© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

RESEARCH ARTICLE

Maternal glucocorticoids have minimal effects on HPA axis activity and behavior of juvenile wild North American red squirrels

Sarah E. Westrick[1,*,‡], Freya van Kesteren[1], Stan Boutin[2], Jeffrey E. Lane[3], Andrew G. McAdam[4] and Ben Dantzer[1,5]

ABSTRACT

As a response to environmental cues, maternal glucocorticoids (GCs) may trigger adaptive developmental plasticity in the physiology and behavior of offspring. In North American red squirrels (*Tamiasciurus hudsonicus*), mothers exhibit increased GCs when conspecific density is elevated, and selection favors more aggressive and perhaps more active mothers under these conditions. We tested the hypothesis that elevated maternal GCs cause shifts in offspring behavior that may prepare them for high-density conditions. We experimentally elevated maternal GCs during gestation or early lactation. We measured two behavioral traits (activity and aggression) in weaned offspring using standardized behavioral assays. Because maternal GCs may influence offspring hypothalamic–pituitary–adrenal (HPA) axis dynamics, which may in turn affect behavior, we also measured the impact of our treatments on offspring HPA axis dynamics (adrenal reactivity and negative feedback), and the association between offspring HPA axis dynamics and behavior. Increased maternal GCs during lactation, but not gestation, slightly elevated activity levels in offspring. Offspring aggression and adrenal reactivity did not differ between treatment groups. Male, but not female, offspring from mothers treated with GCs during pregnancy exhibited stronger negative feedback compared with those from control mothers, but there were no differences in negative feedback between lactation treatment groups. Offspring with higher adrenal reactivity from mothers treated during pregnancy (both controls and GC-treated) exhibited lower aggression and activity. These results suggest that maternal GCs during gestation or early lactation alone may not be a sufficient cue to produce substantial changes in behavioral and physiological stress responses in offspring in natural populations.

KEY WORDS: Coping styles, Development, GCs, Maternal effects, Maternal hormones, *Tamiasciurus hudsonicus*

INTRODUCTION

Maternal effects, or the influence of a mother's phenotype on those of her offspring, contribute to among-individual phenotypic variation, and may induce adaptive developmental plasticity (Lancaster et al., 2007; Reddon, 2012; Rossiter, 1991; Stamps and Groothuis, 2010). For example, gravid female fall field crickets (*Gryllus pennsylvanicus*) exposed to a non-lethal wolf spider (*Hogna helluo*) prior to laying eggs produced more cautious offspring that were more likely to survive in the presence of a lethal wolf spider than control offspring (Storm and Lima, 2010). Maternal glucocorticoids (GCs) have been proposed as one proximate mechanism by which maternal effects shape offspring phenotypes (Kapoor et al., 2008; Meaney, 2001), particularly with regard to the development of their physiological stress response and behavior. GCs are responsive to environmental changes, and therefore may act as an indicator that offspring are cued into and respond to with adaptive changes in phenotypes (Del Giudice et al., 2011; Sih, 2011). Similarly, an increase in maternal corticosterone in pregnant female viviparous lizards (*Zootoca vivipara*) improved odds of survival for male offspring (Meylan and Clobert, 2005).

A consistent finding across taxa is that variation in maternal GCs can induce shifts in offspring behavior (reviewed in Weinstock, 2008). One way in which maternal GCs could lead to adaptive shifts in offspring behavior is through changes in their hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis is a negative feedback system that regulates systemic effector hormones (GCs) (Sapolsky et al., 1985; Spencer and Deak, 2017). Briefly, neural inputs trigger the paraventricular nucleus in the hypothalamus to release corticotropin-releasing factor (CRF), which acts upon the anterior pituitary to release adrenocorticotropic hormone (ACTH), which travels systemically to activate the adrenal cortex to release GCs (cortisol and/or corticosterone) (Packard et al., 2016; Spencer and Deak, 2017). GCs are primarily metabolic steroid hormones, but are often studied because of their release in response to stressful situations and adverse environmental conditions (Charmandari et al., 2005; Sapolsky et al., 2000; Spencer and Deak, 2017; Tsigos and Chrousos, 2002). High levels of systemic GCs induce negative feedback by binding to receptors in the hypothalamus and pituitary to return expression of CRF, ACTH and GCs to basal levels after a response to an acute stressor (Sapolsky et al., 1985; Spencer and Deak, 2017).

There are now many laboratory and field studies demonstrating a strong effect of the early life environment and maternal GCs on offspring behavior and HPA axis physiology (reviewed in Caldji et al., 2011). In an experiment using maternal adrenalectomies and administration of exogenous GCs in rats, Barbazanges et al. (1996) demonstrated the role of excess maternal GCs in mediating the impairment of negative feedback regulation of the offspring's HPA axis. As summarized in Weinstock (2008), maternal stress can raise GCs and catecholamines, and reduce neural GC receptors in offspring. This reduction in feedback regulation of the HPA axis can alter emotion, cognition, attention and learning (Meaney, 2001; Weinstock, 2008). For example, increasing GCs of mothers during

[1]Department of Psychology, University of Michigan, Ann Arbor, MI 48109-1043, USA. [2]Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada, T6G 2E9. [3]Department of Biology, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5E2. [4]Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA. [5]Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1085, USA.
*Present address: Department of Evolution, Ecology, and Behavior, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

‡Author for correspondence (westse@illinois.edu)

S.E.W., 0000-0002-5381-1048; F.v., 0000-0002-3034-9022; S.B., 0000-0001-6317-038X; J.E.L., 0000-0003-0849-7181; A.G.M., 0000-0001-7323-2572; B.D., 0000-0002-3058-265X

Received 2 September 2020; Accepted 29 March 2021

lactation improved learning and reduced fearfulness of offspring in rats (Catalani et al., 2000), whereas higher GCs in milk of rhesus macaques (*Macaca mulatta*) produced more 'nervous' and less 'confident' offspring (Hinde et al., 2015). These laboratory studies reveal the potential for changes in maternal GCs to alter offspring behavior, but studies in natural populations are required to understand if the changes in offspring behavior are adaptive.

North American red squirrels (*Tamiasciurus hudsonicus*, hereafter 'red squirrels') in the Yukon Territory, Canada, experience among-year fluctuations in the availability of their major food source, seeds from white spruce (*Picea glauca*) trees (Fletcher et al., 2010, 2013; Ren et al., 2017). Red squirrels defend individual territories year-round that each contain a hoard of seed-containing white spruce cones (Dantzer et al., 2012; Siracusa et al., 2017). Juvenile red squirrels usually must acquire a territory after weaning (usually in the late spring or summer) to survive their first winter (Larsen and Boutin, 1994). The among-year variation in food abundance causes changes in population density such that juvenile red squirrels experience fluctuations in the degree of competition over vacant territories (Taylor et al., 2014). We have previously shown that red squirrels that grow quickly after birth (Dantzer et al., 2013), and those with mothers that were more aggressive and less active (in standardized behavioral assays; Taylor et al., 2014) tended to be more likely to survive under high-density conditions. We have also previously found that mothers have elevated GCs during high-density conditions (Dantzer et al., 2013; Guindre-Parker et al., 2019), and that elevated GCs during pregnancy result in faster-growing pups (Dantzer et al., 2013, 2020b). This suggests that elevations in maternal GCs during pregnancy may induce adaptive increases in offspring growth, but whether elevations in maternal GCs cause adaptive increases in offspring aggressiveness is not clear.

In this study, we investigated the effects of elevated maternal GCs on the HPA axis and behavior of their offspring. We asked whether changes in maternal GCs induced developmental plasticity in offspring behavior, physiological stress responsiveness, and the interaction between the two by conducting a 3-year GC supplementation experiment in a wild population of red squirrels. We tested the hypothesis that elevated maternal GCs induce an adaptive shift in offspring aggressiveness and activity that prepares them for competitive environments. To do so, we increased maternal GCs, which should increase the transfer of maternal GCs to the offspring across the placenta or through milk (Grey et al., 2013; Kulski and Hartmann, 1981; O'Donnell et al., 2009), and could also influence rates of maternal care (e.g. Nephew and Bridges, 2011; Smith et al., 2004; Vilela and Giusti-Paiva, 2011). Our previous studies indicated that elevations in maternal GCs during pregnancy caused increases in offspring postnatal growth, but elevations in maternal GCs during lactation reduced offspring postnatal growth (Dantzer et al., 2020b). We therefore investigated how elevations in maternal GCs during pregnancy or lactation affected offspring behavior and HPA axis responsiveness.

We characterized the behavior of offspring from GC-treated mothers and control mothers using standardized behavioral assays that measured offspring activity and aggression towards a simulated conspecific in an enclosed arena. Activity and aggression were quantified using open-field trials and mirror image stimulation tests, respectively. Similar to other studies in red squirrels (Boon et al., 2007, 2008; Cooper et al., 2017; Taylor et al., 2012, 2014; Westrick et al., 2019), squirrels were characterized as more active if they spent more time moving around the arena in the open-field trial and were characterized as more aggressive if they attacked their mirror image (simulated conspecific) more often in the mirror image stimulation test. If maternal GCs induce adaptive plasticity in offspring behavior, we predicted that offspring produced by mothers treated with GCs would be more aggressive and less active. Given that increased exposure to maternal GCs can modify the activity of the HPA axis in offspring (see above), and this may in turn cause changes in offspring behavior, we also quantified the impact of experimentally elevated maternal GCs on offspring HPA axis activity. To do so, we conducted stress challenges that measure the ability of the HPA axis to terminate the physiological stress response (negative feedback following dexamethasone administration; van Kesteren et al., 2019) and to mount a physiological stress response (rise in plasma cortisol concentrations following administration of ACTH; van Kesteren et al., 2019). Based upon previous work in other species (see above), we predicted that offspring produced by mothers treated with GCs would exhibit decreased negative feedback and increased stress responsiveness. As some studies have linked HPA axis activity with behavioral characteristics including aggression and activity (Koolhaas et al., 1999; Westrick et al., 2019), we also examined if there was an association between HPA axis activity and aggression or activity in offspring.

Finally, our previous work showed that activity and aggression in female red squirrels are both phenotypically and genetically positively correlated in red squirrels (Taylor et al., 2012), but selection may favor females that are divergent from that population trend (Taylor et al., 2014). Previous studies in other taxa suggest that elevations in maternal GCs may cause adaptive shifts in phenotypic co-variance by either strengthening the degree of co-variation among particular traits (Merrill and Grindstaff, 2015) or lessening the overall degree of phenotypic co-variance (Careau et al., 2014). We therefore investigated whether our GC treatments affected the phenotypic correlation between activity and aggression. We predicted that juveniles produced by mothers with elevated GCs would exhibit a significantly reduced positive correlation between aggression and activity compared with offspring from control mothers.

MATERIALS AND METHODS

Study population

We studied North American red squirrels [*Tamiasciurus hudsonicus* (Erxleben 1777)] in two different study areas on the traditional lands of the Champagne and Aishihik First Nations in the Yukon Territory, Canada (61°N, 138°W). North American red squirrels of both sexes are highly territorial and defend their food cache year-round (Dantzer et al., 2012; Siracusa et al., 2017). To identify adults, we used unique alphanumeric stamped ear tags (National Band and Tag Company, Newport, KY, USA) and unique combinations of colored wire threaded through both ear tags. We used live trapping (Tomahawk Live Trap, Tomahawk, WI, USA) to monitor reproductive status by abdominal palpation to detect fetuses, and manual milk expression to detect lactation (McAdam et al., 2007). Upon identifying a lactating female, we collared her with a VHF radio transmitter (Holohil PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada), and used telemetry to locate the nest containing her pups. We briefly removed the pups from the nest soon after birth, and again ∼25 days post-parturition in order to identify sexes, record masses to calculate post-natal growth, and ear-tag pups with alphanumeric tags and unique combinations of colored discs for identification after emergence at ∼35 days. Red squirrel pups are completely weaned around 70 days (Boutin and Larsen, 1993). For more details on the general population monitoring methods, see McAdam et al. (2007). All work was conducted under animal ethics approvals from the University of Michigan (PRO00005866).

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

Glucocorticoid supplementation experiment

Between February and August of 2015–2017, we conducted a GC supplementation experiment in this population of red squirrels. To simulate chronic increases in GCs, we treated pregnant or lactating females with exogenous GCs mixed with all-natural peanut butter using methods described in Dantzer et al. (2013) and van Kesteren et al. (2019). Control females received the same peanut butter treatments without GCs. To treat individuals with GCs daily, we provisioned them with small amounts of peanut butter (∼8 g) and wheat germ (∼2 g) mixed with dissolved hydrocortisone (hereafter, cortisol) (H4001, Sigma-Aldrich). We first dissolved the cortisol in 1 ml of 100% ethanol before mixing with 5 ml of peanut oil. We let the emulsion sit overnight to evaporate the ethanol. We then combined the peanut oil with 800 g peanut butter and 200 g wheat germ, weighed out individual doses (∼10 g), placed each dose in individual containers, and stored doses at −20°C until needed for provisioning the squirrels. Control treatments were made in exactly the same manner, but did not include cortisol in the peanut oil.

On the territory of each squirrel in our experiment, we hung a 10.5-liter bucket with two holes cut into its sides. Each bucket was covered with a lid and hung ∼7–10 m off the ground at the center of the squirrel's territory. We placed individual peanut butter treatments in the buckets for provisioning each day. We randomly assigned squirrels to either the control treatment (8 g all-natural peanut butter, 2 g wheat germ, no GCs) or GC treatment (8 g all-natural peanut butter, 2 g wheat germ, 8 or 12 mg of GCs). We fed all squirrels treated during pregnancy 8 mg of GCs per day and squirrels treated during lactation either 8 or 12 mg of GCs per day. We selected these dosages of GCs to keep GCs within physiological levels, based on previous studies in red squirrels (Dantzer et al., 2013; van Kesteren et al., 2019) and laboratory rats (Casolini et al., 1997; Catalani et al., 2002). A previous study using the same procedure showed that plasma cortisol levels increase rapidly after consumption of the GC treatments and that cumulative exposure to GCs over a 24 h period was higher than controls (van Kesteren et al., 2019). Wilcoxon rank sum tests on the four response variables (two HPA axis measurements and two behavioral traits) showed no significant differences between 8 and 12 mg of GCs per day in the lactation treatment group (Table S1) and a previous study showed no differences in fecal glucocorticoid metabolite levels between squirrels fed 8 and 12 mg of GCs per day (van Kesteren et al., 2019). Therefore, we combined the 8 and 12 mg treatments into one GC treatment group to increase statistical power (Dantzer et al., 2020b).

To examine whether the timing of an increase in maternal GCs produced unique changes in offspring phenotypes, we treated breeding female squirrels either during late pregnancy or during early lactation. In the pregnancy treatment groups, we aimed to treat mothers for 20 days starting approximately 15 days prior to birth (20 days after conception) until 5 days after birth. Owing to variation in detecting the precise stage of pregnancy via palpation, we actually treated mothers from 10.8±0.7 days (mean±s.d.) prior to birth to 4.6±0.4 days after birth (actual treatment length: 16.3±0.6 days). In the lactation treatment groups, we aimed to treat mothers for 10 days starting 5 days after birth until 15 days after birth. We actually treated mothers during lactation 5.1±0.2 days post-parturition to 14±0.3 days post-parturition (actual treatment length: 10±0.1 days). This experimental design resulted in four treatment groups which we will refer to as: pregnancy control, pregnancy GC, lactation control and lactation GC (see Table 1 for sample sizes). For more detailed information about this manipulation and how it impacts circulating levels of cortisol (the major GC in red squirrels) in plasma, see van Kesteren et al. (2019).

Table 1. Sample sizes

	Pregnancy treatments	Lactation treatments
HPA axis dynamics by treatment group		
GC peanut butter (8 or 12 mg cortisol)	13 mothers supplemented	8 mothers supplemented
	13 litters produced	8 litters produced
	20 pups tested	13 pups tested
Control peanut butter (0 mg cortisol)	13 mothers supplemented	9 mothers supplemented
	13 litters produced	9 litters produced
	21 pups tested	13 pups tested
HPA axis dynamics and behavior by treatment group		
GC peanut butter (8 or 12 mg cortisol)	12 mothers supplemented	7 mothers supplemented
	12 litters produced	7 litters produced
	16 pups tested	12 pups tested
Control peanut butter (0 mg cortisol)	12 mothers supplemented	9 mothers supplemented
	12 litters produced	9 litters produced
	18 pups tested	11 pups tested

The number of red squirrel mothers supplemented, litters produced, and pups tested within each treatment group are given. Two mothers are represented in alternating treatment groups [glucocorticoid (GC) and control] during pregnancy in different years. Not all juvenile red squirrels underwent an open-field/mirror image simulation trial, so the sample sizes for analyses of HPA axis dynamics without any behavioral variables included a few more squirrels than the analyses of HPA axis dynamics and behavior ('activity' and 'aggression')

Behavioral trials

We live-trapped offspring from our experimental females around the age of weaning (67.79±3.76 days old, mean±s.d.; weaning age is ∼70 days old; Boutin and Larsen, 1993). Due to logistical limitations, we aimed to sample a maximum of two individuals per litter. This allowed us to maximize the number of litters we could sample at as close to 70 days old as possible. The maximum number of pups from each litter in our sample is two (N=7 litters with two pups out of 16 total litters). Using a canvas handling bag, we weighed the juvenile squirrels before transferring them to our behavioral assay arena for two trials: open-field trials to measure activity, and mirror image stimulation trials to measure conspecific aggression. Open-field trials are widely used in behavioral neuroscience to measure locomotor–exploratory behavior (Mazzamuto et al., 2019; Perals et al., 2017; Seibenhener and Wooten, 2015; Walsh and Cummins, 1976). Differences in open-field behavior among rodent species appear to be consistent with natural behavioral differences (Wilson et al., 1976). Mirror image stimulation trials are commonly used as a standardized method to measure conspecific aggression in species that do not have self-recognition in a mirror image (Balzarini et al., 2014; Gallup, 1968; Mazzamuto et al., 2019).

For both trials, we used an arena (60×80×50 cm) constructed with white corrugated plastic and a clear acrylic lid, as described in previous studies in this study system (Boon et al., 2007, 2008; Kelley et al., 2015; Taylor et al., 2012, 2014). The arena contained four blind holes that the squirrel could investigate during the trial. We recorded the squirrel's behavior using a digital video camera for later scoring. For the open-field trial, squirrels were in the open arena for 7 min. This also served as the acclimation period for the following mirror image stimulation trial which lasted 5 min after the mirror (45×30 cm) on one side of the arena was exposed. Although the estimated amount of time that had elapsed from the squirrel first entering the trap was variable (Table S2A), there were no significant differences among the treatment groups (Table S2B).

We used JWatcher (Blumstein and Daniel, 2007) to manually score the videos using the same ethogram used in previously published studies of red squirrel personality (Boon et al., 2007, 2008; Taylor et al., 2012, 2014; Westrick et al., 2019). Observers (N=4) were blind to the treatment group of the individual. In our analyses, we only included behaviors that previously showed high inter-observer reliability (Taylor et al., 2012).

HPA axis hormone challenges

Following the behavioral trials, we performed HPA axis hormone challenges by administering dexamethasone (Dex; a GC receptor agonist) and ACTH, as previously described, to offspring from our experimental females (van Kesteren et al., 2019). Briefly, Dex binds to the GC receptors to induce negative feedback of the HPA axis, primarily through acting on the anterior pituitary (De Kloet et al., 1975), which downregulates circulating GC levels, while ACTH acts upon the adrenals to upregulate the production of GCs. We began by collecting a blood sample from a rear toenail using heparinized microcapillary tubes (mean blood collection volume ∼100–150 µl; described in van Kesteren et al., 2019; Dantzer et al., 2020a,b) for measurement of initial concentrations of total cortisol circulating in plasma. It is important to note that this sample was taken after trapping, handling, recording through a behavioral trial, and transporting individuals from their natal or recently claimed territories to our field station. Across all treatment groups, there was variation in the duration of time individuals spent in the trap (Table S2A), both prior to the behavioral trial and prior to the first blood sample. Although this could theoretically influence the responses of squirrels to our stress challenges, there were no systematic differences among the different treatment groups in the amount of time squirrels spent in the trap (Table S2B), and we found that the time offspring spent in the trap prior to the behavioral trial and hormone challenge did not influence either behavior or HPA axis dynamics (Table S3). Additionally, squirrels with higher initial plasma cortisol concentrations did not have lower responses to ACTH (Table S4B), indicating that any biases would be spread across the treatment groups, and that squirrels' adrenal glands were still responsive to ACTH after undergoing substantial handling. Owing to this substantial amount of handling and disturbance, we consider the initial sample levels of cortisol as stress induced. We then injected 3.2 mg kg^{-1} of dexamethasone (VetOne, dexamethasone sodium phosphate, 4 mg ml^{-1} solution in sterile saline) intramuscularly into the squirrel's upper rear leg. We released the squirrel back into the live trap and waited 1 h before taking another blood sample ('Dex bleed'). Next, we injected 4 IU kg^{-1} of ACTH (cosyntropin diluted in saline; Cortrosyn, Amphastar, Rancho Cucamonga, CA, USA) intramuscularly in the alternate upper rear leg. We kept the squirrel in the live trap before taking blood samples 30 min ('ACTH 30') and 1 h post-injection ('ACTH 60'). We have previously shown that these dosages of Dex and ACTH are sufficient to respectively decrease and increase circulating plasma cortisol concentrations in adult red squirrels (Boonstra and McColl, 2000; van Kesteren et al., 2019). We kept all blood samples on wet ice during the challenge. At the conclusion of the challenge, we separated the plasma via centrifugation and then froze the samples at −80°C.

To quantify total plasma cortisol concentrations, we used an ImmuChem cortisol coated tube radioimmunoassay (RIA) kit (MP Biomedicals) following the manufacturer's instructions, with minor modification of sample and tracer volumes, and ran samples in duplicate, when possible. To run as many duplicates as possible with our small plasma volumes, we used 12.5 µl of sample and 500 µl of tracer. We ran 87% of samples in duplicate. On rare occasions, we were unable to collect enough blood to quantify total plasma cortisol at every time point (initial sample: N=5 samples missed from all individuals; Dex bleed, N=3; ACTH 30, N=6; ACTH 60, N=4). To maximize the number of squirrels included in our study, we used the global mean value of total plasma cortisol for that respective time point for these missing time points. Due to the large number of samples, we ran RIAs on four different days across three years. We ran RIAs after each period of sample collection with all plasma samples from that year with maternal treatments spread across all assays. Across all four assays, our average standard and sample intra-assay coefficients of variance (CVs) were 9.5%, our average intra-assay CV for red squirrel plasma samples was 9.3%, and our average inter-assay CV for the five standards provided (10, 30, 100, 300 and 1000 ng ml^{-1} cortisol) was 14%. As our red squirrel plasma volumes were limited, we included a pooled standard of prairie vole (*Microtus ochrogaster*) plasma across all assays instead. Inter-assay CV for the pooled prairie vole plasma sample was 7.8%. The experimenters conducting the HPA axis challenges were not blind to maternal treatments owing to the same researchers mixing the peanut butter treatments, provisioning the mothers and trapping the squirrels. The experimenters were blind to the results of the behavioral trials. The experimenters conducting the RIAs were blind to both the maternal treatments and results of the behavioral trials.

Statistical analyses

We ran all statistical analyses in R version 3.5.2 (https://www.r-project.org/). We used the R package 'ade4' version 1.7-10 (Dray and Dufour, 2007). We ran two distinct principal component analyses with correlation matrices (one for open-field behaviors and one for mirror image stimulation behaviors) to reduce behavioral variables down to one major component for each assay. Based on the loadings (Table 2), we interpreted the first principal component of the open-field trial as 'activity', explaining 30% of variation in open-field behaviors in our dataset. We interpreted the first component of the mirror image stimulation trial as 'aggression', explaining 50% of variation in mirror image stimulation behaviors

Table 2. Principal component analysis loadings for behaviors scored in the open-field trials and mirror image stimulation trials

	Loading
Open-field behavior	
Time spent walking	0.59
Time spent hanging	0.33
Chewing or scratching	0.19
Number of jumps	0.79
Hole head dips	0.29
Time spent grooming	−0.47
Not moving	−0.81
Proportion of variance	0.30
Mirror image stimulation behavior	
Time spent in front of arena	0.77
Number of attacks	0.48
Time spent in back of arena	−0.68
Latency to attack	−0.75
Latency to approach	−0.80
Proportion of variance	0.50

Principal component analysis (PCA) loadings are shown for juvenile red squirrel behaviors with high inter-observer reliability for the first PCA component for both the open-field and mirror image stimulation trials (Taylor et al., 2012). We used PCA loadings over 0.2 for the interpretation of the component. We calculated 'activity' and 'aggression' scores from these loadings.

in our dataset. Previous studies in this system have used the same methods to analyse open-field and mirror image stimulation trials, and also used the same interpretation for the first component for the open-field trial and mirror image stimulation trial (Boon et al., 2007, 2008; Cooper et al., 2017; Kelley et al., 2015; Taylor et al., 2012, 2014; Westrick et al., 2019). Activity measured using open-field trials has some ecological validity in red squirrels, as a previous study showed that red squirrels who were more active in the open-field arena were also live-trapped more often, and at more unique locations than less active individuals (Boon et al., 2008). All subsequent analyses used the individual scores calculated from the principal component loadings for each trial (Table 2). Higher 'activity' scores mean the squirrel spent more time walking, jumping, etc. Higher 'aggression' scores mean the squirrel attacked the mirror more often and spent more time in front of the mirror than lower-scoring squirrels (Table 2).

We calculated the adrenal responsivity, or the net integrated response of cortisol over the 60 min post-ACTH injection, as the area under the curve (AUC) from the Dex bleed to ACTH 60 using the natural cubic spline interpolation (ACTH AUC). AUC is used in other mammalian and avian study systems as a measure of the integrated adrenocortical response to ACTH (Heidinger et al., 2008; Ingram et al., 1997; Janssens et al., 1994; Rich and Romero, 2005; Saltzman et al., 2000). Based on a recent review about calculating HPA axis negative feedback after a Dex injection (Lattin and Kelly, 2020), we calculated the relative decrease in cortisol from the initial sample bleed to the Dex bleed (Dex response). The relative reduction in cortisol is an integrative measure of HPA axis negative feedback and is easily comparable across species (Lattin and Kelly, 2020).

We used the R package 'lme4' version 1.1-19 (Bates et al., 2015) to fit linear mixed-effects models and estimated *P*-values using the R package 'lmerTest' version 3.0-1 (https://cran.r-project.org/package=lmerTest). We used the R package 'multcomp' version 1.4-8 (Hothorn et al., 2008) to run a Tukey's *post hoc* comparison following a linear mixed-effects model of plasma cortisol levels at each of the four sampling time points.

For each response variable (HPA axis and behavior variables), we fitted separate models for pregnancy and lactation treatment groups. We separated the pregnancy and lactation treatments to simplify our model structures and to allow us to statistically control for variation in treatment length, which was unique to pregnancy treatments. To control for the variability in the number of doses mothers in the pregnancy treatment group received (16.3±0.6 days, mean±s.d.), we included maternal treatment length in all pregnancy models. Treatment length among mothers in the lactation groups did not vary considerably (10±0.1 days, mean±s.d.); therefore, we did not include this in the lactation models. We also wanted to separate the lactation and pregnancy models to be consistent with our previous publication using data from this GC manipulation experiment (Dantzer et al., 2020b).

We compared the HPA response variables (ACTH AUC and Dex response) and behavioral response variables (activity and aggression) between juveniles in GC-treated groups with the appropriate control groups using linear mixed-effects models. In the models to predict ACTH AUC, we included treatment group (GC or control), sex, post-Dex injection plasma total cortisol concentration, treatment year (categorical) and age of the juvenile (standardized across all data for all analyses) as fixed effects. In the models to predict the response to Dex, we included treatment group, sex, treatment year and age as fixed effects. In all HPA axis models, we included an interaction between GC treatment group and sex to investigate any sex-specific effects of the GC treatments. In all the pregnancy models, we also included maternal treatment length (standardized across all data for all analyses).

To assess the impact of maternal GCs on behavior and the relationship between HPA and behavior, we fitted separate linear mixed-effects models for activity and aggression. For our aggression models, we included treatment group, sex, treatment year, age, ACTH AUC, response to DEX and an interaction between treatment group and sex as fixed effects. For activity, we included treatment group, sex, treatment year, age, ACTH AUC, response to Dex, treatment group and sex as fixed effects, but also added additional fixed effects of aggression and an interaction between treatment group and aggression. This model allowed us to assess the impact of maternal treatment on the relationship between activity and aggression.

As we included multiple pups from the same litter, we included litter identity (ID) as a random effect whenever possible. In three of the eight total models (pregnancy treatments: negative feedback and aggression; lactation treatments: activity), including litter ID as a random effect resulted in singular fit. In these instances, we used a general linear model without random effects. ANOVAs comparing the linear and linear mixed-effects models showed that the models were nearly identical, so we removed the random effect to simplify the model while maintaining our treatment group interaction terms. To detect any collinearity in the predictors included in our models, we used R package 'car' version 3.0-2 (Fox and Weisberg, 2011) to assess the variance inflation factors. We found $GVIF^{[1/(2\times DF)]}<2$ (where GVIF is generalized variance inflation factor and DF is degrees of freedom) for all predictors across all models. We visually confirmed normality and homoscedasticity of residuals of all linear models.

RESULTS

On average, all treatment groups responded to Dex and ACTH as expected, with a decrease in plasma cortisol concentrations following Dex, and a subsequent increase in plasma cortisol concentrations following ACTH (Fig. 1). A general linear mixed model with Tukey's *post hoc* comparisons showed that plasma cortisol concentrations did not differ between the initial handling stress-induced sample and the ACTH 30 min bleed (ACTH 30 – initial sample: β=−4.77, z=−2.03, P=0.18), and were lowest at the Dex bleed, 1 h after the injection of Dex (Dex bleed – initial sample: β=−36.78, z=−15.67, P<0.001; ACTH 30 – Dex: β=−32.01, z=13.66, P<0.001; ACTH 60 – Dex: β=27.08, z=11.55, P<0.001). The lack of difference between plasma cortisol concentrations in the initial handling stress-induced sample and the samples obtained 30 min after ACTH administration illustrates that the squirrels did not suffer from adrenal exhaustion due to stress associated with capture and handling. Plasma cortisol concentrations did not differ significantly between ACTH 30 min and ACTH 60 min bleeds (ACTH 60 – ACTH 30: β=−4.93, z=−2.10, P=0.15). One individual (out of 57 total individuals) did not respond to Dex. As all other individuals in the study responded to Dex, we excluded it from further analysis, given that it was probably due to an error during Dex administration.

Effect of maternal glucocorticoids on HPA axis activity

Juveniles from mothers treated with exogenous GCs during pregnancy or lactation did not differ in their adrenal response to ACTH, as measured by AUC, compared with controls (pregnancy treatment: β=5.34, P=0.56; lactation treatment: β=−15.48, P=0.11; Table 3, Fig. 2). There were no overall sex differences in the response to ACTH and no significant interaction between sex and

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

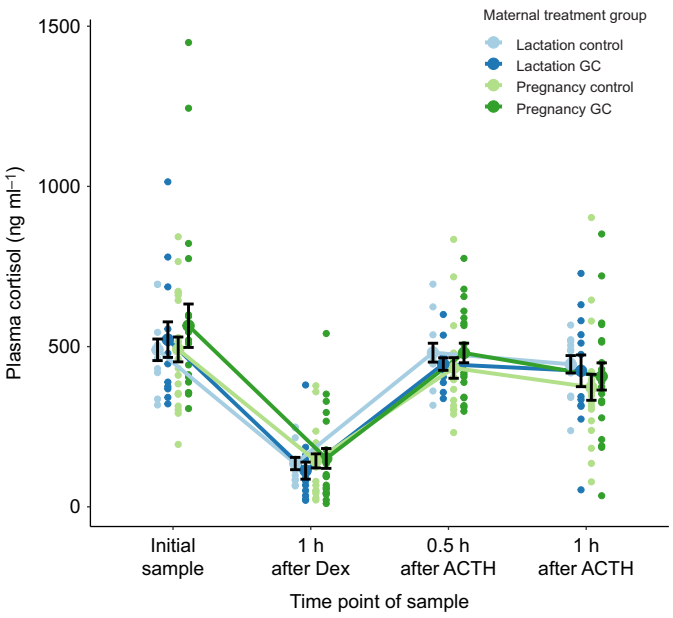

Fig. 1. Hypothalamic–pituitary–adrenal axis hormone challenge curves by treatment group. Plasma cortisol concentrations (ng ml^{-1}) of juvenile red squirrels plotted across the dexamethasone (Dex) and adrenocorticotropic hormone (ACTH) hormone challenge time series. Blue lines and points indicate juveniles from lactation treatments [lactation control, *N*=12 juvenile squirrels; lactation glucocorticoid (GC), *N*=13 juvenile squirrels] and green lines and points indicate juveniles from pregnancy treatments [pregnancy control, *N*=20 juvenile squirrels; pregnancy glucocorticoid (GC), *N*=20 juvenile squirrels]. Data are staggered at each time point by maternal treatment group for ease of visualization. Black bars indicate the standard error around the mean for each group at each time point.

GC treatment (Table 3). Older pups in the lactation treatment group (but not in the pregnancy group) had smaller responses to ACTH (β=−6.67, *P*=0.02). Treatment length for pregnancy treatments did not explain variation in ACTH AUC (β=−2.49, *P*=0.65; Table 3). We found no effect of year on ACTH AUC across the 3 years of this experiment in either pregnancy or lactation treatment groups (Table 3).

The effect of the pregnancy GC treatment on responsiveness to Dex varied by sex, with males, but not females, from mothers treated with GCs during pregnancy showing lower plasma cortisol concentrations 60 min after Dex administration compared with the controls (interaction: β=−30.79, *P*=0.01; pregnancy control versus pregnancy GC treatment groups for males: β=−29.75, *P*=0.002, pregnancy control versus pregnancy GC for females: β=1.04, *P*=0.99; Table 3, Fig. 2B). This indicates enhanced negative feedback in the HPA axis in males from mothers treated with GCs during pregnancy. Juveniles from mothers treated with exogenous GCs during lactation did not differ in the magnitude of their negative feedback response to Dex compared with controls (β=−3.44, *P*=0.78; Table 3, Fig. 2). The age of the juvenile did not contribute significantly to variation in negative feedback (Table 3). For juveniles from mothers treated during lactation, sex and year of treatment did not impact negative feedback (Table 3) but there was significant variation across years in offspring from mothers treated with GCs during pregnancy (2015 versus 2016: β=−23.26, *P*=0.03; 2015 versus 2017: β=−21.26, *P*=0.04; Table 3).

We conducted additional analyses to confirm the lack of treatment effects on offspring HPA axes where we assessed treatment effects on plasma cortisol concentrations in the samples obtained 60 min following the Dex injection, 30 min after the ACTH injection, and 60 min after the ACTH injection. Our results are the same as for our analyses described above using ACTH AUC or the responsiveness to Dex. There were no effects of treatment on plasma cortisol concentrations in the samples obtained 30 or 60 min following ACTH administration (Table S4). Plasma cortisol concentrations in the samples obtained 60 min following Dex did not differ between mothers treated with GCs during lactation or the controls, but male (but not female) offspring from mothers treated with GCs during pregnancy had significantly lower plasma cortisol concentrations 60 min after Dex compared with those from control mothers (Table S4). Except for the latter, there were no sex-specific treatment effects (Table S4).

Association between offspring HPA axis activity and behavior

Among juveniles from mothers treated with GCs during pregnancy and the controls, more active offspring exhibited a lower adrenal response to ACTH (ACTH AUC) than less active offspring (β=−0.02, *P*=0.03; (Table 4, Fig. 3). However, juveniles from mothers treated with GCs during lactation and the controls did not exhibit any relationship between ACTH AUC and activity (β=0.03, *P*=0.16; Table 4, Fig. 3). The negative feedback response to Dex did not predict activity in either pregnancy or lactation treatment groups (Table 4, Fig. 3). Among all juveniles from all treatment groups, more aggressive individuals had a lower adrenal response to ACTH than less aggressive individuals (pregnancy treatment: β=−0.02, *P*=0.02; lactation treatment: β=−0.03, *P*=0.03; Table 4, Fig. 3).

Effect of maternal glucocorticoids on offspring behavioral traits

Juveniles from mothers treated with GCs during pregnancy did not differ in their activity or aggression levels measured in the open-field trial and mirror image simulation tests compared with those from control mothers (Table 4, Fig. 4). Juveniles from mothers treated with GCs during lactation did exhibit slightly, but significantly, higher activity levels than those from lactation control mothers (β=2.49, *P*=0.01; Table 4, Fig. 4), but there was no effect on juvenile aggression levels (Table 4, Fig. 4). There were no other sex-specific effects of the GC treatments on offspring activity and aggression and treatment length; age of the juvenile or year did not contribute to variation in activity or aggression (Table 4). Treatment with GCs during pregnancy or lactation also did not affect the relationship between activity and aggression (Table 4).

DISCUSSION

Endocrine responses to environmental changes or cues are widely acknowledged to induce adaptive phenotypic plasticity (Denver, 2009; Hau et al., 2016; Lema and Kitano, 2013). We have previously shown that female red squirrels exhibit increases in GCs in response to elevated population density (Dantzer et al., 2013; Guindre-Parker et al., 2019), and that increases in GCs in pregnant females induce adaptive plasticity in offspring postnatal growth rates that likely makes offspring better able to survive in high-density environments (Dantzer et al., 2013, 2020b). Here, we tested the hypothesis that elevated maternal GCs induce adaptive plasticity in offspring behavior by modifying their HPA axis. Under high-density conditions, female red squirrels that are more aggressive and less active tend to have higher reproductive success under high-density conditions (Taylor et al., 2014). As activity and aggression are at least partially heritable and experience moderate maternal

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

Table 3. Full results of HPA axis dynamics models

	Pregnancy treatments			Lactation treatments		
Fixed effect	β	95% CI	*P*-value	β	95% CI	*P*-value
Response to ACTH (area under the curve from Dex to ACTH 60)						
Intercept	**75.24**	**55.42 to 95.06**	**<0.001**	**67.45**	**48.64 to 86.27**	**<0.001**
Fed GCs	5.34	−12.48 to 23.16	0.56	−15.48	−33.23 to 2.27	0.11
Sex: male	−6.18	−24.21 to 11.85	0.51	−5.25	−21.31 to 10.82	0.53
Dex [cortisol] (μg dl^{-1})	**1.18**	**0.67 to 1.69**	**<0.001**	**1.64**	**1.26 to 2.01**	**<0.001**
Standardized treatment length (days)	−2.49	−12.93 to 7.95	0.65			
Year: 2016	−22.72	−47.27 to 1.83	0.09	−3.93	−30.35 to 22.49	0.78
Year: 2017	−21.45	−45.83 to 2.93	0.10	0.47	−16.08 to 17.02	0.96
Standardized age (days)	−1.84	−10.75 to 7.06	0.69	**−6.67**	**−11.70 to −1.64**	**0.02**
Fed GCs×Sex: male	7.11	−19.20 to 33.43	0.60	17.57	−3.90 to 39.04	0.13
Random effect: litter ID						
Within-group variance	292.04			53.61		
Between-group variance	66.86			190.13		
Intra-class correlation	0.19			0.78		
Observations	38 pups from 25 litters			25 pups from 17 litters		
Response to Dex (negative feedback; % reduction in plasma cortisol)						
Intercept	88.20	73.10 to 103.30	<0.001	82.66	58.56 to 106.76	<0.001
Fed GCs	−1.04	−16.03 to 13.95	0.89	−3.44	−27.40 to 20.51	0.78
Sex: male	**−26.60**	**−42.27 to −10.94**	**<0.01**	−3.95	−26.95 to 19.04	0.74
Year: 2016	**−23.26**	**−43.20 to −3.32**	**0.03**	−8.51	−35.37 to 18.36	0.55
Year: 2017	**−21.26**	**−41.03 to −1.49**	**0.04**	−4.94	−22.17 to 12.29	0.59
Standardized treatment length (days)	**9.78**	**1.36 to 18.21**	**0.03**			
Standardized age (days)	−4.53	−12.15 to 3.09	0.25	−6.23	−14.38 to 1.92	0.15
Fed GCs×Sex: male	**30.79**	**9.01 to 52.57**	**0.01**	6.98	−25.42 to 39.39	0.68
Random effect: litter ID						
Within-group variance	n/a			347.05		
Between-group variance	n/a			13.37		
Intra-class correlation	n/a			0.04		
Observations	40 pups from 26 litters			25 pups from 17 litters		

We ran four distinct models testing the effect of maternal GCs on the HPA axis dynamics in juvenile red squirrels. We ran separate models for the pregnancy and lactation treatment groups, and the two different aspects of the HPA axis dynamics: adrenal reactivity and negative feedback. We standardized treatment length (days) across all data for the pregnancy group and standardized age of pup (days) across all data. We did not include treatment length as a fixed effect in the lactation models because treatment length was mostly consistent for those litters. We did not include a random effect of litter ID in the negative feedback model for pregnancy treatments. The comparison group for categorical variables is control treatment females from 2015. Bold font indicates statistical significance of $P<0.05$. CI, confidence interval; n/a, not applicable.

effects (Taylor et al., 2012), less active or more aggressive mothers may produce offspring that are less active or more aggressive and consequently have higher survival rates under high-density conditions (Taylor et al., 2014). We did not find support for our prediction that offspring produced by mothers treated with GCs would be less active and more aggressive, which should be beneficial for high-density environments. Activity was significantly higher in offspring from females treated with GCs during lactation compared with those from controls, but there were no differences in activity levels between the pregnancy treatment groups, and offspring aggression was not affected by either of the GC treatments. Although there has been much research on the impacts of maternal GCs and perinatal stress on offspring behavior (Weinstock, 2008), surprisingly few studies have documented their impacts on offspring personality traits measured using standardized behavioral assays as we did here. Our results differ from a previous study in captive birds showing that increased exposure to GCs early in life (via egg injections) increased the overall activity levels of offspring later in life (Zimmer et al., 2013).

Our second prediction from our central hypothesis was that the changes in offspring behavior induced by elevated maternal GCs would result from altering offspring HPA axis activity. Numerous studies indicate associations between HPA axis activity and behavioral traits, including activity and aggression (Raulo and Dantzer, 2018; Weinstock, 2008). Other than finding that male offspring from mothers treated with GCs during pregnancy exhibited less negative feedback in response to dexamethasone, there were minimal effects on offspring HPA axis activity. This is surprising, as it is contrary to numerous studies in laboratory rodents on the effects of exogenous GCs or experimental manipulations that expose breeding females to various stressors (Harris and Seckl, 2011). For example, a recent meta-analysis including 39 studies across 14 vertebrate species found an overall positive relationship between 'prenatal stress' and offspring GC levels (Thayer et al., 2018). In particular, the authors found a stronger effect of prenatal stress on the negative feedback of the HPA axis than on the baseline or peak GC response to a stressor (Thayer et al., 2018). Research on the impacts of elevated postnatal GCs or increased postnatal exposure to stressors is less common. Some studies show that postnatal treatment of mothers with GCs can downregulate the activity of the HPA axis in offspring (Grace and Anderson, 2018; Grace et al., 2020), although we note that the effects of early-life exposure to GCs or stressful experiences on HPA axis reactivity depend upon the species, and that these effects can be sex specific (Dantzer et al., 2019; Grace and Anderson, 2018; Grace et al., 2020; Marasco et al., 2012; Spencer et al., 2009). Although we found that offspring from mothers treated with GCs during pregnancy did not have a more reactive HPA axis, there was a trend for offspring from mothers treated with GCs during lactation to have reduced HPA axis reactivity as measured using AUC ($P=0.11$; Table 3) and plasma

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

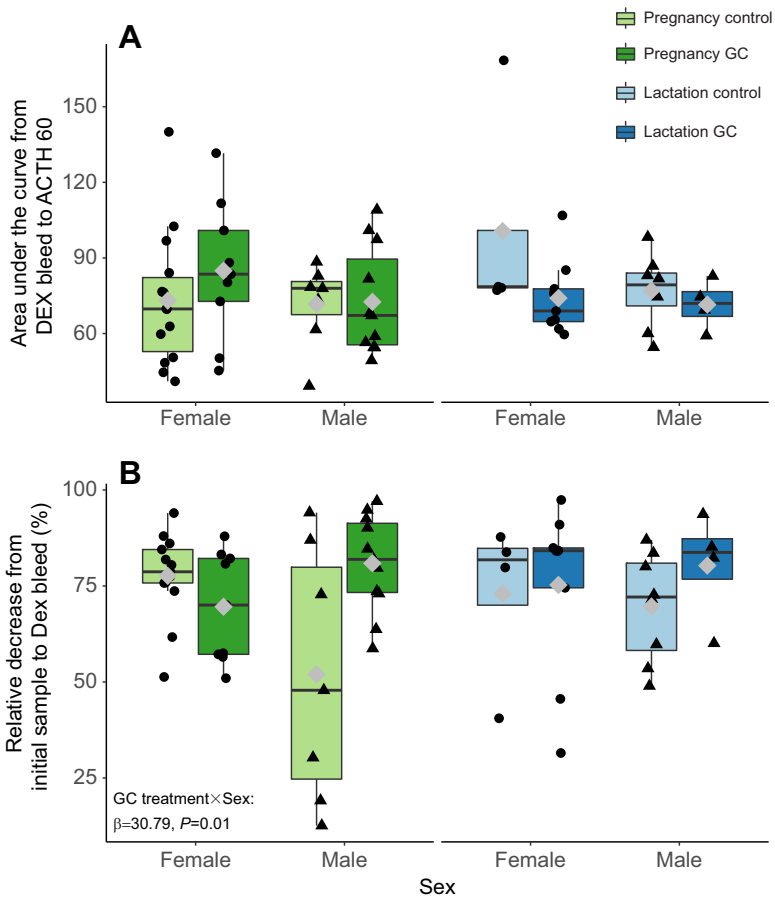

Fig. 2. Effect of treatment group on hypothalamic–pituitary–adrenal (HPA) axis dynamics. (A) Adrenal reactivity [response to adrenocorticotropic hormone (ACTH)] of juvenile red squirrels was not impacted by maternal treatment with exogenous glucocorticoids (GCs) (Table 3). (B) Negative feedback [response to dexamethasone (Dex)] was lower in male juveniles from mothers in the pregnancy control treatment group compared with males from the pregnancy GC group (Table 3). Box plots indicate the lower quartile, median and upper quartile. Whiskers indicate the range of the data. Gray diamonds indicate the group means. Data points represent individual juvenile red squirrels (pregnancy control, N=21; pregnancy GC, N=20; lactation control, N=13; lactation GC, N=13). The shape of the data point indicates the sex of the individual (circle: female red squirrel; triangle: male red squirrel).

cortisol concentrations 30 min after the ACTH injection (P=0.055; Table S4). These latter observations are consistent with previous studies showing that elevated postnatal exposure to GCs or stressors can reduce HPA axis reactivity in offspring (e.g. Grace et al., 2020). Our study and others show that there is not a general pattern among all species where elevated GCs or increased exposure to stressors either prenatally or postnatally enhances HPA axis activity in offspring. They also emphasize the need to assess the effects of elevated maternal GCs or increased exposure to stressors in a greater number of species and to consider sex specificity of their effects (Sheriff et al., 2017). Here, we found that male but not female offspring from mothers treated with GCs during pregnancy exhibited stronger negative feedback, although our previous study using the same experimental methods did not find any sex-specific effects on offspring postnatal growth (Dantzer et al., 2020b). As the number of species examined and the number of studies of free-living animals increases, it seems that there is not an easy or simple explanation that early life adversity enhances HPA axis activity in offspring in wild populations, as may be the case in studies of laboratory rodents.

We found little support for our third prediction from our central hypothesis, that increased maternal GCs would alter the phenotypic correlation between activity and aggression. Activity and aggression in red squirrels are phenotypically and genetically positively correlated (Taylor et al., 2012), yet a previous study suggested that selection favors squirrels that can decouple this positive correlation, as females that are less active and more aggressive tend to have higher reproductive success under high-density conditions (Taylor et al., 2014). Exposures to stressors can release cryptic genetic variation (Badyaev, 2005; Hoffmann and Hercus, 2000; Ledón-Rettig et al., 2010) and exposures to stressors during development can alter the degree of co-variance among different offspring phenotypes (Careau et al., 2014; Merrill and Grindstaff, 2015). If elevated maternal GCs induce an adaptive shift in the correlation between activity and aggression, we should have observed a significant lessening of the positive association between offspring activity and aggression, but we did not observe this.

Although we found little evidence that elevated maternal GCs affected offspring behavior or HPA axis, our results do provide insight into models about the correlated evolution of physiological and behavioral phenotypes. The unidimensional coping styles model (Koolhaas et al., 1999) posits that more active, aggressive individuals should exhibit decreased HPA axis activity, indicating co-variation between these physiological and behavioral phenotypes. Conversely, the two-tier coping styles model proposes that the behavioral and physiological stress responses are distinct and do not co-vary (Koolhaas et al., 2010). We found that offspring from mothers treated during pregnancy (either controls or GC-treated) with higher responsiveness to ACTH were less active and less aggressive. Why these same patterns were not found in offspring of females treated with GCs during lactation is not clear, but could be due to smaller sample sizes. The former results provide some support for the unidimensional model where the physiological stress response and these two behaviors are negatively correlated. Previously, we found support for the two-tier coping style model in adult red squirrels as we found no association between a measure of HPA axis activity (fecal glucocorticoid metabolite concentrations) and behavioral traits (activity and aggression; Westrick et al., 2019). While the conclusions of our two studies differ, they are not directly

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

Table 4. Full results of behavioral trait models

	Pregnancy			Lactation		
Fixed effect	β	95% CI	*P*-value	β	95% CI	*P*-value
Activity in open-field trials						
Intercept	1.66	−0.89 to 4.21	0.22	**−5.12**	**−9.70 to −0.53**	**0.05**
ACTH AUC (imputed)	**−0.02**	**−0.03 to −0.00**	**0.03**	0.03	−0.01 to 0.06	0.16
Negative feedback (%)	0.00	−0.02 to 0.02	0.77	0.01	−0.02 to 0.04	0.40
Fed GCs	0.03	−0.78 to 0.84	0.95	**2.49**	**1.04 to 3.94**	**0.01**
Sex: male	−0.01	−1.04 to 1.02	0.98	1.23	−0.13 to 2.60	0.10
Aggression	−0.09	−0.86 to 0.68	0.83	0.57	−0.14 to 1.28	0.14
Standardized treatment length (days)	−0.03	−0.63 to 0.57	0.92			
Year: 2016	−0.64	−1.87 to 0.59	0.32	1.29	−0.12 to 2.71	0.10
Year: 2017	−0.84	−2.09 to 0.42	0.21	0.05	−0.87 to 0.96	0.92
Standardized age (days)	−0.30	−0.70 to 0.10	0.16	0.41	−0.05 to 0.87	0.11
Fed GCs×Sex: male	0.00	−1.24 to 1.25	0.99	−1.65	−3.62 to 0.32	0.13
Fed GCs×Aggression	0.25	−0.70 to 1.20	0.61	−0.04	−1.01 to 0.94	0.94
Random effect: litter ID						
Within-group variance		0.46			n/a	
Between-group variance		0.14			n/a	
Intra-class correlation		0.23			n/a	
Observations		34 pups from 23 litters			23 pups from 16 litters	
Aggression in mirror image stimulation trials						
Intercept	2.07	−0.09 to 4.23	0.07	4.23	0.20 to 8.27	0.06
ACTH AUC (imputed)	**−0.02**	**−0.03 to −0.00**	**0.02**	**−0.03**	**−0.06 to −0.01**	**0.03**
Negative feedback (%)	−0.00	−0.02 to 0.01	0.76	−0.02	−0.04 to 0.00	0.11
Fed GCs	−0.40	−1.09 to 0.30	0.28	−0.81	−2.39 to 0.76	0.33
Sex: male	−0.73	−1.54 to 0.07	0.09	0.35	−1.16 to 1.86	0.66
Standardized treatment length (days)	−0.06	−0.51 to 0.38	0.78			
Year: 2016	−0.29	−1.31 to 0.72	0.58	−0.27	−2.11 to 1.57	0.78
Year: 2017	−0.59	−1.56 to 0.38	0.24	0.04	−1.16 to 1.23	0.95
Standardized age (days)	−0.13	−0.48 to 0.23	0.49	−0.32	−0.74 to 0.09	0.15
Fed GCs×Sex: male	0.68	−0.42 to 1.77	0.24	0.44	−1.22 to 2.10	0.61
Random effects						
Within-group variance		n/a			0.18	
Between-group variance		n/a			0.96	
Intra-class correlation		n/a			0.84	
Observations		34 pups from 23 litters			23 pups from 16 litters	

We ran four distinct models testing the effect of maternal GCs on activity and aggression in juvenile red squirrels. We ran separate models for the pregnancy and lactation treatment groups and the two behavioral traits: activity and aggression. We standardized treatment length (days) across all data for the pregnancy models and age of pup (days) across all data. We did not include a random effect of litter ID in the activity model for lactation treatments and the aggression model for pregnancy treatments. The comparison group for categorical variables is control treatment females from 2015. Bold font indicates statistical significance of $P<0.05$. n/a, not applicable.

comparable for several reasons. First, in our previous study, although we used the same behavioral methods, we used fecal glucocorticoid metabolite concentrations as an integrative measurement of HPA axis activity (Westrick et al., 2019). It is possible that the relationship between HPA axis activity and behavioral traits was not detectable on that broad scale (but see Montiglio et al., 2012), whereas measuring plasma cortisol in response to a standardized challenge (using Dex and ACTH) could provide for a more precise measurement of the acute HPA axis response. Second, our previous study looked at the behavioral and physiological stress response in adult (>1 year old) squirrels, whereas our current study involves juvenile red squirrels around the period of weaning (∼70 days old). Previous work in this species showed that these behavioral traits 'regress to the mean', meaning there is less variation and a lack of individuals at the extremes of activity and aggression in the adult population compared with the juvenile population (Kelley et al., 2015). This could mean that the wide range in behavioral stress responses in the juvenile population of red squirrels is more consistent with the previous laboratory studies of other species and selection lines for individuals at the extreme ends of the proactive–reactive spectrum (which often support the unidimensional model; Koolhaas et al., 1999; Westrick et al., 2019) compared with the adult population of red squirrels. These two findings in the same study system serve as further evidence of a need for a more generalizable model of the relationship between the behavioral and physiological stress response, as highlighted in Westrick et al. (2019).

Our results showing a relative lack of impact of maternal GCs on offspring HPA axis activity and behavior provide an important contrast for previous studies conducted in the laboratory (especially in laboratory rodents; Harris and Seckl, 2011; Weinstock, 2008). There are multiple non-mutually exclusive potential explanations for why our experimental manipulations of maternal GCs did not have more widespread impacts on offspring behavior and the HPA axis. Two explanations involve potential bias in our experimental design. First, inevitably not all offspring from the pre- and postnatal GC supplementation experiment survived to weaning (van Kesteren et al., 2019). It is also possible that offspring from the experiment did not stay within our study range, although we think this is unlikely given relatively short natal dispersal distances prior to or around weaning (Berteaux and Boutin, 2000; Cooper et al., 2017; Kerr et al., 2007; Martinig et al., 2020). This could have resulted in a survivor bias in our results where individuals who were impacted by our GC treatments either died before weaning or disappeared before

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

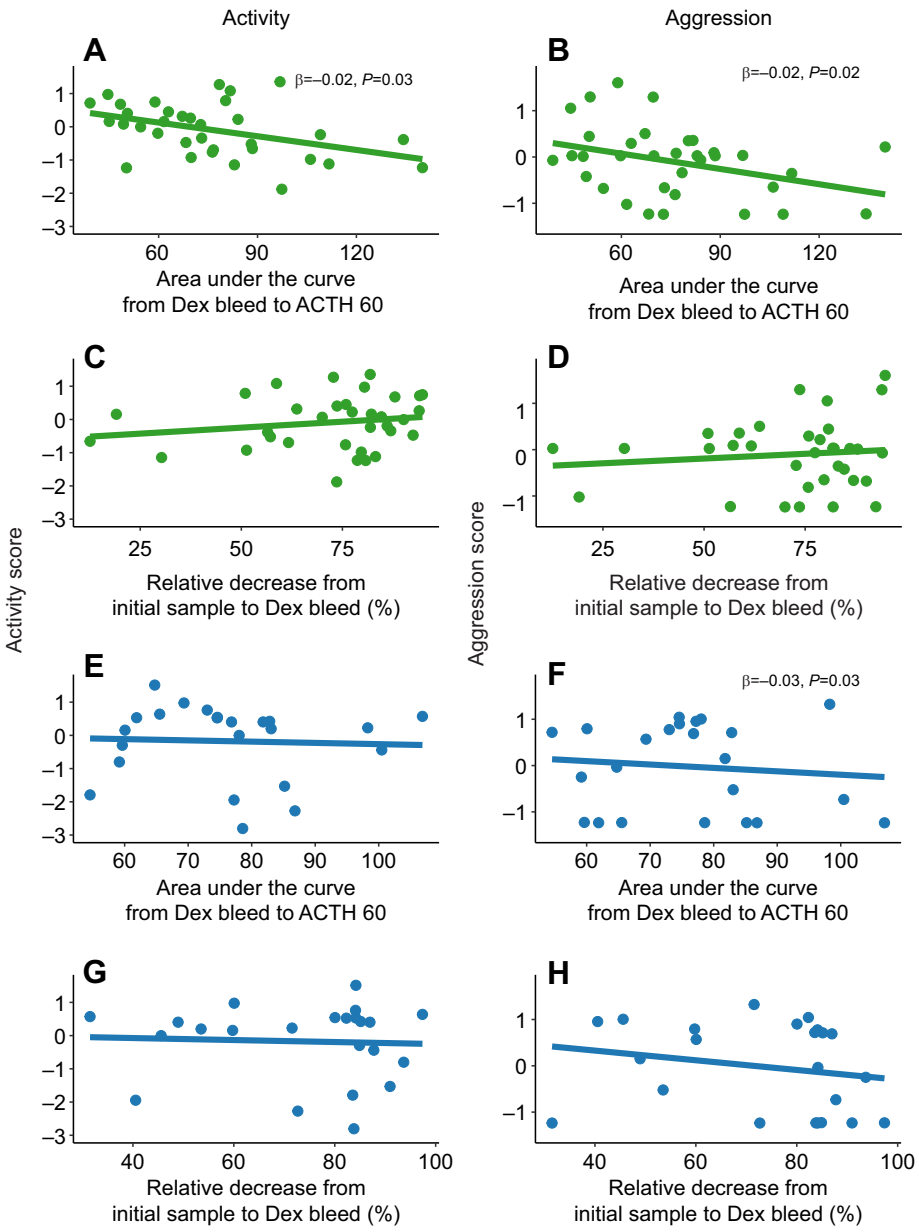

Fig. 3. Relationships between behavioral traits and HPA axis dynamics. More active (A) and aggressive (B) juvenile red squirrels from the pregnancy treatment groups (both GC-treated and controls) exhibited smaller adrenal reactivity (area under the curve from the Dex bleed to the ACTH 60 min bleed; Table 4). More aggressive juveniles from the lactation treatment groups (both GC-treated and the controls) also exhibited smaller ACTH AUC (F; Table 4). (A–D) Green points represent raw data from juveniles from mothers treated during pregnancy (*N*=34). (E–H) Blue points represent raw data from juveniles from mothers treated during lactation (*N*=23). Regression coefficients and *P*-values are shown for models with $P<0.05$. A, C, E and G show the relationship between activity and our two measures of HPA axis dynamics. B, D, F and H show the relationship between aggression and HPA axis dynamics.

we could capture them. Second, despite extensive trapping efforts, differences in behavior among offspring may have resulted in a reduction in our ability to trap less aggressive or active squirrels, and led to under-estimation of effect sizes, which would reduce our power to detect any treatment effects. Differences in trappability of offspring could lead to biases in the types of offspring we captured (Carter et al., 2012; Kelley et al., 2015; but see Michelangeli et al., 2016), such as being less likely to capture offspring that were less active or less aggressive. However, based on the estimated number of juveniles alive at weaning (determined via regular trapping and behavioral observations; Dantzer et al., 2020a), we actually included a majority of the juveniles born to treated females in this study: 68% of the juveniles produced by treated females were included. We sampled 60% of the litters where at least one pup was known to be alive at 25 days of age. The rest of the juveniles (32%) produced by treated females were thought to be alive around weaning (based upon trapping and behavioral observations) but were not sampled. Many of these juveniles who were not sampled were purposely excluded from sampling to avoid collecting data for more than two individuals per litter, as we aimed to maximize sampling effort across different litters. For other individuals, we put in much effort to capture them but were not able to. Thus, it is possible but seems unlikely that the lack of GC treatment effects on offspring were due to biases in our ability to capture offspring from treated mothers.

Another possible explanation for the relative lack of GC treatment effects on offspring HPA axis or behavior has to do with timing, both in terms of the duration of our experimental treatments and when we measured these traits in offspring. Our manipulations during pregnancy lasted ~16 days primarily during the late stages of gestation, whereas our manipulations during lactation lasted approximately the first 10 days after parturition. With an approximate gestation length of 35 days and weaning length of 70 days, this means that developing juveniles only experienced artificially elevated maternal GCs during a short period during late gestation or early lactation. Our previous work shows that this same period of exposure to GCs, either during pregnancy or lactation, affects offspring postnatal growth rates (Dantzer et al., 2013,

Journal of Experimental Biology (2021) 224, jeb236620. doi:10.1242/jeb.236620

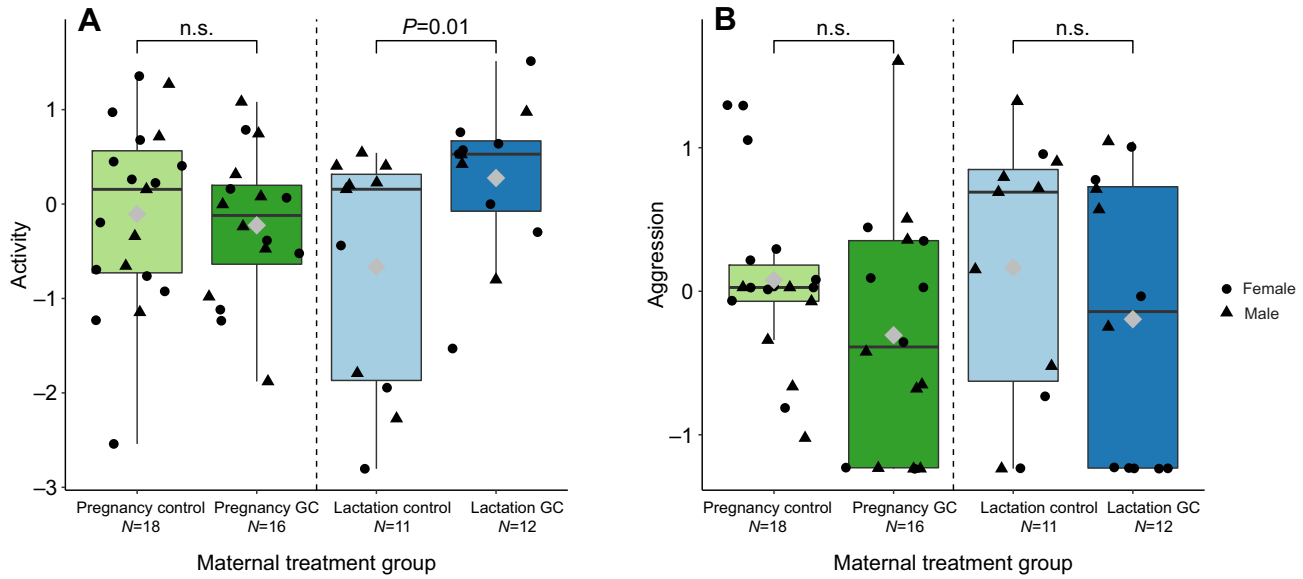

Fig. 4. Effects of treatment group on activity and aggression. (A) Activity and (B) aggression were not strongly impacted by maternal treatment with GCs, although lactation GC juveniles showed significantly higher levels of activity than controls (Table 4). Gray diamonds indicate the group means. Box plots indicate the lower quartile, median and upper quartile. Whiskers indicate the range of the data. Data points represent individual juvenile red squirrels (pregnancy control, *N*=18; pregnancy GC, *N*=16; lactation control, *N*=11; lactation GC, *N*=12). The shape of the data point indicates the sex of the individual (circle: female red squirrel; triangle: male red squirrel). *P*-value of comparison between the corresponding control and GC treatment groups is indicated above the box plot if $P<0.05$ (n.s., not significant).

2020b). This suggests that the timing and duration of our treatments can affect offspring growth but seems to have minimal impacts on offspring HPA axis activity and behavior. This could either suggest that these different traits in offspring (postnatal growth, HPA axis activity, behavior) have different windows when they are sensitive to maternal GCs, or that our treatments were not long enough or did not occur at the correct time to influence these traits in offspring. Secondly, it is also possible that there were impacts of elevated maternal GCs on offspring HPA axis and/or behavior that were not long-lasting or were either diluted or 'overwritten' by subsequent developmental experiences that happened after the maternal GC treatments were completed. We sampled offspring around the time of weaning and the effects of the GC treatments may not have lasted into adolescence due to neural pruning of important brain structures responsible for consistent behavioral traits and reactions to external stressors between early life and adolescence (Groothuis and Trillmich, 2011; Spear, 2000). Previous studies in birds showed that the effects of exposure to developmental stress (such as treating mothers or the offspring directly with exogenous GCs) on offspring HPA axis activity can either be short-term (Lendvai et al., 2009) or long-term (Grace and Anderson, 2018; Marasco et al., 2012). Other studies in laboratory rodents illustrate how the timing of sampling offspring can affect the inferences: offspring from lactating rats treated with exogenous GCs exhibit higher HPA axis reactivity when sampled 11 days after birth but not 16 days after birth (Casolini et al., 1997). Thus, it is possible that the impacts of the GC treatments on offspring HPA axis activity or behavior would have been observed if we had sampled them at multiple time points following birth rather than just at weaning, but this was not possible without extremely large sample sizes as most offspring do not survive after weaning.

Another related possibility is that it may be beneficial for offspring to adjust their behavioral or physiological phenotypes throughout development rather than being canalized based upon a short-term developmental experience. Too much sensitivity to the early life environment may be maladaptive for species in a highly variable environment, as this could result in a mismatch between the parental environment and the offspring environment (Langenhof and Komdeur, 2018). It could be more beneficial for offspring to retain a sensitivity to their own environment during subsequent development that allows any maternal cues to be 'overwritten' by the cues present in their own environment as they become more independent from their mother (Leimar and McNamara, 2015). In our study area, predation risk, resource availability and conspecific competition fluctuate between an individual's birth and first breeding season (Dantzer et al., 2013; Hendrix et al., 2020; McAdam and Boutin, 2003; Taylor et al., 2014). Theoretically, in such variable environments, it may be more beneficial for offspring to be shaped by the parental environment rather than their own environment experienced during development (Leimar and McNamara, 2015). Because among-year fluctuations in these ecological factors are much greater than within-year fluctuations (Dantzer et al., 2020a), we expected that most aspects of the parental environment would be consistent with the environment the offspring will experience as they wean and establish their territory before overwintering. This is why we sampled offspring at weaning as we expected that the maternal environment closely corresponded to the environment offspring experienced during development and weaning. We did find that the HPA axis of weaned juveniles was well developed, as they did show a functional response to both ACTH and Dex, but previous studies show that juvenile red squirrel behavior changes over development (Kelley et al., 2015). Future studies testing offspring at multiple time points, both before and after weaning, will allow us to assess if offspring HPA axis activity and behavior may have been shaped more by their own experiences rather than their early-life environment provided by their mother (Leimar and McNamara, 2015; Nettle and Bateson, 2015).

Our study is one of the first to use an experimental manipulation to understand the effects of the maternal environment, as encoded through maternal GCs, on offspring personality traits in a wild

population. Given the context of our extensive knowledge around the selection pressures acting on these specific behavioral traits (Boon et al., 2007, 2008; Cooper et al., 2017; Kelley et al., 2015; Taylor et al., 2012, 2014) and our knowledge of how maternal GCs shape specific aspects of early development in red squirrels (Dantzer et al., 2013, 2020b), the present study system provided the ideal opportunity to test the developmental role of maternal GCs in personality in a natural environment (Groothuis and Trillmich, 2011; Langenhof and Komdeur, 2018). The fact that we did not find a substantial effect of prenatal or postnatal GCs on HPA axis activity and behavior of juvenile squirrels may indicate maternal GCs are not an ecologically relevant cue for the development of these traits. It may be that, as a short-lived animal in a highly variable environment, juvenile squirrels are less sensitive to maternal GC cues despite the proposed links between maternal GCs, the HPA axis development and behavior. Based on our results, maternal GCs do not adaptively drive developmental plasticity of behavior or the phenotypic correlations between behavior and HPA axis dynamics in recently weaned red squirrels.

Acknowledgements
We thank Agnes MacDonald, her family, and Champagne and Aishihik First Nations for allowing us to conduct our work within their traditional territory. We thank Monica Cooper, Zach Fogel, Claire Hoffmann, Noah Israel, Sean Konkolics, Laura Porter, Matt Sehrsweeny, Sam Sonnega, Jess Steketee and Dylan Yaffy for their assistance in field data collection. We thank Brie Coleman, Deirdre McGovern, Austin Rife and Meg Ryan for their assistance in scoring videos. We thank two anonymous reviewers for their constructive feedback that strengthened the paper. This is publication #114 of the Kluane Red Squirrel Project.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.E.W., F.v.K., B.D.; Methodology: S.E.W., F.v.K., B.D.; Formal analysis: S.E.W.; Investigation: S.E.W., B.D.; Resources: S.B., J.E.L., A.G.M., B.D.; Data curation: S.E.W.; Writing – original draft: S.E.W., B.D.; Writing – review & editing: S.E.W., F.v.K., S.B., J.E.L., A.G.M., B.D.; Visualization: S.E.W.; Supervision: S.E.W., F.v.K., S.B., J.E.L., A.G.M., B.D.; Project administration: S.E.W., F.v.K., S.B., J.E.L., A.G.M., B.D.; Funding acquisition: S.E.W., S.B., J.E.L., A.G.M., B.D.

Competing interests
No competing interests declared.

Funding
This work was supported by the American Society of Mammalogists to S.E.W.; the University of Michigan to S.E.W. and B.D.; National Science Foundation (IOS-1749627 to B.D.); and the Natural Sciences and Engineering Research Council of Canada to S.B., A.G.M. and J.E.L.

Data availability
All data are available from figshare: https://figshare.com/s/9f5846adda84f6e7bb02

References
Badyaev, A. V. (2005). Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. *Proc. R. Soc. B Biol. Sci.* **272**, 877-886. doi:10.1098/rspb.2004.3045

Balzarini, V., Taborsky, M., Wanner, S., Koch, F. and Frommen, J. G. (2014). Mirror, mirror on the wall: the predictive value of mirror tests for measuring aggression in fish. *Behav. Ecol. Sociobiol.* **68**, 871-878. doi:10.1007/s00265-014-1698-7

Barbazanges, A., Piazza, P. V., Moal, M. L. and Maccari, S. (1996). Maternal glucocorticoid secretion mediates effects of prenatal stress. *J. Neurosci.* **16**, 3943-3949. doi:10.1523/JNEUROSCI.16-12-03943.1996

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48. doi:10.18637/jss.v067.i01

Berteaux, D. and Boutin, S. (2000). Breeding dispersal in female North American red squirrels. *Ecology* **81**, 1311-1326. doi:10.1890/0012-9658(2000)081[1311:BDIFNA]2.0.CO;2

Blumstein, D. T. and Daniel, J. C. (2007). *Quantifying behavior the JWatcher way*. Sunderland, MA: Sinaeur Associates, Inc.

Boon, A. K., Réale, D. and Boutin, S. (2007). The interaction between personality, offspring fitness and food abundance in North American red squirrels. *Ecol. Lett.* **10**, 1094-1104. doi:10.1111/j.1461-0248.2007.01106.x

Boon, A. K., Reale, D. and Boutin, S. (2008). Personality, habitat use, and their consequences for survival in North American red squirrels *Tamiasciurus hudsonicus*. *Oikos* **117**, 1321-1328. doi:10.1111/j.0030-1299.2008.16567.x

Boonstra, R. and McColl, C. J. (2000). Contrasting stress response of male Arctic ground squirrels and red squirrels. *J. Exp. Zool.* **286**, 390-404. doi:10.1002/(SICI)1097-010X(20000301)286:4<390::AID-JEZ7>3.0.CO;2-O

Boutin, S. and Larsen, K. (1993). Does food availability affect growth and survival of males and females differently in a promiscuous small mammal, *Tamiasciurus hudsonicus*? *J. Anim. Ecol.* **62**, 364-370. doi:10.2307/5367

Caldji, C., Liu, D., Sharma, S., Diorio, J., Francis, D., Meaney, M. J. and Plotsky, P. M. (2011). Development of individual differences in behavioral and endocrine responses to stress: role of the postnatal environment. In *Comprehensive Physiology* (ed. R. Terjung), pp. 271-292. Hoboken, NJ: John Wiley & Sons, Inc.

Careau, V., Buttemer, W. A. and Buchanan, K. L. (2014). Developmental stress can uncouple relationships between physiology and behaviour. *Biol. Lett.* **10**, 20140834. doi:10.1098/rsbl.2014.0834

Carter, A. J., Heinsohn, R., Goldizen, A. W. and Biro, P. A. (2012). Boldness, trappability and sampling bias in wild lizards. *Anim. Behav.* **83**, 1051-1058. doi:10.1016/j.anbehav.2012.01.033

Casolini, P., Cigliana, G., Alemà, G. S., Ruggieri, V., Angelucci, L. and Catalani, A. (1997). Effect of increased maternal corticosterone during lactation on hippocampal corticosteroid receptors, stress response and learning in offspring in the early stages of life. *Neuroscience* **79**, 1005-1012. doi:10.1016/S0306-4522(96)00668-9

Catalani, A., Casolini, P., Cigliana, G., Scaccianoce, S., Consoli, C., Cinque, C., Zuena, A. R. and Angelucci, L. (2002). Maternal corticosterone influences behavior, stress response and corticosteroid receptors in the female rat. *Pharmacol. Biochem. Behav.* **73**, 105-114. doi:10.1016/S0091-3057(02)00755-4

Catalani, A., Casolini, P., Scaccianoce, S., Patacchioli, F. R., Spinozzi, P. and Angelucci, L. (2000). Maternal corticosterone during lactation permanently affects brain corticosteroid receptors, stress response and behaviour in rat progeny. *Neuroscience* **100**, 319-325. doi:10.1016/S0306-4522(00)00277-3

Charmandari, E., Tsigos, C. and Chrousos, G. (2005). Endocrinology of the stress response. *Annu. Rev. Physiol.* **67**, 259-284. doi:10.1146/annurev.physiol.67.040403.120816

Cooper, E. B., Taylor, R. W., Kelley, A. D., Martinig, A. R., Boutin, S., Humphries, M. M., Dantzer, B., Lane, J. E. and McAdam, A. G. (2017). Personality is correlated with natal dispersal in North American red squirrels (*Tamiasciurus hudsonicus*). *Behaviour* **154**, 939-961. doi:10.1163/1568539X-00003450

Dantzer, B., Boutin, S., Humphries, M. M. and McAdam, A. G. (2012). Behavioral responses of territorial red squirrels to natural and experimental variation in population density. *Behav. Ecol. Sociobiol.* **66**, 865-878. doi:10.1007/s00265-012-1335-2

Dantzer, B., Dubuc, C., Goncalves, I. B., Cram, D. L., Bennett, N. C., Ganswindt, A., Heistermann, M., Duncan, C., Gaynor, D. and Clutton-Brock, T. H. (2019). The development of individual differences in cooperative behaviour: maternal glucocorticoid hormones alter helping behaviour of offspring in wild meerkats. *Phil. Trans. R. Soc. B: Biol. Sci.* **374**, 20180117. doi:10.1098/rstb.2018.0117

Dantzer, B., McAdam, A. G., Humphries, M. M., Lane, J. E. and Boutin, S. (2020a). Decoupling the effects of food and density on life-history plasticity of wild animals using field experiments: insights from the steward who sits in the shadow of its tail, the North American red squirrel. *J. Anim. Ecol.* **89**, 2397-2414. doi:10.1111/1365-2656.13341

Dantzer, B., Newman, A. E. M., Boonstra, R., Palme, R., Boutin, S., Humphries, M. M. and McAdam, A. G. (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. *Science* **340**, 1215-1217. doi:10.1126/science.1235765

Dantzer, B., van Kesteren, F., Westrick, S. E., Boutin, S., McAdam, A. G., Lane, J. E., Gillespie, R., Majer, A., Haussmann, M. F. and Monaghan, P. (2020b). Maternal glucocorticoids promote offspring growth without inducing oxidative stress or shortening telomeres in wild red squirrels. *J. Exp. Biol.* **223**, jeb212373. doi:10.1242/jeb.212373

De Kloet, R., Wallach, G. and McEwen, B. S. (1975). Differences in corticosterone and dexamethasone binding to rat brain and pituitary. *Endocrinology* **96**, 598-609. doi:10.1210/endo-96-3-598

Del Giudice, M., Ellis, B. J. and Shirtcliff, E. A. (2011). The adaptive calibration model of stress responsivity. *Neurosci. Biobehav. Rev.* **35**, 1562-1592. doi:10.1016/j.neubiorev.2010.11.007

Denver, R. J. (2009). Stress hormones mediate environment-genotype interactions during amphibian development. *Gen. Comp. Endocrinol.* **164**, 20-31. doi:10.1016/j.ygcen.2009.04.016

Dray, S. and Dufour, A.-B. (2007). The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* **22**, 1-20. doi:10.18637/jss.v022.i04

Fletcher, Q. E., Boutin, S., Lane, J. E., LaMontagne, J. M., McAdam, A. G., Krebs, C. J. and Humphries, M. M. (2010). The functional response of a

hoarding seed predator to mast seeding. *Ecology* **91**, 2673-2683. doi:10.1890/09-1816.1
Fletcher, Q. E., Landry-Cuerrier, M., Boutin, S., McAdam, A. G., Speakman, J. R. and Humphries, M. M. (2013). Reproductive timing and reliance on hoarded capital resources by lactating red squirrels. *Oecologia* **173**, 1203-1215. doi:10.1007/s00442-013-2699-3
Fox, J. and Weisberg, S. (2011). *An R companion to applied regression*, 2nd edn. Thousand Oaks, CA: Sage.
Gallup, G. G. (1968). Mirror-image stimulation. *Psychol. Bull.* **70**, 782-793. doi:10.1037/h0026777
Grace, J. K. and Anderson, D. J. (2018). Early-life maltreatment predicts adult stress response in a long-lived wild bird. *Biol. Lett.* **14**, 20170679. doi:10.1098/rsbl.2017.0679
Grace, J. K., Parenteau, C. and Angelier, F. (2020). Post-natal corticosterone exposure downregulates the HPA axis through adulthood in a common passerine. *Gen. Comp. Endocrinol.* **292**, 113421. doi:10.1016/j.ygcen.2020.113421
Grey, K. R., Davis, E. P., Sandman, C. A. and Glynn, L. M. (2013). Human milk cortisol is associated with infant temperament. *Psychoneuroendocrinology* **38**, 1178-1185. doi:10.1016/j.psyneuen.2012.11.002
Groothuis, T. G. G. and Trillmich, F. (2011). Unfolding personalities: the importance of studying ontogeny. *Dev. Psychobiol.* **53**, 641-655. doi:10.1002/dev.20574
Guindre-Parker, S., Mcadam, A. G., van Kesteren, F., Palme, R., Boonstra, R., Boutin, S., Lane, J. E. and Dantzer, B. (2019). Individual variation in phenotypic plasticity of the stress axis. *Biol. Lett.* **15**, 20190260. doi:10.1098/rsbl.2019.0260
Harris, A. and Seckl, J. (2011). Glucocorticoids, prenatal stress and the programming of disease. *Horm. Behav.* **59**, 279-289. doi:10.1016/j.yhbeh.2010.06.007
Hau, M., Casagrande, S., Ouyang, J. Q. and Baugh, A. T. (2016). Glucocorticoid-mediated phenotypes in vertebrates. In *Advances in the Study of Behavior (ed. M. Naguib, J. C. Mitani, L. W. Simmons, L. Barrett, S. Healy and M. Zuk)*, pp. 41-115. Elsevier.
Heidinger, B. J., Nisbet, I. C. T. and Ketterson, E. D. (2008). Changes in adrenal capacity contribute to a decline in the stress response with age in a long-lived seabird. *Gen. Comp. Endocrinol.* **156**, 564-568. doi:10.1016/j.ygcen.2008.02.014
Hendrix, J. G., Fisher, D. N., Martinig, A. R., Boutin, S., Dantzer, B., Lane, J. E. and McAdam, A. G. (2020). Territory acquisition mediates the influence of predators and climate on juvenile red squirrel survival. *J. Anim. Ecol.* **89**, 1408-1418. doi:10.1111/1365-2656.13209
Hinde, K., Skibiel, A. L., Foster, A. B., Del Rosso, L., Mendoza, S. P. and Capitanio, J. P. (2015). Cortisol in mother's milk across lactation reflects maternal life history and predicts infant temperament. *Behav. Ecol.* **26**, 269-281. doi:10.1093/beheco/aru186
Hoffmann, A. A. and Hercus, M. J. (2000). Environmental stress as an evolutionary force. *Bioscience* **50**, 217. doi:10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2
Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biomet. J.* **50**, 346-363. doi:10.1002/bimj.200810425
Ingram, J. R., Matthews, L. R., Carragher, J. F. and Schaare, P. R. (1997). Plasma cortisol responses to remote adrenocorticotropic hormone (ACTH) infusion in free-ranging red deer (*Cervus elaphus*). *Domest. Anim. Endocrinol.* **14**, 63-71. doi:10.1016/S0739-7240(96)00095-1
Janssens, C. J. J. G., Helmond, F. A. and Wiegant, V. M. (1994). Increased cortisol response to exogenous adrenocorticotropic hormone in chronically stressed pigs: influence of housing conditions. *J. Anim. Sci.* **72**, 1771-1777. doi:10.2527/1994.7271771x
Kapoor, A., Petropoulos, S. and Matthews, S. G. (2008). Fetal programming of hypothalamic–pituitary–adrenal (HPA) axis function and behavior by synthetic glucocorticoids. *Brain Res. Rev.* **57**, 586-595. doi:10.1016/j.brainresrev.2007.06.013
Kelley, A. D., Humphries, M. M., McAdam, A. G. and Boutin, S. (2015). Changes in wild red squirrel personality across ontogeny: activity and aggression regress towards the mean. *Behaviour* **152**, 1291-1306. doi:10.1163/1568539X-00003279
Kerr, T. D., Boutin, S., LaMontagne, J. M., McAdam, A. G. and Humphries, M. M. (2007). Persistent maternal effects on juvenile survival in North American red squirrels. *Biol. Lett.* **3**, 289-291. doi:10.1098/rsbl.2006.0615
Koolhaas, J. M., de Boer, S. F., Coppens, C. M. and Buwalda, B. (2010). Neuroendocrinology of coping styles: towards understanding the biology of individual variation. *Front. Neuroendocrinol.* **31**, 307-321. doi:10.1016/j.yfrne.2010.04.001
Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., De Jong, I. C., Ruis, M. A. W. and Blokhuis, H. J. (1999). Coping styles in animals: current status in behavior and stress-physiology. *Neurosci. Biobehav. Rev.* **23**, 925-935. doi:10.1016/S0149-7634(99)00026-3
Kulski, J. K. and Hartmann, P. E. (1981). Changes in the concentration of cortisol in milk during different stages of human lactation. *Austral. J. Exp. Biol. Med. Sci.* **59**, 769-778. doi:10.1038/icb.1981.66
Lancaster, L. T., McAdam, A. G., Wingfield, J. C. and Sinervo, B. R. (2007). Adaptive social and maternal induction of antipredator dorsal patterns in a lizard with alternative social strategies. *Ecol. Lett.* **10**, 798-808. doi:10.1111/j.1461-0248.2007.01069.x
Langenhof, M. R. and Komdeur, J. (2018). Why and how the early-life environment affects development of coping behaviours. *Behav. Ecol. Sociobiol.* **72**, 34. doi:10.1007/s00265-018-2452-3
Larsen, K. W. and Boutin, S. (1994). Movements, survival, and settlement of red squirrel (*Tamiasciurus hudsonicus*) offspring. *Ecology* **75**, 214-223. doi:10.2307/1939395
Lattin, C. R. and Kelly, T. R. (2020). Glucocorticoid negative feedback as a potential mediator of trade-offs between reproduction and survival. *Gen. Comp. Endocrinol.* **286**, 113301. doi:10.1016/j.ygcen.2019.113301
Ledón-Rettig, C. C., Pfennig, D. W. and Crespi, E. J. (2010). Diet and hormonal manipulation reveal cryptic genetic variation: implications for the evolution of novel feeding strategies. *Proc. R. Soc. B Biol. Sci.* **277**, 3569-3578. doi:10.1098/rspb.2010.0877
Leimar, O. and McNamara, J. M. (2015). The evolution of transgenerational integration of information in heterogeneous environments. *Am. Nat.* **185**, E55-E69. doi:10.1086/679575
Lema, S. C. and Kitano, J. (2013). Hormones and phenotypic plasticity: implications for the evolution of integrated adaptive phenotypes. *Curr. Zool.* **59**, 506-525. doi:10.1093/czoolo/59.4.506
Lendvai, Á. Z., Loiseau, C., Sorci, G. and Chastel, O. (2009). Early developmental conditions affect stress response in juvenile but not in adult house sparrows (*Passer domesticus*). *Gen. Comp. Endocrinol.* **160**, 30-35. doi:10.1016/j.ygcen.2008.10.004
Marasco, V., Robinson, J., Herzyk, P. and Spencer, K. A. (2012). Pre- and post-natal stress in context: effects on the stress physiology in a precocial bird. *J. Exp. Biol.* **215**, 3955-3964. doi:10.1242/jeb.071423
Martinig, A. R., McAdam, A. G., Dantzer, B., Lane, J. E., Coltman, D. W. and Boutin, S. (2020). The new kid on the block: immigrant males win big whereas females pay fitness cost after dispersal. *Ecol. Lett.* **23**, 430-438. doi:10.1111/ele.13436
Mazzamuto, M. V., Cremonesi, G., Santicchia, F., Preatoni, D., Martinoli, A. and Wauters, L. A. (2019). Rodents in the arena: a critical evaluation of methods measuring personality traits. *Ethol. Ecol. Evol.* **31**, 38-58. doi:10.1080/03949370.2018.1488768
McAdam, A. G. and Boutin, S. (2003). Effects of food abundance on genetic and maternal variation in the growth rate of juvenile red squirrels. *J. Evol. Biol.* **16**, 1249-1256. doi:10.1046/j.1420-9101.2003.00630.x
McAdam, A. G., Boutin, S., Sykes, A. K. and Humphries, M. M. (2007). Life histories of female red squirrels and their contributions to population growth and lifetime fitness. *Ecoscience* **14**, 362-362. doi:10.2980/1195-6860(2007)14[362:LHOFRS]2.0.CO;2
Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Ann. Rev. Neurosci.* **24**, 1161-1192. doi:10.1146/annurev.neuro.24.1.1161
Merrill, L. and Grindstaff, J. L. (2015). Pre- and post-natal antigen exposure can program the stress axis of adult zebra finches: evidence for environment matching. *Brain Behav. Immun.* **45**, 71-79. doi:10.1016/j.bbi.2014.12.013
Meylan, S. and Clobert, J. (2005). Is corticosterone-mediated phenotype development adaptive? Maternal corticosterone treatment enhances survival in male lizards. *Horm. Behav.* **48**, 44-52. doi:10.1016/j.yhbeh.2004.11.022
Michelangeli, M., Wong, B. B. M. and Chapple, D. G. (2016). It's a trap: sampling bias due to animal personality is not always inevitable. *Behav. Ecol.* **27**, 62-67. doi:10.1093/beheco/arv123
Montiglio, P.-O., Garant, D., Pelletier, F. and Réale, D. (2012). Personality differences are related to long-term stress reactivity in a population of wild eastern chipmunks, *Tamias striatus*. *Anim. Behav.* **84**, 1071-1079. doi:10.1016/j.anbehav.2012.08.010
Nephew, B. C. and Bridges, R. S. (2011). Effects of chronic social stress during lactation on maternal behavior and growth in rats. *Stress* **14**, 677-684. doi:10.3109/10253890.2011.605487
Nettle, D. and Bateson, M. (2015). Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve? *Proc. R. Soc. B Biol. Sci.* **282**, 20151005.
O'Donnell, K., O'Connor, T. G. and Glover, V. (2009). Prenatal stress and neurodevelopment of the child: focus on the HPA axis and role of the placenta. *Dev. Neurosci.* **31**, 285-292. doi:10.1159/000216539
Packard, A. E. B., Egan, A. E. and Ulrich-Lai, Y. M. (2016). HPA axis interactions with behavioral systems. *Comprehensive Physiology* **6**, 1897-1934. doi:10.1002/cphy.c150042
Perals, D., Griffin, A. S., Bartomeus, I. and Sol, D. (2017). Revisiting the open-field test: what does it really tell us about animal personality? *Anim. Behav.* **123**, 69-79. doi:10.1016/j.anbehav.2016.10.006
Raulo, A. and Dantzer, B. (2018). Associations between glucocorticoids and sociality across a continuum of vertebrate social behavior. *Ecol. Evol.* **8**, 7697-7716. doi:10.1002/ece3.4059
Reddon, A. R. (2012). Parental effects on animal personality. *Behav. Ecol.* **23**, 242-245. doi:10.1093/beheco/arr210
Ren, T., Boutin, S., Humphries, M. M., Dantzer, B., Gorrell, J. C., Coltman, D. W., McAdam, A. G. and Wu, M. (2017). Seasonal, spatial, and maternal effects on

gut microbiome in wild red squirrels. *Microbiome* **5**, 163. doi:10.1186/s40168-017-0382-3

Rich, E. L. and Romero, L. M. (2005). Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am. J. Physiol. Regul. Integrat. Comp. Physiol.* **288**, R1628-R1636. doi:10.1152/ajpregu.00484.2004

Rossiter, M. C. (1991). Maternal effects generate variation in life history: consequences of egg weight plasticity in the gypsy moth. *Funct. Ecol.* **5**, 386. doi:10.2307/2389810

Saltzman, W., Prudom, S. L., Schultz-Darken, N. J. and Abbott, D. H. (2000). Reduced adrenocortical responsiveness to adrenocorticotropic hormone (ACTH) in socially subordinate female marmoset monkeys. *Psychoneuroendocrinology* **25**, 463-477. doi:10.1016/S0306-4530(00)00003-2

Sapolsky, R. M., Meaney, M. J. and McEwen, B. S. (1985). The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation. *Develop. Brain Res.* **18**, 169-173. doi:10.1016/0165-3806(85)90261-5

Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Rev.* **21**, 55-89. doi:10.1210/er.21.1.55

Seibenhener, M. L. and Wooten, M. C. (2015). Use of the open field maze to measure locomotor and anxiety-like behavior in mice. *J. Vis. Exp.* **96**, e52434. doi:10.3791/52434

Sheriff, M. J., Bell, A., Boonstra, R., Dantzer, B., Lavergne, S. G., McGhee, K. E., MacLeod, K. J., Winandy, L., Zimmer, C. and Love, O. P. (2017). Integrating ecological and evolutionary context in the study of maternal stress. *Integrat. Comp. Biol.* **57**, 437-449. doi:10.1093/icb/icx105

Sih, A. (2011). Effects of early stress on behavioral syndromes: an integrated adaptive perspective. *Neurosci. Biobehav. Rev.* **35**, 1452-1465. doi:10.1016/j.neubiorev.2011.03.015

Siracusa, E., Morandini, M., Boutin, S., Humphries, M. M., Dantzer, B., Lane, J. E. and McAdam, A. G. (2017). Red squirrel territorial vocalizations deter intrusions by conspecific rivals. *Behaviour* **154**, 1259-1273. doi:10.1163/1568539X-00003467

Smith, J. W., Seckl, J. R., Evans, A. T., Costall, B. and Smythe, J. W. (2004). Gestational stress induces post-partum depression-like behaviour and alters maternal care in rats. *Psychoneuroendocrinology* **29**, 227-244. doi:10.1016/S0306-4530(03)00025-8

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* **24**, 417-463. doi:10.1016/S0149-7634(00)00014-2

Spencer, R. L. and Deak, T. (2017). A user's guide to HPA axis research. *Physiol. Behav.* **178**, 43-65. doi:10.1016/j.physbeh.2016.11.014

Spencer, K. A., Evans, N. P. and Monaghan, P. (2009). Postnatal stress in birds: a novel model of glucocorticoid programming of the hypothalamic-pituitary-adrenal axis. *Endocrinology* **150**, 1931-1934. doi:10.1210/en.2008-1471

Stamps, J. A. and Groothuis, T. G. G. (2010). Developmental perspectives on personality: implications for ecological and evolutionary studies of individual differences. *Philos. Trans. R. Soc. B* **365**, 4029-4041. doi:10.1098/rstb.2010.0218

Storm, J. J. and Lima, S. L. (2010). Mothers forewarn offspring about predators: a transgenerational maternal effect on behavior. *Am. Nat.* **175**, 382-390. doi:10.1086/650443

Taylor, R. W., Boon, A. K., Dantzer, B., Réale, D., Humphries, M. M., Boutin, S., Gorrell, J. C., Coltman, D. W. and McAdam, A. G. (2012). Low heritabilities, but genetic and maternal correlations between red squirrel behaviours. *J. Evol. Biol.* **25**, 614-624. doi:10.1111/j.1420-9101.2012.02456.x

Taylor, R. W., Boutin, S., Humphries, M. M. and McAdam, A. G. (2014). Selection on female behaviour fluctuates with offspring environment. *J. Evol. Biol.* **27**, 2308-2321. doi:10.1111/jeb.12495

Thayer, Z. M., Wilson, M. A., Kim, A. W. and Jaeggi, A. V. (2018). Impact of prenatal stress on offspring glucocorticoid levels: a phylogenetic meta-analysis across 14 vertebrate species. *Sci. Rep.* **8**, 4942. doi:10.1038/s41598-018-23169-w

Tsigos, C. and Chrousos, G. P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J. Psychosom. Res.* **53**, 865-871. doi:10.1016/S0022-3999(02)00429-4

van Kesteren, F., Delehanty, B., Westrick, S. E., Palme, R., Boonstra, R., Lane, J. E., Boutin, S., McAdam, A. G. and Dantzer, B. (2019). Experimental increases in glucocorticoids alter function of the HPA axis in wild red squirrels without negatively impacting survival and reproduction. *Physiol. Biochem. Zool.* **92**, 445-458. doi:10.1086/705121

Vilela, F. C. and Giusti-Paiva, A. (2011). Glucocorticoids disrupt neuroendocrine and behavioral responses during lactation. *Endocrinology* **152**, 4838-4845. doi:10.1210/en.2011-1096

Walsh, R. N. and Cummins, R. A. (1976). The open-field test: a critical review. *Psychol. Bull.* **83**, 482-504. doi:10.1037/0033-2909.83.3.482

Weinstock, M. (2008). The long-term behavioural consequences of prenatal stress. *Neurosci. Biobehav. Rev.* **32**, 1073-1086. doi:10.1016/j.neubiorev.2008.03.002

Westrick, S. E., van Kesteren, F., Palme, R., Boonstra, R., Lane, J. E., Boutin, S., McAdam, A. G. and Dantzer, B. (2019). Stress activity is not predictive of coping style in North American red squirrels. *Behav. Ecol. Sociobiol.* **73**, 113. doi:10.1007/s00265-019-2728-2

Wilson, R. C., Vacek, T., Lanier, D. L. and Dewsbury, D. A. (1976). Open-field behavior in muroid rodents. *Behav. Biol.* **17**, 495-506. doi:10.1016/S0091-6773(76)90901-9

Zimmer, C., Boogert, N. J. and Spencer, K. A. (2013). Developmental programming: cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behaviour in adulthood. *Horm. Behav.* **64**, 494-500. doi:10.1016/j.yhbeh.2013.07.002

Table S1. Comparison of lactation treatment dosages. We ran a Wilcox Rank Sum Test for each of the four response variables measured, two HPA axis measurements, and two behavioral measurements, to test for an effect of GC treatment dosage (8 mg or 12 mg) in the lactation treatment group. We found no significant difference between the treatment dosage groups, and therefore combined them in all further analyses.

Dependent variable	W	*p*-value
Area under the curve (DEX to ACTH 60 min)	14	0.81
Relative decrease from initial handling-stressed sample to DEX (%)	17	0.93
Activity	27	0.07
Aggression	8	0.21

Table S2. Analyses with time spent in trap by treatment group. Using data from 2016 and 2017 (data from 2015 were not available), we ran two independent ANOVAs to test for any treatment group biases in the estimated amount of time individual squirrels spent in the trap (B) prior to the behavioral trials and (C) prior to the first blood sample. Time spent in the trap is an estimate based on the time the trap was last seen empty for "time in trap prior to behavioral trials". Although we do not know the precise time the squirrel entered the trap, we do know they entered it sometime after that moment, and it is therefore the *maximum* amount of time the squirrel could have spent in the trap, though it is likely the actual amount of time spent in traps is lower. Time in trap prior to first blood sample is the time elapsed since the trap was last seen empty to the start of the first blood sample, which includes the time in trap prior to behavioral trials, and the time that elapsed during the handling of the squirrel and behavioral trials. Summary statistics are provided (A). The ANOVAs and Tukey post-hoc comparisons showed no significant treatment group differences in either duration of time spent in the trap (B) prior to behavioral trials or (C) prior to the first blood sample being drawn.

A) Summary Statistics

	time in trap prior to behavioral trials (min)	**time in trap + handling time prior to first blood sample (min)**
Treatment Group	***mean (± SD)***	***mean (± SD)***
lac control	89.5 (± 45.1)	204 (± 49.7)
lac GC	91 (± 53.8)	151 (± 53.9)
preg control	81.8 (± 44.6)	164 (± 53.2)
preg GC	105 (± 37.7)	195 (± 59.7)

B) Duration of time (min) in trap prior to behavioral trials

ANOVA	**df**	**sum sq**	**mean sq**	***f*-value**	***p*-value**
Treatment Group	3	6318	2106	1.41	0.26
Residuals	42	62962	1499		

Tukey comparison group	**ß**	**SE**	***t*-value**	***p*-value**
lac GC – lac control	8.89	20.41	0.44	0.97
preg control – lac control	5.12	16.13	0.32	0.99
preg GC – lac control	28.76	16.33	1.76	0.30
preg control – lac GC	-3.77	18.54	-0.20	0.99
preg GC – lac GC	19.87	18.70	1.06	0.71
preg GC – preg control	23.64	13.92	1.70	0.33

C) Duration of time (min) in trap prior to the first blood sample

ANOVA	**df**	**sum sq**	**mean sq**	***f*-value**	***p*-value**
Treatment Group	3	18928	6309	2.08	0.12
Residuals	44	133196	3027		

Tukey comparison group	**ß**	**SE**	***t*-value**	***p*-value**
lac GC – lac control	-52.95	27.73	-1.91	0.24
preg control – lac control	-39.92	22.93	-1.74	0.31
preg GC – lac control	-8.54	22.93	-0.37	0.98
preg control – lac GC	13.04	24.93	0.52	0.95
preg GC – lac GC	44.41	24.93	1.78	0.29
preg GC – preg control	31.38	19.45	1.61	0.38

Table S3. Time in trap as predictor of behavior and HPA axis measurements. Using data from 2016 and 2017 (data from 2015 were not available), we ran general linear models to estimate the impact the total maximum time offspring spent in the trap on the (A) behavioral traits and (B) HPA axis measurements, prior to either the behavioral trials or HPA axis hormone challenge respectively. In the model for ACTH area under the curve (AUC), we included the plasma cortisol concentration at the DEX blood sample to control for starting concentrations of cortisol (see statistical methods in main text). Although the amount of time offspring spent in the trap was variable (Table S2), it did not significantly impact either behavior or HPA axis dynamics.

A) Behavioral traits

		Activity			**Aggression**	
Fixed Effect	**ß**	***CI (95%)***	***p-value***	**ß**	***CI (95%)***	***p-value***
Intercept	0.24	-0.43 – 0.91	0.49	0.34	-0.35 – 1.04	0.34
minutes in trap *before behavioral trials*	-0.01	-0.01 – 0.00	0.11	-0.00	-0.01 – 0.00	0.19
Observations	39			39		
R^2 / adjusted R^2	0.07 / 0.041			0.05 / 0.02		

B) HPA axis measurements

		ACTH AUC			**Negative Feedback (%)**	
Fixed Effect	**ß**	***CI (95%)***	***p-value***	**ß**	***CI (95%)***	***p-value***
Intercept	47.75	31.92 – 63.57	**<0.001**	76.59	56.69 – 96.49	**<0.001**
minutes in trap *before first blood sample*	0.05	-0.03 – 0.14	0.25	-0.03	-0.14 – 0.08	0.58
plasma cortisol at DEX (µg/dL)	1.24	0.85 – 1.63	**<0.001**			
Observations	46			46		
R^2 / adjusted R^2	0.52 / 0.50			0.01 / -0.02		

Table S4. Full model results for each hormone challenge time point independently We ran independent general linear models to test for the effect of maternal GC treatment on the raw plasma cortisol concentration values at each of the three time points in the HPA axis hormone challenge: (A) 1 h post-dexamethasone (DEX) injection, (B) 30 mins post-ACTH injection, and (C) 1 h post-ACTH injection. On rare occasions, we did not get a plasma sample at each time point for an individual squirrel. These models only included measured data (no imputed values), therefore the sample size for each model is different. For each time point, we ran independent models for pregnancy and lactation treatment groups. We included the following fixed effects for pregnancy treatment models: treatment group (control or GC), sex (F or M), initial blood sample cortisol concentration, treatment length in days (standardized), year (as categorical factor), offspring age (standardized), and the interaction between treatment group and sex. We included the same fixed effects for lactation treatment models, but we excluded treatment length since there was almost no variation in treatment length for the lactation treated litters. We did not include a random effect for litter identity in these models due to evidence of overfitting if they were included (singular fit). The reference group is control females from 2015.

Click here to download Table S4