may be physiological and/or biological in nature (reviewed by Palme, 2019). Physiological validations involve feeding or injecting individuals with adrenocorticotropic hormone (ACTH) to pharmacologically induce elevations in circulating GCs, whereas biological validations involve exposure to an assumed stressful event (Goymann, 2005; Jepsen et al., 2019; Ngcamphalala et al., 2023). In both physiological and biological validations, the aim is to establish whether the resulting alterations in circulating GC concentrations are reflected in alterations in related fGCM concentrations, and the post-stressor lag time (Touma and Palme, 2005; Palme, 2019; Ngcamphalala et al., 2023).

In this study, we evaluated how the red-winged starling (*Onychognathus morio*), an avian urban exploiter, responds to fluctuating numbers of humans in an urban setting. Red-winged starlings are known to thrive in human-dominated landscapes including highly urbanised city centres (Craig, 2005; du Plessis, 2005), where they exploit energy-rich anthropogenic food sources (Stofberg et al., 2019; Risi et al., 2021). Red-winged starlings also display behavioural flexibility in urban environments, increasing the proportion of anthropogenic food in their diets during periods of high human numbers (e.g. weekdays in urban centres), but switching diet to naturally available food sources during quieter periods (e.g. weekends; Stofberg et al., 2019, Catto et al., 2021). Food availability has been shown to affect circulating GC concentrations, for example, in urban white ibises (*Eudocimus albus*), baseline GC concentrations were lowest in the post-breeding season when they consumed more anthropogenic food (Murray et al., 2018; Cummings et al., 2020) and in Florida scrub-jays (*Aphelocoma coerulescens*) the provision/supplementation of food in suburban birds resulted in reduced GCs (Schoech et al., 2007; Schoech et al., 2008). This suggests that consuming anthropogenic food may alleviate the challenges associated with foraging and decrease GC secretion (Cummings et al., 2020). Therefore, the presence of humans in high numbers within the starlings' environment could elevate circulating GCs (corticosterone in birds) if they perceive humans as a threat or reduce GC concentrations if the birds benefit from human presence due to anthropogenic food discards or protection from natural predators.

We collected faecal samples from free-living red-winged starlings at the University of Cape Town's Upper Campus on weekdays and weekends to quantify variations in fGCM concentrations in response to fluctuating human numbers. In this study we set out to test the prediction that fGCM concentrations would be lower during periods of higher human presence. We made this prediction because the population of red-winged starlings involved in this study heavily utilizes anthropogenic food sources when available i.e., anthropogenic food can make up to 92.3 % of their diet on weekdays compared to 46.3 % on weekends (Stofberg et al., 2019), and because human presence may deter predators (Shannon et al., 2014). As a prerequisite for the use of fGCM quantification in the species, we conducted a physiological validation test on red-winged starlings to 1) identify a suitable enzyme immunoassay for quantifying fGCMs in the species, and 2) validate whether elevations in circulating GC levels are reflected in fGCM levels and assess lag time.

2. Materials and methods

2.1. Study site

This study was carried out on the red-winged starling *Onychognathus morio* (hereafter 'starling') population resident on the University of Cape Town's (UCT) Upper Campus (33°57′31.5" S, 18°27′36.4″E), and in the neighbouring suburb of Mowbray (33°0.94′65.4" S, 18°0.47′01.8" S), Western Cape, South Africa. The study area is located on the lower eastern slopes of Table Mountain and adjacent flat land and is highly urbanised. The landscape comprises the main M3 highway into the Cape Town CBD from the south and mixed high-rise residential and commercial properties including a high density of concrete buildings and sealed surfaces. The Mowbray site was used for the capture of starlings for the adrenocorticotropic hormone (ACTH) challenge (see below), and the UCT Upper Campus site for collection of faecal samples from free-living starling individuals. The UCT Upper Campus site is characterized by high human density (students and staff of the University) during weekdays and low human density on weekends (see Stofberg et al. (2019) for confirmation that human presence fluctuates in a predictable weekly cycle, and Risi et al. (2021) for confirmation that human presence is associated with anthropogenic food supply).

2.2. Study species

Red-winged starlings are common, medium-sized omnivorous birds, distributed from Ethiopia in East Africa to the Cape in Southern Africa

(Craig, 2005). They are sexually dichromatic: females have a grey head and males a glossy black head; both sexes have glossy blue-black body plumage and the eponymous red flight feathers. Immature birds have a dull black head, and sex cannot be determined from plumage characteristics for the first year (Fry and Keith, 2020). Non-breeding birds form large flocks, while breeding pairs defend nest sites during summer. Their natural diet consists mainly of fruits, seeds, nectar and arthropods and they nest on natural cliffs, but also anthropogenic structures like building ledges (Craig, 2005). At UCT, high human presence on campus during the week is strongly associated with increased availability of anthropogenetic food discards, which are ~5 times higher on weekdays (3.5 g per 100 m^2) than weekends (0.7 g per 100 m^2; Risi et al., 2021). The UCT red-winged starling population relies heavily on this anthropogenic food source, with 80–99 % of the weekday diet anthropogenic in origin (Stofberg et al., 2019; Catto et al., 2021). Red-winged starlings respond to anthropogenic food scarcity on low human presence days (e.g. weekends, vacations) by switching to naturally occurring food sources such as nectar and fruit of plants found in gardens around residency buildings, small and large arthropods, and geckos, but this results in reduced body mass gain (Stofberg et al., 2019; Bates et al., 2021; Catto et al., 2021). The UCT population of red-winged starlings is habituated to close human proximity due to high human presence on campus during weekdays in term time. The birds will approach humans closely (especially if they have food) and many individuals tolerate proximity of people <1 m. Red-winged starlings have been colour-ringed for research on the UCT Upper Campus since 2017, and the population contains ~200 colour-ringed individuals (for details of capture and colour-ringing, see Stofberg et al., 2019).

2.3. Adrenocorticotropic hormone (ACTH) challenge

2.3.1. Capture and housing

Ten red-winged starlings (4 females and 6 males, ranging in body mass from 110 to 161 g, mean 134 ± 4 g) were caught using baited spring traps in Mowbray in June 2021. The starlings were caught in the morning (08 h00 – 10 h00), to allow 8 h of captivity for the adrenocorticotropic hormone (ACTH) challenge test (see below) and safe release before nightfall. Once caught, each individual bird was removed immediately from the trap and placed in a soft cloth bird bag sewn from dark fabric for transportation to the research station. The starlings were transferred to individual plastic mesh cages (0.4 m × 0.5 m × 0.7 m) with a sliding wooden floor for ease of collection of faecal samples. Each cage was equipped with perches, food, and water dishes and food and water were provided ad libitum. Starlings were held for a minimum of 7 h and a maximum of 8 h.

2.3.2. ACTH administration, faecal sampling and blood sample collection

Faecal samples were collected at hourly intervals from the cage floor for 3 h. The birds were then captured, and each bird received an intramuscular injection to the pectoral muscle containing approximately 2 IU/kg Synacthen® Depot (Novartis) mixed with saline solution, to initiate an increase in GC production. Following injection, birds were returned to the cages and faecal samples were collected from the cage floor hourly for a further 4 h to quantify ACTH-induced changes in faecal glucocorticoid metabolite (fGCM) concentrations. Hourly collection of faecal samples was done to ensure that changes in fGCM concentrations could be tracked through time (as per Palme, 2019). Samples were frozen immediately after collection to avoid further bacterial alterations in fGCM composition.

Two blood samples (maximum 200 μL each) were taken from each bird, the first immediately before and the second 30 min after the ACTH injection was administered. Blood was collected within two minutes of removal of the bird from the cage, and chilled at ~4 °C to allow clotting and centrifuged to separate serum from red blood cells within 4 h of collection.

2.3.3. Serum glucocorticoid analysis

To determine serum glucocorticoid concentrations, all serum samples were analysed using an enzyme-immunoassay (EIA), utilizing antibodies against corticosterone-3-CMO: BSA (Palme and Möstl, 1997). For analyses, untreated sera were diluted between 1:500 and 1:10000 and the sensitivity for the Corticosterone EIA was 80 $pgmL^{-1}$. Assay details including components, and antibody cross-reactivities are provided by Palme and Möstl (1997).

2.3.4. Steroid extraction and fGCM quantification

Forty faecal samples from ten birds were available for fGCM quantification. The frozen faecal samples were lyophilized for 3 days and pulverized before adding 1.5 mL 80 % ethanol to each faecal powder sample weighing between 0.037 and 0.055 g, 1 mL ethanol was added to samples weighing between 0.02 and 0.037 g and 0.5 mL ethanol was added to samples weighing >0.01 g to facilitate steroid extraction as described by Ganswindt et al. (2002). Four different enzyme immunoassays (EIAs): (i) corticosterone, (ii) 5α-pregnane- 3β,11β,21-triol-20-one (measuring 3β,11β-diol-cortisol metabolites), (iii) tetrahydrocorticosterone (5β-pregnane-3α,11β,21-triol-20-one), and (iv) 11-oxoetiocholanolone (detecting fGCMs with a 5β-3α-ol-11-one structure), were tested for their suitability to detect fGCM concentrations in red-winged starling excreta, using a subset of 24 faecal samples from five birds. Assay characteristics, including antibody cross-reactivity, have been provided by Palme and Möstl (1997) for the corticosterone EIA, by Touma et al. (2003) for the 5α-pregnane3β, 11β, 21-triol-20-one EIA, by Quillfeldt and Möstl (2003) for the tetrahydrocorticosterone EIA, and by Möstl et al. (2002) for the 11-oxoetiocholanalone EIA.

Inter- and intra-assay coefficients of variation (CV), determined by repeated measurements of high- and low-quality controls, and sensitivities (i.e., smallest change detected by assay) of each assay are provided in Table 1. Additionally, a parallelism test was conducted for the 11-oxoaetiocholanolone EIA i.e., serial dilutions of faecal extracts gave a displacement curve that was parallel to the respective standard curve (relative variation of the slope of respective trend lines <4 %).

Once the best performing assay was identified the EIA was used to analyse the remaining 16 ACTH challenge faecal samples and those collected from free-ranging birds on UCT's upper campus ($n = 56$, see below).

2.4. Red-winged starling fGCMs in relation to human presence

2.4.1. Faecal sample collection

Red-winged starling faecal samples were collected between March and May 2021 (austral autumn, non-breeding season) from the colour-ringed urban red-winged starling population comprising free-living individuals resident on UCT Upper Campus. Samples were collected on weekdays ($n = 27$ samples, female = 13, male = 11, immature = 3) and weekends ($n = 29$ samples, female = 6, male = 13, immature = 10) to ensure wide variation in numbers of people on campus. Each day, we visited a different randomly-selected location on Upper Campus to avoid introducing sampling bias. Droppings were collected by closely observing starlings encountered at the specified location for periods of

Table 1
Coefficients of variance (%) and sensitivity of EIAs (ng/g) used to quantify faecal glucocorticoid metabolite (fGCM) concentrations in a subset of the red-winged starling ACTH faecal samples ($n = 24$) using four different fGCM enzyme immunoassays (EIAs).

EIAs	Intra-assay variance (%)	Inter-assay variance (%)	Sensitivities of EIAs (ng/g)
Corticosterone	4.57 & 5.74	8.32 & 10.57	2.4
5α-pregnane3β, 11β, 21-triol-20-one	3.15 & 3.61	4.09 & 8.09	2.4
Tetrahydrocorticosterone	3.36 & 4.38	7.36 & 8.52	9.6
11-oxoetiocholanolone	4.94 & 6.84	11.45 & 12.97	0.75

≤15 min until they produced excreta. These were immediately transferred within seconds after defecation into a plastic Eppendorf tube. Samples were frozen at −20 °C within 3 h of collection.

2.4.2. Human presence data collection

Human presence was measured using a metric of 'foot traffic', the number of people passing fixed counting points per hour. This was estimated by counting the number of people that entered a designated 20 m radius at three sites, one each in the northern, central and southern parts of campus. People were counted simultaneously at each of the 3 sites with the help of 3 volunteers for intervals of 15 min in the morning (08 h00 - 08 h15), afternoon (12 h00 - 12 h15) and evening (15 h45 -16 h00). These counts were used as an indication of the number of people (count h^{-1}) on campus throughout the day on weekdays and weekends.

2.5. Statistical analyses

All analyses were conducted in R 3.4.3 (2016, R Foundation for Statistical Computing, Vienna, Austria). *P*-values <0.05 were taken as statistically significant.

2.5.1. Adrenocorticotropic hormone (ACTH) challenge

2.5.1.1. Serum corticosterone analysis. A two-tailed paired *t*-test was used to determine the differences in circulating serum glucocorticoid concentrations pre- and post-ACTH injection. Pairs plots were constructed using the ggplot () function (ggplot2 version 3.4.4; Wickham et al., 2016), to illustrate the difference in glucocorticoid concentration between paired pre- and post-injection readings.

2.5.1.2. Faecal GCM analysis. The best performing enzyme immunoassay (EIA) was determined using data from the subsample of 24 excreta (from 5 birds), analysed with the four different candidate EIAs. Data for each EIA were compared by a) calculating the percent increase in fGCMs at each time lag relative to the baseline faecal samples at −2 h pre-injection (the first set of faecal samples collected) for each EIA and b) making boxplots of the fGCM concentrations in the samples detected by each EIA at each time lag. The best-performing EIA was determined on the following criteria: (1) that it detected the highest increase in fGCMs post-injection compared to the baseline, (2) that it presented the clearest profile of change in fGCMs pre- and post-injection (increasing fGCMs post-injection and a decline after a distinct peak), on visual examination of the boxplots and (3) that it had the highest sensitivity i.e., could detect the smallest changes in fGCM concentrations per g of faecal powder.

A linear mixed effect model with Gaussian error distribution (package nlme version 3.1–162; Pinheiro et al., 2013) was fitted to the full set of faecal extracts analysed using the best performing assay data to establish the time lag at which peak fGCMs were detected post ACTH injection. The model included fGCM concentration as the response variable, time lag, sex (male or female) and mass as explanatory variables and bird identity as a random factor. Model residuals were checked to ensure the assumption of normality was met. Tukey post hoc tests were conducted, using package emmeans (version 1.8.5; Russell, 2018) to test for significant differences in fGCMs between each time lag.

2.5.2. Red-winged starling fGCMs in relation to human presence

Foot traffic data were modelled as a function of day status (weekday versus weekend) using a two-tailed *t*-test, to confirm that human presence on campus was significantly related to day status during the period of the study.

Data on fGCM concentrations in the faecal samples collected on UCT upper campus were log-transformed for normality and fitted with a linear model with Gaussian error distribution including day status and sex (male, female or immature – immature birds cannot be sexed based on plumage characters before the first year) as predictor variables.

3. Results

3.1. Adrenocorticotropic hormone (ACTH) challenge

3.1.1. Serum analysis

In one of the 10 red-winged starlings, both pre- and post-injection circulating GC concentrations were > 6-fold higher than the mean GC concentrations of the other nine birds. When this outlier individual was excluded from analyses, serum glucocorticoid concentrations (GCs) averaged 315.1 ng mL^{-1} (range = 105.26–563.23 ng mL^{-1}) in pre-injection samples, and 716.5 ng mL^{-1} (range = 336.20–1113.0 ng mL^{-1}) in post-injection samples, a 127.4 % increase (Fig. 1; paired Wilcoxon test: v = 0, $p = 0.003$) following the ACTH injection.

Serum glucocorticoid concentrations for the outlier individual were 2611 ng mL^{-1} before and 4393.8 ng mL^{-1} after the ACTH injection, and retention of this bird in the dataset gave a similar result (198.9 % increase in plasma GCs, paired Wilcoxon test: v = 0, $p = 0.001$).

3.1.2. Enzyme immunoassays (EIA) selection

In a subset of $n = 24$ faecal samples from 5 birds, red-winged starlings showed a mean post ACTH-injection increase in fGCM concentration of 134 % using the corticosterone EIA, whereas the 5α-pregnane-3β, 11β, 21-triol-20-one EIA revealed an increase of 170 %, and the tetrahydrocortiocosterone and 11-oxoaetiocholanolone EIAs showed overall increases of 258 % and 310 % respectively (Fig. 2).

The full set of 40 faecal samples (from all 10 birds) were analysed for fGCM concentrations using 11-oxoaetiocholanolone EIA. One clear outlier at 1 h pre-injection (Time − 1) was subsequently removed from analysis, because it was 7 x higher than the mean of all other samples at this time lag, > 4 x higher than the mean of all samples across all time lags, and > 15 x higher than the mean of all other samples from this individual bird, suggesting an error may have occurred during sample processing.

Maximum fGCM concentrations occurred 1 h post-injection ($n = 39$ faecal samples from 10 birds). Peak fGCM concentrations at 1 h following ACTH administration were significantly higher than values at −2 h, −1 h and 0 h prior to the injection (Tukey post-hoc pairwise

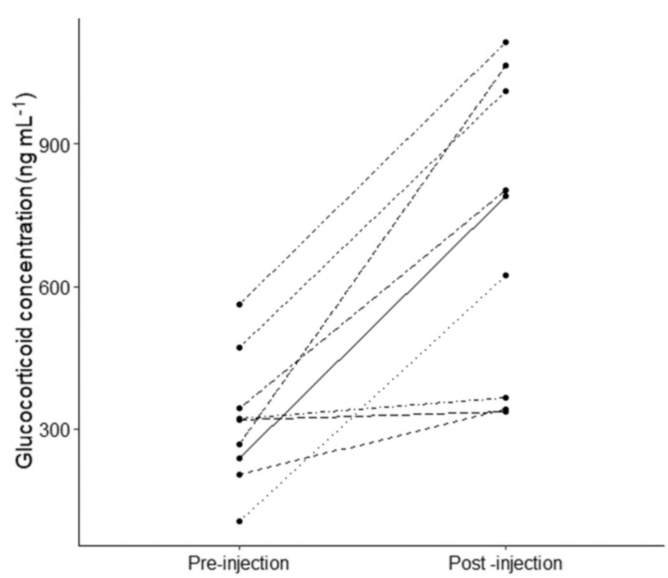

Fig. 1. ***Pairs plot showing an increase in serum glucocorticoid concentrations post -injection with an adrenocorticotropic hormone containing chemical (Synacthen®), compared to pre-injection,*** *in nine urban red-winged starlings (Onychognathus morio) captured in Cape Town, South Africa. Different line styles indicate different individual birds.*

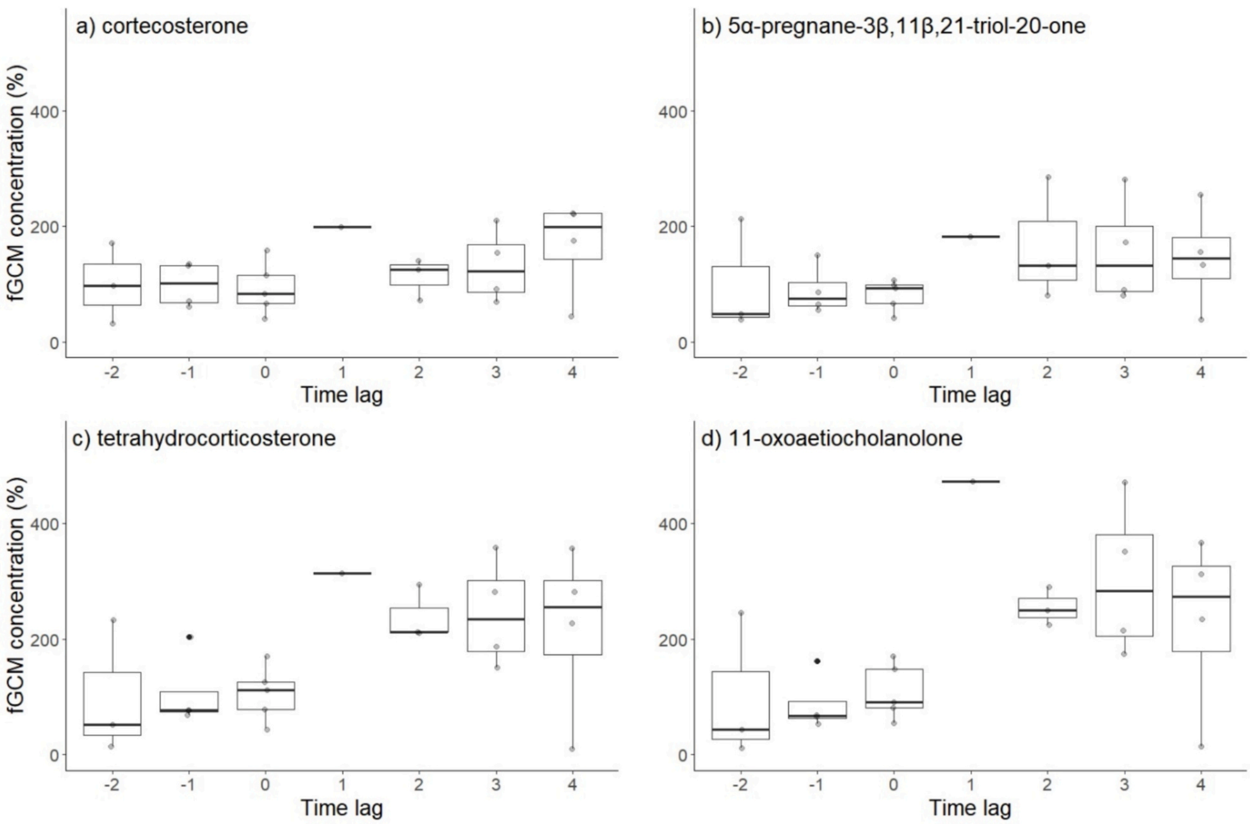

Fig. 2. ***Faecal glucocorticoid metabolite (fGCM) concentrations in urban Red-winged Starlings (Onychognathus morio) over time (hr) during the period pre- and post- adrenocorticotropic hormone injection,*** *when evaluated using (a) corticosterone EIA, (b) 5 α-Pregnane-3β, 11β, 21-triol-20-one EIA, (c) tetrahydrocorticosterone EIA, and (d) 11-oxoaetiocholanolone II EIA. fGCM concentrations are presented as a percentage (%) of the mean fGCM concentration recorded at baseline time lag 0, light grey dots represent raw data.*

comparisons of percentage increase in fGCM concentrations: −2 h to 1 h, 429 % higher, $t = 4.20$, $p = 0.005$; −1 h to 1 h, 414 % higher, $t = 3.71$, $p = 0.02$; 0 h to 1 h, 398 % higher, $t = 3.92$, $p = 0.01$; Table 2, Fig. 3). There was no significant difference in fGCM concentrations at 2 h, 3 h and 4 h post-injection compared to pre-injection values or to each other (Tukey post-hoc pairwise comparisons all $p > 0.05$; Fig. 3). Neither sex nor body mass influenced fGCM concentrations (Table 2).

Table 2
Model outputs from a linear mixed-effects model of the difference in red-winged starling (Onychognathus morio) faecal glucocorticoid metabolite (fGCM) concentration with time lag (Time, in hours) after the adrenocorticotropic hormone (ACTH) injection, sex and mass (g) as explanatory variables, and percentage increase in fGCMs concentrations relative to Time − 2 h as the response variable. Bird identity was included as a random factor. Model outputs indicate significant increase in fGCM concentrations post ACTH injection at Time 0, relative to Time − 2. Baseline values are Time − 2, and Sex female. Significant effects (p < 0.05) are highlighted in bold. N = 39 samples from 10 birds.

Fixed effects	Estimate	Standard error	Degrees of freedom	Test statistic	p-value
Intercept	*−628.00*	*552.56*	*23*	*−1.13*	*0.267*
Time − 1	14.83	88.04	23	0.17	0.868
Time 0	30.29	81.02	23	0.37	0.712
Time 1	**428.71**	**101.98**	**23**	**4.20**	**< 0.001**
Time 2	**298.05**	**101.11**	**23**	**2.95**	**0.007**
Time 3	**276.96**	**93.36**	**23**	**2.97**	**0.007**
Time 4	**297.50**	**99.28**	**23**	**3.00**	**0.006**
Sex ‘male’	−178.81	127.57	7	−1.40	0.204
Mass (g)	6.16	4.50	7	1.37	0.213

3.2. *Red-winged starling fGCMs in relation to human presence*

Human ‘foot traffic’ i.e., the number of people passing fixed counting points per hour on the University of Cape Town’s Upper Campus was significantly higher on weekdays ($\bar{x} = 503.6 \pm 60.4$ people h^{-1}, $n = 3$ days) than weekends ($\bar{x} = 40.7 \pm 8.4$ people h^{-1}, n = 3 days), ($t = 7.58$, df = 2.08, p - value = 0.015).

Free-living red-winged starlings on Upper Campus had lower fGCM concentrations on weekdays (back-transformed model estimate: $\bar{x}$ = 0.039 ng/g DW, 95 % CI: 0.031–0.049, $n = 27$) than weekends (0.056 ng/g DW, 95 % CI: 0.046–0.069, $n = 29$; Table 3, Fig. 4). Sex (female, male or immature) did not correlate with fGCM concentrations.

4. Discussion

Red-winged starlings’ faecal glucocorticoid metabolite (fGCM) concentrations were negatively correlated with human presence on the University of Cape Town’s Upper Campus. This suggests that these urban birds may experience reduced stress on weekdays when human foot traffic is higher, and provides a proof of the concept that fGCMs may be a useful matrix for estimating changes in circulating glucocorticoids (GCs) in response to ecological changes, in this species. Our results are consistent with our prediction that high availability of anthropogenic food associated with high human presence, or refuge from natural predators provided by human proximity, might reduce GC levels in this population. Consistent with previous studies on other bird species (e.g. Goymann et al., 2002; Dehnhard et al., 2003; Bouwer et al., 2021; Jepsen et al., 2019), the starlings responded to an ACTH challenge by increasing blood serum GC (corticosterone) levels (by 127.4 %). This increase in blood serum GCs was reflected in an increase in fGCMs

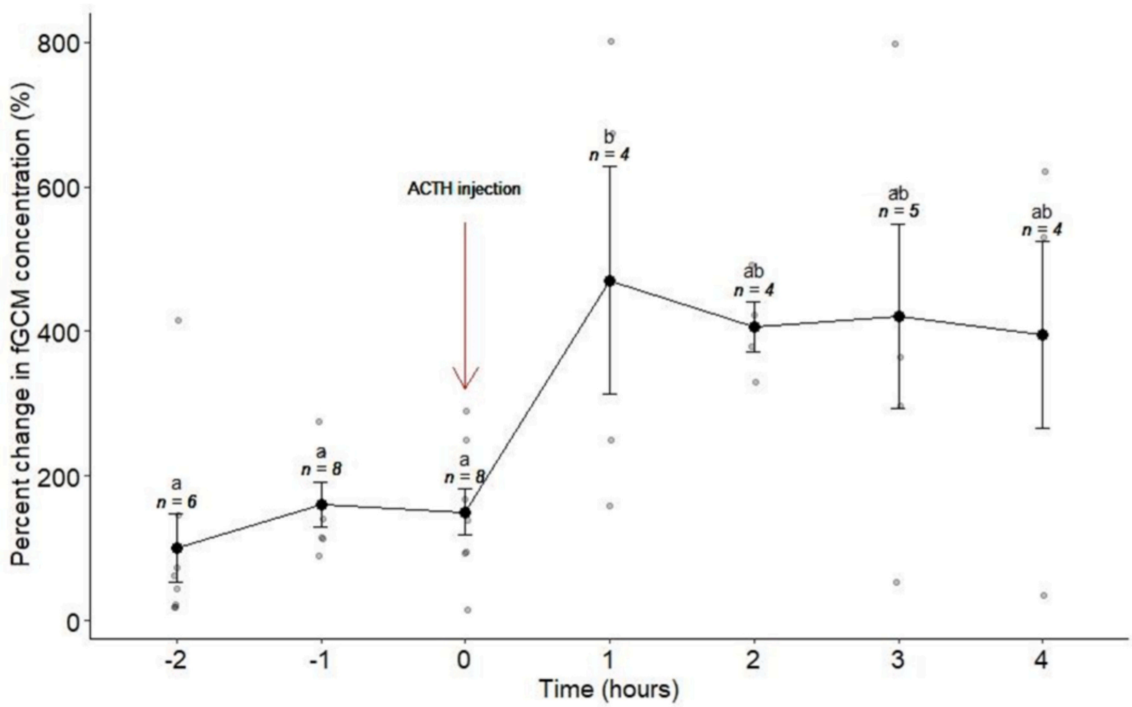

Fig. 3. ***Percent change in faecal glucocorticoid metabolite (fGCM) concentrations of 10 urban red-winged starlings (Onychognathus morio) before and after ACTH injection (at Time 0), evaluated using 11-oxoaetiocholanolone II EIA.*** *Grey points = raw data values, black points = mean values at each time interval, error bars = 1 standard error. Letters indicate significant differences at a 95 % level ($p < 0.05$) from a pairwise post-hoc Tukey test.*

Table 3

Model outputs from a linear model of the relationship between faecal glucocorticoid metabolites (fGCMs) of free-living red-winged starlings (Onychognathus morio) on the University of Cape Town's Upper Campus, and day status (weekday versus weekend; a factor significantly correlated with human presence on campus). 'Weekend' is set as the baseline for the 'day status' variable. The model was fitted to log-transformed fGCM data, and also included the fixed factor 'sex' (female, male, immature) with 'female' set as the baseline. Significant effects ($p < 0.05$) are highlighted in bold. Model outputs indicate lower fGCM concentrations on weekdays than weekends.

Model 1: log (fGCM) concentrations on weekdays ($n = 27$) versus weekends ($n = 29$)				
	Estimate	Standard error	t-value	p-value
Intercept	**−2.72**	**0.16**	**−16.66**	**>0.001**
Day status 'weekday'	**−0.37**	**0.15**	**−2.40**	**0.020**
Sex 'immature'	−0.21	0.20	−1.02	0.311
Sex 'male'	−0.26	0.17	−1.54	0.131

within 1 h after ACTH injection, confirming that faecal samples are suitable for monitoring GC levels non-invasively in this species.

Free-living birds and other wildlife generally display elevations in GCs associated with human disturbance (e.g. Creel et al., 2002, 2013; Casas et al., 2016; Coppes et al., 2018; Strasser and Heath, 2013), even in habituated individuals (e.g. in primates, Muehlenbein et al., 2012; Shutt et al., 2014). However, this response is not ubiquitous (Pérez-Ortega and Hendry, 2023). For example, work in penguins has shown that habituation can lead to attenuation of the stress response to human presence (e.g. Fowler, 1999), and African penguin (*Spheniscus demersus*) chicks regularly exposed to high human presence may even have lower fGCMs than those experiencing little to no human presence (Scheun et al., 2021). Similarly, two chough species (alpine and red-billed chough *Pyrrhocorax graculus* and *P. pyrrhocorax*), in habitats exposed to tourists had lower fGCMs than conspecifics in non-tourist habitats (Jiménez et al., 2011). Whereas some bird species show increased or no variation in baseline GCs between urban- and rural-dwelling individuals (Fokidis et al., 2009; Zhang et al., 2011; Atwell et al., 2012), several

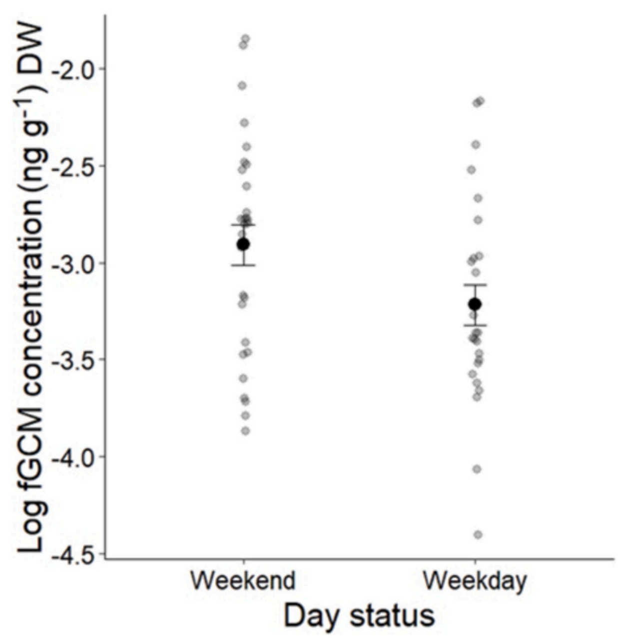

Fig. 4. ***Faecal glucocorticoid metabolite concentrations (fGCM, ng/g DW) in free-living red-winged starlings (Onychognathus morio) on the University of Cape Town's Upper Campus were lower on weekdays ($n = 27$) than weekends ($n = 29$).*** *Black filled circles indicate mean fGCM concentrations, and error bars indicate 1 standard error above and below the mean. Grey points show raw data.*

studies have shown lower baseline GC and fGCM concentrations in urban populations compared to rural conspecifics (Schoech et al., 2004, 2007; Fokidis et al., 2009; Cummings et al., 2020). This is often attributed to increased foraging efficiency associated with reduced predation risk (Rolando et al., 2001; Jiménez et al., 2011) and reduced GC secretion in response to high food availability (Schoech et al., 2007), but may also be associated with habituation (Møller, 2008; Atwell et al., 2012; Pérez-Ortega and Hendry, 2023). High human densities on UCT's Upper Campus throughout much of the year have resulted in high levels of habituation in our red-winged starling study population, with many starlings tolerating human presence at <1 m (S.J. Cunningham, P. Sumasgutner, C.A. Ngcamphalala and K.B. Nhlapo's pers. obs.). These high levels of habituation may result in individuals downregulating their physiological responses to human presence (Fowler, 1999; Partecke et al., 2006; Møller, 2008), allowing other factors associated with high human foot traffic in this urban environment to become more prominent drivers of GC concentrations than the humans themselves.

There are several potential explanations for the negative relationship between human presence and fGCM concentrations in our study population. First, elevation of circulating GC levels is a common mechanism used by birds when food availability is reduced, to maintain energy stores and promote self-maintenance (Kitaysky et al., 2007; Jenni-Eiermann et al., 2008). The abrupt decrease of anthropogenic food availability on weekends (Risi et al., 2021) could therefore explain the elevated fGCM concentrations at times of low human foot traffic. Second, the observed relationship between human presence and fGCM concentrations could arise from the presence of large numbers of people on campus deterring starlings' natural predators, including peregrine falcons (*Falco peregrinus*; Jenkins and Avery, 1999) and black sparrowhawks (*Accipiter melanoleucus*; Suri et al., 2017), reducing the starlings' perceived predation risk (Møller, 2012). Reduced predation pressure in association with high human densities has been reported elsewhere, including lower predation risk for urban compared to rural bird populations in Europe, North America and Australia (Eötvös et al., 2018; Noske et al., 2008, but see Valcarcel and Fernández-Juricic, 2009). Evidence that wildlife might perceive proximity to humans as providing a refuge from natural predators also comes from studies of wildlife in more natural settings (Shannon et al., 2014). The potential for humans to reduce perceived predation risk for starlings in our study population could be assessed by a) evaluating whether activity of natural predators like falcons and sparrowhawks on the UCT campus varies with human presence, and b) quantifying perceived predation risk through starling vigilance and alarm calling behavior.

Our study population of red-winged starlings relies heavily on anthropogenic food sources during the week (80–99 % of their diet) but increase the proportion of natural food sources in their diet when anthropogenic food is scarce (Stofberg et al., 2019; Catto et al., 2021; Bates et al., 2021). There is also some evidence that diet itself may influence fGCM concentrations in the excreta of birds and mammals (Goymann, 2005; Dantzer et al., 2011). For example, gull-billed tern chicks (*Gelochelidon nilotica*) fed fish had higher fGCM concentrations and total daily excreted metabolites than insect-fed chicks (Albano et al., 2015). Additionally, an increase in dietary fibre resulted in reduced testosterone and glucocorticoid metabolites in European stonechats (*Saxicola rubicola*); likely due to differences in excreta volume (rather than deposition rate of hormone metabolites in excreta; Goymann, 2005). Thus, we cannot rule out the possibility that the negative relationship between human presence and fGCM concentrations we observed is simply a result of differences in the red-winged starling diet with varied human traffic, especially given that anthropogenic food has lower nutritional value and dietary fibre than the insects, fruits and seeds of the natural diet (Isaksson and Andersson, 2007; Murray et al., 2015; Giraudeau et al., 2018).

One of the limitations of our study is that different individuals were sampled during weekends and weekdays which could result in sampling bias due to individual personality traits. Given that circulating GCs vary widely among individuals (Cockrem, 2013; Guindre-Parker, 2020) and boldness is often associated with low GC concentrations in birds (Cockrem, 2013), it is possible that the birds sampled during weekdays and high human presence days were bolder than individuals sampled during weekends. However, we think this is unlikely, as the birds in our study population are habituated to human presence (Risi et al., 2021) and habituated birds often do not perceive humans as a threat, evident as shorter flight initiation distances and lower circulating GCs (Vincze et al., 2016; Tablado et al., 2021). Furthermore, assessment of our long-term resightings database of colour-ringed starlings on campus shows >85 % overlap in the individuals encountered on weekdays and weekends (among birds seen at least twice, S.J. Cunningham, P. Sumasgutner, *unpubl. data*) suggesting it is unlikely that most shier individuals avoid campus on weekdays. Finally, two adult male colour-ringed starlings in our study were sampled both on LHP and HHP days and both had higher fGCM concentrations on the LHP days.

All four of the tested enzyme immunoassays (EIAs) have been validated and used for fGCM analysis in previous studies on several bird species. The corticosterone EIA has been validated and used for quantifying fGCMs in greylag geese (*Anser anser*; Kotrschal et al., 1998; Frigerio et al., 2001), ravens (*Corvus corax*; Stöwe et al., 2008) as well as in white-backed munia (*Lonchura striata*) and its domesticated strain, the Bengalese finch (*Lonchura striata* var. *domestica*; Suzuki et al., 2012). Whereas the 5α-pregnane-3β,11β,21-triol-20-one EIA was established to measure fGCMs in mice, utilizing antibodies against metabolites of corticosterone (Touma et al., 2003), it emerged as the most suitable EIA for measuring fGCMs in the laughing doves (*Spilopelia senegalensis*; Ngcamphalala et al., 2023). Furthermore, the tetrahydrocorticosterone EIA has been successfully used for monitoring adrenocortical activity in a wide range in species including Adelie penguins (*Pygoscelis adeliae*; Nakagawa et al., 2003), Wilson's storm petrels (*Oceanites oceanicus*; Quillfeldt and Möstl, 2003), greylag geese (*A. anser*; Scheiber et al., 2015), southern pied babblers (*T. bicolor*; Jepsen et al., 2019) and southern yellow-billed hornbills (*Tockus leucomelas*; Bouwer et al., 2021). The 11-oxoaetiocholanolone EIA has been shown to reliably detect fGCM concentrations in southern pied babblers (*Turdoides bicolor*, Jepsen et al., 2019), upland geese (*Chloephaga picta*, Koch et al., 2009) and little auks (*Alle alle*, Kidawa et al., 2014). In the study, this 11-oxoaetiocholanolone EIA was the most sensitive and measured the highest fGCM concentrations in the red-winged starlings.

Faecal GCM concentrations were highest 1 h post-ACTH injection and remained elevated until the end of the experiment (4 h post injection). Peak fGCM concentrations were detected earlier in this species compared to other small-to-medium sized wild bird species like laughing doves (*Spilopelia capensis*), southern yellow-billed hornbills (*Tockus leucomelas*), or European stonechats (*Saxicola torquata rubicola*), which show a peak in fGCM concentrations after 2 h, 3 h and 2 to 4 h respectively (Goymann et al., 2002; Bouwer et al., 2021; Ngcamphalala et al., 2023). However, like the starlings in this study, all these species had higher than baseline fGCM concentrations between 1 h and 4 h post injection. The differences in the lag time associated with peak fGCM concentrations may be due to the species-specific nature of GC metabolism (Goymann, 2005; Touma and Palme, 2005), differences in diet (Karasov and Levey, 1990) or the amount of food present in the gut (Afik and Karasov, 1995).

5. Conclusion

Our small sample size precludes us from making generalized conclusions on the correlation between human presence and fGCMs in urban birds. However, the starlings in this study, appear to benefit from high human presence on campus, as increased human presence correlates with lower fGCM levels. Previous research on this population has shown that high human presence correlates with increased daily body mass gain (e.g. Catto et al., 2021) and may allow starlings to maintain higher body condition (e.g. known colour-marked individuals were consistently lighter during the strict covid-19 lockdowns in 2020 compared to other years; Bates et al., 2021). The correlation between human presence and fGCMs may be driven by the anthropogenic food scraps people discard on campus, but also the protection from predation that human presence provides. However, differences in food type/

sources (anthropogenic vs natural) associated with day status (Stofberg et al., 2019; Catto et al., 2021) cannot be ruled out as a confounding reason behind our observations. Future studies should aim to disentangle the mechanisms driving this association, assess whether this finding is repeatable in other study populations and species, and provide insights into how urban spaces can be made more welcoming for wildlife.

CRediT authorship contribution statement

Kagiso B. Nhlapo: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Susan J. Cunningham:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Petra Sumasgutner:** Writing – review & editing, Resources, Methodology. **Andre Ganswindt:** Writing – review & editing, Resources, Methodology, Conceptualization. **Andrew E. McKechnie:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Celiwe A. Ngcamphalala:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The datasets generated and analysed during the study titled "High human presence is correlated with lower faecal glucocorticoid metabolite levels in an urban bird population." are available as electronic material on UCT's ZivaHub: https://doi.org/10.25375/uct.28643384.v1.

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