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Faecal glucocorticoid metabolites and alarm calling in free-living yellow-bellied marmots

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When individuals of a variety of species encounter a potential predator, some, but not all, emit alarm calls. To explain the proximate basis of this variation, we compared faecal glucocorticoid metabolite concentrations in live-trapped yellow-bellied marmots (*Marmota flaviventris*) between occasions when they did and did not emit alarm calls. We found that marmots had significantly higher glucocorticoid levels when they called than when they did not call, suggesting that stress or arousal may play an important role in potentiating alarm calls. Marmots are sensitive to variation in the reliability of callers. The present finding provides one possible mechanism underlying caller variation: physiological arousal influences the propensity to emit alarm calls.

Keywords: alarm calling; glucocorticoids; stress

1. INTRODUCTION

When individuals of a variety of taxa encounter predators, they may emit alarm calls (Klump & Shalter 1984). Such calls may be directed to the predator to discourage pursuit, or directed towards conspecifics either to create pandemonium or to warn them about impending risk (Blumstein 2004, in press). Interestingly, individuals do not always call when they encounter a predator. This variation could be explained, in part, by variation in risk (MacWhirter 1992). In some species, animals call only when they are in safe locations (Hoogland 1996; Blumstein & Armitage 1997; Randall *et al.* 2000), or in response to certain predators (Owings *et al.* 1986). Additional variation might be explained by the direct and indirect fitness benefits (Brown 1987) of calling. Thus, mothers with vulnerable offspring are more likely to call because they have more to gain from warning their young (Blumstein & Armitage 1997) compared to individuals with no kin within earshot (Sherman 1977).

Further variation might be explained by the underlying physiological state of a potential victim. For example, animals may be more likely to call when they are more aroused or anxious than at other times. Thus, it may be predicted that alarm calling may be associated with, and perhaps even potentiated by, physiological indices of arousal or anxiety, such as elevated concentrations of glucocorticoid hormones (e.g. corticosterone or cortisol). Glucocorticoids are secreted by the adrenal cortex in response to stressors, play a critical role in energy metabolism, and may influence the expression of behaviour (Wingfield & Romero 2001). Motivational state or arousal is known to influence vocalization structure (e.g. Fischer *et al.* 1995, 2001; Weary & Fraser 1995; Fichtel *et al.* 2001). In support of the hypothesis that glucocorticoids potentiate calling, a previous study found that captive rhesus macaques (*Macaca mulatta*) treated with metyrapone, an inhibitor of glucocorticoid biosynthesis, were less likely to emit alarm calls than those treated with vehicle (Bercovitch *et al.* 1995). This study was based on only 12 captive animals, however; no studies have been conducted in the wild to determine whether glucocorticoids potentiate alarm calls in free-living animals.

We studied free-living yellow-bellied marmots. Individuals of this species often call when encountering potential predators but vary considerably in whether or not they call in a particular encounter (Blumstein & Armitage 1997; Blumstein *et al.* 1997). Moreover, when individuals are live-trapped, the pattern of alarm calling towards humans is similar to the pattern of calling under natural conditions (Blumstein *et al.* 1997; unpublished data 1980, 1985, 1991, 1995). Thus, trap-elicited calling is an ideal assay with which to study the proximate basis of calling.

2. MATERIAL AND METHODS

We studied marmots around the Rocky Mountain Biological Laboratory, Gothic, Colorado, USA (details in Blumstein & Armitage 1997). Between 2002 and 2004, marmots were routinely (approximately every other week) live-trapped in Tomahawk live traps set at burrow entrances (Armitage 1982). We noted whether subjects emitted alarm calls when we approached them in the trap. Animals were then transferred into a canvas handling bag and, over the next 2–10 min, were weighed, sexed, and if necessary, marked with ear tags and fur dye. Faeces found in traps, when we reached them, were collected in a plastic bag, immediately placed on ice and frozen at $-20\,^{\circ}\mathrm{C}$ within 2 h of collection. We assume (because marmots have relatively slow gut passage rates: Hume *et al.* 1993) that faecal glucocorticoid metabolites reflect levels pooled over time and, unlike concentrations in blood samples, are not influenced acutely by the capturing procedure. The lag time between secretion of hormones into the blood and excretion in the faeces is not known for marmots, but is 6–12 h in small rodents (mice and voles; Harper & Austad 2000).

We collected data from each of 29 adult (aged 2 years and older) female marmots on at least one occasion when it emitted alarm calls in the trap and at least one occasion when it did not call. For 12 animals, the 'calling' observation preceded the 'no calling' observation (18 ± 13 days, mean$\pm$s.d. latency between observations); for the remaining 17 animals, the 'no calling' observation preceded the 'calling' observation (9 ± 7 days). The Julian date did not differ (Wilcoxon $p=0.931$) between the 'no calling' (167 ± 21) and 'calling' (165 ± 14) observations, suggesting that seasonal variation in glucocorticoid levels (Place & Kenagy 2000) did not confound our results.

Faecal glucocorticoid metabolites were measured at the San Diego Zoological Society's research facility using a double-antibody ^{125}I radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA). This assay detects a variety of faecal metabolites of both corticosterone and cortisol commonly found in mammals (Wasser *et al.* 2000). Details of sample preparation and assay validation are in appendix A.

We treated individual marmots studied in different years as independent data points because more variation was explained by identity within a year than across years (see §3). If subjects called

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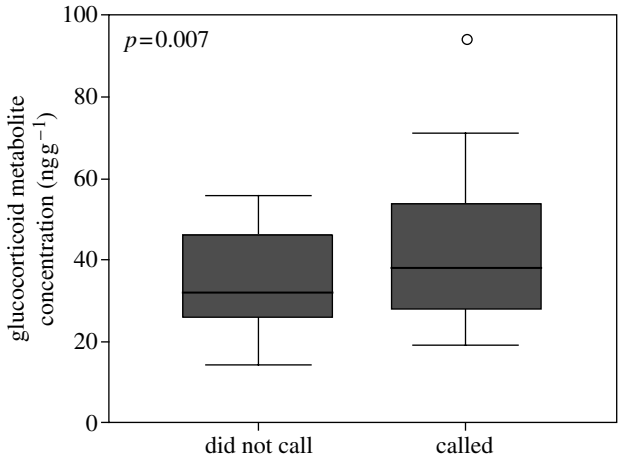

Figure 1. Box plots illustrating faecal glucocorticoid metabolite levels in yellow-bellied marmots on occasions when they did or did not emit alarm calls while restrained in a live trap. The circle illustrates an outlier.

on more than one occasion in a given year, we randomly selected one calling observation for analysis. We selected the 'no calling' observation randomly, subject to the following constraint: if possible, we did not select an observation that occurred on the day after a calling observation on the same animal, to avoid potential carry-over effects of trapping and restraint across days. In a separate dataset, however, faecal glucocorticoid metabolite concentrations were not altered by trapping and handling on the preceding day (day 1, 175.5 ± 66.0 ng g^{-1}; day 2, 195.5 ± 80.0 ng g^{-1}; Wilcoxon $p=0.421$, $n=13$).

3. RESULTS

The likelihood of calling showed a moderately strong (Cohen 1988) association with faecal glucocorticoid metabolite concentrations (Wilcoxon $p=0.007$, $d=0.52$; figure 1). Individual marmots had higher glucocorticoid metabolite levels when they emitted alarm calls than when they did not (Wilcoxon $p=0.007$, $n=29$). Removal of one outlier (an exceptionally high glucocorticoid value) did not influence these results ($p=0.013$, $n=28$).

Individual identity explained some, but not all, of the variation in glucocorticoid metabolites. For the 18 subjects measured six or more times in a given year (153 observations in total), we fitted a linear model with identity-year as the independent variable (i.e. a subject with six measurements or more in each of the 3 years was entered three times in this analysis) and glucocorticoid metabolite level as the dependent variable. We found that identity-year significantly ($p=0.032$) explained 18.6% of the variation in faecal glucocorticoid metabolites. When we combined years and focused only on subjects with six measurements or more (281 observations on 27 subjects), we found that no significant variation in glucocorticoid metabolites was explained by animal identity ($p=0.254$, $R^2=0.108$). Thus, marmots exhibited some individual consistency in their levels of faecal glucocorticoid metabolites within but not across years.

Glucocorticoid levels fluctuate throughout the day (e.g. Reeder & Kramer 2005). For 11 of our 29 subjects, the 'calling' observation occurred later in the day than the 'no calling' observation. There was no relationship, however, between a subject having higher faecal glucocorticoid levels when calling and whether or not the calling observation occurred later in the day (Fisher's exact probability $p=0.092$).

Reproductive status may also influence glucocorticoid levels (Reeder & Kramer 2005). Twenty-three of our 29 female subjects reproduced in the year they were studied. There was no relationship, however, between a subject's having higher faecal glucocorticoid metabolites when calling and whether or not the subject reproduced that year (Fisher's exact probability $p=0.237$).

4. DISCUSSION

We found that on occasions when individual yellow-bellied marmots emitted alarm calls in traps, they had systematically higher concentrations of faecal glucocorticoid metabolites than on occasions when they did not call. This result could not be explained by differences in time of season, time of day, or reproductive status between the 'calling' and 'no calling' observations. In addition to seasonal, circadian, and reproductive effects, glucocorticoid concentrations change acutely in response to physical and psychosocial stressors and are associated with arousal and anxiety (Reeder & Kramer 2005). Although we have not experimentally manipulated glucocorticoids, our findings are, to our knowledge, the first demonstration that variation in the propensity to emit alarm calls is associated with spontaneously occurring variation in glucocorticoid concentrations. Thus, in conjunction with Bercovitch *et al.*'s (1995) findings that pharmacological suppression of glucocorticoid synthesis inhibited alarm calling in rhesus macaques, our results suggest that intra-individual variation in glucocorticoids may be a significant source of variation in the likelihood that free-living animals will emit alarm calls. It is also possible that differences in glucocorticoid levels may contribute to inter-individual variation in calling, although confirmatory studies are required.

Our results further suggest that variation in glucocorticoid concentrations provides a plausible mechanism explaining variation in caller reliability, which in turn explains the maintenance, and perhaps the evolution of individuals' recognition of or differential responsiveness to individually specific alarm calls (Blumstein *et al.* 2004). Although the effect size was small, some variation in glucocorticoid metabolite concentrations was explained by caller identity. Thus, those individuals with systematically higher glucocorticoid levels may be more likely to emit calls and, by having a relatively low calling threshold, may emit calls in situations where there is little risk.

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APPENDIX A. DETAILS OF SAMPLE PREPARATION AND GLUCOCORTICOID ASSAY VALIDATION

(a) *Sample preparation*

A 0.2 g sample of faeces was mixed with 5 ml of 90% aqueous ethanol (ETOH, Gallade Chemical, Inc., Escondido, CA) in an 8 ml polypropylene vial (Sarstedt, Inc., Newton, NC). Samples were boiled for 20 min at 80 °C, then centrifuged for 20 min at 1500*g* at room temperature. The supernatant was decanted into 16×100 mm tubes and the pellet was suspended in 5 ml 90% ethanol, vortexed for 1 min, and again centrifuged. Supernatants were combined and dried in a vacuum centrifuge (Savant, Holbrook, NY), reconstituted in 1 ml of 100% ethanol (Gallade Chemical, Inc., Escondido, CA) and stored at 4 °C. For sample analysis, 25 µl of faecal neat was dried down and reconstituted in assay buffer.

(b) *Glucocorticoid assay*

Faecal glucocorticoid metabolites were measured with a double-antibody ^{125}I radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA). The primary antibody in this kit was raised against corticosterone and cross-reacts with a variety of faecal metabolites of both corticosterone and cortisol found in birds and mammals (Wasser *et al*. 2000).

(c) *Assay validation*

We validated the assay by assessing activity in sample-free media, and demonstrating parallelism between standard and unknowns, as well as by recovering glucocorticoids added to faecal samples. Limits were defined for assay sensitivity, accuracy, inter-assay variation and intra-assay variation.

In the glucocorticoid assay, buffer blanks had an immunoreactive content below the assay sensitivity. A serial dilution of the faecal extract was parallel with the glucocorticoid standard ($r=0.984$). The mean ($\pm$s.d.) recovery of hormone was $40.1\pm3.8\%$ ($n=10$) . Assay sensitivity was 16.94 pg per tube (calculated as mean pg per tube at 90% B/B_0, $n=10$). Accuracy was determined as $98.02\pm2.63\%$ by recovery of five known quantities of standard that were equivalent to 75% of the quantities used in the standard curve (25–1000 pg), plus a pool of faecal extract that had an immunoreactive content above the sensitivity of the assay (35 pg per tube). The inter-assay coefficient of variation (CV) was 7.37% ($n=9$), based on duplicates of a high pool binding at $30.5\pm1.7\%$, and 18.29% based on duplicates of a low pool binding at $58.0\pm4.2\%$. Estimates of the intra-assay CV, calculated from 10 replicates of the same pool in a single RIA, were 6.2% for the low pool and 12.3% for the high pool.

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