# Experimental increases in glucocorticoids alter function of the neuroendocrine stress axis in wild red squirrels without negatively impacting survival and reproduction

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**Abstract**

Hormones including glucocorticoids (stress hormones) are well known for their effects on animal behavior and life history traits, and this understanding has come through both correlative and manipulative studies. While the latter offers a higher level of control (and ability to assign causality), there are important methodological concerns that are often not considered when manipulating hormones, including glucocorticoids, in wild animals. In this study, we examined how experimental elevations of cortisol concentrations in wild North American red squirrels (*Tamiasciurus hudsonicus*) affected their hypothalamic–pituitary–adrenal (HPA) axis reactivity, and life history traits including body mass, litter survival, and adult survival. The effects of exogenous cortisol on plasma cortisol concentrations depended on the time between treatment consumption and blood sampling. In the first nine hours after consumption of exogenous cortisol, individuals had significantly higher true baseline plasma cortisol concentrations, but adrenal gland function was impaired. Approximately 24 hours after consumption of exogenous cortisol, individuals had much lower plasma cortisol concentrations than controls, but adrenal function was restored. Corticosteroid binding globulin (CBG) concentrations were also significantly reduced in squirrels treated with cortisol. Despite these profound shifts in the functionality of the neuroendocrine stress axis, fitness proxies including squirrel body mass, offspring survival, and adult survival were unaffected by experimental increases in cortisol concentrations. Our results highlight that even short-term experimental increases in glucocorticoids can affect adrenal gland functioning and CBG concentrations, but may have no side-effects on proxies of fitness.

**Keywords:** cortisol, hormone manipulations, hypothalamic–pituitary–adrenal (HPA) axis, North American red squirrels

## 1. Introduction

Associations between glucocorticoids (stress hormones) and life history or behavioral traits are being increasingly studied, due to their role as a mechanistic link between the genome and the environment, and to uncover general relationships between hormones and fitness (Breuner et al., 2008; Dantzer et al., 2016). Glucocorticoids (hereafter GCs), in particular, are receiving heightened focus, because of the widespread relevance of the stress response mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000). This includes documented effects of GCs on behavior and other traits (e.g. DeNardo and Sinervo, 1994; Ebensperger et al., 2011). Correlational studies have helped advance our understanding of the relationships between GCs and phenotypic traits, but establishing the causality of such relationships requires experimental manipulation (e.g. artificially elevating GCs). In laboratory settings, hormone manipulations are logistically feasible (e.g. Karatsoreos et al., 2010; Lussier et al., 2009), but experimental studies conducted in wild populations are likely to provide better insights into the ecologically relevant effects of GCs on life history variation.

Hormone manipulations in wild animals are more challenging than in the laboratory, but several methods have been developed (see Sopinka et al., 2015). That said, exogenous GCs may have unintended physiological side effects, which may influence or skew interpretation of the results obtained from manipulative studies. Although detailed studies about the potential complications of manipulating hormones in wild animals have not been performed, these issues were highlighted 10 years ago (Fusani, 2008). One potential problem with hormone manipulations is related to the fact that the endocrine system is a homeostatic system that is controlled by negative feedback mechanisms, and tends to compensate for disruption. Therefore, if animals are treated with a hormone, the endogenous production of the hormone may be reduced after a few days, and longer treatment duration may lead

to the regression of the endocrine gland, and have important consequences for endocrine homeostasis (Fusani, 2008). Such effects are well documented in humans, as both cortisol and synthetic GCs (which may be more potent, see Meikle and Tyler, 1977), are used to treat a range of ailments (Arabi et al., 2010; Kirwan et al., 2007). Such treatments may lead to side-effects, including suppression of the HPA axis and reduced adrenal function (Broide et al., 1995; Feiwel et al., 1969; Jacobs et al., 1983). Although such side-effects are usually temporary (Morris and Jorgensen, 1971; Streck and Lockwood, 1979), in extreme cases, patients may develop more severe and long term conditions such as Cushing's syndrome (symptoms include obesity, poor wound healing, and hypertension, see Axelrod, 1976). In other cases, GC therapy may cause secondary adrenal insufficiency and lead to Addison's disease (Arlt and Allolio, 2003). If GC manipulations affect the adrenal glands, endogenous production of GCs, and endocrine homeostasis, this may lead to unintended consequences in wild animals. This could jeopardize the value of performing such studies, as they could adversely influence survival and reproduction. Indeed, some studies indicate that elevations in stress hormones reduce estimates of fitness (Bonier et al., 2009; Breuner et al., 2008; Wingfield et al., 1998), but it is unclear if this is due to an unintended complication from the manipulation rather than a natural consequence of increased stress hormones.

We examined how exogenous cortisol affected the HPA axis and life history traits of North American red squirrels (*Tamiasciurius hudsonicus*). We expected that exogenous cortisol would increase plasma cortisol concentrations, but that, as may be the case in humans receiving GC therapy, HPA axis responsiveness would decrease. We expected that exogenous cortisol might lead to increased body mass in squirrels (Axelrod, 1976), but did not expect our treatment dosages to be sufficiently high to cause anorexia through sustained adrenal impairment (Arlt and Allolio, 2003). As we aimed to keep

physiological stress within a 'normal' range for this species, we did not expect to see negative effects of our treatments on body mass or adult or litter survival.

## 2. Materials and Methods

### *2.1 Study population*

We studied a natural population of red squirrels in the Yukon, Canada (61°N, 138°W) that has been monitored since 1987 (Boutin et al., 2006; McAdam et al., 2007). All squirrels in this population are individually identified by a unique ear tag in each ear, as well as a unique color combination of colored wires attached to each ear tag which allow researchers to identify individuals from a distance. Squirrels were live-trapped (Tomahawk Live Trap Co., WI, USA), during which they were weighed using a Pesola spring balance, and fecal samples were collected from underneath traps, placed on ice, and stored at -20 °C upon return to the field station (Dantzer et al., 2010). Female and male reproductive condition was assessed through abdominal palpation (see McAdam et al., 2007).

### *2.2 GC manipulations*

In 2015, and 2016, squirrels were randomly allocated to either control (8 g all natural peanut butter, 2 g wheat germ, no cortisol) or cortisol treatments (8 g peanut butter, 2 g wheat germ with 6, 8, or 12 mg of cortisol [H4001, Sigma Aldrich, USA]). Dosages of 0, 6, and 12 mg cortisol were selected following Dantzer et al. (2013), who based their selected dosages on previous studies in similar sized rodents that showed that a dose of 12 mg/day of cortisol induces chronic mild stress (Casolinia et al., 1997; Catalani et al., 2002; Mateo, 2008). We also added an intermediate dose of 8 mg cortisol/day. Cortisol was provided to squirrels as 10 g dosages placed in buckets hung from trees in squirrel territories (for details, see Dantzer et al., 2013). To ensure that target squirrels (identifiable through ear tags/radio-

collars) were consuming the treatments, camera traps (Reconyx PC900 HyperFire Professional Covert IR) were placed by the buckets of 31 squirrels for 5 days. Out of 155 d of camera trapping, conspecific pilferage was only observed ten times, and there was one case of heterospecific pilferage by a grey jay (*Perisoreus canadensis*). Consumption of each treatment was estimated daily by checking buckets for any leftovers and estimating these as a percentage. Squirrels consumed on average 91.9% of their total peanut butter treatments (median = 100%, SD = 12.13%, range = 43-100%).

*2.3 Effects of exogenous cortisol on fecal glucocorticoid metabolites (FGM)*

To evaluate the effects of cortisol treatments on FGM, fecal samples were collected in 2015 and 2016 from male and female squirrels fed with 0, 6, 8, and 12 mg cortisol/day (Table 1). Glucocorticoid metabolites from fecal samples were extracted and assayed as previously validated and described (Dantzer et al., 2011, 2010) using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (Touma et al., 2003). Intra- and inter-assay CVs for pools diluted 1:250 (n = 13 plates) were 7.4% and 15.4%. For pools diluted 1:500 (n = 13 plates) this was 7.5% and 17.9%. Pools diluted 1:100 (n = 9 plates) had intra and inter-assay CVs of 10.0% and 17.9%, and for pools diluted at 1:700 (n = 9 plates) this was 6.4% and 18.9%. Samples from control (n = 135) or cortisol (n = 237) treated squirrels included those collected before, during, and after treatment.

*2.4 Effects of exogenous cortisol on plasma cortisol, corticosteroid binding globulin, and HPA axis*

Non-breeding male squirrels were fed cortisol (8 mg/day) or control treatments for one (n = 40) or two weeks (n = 26). Eighteen squirrels were included in both spring/summer (mid-April to mid-July) and autumn 2016 (mid-September to early October, with treatments switched between periods, with the exception of two squirrels fed GCs twice and one squirrel fed control treatments twice, due to human

error). The time that squirrels consumed their treatments was estimated by checking buckets at intervals between 25 minutes and a few hours (shown as hours:min, mean = 1:53 hrs, SD = 1:12 hrs). Squirrels were either blood sampled the same day as confirming they consumed their last treatment (n = 36, mean = 3:30 hrs, range = 0:57-8:55 hrs, referred to as '*same day bleeds*' hereafter) or the next day (n = 30, mean = 22:46 hrs, range = 14:57-30:55 hrs, referred to as '*next day bleeds*' hereafter. Note that for 19 next day bleed squirrels, the time of treatment consumption was not recorded). Blood samples obtained within 3 min of squirrels entering a trap (n = 54) are referred to as *true baseline* samples (Romero et al. 2005). If the first blood sample was obtained >3 min after squirrels went into traps (n = 12), this is referred to as *nominal baseline* samples.

Blood samples were obtained from the nailbed and collected into heparinized capillary tubes. To determine how exogenous cortisol affected the HPA axis, we used two different methods. We either 1) subjected squirrels to dexamethasone and adrenocorticotropic hormone challenges (n = 32, hereafter referred to as DEX/ACTH challenges) following previously described protocols (Boonstra and McColl, 2000), with modified concentrations of dexamethasone (3.2 mg/kg, hereafter, DEX) and adrenocorticotropic hormone (4 IU/kg, hereafter ACTH), or 2) bled them at intervals of 0-3 min (samples shown as same day/next day: squirrel n = 23/11), 3-6 min (n = 14/6), 6-12 min (n = 15/7), and 18-22 min (n = 16/11, hereafter referred to as *timed bleeds*) to assess the response to handling stress.

Total plasma cortisol concentrations were assayed using an ImmuChem coated tube cortisol radio-immunoassay (MP Biomedicals, New York, USA) following the manufacturer's instructions, with the exception that, due to small sample volumes, plasma and tracer volumes of 12.5 µL and 500 µL were used. Linearity was tested by pooling several samples and serially diluting these from 1 (neat) to 1:64.

Results were plotted, visually inspected, and evaluated with linear regression ($R^2$ adj = 0.991, $p < 0.001$). According to the manufacturer, the assay detection limit is 1.7 ng/ml, and samples that read below this value (n = 8) were set at 1.7 ng/ml. Most samples were run in duplicate, but because of small plasma volumes only one estimate was obtained for 33.9% of samples. Average standard and sample intra-assay CVs were 7.9% (n = 4 assays). Inter-assay CVs for the five standards provided (10, 30, 100, 300 and 1000 ng/ml cortisol) were 11.1%, 15.4%, 8.8%, 4.0% and 7.7%.

Corticosteroid binding capacity was measured in plasma stripped of endogenous steroids using dextran-coated charcoal (DCC) and diluted to a final dilution of 1/50 in phosphate buffered saline with 0.1% gelatin (PBS). Three tubes (final volume of 150 µL) were prepared for each sample: two containing 160 nM cortisol (10% 1,2,6,7-$^3$H-cortisol, Perkin Elmer, Waltham, MA, and 90% non-labeled cortisol, C-106, Sigma-Aldrich) to measure total binding, and one containing an additional 4 µM non-labeled cortisol to measure nonspecific binding (primarily by albumin). After incubating tubes overnight, 300 µL of ice-cold DCC was added and left for 15 minutes to strip free cortisol from the plasma mixture. The tubes were then centrifuged at 2000 x *g* at 4 °C for 12 minutes. The supernatant (containing bound cortisol) was decanted into scintillation vials, to which 4 mL of scintillation fluid (Emulsifier-Safe cocktail, Cat. No. 6013389, Perkin Elmer, Groningen, Netherlands) was added. Vials were counted in a scintillation counter. Specific binding by CBG was calculated by subtracting nonspecific binding counts from total binding counts. Specific binding scintillation counts were converted to nM binding by measuring the total counts in the 150 µL of the 160 nM solution and adjusting for the plasma dilution. Some CBG-bound hormone is lost to the DCC during the 15 minute DCC exposure. Using pooled plasma exposed to DCC for 5-20 minutes, we calculated the rate of loss of CBG-bound cortisol (data not shown). From this, we calculated that the 15 minute DCC exposure resulted in the loss of 28.5% of CBG-bound hormone, and all our

specific binding measurements were corrected accordingly. To calculate the percent free cortisol, we estimated free cortisol concentrations (i.e. not bound by CBG) using the total cortisol concentration, the equilibrium dissociation constant for red squirrels of 61.1 nM (Delehanty et al., 2015), and the equation in Barsano and Baumann (1989). As plasma volumes were limited, only 58 samples could be assayed for both CBG/percent free cortisol and total cortisol.

*2.5 Effects of exogenous cortisol on squirrel body mass, litter survival, and adult survival*

Squirrels were trapped on average once per week (range 1-25 d) and weighed to the nearest 5g with a spring scale. We compared non-breeding squirrel mass in cortisol treated and control squirrels sampled in 2015 and 2016 before (range = 0-20 d, mean = 6 d, SD = 5 d) and during treatment (range = 1-33 days after treatments started, mean = 12 days, SD = 7). Because data on litter survival in 2015 and 2016 were limited, we also included data on litter fate collected in 2012 from squirrels fed the same dosages (0, 6, 12 mg cortisol/day) for similar periods of time (see Dantzer et al., 2013). When females gave birth, their nests were located within a few days of parturition (first nest entry) and again when pups were ~25 d old (second nest entry) following McAdam et al. (2007). We examined how our treatments affected whether females treated during pregnancy (control n = 24, 6 mg cortisol/day n = 9, 8 mg cortisol/day n = 16, 12 mg cortisol/day n = 22) or lactation (control n = 8, 12 mg cortisol/day n = 9) lost their litters before the first nest entry or between the first and second nest entry, as determined by abdominal palpation. Females treated during pregnancy were treated from the estimated last third of pregnancy (based on abdominal palpation), until five days post parturition (treatment duration range = 8 – 25 days, mean = 18, SD = 4). Females treated during lactation were treated for 10 days, from days 5 to 15 post parturition, although due to human error and field conditions, one female was treated for 8, and another

for 11 days (range = 8 -11 days, mean = 10, SD = 0.6). Adult squirrel survival was monitored through regular live trapping and behavioral observations (McAdam et al., 2007). Survival data were only available from squirrels studied in 2015 (n = 50, including 41 females and nine males). These squirrels were fed either control treatments (n = 25, 10 – 26 days, mean = 19, SD = 7) or 12 mg cortisol/day (n = 25, 8 -35 days, mean = 20, SD = 7). Although we did not know the ages of all squirrels, there was no age bias between squirrels fed control (eight known ages, mean = 4.05, SD= 1.05 years) and those fed cortisol (nine known ages, mean = 3.97, SD = 0.87 years, $t_{13.8}$= 0.18, p = 0.86). We estimated survival until exactly 1 year after the treatments were stopped.

*2.6 Statistical analyses*

Analyses were conducted using R statistical software (v 3.3.3, R Core Team, 2017). Where there were multiple measures for individual squirrels, linear mixed-effects models (LMMs) were conducted using packages 'lme4' (v 1.1.10, Bates et al., 2015) and all such models contained 'squirrel ID' as a random intercept term. If there were no repeated measures, general linear models (GLM) were used. To make comparisons between groups, we used the 'glht' function in R package 'multcomp' (Hothorn et al., 2017). Model residuals were plotted to check for conformity with homogeneity of variance and normality (Zuur et al., 2010). Where necessary, data were ln transformed. Regression lines were visualized using R package 'visreg' (v 2.2.2, Breheny and Burchett, 2016).

We tested effects of treatments on FGM concentrations using LMMs, analyzing female and male data separately due to differences in reproductive states. Models for females included dose (0, 6, 8, 12 mg of cortisol/day), reproductive state (non-breeding, pregnant, lactating), Julian date, and whether the squirrel was treated on the sampling day (yes/no) as fixed effects, with an interaction term for dose and

treatment (yes/no). Models for males included the same variables (but only doses of 0 and 8 mg) except reproductive state (all were non-breeding). To test for lasting effects of treatments on FGM, we analyzed samples collected between 1-21 d after treatments stopped. We included an interaction between dose and days after treatments ended in an LMM, as above.

Regarding blood sample collection, there was no daytime sampling bias between cortisol treated (between 9:41 and 18:07, mean = 13:23) and control squirrels (between 9:33 and 17:31, mean = 13:50, t-test, $t_{42.15}$= 1.14, p = 0.26), and no effect of sampling time on plasma cortisol (linear regression, b = 0.008, $t_{0.07}$ = 0.11, p = 0.91), indicating that there were no effects of circadian patterns in our dataset.

Squirrels were either subjected to DEX/ACTH challenges, or squirrels were subjected to timed bleeds (and blood samples were collected between 2 and 28 minutes after trap doors closed). LMMs were run separately for DEX/ACTH challenges and timed bleeds. Models included fixed effects for treatment and bleed time. For DEX/ACTH challenges bleed times included the categorical variables 'true baseline', 'nominal baseline', '60 minutes after DEX injection' (hereafter; DEX), '30 minutes after ACTH injection' (hereafter; ACTH30), and '60 minutes after ACTH injection' (hereafter; ACTH60); for timed bleeds, bleed times were expressed as minutes since the squirrel was trapped (continuous variable, standardized following Schielzeth, 2010). Two plasma samples with very low binding (<10%) were excluded from the analysis. Some models included squirrels treated for either 1 or 2 weeks, and some included squirrels that were treated in both spring and autumn. Where this was the case, treatment duration (1/2 weeks) and whether or not squirrels had been treated before (yes/no) was included in the models. However, these variables were not significant in any of the models, and are not discussed further.

To assess effects of treatments on CBG concentrations and percent free cortisol, we subset samples into those from DEX/ACTH challenges conducted the same day as consumption of the last treatment (n = 16) and those collected the next day (n = 27), and timed bleeds. Due to limited data (only 58 samples were analyzed for CBG, across all categories), only the effects of treatment (control or 8 mg cortisol/day) on CBG and percent free cortisol were tested for squirrels DEX/ACTH challenged on the same day as consuming their last treatments. Models for squirrels ACTH challenged the day after consuming their last treatments included interactions between sample time (nominal baseline, DEX, ACTH30, ACTH60) and treatment (control or 8 mg cortisol/day). For timed bleeds, data from samples collected on the same day (n = 12) and the day after (n = 3) the last treatment was consumed were pooled. Models included an interaction between the sampling day (same/next) and treatment (control or 8 mg cortisol/day).

To estimate the total plasma cortisol in a 24 h period, true baseline cortisol was plotted against the time since treatment was consumed. Regression line equations were used to calculate the area under these lines for both control and cortisol treated squirrels, using the 'trapzfun' command in package 'pracma' (Borchers, 2018), and areas under the curve were compared with $\chi^2$ tests.

Data on body mass were subset into those collected in spring (females fed 0 or 12 mg cortisol/day) and autumn (males fed 0 or 8 mg cortisol/day). Body masses were compared using LMMs including a two-way interaction between treatment, and time (before/during treatment). To assess differences between litter survival (lost/not lost), and adult survival (yes/no) GLMs were applied using binomial errors. Models included treatment (12 mg cortisol/day or control) and sex. Dispersion parameters (using R package blemco, Korner-Nievergelt et al., 2015) between 0.75 and 1.4 were taken to accept overdispersion was not problematic.

## 3. Results

### *3.1 Effects of treatments on fecal glucocorticoid metabolite concentrations*

Overall, squirrels fed cortisol treatments (6, 8, 12 mg/day) had significantly higher FGM concentrations than when they were not being fed ($F_{3,263.8}$= 11.5, p < 0.001, Fig. 1), but the magnitude of increase depended on the dosage. Squirrels fed plain peanut butter with no cortisol did not have higher FGM concentrations when they were being fed their treatments compared to when they were not being fed their treatments (females: b = -0.03, SE = 0.15, z = 0.22, p = 1.0; males: b =0.14, SE= 0.36, z = 0.41, p = 0.96). FGM concentrations in squirrels fed 6, 8, or 12 mg cortisol/day in both females and males were significantly higher compared when they were being fed compared to when they were not being fed (6 mg: b = 0.78, SE = 0.28, z = 2.8, p = 0.032; 8 mg: females: b = 0.79, SE = 0.21, z = 3.9, p < 0.001; males: b =1.37, SE= 0.49, z = 2.82, p = 0.013; 12 mg: b = 1.39, SE = 0.17, z = 8.0, p < 0.001). Concentrations of FGM during treatment in female squirrels treated with 12 mg vs 8 mg (b = 0.39, SE = 0.28, z = 1.4, p = 0.63), 6 mg vs 8 mg (b = 0.36, SE = 0.36, z = 1.0, p = 0.88), and 6 mg vs 12 mg cortisol/day (b = 0.74, SE = 0.35, z = 2.1, p = 0.18) were not significantly different. Julian date did not affect FGM concentrations in females ($F_{1,284.9}$ = 3.44, p = 0.06) or males ($F_{1,15.2}$ = 0.73, p = 0.41). Reproductive condition did not affect FGM in this dataset, possibly because of limited sample numbers on some reproductive states (see Table 1, $F_{2,105.0}$ = 1.37, p = 0.26).

There was little evidence that the GC treatments had a lasting influence on FGM concentrations in squirrels. FGM concentrations in control females (b = -0.03, SE = 0.03, t = -1.16, DF = 53.9, p = 0.25) and males (b = -0.25, SE = 0.16, t = -1.61, DF = 8.4, p = 0.14) did not change in the 1-21 days after treatments stopped, nor did FGM concentrations from females (b = -0.01, SE = 0.04, t = 1.48, DF = 53.3,

p = 0.14) and males (b = 0.19, SE = 0.16, t = 1.2, DF = 8.4, p = 0.26) treated with 8 mg cortisol/day (although sample sizes from males were small). FGM concentrations in females treated with 6 mg cortisol/day also did not significantly change in the 1-21 days after treatments stopped (b = -0.01, SE = 0.06, t = -0.21, DF = 53.9, p = 0.14), but FGM concentrations in females treated with 12 mg of cortisol/day significantly increased after treatments stopped (b = 0.08, SE = 0.04, t = 2.31, DF = 49.8, p = 0.02, Fig. 2).

*3.2 Squirrels DEX/ACTH challenged the same day as consuming their last treatment*

Squirrels treated with cortisol (8 mg/d) that were DEX/ACTH challenged the same day as consuming their last treatment had a significantly different response to DEX and ACTH compared to control squirrels DEX/ACTH challenged the same day as consuming their last treatment ($F_{9,29.8}$ = 6.4, p < 0.001). Squirrels treated with cortisol (8 mg/day) had significantly higher true baseline cortisol concentrations (611.4±104.4 ng/ml) than control squirrels (214.5±41.3 ng/ml, b = 394.7, SE = 88.7, z = 4.4, p < 0.001, Fig. 3A). Both cortisol treated (205.8±50.2 ng/ml) and control (292.2±44.5 ng/ml) squirrels responded to DEX, as indicated by the reductions in their plasma cortisol concentrations 60 min after the DEX injection, although this effect was not significant (control: b = -33.9, SE = 81.2, z = -0.44, p = 1.0; cortisol treated: b = -139.6, SE = 88.7, z = -1.57, p = 0.62). Control squirrels had significantly higher plasma cortisol concentrations in samples taken 30 minutes after ACTH injection (604.9±93.6 ng/ml, b = 321.1, SE = 81.2, z = 3.97, p < 0.001), although concentrations started to decrease again 60 min after ACTH injection (404.6±76.3 ng/ml, b = -213.4, SE = 85.1, z = -2.51, p = 0.10). However, in cortisol treated squirrels, plasma cortisol concentrations decreased 30 minutes after ACTH injection (153.5±22.1 ng/ml, b = -51.8, SE = 88.7, z = -0.58, p = 1.0), and increased slightly 60 minutes after ACTH injection (163.1±36.7 ng/ml, b = 9.5, SE = 83.5, z = 0.11, p =1.0), although these effects were non-significant.

### *3.3 Squirrels DEX/ACTH challenged the day after consuming their last treatment*

Squirrels treated with cortisol (8 mg/d) that were DEX/ACTH challenged the day after consuming their last treatment had a significantly different response to DEX and ACTH compared to control squirrels DEX/ACTH challenged the same day as consuming their treatment ($F_{4,46.5}$ = 9.2, $p < 0.001$). Cortisol treated squirrels that were sampled the day after consuming their last treatment had lower plasma cortisol concentrations than controls at all sampling times. Nominal baseline plasma cortisol concentrations (35.9±21.2 ng/ml) were on average 92.3% lower in cortisol treated squirrels than in control squirrels (468.5±34.8 ng/ml, b = -404.8, SE = 67.4, z = -6.0, $p < 0.001$, Fig. 3B). Plasma cortisol concentrations after the DEX injection were also lower in cortisol treated squirrels (56.5±34.6 ng/ml) than controls (239.8±24.8 ng/ml, a 76.4% difference, b = -183.3, SE = 53.7, z = -3.4, p = 0.006). Thirty minutes after the ACTH injection, cortisol treated squirrels (mean = 97.9±19.9 ng/ml) had plasma cortisol concentrations that were on average 80.4% lower than in control squirrels (mean = 498.8±34.6 ng/ml, b = 398.1, SE = 57.5, z = -6.9, $p < 0.001$). This difference remained 60 min after the ACTH injection (cortisol mean = 101.7±11.4 ng/ml, control mean = 469.0±89.2 ng/ml, a difference of 78.3%, b = -368.2, SE = 56.8, z = -6.5, $p < 0.001$, Fig. 3B).

In squirrels sampled the day after receiving their last treatment, control squirrels (b = -211.9, SE = 53.7, z = -3.9, $p < 0.001$), but not cortisol treated squirrels (b = 9.6, SE = 49.4, z = 0.2, p = 1.0), had significantly lower plasma cortisol concentrations 60 minutes after DEX injections compared to nominal baselines. Thirty minutes after the ACTH injection (ACTH30), control squirrels (b = 257.3, SE = 45.6, z = 5.6, $p < 0.001$), had significantly higher plasma cortisol concentrations than 60 minutes after DEX injections. In cortisol treated squirrels, plasma cortisol concentrations were higher at 30 minutes after

313 ACTH injection (ACTH30) than 60 minutes after DEX injection, but this was not significant (b = 42.6, SE =
314 44.7, z = 0.95, p = 0.95, Fig. 3B).

315 *3.4 Effects of treatments on stress response to capture and handling*

316 Squirrels were bled at intervals of 0-3, 3-6, 6-12 and 18-22 min after trap doors closed (timed
317 bleeds) either the same day as confirming they ate their last treatment (mean time elapsed = 3:36 hrs,
318 range = 0:57-8:55 hrs, cortisol n = 10, control n = 13) or the next day (mean time elapsed = 22:46, hrs
319 range = 14:57-30:55 hrs, cortisol n = 5, control n = 6). Treatment (cortisol or control) affected how
320 handling stress affected squirrel plasma cortisol concentrations in squirrels bled on the same day as
321 consuming their last treatment. Plasma cortisol concentrations were generally higher in cortisol treated
322 squirrels, but concentrations decreased as time since the squirrel went in the trap increased (b = -0.56,
323 SE = 0.17, t = 3.4, DF = 62.3 p = 0.001). In control squirrels, on the other hand, plasma cortisol
324 concentrations increased as time since the squirrel went in the trap increased (b = 0.31, SE = 0.11, t = 2.9,
325 DF = 62.5, p = 0.006, Fig. 4A).

326 Plasma cortisol concentrations were generally lower in cortisol treated squirrels that were bled
327 the day after consuming their last treatment than in control squirrels (b = -1.8, SE = 0.81, t = -2.2, DF =
328 8.4, p = 0.057). Handling time generally increased plasma cortisol concentrations (b = 0.42, SE = 0.20, t =
329 2.1, DF = 19.2, p = 0.048), and this was not affected by treatment (b = 0.40, SE = 0.1.4, DF= 18.7, p = 0.16).

330 The time since squirrels consumed their last treatments significantly affected true baseline
331 plasma cortisol in cortisol treated squirrels (b = -0.27, SE = 0.04, t = -6.1, p < 0.001) but not controls (b =
332 -0.02, SE = 0.03, t = -0.58, p = 0.57, Fig. 5). Plasma cortisol increased after squirrels consumed their
333 cortisol treatments, but declined with time post-consumption, until, ~20 hours post-consumption,
334 plasma cortisol was much lower in cortisol fed than in control squirrels (Fig. 5). Overall, squirrels fed

cortisol experienced significantly higher plasma cortisol (total area = 7907.4 units) than controls (total area = 4110.8 units, $\chi$=614.4, DF = 1, $p < 0.001$, Fig. 5) in a 24 h period.

*3.5 Effects of treatments on CBG*

Cortisol treatment significantly lowered CBG concentrations in squirrels DEX/ACTH challenged on the same day as consuming their last treatments ($F_{1,5.0} = 51.0$, $p < 0.001$, Fig. 6A). Cortisol treatment also significantly lowered CBG concentrations in squirrels DEX/ACTH challenged the day after consuming their last treatment ($F_{1,12.9} = 29.3$, $p < 0.001$). Consequently, cortisol treatment significantly increased the proportion of free cortisol in plasma in squirrels DEX/ACTH challenged on the same day ($F_{1,5.0} = 7.6$, $p = 0.04$) and the day after consuming their last treatments ($F_{1,12.1} = 15.3$, $p = 0.002$, Fig. 6B). Treatment did not affect plasma CBG concentrations at different sample times (nominal baseline, DEX, ACTH30, ACTH60) in squirrels sampled the day after consuming their last treatment ($F_{3,6.2} = 1.0$, $p = 0.46$), nor percent free cortisol ($F_{3,12.3} = 0.5$, $p = 0.71$). Cortisol treatment also lowered CBG concentrations in squirrels subjected to timed bleeds ($F_{1,7.0} = 24.5$, $p = 0.002$, Fig. 6A), and increased percent free cortisol ($F_{7.0} = 2.3$, $p = 0.17$). The effect of treatment on plasma CBG concentrations ($F_{7.0} = 1.3$, $p = 0.30$) and percent free cortisol ($F_{1,7.0} = 1.9$, $p = 0.21$) was not different between squirrels sampled the same day or the day after last treatment.

*3.6 Effects of treatments on body mass*

There was no effect of the interaction between treatment (control or 12 mg cortisol/day) and time of sampling (during treatment yes/no) on masses of control females (n = 24 before, n = 23 during treatment records) and females fed 12 mg cortisol/day (n = 30 before, n = 31 during treatment records, b= - 3.9, SE = 4.9, $t_{87.9} = -0.79$, $p = 0.43$). There was also no effect of the interaction between treatment and time of sampling on masses of males fed 8 mg cortisol/day (n = 17 before, n = 11 during treatment

357 records) and males fed control treatments (n = 20 before, n = 17 during treatment records, b = 6.9, SE =
358 4.8, $t_{29.3} = 1.4$, p = 0.16).

359 *3.7 Effects of treatments on litter survival*

360 There was no significant difference in litter survival rates before the first nest entry between
361 females treated with cortisol during pregnancy (14/47 lost) and controls (7/24 lost; $z_{0.55} = 0.13$, p = 0.89).
362 When dosages (0, 6, 8, 12 mg/day) were analyzed separately, there also was no evidence of any dosage
363 significantly affecting litter survival prior to the first nest entry (6 mg: $z_{0.81} = -1.4$, p = 0.17: 8 mg; $z_{0.78} =$
364 0.74, p = 0.46; 12 mg: $z_{0.68} = 0.50$, p = 0.62).

365 There were also no significant differences in litter survival between the first and second nest
366 entry between females treated with cortisol during pregnancy (17/47) and controls (4/24: $z_{0.55} = -0.05$, p
367 = 0.96). There was no evidence of dosages of 6, 8, or 12 mg/day significantly affecting litter survival
368 between the first and second nest entries (6 mg: $z_{0.81} = -1.38$, p = 0.17; 8 mg: $z_{0.73} = 0.29$, p = 0.77, 12 mg:
369 $z_{0.68} = 0.50$, p = 0.62).

370 For females treated during lactation (n = 17), there was no significant difference in litter survival
371 between the first and second nest entry, with 1/8 control females losing their litter and 3/9 cortisol fed
372 (12 mg/day) females losing their litter ($z_{1.3} = -0.98$, p = 0.33).

373 *3.8 Effects of treatments on adult survival*

374 There was no difference in survival to one year following cessation of the treatments between
375 controls (18/25 survived to one year) and those fed cortisol (14/25 survived to one year; $z_{0.6} = -1.13$, p =
376 0.26). There was no difference in survival between males (7/9 survived to one year), and females (25/41
377 survived to one year, $z_{0.9} = 0.89$, p = 0.37).

378

## 4. Discussion

Our results on cortisol manipulations in wild red squirrels, spanning a range of dosages, life history stages, and including both sexes, provide important information regarding the response of wild animals to such hormone manipulation. Squirrels treated with cortisol had higher FGM concentrations and plasma cortisol concentrations over a 24 h period. However, our results highlight that exogenous GCs can cause the adrenals to stop responding to handling stress or pharmaceutical (DEX/ACTH) challenges, although these effects were short-lived and did not affect fitness proxies, including body mass, and offspring or adult survival.

Concentrations of CBG were significantly reduced in squirrels treated with cortisol for one or two weeks, suggesting that chronically elevated GCs reduce CBG concentrations. This reduction in CBG is likely to result in a higher bioavailability of plasma cortisol (Breuner et al., 2013). Studies in rats have shown that administration of exogenous GCs can inhibit the rate of CBG production and secretion in the liver (Feldman et al., 1979), and one study found that, 24 hours after acute stress, CBG concentrations were reduced in rats (Fleshner et al., 1995). Chronic stress has also been shown to lead to reduced CBG concentrations in most species studied to date (Armario et al., 1994; Breuner et al., 2013). A previous study found that CBG concentrations in red squirrel plasma (which were initially high) started to decrease as quickly as four hours after the start of DEX/ACTH challenges, suggesting that although high concentrations of CBG may buffer squirrels from the effects of high concentrations of free cortisol caused by acute stressors, these concentrations decline rapidly when the duration of the stressor is longer than a few hours (Boonstra and McColl, 2000). However, this does not seem to carry any noticeable cost as there were no changes in body mass or litter and adult survival in response to our treatments.

Previous reviews have emphasized the importance of maintaining hormone concentrations within a physiological range when performing hormone manipulations (Crossin et al., 2016; Fusani, 2008; Zera, 2007). Studies in mammals have shown that acute experimental challenges can cause increases in plasma cortisol that are comparable to those achieved by our treatments. For example, in both laboratory rats and wild animals, physical restraint, open field trials, and maze tests may cause >10 fold increases in plasma glucocorticoid concentrations (Cockrem, 2013). In control squirrels, plasma cortisol concentrations increased up to 10.4 fold from true baseline concentrations in timed bleeds, indicating that plasma cortisol could increase by this much without chemical stimulation. In this study, the highest recorded true baseline plasma cortisol concentration in cortisol treated squirrels was approximately seven times higher than the average control true baseline plasma cortisol concentration. This suggests that the increase in plasma cortisol caused by our 8 mg cortisol/day treatment is within the physiological range for a squirrel. However, it is possible that the duration of elevated plasma cortisol caused by our treatments is longer than that caused by natural stressors. Studies in rats show that plasma glucocorticoids increase quickly in response to acute stress, but returns to baseline concentrations within 2-5 hours after the stressor is removed (Marin et al., 2007; Mizoguchi et al., 2001), but in cortisol treated squirrels, plasma cortisol remained elevated, compared to control squirrels, for an estimated 17 hours post-treatment.

Our results highlight the importance of regularly provisioning individuals with treatments to sustain increases in hormone concentrations. Although we did find that squirrels fed cortisol had significantly higher concentrations of plasma cortisol over a 24 hr period than the controls, it was important to provision individuals with the treatments every 24 hrs. This was because plasma cortisol in cortisol treated squirrels did decrease to concentrations well below those of control squirrels >20 hrs

423 after consuming their treatments. When it is feasible, daily supplementation may be effective in
424 maintaining sustained elevations in hormone concentrations and provide an alternative to other
425 methods like implants that carry some disadvantages (Sopinka et al., 2015).

426 Our results also highlight the potentially adverse consequences that may occur when ending
427 hormone manipulations in wild animals. When sampled less than a day after the end of cortisol
428 treatment, squirrels did not respond to handling stress or ACTH injection, and appeared to have impaired
429 adrenal function and lower CBG concentrations. Data collected from cortisol treated squirrels the day
430 after consuming their last treatment showed that plasma cortisol was very low compared to control
431 squirrels, suggesting exogenous GCs have been excreted but endogenous GCs were being produced at a
432 lower rate than in control animals. However, plasma cortisol did increase with handling stress, suggesting
433 some recovery of adrenal function within 24 hrs of stopping the treatments. Data on FGM collected
434 between 0-21 d after treatments were stopped suggest that in squirrels fed 6 or 8 mg cortisol/day there
435 were no longer-term treatment effects, although squirrels fed 12 mg/day showed increases in FGM in
436 the 21 d following the end of treatment. Our results suggest that the adrenal gland may need time to
437 recover from treatment, and endogenous cortisol production may not return to pre-treatment levels for
438 several days.

439 Hormone manipulations can provide powerful tools to study relationships between hormones
440 and life history traits, and in recent years methods have been developed to achieve this (Sopinka et al.,
441 2015). Many studies aim to experimentally elevate GCs to test the “cort-fitness hypothesis”, which
442 proposes that elevations in baseline GCs decreases survival or reproduction (Bonier et al., 2009). We
443 show that elevation of plasma cortisol concentrations within the physiological range for 1-2 weeks had
444 profound effects on measures of HPA axis reactivity and CBG concentrations. Despite these shifts in the

445 functionality of the neuroendocrine stress axis and the sustained elevations in GCs, we found no change
446 in body mass or offspring and adult survival. This indicates that some species can tolerate bouts of
447 increased GCs and rapid reorganization of the stress axis without negatively impacting survival and
448 reproduction.

449

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458

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587

588

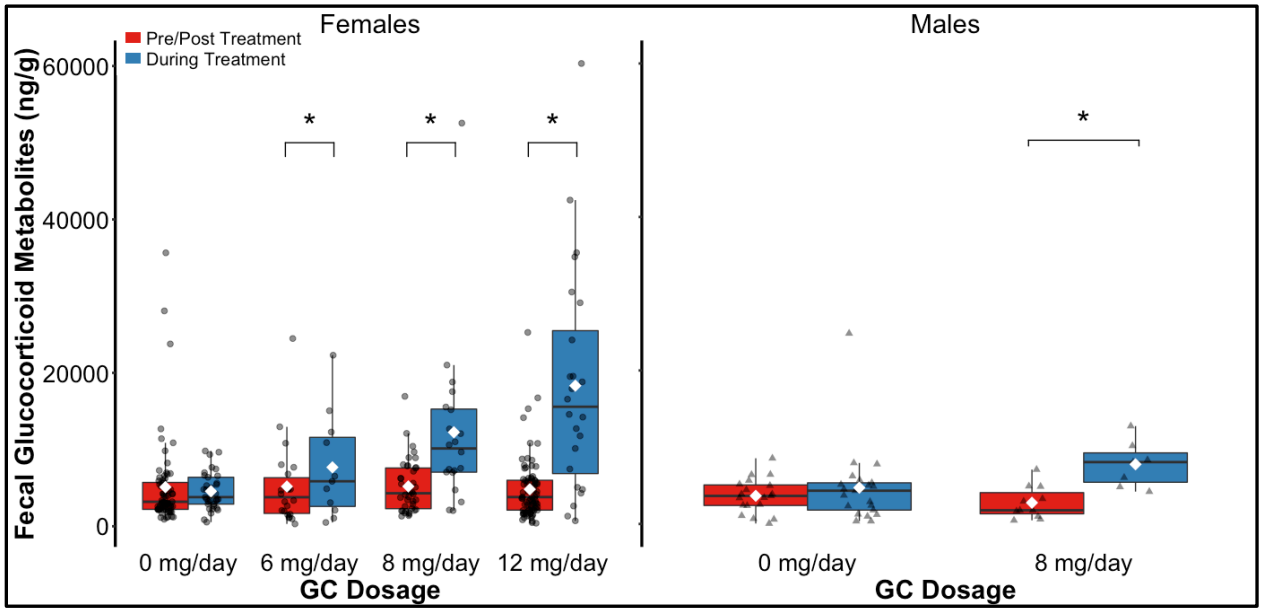
589 **Figure captions**

590 **Fig 1**. FGM concentrations in squirrels treated with control (0 mg/day) or with cortisol (6, 8, or 12 mg/day)
591 treatments. Asterisks (*) indicate significant differences ($p < 0.05$). Upper and lower hinges correspond
592 to the first and third quartiles. Upper/lower whiskers extend from the hinge to the highest/lowest value
593 that is within 1.5x the interquartile range. White diamonds indicate means.

594 **Fig 2.** Effect of number of days since A) female or B) male squirrels were last treated with cortisol (6, 8,
595 12 mg/day) or control peanut butter on partial residuals of fecal glucocorticoid metabolite (FGM)
596 concentrations. Values on the x-axis correspond to the number of days elapsed since treatments ended,
597 and values on y-axis are partial residuals from a LMM that included an interaction between the number
598 of days after treatment ended and the dosage (0, 6, 8, 12 mg cortisol/day).

599 **Fig 3**. Plasma cortisol from DEX/ACTH challenges conducted on males treated with 0 mg (Control) or 8
600 mg cortisol/day for 7-14 days (GCs). A) Males were trapped the same day as confirming they consumed
601 their last treatment. B) Males were trapped the day after being fed their last treatment, but note that
602 the time they consumed their treatments was not recorded. Note that true baseline samples for cortisol
603 treated squirrels were highly variable (two samples of 321.2 and 412.7 ng/ml, and two of 1.7 ng/ml).
604 Means and standard errors are shown.
605 **Fig. 4.** Effect of time elapsed since squirrels entered traps on plasma cortisol concentrations from control
606 squirrels (0 mg/day), or those treated with GCs (8 mg/day) and (A) sampled the same day as consuming
607 their last treatment or (B) sampled the day after consuming their last treatment.
608 **Fig. 5.** True baseline plasma cortisol concentrations in squirrels fed cortisol (GC, 8 mg/day) and controls
609 (0 mg/day) sampled between 1 to 31 hours after confirming they consumed their last treatments.
610 Different points correspond to different individual squirrels.
611 **Fig. 6.** A) Plasma corticosteroid binding globulin (CBG) and B) percent free cortisol in control and cortisol
612 treated squirrels subjected to both DEX/ACTH challenges (DEX/ACTH Challenge) and timed bleeds at
613 intervals of 0-3, 3-6, 6-12 and 18-22 minutes (Timed bleeds). The figure includes squirrels that were
614 sampled both on the same day as consuming their last treatment and the day after consuming their last
615 treatment.
616

617 **Fig. 1**

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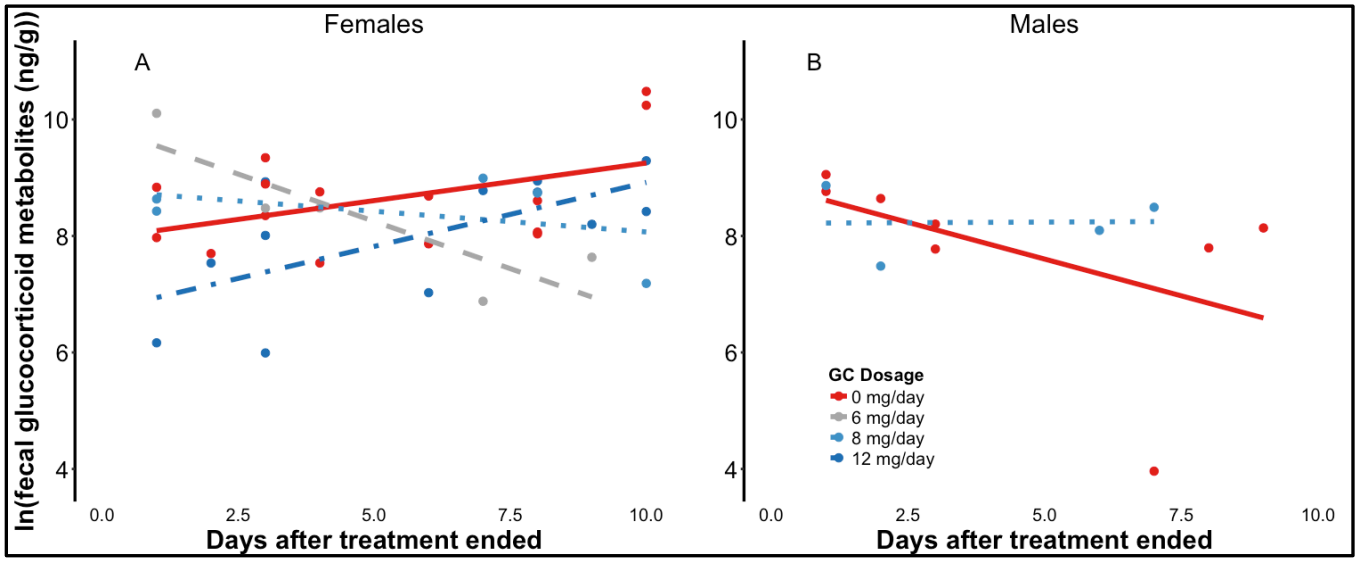
620 **Fig. 2**

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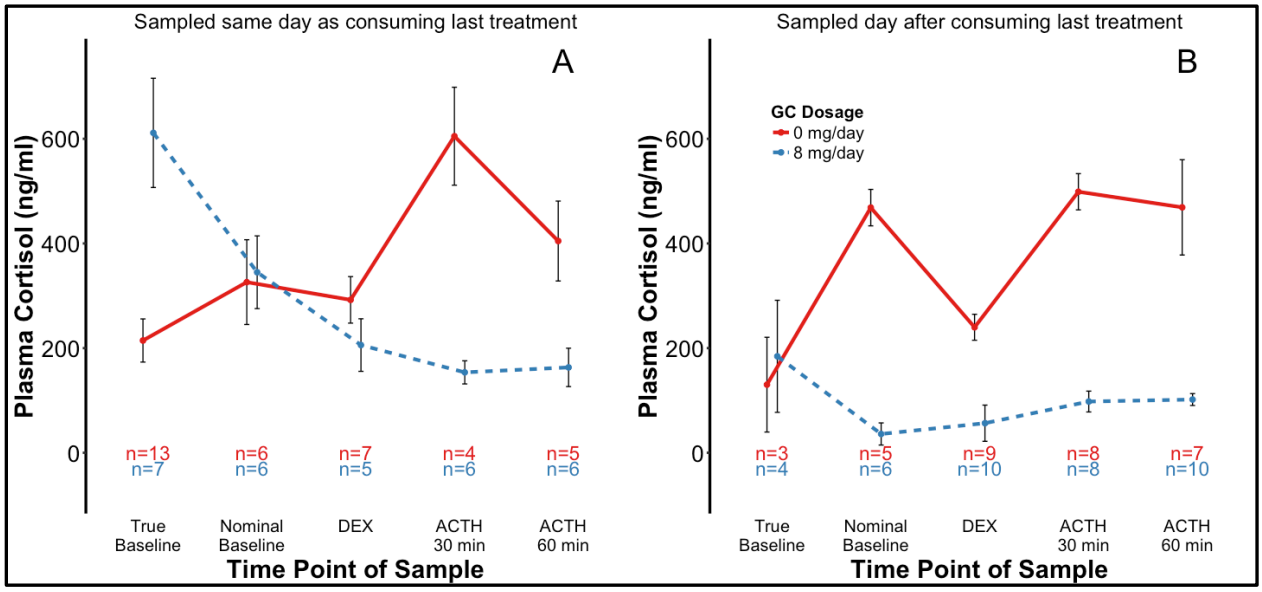
623 **Fig. 3**

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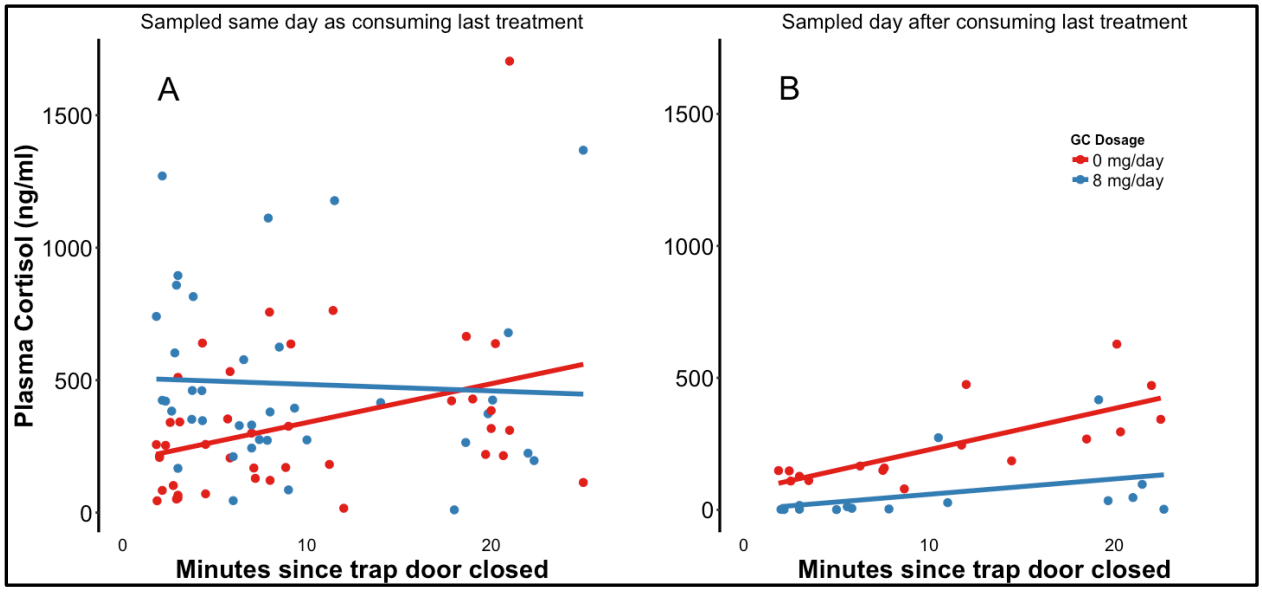
626 **Fig. 4**

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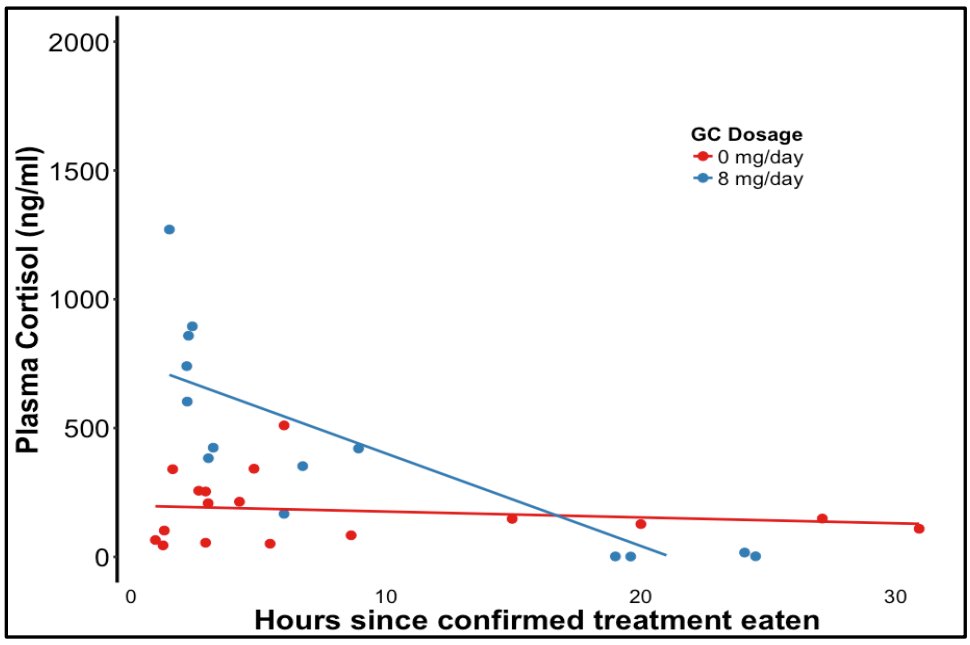
629 **Fig. 5**

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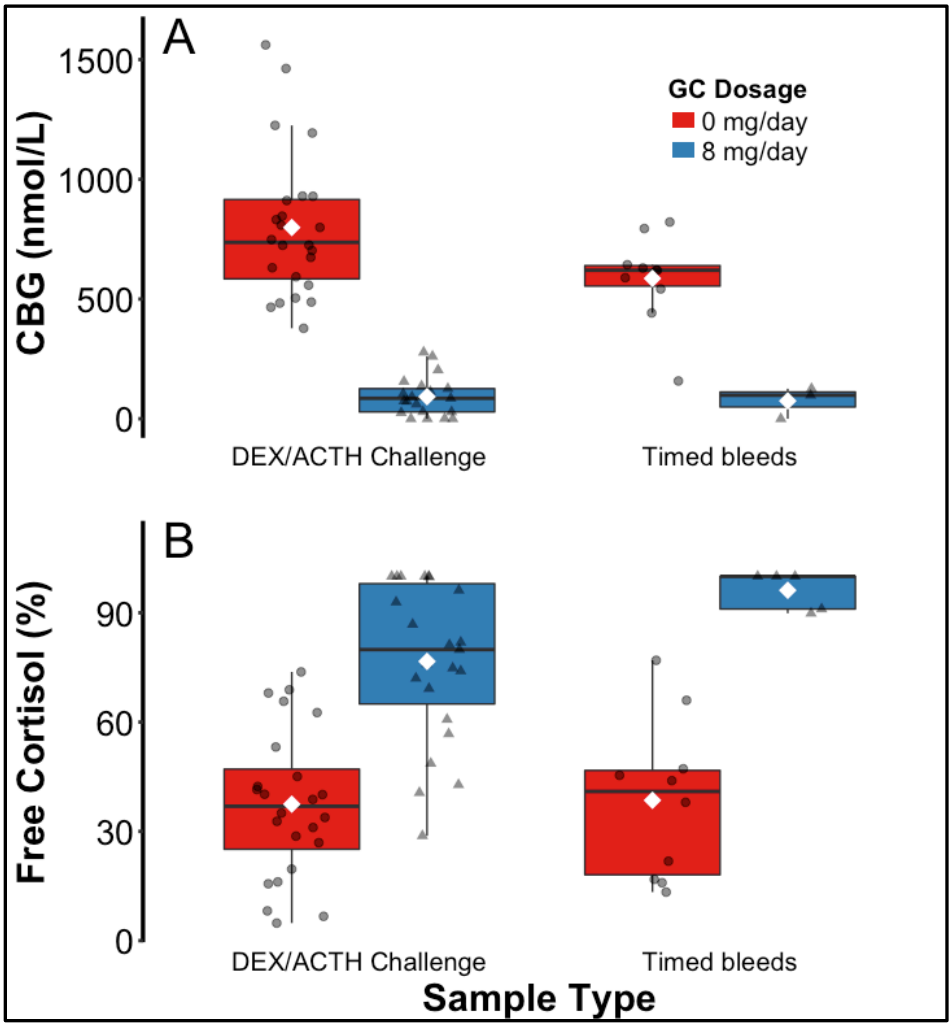
632 **Fig. 6**




633

634

635 **Table 1:** Sample sizes used in this study. Period refers to when squirrels were treated. Preg: females
636 treated from the estimated last third of pregnancy until 5 days post parturition. Lac: females treated
637 from days 5 -15 post parturition. NB: males and females treated outside of the breeding season.
638 Treatment duration is shown as the range and mean ± SEM. Samples (n) refers to the number of fecal
639 samples. F refers to females, M refers to males, SD refers to standard deviation.

| Sex | Period | Dose cortisol (mg/day) | Squirrels (n) | Fecal samples (n) | Treatment duration range, mean and SD (days) | Samples (n) during/pre+post treatment |
|---|---|---|---|---|---|---|
| F | Preg | 0 | 8 | 34 | 8 – 24 (18.13 ± 1.74) | 11/23 |
| F | Preg | 6 | 3 | 21 | 8 – 22 (13.33 ± 4.37) | 7/14 |
| F | Preg | 8 | 13 | 60 | 10 – 19 (15.00 ± 0.69) | 20/40 |
| F | Preg | 12 | 2 | 11 | 15 – 21 (18.00 ± 3.00) | 2/9 |
| F | Lac | 0 | 4 | 20 | 10 (10.00 ± 0.00) | 2/108 |
| F | Lac | 12 | 5 | 42 | 10 (10.00 ± 0.00) | 7/35 |
| F | NB | 0 | 8 | 49 | 19 – 24 (22.13 ± 0.67) | 17/32 |
| F | NB | 6 | 1 | 12 | 21 (21.00 ± 0.00) | 4/8 |
| F | NB | 12 | 9 | 48 | 15 – 34 (23.33 ± 1.69) | 17/31 |
| M | NB | 0 | 22 | 40 | 6 – 26 (13.14 ± 1.50) | 21/19 |
| M | NB | 8 | 12 | 18 | 6 – 15 (10.08 ± 1.04) | 7/11 |

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