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LETTER

The role of herbivory in the macroevolution of vertebrate hormone dynamics

Phoebe D. Edwards,[1,2]* Nicholas Sookhan[1,2] and Rudy Boonstra[1,2]

[1]*Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, M5S 1A1, Canada*

[2]*Department of Biological Sciences, University of Toronto Scarborough, Toronto, ON, M1C 1A4, Canada*

**Correspondence: E-mail: phoebe.edwards@mail.utoronto.ca*

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Abstract

Vertebrates have high species-level variation in circulating hormone concentrations, and the functional significance of this variation is largely unknown. We tested the hypothesis that interspecific differences in hormone concentrations are partially driven by plant consumption, based on the prediction that herbivores should have higher basal hormone levels to 'outcompete' plant endocrine disruptors. We compared levels of glucocorticoids (GCs), the hormones with the most available data, across 166 species. Using phylogenetically informed comparisons, we found that herbivores had higher GC levels than carnivores. Furthermore, we found that the previously described negative relationship between GC levels and body mass only held in herbivores, not carnivores, and that the effect of diet was greatest at extreme body sizes. These findings demonstrate the far-reaching effects of diet on animal physiology, and provide evidence that herbivory influences circulating hormone concentrations. We urge future direct testing of the relationship between phytochemical load and GC levels.

Keywords

Chemical ecology, comparative biology, corticosteroid-binding globulin, cortisol, glucocorticoids, herbivory, phytochemicals, plant-herbivore interactions, secondary plant compounds.

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INTRODUCTION

Vertebrates have high variation in circulating hormone levels across species, and the evolutionary and functional drivers of these species differences are of great interest. Although such variation applies to many endocrine traits, species differences in glucocorticoid (GC) levels has arguably generated the most interest, and has been the subject of several recent meta-analyses (e.g. Jessop *et al*. 2013; Haase *et al*. 2016;Francis *et al*. 2018; Johnson *et al*. 2018; Martin *et al*. 2018; Vitousek *et al*. 2019). Glucocorticoids (corticosterone, cortisol, or both, depending on the species), are vertebrate steroid hormones that have pleiotropic effects primarily related to energy use. GCs are secreted in a circadian pattern to regulate activity patterns and waking behaviours, stimulating appetite and exploratory behaviour as well as basic metabolic processes and regulation of glucose availability (McEwen *et al*. 1988; Hau *et al*. 2016). They also play a well-documented role in responding to stressors by adaptively diverting energy towards survival and away from costly and non-immediately essential processes (Sapolsky *et al*. 2000). These physiological and behavioural effects are executed when GCs pass from the blood into tissues and bind to intracellular receptors, which act as transcription factors and affect the expression of thousands of genes (Sacta *et al*. 2016).

Despite the critical role of GCs, levels of circulating GCs show extreme inter-specific differences. For example, marmosets (*Callithrix argentata*) have blood GC concentrations approximately 80 times those of humans (Desantis *et al*. 2013). To explain such differences, several meta-analyses have focused on body size and metabolic rate as drivers of species variation, due to the essential role of GCs in modulating use of energy reserves. However, conclusions have been mixed. Baseline ('unstressed') GCs were found to be negatively related to body size in mammals and in in tetrapods as a whole, that is, smaller animals tend to have higher GC levels (Haase *et al*. 2016; Francis *et al*. 2018; Vitousek *et al*. 2019). Yet, no relationship was found between baseline GCs and body size in birds, reptiles, or amphibians analysed alone (Francis *et al*. 2018). Furthermore, no relationship between metabolic rate and baseline or stress-elevated GCs has been found across tetrapods nor within any group of tetrapods (Francis *et al*. 2018; Vitousek *et al*. 2019). These meta-analyses have revealed a great deal about the evolutionary and environmental factors that predict species GC levels, but there are still major unknown and untested drivers of species differences in GCs to explain the broad scale patterns. For example, to explain species differences in GCs in birds, Jessop *et al*. (2013) considered the roles of body mass, latitude, elevation, temperature and net primary productivity of the environment. Their best model could only account for 14% of the species variation in GC levels.

We propose that a key driver of species differences in GC levels is diet: specifically herbivory and therefore consumption of phytochemicals with allelopathic or endocrine disrupting effects. Herbivores, but not carnivores, need to buffer phytochemicals from passing into the tissues. With this, the function of their endogenous endocrine systems would be less effective, and therefore herbivorous species would need to compensate by increasing their levels of circulating hormones.

Phytochemicals (plant secondary metabolites) are biologically active compounds produced by plants. Plants produce many phytochemicals that are similar in structure to vertebrate hormones, and can thus interact with vertebrate endocrine receptors and binding proteins as endocrine disruptors (Lambert & Edwards 2017). Such hormonally active

phytochemicals have been found in all plant tissue types - roots, stems, leaves, seeds, flowers, and fruit- and their tissue-specific distribution depends on the species, plant life-stage, and environmental triggers (Lambert & Edwards 2017). Although most research in endocrine disrupting phytochemicals has focused on phytoestrogens, there is also evidence that there are a variety of plant metabolites that act as phytoglucocorticoids (Leung *et al.* 2007; Wasserman *et al.* 2013; Martini *et al.* 2016), and that these are common in some animal diets (Wasserman *et al.* 2013).

Vertebrates that consume plants have two direct options to prevent endocrine disrupting phytochemicals from binding to their hormone receptors: either increase levels of binding proteins in the blood as a phytochemical sink or decrease tissue sensitivity to prevent phytochemicals from entering cells or interacting with receptors. The first strategy has been proposed to explain the evolution of albumin, which is the most abundant carrier protein in the blood, but has a low specificity (Peters 1995). This results in albumin's general binding or 'fuzzy recognition' of a variety of hormones and other lipophilic compounds. Baker (1998, 2002) proposed that due to this generality, albumin acts as a sink for phytochemicals and other endocrine disruptors in the environment by binding to them and thus preventing them from interacting with receptors. Supporting this hypothesis is experimental evidence that the presence of albumin decreases xenoestrogen binding to oestrogen receptors (Arnold *et al.* 1996). The same principle could be extended to the GC carrier protein, corticosteroid-binding globulin (CBG), which has a high specificity and affinity for GCs (Seal & Doe 1966; Westphal 1986). Phytochemicals that are similar enough in structure to glucocorticoids should be able to be compete for and bind to CBG. Although the binding of phytoglucocorticoids to CBG has not been specifically tested to our knowledge, some phytoestrogens are known to bind to sex-hormone binding globulin, which, like CBG, also has high specificity and high affinity relative to albumin (Jury *et al.* 2000).

If herbivores use the first strategy and increase CBG levels as a phytochemical sink, they should have higher levels of CBG than carnivores. If herbivores use the second strategy and decrease tissue sensitivity, they should have no difference in CBG levels relative to carnivores. However, in both cases, herbivores should have higher levels of circulating GCs than carnivores, because of the disrupted access to GC receptors whether by increased CBG binding or by mechanisms within the tissues. We tested these predictions with previously published databases (Desantis *et al.* 2013; Vitousek *et al.* 2019) of species levels of total circulating GCs (both bound and unbound GCs), free GCs (unbound only), and CBG levels. We compared GC levels of species that were herbivorous, eating almost entirely plant matter, to species that were carnivorous, eating virtually no plant matter. 166 species in these GC datasets fell into these dietary categories, with 53 herbivores and 113 carnivores (Fig. 1). We used generalised linear mixed effect models with Markov Chain Monte Carlo sampling (MCMCglmm) to compare species levels of total GCs, free GCs, and CBG by diet type, accounting for phylogeny and body mass. For a portion of species in the dataset ($n = 117$ species), additional factors were reported in the original studies during hormone sample collection: individual sex, breeding status, dominant GC type (cortisol or corticosterone), and whether the samples were collected as true baseline levels or collected after potential trapping stress ('nominal baseline'). With this smaller dataset, we tested if the same dietary patterns held when accounting for these factors.

METHODS

Species information

To test the association among species diet and total GCs levels, hormone data were compiled from Desantis *et al.* (2013) and from HormoneBase (Vitousek *et al.* 2018). HormoneBase contains not only species with baseline GC data, but also species with stress-induced GC data and testosterone data. We used a subset of HormoneBase which included only the baseline total GC data, published by Vitousek *et al.* (2019). Species levels of CBG and free GCs were taken from Desantis *et al.* (2013) only, as HormoneBase does not contain information on CBG levels. CBG levels in Desantis *et al.* (2013) are reported as maximum corticosteroid-binding capacity (MCBC); this measure is described in detail in Delehanty & Boonstra (2009). 13 species were duplicated in both the Desantis *et al.* (2013) database and the Vitousek *et al.* (2019) database, the values from Vitousek *et al.* were dropped to avoid repeated sampling of the same study. This combined dataset resulted in 269 species.

We compiled dietary information for each species from EltonTraits (Wilman *et al.* 2014), The Birds of North American (Rodewald 2015), AmphiBIO (Oliveira *et al.* 2017), Animal Diversity Web (https://animaldiversity.org), and the primary literature. Primary studies were those where stomach contents were dissected and separated into percent composition of contents, or time foraging was separated into percent of time spent on particular food items. Adult diet was used. References for all dietary information can be found in the Supplementary materials. We then grouped food items into the following dietary categories: animal matter (vertebrate and invertebrate), and plant matter (of all types, including shoots, stems, leaves, fruits, seeds, exudates). Fungi were included in the plant matter category, as fungal consumption was typically not reported as its own category in dietary studies (e.g. O'Brien & Kinnaird 1997; Okecha & Newton-Fisher 2006) and thus there were not enough data on fungi alone to include them separately. Fungi also produce a variety of secondary compounds and chemical defences (mycotoxins), some of which are known to be endocrine disrupting. For example, the mycotoxin Zearalenone is a notorious estrogenic endocrine disruptor, and its metabolites have also been found to induce the production of cortisol (Kowalska *et al.* 2016). Some studies have proposed that many phytochemicals in terrestrial plants may be derived from fungi, either by direct transfer or horizontal gene transfer (Lehtonen *et al.* 2005; Wink 2008). Hence, we determined that combining fungi and plants consumption as a source of phytotoxins was warranted from both a biological and methodological standpoint.

We recorded the maximum percent of animal or plant matter that made up in the diet for each species, as our goal was to examine maximum potential exposure to plant phytochemicals. For example, if a species had a diet of 20% plant matter

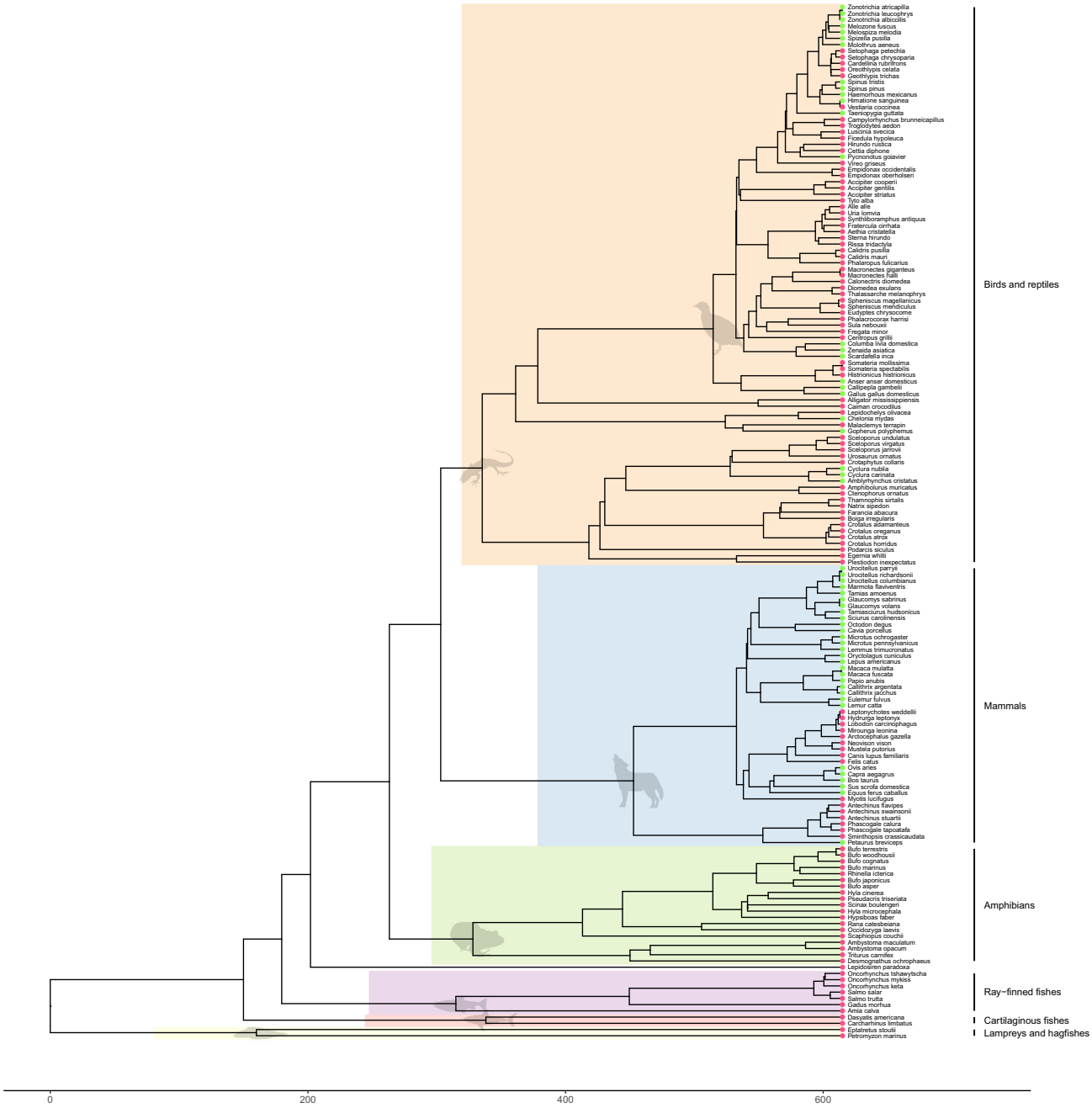

Figure 1 Time-calibrated ultrametric phylogeny of herbivorous (green dots) and carnivorous (red dots) species from Desantis et al. (2013) and Vitousek et al. (2019). 166 species are represented: 53 herbivores and 113 carnivores.

in the winter, and 60% plant matter in the summer, the maximum percent plant matter was recorded as 60%. We then categorised carnivores and herbivores in the dataset. We considered a species to be a carnivore if it had 10% or less maximum plant matter consumption (we assumed values of 10% or less constituted accidental ingestion). In the same way, we considered a species to be a herbivore if it had 10% or less maximum animal matter consumption. Species that did not fall into either dietary category were not used in the main analysis, but we include an analysis with a mixed omnivorous category in the supplemental material (Fig. S1, Table S1 and S2). The herbivore and carnivore categorisation resulted in a total GC dataset of 166 species: 45 mammals, 63 birds, 27 reptiles, 19 amphibians, and 12 fish (Fig. 1). 53 of these species were herbivores and 113 were carnivores. The CBG dataset contained 72 species, with 34 herbivores and 38 carnivores, and the free GC dataset contained 45 species, with 22 herbivores and 23 carnivores. All dietary breakdowns and assignments can be found in the associated supplemental dataset. If dietary information for a given species could not be found (n = 25 species out of the original 269 in the combined dataset), the species was not used and is not included in the above

sample sizes. Species excluded for this reason are noted in the supplemental dataset, with further notes given.

Species body mass was taken directly from the Vitousek *et al.* (2019) dataset and from the above databases (Animal Diversity web, AmphiBIO, etc.). If body mass was not listed for a given species in any of these sources, the reference for body mass is given in the supplemental dataset. Information for other factors (sex, nominal or true base, breeding status) were taken directly from the Vitousek *et al.* (2019) and Destantis et al. (2013) datasets. Many types of breeding status were reported in the latter dataset, and we consolidated them to simply 'breeding' or 'not breeding'. For example, when an animal was listed as pregnant or lactating, we assigned them to "breeding." These assignments are also reported in the supplemental dataset.

Phylogeny construction

An ultrametic time-calibrated phylogeny containing the 166 species was assembled. Lineage specific phylogenies were grafted onto a backbone phylogeny that contained a representative species of each lineage found in the dataset. The backbone phylogeny was constructed using the Hedges *et al.* 2015 supertree, using the 'TIMETREE' online tool (http://www.timetree.org). Hagfish and cartilaginous fish are not represented in the Hedges *et al.* 2015 supertree. Representative species for these lineages were manually grafted onto the backbone phylogeny, node dates were estimated using the 'NODETIME' tool (http://www.timetree.org) developed by Kumar *et al.* (2017). The following lineage species ultrametric time-calibrated phylogenies were used: cartilaginous fishes (Stein *et al.* 2018), ray-finned fishes (Rabosky *et al.* 2018), amphibians (Jetz & Pyron 2018), turtles (Jaffe 2011), crocodilians (Oaks 2011), squamates (Zheng & Wiens 2016), birds (Jetz *et al.* 2012), and mammals (Upham et al. 2019).

Statistical analyses

We first tested the effect of diet type on species levels of total GCs, CBG, and free GCs, while accounting for phylogeny and body mass. Generalised linear mixed effect models with Markov Chain Monte Carlo sampling (MCMCglmm) were constructed to estimate the relationship between these endocrine traits and diet. Analyses were conducted using the package MCMCglmm (Hadfield 2010) in the R v3.5.2 statistical environment (R Core Team 2019). Hormone data (ng/ml) were natural log transformed prior to the fitting of models. Body mass (g) data were also log transformed. Dietary category, body mass, and the dietary category and body mass interaction effect were fit as a fixed effect with carnivores treated as the intercept.

Phylogenetic structure was accounted for by fitting a species level inverted relatedness matrix as a random effect. Priors for random effects were set to an inverse gamma distribution (V = 1, nu = 0.002). The error distribution was modelled with the Gaussian family. Models were run for 1,000,000 iterations with a burn-in period of 50 000 and a thinning interval of 200. Trace, density and auto-correlation function (ACF) plots were inspected to assess stationarity in the MCMC chains. MCMC chains were assessed to ensure autocorrelation values less than 0.1 (Hadfield 2019). The models were fit two subsequent times and the Gelman-Rubin statistic diagnostic plots were used to assess convergence of the three MCMC chains (Gelman & Rubin 1992). Deviance information criterion (DIC) was used to compare fitted models to intercept-only models. Post-hoc analysis of diet categories was completed through pairwise comparisons of the marginal estimate for each dietary category. The calculation of marginal estimates was completed using the emmeans package (Lenth 2018). Models were re-fit with strong priors (V = 1, nu = 1) to ensure that results were not sensitive to prior specification (Wilson *et al.* 2010).

We additionally aimed to test for the effects of other potentially important factors related to GC levels: sex, breeding status, primary glucocorticoid type (cortisol or corticosterone) and if the hormone samples were taken at true baseline or nominal. True baseline indicates the animal was sampled in < 3 min of capture, and thus samples should not reflect trapping stress, and nominal baseline indicates that these animals were sampled > 3 min after capture and thus these levels may reflect stress caused by live-trapping. Out of the 166 species in the total GC dataset, all of these factors were reported for 117. Out of the 72 species in the CBG dataset, and the 45 species in the free GC dataset, these factors were only reported for 22 and 21 species respectively. Thus, we did a second analysis of these smaller, filtered datasets where all factors could be included. In these models, the response factors were log GCs, CBG and free GCs, and the fixed effects were dietary category, log body mass (g), the dietary category and body mass interaction, sex, reproductive status (breeding or not breeding), sampling type (nominal or true base) and primary glucocorticoid type (cortisol or corticosterone). Phylogenetic structure was accounted for in the same manner as described above.

RESULTS

Species level diet and glucocorticoids

This first analysis examined the effect of dietary category on levels of total GC (n = 166 species), free GC (n = 72 species) and MCBC levels (n = 45 species) while accounting for body mass and phylogeny by MCMCglmm. Model summaries of the best-fit model (according to DIC score) for each response variable are reported in Table 1. Data are presented as the posterior mean and 95% CI. For total GCs, the best-fit model was the one which included diet, body mass, and the diet and body mass interaction (DIC = 508.92). This was relative to the diet only model (DIC = 509.09), the null model (DIC = 509.26), and the mass only model (DIC = 509.78). Herbivores had higher total GC levels than carnivores (β = 1.52 [0.50, 2.60] ng/ml, $P < 0.01$). The effect of body mass on species total GC levels was dietary category dependent (Fig. 2a), with a significant dietary category and mass interaction (β = −0.22 [−0.39, −0.07] ng/ml, $P < 0.01$). Thus, the effect of body mass on total GCs was negative for herbivores (slope of the fit-line and credible interval: −0.17 [−0.31, −0.03]) but neutral for carnivores (0.05 [−0.07, 0.15]) as the credible interval crosses zero. This means that small

Table 1 Summaries from MCMCglmm where glucocorticoid levels (ng/ml) were fit to dietary category (carnivore or primarily herbivore), log body mass (g), and the interaction effect of dietary category and log body mass. Data were binned at the species level.

Response variable	Parameter	Posterior mean [95% CI]	Effective sampling	*p* MCMC
Total glucocorticoids (log ng/ml)	**Intercept**	**3.85 [1.81, 5.84]**	**4750**	**< 0.01**
	Diet–herbivore	**1.52 [0.50, 2.60]**	**4750**	**< 0.01**
	Mass (log g)	0.05 [−0.07, 0.15]	4967	0.43
	Diet × mass	**−0.22 [−0.39, −0.07]**	**4750**	**< 0.01**
	Phylogeny	3.44 [1.48, 5.60]	4750	–
	Residual	0.95 [0.65, 1.26]	4750	–
Free glucocorticoids (log ng/ml)	Intercept	1.56 [−3.72, 7.13]	4332	0.51
	Diet–herbivore	3.53 [−1.01, 7.70]	4750	0.11
	Mass (log g)	0.02 [−0.34, 0.42]	4750	0.90
	Diet x mass	−0.41 [−0.94, 0.13]	4750	0.13
	Phylogeny	21.23 [2.32, 51.63]	3983	–
	Residual	1.16 [0.00, 2.56]	4483	–
Maximum corticosteroid-binding capacity (log ng/ml)	Intercept	2.01 [−0.71, 4.58]	5034	0.12
	Diet–herbivore	1.63 [−0.31, 3.37]	4750	0.09
	Mass (log g)	0.15 [−0.01, 0.31]	4750	0.05
	Diet × mass	**−0.25 [−0.49, 0.04]**	**4750**	**0.03**
	Phylogeny	5.24 [0.56, 10.36]	4380	–
	Residual	0.57 [0.16, 1.08]	4471	–

MCMCglmm were fit to: **(a)** log total glucocorticoid levels (n = 166, $r^2_{(m)}$ = 0.02, $r^2_{(c)}$ = 0.79), (b) log free glucocorticoid levels (n = 45, $r^2_{(m)}$ = 0.03, $r^2_{(c)}$ = 0.95), and **(c)** log maximum corticosteroid-binding capacity levels (n = 72, $r^2_{(m)}$ = 0.03, $r^2_{(c)}$ = 0.90).
Bold parameters indicate p MCMC < 0.05.

herbivores tend to have higher GC levels than large herbivores, but carnivores have no change in GC levels across body sizes. For MCBC and free GCs, the full model was again the best-fit model. There was a dietary category and mass interaction effect on species MCBC levels (β = −0.25 [−0.49, −0.04] ng/ml, P < 0.05). However, no significant effect of dietary category alone was found on species MCBC or free GC levels (P = 0.09 and P = 0.11 respectively).

Additional factors affecting glucocorticoid levels

In the subset of the dataset where additional factors during sample collection were reported, we tested the effects of diet category, body mass, sex, breeding status, sampling type (nominal vs. true base) and dominant GC type (cortisol vs. corticosterone). This subset of the dataset included 117 species with total GC data, 22 species with MCBC data, and 21

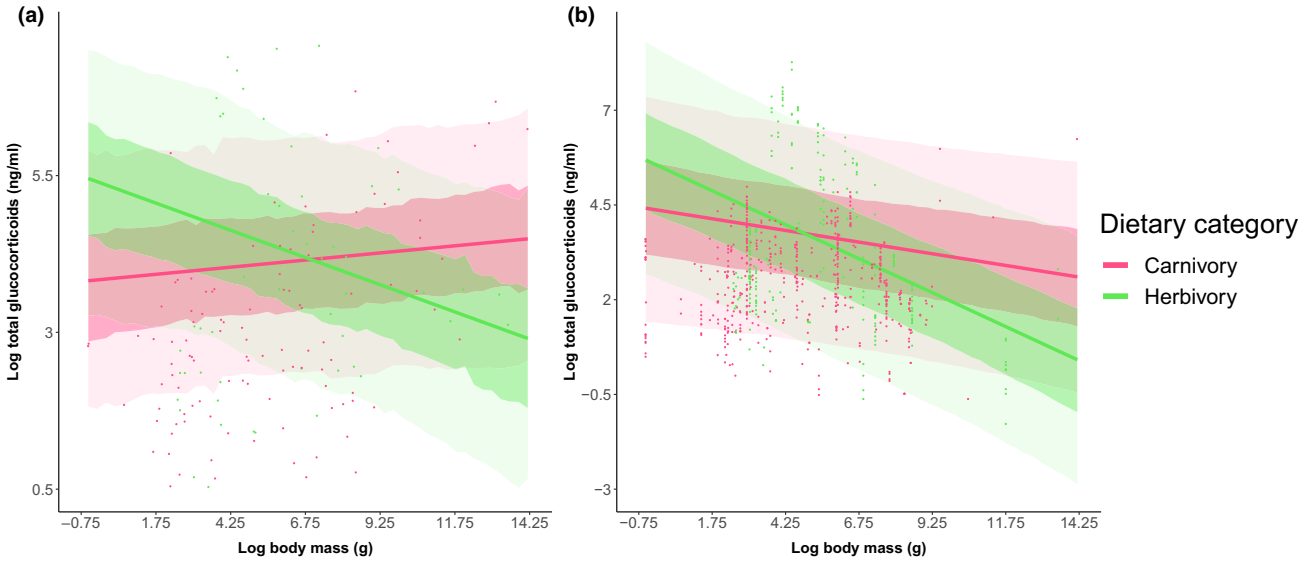

Figure 2 (a) MCMCglmm model estimated effect of dietary category by log body mass (g) on log total glucocorticoid levels (ng/ml). Data were binned at the species level. Ribbons denote the 95% credible interval of the estimate. Herbivores (n = 53) and carnivores (n = 113) are colored green and red, respectively. (b) MCMCglmm model estimated effect of dietary category by log body mass (g), sex, breeding status, sampling type (true or nominal base), and glucocorticoid type (cortisol or corticosterone) on log total glucocorticoid levels (ng/ml). Data were binned at the individual level (n = 723 sampling points) and cover 33 herbivores and 84 carnivores.

Table 2 Summaries from MCMCglmm where glucocorticoid levels (ng/ml) were fit to dietary category (carnivore or herbivore), log body mass (g), the interaction effect of dietary category and log body mass, sex (female, male), base (true, nominal) and breeding status (not breeding, breeding). Data were binned at the individual level.

Response variable	Parameter	Posterior mean [95% CI]	Effective sampling	*p* MCMC
Total glucocorticoids (log ng/ml)	**Intercept**	**3.77 [0.62, 6.79]**	**4691**	**0.02**
	Diet - herbivore	**1.15 [0.10, 2.18]**	**4750**	**0.03**
	Mass (log g)	−0.12 [−0.27, 0.02]	4750	0.10
	Sex - female	−0.06 [−0.18, 0.06]	5188	0.33
	Status - breeding	**0.24 [0.09, 0.40]**	**4964**	**< 0.01**
	Sampling - nominal base	**1.30 [0.90, 1.68]**	**4750**	**< 0.01**
	GC type - corticosterone	-0.32 [−2.19, 1.73]	4750	0.74
	Diet × mass	**-0.24 [−0.42, −0.04]**	**4750**	**0.02**
	Phylogeny	7.05 [4.35, 10.03]	5338	–
	Residual	0.48 [0.42, 0.53]	4750	–
Free glucocorticoids (log ng/ml)	Intercept	-0.30 [−11.33, 9.73]	5216	0.98
	Diet - herbivore	3.57 [−5.58, 11.72]	4750	0.41
	Mass (log g)	0.19 [−0.56, 0.91]	5095	0.60
	Sex - female	-0.34 [−0.75, 0.06]	4806	0.10
	Status - breeding	-0.27 [−0.76, 0.22]	4750	0.28
	Sampling - nominal base	**1.88 [1.23, 2.59]**	**4750**	**< 0.01**
	GC type - corticosterone	-0.70 [−6.62, 5.21]	4951	0.80
	Diet x mass	-0.52 [−1.47, 0.38]	4750	0.25
	Phylogeny	44.80 [12.12, 87.43]	4750	-
	Residual	0.86 [0.60, 1.13]	4420	-
Maximum corticosteroid-binding capacity (log ng/ml)	**Intercept**	**4.69 [1.39, 8.11]**	**4750**	**0.01**
	Diet - herbivore	−1.00 [−2.89, 0.95]	4750	0.26
	Sex - female	**0.58 [0.32, 0.83]**	**4558**	**< 0.01**
	Status - breeding	-0.29 [−0.59, 0.00]	4750	0.06
	Sampling – nominal base	0.17 [−0.23, 0.54]	4750	0.40
	GC type - corticosterone	1.90 [−0.39, 4.12]	4750	0.09
	Phylogeny	6.76 [1.32, 14.58]	4523	–
	Residual	0.40 [0.29, 0.53]	4523	–

MCMCglmm were fit to: (a) log total glucocorticoid levels (n = 117 species, 723 sampling points, $r^2_{(m)}$ = 0.07, $r^2_{(c)}$ = 0.94), (b) log free glucocorticoid levels (n = 21 species, 105 sampling points, $r^2_{(m)}$ = 0.02, $r^2_{(c)}$ = 0.98), and (c) log maximum corticosteroid-binding capacity levels (n = 22 species, 117 sampling points, $r^2_{(m)}$ = 0.09, $r^2_{(c)}$ = 0.95).
*Bold parameters indicate p MCMC < 0.05.

species with free GC data. Best-fit model summaries are reported in Table 2. For total GCs, the best-fit model was the omnibus model (i.e. all factors; DIC = 1603.75), relative to the model with all factors except diet (DIC = 1606.09), the model with all factors except the diet/mass interaction effect (DIC = 1606.26), and the null model (DIC = 1651.12). Again, herbivores had higher total GC levels than carnivores (β = 1.15 [0.10, 2.18] ng/ml, $P < 0.05$) and there was an interaction between dietary type and body mass (β = −0.24 [−0.42, −0.04] ng/ml, $P < 0.05$) indicating that the relationship between body mass and total GCs was dependent on dietary category (Fig. 2b). In this model, again herbivores had a negative relationship between GC levels and mass (slope and credible interval: −0.36 [−0.53, −0.17]), and carnivores had no change in GC levels across body sizes (−0.12 [−0.27, 0.02]). Sampling type and breeding status also affected total GC levels, with higher levels at nominal base than true base (β = 1.30 [0.90, 1.68], $P < 0.01$) and higher levels in breeding than nonbreeding individuals (β = 0.24 [0.09, 0.40] ng/ml, $P < 0.01$). Sex and GC type did not have an effect on total GC levels (P = 0.33 and P = 0.74 respectively).

The best-fit model for MCBC included diet and the other factors (sex, breeding, etc.), but not body mass or the body mass and diet interaction. The only factor that had an effect on MCBC levels was sex, with females being higher (β = 0.58 [0.32, 0.83], $P < 0.01$). The best-fit model for free GCs was the omnibus model and the only factor that had an effect on free GCs was sampling type, with nominal base being higher (β = 1.88 [1.23, 2.59], $P < 0.01$).

DISCUSSION

We have shown that diet is a key factor associated with the macroevolution of species differences in total GC levels. Phylogenetically informed comparisons supported the relationship between diet type and total GC levels, with herbivores having higher total GC levels than carnivores. Furthermore, the best-fit models always included dietary category. The individual-level analysis on the smaller dataset (Table 2) highlighted that sampling type (nominal or true base) and breeding status do have an effect on total GC levels across vertebrates, and these factors are critical to record and account for. However, ultimately both the species level total GC analysis and the individual level total GC analysis supported our central prediction: that herbivores have higher GC levels than carnivores.

We found no species differences in CBG and free GCs for the two dietary categories, and therefore no evidence that changes in glucocorticoid binding generally acts as a phytochemical

buffer in herbivorous species. The only factor tested in these analyses that affected CBG levels was sex, with females having higher levels than males. While there could be species differences in albumin levels based on diet, and albumin binds to GCs, we do not expect that interspecific changes in albumin should drive changes in circulating hormone concentrations. Fluctuations in albumin levels do not appear to greatly affect free GC levels due to the low affinity/high dissociation between albumin and GCs (Gudmand-Hoeyer & Ottesen 2018). Yet, it is important to acknowledge the small sample size in the CBG and free GC analyses, which had only a fraction of the species that were present in the total GC analysis. With species level *p*MCMC values of 0.09 for CBG dietary category and 0.11 for free GC dietary category, we question if the effect of diet may have reached significance with a more robust, high sample size dataset. However, no such data exist, and we urge the collection of CBG data in future studies.

The impact of dietary category on total GC levels supports predictions for the herbivore strategy based on reduction of tissue sensitivity and compensatory increase in GC levels. A potential example of this strategy can be seen in the New World monkeys, which have exceptionally high GCs levels and low CBG binding capacity (Pugeat *et al*. 1984; Fuller *et al*. 2004). New World monkeys are known to have tissue-level resistance to GCs and several other hormones (Fuller *et al*. 2004). The mechanism for GC resistance has been demonstrated in three species of New World monkey which have reduced glucocorticoid receptor function due to an over-expression of the immunophilin FKBP51, which inhibits transcriptional activity (Scammell *et al*. 2001; Stechschulte & Sanchez 2011). These relationships demonstrate that to understand the evolution of species circulating hormone levels, it is critical to look at other components of endocrine signalling systems, not just total circulating hormone concentrations. Here we test only the signal in plasma, not the reception at the cellular level. While such testing remains difficult because of many possible mechanisms that could exist to limit receptor action, it would be informative to compare receptor expression levels and co-factor levels across a subset of species, or to examine specific nucleotide and amino acid changes in endocrine traits to test for directional selection (Bonett 2016).

Another major result of our analyses is the interaction between dietary category and body mass on total GC levels (Fig. 2). We found that the previously reported negative relationship between total GC levels and body mass is only present in herbivores, not carnivores, in this dataset. Because of this dietary category and body mass interaction, the effect of diet on total GC levels is greatest in small-bodied and large-bodied vertebrates (Fig. 2). Whether higher GC levels in small herbivores could potentially be related to phytochemical consumption is unclear. On one hand, a given dose of a phytochemical would be more concentrated in the blood of small animals and more dilute in large ones (Lambdon & Hassall 2001). Thus, small amounts of consumed endocrine disruptors in a small-bodied animals should have greater ramifications for the endocrine system. On the other hand, some have argued that smaller animals should be more phytochemically tolerant, due to faster metabolism and detoxification rates (Freeland 1991), though others have argued against this relationship (Clauss *et al*. 2013). This diet and body mass interaction effect could also partially explain why the negative body mass and GC relationship has been found in mammals (Haase *et al*. 2016; Francis *et al*. 2018), but not other vertebrate taxa analysed alone (Francis *et al*. 2018), as herbivory is more prevalent in mammals than other vertebrate groups (Dearing *et al*. 2005). In any case, the simplest conclusion is that our analysis demonstrates again that the relationship between body size and GC is not robust across vertebrates. Furthermore, body mass alone had no significant effect on total GC levels (Tables 1 and 2).

While this meta-analysis demonstrates the effect of a herbivory on species total GC levels, we suggest, but cannot conclusively demonstrate, that this relationship may be driven by ingestion of phytochemicals. Though herbivory has other associations that may explain higher GC levels aside from phytochemicals, some of these major alternative explanations can be rejected. For example, it could be hypothesised that herbivores have higher GC levels because they tend to be prey species and experience chronic stress of predation. We can reject this on two levels. First, our dataset includes many small-bodied carnivores (Fig. 2) which are also prey themselves. For example, some of the lowest GC levels in the carnivore dataset are from brush-tailed phascogales (*Phascogale tapoatafa*), American mink (*Neovision vision*), and ornate tree lizards (*Urosaurus ornatus*), which are also all prey of other predators. Second, while a direct encounter with a predator will acutely raise GC levels, different species vary in whether or not they experience chronic stress in response to predation. Some species show elevation of GC levels in response to increased predation risk (Dulude-de Broin *et al*. 2020), whereas others do not (Boonstra 2013).

More specific testing of this phytochemical hypothesis is needed. The dietary separations in these analyses are very coarse and do not take into account species differences in specialised digestive or behavioural adaptations for dealing with phytochemicals. Comparing the GC levels of closely related species that are known to ingest different amounts of phytochemicals would be informative. In addition, GC levels of specialist and generalist herbivores could be compared. While specialist herbivores tend to consume plants with more phytotoxins than generalists do, they also have evolved more effective adaptations to prevent the absorption of these toxins into the blood (Sorenson & Dearing 2003; Shipley *et al*. 2012). Conversely, generalists ingest a variety of toxin types and are more likely to have a broad anti-phytochemical response like decreasing tissue sensitivity once the phytochemical is already circulating, and therefore should have higher circulating hormone levels to compensate. Finally, the same rules should apply to other types of circulating hormones in addition to GCs (e.g. reproductive hormones). Wynne-Edwards (2001) made such a prediction about sex hormone levels in carnivores compared with herbivores, proposing that herbivores should have increased levels of oestrogens to decrease the effect of ingested phytoestrogens and other endocrine disruptors (changing the 'signal to noise ratio'). To the best of our knowledge, this relationship has not been tested, as there are no equivalent datasets of species average levels of oestrogens.

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AUTHORSHIP

PE designed the study and compiled data. NS constructed the phylogeny and performed analyses. PE and RB wrote the manuscript. All authors contributed to revising the manuscript.

DATA AVAILABILITY STATEMENT

Data are available from the Figshare Repository: https://doi.org/10.6084/m9.figshare.12203249

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SUPPORTING INFORMATION

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