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SHORT COMMUNICATION

Hot days are associated with short-term adrenocortical responses in a southern African arid-zone passerine bird

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ABSTRACT

Relatively little effort has been directed towards elucidating the role of physiological stress pathways in mediating avian responses to global heating. For free-ranging southern pied babblers, *Turdoides bicolor*, daily maximum air temperatures (T_{max}) between ~35 and ~40°C result in reduced foraging efficiency, loss of body mass and compromised breeding success. We tested the hypothesis that very hot days are experienced as stressors by quantifying relationships between T_{max} and faecal glucocorticoid metabolite (fGCM) levels in naturally excreted droppings. On days when T_{max}<38°C, fGCM levels were independent of T_{max} (mean±s.d. 140.25±56.92 ng g^{-1} dry mass). At T_{max}>38°C, however, fGCM levels increased linearly with T_{max} and averaged 190.79±70.13 ng g^{-1} dry mass. The effects of T_{max} on fGCM levels did not carry over to the following morning, suggesting that very hot days are experienced as acute stressors.

KEY WORDS: Air temperature, Desert, Faecal glucocorticoid metabolites, Fitness costs, Heat stress

INTRODUCTION

Stress responses mediated by the endocrine system are a vital component of animals' reactions to environmental perturbations (Sheriff et al., 2011), spanning physiological and behavioural changes (Agarwal and Prabakaran, 2005; Stott, 1981) that contribute to an animal's ability to respond appropriately to environmental change and therefore its likelihood of survival (Asres and Amha, 2014; Jensen et al., 2019). Acute stress is associated with the rapid, transient release of glucocorticoids (GCs), often to levels far above baseline, in response to a specific stimulus (Beiko et al., 2004). These short-term elevations can be beneficial, supporting immune responses and mobilizing energy reserves,

which may, for example, allow an individual to escape a potentially dangerous situation such as attempted predation (Buchanan, 2000).

High environmental temperatures can be experienced as a stressor (de Bruijn and Romero, 2011; Jessop et al., 2016; Krause et al., 2016; Ruuskanen et al., 2020; Xie et al., 2017), with temperature increases usually associated with elevations in circulating GC concentrations (de Bruijn and Romero, 2018). Avian adrenocortical responses to periods of increased environmental temperature often, but not always, involve increases in circulating corticosterone (de Bruijn and Romero, 2018). For instance, plasma corticosterone concentrations in Sonoran Desert birds were not elevated during summer compared with winter, even during a year when summer temperatures were higher than normal (Wingfield et al., 1992). Among three Australian arid-zone species, one exhibited elevated corticosterone levels after acute heat exposure under laboratory conditions, whereas two others did not (Xie et al., 2017). Avian adrenocortical responses to heat exposure have been examined under laboratory conditions in several domestic and wild species (reviewed by de Bruijn and Romero, 2018), but investigations of the effects of periods of higher temperatures among free-ranging wild birds experiencing natural weather fluctuations are rare (Fairhurst et al., 2012; Viblanc et al., 2014).

Birds inhabiting habitats characterized by high summer air temperatures trade off heat dissipation behaviour (e.g. panting and/or inactivity in shaded microsites) and foraging (Cunningham et al., 2015; du Plessis et al., 2012; Edwards et al., 2015; van de Ven et al., 2019). Reduced foraging effort and efficiency on very hot days are associated with body mass (M_b) loss (du Plessis et al., 2012; Sharpe et al., 2019; van de Ven et al., 2019), reduced breeding success (Bourne et al., 2020b; DuRant et al., 2019; Salaberria et al., 2014; Sharpe et al., 2019; van de Ven et al., 2020; Wiley and Ridley, 2016) and reduced survival of adults (Bourne et al., 2020c; Sharpe et al., 2019). These fitness costs associated with high air temperatures raise the possibility that very hot days are perceived by birds as stressors, a possibility that we evaluate here.

We hypothesized that high maximum daily air temperatures (T_{max}), which are associated with compromised foraging and net 24 h mass loss (du Plessis et al., 2012) as well as failed reproduction (Bourne et al., 2020b) in southern pied babblers, *Turdoides bicolor* (hereafter, babblers), act as a stressor for this species. Babblers are known to lose mass over 24 h periods and reduce provisioning to nestlings when T_{max}≥35.5°C (du Plessis et al., 2012; Wiley and Ridley, 2016) and are unable to breed successfully at average T_{max}≥38°C (Bourne et al., 2020b). We therefore predicted temperatures in the mid- to high-30°C range would trigger an acute stress response, indicated by elevated GC secretion.

We examined the relationship between temperature and faecal glucocorticoid metabolite (fGCM) concentrations in free-ranging babblers in the Kalahari Desert of southern Africa. The concentration of fGCMs has proved useful for quantifying

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physiological responses to stressors among free-ranging animals (Crino et al., 2017; Dantzer et al., 2010; Ganswindt et al., 2010; Sheriff et al., 2011). Once an animal perceives a stressor, GC concentrations start to increase above individual baseline levels (de Bruijn and Romero, 2018). Increases in fGCMs in response to a specific stressor can be further influenced by a variety of factors, such as food and water intake, bacterial alteration of the hormone matrix and sample exposure to the environment (Goymann and Wingfield, 2004; Kalliokoski et al., 2015; Wilkening et al., 2016). Although increasing in popularity among researchers, non-invasive sampling of fGCMs via the collection of droppings (especially from free-ranging birds) has been used less frequently than in studies of mammals and reptiles (Palme, 2019).

To evaluate the time scale of elevations in fGCM levels and thus whether extremely hot days represent an acute stressor, we investigated whether T_{max} predicted fGCM levels during and immediately after the hottest part of the day, or those of the following morning when conditions were cooler. We reasoned that if an extremely hot day triggers an acute stress response, T_{max} would influence babblers' fGCM levels during that afternoon but not the following morning.

MATERIALS AND METHODS
Study site
The study was conducted at Kuruman River Reserve (KRR), located in the southern Kalahari Desert, 28 km west of Van Zylsrus town in the Northern Cape Province, South Africa (26°58′S, 21°49′E). T_{max} recorded during summer at KRR had a mean (±s.d.) of 34.2±0.9°C between 2005 and 2019 (range of means across years: 32.4–36.5°C), with mean annual rainfall of 185.4±86.2 mm (range: 64.4–352.1 mm; Bourne et al., 2020a). The 33 km² reserve is flat to undulating, with a sandy substrate supporting semi-arid savannah vegetation (Mucina and Rutherford, 2006).

Study species
Southern pied babblers are medium-sized (60–90 g) cooperatively breeding passerines. Wild babblers at KRR are habituated to the presence of human observers, permitting observation from distances of 1–5 m (Ridley and Raihani, 2007) and facilitating the collection of droppings shortly after excretion (Bourne et al., 2019). During the study, babbler group sizes ranged from 2 to 8 adults (individuals aged ≥12 months; Ridley and Raihani, 2007). Each group has a dominant female and male, with the remaining individuals being subordinates (Nelson-Flower et al., 2011). Babblers have high reproductive skew, with 95% of young produced by the dominant pair (Nelson-Flower et al., 2011). Babblers are highly vocal and primarily terrestrial foragers that inhabit open woodlands; these are characteristics that make them easy to observe for research purposes (Ridley, 2016). The birds are ringed with metal and coloured rings for individual identification, making it straightforward to assign each faecal sample to an individual. All experimental procedures were approved by the Animal Ethics Committee of the University of Pretoria (protocol NAS359/2019), the Research Ethics and Scientific Committee of the South African National Biodiversity Institute (protocol P19-22) and the University of Cape Town's Science Faculty Animal Ethics Committee (protocol 2016/v6/SC).

Data collection
Droppings were collected by following the birds and sampling excreta within 1 min of defecation by a known individual, with the droppings immediately transferred to a screw-cap, plastic

Eppendorf tube sealed with parafilm following Bourne et al. (2019). Each babbler group was visited weekly during the breeding season and monitoring visits lasted up to 90 min, sufficient for identification of individuals and collection of droppings. Faecal samples were collected throughout the day during the austral summer breeding season of November 2018 to February 2019 (n=898 samples in total). Samples were frozen at −18°C within 1.81 ±1.07 h of collection (mean±s.d.). Samples were collected from approximately 71 individual pied babblers in 18 groups, including dominant and subordinate adults of both sexes. Weather data were obtained from a weather station onsite (Vantage Pro2, Davis Instruments, Hayward, CA, USA), which recorded air temperature (°C), wind speed (m s⁻¹), rainfall (mm) and solar radiation (W m⁻²) at 10 min intervals throughout the study period (van de Ven et al., 2019).

fGCM analysis
A subset of 228 samples were selected for analysis, comprising all samples collected between 15:05 h and 19:42 h (afternoon samples, n=114) and an additional 114 samples collected between 05:10 h and 08:58 h (morning samples). Using afternoon samples collected between 15:05 h and 19:42 h allowed sufficient time between exposure to a temperature stressor during the hottest time of the day and detecting a measurable response in fGCM levels in babblers ~2 h following increases in circulating corticosterone levels (Jepsen et al., 2019). Afternoon samples were collected on days with T_{max} varying between 28 and 41°C and from individuals from various group sizes (2–8 adults), sexes (n=43 males, n=63 females, n=8 unknown sex), ranks (n=52 dominant, n=62 subordinate) and breeding stages (n=45 from breeding groups, 69 from non-breeding groups). The 114 morning samples represented the same maximum temperature range of 28–41°C on the previous afternoon ($T_{max,prev}$) and were similarly distributed across group sizes (3–8 adults), sex (n=58 males, n=56 females), rank (n=52 dominant, n=62 subordinate) and breeding stage (n=45 breeding, n=69 non-breeding). The subset of morning samples was randomly selected within the categories breeding stage, group size, sex and rank. Individuals from pairs (group size=2 adults) were excluded from statistical analysis because the samples were too few (n=1). Morning and afternoon samples were not paired.

Frozen faecal samples were lyophilized, pulverized and sieved to remove undigested material before adding 1.5 ml of 80% ethanol in distilled water to 0.050–0.055 g of faecal powder and vortexing for 15 min to facilitate steroid extraction (Ganswindt et al., 2002). After centrifuging the samples for 10 min, the supernatants were transferred into microcentrifuge tubes and stored at −20°C (Jepsen et al., 2019). Immunoreactive fGCMs were quantified using an enzyme immunoassay (EIA) utilizing an antibody against 5β-pregnane-3,11β,21-triol-20-one-CMO:BSA (tetrahydrocorticosterone). Characteristics of the EIA including cross-reactivities are given in Quillfeldt and Möstl (2003). This EIA was validated for the reliable quantification of fGCMs in pied babblers by Jepsen et al. (2019). Intra-assay coefficients of variation (CV), determined by repeated measures of high- and low-quality controls, were 6.33% and 6.64%, while respective inter-assay CVs were 13.26% and 13.62%, respectively. The sensitivity of the EIA was 9 ng g⁻¹ faecal dry mass.

Statistical analyses
R version 3.6.3 (http://www.R-project.org/) was used for all analyses and model fits were checked using Q–Q plots and histogram residuals. The variance inflation factor (VIF) was used to assess collinearity between numeric variables (Harrison et al.,

2018), with all VIF<2. Collinearity between categorical and numeric predictor variables was tested using analysis of variance (ANOVA). In the afternoon samples, sex and group size were associated ($F_{1,99}$=5.920, P=0.017) and rank and group size were also associated ($F_{1,105}$=4.203, P=0.043), with more subordinate birds and specifically more females than males in the group sizes of 4–8 individuals. Group size and breeding stage were associated in the morning samples ($F_{1,101}$=5.098, P=0.026), with larger groups more likely to be breeding (see also Bourne et al., 2020a; Ridley, 2016). Correlation between categorical predictors was checked using a chi-square test and no pairs of categorical variables were correlated. Correlated predictors were not included in the same additive models (Harrison et al., 2018). Sample sizes reflect complete datasets after removing records containing any missing values.

The effects of T_{max} (for afternoon samples), $T_{max,prev}$ (for morning and afternoon samples), breeding stage, group size, rank, sex and the interaction between rank and sex on fGCM levels were tested in linear mixed-effects models (LMMs) fitted using the lme4 package (Bates et al., 2014; Harrison et al., 2018). Group identity was included as a random term. The inclusion of individual identity as a random term in addition to group identity resulted in unstable models and of the two random terms, group identity explained the greatest proportion of variation while avoiding destabilizing the models (Grueber et al., 2011; Harrison et al., 2018). Effects of both T_{max} and $T_{max,prev}$ were tested in an additive model for afternoon samples to explore the importance of acute (same-day T_{max}) responses. The interaction between rank and sex was included to confirm the association found by Jepsen et al. (2019) using a smaller dataset collected in a different year, whereby dominant male babblers exhibited higher fGCM levels. The three continuous explanatory variables were rescaled by centring and standardizing by the mean of the variables for model comparisons (Harrison et al., 2018). Model terms with confidence intervals (CIs) not intersecting zero were considered to explain significant patterns in our data (Grueber et al., 2011). Models were compared using Akaike's
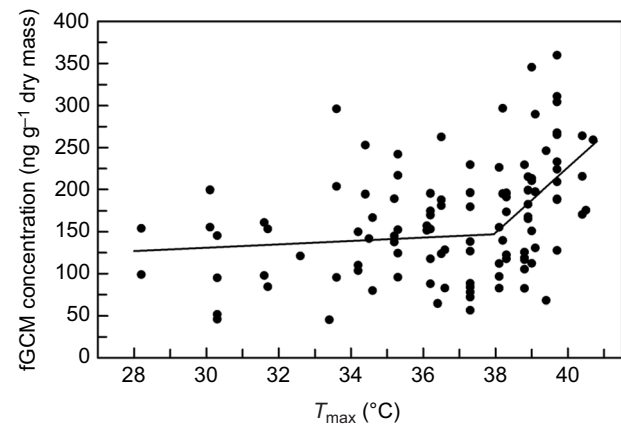

Fig. 1. Relationship between afternoon faecal glucocorticoid metabolite (fGCM) levels in southern pied babblers (*Turdoides bicolor*) and daily maximum air temperature (T_{max}) in the Kalahari Desert. An inflection occurred at T_{max}=38.0°C (95% confidence interval: 36.876, 39.122). Data represent 101 samples collected from 56 individual southern pied babblers from 18 groups, including dominant and subordinate adults of both sexes.

information criterion (AIC) with the *MuMIn* package (https:// CRAN.R-project.org/package=MuMIn; Harrison et al., 2018).

When visual inspection of the data suggested a non-linear response, we supplemented the above LMM analyses with a segmented linear regression using the R package *segmented* (Muggeo, 2008). Segmented regression can be used to identify temperature thresholds ('breakpoints') above which fGCM levels begin to increase. The effect of T_{max} was separately analysed above and below the identified breakpoints, including group as a random term. Pearson product moment correlations were used to test the relationship between fGCM concentration and the time required to freeze samples (Jepsen et al., 2019).

RESULTS AND DISCUSSION

Among afternoon samples, T_{max} significantly predicted fGCM levels, with higher fGCM levels on hotter days (Table 1, Fig. 1). The single best-fit model explaining variation in fGCM levels had a model weight of 0.856 (Table 1) and included both T_{max} and $T_{max,prev}$. We identified a breakpoint at T_{max}=38.0°C (Fig. 1). There was no effect of T_{max}<38.0°C on fGCM levels (Estimate 3.438 ±2.987, 95% CI −2.533 to 9.278), but fGCM levels increased linearly with increasing T_{max}>38.0°C (Estimate 35.14±13.88, 95% CI 8.285 to 64.050). We found no significant effect of group size, breeding stage, rank, sex or the interaction between rank and sex on afternoon fGCM levels (Table 1). For morning samples, there was no significant relationship between fGCM levels and any of the potential predictors (Table 2). The single best-fit model explaining variation in fGCM levels had a model weight of 0.990 and contained sex, rank and the interaction between the two. Average fGCM levels were higher in dominant females than in other individuals, but the difference was not statistically significant. There was no correlation between fGCM concentration and time required for freezing samples for morning (r=0.100) or afternoon samples (r=0.026).

Our results reveal that very hot days are associated with elevated fGCM levels indicative of acute heat stress in babblers. Specifically, we found a significant effect of T_{max} on fGCM levels in afternoon samples, but no effect on fGCM levels in samples collected the following day, revealing that high T_{max} triggers an acute response.

Table 1. Model outputs for analyses of afternoon faecal glucocorticoid metabolite levels in southern pied babblers (*Turdoides bicolor*)

Model	AICc	ΔAICc	w_i
T_{max}+$T_{max,prev}$	1110.1	0.00	0.856
T_{max}	1114.3	4.17	0.107
Rank×Sex	1116.5	6.35	0.036
$T_{max,prev}$	1123.6	13.48	0.001
Sex	1128.0	17.91	0.000
Breeding stage	1128.2	18.05	0.000
Group size	1128.3	18.20	0.000
Rank	1128.5	18.38	0.000
Null model	1133.6	23.52	0.000

Effect size of explanatory terms in the top model	Estimate	s.e.	95% CI
Intercept	165.266	9.554	146.349, 184.618
T_{max}	**28.197**	**8.975**	**10.592, 45.629**
$T_{max,prev}$	−3.715	8.906	−21.053, 13.657

Data are from 101 dropping samples collected in the afternoon from 56 individuals in 18 social groups. Estimate, s.e. and confidence interval (CI) are shown for the top model, with bold indicating significant terms. Null models are shown for comparison with top model sets. Group identity was included as a random term. AIC, Akaike's information criterion; AICc, Akaike's information criterion with correction for small sample sizes; w_i, Akaike weight; T_{max}, daily maximum air temperature; $T_{max,prev}$, daily maximum air temperature of the previous day.

Table 2. Model outputs for analyses of morning faecal glucocorticoid metabolite levels in southern pied babblers (*Turdoides bicolor*)

Model	AICc	ΔAICc	w_i
Rank×Sex	1043.4	0.00	0.990
Rank	1054.6	11.23	0.004
Sex	1055.6	12.29	0.002
Breeding stage	1056.1	12.79	0.002
$T_{max,prev}$	1056.4	13.00	0.001
Group size	1157.6	14.21	0.001
Null model	1060.2	16.81	0.000

Effect size of explanatory terms in the top model	Estimate	s.e.	95% CI
Intercept	199.532	9.228	101.633, 137.431
Rank (rank=subordinate)	−21.200	11.836	−44.158, 1.757
Sex (sex=male)	−18.110	12.175	−41.725, 5.506
Rank×Sex	18.423	16.472	−13.525, 50.371

Data are from 104 dropping samples collected in the morning from 61 individuals in 18 social groups, with all samples collected before 09:00 h. Estimate, s.e. and CIs are shown for the top model; there were no significant terms. Null models shown for comparison with top model sets. Group identity was included as a random term.

The T_{max} inflection of 38°C, above which fGCMs increased linearly, is similar to thresholds related to the effects of T_{max} on M_b and breeding success in the same study population. du Plessis et al. (2012) found that $T_{max} \geq 35.5°C$ has the potential to compromise the maintenance of M_b. Babblers typically lose ~4% of M_b overnight, with reduced foraging success on hot days, creating conditions under which they may fail to regain those losses on days with $T_{max} \geq 35.5°C$, resulting in progressive loss of condition during periods of sustained hot weather (du Plessis et al., 2012). Wiley and Ridley (2016) reported reduced provisioning to babbler nestlings when $T_{max} \geq 35.5°C$ and Bourne et al. (2020b), using 15 years of life history data, found that no chicks survived to fledging when mean T_{max} during the nestling period exceeded 38°C. The rapid increases in fGCM levels at $T_{max} > 38°C$ we document here thus support our hypothesis that hot days, known to incur fitness costs in this species, are experienced as stressors by the babblers.

Thermal thresholds above which fitness costs are incurred have been documented in several species, including a significant decline in foraging effort at air temperatures >27°C in western Australian magpies, *Cracticus tibicen dorsalis* (Edwards et al., 2015), cessation of foraging, provisioning of nestlings or even incubating at air temperatures >38°C in jacky winters, *Microeca fascinans* (Sharpe et al., 2019; see also Bayter, 2019), zero diurnal M_b gain at $T_{max} > 38.4°C$ in southern yellow-billed hornbills, *Tockus leucomelas* (van de Ven et al., 2019), and reductions in tarsus length and extended nestling periods associated with $T_{max} > 37°C$ and $T_{max} > 35°C$, respectively, in common fiscals, *Lanius collaris* (Cunningham et al., 2013). The existence of threshold temperatures similar to those of babblers in most of the species investigated to date raises the possibility that temperature-related sublethal fitness costs may be associated with physiological stress responses among birds more generally, a possibility with far-reaching implications for understanding the role of physiological stress pathways in avian responses to hot weather under current and future climates.

One potential limitation of our study is that delays between collecting and freezing the samples could affect measurements of fGCM levels. Exposure to biotic and abiotic elements has influenced measured fCGM levels in some other studies (Lafferty et al., 2019). Measured fGCM levels may also be influenced by environmental factors; for example, ambient temperature and humidity between excretion and freezing of the sample can affect bacterial metabolism, potentially resulting in alteration of fGCM levels (Palme et al., 2013; Terio et al., 2002; Washburn and Millspaugh, 2002). However, we did not find any significant influence of the period between collection and freezing on fGCM levels in this study (see also Jepsen et al., 2019). By collecting droppings after they were naturally excreted and after the bird had moved away of its own accord (Bourne et al., 2019; Jepsen et al., 2019), we entirely avoided handling the study animals and thereby eliminated the potentially confounding effect of capture and handling stress (Harper and Austad, 2000; Millspaugh et al., 2001; Touma and Palme, 2005). We are therefore confident that the change detected in fGCM levels measured in samples from free-living babblers represents a biologically meaningful response to a natural stressor, high T_{max}.

The increases in fGCMs in free-ranging babblers at $T_{max} > 38°C$ we report here suggest the hypothalamic–pituitary–adrenal (HPA) axis could be involved in mediating avian responses to periods of very hot weather over time scales of hours. Moreover, as nest failures in this population also occur at $T_{max} > 38°C$ (Bourne et al., 2020b), our results raise the possibility that increases in circulating GCs – and thus fGCMs – have the potential to serve as indicators of conditions under which birds incur fitness costs related to survival and/or reproduction. Further work is needed to establish whether temperature-dependent increases in GCs and fGCMs corresponding with threshold values for fitness costs are a general feature of birds inhabiting hot environments. If so, non-invasive hormone monitoring over a range of T_{max} might provide the basis for rapid assessment of species' relative sensitivity to sublethal fitness costs, which have the potential to drive substantial declines in arid-zone birds in coming decades on account of rapid anthropogenic global heating (Conradie et al., 2019). Quantifying heat stress could also prove a useful tool for managers and conservationists seeking to evaluate the effect of management interventions such as supplementary feeding or the provision of artificial water or shade, particularly for threatened, range-restricted species in hot climates. Although our study involved just one species at one study site, the grave threat posed to desert birds by climate change (Albright et al., 2017; Conradie et al., 2019; 2020; Riddell et al., 2021; Riddell et al., 2019) underscores the urgent need to better understand the role of endocrine stress pathways in avian responses to hot weather.

Author contributions
Conceptualization: A.R.B., S.J.C., A.R.R., A.E.M.; Methodology: L.L.M., A.R.B., C.A.N., A.G.; Formal analysis: L.L.M., A.R.B.; Investigation: L.L.M., A.R.B.; Resources: S.J.C., A.E.M.; Data curation: L.L.M., A.R.B.; Writing - original draft: L.L.M.; Writing - review & editing: A.R.B., S.J.C., R.J., C.A.N., A.G., A.R.R., A.E.M.;

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