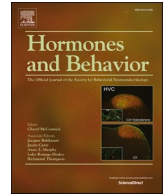




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Effect of exogenous manipulation of glucocorticoid concentrations on meerkat heart rate, behaviour and vocal production

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ABSTRACT

Encoding of emotional arousal in vocalisations is commonly observed in the animal kingdom, and provides a rapid means of information transfer about an individual's affective responses to internal and external stimuli. As a result, assessing affective arousal-related variation in the acoustic structure of vocalisations can provide insight into how animals perceive both internal and external stimuli, and how this is, in turn, communicated to conspecifics. However, the underlying physiological mechanisms driving arousal-related acoustic variation remains unclear. One potential driver of such variation in behaviour and vocal production are glucocorticoids. Through exogenous glucocorticoid manipulation, we aimed to gain insight on the relationship between arousal and physiological parameters, behaviour and vocal production in wild meerkats (*Suricata suricatta*). To this aim, we administered glucocorticoids to wild meerkats, and recorded their heart rate, vigilance behaviour, call rate and acoustic structure during natural behavioural contexts. The results suggest that, although the glucocorticoid treatment did increase plasma glucocorticoid levels, this did not result in observable changes in heart rate, vigilance, or vocal production. This lack of treatment effect suggests that, while glucocorticoids may be a significant component and correlate of the arousal response, they are not the direct drivers of affective arousal related changes in heart rate, behaviour, or vocal production.

1. Introduction

Emotions, defined as short term affective responses to eliciting stimuli, influence how animals, across taxa, process and respond to the world around them (Mendl et al., 2011; Mendl et al., 2010). Emotions can be described across two dimensions of valence (positive or negative) and arousal (bodily activation; high or low) (Mendl et al., 2010; Russell, 1980). Emotional states can influence observable responses, such as behaviour or vocal production (Briefer, 2020; Briefer, 2012). Many animals have been shown to produce distinct call types in different behavioural contexts and related underlying emotional states (Liao et al., 2018; Maigrot et al., 2018; Manser, 2001), and also to encode information within call types related to current emotions (Stomp et al., 2018; Szpl et al., 2017). The encoding of emotional states within call types can transfer potentially more nuanced details on both the general

context, and the producers' current emotional state. In humans, *Homo sapiens*, this can be illustrated in speech, with words and syntax signalling the meaning and the prosodic structure (intonation, stress, tone, rhythm) providing additional information on the speaker's emotional state (Hammerschmidt and Jürgens, 2007; Johnstone and Scherer, 2000; Pell and Kotz, 2011).

Arousal-related variation in vocalisations has been extensively studied. Across mammalian taxa, increases in arousal are consistently associated with increases in fundamental frequency (lowest frequency of the vocalisation; f_0), f_0 modulation and call rates (e.g. Illmann et al., 2013; Linhart et al., 2015; Saito et al., 2019; Klenova et al., 2021; Martin et al., 2022; see review Briefer, 2012). The acoustic variation associated with arousal states likely results from changes in respiration rate, salivation and muscle tension, affecting notably subglottal pressure and vocal tract resonance properties (Briefer, 2012; Scherer, 1986).

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However, the role and specific physiological drivers behind arousal-related changes in specific acoustic parameters remain unclear, as studies linking potential physiological correlates, such as heart rate or glucocorticoid concentrations, and acoustic structure, are scarce (but see Blumstein and Chi, 2012; Perez et al., 2012).

Glucocorticoids are a key arousal-associated hormone; their levels are thus commonly linked to changes in arousal state (Sapolsky et al., 2000; Sopinka et al., 2015). Physiological stress responses in mammals are mediated by the sympathetic-adrenal-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis (Hawlena and Schmitz, 2010). SAM axis activation constitutes the immediate response to a stressor, in a matter of seconds, resulting in the release of catecholamines (i.e., epinephrine and norepinephrine) and associated physiological changes (e.g., increased heart rate, blood pressure and respiration rate). HPA activation happens over a slightly longer period, minutes to hours, leading to a cascade of reactions resulting in the secretion of glucocorticoids (e.g., cortisol, corticosterone) by the adrenal cortex (Hawlena and Schmitz, 2010; Sapolsky et al., 2000). These changes in glucocorticoid levels can also mediate the cardiovascular response (e.g. increase in heart rate; Sapolsky et al., 2000). Studies have shown differing natural levels of glucocorticoids to be associated with changes in vocal behaviour and acoustic structure (Blumstein and Chi, 2012; Cassetari et al., 2022; Crocker-Buta and Leary, 2018; Leary and Crocker-Buta, 2018; Schrader and Todt, 1998). In yellow-bellied marmots, *Marmota flaviventris*, individuals with higher levels of faecal glucocorticoid metabolites produce less noisy calls (Blumstein and Chi, 2012). In addition, a small number of studies performed manipulations of glucocorticoid levels to investigate the presence of a direct link between circulating glucocorticoid levels and acoustic structure, either in a causal or reactive way (Leary et al., 2006; Leary and Crocker-Buta, 2018; Perez et al., 2015; Perez et al., 2012; Sehrsweeney et al., 2019; Simola et al., 2018). For example, green tree frogs, *Hyla cinerea*, reduce call duration and overall call effort following glucocorticoid injection (Leary and Crocker-Buta, 2018). In zebra finches, *Taeniopygia guttata*, increases in glucocorticoid levels are associated with reduced vocal activity and longer calls with higher f_0 (Perez et al., 2012). These changes could be explained by direct or indirect effects of glucocorticoids on the vocal apparatus; glucocorticoids may influence acoustic structure either directly by acting on the vocal folds or indirectly by affecting muscle tension (Briefer, 2020; Mukudai et al., 2015). Alternatively, glucocorticoids could influence vocal motor output via neural regulation (Remage-Healey and Bass, 2004).

In this study, we aimed to expand research on the link between alteration in glucocorticoid concentration and vocalisations, using a highly vocal species as a model, meerkats, *Suricata suricatta*. Meerkats are a social, cooperatively breeding species. In all aspects of life, meerkats emit vocalisations to coordinate group movement and cohesion, elicit food provisioning to offspring, manage social interactions and communicate current predation risk, including the presence of a threat (Manser et al., 2014). The use of vocal communication is particularly beneficial to meerkats while foraging, as during this time the group moves through their territory as a group, but forage independently with their heads often below the ground digging for prey. The limited visual contact between group members makes vocalisations the primary mode of communication at this time. During foraging, meerkats produce a range of different call types, including close calls, to communicate current location and monitor the location of others (Engesser and Manser, 2022; Gall and Manser, 2017; Townsend et al., 2011); move and lead calls, to initiate and direct changes in foraging location (Bousquet et al., 2011; Gall et al., 2017); and sentinel calls, produced by a single individual performing vigilance for the rest of the group, to communicate the absence of threats (Manser, 1999; Rauber and Manser, 2017).

As a highly vocal species, with a well studied vocal repertoire, meerkats provide an ideal study system to investigate how individual emotional arousal influences vocal production. Previous work has

shown that meerkat alarm calls vary both with predator type (aerial/terrestrial) and urgency (high/low), which is likely related to the producer's arousal level (Manser et al., 2002). However, the underlying processes behind these changes in acoustic structure have yet to be investigated. To explore glucocorticoid-related changes in their vocalisations, we orally administered cortisol to increase meerkats' glucocorticoid levels. Previous work has demonstrated that glucocorticoid manipulation influences several aspects of meerkat behaviour, including reducing foraging behaviour and increasing proximity to pups (Santema et al., 2013), increased latency to resume foraging after alarm events (Voellmy et al., 2014), and increased cooperative pup feeding behaviour (Dantzer et al., 2017b). We recorded meerkats during foraging to assess how this glucocorticoid manipulation influenced the acoustic structure of a range of call types. In addition, we assessed the resulting changes in circulating glucocorticoid levels on heart rate and vigilance behaviour, in order to understand the overall effect and confirm validity of the manipulation. We predicted that, if increases in glucocorticoids, as part of the arousal response, play a functional role in vocal production, there would be consistent increases in f_0 , call rate and potentially call duration across call types (including close calls, post-vigilance close calls, move/lead calls and sentinel calls) in individuals with artificially increased glucocorticoid levels. By focussing mainly at within call type variation, we aimed to investigate the effect of glucocorticoids on fine-grained differences in acoustic structure within contexts.

2. Methods

2.1. Study population

Our study was conducted at the Kuruman River Reserve, Northern Cape, South Africa on a population of habituated meerkats studied for over 25 years (Clutton-Brock and Manser, 2016). Each individual was followed throughout their lifetime and uniquely identifiable from PIT tags and dye marks (Jordan et al., 2007). All members of the study population were habituated to being followed at a distance of <1 m allowing for detailed observational recordings and experimental manipulations. Data was collected between March 2021 and June 2023.

2.2. Ethical statement

All experimental manipulations and recordings were carried out following methodology approved by the ethical committee of University of Pretoria (NASO29-2022) in line with ARRIVE guidelines (du Sert et al., 2020), and the Northern Cape Conservation Service, South Africa (FAUNA 0906/2022). All individuals were only used once per experimental manipulation and closely monitored throughout. Only subordinate adult (>1 year) meerkats were used to limit any potential influence on general group dynamics.

2.3. Preparation and administration of cortisol and control solutions

To artificially increase individual glucocorticoid levels, we prepared dead scorpions injected with cortisol suspended in coconut oil for to provision the meerkats. Cortisol dosages, of 14 mg (approximately 20 mg/kg; hydrocortisone, Sigma H4126), were prepared in line with previous used and validated methods (Dantzer et al., 2017b). This dosage has been shown to temporarily increase circulating cortisol levels and be metabolised within 25 h (Braga Goncalves et al., 2016; Dantzer et al., 2017b). To prepare the dosages, 140 mg of cortisol was combined with 1 ml of 100 % coconut oil (sigma, C1758) to achieve a standardised concentration for cortisol (0.14 mg/ μ l). The solutions were vortexed to suspend the cortisol. The control solution consisted of coconut oil only, and was also measured out and vortexed following the same method. In total, 100 μ l of either the cortisol or control emulsions were injected into the abdomen of a dead scorpion. Cortisol/control solutions and scorpions were prepared approximately 16 h prior to use

in the experiments and stored at 4 °C. Meerkats were fed within 30 min of starting foraging in the morning. Scorpions were provisioned by attaching them to a length of fishing line and presenting them to the focal individuals while they were digging in a foraging hole. Meerkats were observed to ensure that the entire scorpion, and therefore treatment, was consumed.

In total, 32 subordinate adult (>1 year) individuals from 12 groups were used in three separate experimental manipulations (see details in the sections below). Each individual was treated only once with cortisol, and no individual was used in more than one experimental manipulation study. Firstly, to investigate the effect of cortisol on circulating glucocorticoid levels, we treated six individuals (three female, three male) with cortisol solutions and six other individuals (three female, three male) with control solutions ($n = 12$ individuals from six groups in total). Secondly, to investigate the influence of cortisol treatment on heart rate, we treated eight meerkats from six groups with cortisol only (three female, five male). Thirdly, to test the effect of glucocorticoid treatment on vocalisations and behaviour, we treated 12 individuals from six groups with both cortisol and control treatments (six female, six male).

2.4. Measuring effect of glucocorticoid treatment

2.4.1. Circulating plasma glucocorticoid levels

To validate the effect of exogenous glucocorticoid treatment, we compared blood samples of cortisol- and control-treated meerkats to confirm that the treatment did influence circulating glucocorticoid levels. To do so, we provisioned the individuals between November 2022 and June 2023. Pairs of same-sex litter mates were selected, treating one with control and one with cortisol scorpions following the methods outlined above. We then caught both members of the pair in a random order, and took blood samples on average 66 min ($n = 6$ pairs, 40–95 min) after consuming the treatment. The times of sampling were chosen to determine the circulating glucocorticoid levels across a longer time period, and not only immediately following treatment, thus allowing us to confirm the treatment effect over the duration of the recording period for heart rate, behaviour, and vocalisations. Meerkats were caught by grasping them by the base of the tail and placing them in a sterilised pillow case. They were then brought back to a car approximately 50–100 m from the group and placed under anaesthesia (Isoflurane and oxygen, 1–4 %) within 1 ± 0.21 min of capture (0–2 min). We took 2–5mls of blood from the jugular vein for each individual, taken within 4 ± 0.67 min (2–10 min) of capture, which we stored on ice. Individuals were then placed in a dark box to recover from the anaesthesia before releasing back to the group, with 13 ± 1.15 min (6–21 min) between capture and release. Blood samples were centrifuged upon return from the field, with plasma and red blood cells separated and frozen at

at -20 °C. The plasma cortisol levels were determined using a cortisol enzyme-immunoassay already validated for reliably quantifying cortisol levels in plasma samples of meerkats (Dantzer et al., 2017b). Detailed assay characteristics, including full descriptions of the assay components and antibody cross-reactivities are provided in Palme and Mostl (1997). The sensitivity of the assay was 25 pg/ml, and the intra- and inter-assay coefficient of variance, determined by repeated measurements of high- and low value controls, was 4.89 % and 5.83 % and 8.48 % and 9.21 %, respectively. Assays were performed on microtiter plates following established protocols (Ganswindt et al., 2002) at the Endocrine Research Laboratory, University of Pretoria, South Africa.

2.4.2. Heart rate

To investigate the effect of exogenous glucocorticoid treatment on heart rate, we treated meerkats implanted with heart rate loggers with cortisol. We treated the implanted individuals with the cortisol solution between November 2022 and June 2023, following the methods outlined above. As part of a larger project data-loggers (Star-Oddi micro-

HRT, 3.3 g, cylindrical, 2.5 cm long) were surgically implanted subcutaneously at the base of the sternum, in 14 meerkats total (six females, eight males), recording the heart rate at 600 Hz every 15 s, for 6 h per day (see Appendix for procedure details). From these devices, we extracted the heart rate data collected on the day before treatment (untreated) and day of treatment (cortisol-treated). To control for variation relating to time of day, we included heart rate measured from 30 min after the time of treatment, and selected data from the equivalent time period for the untreated day before. We then collected behavioural data on the implanted individuals using a custom-made pendragon form [Pendragon Software Corporation, USA] on a tablet (Lenovo Table V7) on the day before treatment with cortisol (untreated) and on the day of cortisol treatment (cortisol-treated). This allowed us to calculate the average heart rate for each behaviour performed, in order to compare the effect of cortisol treatment within an individual, whilst controlling for activity-related differences between types of behaviours. We selected heart rates with at least five seconds between the behaviour start and when the heart rate was recorded, to limit the possible influence of behavioural activity from the previous behaviour.

2.5. Vigilance and vocalisations

2.5.1. Acoustic and vigilance recordings

To study the effects of the glucocorticoid administration, on meerkat vigilance and vocal production, pairs of same-sex littermates were treated with the cortisol (cort) or control solution and followed simultaneously. We provisioned individuals between March 2021 and July 2021. Each pair was followed twice for periods of two days, during which the vocalisations and vigilance of both members of the pair were recorded on the day before treatment and the day of treatment. On the day of treatment, one member of the pair was randomly provisioned with a cortisol-treated scorpion and the other a control scorpion. After an interval of two-weeks, the two-days of follows and treatment were repeated, with treatment type reversed within the pair.

Following scorpion provisioning, each member of the pair was followed by one observer (two observers in total) at a distance of 0.3–1 m with a Sennheiser directional microphone (ME66/K6) connected to a Marantz PMD-670 solid-state recorder (Marantz Japan Inc.; sampling frequency 44.2 kHz, 16 bits accuracy) for approximately 2.5 h (02:17 ± 00:19). During this time, all calls made by the focal were verbally annotated on the second channel of the recorder and contextual information, such as behavioural context, vigilance behaviour and predator encounters, were recorded on a tablet using a custom-made pendragon form [Pendragon Software Corporation, USA]. Behavioural vigilance data was defined as three types: quadrupedal vigilance (briefly scanning on all four legs), bipedal vigilance (vigilance whilst stood on two legs), and raised guard (vigilance while in a raised position, such as on a bush or log) (Manser, 1999; Rauber and Manser, 2017). Quadrupedal vigilance was recorded as a one second point event due to its brief duration, whereas bipedal vigilance and guarding were recorded as durations. Observers following each individual in a pair switched every 30 min to minimize effect of observer bias. One observer was completely blind to the treatment type, the other had prepared the treated scorpions the day before, assigning scorpions as A and B to each individual ID, with no indication of treatment type when provisioning the scorpions.

2.5.2. Acoustic analysis

Acoustic recordings were processed in Adobe Audition [Build 13.0.6.38] and all calls given by the focal were annotated. We analysed five types of call, including close calls, post-vigilance close calls, move/lead calls, single note sentinel calls and long sentinel calls (see Fig. 1 for examples). For each call type, we selected a random subset of maximum 20 calls per individual, treatment and day, to ensure that a similar number of calls was analysed across individuals and treatments (Table A2). Individual calls were cut at zero-crossings and filtered (hann band-pass between 0.05 and 12 kHz) for analysis in PRAAT ([version

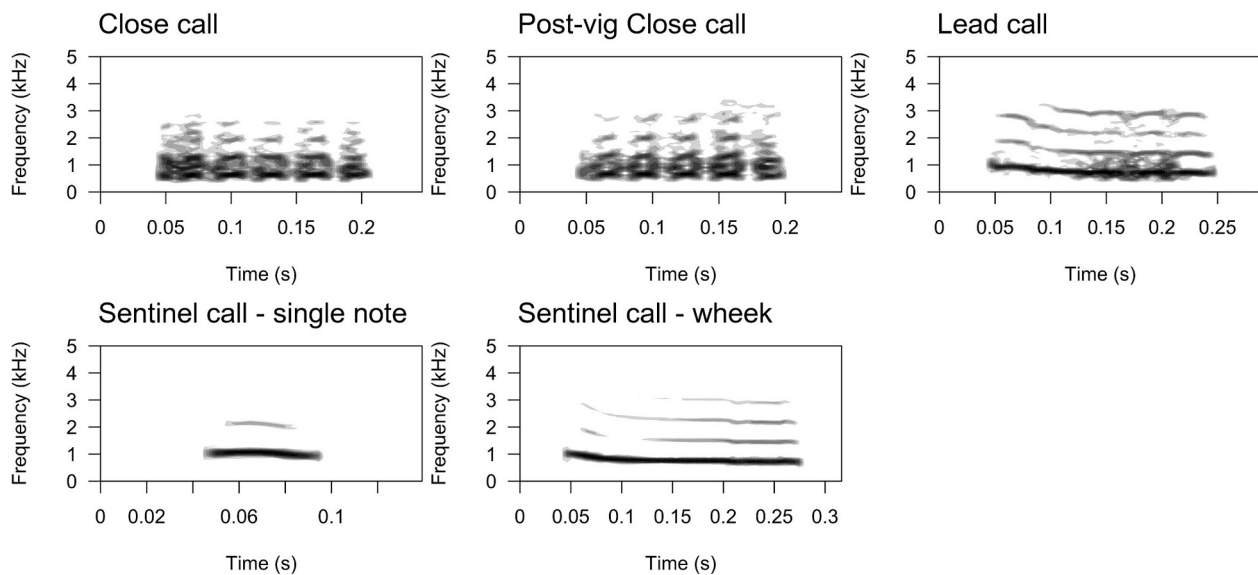


Fig. 1. Spectrograms of the various call types analysed as part of the study.

6.1.53] Boersma, 2001). In total, 30 acoustic parameters (Table A1) were extracted using a custom built PRAAT script (Reby and McComb, 2003; Charlton et al., 2009; Briefer et al., 2019). We also calculated call rate for each call type analysed using all of the annotated calls. Close calls, post-vigilance close calls and move/lead calls rate was calculated by dividing the number of calls of each type by the recording period minus time spent vigilant, as these call types are not produced during vigilance behaviour. Both sentinel call rates (short and long) were calculated by dividing the number of calls produced by the time spent on bipedal vigilance or on guard.

2.6. Statistical analysis

To test the effect of glucocorticoid treatment on circulating plasma glucocorticoid levels, heart rate, vigilance, call rate and vocalisation acoustic structure, we ran Linear Mixed Effects Models (LMM) and Generalised Linear Mixed Effects Models (GLMM). 1) To analyse the effect of cortisol treatment on circulating plasma cortisol levels, we used a LMM with cortisol concentration as the response variable, treatment (cortisol or control) as the fixed effect, and pair ID as a random effect. 2) We used LMMs to analyse the effect of cortisol treatment on heart rate on the day before treatment and day of treatment. Models were fitted with either mean heart rate per behaviour or standard deviation in heart rate per behaviour as a response variable, treatment (untreated or cortisol-treated) and behavioural category of common behaviours predicted to have variation in affective arousal state (foraging, vigilant, digging bolthole, moving, grooming, resting) and their interaction as fixed effects (lmer function, lme4 library (Bates et al., 2015)). Individual identity was fitted as a random effect. 3) To test the effect of cortisol treatment on vigilance and vocalisations, we ran LMMs and GLMMs, with the follow day (before or on) and treatment (cortisol or control) and the interaction between them as fixed effects. The response variable consisted of the vigilance behaviour, call rate or acoustic variables (see description below). Individual identity and date were fitted as crossed random effects to control for individual and day-specific related differences.

(1) Vigilance

We analysed vigilance behaviour, by calculating the total time spent vigilant, adding up the duration all three types of vigilance, with each quadrupedal event as one second, divided by the period of recording in

the session. We used generalised linear mixed models (GLMM) with a binomial error structure to investigate how vigilance varied as a function of the interaction between the follow day (before or after) and treatment (cortisol or control), weighting for the total recording time (glmer function, lme4 library (Bates et al., 2015)).

(2) Call rate

We analysed the rate of close calls, post-vigilance close calls, move/lead calls, short note (single- and double-note) sentinel calls and long (di-drr and wheek) sentinel calls over the recording period. We then used a LMM with call rate $\log+1$ transformed as the response variable, and the same fixed and random effects as described above (lmer function, lme4 library (Bates et al., 2015)).

(3) Acoustic structure

We used principal component analyses (PCA) to determine which of the 30 measured acoustic parameters accounted for the variance in the data and to control for correlations between parameters. Highly correlated variables (>0.8) were removed prior to PCA analysis to improve interpretability of results. We entered close calls, post-vigilance close calls, move/lead calls, single-note sentinel calls and long (di-drr and wheek) sentinel calls in separate PCAs. For each PCA, principal components (PC) with eigenvalues greater than one (Kaiser's criterion) were kept and their PC scores were entered as response variables in separate LMMs (lmer function, lme4 library (Bates et al., 2015)). In the case of a significant effect of the interaction on the PC scores, we also ran the model with the top 3 loading acoustic parameters to confirm the effect on the raw variables. The LMMs for all call types were fitted with the same explanatory and fixed and random as described above.

2.7. Assumptions and p-value extractions

To assess model assumptions for normality and heteroscedasticity, we tested model residuals distribution (KS test; *testResiduals* function, DHARMA library (Hartig, 2022)), dispersion (*testResiduals* function, DHARMA library), and outliers (*testResiduals* function, DHARMA library). If model residuals did not fit a normal distribution or were over- or under-dispersed, we transformed the response variables or used a GLMM instead of LMM depending on best residual fit. LMMs were used unless specified otherwise above. We used parametric bootstrapping

(PBmodcomp function, pbkrtest library) to test the statistical significance of the factors using a likelihood-ratio test. We then performed post-hoc comparisons using Tukey HSD to test the effect of the treatment (glht function, multcomp library (Hothorn et al., 2008)). For the models on vigilance behaviour and vocalisations, we only tested the statistical significance by comparing models with and without the interaction. For these analyses the interaction between follow day and treatment was the only term of interest, as this would indicate whether the glucocorticoid treatment had an effect, while controlling for within and between individual differences. When an interaction effect was shown, further post-hoc comparisons were performed using a Tukey HSD test (glht function, multcomp library (Hothorn et al., 2008)).

3. Results

3.1. Physiological measurements

Glucocorticoid treated meerkats had approximately 16 times greater plasma cortisol levels than the paired control individuals, but no significant effect on heart rate was observed (Fig. 2). Analysis of the plasma samples showed significantly higher plasma cortisol levels in cortisol-treated individuals with an average level of 445.9 ± 84.5 ng/ml compared to 27.4 ± 8.0 ng/ml for control individuals (Fig. 2a, LMM; estimate(S.E.) = $2.93(0.37)$, $Z = 7.91$, 95 % CI (1.98, 3.88); PBTest = 22.14, $p < 0.001$). The LMMs did not reveal any significant difference in the average heart rate, or in the standard deviation of meerkat heart rates between cortisol-treated and untreated days, within each behavioural category, (Fig. 2b, Table A3 & A4).

3.2. Vigilance behaviour

There was a significant effect of the interaction between day and treatment on the proportion of total time spent vigilant during the recording period, combining all three vigilance types (Fig. 3; Table A5; GLMM: estimate(S.E.) = $0.42(0.02)$, $Z = 20.11$, 95 % CI (0.38, 0.46); PBtest = 404.47, $p < 0.001$). Post-hoc contrast analyses showed that this significant difference was not driven by the cortisol treatment, as there were no consistent significant differences between the day of treatment with cortisol and the other three day/treatment combinations. Instead, there was a significantly lower vigilance in the cortisol and control individuals on the day before the treatment (i.e. when untreated; Table A6; Tukey HSD: estimate(S.E.) = $-0.49(0.02)$, $Z = -32.15$, $p < 0.001$), and a lower vigilance on the day of treatment for cortisol

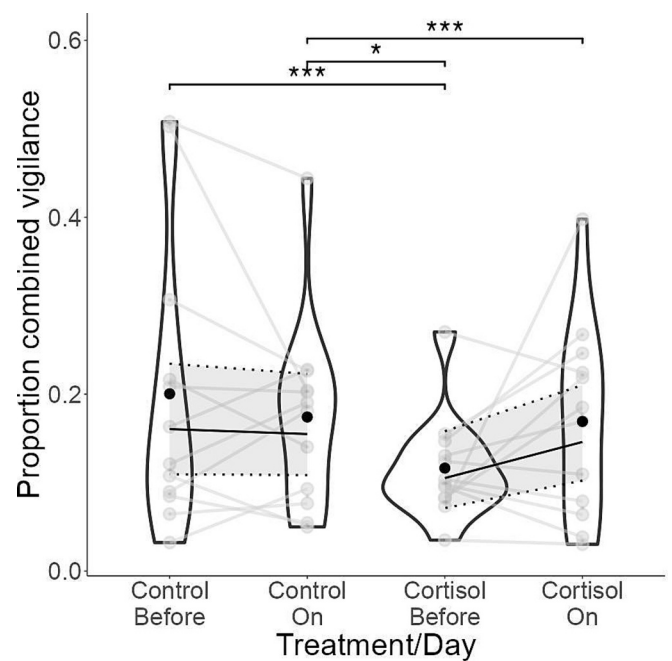


Fig. 3. - Effect of treatment type (cortisol or control) and recording day (before or on) on the proportion of time spent vigilant ($n = 12$). Centre black horizontal line shows the median, boxes represent the interquartile range, with whiskers extending to 1.5 times the interquartile range above the upper quartile or below the lower quartile. Small points indicate outliers and large black points show the mean. The solid black lines with shaded sections and dotted line represent model estimates with confidence intervals. Only significant contrasts are marked with an asterix.

compared to control individuals (Table A6; estimate(S.E.) = $-0.07(0.01)$, $Z = -4.95$, $p < 0.001$). There was also a significantly lower vigilance on the day before cortisol treatment than on the day of control treatment (Table A6; estimate(S.E.) = $-0.45(0.18)$, $Z = -2.44$, $p = 0.045$). These results do not support that the differences were driven by the effect of cortisol treatment. Indeed, in this case, there would have only been a significant difference between the day of cortisol treatment and all other days/treatment combinations, and, particularly, no significant difference between treatments on the day before. Instead, the results hence suggest an overall effect of between individual and

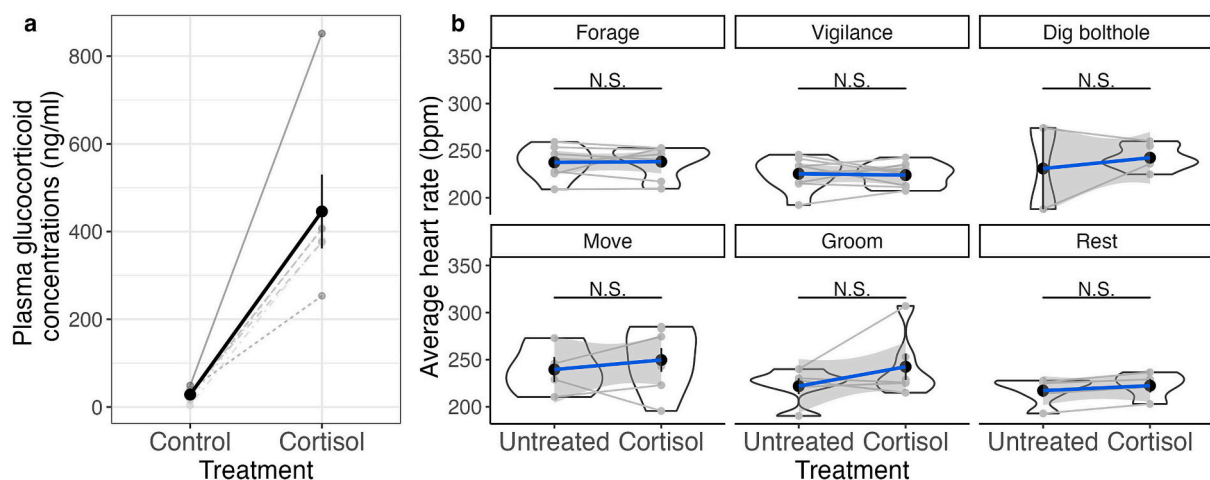


Fig. 2. (a) The effect of control versus cortisol treatment on circulating plasma glucocorticoid concentration (control: $n = 6$, cortisol $n = 6$; solid black line and points with standard error show the overall mean, grey solid/dashed/dotted lines and points represent each pair of individuals treated). (b) The average heart rates of individuals ($n = 8$) when untreated or cortisol-treated for each behavioural category (black points indicate mean with standard error, and blue line with grey shading shows regression line with standard error). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between day variation.

3.3. Rate of the different call types

There was a significant effect of the interaction between recording day and treatment on close call rate per minute (Fig. 4a; Table A7; LMM: estimate(S.E.) = 0.23(0.1), $Z = 2.33$, 95 % CI (0.04, 0.43); PBtest = 5749.6, $p < 0.001$). Post-hoc contrast analyses revealed that there was a significantly higher close call rate on the day before treatment for the control compared to the cortisol-treated individuals (Table A8; Tukey HSD: estimate(S.E.) = $-0.22(0.09)$, $Z = -2.59$, $p = 0.05$). In a similar way as for the vigilance results, these results do not support that the differences were driven by the effect of cortisol treatment. In addition, there was no statistical support for an effect of the interaction between treatment and day for the rates of all other call types; post-vigilance close calls, move/lead calls, all sentinel calls combined, short-note sentinel calls and long sentinel calls (Fig. 4b-3f; Table A7).

3.4. Acoustic structure of the different call types

Glucocorticoid treatment only had a slight effect on the acoustic structure of the call types analysed (close calls, post-vigilance close calls, move/lead calls or single-note and long-note sentinel calls) (Fig. 5a-e). There was only a significant effect of the interaction between treatment (cortisol/control) and day (before/on) for three principal components across the combined 27 principal components analysed across all call types (Table 1 for summary; Table A9-S13 for PC loadings; Table A14-A25 for detailed model outputs). For close calls, there was a significant effect of the interaction between treatment and day on PC6 (Table 1; Table A14), but post-hoc tests did not reveal any significant two-by-two comparisons (Table A15). For post-vigilance close calls, there was a significant effect of the interaction between treatment and day on PC2 (Table 1; Table A16); PC2 scores were higher in the control individuals than the cortisol treated ones on the treatment day (Fig. 5b; Table A17;

Tukey HSD: estimate(S.E.) = 0.89(0.29), $Z = 3.11$, $p = 0.009$). Based on the highest loadings of the acoustic parameters on PC2 (Table A10), this suggests that individuals treated with cortisol produced post-vigilance close calls with higher standard deviation in f_0 , lower minimum f_0 and overall variation in f_0 . However, post-hoc tests did not show a significant effect of cortisol treatment for the top three loading raw acoustic parameters, there was only a significant effect of the interaction between treatment and day on minimum f_0 (Table A18), and no significant effect of cortisol treatment on minimum f_0 in two-by-two comparisons (Table A19). For long-note sentinel calls, there was a significant effect of the interaction between treatment and day on PC2 (Table 1; Table A22); PC2 scores were significantly lower for individuals on cortisol treatment on the treatment day than the day before (Fig. 5c; Table A23; Tukey HSD: estimate(S.E.) = $-1.76(0.6)$, $Z = -2.94$, $p = 0.017$), and cortisol treated individuals had significantly lower PC2 scores than control individuals the treatment day (Table A23; estimate (S.E.) = 1.34(0.48), $Z = 2.78$, $p = 0.027$). Based on the highest loadings of the acoustic parameters on PC2 for long-note sentinel calls (Table A13), this suggests that individuals treated with cortisol produced calls with a lower range in f_0 and higher variation between f_0 points and higher harmonicity. However, again, post-hoc tests did not show a relevant significant effect of cortisol treatment for the top loading raw acoustic parameters, there was a significant effect of the interaction on the top three raw parameters (Table A24), but no significant result indicating an effect of cortisol treatment in two-by-two comparisons (Table A25). The lack of cortisol effect on the majority of acoustic parameters analysed across call types suggests that glucocorticoid treatment only had a limited overall effect on the acoustic structure. There was not a consistent significant contrast between the day of cortisol treatment and the other three treatment/day combinations and little effect on the raw acoustic parameters, suggesting that the variation in PC2 for post-vigilance close calls and long-note sentinel calls was not driven by cortisol treatment, and any of the variation detected was driven by other factors.

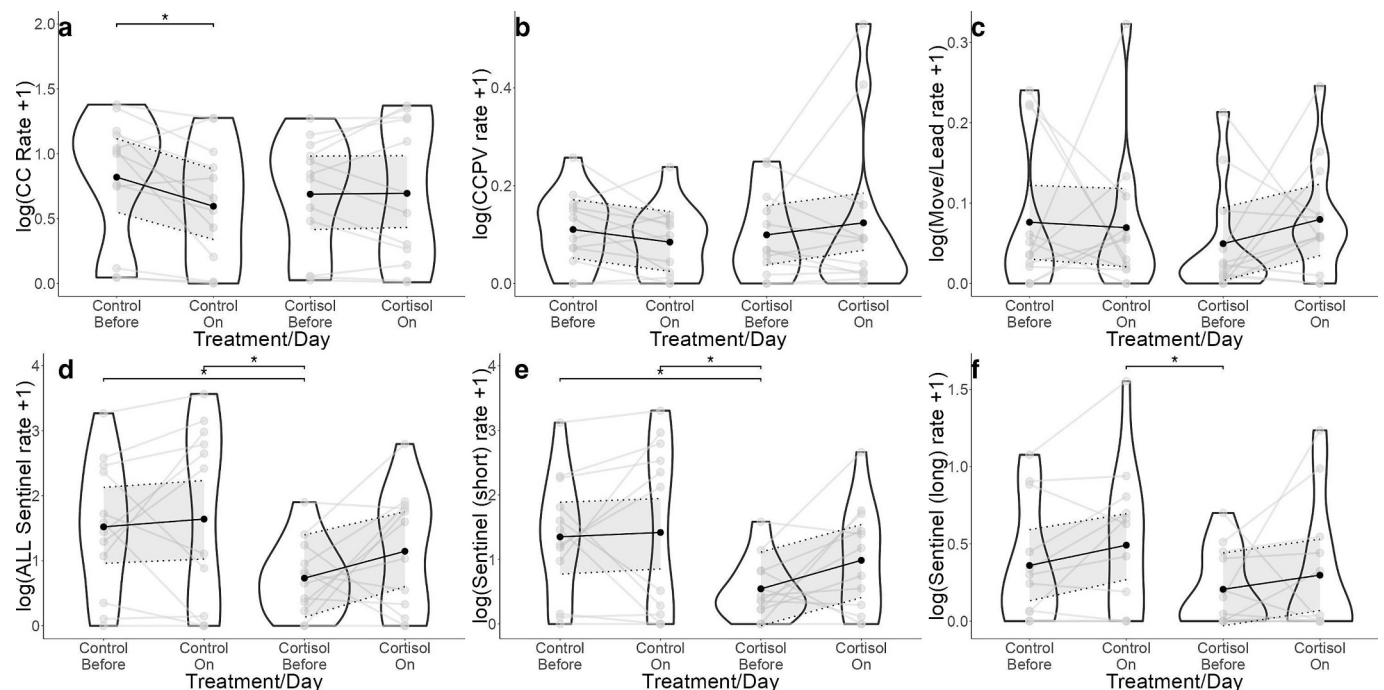


Fig. 4. – Effect of treatment type (cortisol or control) and recording day (before or on) on the call rate for different call types ($n = 12$; close calls (a), post-vigilance close calls (b), move/lead calls (c), all sentinel calls (d), short-note sentinel calls only (e), long-note sentinel calls only (f)). Centre black horizontal line shows the median, boxes represent the interquartile range, with whiskers extending to 1.5 times the interquartile range above the upper quartile or below the lower quartile. Small points indicate outliers and large black points show the mean. The solid black lines with shaded sections and dotted line represent model estimates with confidence intervals. Only significant contrasts are marked with an asterisk.

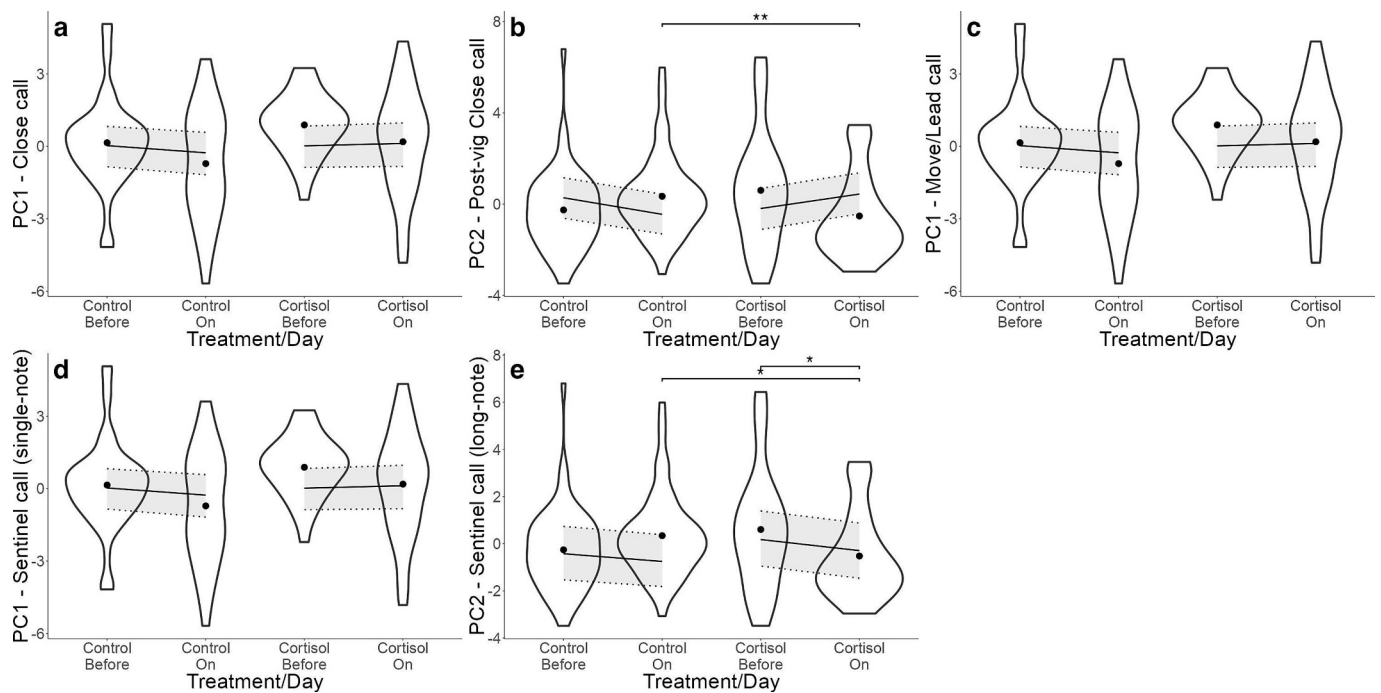


Fig. 5. Effect of treatment type (cortisol or control) and recording day (before or on) on the first principal component, unless there was a significant effect for another principal component, of the acoustic structure for different call types ($n = 12$, close calls (a), post-vigilance close calls (b), move/lead calls (c), short-note sentinel calls only (d), long-note sentinel calls only (e)). Centre black horizontal line shows the median, boxes represent the interquartile range, with whiskers extending to 1.5 times the interquartile range above the upper quartile or below the lower quartile. Small points indicate outliers and large black points show the mean. The solid black lines with shaded sections and dotted line represent model estimates with confidence intervals. Only significant contrasts are marked with an asterix.

4. Discussion

An individual's arousal state can influence vocal production, both in terms of the type of call produced and call rate, but also the acoustic structure of the call itself (Briefer, 2020; Briefer, 2012). We investigated whether artificially increasing plasma glucocorticoid levels, considered an arousal-associated hormone, would result in changes in heart rate, vigilance and vocalisations produced by wild meerkats. Our results suggest that, while exogenous glucocorticoid treatment did increase circulating glucocorticoid levels, it did not influence heart rate, vigilance, call rate or call acoustic structure. This suggests that there was no direct effect of our manipulation of circulating plasma glucocorticoid levels on heart rate or behaviours that have previously been described to correlate with affective arousal states.

There was no detectable effect of glucocorticoid treatment on vigilance behaviour, call rate, or call acoustic structure within individuals (day: before/on or treatment: cortisol/control) or between individuals experiencing the same environmental and contextual conditions (day of treatment: cortisol/control). There was some variance related to the treatment type and day, but without any discernible patterns of a difference for the day of cortisol treatment compared to all other treatment combinations, suggesting that this variance was likely driven by something outside the scope of the experimental design. In addition, there was no difference between the heart rate of individuals when treated with cortisol compared to when they were untreated (day before). Our validation of the glucocorticoid dosage and administration showed that the treatment did increase circulating plasma cortisol levels, with 16-fold increase on average. This effect is consistent with previous glucocorticoid treatments and validation in meerkats (Dantzer et al., 2017b; Santema et al., 2013), and although levels are on the high-end, they are within the biological range for post-stressor glucocorticoid levels in meerkats (Carlson et al., 2006; Carlson et al., 2004; Dantzer et al., 2017a). Therefore, although glucocorticoid treatment did elevate circulating glucocorticoid levels, this did not have a clear, detectable

effect on heart rate, vigilance behaviour, or vocalisations.

The absence of an effect of glucocorticoid treatment on vocal production suggests a lack of arousal manipulation, rather than a lack of emotional arousal effect on vocalisations, in both call rate or within-call-type variation in acoustic structure in meerkats. This is further supported by the lack of treatment effect on heart rate and vigilance behaviour. Due to vocal modification occurring under short time frames, and the often rapid, reactionary nature of their production, it is possible that glucocorticoid levels are not altered rapidly enough to directly influence vocalisations. Vocal production has been shown to vary with arousal state in many species (Briefer, 2012). For example, in rhesus macaques, *Macaca mulatta*, increases in arousal result in increases in f_0 in two different call types; coos that function as separation calls given in a range of contexts and screams produced during agonistic interactions (Schwartz et al., 2022). Additionally, pigs, *Sus scrofa domesticus*, increase both grunt and scream rates with increasing arousal (Linhart et al., 2015), and Cape fur seals, *Arctocephalus pusillus*, increase bark rate in high arousal contexts (Martin et al., 2022). It is therefore clear that arousal-related variation in acoustic structure exists throughout the animal kingdom, but the exact mechanisms underlying this variation remains uncertain.

The lack of glucocorticoid treatment effect, despite confirmed increased circulating levels, aligns with considerable variation in treatment effects shown in other studies and species. While several studies do report effects of glucocorticoid treatment on behaviour and vocalisations, many report no effect at all (see review, Sopinka et al., 2015). Glucocorticoid treatment has been shown to reduce vocal output and modify structure (Leary and Crocker-Buta, 2018; Perez et al., 2012), to both reduce (Bliley and Woodley, 2012; Van Kesteren et al., 2019; Torres-Medina et al., 2018) or increase responsiveness to stressors (Thaker et al., 2009; Voellmy et al., 2014), and to increase (Crossin et al., 2012) or decrease foraging behaviour (Busch et al., 2008). In addition, glucocorticoid treatment has also been shown to not affect call rate (Simola et al., 2018), acoustic structure (Sehrsweeney et al., 2019),

Table 1

- Summary table showing the percentage of variance explained by each of the top principal components analysed, with the model summaries and parametric bootstrapping test results for the interaction between treatment type and recording day on acoustic structure for the different call types. Significant *p* values appear in bold.

	PC variance	LMM				PBTest	
		Estimate	SE	Z	95 % CI	stat	p-value
Close call							
PC1	0.25 %	0.395	0.227	1.739	(-0.05, 0.84)	3.014	0.079
PC2	0.17 %	-0.134	0.233	-0.574	(-0.59, 0.32)	0.329	0.542
PC3	0.11 %	-0.298	0.173	-1.726	(-0.64, 0.04)	2.971	0.087
PC4	0.10 %	-0.035	0.187	-0.185	(-0.40, 0.33)	0.034	0.850
PC5	0.08 %	0.226	0.162	1.401	(-0.09, 0.54)	1.940	0.175
PC6	0.07 %	0.426	0.148	2.875	(0.14, 0.72)	8.192	0.004
Post-vigilance close call							
PC1	0.23 %	0.507	0.437	1.160	(-0.35, 1.36)	1.324	0.231
PC2	0.19 %	1.361	0.403	3.380	(0.57, 2.15)	11.105	0.001
PC3	0.11 %	0.188	0.312	0.603	(-0.42, 0.8)	0.361	0.547
PC4	0.10 %	0.188	0.342	0.549	(-0.48, 0.86)	0.300	0.564
PC5	0.08 %	0.033	0.293	0.113	(-0.54, 0.61)	0.013	0.911
PC6	0.07 %	0.226	0.293	0.771	(-0.35, 0.80)	0.592	0.448
Move/Lead call							
PC1	0.23 %	-0.945	0.634	-1.490	(-2.19, 0.3)	2.203	0.143
PC2	0.20 %	1.098	0.643	1.709	(-0.16, 2.36)	2.763	0.122
PC3	0.12 %	-0.160	0.478	-0.335	(-1.1, 0.78)	0.073	0.796
PC4	0.11 %	0.720	0.424	1.698	(-0.11, 1.55)	2.852	0.107
PC5	0.09 %	-0.162	0.427	-0.379	(-1, 0.68)	0.139	0.707
PC6	0.05 %	-0.062	0.323	-0.190	(-0.7, 0.57)	0.035	0.854
Sentinel call (single-note)							
PC1	0.24 %	0.356	0.458	0.778	(-0.54, 1.25)	0.598	0.481
PC2	0.21 %	0.027	0.401	0.068	(-0.76, 0.81)	0.004	0.958
PC3	0.12 %	-0.154	0.549	-0.280	(-1.23, 0.92)	0.073	0.797
PC4	0.10 %	-0.141	0.350	-0.402	(-0.83, 0.55)	0.152	0.754
Sentinel call (long-note)							
PC1	0.24 %	-0.143	0.554	-0.258	(-1.23, 0.94)	0.066	0.813
PC2	0.21 %	-2.247	0.637	-3.530	(-3.5, -1)	11.647	0.001
PC3	0.12 %	-0.154	0.549	-0.280	(-1.23, 0.92)	0.018	0.905
PC4	0.10 %	0.063	0.409	0.153	(-0.74, 0.86)	0.024	0.893
PC5	0.08 %	-0.254	0.405	-0.626	(-1.05, 0.54)	0.389	0.543

locomotion and feeding behaviour (Ricciardella et al., 2010), anti-predator behaviour (Brachetta et al., 2020), or exploratory behaviour (Carbajal et al., 2023). To the best of our knowledge, exogenous glucocorticoid treatment has only been shown to affect birds (Gaviraghi Mussoi et al., 2024; Perez et al., 2012) and amphibians (Leary et al., 2006; Leary and Crocker-Buta, 2018), with no treatment-related effect reported in mammals (Sehrsweeney et al., 2019; Simola et al., 2018). Together with our results, these findings suggest that, at least in mammals, glucocorticoids and vocalisations are discrete indicators of emotional arousal, without a direct functional interaction.

The inconsistent and often contrasting findings in the positive, negative, or total lack of effect from exogenous glucocorticoid administration, suggests that the physiological processes underlying the arousal response is more complex than often considered. A lack of effect of artificially increasing glucocorticoid levels may result from the fact that glucocorticoids are a product of the HPA axis activation response, rather than the sole driver of subsequent changes in physiology, behaviour and vocal production. Glucocorticoid levels do not create stress/increase arousal; instead, their production is part of the long-term body coping mechanism in response to a stressor (MacDougall-Shackleton et al., 2019). Glucocorticoids act to facilitate a shift in energy balance in order to minimize the effect of a stressor through changes in behaviour and physiology, and to prepare the body for future stressors (MacDougall-Shackleton et al., 2019; Romero and Wingfield, 2015; Sapolsky et al., 2000). Elevating glucocorticoid levels in the absence of a stressor, and other related products, may not be enough to elicit changes in affective arousal and observable responses. In addition, the

inconsistencies in findings resulting from exogenous glucocorticoid manipulation may be due to differences in administration method and dosages between studies.

Glucocorticoid levels are nevertheless an informative indicator of the scale of an individuals' response to a situation or stressor. Many studies have demonstrated the myriad of behavioural and vocal responses correlated to naturally occurring glucocorticoid levels. Changes in antipredator behaviour (Brachetta et al., 2020; Mateo, 2007), acoustics structure (Blumstein and Chi, 2012), provisioning rate (Carlson et al., 2004), foraging activity (Chmura et al., 2016; Zhang et al., 2020), mate choice choosiness (Baugh et al., 2021), vigilance behaviour (Voellmy et al., 2014) and risk-taking behaviour (Martins et al., 2007), have all been shown to correlate with glucocorticoid levels. While glucocorticoid levels can be meaningful in assessing the magnitude of an arousal response, glucocorticoids themselves seem not to be the sole drivers. As such, manipulation of glucocorticoid levels on its own may not be sufficient to alter an animal's physiological state to investigate arousal-based changes, and other physiological measures and manipulations may be more informative. In starlings, both heart rate and glucocorticoid levels did increase in response to stressors, however glucocorticoid responses were more consistent across different stressor types, whereas heart rate showed greater variation in relation to stressor type, possibly providing a better insight into the perception and risk of stressors (Nephew et al., 2003). In addition, in mice, *Mus musculus C57BL/6*, manually increasing heart rate using an optogenetic pacemaker to mimic an arousal response did not result in general increases in anxiety-like behaviours, but only in the presence of a stressor (Hsueh et al.,

2023). Previous work in meerkats has shown that, while natural faecal glucocorticoid levels are associated with higher vigilance levels, increasing exogenous glucocorticoid levels did not change vigilance levels, in line with our findings, and only resulted in an increase in latency to resume foraging following an alarm event (Voellmy et al., 2014). This suggests that glucocorticoid manipulation may not result in generalised baseline changes in behaviour, but may simply influence reactivity to stressors. Unfortunately, during our recording periods, not enough naturally occurring alarm events occurred to be able to examine the effects of glucocorticoid treatment on responses to stressors. It would be beneficial for future studies to simulate predator encounters, in order to compare the basal and stressor-related effects of glucocorticoid treatment.

Overall, elevating circulating plasma glucocorticoid concentrations was not sufficient in manipulating arousal-mediated physiological, behavioural or vocal responses in wild meerkats. The results add to the growing body evidence that suggests that studies should avoid simply conflating glucocorticoids and stress (MacDougall-Shackleton et al., 2019). To our knowledge, few other studies have taken such a combined approach in looking at the effect of glucocorticoids on physiology, behaviour and vocalisations in a wild, naturalistic setting. Future research should take a more integrative, holistic approach of physiological processes to improve our understanding of the precise arousal-related changes and how these are reflected in acoustic structure and behaviour in general.

CRedit authorship contribution statement

Isabel Driscoll: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elodie F. Briefer:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Andre Ganswindt:** Writing – review & editing, Resources. **Marta B. Manser:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2025.105676>.

Data availability

Effect of exogenous manipulation of glucocorticoid concentrations on meerkat heart rate, behaviour and vocal production (Original data) (Mendeley data)

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