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Cortisol and corticosterone independence in cortisol-dominant wildlife

Lee Koren [a,c,*], Douglas Whiteside [a,d], Åsa Fahlman [a,e], Kathreen Ruckstuhl [b], Susan Kutz [a], Sylvia Checkley [a], Mathieu Dumond [f], Katherine Wynne-Edwards [a,c]

[a] Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada
[b] Department of Biological Sciences, University of Calgary, Alberta, Canada
[c] Hotchkiss Brain Institute, University of Calgary, Alberta, Canada
[d] Calgary Zoo, Alberta, Canada
[e] Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden
[f] Government of Nunavut, Canada

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ABSTRACT

Species have traditionally been defined as cortisol-dominant or corticosterone-dominant, depending on the glucocorticoid that is reported. To assess the degree of covariance versus independence between cortisol and corticosterone, 245 serum samples belonging to 219 individuals from 18 cortisol-dominant, non-domesticated species (6 mammalian orders) were compared by mass spectrometry. In these samples, which were elevated above baseline, concentration ranges were overlapping for cortisol and corticosterone although cortisol was dominant in every sample except one of 17 bighorn sheep with a corticosterone-biased cortisol-to-corticosterone ratio of 0.17. As expected, cortisol and corticosterone were strongly associated among species ($r^2 = 0.8$; species with high absolute cortisol tend to have high absolute corticosterone concentrations), with wide variation in the species-average cortisol-to-corticosterone ratio (range 7.5–49) and an even wider ratio range across individuals (0.2–341). However, only 9 out of 13 species with >7 individuals showed a positive association between cortisol and corticosterone among individuals, and repeated measures of the cortisol-to-corticosterone ratio within individuals were weakly associated (CV range 3–136%). We conclude that corticosterone, although at lower concentrations, has the potential to signal independently of cortisol, and should be included in integrated endocrine models of stress responses.

1. Introduction

The mammalian adrenal gland synthesizes multiple steroids with the primary end products being a glucocorticoid, a mineralocorticoid, and a weak androgen. In general, the predominant glucocorticoid produced is species-specific, and is either cortisol (e.g. humans) or corticosterone (e.g. rats, birds) [44]. However, even when cortisol is dominant, corticosterone is still synthesized as an essential intermediate in the mineralocorticoid synthesis pathway. Cortisol and corticosterone synthesis pathways also share enzymes, except that cortisol synthesis requires 17α-hydroxylase. Since 17α-hydroxylase is also essential for androgen and estrogen synthesis, the enzyme is present in the gonad of all species [16]. 17α-Hydroxylase is also essential for dehydroepiandrosterone (DHEA) synthesis so that complete suppression of 17α-hydroxylase activity in the adrenal cortex would also eliminate adrenal DHEA synthesis. Thus, corticosterone must be synthesized in cortisol-dominant species and cortisol is likely to be synthesized in corticosterone-dominant species.

Glucocorticoid synthesis is also not limited to the adrenal cortical tissues. Peripheral tissues and organs can locally synthesize glucocorticoids *de novo* from cholesterol (e.g., [37,41]). Some evidence also suggests that cortisol and corticosterone can vary independently in response to stress. For example, in tuco-tucos (*Ctenomys talarum*), cortisol and corticosterone exhibit different seasonal variation and responses to acute stress and captivity [43]. In several other small rodents, cortisol and corticosterone show independent seasonal changes (e.g., [7]). Older studies in pigs and golden hamsters also show independent cortisol and corticosterone circadian rhythms [2,8], and a switch from corticosterone-dominance to cortisol-dominance in rabbits in response to chronic stress [17,20]. Unfortunately, the literature reporting both cortisol and corticosterone concentrations from the same samples is limited in the scope of species represented (e.g., [21,29,31,32,34]). Thus, there is a gap in our understanding of covariation versus independence in the concentrations of the two glucocorticoids.

One of the challenges in closing this gap is methodological [24]. Glucocorticoid discrimination using antibody-based assay methods requires sample separation before quantitation because the

* Corresponding author. Address: Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1.
E-mail address: lee.koren@gmail.com (L. Koren).

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antibodies tend to cross-react with both glucocorticoids, which is problematic when the non-dominant glucocorticoid is at a much lower, or unknown, concentration. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) eliminates cross-reactivity since each steroid has a unique molecular mass-to-charge ratio, thus ensuring high specificity [13,24,38–40]. Another advantage of LC–MS/MS is its ability to analyze multiple steroids simultaneously [18,24], since sample volume is often limited (e.g. wildlife samples). Spiking with a bio-identical deuterated steroid before sample extraction and preparation can be used to correct for sample losses and yield precise quantitation.

The current study was conducted to (a) use the unambiguous discrimination of LC–MS/MS to determine the diversity in cortisol-to-corticosterone ratio and absolute concentrations in a range of mammalian species, and (b) test the hypothesis that cortisol and corticosterone would be positively associated within single, non-baseline samples as the result of linked synthesis and release pathways.

2. Materials and methods

Sample preparation and LC–MS/MS method for quantitation of cortisol and corticosterone, in a single run, was standardized among species using a method optimized for use in diverse mammalian and avian serum and plasma samples with unknown and variable interfering compounds, including serum lipids [24]. Details follow.

2.1. Chemicals and reagents

Cortisol and corticosterone were obtained from Sigma–Aldrich (St. Louis, MO, USA). Deuterium labeled internal standards cortisol-9,11,12,12-d4 (cortisol-d_4) and corticosterone-2,2,4,6,6,17α,21,21-d_8 (corticosterone-d_8) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). Optima-grade ethyl acetate, hexane, methanol and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Bond Elut® C18 (100 mg, 1 mL endcapped) solid phase extraction (SPE) cartridges were from Agilent Technologies (Santa Clara, CA, USA).

2.2. Preparation of calibrators, internal standards and quality controls

Defibrinated and 4× charcoal-stripped human serum (BioChemed Services, Winchester, VA) was used to prepare the calibration curves for quantitation of all serum samples. Stock solutions of each steroid and internal standard (IS) were prepared separately in methanol at 1.0 mg/mL. For the calibration curve, a mixture of the two steroids was diluted with stripped human serum to obtain a working concentration of 500 ng/mL cortisol and 50 ng/mL corticosterone. An eight-point calibration curve (7 calibrators and a blank) was prepared by further dilution in stripped human serum. In-house quality controls (QC) were independently combined and diluted into stripped human serum and aliquotted as a stock containing 10 ng/mL cortisol and 10 ng/mL corticosterone. Deuterated internal standards were combined and prepared in methanol to obtain a stock concentration of 20 ng/mL cortisol-d_4 and 10 ng/mL corticosterone-d_8.

2.3. Sampling

Samples from 18 species belonging to 6 mammalian orders were obtained from the Collaborative Group for Wildlife Samples. The cow, sheep, horse, cat and ground squirrels report cortisol values in the literature, hence the species sample was expected to yield a strong bias towards cortisol-dominance (cortisol-to-corticosterone ratio >1). For each species, samples were screened to contain only males and non-pregnant females over the age of one year. In addition, all samples were screened to exclude all steroid hormone manipulations (steroid therapies or contraception). Wood bison (*Bison bison athabascae*), Bactrian camel (*Camelus bactrianus*), Patagonian cavy (*Dolichotis patagonum*), Sri Lankan elephant (*Elephas maximus maximus*), Rocky mountain goat (*Oreamnos americanus*), Przewalski's wild horse (*Equus caballus przewalskii*), Vancouver Island marmot (*Marmota vancouverensis*), moose (*Alces alces*), red panda (*Ailurus fulgens*), Bengal tiger (*Panthera tigris tigris*), Siberian tiger (*Panthera tigris altaica*), and Grevy's zebra (*Equus grevyi*) samples were obtained from the Calgary Zoo's serum bank. These species were chosen because their reproduction is recorded but not manipulated. Captive reindeer (*Rangifer tarandus*) samples were from the University of Calgary, wild brown bear (*Ursus arctos*) samples were from Sweden, wild rock hyrax (*Procavia capensis*) were sampled in Israel, wild muskoxen (*Ovibos moschatus*) were sampled in Nunavut, Canada, captive white-tailed deer (*Odocoileus virginianus*) were from Saskatchewan, Canada, and wild bighorn sheep (*Ovis canadensis*) were from Alberta, Canada.

All blood samples were obtained opportunistically from ongoing sampling protocols. Various capture methods, restraint, and anesthetic protocols were used, depending on the species. For example, hunters on snowmobiles shot free-ranging muskoxen and samples were collected post-mortem; reindeer were sampled while restrained in a chute without anesthesia; rock hyrax were anesthetized following live trapping [22]; brown bears were darted from a helicopter; and bighorn sheep were ground-darted. In some free-ranging species, there was an intensive pursuit (e.g., [15]). Thus, given the well-established response of glucocorticoids to stress, all samples were assumed to represent an elevated, as opposed to a baseline, state for glucocorticoids.

2.4. Sample preparation

Blood was drawn from species-specific locations and serum was stored between −20 and −80 °C until analyzed. One hundred microliters of serum, calibrator, or quality control was spiked with 20 μL of deuterated IS followed by 400 μL of water. Sample preparation was done on an automated solid phase extraction (SPE) system from Gilson Inc. (GX-274 ASPEC™, Gilson, Middleton, WI). Briefly, each sample was applied to a 1 mL Bond Elut® C18 SPE cartridge previously conditioned with methanol and water. The sample-loading rate was 0.1 mL/min, and the samples were washed with 1 mL of water followed by 1 mL of hexane at a flow rate of 1 mL/min. The SPE cartridges were then dried for approximately 2 min and eluted with 1 mL of ethyl acetate at a flow rate of 0.1 mL/min. Solvents were evaporated to dryness under a stream of high purity nitrogen (Parker-Balston LCMS-5000NA Nitrogen Generator, Haverhill, MA) at 45 °C using a sample concentrator (Techne Inc., Burlington, NJ). The dry extracts were reconstituted in 100 μL of 50:50 methanol:water. Sample processing was conducted as 10 batched runs containing up to 36 samples per run.

2.5. LC–MS/MS conditions

Following sample preparation, 40 μL of the reconstituted sample (representing 40 μL of original serum sample) was injected into a 100 × 3.00 mm, 2.6 μm Kinetex® C18 HPLC column (Phenomenex, Torrance, CA) using an Agilent 1200 SL LC system with a thermostatted autosampler set at 4 °C (Agilent Technologies, Santa Clara, CA). The chromatographic separation was performed by a gradient elution (15 min) at a flow rate of 0.55 mL/min using water (mobile phase A) and methanol (mobile phase B). The LC system was coupled to an AB SCIEX QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Concord, ON, Canada) fitted with an

atmospheric pressure chemical ionization (APCI) source. The nebulizer current was set at 5 μA with a source temperature of 500 °C. Nitrogen was utilized as the curtain, drying and collision gases. The two steroids were monitored in positive ion mode using multiple reaction monitoring (MRM). Analyst software version 1.5 (AB SCIEX, Concord, ON) was employed for data acquisition and peak area integration. Quantitative results were calculated as an area ratio between the sample peak and the corresponding internal standard.

2.6. Method validation

Limit of quantification (LOQ: the lowest standard that we quantified; signal-to-noise ratio $\geqslant 10$) were 0.1 ng/mL for cortisol and 0.05 ng/mL for corticosterone. However, the limit of detection (LOD; signal to noise ratio $\geqslant 3$) for cortisol was lower, allowing us to assign two samples that had cortisol concentrations below the LOQ a concentration of 0.08 ng/mL.

Independent preparation of calibrators and QC from the same stock solution yielded an average accuracy of 104% for cortisol and 92.8% for corticosterone over the 8 runs. Intra-assay precision was determined by analyzing 6 replicates of a quality control sample in a single LC–MS/MS run. Co-efficient of variation was 5% for cortisol and 6.2% for corticosterone. Inter-assay variation was determined by running 8 replicates of a quality control sample on 8 different days, and was 11.3% for cortisol and 12% for corticosterone. For 15 of 18 species, all samples were processed within the same batch and day.

Extraction recoveries were calculated by comparing stripped human serum spiked before SPE (at the QC concentrations) with samples that were spiked after SPE. Recovery was 96% for cortisol and 101% for corticosterone. Carryover was determined by running a blank solvent after the highest calibrator and by injecting a QC after an animal serum sample. No carry-over was detected.

2.7. Statistical analysis

Descriptive statistics were employed as detailed in Section 3. To test the effects of order, family, species, and sex on the cortisol-to-corticosterone ratio we constructed several generalized linear models. We used the Akaike Information Criterion (AIC) to rank models [11,19]. This approach weighs models by the amount of the variance explained and model complexity (i.e., number of model parameters, K). When $n/K < 40$ the AIC values were corrected for small sample size (AIC_c) using the equation in Burnham and Anderson [11]. Level of support for an AIC_c value was evaluated by ΔAIC_c (i.e., $AIC_c = AIC_i - AIC_{min}$). Models with ΔAIC_c values of 0–2 are equally likely, whereas those with $\Delta AIC_c > 2$ are not supported [11]. We used the software packages JMP 8 (SAS Institute Inc.) and SPSS Statistics (IBM, Massachusetts Institute of Technology, USA).

3. Results

3.1. Detectable concentrations

In total, 245 samples from 219 individuals over 1 year of age, and neither pregnant nor reproductively manipulated, were used in analyses. Cortisol was detectable in all samples. However, corticosterone was below the LOD in 9 samples (all four Vancouver Island marmots, four brown bears and one bighorn sheep) and those samples were assigned a concentration of 0 ng/ml, precluding calculation of a glucocorticoid ratio. Concentration ranges were overlapping for cortisol (range 0.08–426 ng/ml) and detectable corticosterone (range 0.05–37.6 ng/ml).

3.2. Cortisol dominance

All species in the current study were cortisol dominant (glucocorticoid ratio = cortisol/corticosterone > 1) with the mean glucocorticoid ratio across species ranging from 7.5 to 49. Excluding the Vancouver Island marmots and other individuals without a calculable glucocorticoid ratio, the highest ratio seen (=341) was in an adult female moose (Fig. 1). The sole exception to this cortisol dominance pattern was a 3-year-old male bighorn sheep with a corticosterone-biased ratio of 0.17, which corresponds to a 5.9-fold surplus of corticosterone (Fig. 1). The other 16 bighorn sheep were cortisol dominant, including a single individual without detectable corticosterone. Although no species had all individual cortisol-to-corticosterone ratios below 10, five individuals representing two species had a cortisol-to-corticosterone ratio <2 and a further 25 individuals representing nine species had cortisol/corticosterone ratios between 2 and 10. Thus, there was a wide range of cortisol-to-corticosterone ratios between and within species (Fig. 1).

A nested generalized linear model with exponential distribution was the most appropriate (lowest $AIC_c = 1951.7$) to test the effects of order, family, species, and sex on the cortisol-to-corticosterone ratio. The overall model was significant ($p = 0.01$). Species ($\chi^2 = 24.2$; $p = 0.001$) and order ($\chi^2 = 17.11$; $p = 0.004$) were the only significant effects (all other $p > 0.1$).

3.3. Cortisol and corticosterone concentrations between species

For comparisons between species, multiple samples within individuals were averaged to enter a single value for each individual. Results were then averaged to yield a single value for each species. There was a strong, positive association between cortisol and corticosterone among species ($r^2 = 0.8$; $p < 0.0001$; Fig. 2). In other words, species at the highest cortisol concentrations also tended to have the highest corticosterone concentrations, and *vice versa*. Grevy's zebra, with 10 individuals represented, had the highest concentrations.

3.4. Cortisol and corticosterone concentrations within species

Of the 18 species in our database, 5 species were represented by four or fewer individuals and were excluded from subsequent analyses, leaving 13 species with at least 7 individuals (Fig. 3). Nine of the 13 species were well represented by a linear regression model in which cortisol and corticosterone were positively associated ($0.31 \leqslant R^2 \leqslant 0.91$, all $p \leqslant 0.05$: Table 1). For the remaining 4 species, however, there was no evidence supporting an association between cortisol and corticosterone within species. As the literature is inconsistent about the necessity for log transformation for glucocorticoid concentrations, several alternate transformations were also tested but were not superior models. Thus, the prediction that individuals with high cortisol would also have high corticosterone was only supported in nine of 13 species.

3.5. Sex differences

In our model, sex was not a predictor of glucocorticoid ratios. However, in brown bears (panel i of Fig. 3), for example, the highest concentrations for both glucocorticoids were found in samples from females. At a threshold alpha of 0.05, there were nine sex differences in cortisol-to-corticosterone ratio ($N = 1$ species), cortisol ($N = 5$ species), and corticosterone ($N = 3$ species), all of which were biased towards higher values in females. However, 8 out of 9 differences were at an alpha level between 0.03 and 0.05. Following a Bonferroni correction, the appropriate alpha threshold is 0.0013 for the 41 comparisons, so that even the higher female corticosterone in Przewalski's wild horse ($p \leqslant 0.002$) was not significant.

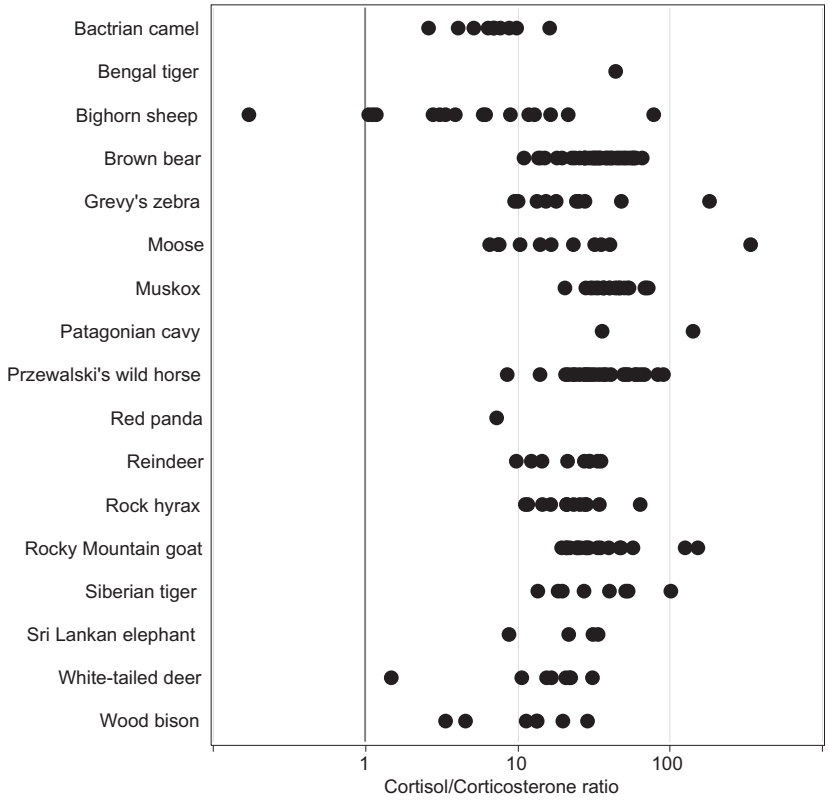

Fig. 1. Glucocorticoid ratio (cortisol/corticosterone on a logarithmic scale) for each individual, arranged by species. Multiple samples were averaged for individuals that had more than one sample. All values >1.0 show cortisol-dominance. Only one bighorn sheep falls in the corticosterone-dominant zone.

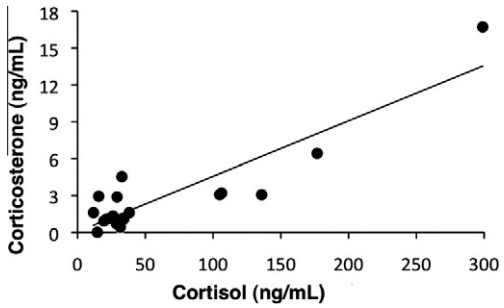

Fig. 2. Positive association among cortisol and corticosterone between 18 species. The equation for the linear regression is corticosterone = 0.048 + 0.045*x* cortisol.

Thus, the sole indication of sex differences was the absence of male-bias (0/41) and the preponderance of female-bias (9/41) relative to the null expectation (1/41 biased in each direction; $\chi^2 = 9.4$; df = 2; $p < 0.01$).

3.6. Cortisol and corticosterone concentrations within individuals

For 17 individuals, representing 6 species, we had two or more samples separated by at least 15 days, and often more than one year. The coefficient of variation (CV) for repeated measures ranged widely for each glucocorticoid and for the cortisol-to-corticosterone ratio (Table 2). On average, the CV for each parameter was approximately 50% of the mean value (range 0.6–141%). As species were processed within the same batch, the relevant intra-assay quantitation CVs were 5% for cortisol and 6.2% for corticosterone. Quantitation error was, therefore, not responsible for the within individual and species CVs.

4. Discussion

As expected, the LC–MS/MS method was successful in measuring both cortisol and corticosterone across a wide range of individuals and species [24]. Among species, high cortisol tended to be associated with high corticosterone, and *vice versa*, so that the relationship among species was well explained by a linear regression. The robustness of the linear regression is also supported by independent research quantifying both cortisol and corticosterone. For example, samples 30 min after handling in non-breeding yellow-pine chipmunks (*Tamias amoenus*), and Golden-mantled ground squirrels (*Spermophilus saturatus*) [21,29,34], yield results that fall close to the regression line, using alternate methods of glucocorticoid quantitation. This association was not surprising because the intrinsic activity levels of the shared biosynthesis and clearance enzymes between cortisol and corticosterone would tend to place each species at a similar relative position for each glucocorticoid [12]. It remains to be seen whether a reciprocal relationship is also present among corticosterone-dominant species.

Similarly, there was an *a priori* expectation that individuals within a species whose adrenal glands had high cortisol output were also likely to have high corticosterone output relative to individuals whose adrenals produced less [28]. The potential for differential individual stress responses was expected to enhance this positive relationship. Thus, an individual perceiving the handling situation as highly stressful was expected to have high cortisol, as well as high corticosterone, concentrations relative to other individuals of their species. This expected relationship, however, was confirmed in only 9 of 13 species. When present, the linear relationship explained between 31% (Przewalski's wild horse) and 91% (white-tailed deer) of the within species variation.

The remaining four species did not exhibit the expected positive association. Although Grevy's zebra was the species with the

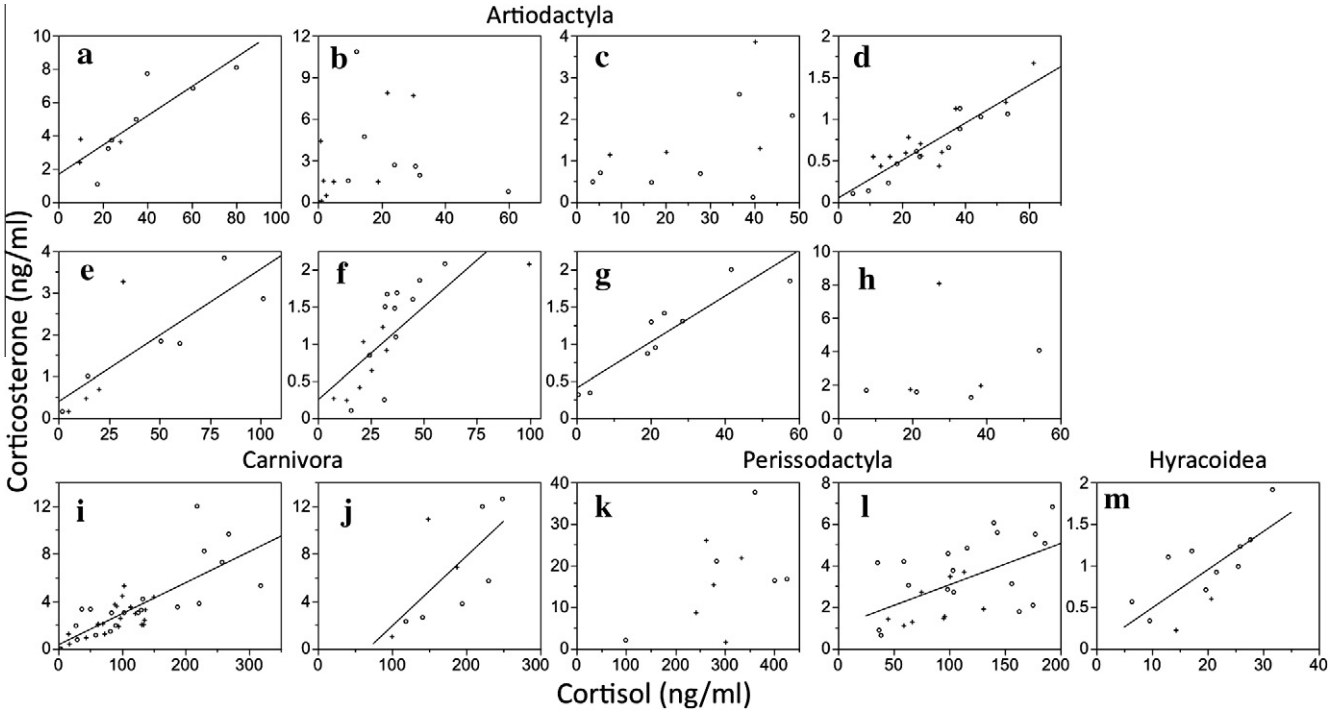

Fig. 3. The relationship between corticosterone and cortisol within 13 species. Species are: (a) Bactrian camel; (b) bighorn sheep; (c) moose; (d) muskox; (e) reindeer; (f) Rocky Mountain goat; (g) white-tailed deer; (h) wood bison; (i) brown bear; (j) Siberian tiger; (k) Grevy's zebra; (l) Przewalski's wild horse; (m) rock hyrax. Note that *x*-axis is cortisol (ng/ml), and *y*-axis is corticosterone (ng/ml) but each axis is scaled to display each species. Statistics relating to line-fitting (N, R^2, p) are presented in Table 1. Open circles indicate adult non-pregnant females and crosses indicate adult males.

Table 1
Association between cortisol and corticosterone within 13 mammalian species with at least seven individuals contributing samples to the database.

Fig. 3 panel	Order	Species	N	R^2 linear fit (p)
a	Artiodactyla	Bactrian camel	10	0.72 (0.002)
b	Artiodactyla	Bighorn sheep	17	0.03 (0.5)
c	Artiodactyla	Moose	11	0.26 (0.3)
d	Artiodactyla	Muskox	23	0.81 (<0.0001)
e	Artiodactyla	Reindeer	10	0.66 (0.004)
f	Artiodactyla	Rocky Mountain goat	19	0.6 (<0.0001)
g	Artiodactyla	White-tailed deer	9	0.91 (0.0007)
h	Artiodactyla	Wood bison	7	0.04 (0.65)
i	Carnivora	Brown bear	43	0.63 (<0.0001)
j	Carnivora	Siberian tiger	9	0.48 (0.04)
k	Perissodactyla	Grevy's zebra	10	0.24 (0.2)
l	Perissodactyla	Przewalski's wild horse	27	0.31 (0.003)
m	Hyracoidea	Rock hyrax	12	0.56 (0.005)

highest glucocorticoid concentrations, no relationship was found between cortisol and corticosterone. The absolute range of concentrations for each glucocorticoid was widest in this species, rather than restricted. Like the Grevy's zebra, two other species without a cortisol–corticosterone association, the wood bison and the moose, were sampled in the course of annual or bi-annual health assessment at the Calgary Zoo. Neither species was distinguished as an outlier for concentration range, relative to other members of the Order Artiodactyla or relative to a comparison of zoo versus non-zoo populations. The fourth species that failed to show the expected association was bighorn sheep. It is possible that sampling conditions were unique for this species because this wild population of bighorn sheep were acclimated to the presence of the researchers and generally remained calm while approached on foot or in a car before being darted (Fahlman, personal communication). However, like the wood bison and moose, the bighorn sheep were not distinctive in concentration range. Instead, they were distinguished by the diversity of cortisol-to-corticosterone ratios, from an individual with a strong corticosterone bias to an individual with undetectable corticosterone. Thus, in four of 13 species, the absence of the positive association between cortisol and corticosterone, which was expected based on shared level of stress-response and commonality of synthesis pathways, was not seen. At the opposite end of the spectrum, only three of the 13 species had a positive association between cortisol and corticosterone that explained at least 75% of the variance.

The current study was not designed to assess variability across repeated measures. Nevertheless, repeatability within individuals was notably weak with standard deviation typically around 50% of the average concentration. This pattern persisted in the coefficient of variation for the cortisol-to-corticosterone ratio. Thus, the few repeated measures available suggest that the cortisol-to-corticosterone ratio within individuals is as labile as the actual concentration. Variability between samples could reflect differential stress-axis activation, as well as other parameters as diverse as differential cortisol and corticosterone responses to anesthesia [30], seasonal and diurnal variations [25,33], and effects of social rank or body condition [3]. Although clearly not conclusive, these results suggest that a study design directed at repeated sampling within individuals might reveal additional lack of association or 'yoking' between cortisol and corticosterone concentrations across repeated measures.

Results, therefore, did not support the null hypothesis that concentrations of the non-dominant glucocorticoid would be explained by concentrations of the dominant glucocorticoid (e.g., [21,29,31]). Instead, results support the emerging literature recognizing independent concentrations [2,8,42,43]. Unfortunately, in the absence of an extensive literature with reliable, independent, measures of both glucocorticoids in the same sample, it is not yet possible to interpret the biological implications of this glucocorticoid 'independence'. Certainly, there are mechanisms that could dissociate the

Table 2
Coefficient of variation (CV in %) measures for within-individual repeatability. Glucocorticoid ratio is defined as cortisol/corticosterone.

Species	Number of samples	CV Cortisol	CV Corticosterone	CV Glucocorticoid ratio
Moose	2	38.8	82.0	104.2
Brown bear	2	113.5	100.6	30.0
Brown bear	2	118.6	141.4	136.2
Brown bear	2	114.8	116.0	3.3
Sri Lankan elephant	5	40.8	24.1	34.5
Sri Lankan elephant	7	54.1	58.6	31.8
Sri Lankan elephant	2	95.2	118.6	53.7
Rocky Mountain goat	2	45.9	70.3	29.1
Przewalski's horse	2	5.2	20.9	15.7
Przewalski's horse	2	0.6	6.2	6.8
Przewalski's horse	2	113.0	19.8	105.0
Przewalski's horse	2	2.5	20.9	18.4
Przewalski's horse	2	39.5	36.3	70.8
Przewalski's horse	3	25.5	43.9	17.5
Przewalski's horse	2	44.2	10.1	34.8
Grevy's zebra	2	17.5	32.3	48.4
Grevy's zebra	2	24.6	4.7	19.7
Mean CV	17	52.6	44.7	53.3

concentrations. For example, adrenal 17α-hydroxylase activity could be rate-limiting or corticosterone concentration could be subject to feedback regulation through mechanisms linked to aldosterone homeostasis [1]. The enzyme 11β-hydroxysteroid dehydrogenase might also have differential activity that would alter clearance. Sensitivity levels of cortisol and corticosterone to ACTH might also be different under diverse environmental conditions [43]. After all, gonadal steroids, angiotensin II, neuropeptides, neurotransmitters, opioids, growth factors, cytokines, adipokines and bacterial ligands, among others, are known to modulate adrenal glucocorticoid production, clearance, and release (e.g., [1,6]).

There are also mechanisms through which cortisol and corticosterone could affect differential signaling while simultaneously circulating in blood. For example, the majority of available glucocorticoids are bound to corticosteroid-binding globulins (CBG), whose concentrations and binding capacities vary widely with species, as well as diurnally, seasonally, in the presence of steroid hormones, pathological states, pH, and temperature [4,34,45]. CBGs generally display greater specificity for the dominant glucocorticoid [45]. Therefore, if CBG binding sites were preferentially occupied by cortisol in these species, free corticosterone might be more available in the circulation. Further, since plasma CBG capacity decreases with stress [9,46] (although albumin binding increases [5]), the binding relationship might also be altered within individuals as the stress response is activated. Thus, if cortisol and corticosterone have differential affinity for the binding globulins, they might have differential representation in the 'free' as opposed to 'bound' pool of glucocorticoids available for cellular uptake and receptor binding. Secondarily, competitive binding and different affinities for glucocorticoid and mineralocorticoid receptors could lead to different second messenger cascades. These two receptors share a common ancestor [26], substantial homology [10], and affinity for synthetic glucocorticoids used in medicine [27], all of which points to the potential for independent signaling [36] and independent functional roles [35].

Finally, there is likely to be a temporal component to the independence in cortisol and corticosterone concentrations. Blood samples reflect a narrow time frame, which can change in minutes, if not seconds [14]. Measures that integrate glucocorticoids over longer time frames (e.g., hair-testing; [23]) might therefore find a stronger association between glucocorticoids than serum, plasma, or saliva sampling. Multiple factors, from the half life, to circadian and annual rhythms, to life stage changes, may effect glucocorticoid concentrations [2,8,20,29,33,37,43] possibly in different directions, influencing the cortisol-to-corticosterone ratio within individuals and species. Temporal differentiation between glucocorticoids might, therefore, be important across a wide range of scales.

In this opportunistic sampling of mammalian species, the ability of LC–MS/MS to provide unambiguous discrimination between cortisol and corticosterone clearly identified a degree of independence between the two glucocorticoids that is worthy of further investigation. The extent of cortisol-to-corticosterone ratio variability within and between individuals was substantial. Given the potential mechanisms to transduce concentration differences into signaling differences, it is possible that the non-dominant glucocorticoid, in spite of its lower concentration, is an important adrenal steroid relevant to acute and chronic stress responses and their consequences for the health of humans and wildlife.

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