

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

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### Abstract

Non-invasive methods for investigating the biological effects of environmental variables are invaluable for understanding potential impacts of climate change on behavioural and physiological stress responses of free-ranging animals. Foraging efficiency, body mass maintenance and breeding success are compromised in Southern pied babblers *Turdoides bicolor* exposed to air temperatures between ~35°C and ~40°C. We tested the hypothesis that these very hot days are acute stressors for free-ranging babblers by quantifying the relationship between daily maximum air temperature ( $T_{\max}$ ) and faecal glucocorticoid metabolite (fGCM) levels. We collected naturally-excreted droppings from free-ranging pied babblers and analysed fGCM levels. Levels of fGCMs in droppings collected after 3pm were independent of same-day  $T_{\max} < 38$  °C and averaged  $140.25 \text{ ng g}^{-1} \text{ Dry Weight} \pm 56.92 \text{ ng g}^{-1} \text{ DW}$  (mean  $\pm$  SD) over this range. Above an inflection  $T_{\max} = 38$  °C, however, fGCM levels increased linearly with same-day  $T_{\max}$  and averaged  $190.79 \text{ ng g}^{-1} \text{ DW} \pm 70.13 \text{ ng g}^{-1} \text{ DW}$ . There was no relationship between  $T_{\max}$  on the previous day and fGCM levels in droppings collected the following morning. Group size, breeding stage, sex and rank did not predict variation in fGCM levels. These results suggest that very high  $T_{\max}$  may be linked to acute, but not chronic, heat stress responses. The fGCM levels we measured are likely to represent a biologically meaningful response to an environmental stressor (high  $T_{\max}$ ), suggesting a

physiological mechanism underlying observed changes in behaviour and reproductive success at high temperatures in this species.

## **Introduction**

Stress responses mediated by the endocrine system are a vital component of animals' reaction to environmental perturbations (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). Responses triggered by exposure to a stressor also include physiological and behavioural changes (Agarwal & Prabhakaran, 2005; Stott, 1981), which contribute to an animal's ability to respond appropriately to environmental change (Jensen, Moseby, Paton, & Fanson, 2019) and its likelihood of survival (Asres & Amha, 2014). Environmental temperature is an important determinant of stress in animals (de Bruijn & Romero, 2011; Jessop et al., 2016; Krause et al., 2016; Xie et al., 2017; Ruuskanen 2019), and understanding species-specific stress responses to increasingly frequent and severe heat waves is important for effective conservation and management under advancing climate change.

Glucocorticoids are a class of steroid hormones important for energy mobilisation, immune function, and metabolism and are a crucial part of stress responses (MacDougall-Shackleton, Bonier, Romero, & Moore, 2019). There are two primary glucocorticoids: i) cortisol, found in ungulates, carnivores and primates, and ii) corticosterone, found in rodents, birds and reptiles (Palme et al 2005; Touma & Palme 2005). Quantification of these glucocorticoids is an established and popular method that has been used to study stress responses in animals in relation to external stressors (Ganswindt et al., 2012; Crino et al., 2016; Dantzer et al., 2010 & Sheriff et al., 2011), including high temperatures (Xie et al., 2017).

Stress responses are usually categorised as either acute or chronic. Acute stress is associated with the rapid, transient release of glucocorticoids, 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 mobilising energy reserves which may, for example, allow an individual to escape a potentially dangerous situation such as an attempted predation (Buchanan, 2000). Chronic stress, on the other hand, occurs over longer periods and involves prolonged elevation of glucocorticoid levels in response to ongoing exposure to stressors. Negative effects of chronic stress include disrupted cognition, immune function, and reproduction, which potentially have long term consequences such as reduced breeding success (McEwen, 2004; Dhabhar, 2009 & Lupien et al., 2009).

Levels of circulating glucocorticoids have often been determined directly using blood samples (Crino et al., 2020; MacDougall-Shackleton et al., 2019). However, blood collection can be stressful for the study

animal (Pavlova et al., 2018; Small et al., 2017), and handling stress risks artificially elevating measurements of stress hormones. Alternative non-invasive methods based on quantifying glucocorticoid metabolites in droppings, hair, feathers, or saliva have attracted much attention and have been validated for an increasing number of species (Sheriff et al., 2011; Palme, 2019; Dantzer et al., 2010; Hämäläinen, Heistermann, Fenosa & Kraus, 2014), including Southern pied babblers (*Turdoides bicolor*; Jepsen et al., 2019). Measurement of adrenocortical activity via faecal glucocorticoid metabolites (fGCMs) can eliminate the need for handling of study animals altogether, thereby avoiding artificially increasing the circulating glucocorticoid levels of the study animals (Hodges et al. 2010), provided that the collection of droppings is not stressful to the animal. In some study populations, collecting droppings also presents an opportunity to sample more frequently and under natural conditions (Jepsen et al., 2019). Non-invasive measurements of stress responses have important conservation applications because they can be used to investigate the sub-lethal effects of social and ecological stressors under natural conditions (Wikelski & Cooke, 2006; Sherif et al., 2011; Narayan, 2013 & Dantzer et al., 2014). Although increasing in popularity among researchers, non-invasive sampling of glucocorticoids in avian droppings (especially from free-ranging birds) have been used less frequently than in studies of mammals and reptiles (Palme, 2019).

Little is known about avian stress responses to high air temperatures. We know that chronic exposure to heat can have strongly negative effects on birds. These effects include a trade-off between shade-seeking, resting, heat dissipation behaviour and foraging, leading to reduced foraging effort, efficiency and success (du Plessis et al. 2012, Edwards et al 2015, Cunningham et al 2015, van de Ven et al 2019), body mass loss at high temperatures (du Plessis et al 2012, Sharpe et al 2019, van de Ven 2019), reduced breeding success (Salaberria et al 2014, Wiley & Ridley 2016, DuRant et al 2019, Sharpe et al 2019, van de Ven et al 2020, Bourne et al 2020a), reduced survival of adults (Bourne et al 2020c, Sharpe et al 2019), mass mortality events (McKechnie & Wolf 2010 & Conradie et al 2020) and population declines (Iknayan & Beissinger 2018). Acute exposure to heat can also result in elevated metabolic rates, dehydration, and death (McKechnie 2019). A study on Sonoran Desert birds showed that plasma corticosterone was not elevated during summer compared to winter, even during a year when summer temperatures were higher than normal (Wingfield et al., 1992). However, an Australian study found elevated corticosterone levels after exposure to heat in some species but not others (Xie et al 2017).

In this study, we test the relationship between temperature and fGCM levels in free-ranging Southern pied babblers (*Turdoides bicolor*). Specifically, we test the hypothesis that high maximum daily air

temperatures ( $T_{\max}$ ), associated with compromised foraging and net 24-hr mass loss (du Plessis *et al.*, 2012) as well as failed reproduction (Bourne *et al.*, 2020a) in pied babblers, will trigger an acute stress response in this species. Pied babblers are known to lose mass overnight and reduce provisioning to nestlings when  $T_{\max} \geq 35.5^{\circ}\text{C}$  (du Plessis *et al.* 2012, Wiley & Ridley 2016) and are unable to breed successfully at average  $T_{\max} \geq 38^{\circ}\text{C}$  (Bourne *et al.*, 2020a). We therefore tested a specific hypothesis that temperatures in the mid- to high-30s ( $^{\circ}\text{C}$ ) would trigger an acute stress response in this species. We expected fGCM levels to increase during hot weather. In order to elucidate the time scale over which fGCM levels were elevated in response to hot weather and the extent to which stress responses to an extremely hot day are acute or chronic, we compared the effect of  $T_{\max}$  on fGCM levels in samples collected that afternoon, and the effect of  $T_{\max}$  of the preceding day ( $T_{\max\text{Prev}}$ ) on fGCM levels in samples collected in the morning. We reasoned that, if an extremely hot day triggers an acute stress response,  $T_{\max}$  would influence pied babblers' fGCM levels in the afternoon of the same day but not in the morning on the following day. Alternately, if an extremely hot day triggers a chronic stress response with carry-over effects to the subsequent day, then elevated fGCM levels would remain detectable the following day.

## Methods and Materials

### *Study site*

The study was conducted at Kuruman River Reserve, located in the southern Kalahari Desert, 28 km west of Van Zylsrus town in the Northern Cape Province, South Africa (S 26° 58' E 21° 49'). The study site's annual average summer maximum temperature from 2005 to 2019 was  $34.2 \pm 0.9^{\circ}\text{C}$  (range: 32.4–36.5  $^{\circ}\text{C}$ ) and summer rainfall averaged  $185.4 \pm 86.2$  mm (range: 64.4–352.1 mm; Bourne *et al.*, 2020b). The 33-km<sup>2</sup> reserve is flat with a sandy substrate supporting semi-arid savannah vegetation (Mucina and Rutherford, 2006).

### *Study species*

Pied babblers are medium-sized (60 – 90g) cooperatively breeding passerines (Ridley 2016, Bourne *et al.*, 2019). As part of an on-going study of the behaviour of wild pied babblers habituated to the presence of human observers, the birds were observed under natural conditions (Ridley, 2016). During our study, pied babbler group sizes ranged from 2 – 8 adults. Adults are defined as individuals aged  $\geq 12$  months (Raihani & Ridley, 2007). Habituation of the pied babblers in the study population allows human

observation from distances of 1 – 5m (Ridley & Raihani, 2007), enabling collection of droppings (Bourne et al., 2019). The birds are ringed with metal and coloured rings for individual identification, which makes it easy to assign each faecal sample to an individual. Each group has a dominant female and male, with the remaining individuals being subordinates (Nelson-Flower et al., 2011). Pied babblers have high reproductive skew, with 95% of young produced by the dominant pair (Nelson-Flower et al., 2011). The pied 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).

### *Data collection*

Droppings were collected by following the birds and sampling excreta within 1 min after defecation by a known individual bird, with the droppings immediately transferred to a screw-cap, plastic Eppendorf tube sealed with parafilm (following Bourne et al., 2019). The date and time of collection of the sample, and the identity of each bird and group were recorded (following Jepsen et al., 2019). Each babbler group was visited weekly during the breeding season and monitoring visits lasted up to 90 min; which was sufficient for proper identification of individuals and collection of the droppings. Faecal samples were collected throughout the day during one austral summer breeding season from November 2018 to February 2019 (n = 898 samples in total). Samples were frozen at -18°C within  $1.81 \pm 1.07$  h of collection (mean  $\pm$  SD hours, range = 0.02 – 11.38 h). Samples were collected from approximately 71 individual pied babblers from 18 groups, including dominant and subordinate adults of both sexes. Weather data were obtained from a weather station onsite (Vantage Pro2, Davis Instruments, Hayward, U.S.A.), which recorded air temperature ( $T_a$ ; °C), wind speed ( $\text{m s}^{-1}$ ), rainfall (mm) and solar radiation ( $\text{W m}^{-2}$ ) at 10-min intervals throughout the study period (van de Ven, McKechnie & Cunningham, 2019).

### *Faecal glucocorticoid metabolites analysis*

Of the 898 samples collected, we selected a subset of 228 for analysis. We selected all of the samples collected after 3 pm (afternoon samples, n = 114) and then selected another 114 from the samples collected before 9 am (morning samples). Using afternoon samples collected after 3pm allowed for 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 after ~ 2h (see Jepsen et al., 2019). Afternoon samples were collected on days distributed across a range of  $T_{\text{max}}$  (28 °C – 41 °C), and from individuals from different group sizes (2-8 adults), sexes (n = 43 males, 63 females, 8 unknown sex), ranks (n = 52 dominant, 62 subordinate) breeding stages (n = 45 from breeding groups, 69 from non-

breeding groups). In order to identify whether any stress response detected represented an acute or chronic response, we further analysed 114 morning samples across the same temperature range of 28 °C – 41 °C, recorded on the previous day ( $T_{\max\text{Prev}}$ ), and similarly distributed across group sizes (3-8 adults), sex ( $n = 58$  males, 56 females), rank ( $n = 52$  dominant, 62 subordinate), and breeding stage ( $n = 45$  breeding, 69 non-breeding). Morning samples were 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 for 10 min, the samples 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 5 $\beta$ -pregnane-3,11 $\beta$ ,21-triol-20-one-CMO:BSA (tetrahydrocorticosterone). Characteristics of the EIA including cross-reactivities are given by Quillfeldt and Möstl (2003). This EIA was validated for the reliable quantification of fGCMs in pied babblers by Jepsen et al. (2019).

The coefficients of variation (CV) for the inter-assay variance of the subset of 32 afternoon samples ranged from 10.17% to 10.36% and the CV for intra-assay ranged from 6.33% to 6.64%, while the remaining 82 afternoon samples had CV for inter-assay that were 9.03% to 11.17% and the CV for intra-assay variance ranged from 6.33% to 6.64%. The 114 morning samples CV for the inter-assay variance were 12.5% and 14.5% while CV for intra-assay variance were 5.30% and 7.75%. The combined CV for inter-assay variance for all 228 samples were 13.26% and 13.62%. The sensitivity of the enzyme immune-assay used for all 228 samples is 9 ng/g faecal dry weight.

### **Statistical analyses**

We used R version 3.6.3 for all analyses (R Core Team, 2020). We checked model residuals of all the variables for normality using QQ plots. The variance inflation factor (VIF) was used to assess collinearity between numeric variables (Harrison et al., 2018) and all  $VIF \leq 2$ . Collinearity between categorical and numeric predictor variables was tested using analysis of variance (ANOVA). In the subset of the afternoon samples, sex and group size were associated ( $F_{1,99} = 5.920$ ,  $p = 0.016$ ), with more subordinate females than males in the group sizes ranging from 4 to 8 individuals. Group size and breeding stage

were associated in the morning samples ( $F_{2,100} = 4.830$ ,  $p = 0.009$ ), with larger groups more likely to be breeding (also see Ridley 2016, Bourne et al. 2020b). Correlation between two 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) and  $T_{\max}$  and  $T_{\max\text{Prev}}$  were not correlated.

We tested the effects of  $T_{\max}$  (for afternoon samples),  $T_{\max\text{Prev}}$  (for morning and afternoon samples), breeding stage, group size, rank, sex and the interaction between rank and sex on fGCM levels in linear mixed-effects models (LMMs) fitted using the *lme4* package (Bates et al., 2015b, Harrison et al., 2018). We included group identity included as a random term. The inclusion of individual identity as a random terms 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 destabilising the models (Grueber, Nakagawa, Laws, & Jamieson, 2011; Harrison et al., 2018). Effects of both  $T_{\max}$  and  $T_{\max\text{Prev}}$  were tested in an additive model for afternoon samples in order to explore the relative importance of acute (same day temperature) vs chronic (temperature on previous day) responses. The interaction between rank and sex was included to confirm the association found by Jepsen et al (2019), whereby dominant male pied babblers exhibited higher fGCM levels, using a larger dataset collected in a different year. Data are presented as mean estimate  $\pm 1$  standard error (SE) unless otherwise stated. The three continuous explanatory variables were rescaled by centring and standardising by the mean of the variables for model comparisons (Harrison et al., 2018). Model terms with confidence intervals not intersecting zero were considered to explain significant patterns in our data (Grueber et al., 2011). Models were compared using Akaike's Information Criterion (AIC) with the *MuMIn* package and competing models within  $\Delta\text{AICc}$  2.0 of the top model were averaged (Bartoń, 2016, Harrison et al., 2018). Model-averaged coefficients are presented following van de Ven et al., (2020) & 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. We analysed the effect of  $T_{\max}$  separately above and below the identified breakpoints, including the random term for group.

## Results

For afternoon samples,  $T_{max}$  significantly predicted fGCM levels, with higher fGCM levels at higher maximum temperatures (Table 1, Figure 1). The single best-fit model explaining variation in fGCM levels had a model weight of 0.779 (Table 1) and included both  $T_{max}$  and  $T_{maxPrev}$ . We identified a breakpoint at  $T_{max} = 38.0$  °C (Figure 1). There was no effect of  $T_{max}$  at temperatures < 38.0 °C but fGCM levels increased with increasing  $T_{max}$  at temperatures > 38.0 °C. 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). Dominant females had higher fGCM levels on average than the other birds but the trend was not statistically significant.

We followed procedures similar to those described in Jepsen et al (2019) and we found no relationship between the amount of time between collection and freezing and measured fGCM levels for either morning ( $F_{1,101} = 1.127$ ;  $p = 0.312$ ) or afternoon samples ( $F_{1,107} = 0.087$ ,  $p = 0.769$ ).

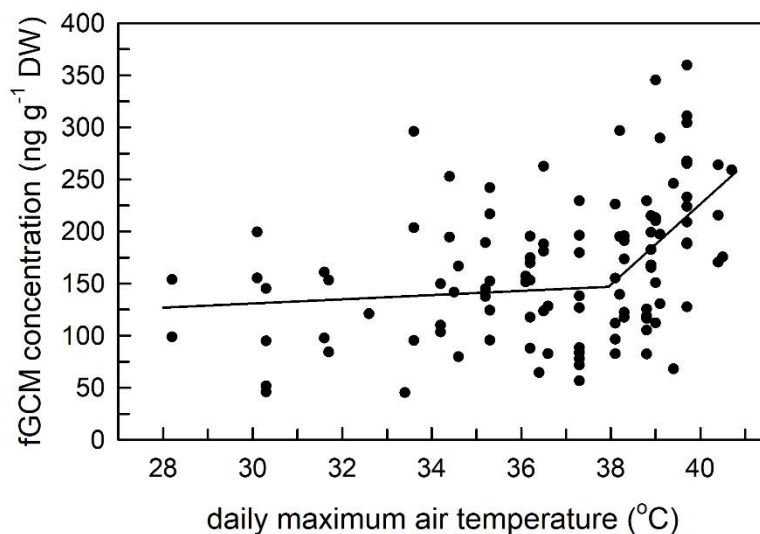


Figure 1: Relationship between afternoon fGCM levels and  $T_{max}$  with increasing fGCM levels as  $T_{max}$  increases in Southern Pied Babblers (*Turdoides bicolor*), with a non-linear effect of  $T_{max}$  on fGCM levels. The break point has been identified at  $T_{max} = 38.0$  °C (95% CI: 36.876, 39.122). Data represented was taken from 144 samples collected from 71 individual Southern Pied Babblers from 18 groups, including dominant and subordinate adults of both sexes.



Table 1: Model outputs for analyses of fGCM levels for afternoon samples. Data comes from 101 afternoon samples from 48 different individuals in 15 groups. Estimate, standard error and confidence intervals are shown for the top model. Significant terms are shown in bold. Null models shown for comparison with top model sets. Random terms: 1 | Group.

<b>Models</b>	<b>AICc</b>	<b><math>\Delta</math>AICc</b>	<b><math>\omega_i</math></b>
T <sub>max</sub> + T <sub>maxPrev</sub>	1110.1	0.00	0.779
T <sub>max</sub>	1114.3	4.17	0.097
Breeding Stage	1114.4	4.30	0.091
Rank*Sex	1116.5	6.35	0.033
T <sub>maxPrev</sub>	1123.6	13.48	0.001
Sex	1128.0	17.91	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

<b>Effect size of explanatory terms in the top model</b>	<b>Estimate</b>	<b>SE</b>	<b>95% CI</b>
Intercept	165.266	9.554	146.349/184.618
<b>T<sub>max</sub></b>	<b>28.197</b>	<b>8.975</b>	<b>10.592/45.629</b>
T <sub>maxPrev</sub>	-3.715	8.906	-21.055/13.657

Table 2: Model outputs for analyses of fGCM levels for morning samples. Data comes from 103 morning samples from 56 different individuals in 18 groups, collected before 9am. Estimate, standard error and confidence intervals are shown for the top model. Significant terms are shown in bold. Null models shown for comparison with top model sets. Random terms: 1 | Group.

<b>Models</b>	<b>AICc</b>	<b><math>\Delta</math>AICc</b>	<b><math>\omega_i</math></b>
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 <sub>maxPrev</sub>	1056.4	13.00	0.001
Group Size	1157.6	14.21	0.001
Null model	1060.2	16.81	0.000
<b>Effect size of explanatory terms in the top model</b>	<b>Estimate</b>	<b>SE</b>	<b>95% CI</b>
Intercept	199.532	9.228	101.633/137.431
Rank (rank = SUB)	-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

## Discussion

Our results reveal that high daily maximum air temperatures are associated with elevated fGCM levels and may be linked to acute heat stress in pied babblers. Specifically, we found a significant effect of  $T_{\max}$  on fGCM levels of samples collected that afternoon, and no significant effect of  $T_{\max\text{Prev}}$  on fGCM levels of samples collected either in the morning or the afternoon of the following day, suggesting that high  $T_{\max}$  triggers an acute stress response in the pied babblers. We found no relationship between fGCM levels and group size or breeding stage and, unlike the Jepsen et al (2019) study, we did not detect a significant relationship between fGCM levels and rank or sex, or the interaction between the two. A significant increase in fGCM levels was evident at  $T_{\max} \geq 38^{\circ}\text{C}$ . This  $T_{\max}$  is similar to several thresholds related to the effects of  $T_{\max}$  on body mass and breeding success in the same population of pied babblers. Du Plessis et al. (2012) found that  $T_{\max} \geq 35.5^{\circ}\text{C}$  has the potential to compromise the ability of pied babblers to maintain body condition. Pied babblers typically lose ~4% of their body mass overnight, but due to reduced foraging success on hot days they may fail to gain at least that amount during the day on days  $\geq 35.5^{\circ}\text{C}$  and this can result in gradual deterioration of body condition over time (du Plessis et al., 2012). Wiley & Ridley (2016) reported reduced provisioning to pied babbler nestlings at temperatures  $\geq 35.5^{\circ}\text{C}$  and Bourne et al (2020a) recently reported, using 15 years of life history data, that no pied babbler young survived when mean  $T_{\max}$  during the nestling period exceeded  $38^{\circ}\text{C}$ . Together, these observational studies suggest that pied babblers may experience heat stress when daily  $T_{\max}$  exceeds  $35.5^{\circ}\text{C}$  and approaches  $40^{\circ}\text{C}$ . Our finding, that fGCM levels increase sharply in free-living pied babblers under natural conditions when  $T_{\max}$  exceeds  $38^{\circ}\text{C}$ , is consistent with these observed temperature thresholds, and suggests a physiological mechanism.

Evidence for the existence of critical air temperatures above which costs are incurred is present in several other bird species. For example, Edwards et al. (2015) found that temperature had a significant effect on the behaviour of Western Australian Magpie *Cracticus tibicen dorsalis*, with temperatures exceeding  $27^{\circ}\text{C}$  resulting in a significant decline in foraging effort. Sharpe et al. (2019) found that Jacky Winters *Microeca fascinans* (a small Australian passerine), were never observed foraging, provisioning nestlings or even incubating, at air temperatures  $> 38^{\circ}\text{C}$  (also see Bayter, 2019). Similarly, van de Ven et al. (2019) showed that foraging efficiency in Yellow-billed Hornbills was negatively affected by both panting and increasing time spent in cooler microsites, resulting in lower numbers of prey captured overall at high air temperature despite no change in foraging effort. The threshold temperature above which breeding male hornbills did not gain any mass over a 12 h day was  $38.4^{\circ}\text{C}$ . In common fiscals

*Lanius collaris*, increasing numbers of days of  $T_{\max} > 37$  °C negatively influenced fledgling tarsus length and increasing number of days of  $T_{\max} > 35$  °C were associated with nestlings having to stay in the nest longer before they were ready to fledge (Cunningham et al., 2013). The presence of threshold temperatures similar to those of pied babblers in other species suggests that these other species might also be experiencing acute physiological stress detectable in fGCM levels.

Although our data were all collected within a single summer, we were able to collect samples across a range of daily maximum temperatures and from a large number of different individuals. In addition, by sampling individuals varying in sex, rank, breeding stage and group size, we were able to evaluate potential causes of individual variation in fGCM levels, to differentiate this from temperature effects. A 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 fGCM 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 that can result in possible increases or decreases in the fGCM levels (Terio et al., 2002, Washburn & Millspaugh, 2002, Palme et al., 2013). Differences in microclimates across the range of a mammal species, the American pika *Ochotona princeps*, influenced fGCM levels when droppings were not immediately collected and preserved after defecation (Wilkening, 2016). Despite these risks, we did not find any significant influence of the period between collection and freezing on fGCM levels in this study (also see Jepsen et al., 2019).

The use of a non-invasive sampling technique with a free-living study population avoids the potential confounds associated with the influence of capture, handling, and captivity on fGCM levels (Wingfield et al., 1995; Dickens et al., 2009). By collecting droppings from the ground after they were naturally excreted by the bird and after the bird had moved away on its own (Bourne et al., 2019; Jepsen et al., 2019), we entirely avoided handling the study animals and eliminated the potentially confounding effect of capture stress (Harper & Austad 2000, Millspaugh 2001 & Touma & Palme 2005). Jepsen et al.'s (2019) study compared captive and free-ranging wild pied babblers and found that wild individuals had significantly lower baseline fGCM levels, even after captive birds had been habituated to captivity for four days. Our measured fGCM levels, collected from free-living pied babblers, are likely to represent a biologically meaningful response to an environmental stressor – in this case high  $T_{\max}$  – experienced under natural conditions.

Non-invasive physiological methods can be very useful for behavioural ecologists who wish to explore physiological correlates of behavioural strategies without disrupting the behaviour of their study organisms to collect these data (Bourne et al., 2019). FGCM levels can be correlated directly with observed natural behaviour and environmental variables. Wider application of non-invasive techniques could open new avenues for assessing behavioural and physiological responses concurrently in wild animals under natural conditions, an increasingly important capability as ecologists seek to understand the impacts of global change pressures on local animal populations (Stillman, 2019). Measured fGCM levels in droppings are less affected by episodic fluctuations of hormone secretion than those in blood and might, therefore, represent the animal's hormonal status more accurately than blood samples (Touma et al., 2004). Droppings are also a particularly useful matrix for measuring fGCM levels because they are relatively abundant and can be collected with minimal disturbance to study animals (Möstl & Palme, 2002, Millspaugh, & Washburn, 2004 and Palme, 2019). FGCM levels in faecal samples can be a reliable indicator of the levels present in blood (Palme, 2019) and birds make particularly useful study subjects as most bird species excrete both urine and droppings together – effectively resulting in a double peak of hormone levels.

The avian upper lethal body temperature is 45 - 46°C, just 4 – 5 °C above resting body temperature (Dawson & Schmidt – Nielsen 1964, Prinzinger et al., 1991). Our study showed that non-invasive methods through faecal sampling can help us understand the physiological impacts of high temperatures on avian species. We demonstrated that fGCM levels in free-living pied babblers, are elevated above a threshold maximum daily air temperature of 38 °C, a result that is not confounded with or influenced by handling or captivity stress. This threshold temperature is very similar to previously published threshold temperatures for pied babblers which demonstrate that high temperatures can be detrimental for reproductive success and survival (Bourne et al 2020a, 2020c). Behavioural adjustments and compromised survival may, therefore, be partially explained by an acute physiological stress response to high temperatures. As anthropogenic climate change is advancing rapidly (IPCC, 2014), studying the biological effects of climate change on free-ranging animals using non-invasive methods can be helpful in terms of understanding the potential impact on their behavioural and physiological responses.

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