Stress activity is not predictive of coping style in North American red squirrels

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Individuals vary in their behavioral and physiological responses to environmental changes. These behavioral responses are often described as 'coping styles' along a proactive-reactive continuum. Studies in laboratory populations often find that behavioral responses and physiological responses to stressors covary where more proactive (more aggressive and active) individuals have a lower physiological stress response, specifically as measured by hypothalamic-pituitary-adrenal (HPA) axis activity. These studies support the possibility of hormonal pleiotropy underlying the presentation of behaviors that make up the proactive-reactive phenotype. However, recent research in wild populations is equivocal, with some studies reporting the same pattern as found in controlled laboratory studies, whereas others do not. We tested the hypothesis that physiological and behavioral stress responses are correlated in wild adult North American red squirrels (*Tamiasciurus hudsonicus*). We used fecal cortisol metabolites (FCMs) as a non-invasive, integrated estimate of circulating glucocorticoids for our measurement of HPA axis activity. We found that FCM concentrations were not correlated with three measures of behavioral coping styles (activity, aggression, and docility) among individuals. This does not support the hypothesis that hormonal pleiotropy underlies a proactive-reactive continuum of coping styles. Instead, our results support the "two-tier" hypothesis that behavioral and physiological stress responses are independent and uncorrelated traits among individuals in wild populations that experience naturally varying environments rather than controlled environments. If also found in other studies, this may alter our predictions about the evolutionary consequences of behavioral and endocrine coping styles in free-living animals.

coping styles | North American red squirrels | stress response | behavior | glucocorticoids

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Introduction

Organisms can respond to fluctuating environmental challenges and aversive stimuli both through behavioral responses and physiological stress responses. Laboratory studies often find these responses to be associated with one another. In behavioral ecology and behavioral neuroscience, 'coping styles' have been recognized as one method of categorizing behavioral reactions to environmental challenges and stressors. Coping styles refer to a consistent set of behavioral responses to a stressor (1–4). Furthermore, the suite of behaviors that make up an individual's coping style is theorized to be mediated by hormones that exert pleiotropic actions (5, 6).

This unidimensional model has been repeatedly supported by studies describing how the hypothalamic-pituitary-adrenal (HPA) axis mediates coping styles (5). These studies have used selected lines or in other studies under controlled conditions in the laboratory. The conclusion from this model is that the behavioral stress response and physiological stress response run along the same axis. This hypothesis suggests a unidimensional response along a proactive-reactive continuum, where 'proactive' individuals are highly aggressive, highly active, and exhibit lower HPA axis reactivity compared to 'reactive' individuals (5, 7, 8). The vast majority of these studies have been conducted using laboratory animals or wild animals selected for specific behavioral phenotypes, producing individuals at the extremes of this behavioral continuum. For example, in wild great tits (*Parus major*) lines selected for divergent personality types show the predicted unidimensional relationship between behavioral and stress responses in that more proactive birds exhibited lower HPA axis reactivity in response to capture and restraint (9) and lower baseline corticosterone metabolites (10).

In contrast to laboratory studies, those in the wild have been equivocal. Whereas there is some support for the unidimensional model in wild animals (see table 1), recent studies that have used this coping style paradigm to test the relationship between behavior and HPA axis reactivity in free-living animals have found that the proactive-reactive continuum is not predictive of the physiological stress response (11–13). For example, though laboratory selection line results are consistent with predictions of the unidimensional model, when testing great tits in the lab with natural, non-selected variation in exploratory behavior, the relationship is no longer holds (9).

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Table 1. Review of field studies testing for the covariance in behavioral coping styles and HPA activity This non-comprehensive table includes studies conducted with natural populations testing the unidimensional (5) and two-tier models (3) of the among individual relationship between behavioral coping styles and HPA axis activity. The first section includes studies that are contrary to the main prediction from the unidimensional model that more proactive individuals would have lower HPA axis activity. These studies do show that behavior and HPA activity covary, but the relationship is in the opposite of the direction predicted by the unidimensional model. The second section includes studies that support the main prediction from the unidimensional model that more proactive individuals have lower HPA axis activity. The third section includes studies that support the two-tier model that predicts that behavior and HPA axis activity do not covary. Correlations or estimates are included if available in the corresponding manuscript. Confidence/credible intervals are included if available; if not, p-values are included when available. If the confidence/credible interval overlapped zero, we interpreted this as the behavior trait measured and the physiological measurement did not covary. (n = sample size of individuals, NS = non-significant ($p > 0.05$), FCM = fecal cortisol metabolites, DEX = dexamethasone (synthetic corticosteroid), ACTH = adrenocorticotropic hormone)

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
Evidence in the opposite direction of predictions of the unidimensional model				
Poeciliid fish (*Brachyrhaphis episcopi*) (Archard et al. 2012)(14)	Exploration, Activity	Water-borne cortisol (*open-field stress-induced*)	96	Pearson's r = -0.29, $p = 0.005$
Great tits (*Parus major*) (Baugh et al. 2013)(15)	Exploration	Blood corticosterone (*true baseline*)	82	b = 0.536, $p = 0.003$
Alpine marmots (*Marmota marmota*) (Costantini et al. 2012)(16)	Exploration, Activity	Blood cortisol (*nominal baseline*)	28	b = 0.54, Fischer C-statistic model selection
Graylag geese (*Anser anser*) (Kralj-Fišer et al. 2009)(17)	Aggression	FCM (*handling stress-induced*)	10	Spearman's r = 0.782, $p = 0.008$
Evidence supporting the predictions of the unidimensional model				
Great tits (*Parus major*) (Baugh et al. 2017)(18)	Exploration	Blood corticosterone (*ACTH challenge induced*)	85	$R^2 = 0.051$, $p = 0.04$
Great tits (*Parus major*) (Baugh, van Oers, Naguib, & Hau, 2013)(15)	Exploration	Blood corticosterone (*90-min handling-restraint stress-induced*)	16	b = -0.417, $p = 0.015$
	Exploration	Blood corticosterone (*area under the stress-induced curve*)	16	b = -24.66, $p = 0.007$
Belding's ground squirrels (*Urocitellus belingi*) (Clary et al. 2014) (19)	Vigilance	FCM (*nominal baseline*)	12	b = 2.109 [0.17, 4.05]
Brook charr (*Salvelinus fontinalis*) (Farwell et al. 2014)(20)	Activity	Whole-body cortisol (*baseline and handling/novel object stress-induced samples*)	66	NA*
House sparrows (*Passer domesticus*) (Lendvai et al. 2011)(21)	Hovering	Blood corticosterone (*30 min handling-restraint stress-induced*)	18	Pearson's r = -0.58, $p = 0.017$
Eastern chipmunks (*Tamias striatus*) (Montiglio et al. 2012)(22)	Exploration	FCM (*FCM (coefficient of variation)*)	58	b = -13.68 [-27.62, -4.45]
Plateau pika (*Ochotona curzoniae*) (Qu et al. 2018)(23)	Shyness	Blood cortisol (*true baseline*)	292	posterior R = 0.45 [0.09, 0.66]
Evidence supporting the predictions of the two-tier model				
Great tits (*Parus major*) (Baugh, Davidson, Hau, & van Oers, 2017)(18)	Exploration	Blood corticosterone (*handling-restrain stress-induced*)	85	$R^2 = 0.03$, $p = 0.14$
	Exploration	Blood corticosterone (*DEX challenge response*)	85	$R^2 = 0.02$, $p = 0.20$
Belding's ground squirrels (*Urocitellus belingi*) (Clary et al., 2014)(19)	Exploration	FCM (*nominal baseline*)	12	b = -0.76 [-1.12, 0.98]

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Table 1 – *Continued from previous page*

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
Alpine marmots (*Marmota marmota*) (Costantini et al., 2012)(16)	Exploration, Activity	Blood cortisol (*pre-restraint to post-restraint response*)	28	NA**
Belding's ground squirrels (*Urocitellus belingi*) (Dosmann et al. 2015)(11)	Activity	FCM (*nominal baseline*)	157	posterior R = -0.007, [-0.123, 0.095]
	Exploration	FCM (*nominal baseline*)	157	posterior R = -0.046, [-0.175, 0.036]
	Docility	FCM (*nominal baseline*)	157	posterior R = 0.016, [-0.077, 0.139]
Alpine marmots (*Marmota marmota*) (Ferrari et al. 2013)(12)	Activity	Blood cortisol (*nominal baseline*)	146	posterior R = 0.04, [-0.56, 0.71]
	Impulsivity	Blood cortisol (*nominal baseline*)	146	posterior R = 0.08, [-0.68, 0.62]
	Docility	Blood cortisol (*nominal baseline*)	146	posterior R = 0.14, [-0.64, 0.63]
Collard flycatchers (*Ficedula albicollis*) (Garamszegi et al. 2012)(13)	Novel object avoidance	FCM (*nominal baseline*)	51	Pearson's r = -0.017, [-0.291, 0.260]
	Aggression	FCM (*nominal baseline*)	56	Pearson's r = -0.076, [-0.332, 0.191]
	Risk-taking	FCM (*nominal baseline*)	54	Pearson's r = 0.074, [-0.198, 0.335]
Nazca boobies (*Sula granti*) (Grace and Anderson 2014)(24)	Gardening (non-social)	Blood corticosterone (*true baseline*)	222	b = 0.26, [-0.09, 0.61]
	Shaking (non-social)	Blood corticosterone (*true baseline*)	222	b = -0.06, [-0.25, 0.13]
	Aggression (non-social)	Blood corticosterone (*true baseline*)	222	b = 0.19, [-0.07, 0.45]
	Gardening (non-social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = -0.30, [-0.98, 0.38]
	Shaking (non-social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = -0.08, [-0.44, 0.28]
	Aggression (non-social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = -0.07, [-0.35, 0.21]
	Gardening (non-social)	Blood corticosterone (*area under the curve across 4 time points during capture-restraint*)	222	b = -0.16, [-0.7, 0.38]
	Shaking (non-social)	Blood corticosterone (*area under the curve across 4 time points during capture-restraint*)	222	NA, CI in figure includes 0
	Aggression (non-social)	Blood corticosterone (*area under the curve across 4 time points during capture-restraint*)	222	NA, CI in figure includes 0

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Table 1 – *Continued from previous page*

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
	Gardening (social)	Blood corticosterone (*true baseline*)	222	b = 0.20, [0.02, 0.38]
	Shaking (social)	Blood corticosterone (*true baseline*)	222	b = -0.04 [-0.18, 0.1]
	Aggression (social)	Blood corticosterone (*true baseline*)	222	b = -0.02 [-0.11, 0.07]
	Gardening (social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = -0.03 [-0.23, 0.17]
	Shaking (social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = 0.04 [-0.2, 0.28]
	Aggression (social)	Blood corticosterone (*maximum value across 4 time points during capture-restraint*)	222	b = -0.01 [-0.02, 0]
Graylag geese (*Anser anser*) (Kralj-Fišer et al. 2009)(17)	Aggression	FCM (*nominal baseline*)	10	Spearman's r = -0.503, NS
	Sociability	FCM (*nominal baseline*)	10	Spearman's r = -0.212, NS
	Sociability	FCM (*handling stress-induced*)	10	Spearman's r = -0.127, NS
House sparrows (*Passer domesticus*) (Lendvai et al. 2011)(21)	Exploration	Blood corticosterone (*30 min handling-restraint stress-induced*)	18	Pearson's r = -0.07, p = 0.797
	Shy-bold	Blood corticosterone (*30 min handling-restraint stress-induced*)	18	Pearson's r = -0.04, p = 0.866
	Exploration	Blood corticosterone (*true baseline*)	18	Pearson's r = -0.18, p = 0.487
	Shy-bold	Blood corticosterone (*true baseline*)	18	Pearson's r = -0.07, p = 0.801
	Hovering	Blood corticosterone (*true baseline*)	18	Pearson's r = -0.14, p = 0.573
Plateau pika (*Ochotona curzoniae*) (Qu et al. 2018)(23)	Docility, in cage	Blood cortisol (*true baseline*)	292	posterior R = 0.25 [-0.02, 0.63]
	Docility, in bag	Blood cortisol (*true baseline*)	292	posterior R = -0.06 [-0.28, 0.31]
	Exploration	Blood cortisol (*true baseline*)	292	posterior R = -0.16 [-0.52, 0.05]
	Docility, in cage	Blood cortisol (*40 min post-capture change from baseline*)	292	posterior R = 0.22 [-0.06, 0.47]
	Docility, in bag	Blood cortisol (*40 min post-capture change from baseline*)	292	posterior R = 0.16 [-0.07, 0.48]
	Exploration	Blood cortisol (*40 min post-capture change from baseline*)	292	posterior R = 0.10 [-0.42, 0.24]

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Table 1 – *Continued from previous page*

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
	Shyness	Blood cortisol (*40 min post-capture change from baseline*)	292	posterior R = -0.23 [-0.28, 0.22]

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The discrepancy between observed relationships in the wild and the simple unidimensional model (5), has recently led to the development of a "two-tier" coping style model. This two-tier model proposes that individuals in a population can vary independently in both behavioral responses and physiological responses to environmental challenges independently (3). This model of coping styles reframed the original model to establish behavioral coping strategies on a continuum independent of physiological coping strategies (3). The distinction between the unidimensional and two-tier coping style models is significant in assessing the ecological and evolutionary consequences of variation in response to stressors. If the phenotypic correlation between behavioral and physiological stress responses (assumed by the unidimensional coping styles model) reflects an underlying correlation mediated by the effects of a hormone, this may present a limitation in the ability of populations to adapt to changing environmental stressors (25, 26). Alternatively, if the two-tier model of coping style is supported, and there are two separate axes for behavioral and physiological stress responses, this suggests the potential for each trait to be an independent target of selection, potentially facilitating rapid adaptation to new environmental challenges (6, 27). Exploring how coping styles relate to the physiological stress response in wild populations allows us to test across the entire spectrum of naturally occurring individual variation in behavioral coping styles, thus informing our perspective on how these mechanisms function in wild populations (2, 12).

We investigated the relationship between three behavioral traits and one measure of HPA axis activity (concentrations of fecal cortisol metabolites, FCM) in a natural population of North American red squirrels (*Tamiasciurus hudsonicus*). Previous studies in this species showed that there was a repeatable, correlated suite of behavioral traits, specifically aggression, activity, and docility, across the adulthood of an individual (28, 29). These suites of behavioral traits can also be placed along the proactive-reactive continuum as coping styles, with the more active, aggressive, and less docile individuals at the proactive end of the continuum. Differences in coping styles in red squirrels have clear environment-dependent fitness correlates (28, 30, 31), and variation in heritable coping styles among individuals in this population may be maintained through fluctuating selection caused by changing environmental conditions (31). We used fecal samples as a non-invasive proxy for HPA axis activity, which is unaffected by trapping-induced stress (32). It is important to note that glucocorticoids are metabolic hormones and only one mediator of the reactive physiological stress response of an individual (33). While this is not a perfect measure of the overall physiological stress response of an individual, glucocorticoids are an important physiological mediator of the stress response (33, 34). Glucocorticoids are secreted to mobilize energy in response to a stressor in the environment, but also exert pleiotropic effects (34). For example, fluctuating baseline glucocorticoids act as a mediator of reproductive investment in European starlings (*Sturnus vulgaris*) by preparing individuals for energetically expensive reproductive seasons (35). Given that red squirrels in this region experience a fluctuating environment in terms of competitors (36), food (37), and predators (38, 39), having behavioral and physiological responses that are correlated may constrain or facilitate the population in adapting to this environmental variability.

To test the unidimensional and two-tier models of the overall stress response, we measured FCM as a non-invasive marker of HPA axis activity and the behavior of individuals using three behavioral assays (open-field trial, mirror-image stimulation trial, and handling docility assay) to measure coping style. We then compared the FCM concentrations to the behavioral coping style of individual squirrels. A relationship between FCMs and behavioral coping style across individuals would support the unidimensional model, whereas a lack of relationship would support the two-tier model.

Methods

Study species. North American red squirrels are a sexually monomorphic species of arboreal squirrels (40). Females and males are both territorial of their food-cache year-round (41, 42). Red squirrels in the region of our study rely on the white spruce (*Picea glauca*) as their primary food source (43). Local squirrel density is closely associated with mast seeding of the white spruce, or episodes of booms and busts in food availability (43, 44). Red squirrels have one litter per year, with the exception of mast years (37, 45).

This study was conducted as a part of the Kluane Red Squirrel Project, a long-term study of wild population of red squirrels within Champagne and Aishihik First Nation traditional territory along the Alaska Highway in the southwest Yukon, Canada (61°N, 138°W). Each squirrel was tagged with a unique set of alphanumeric stamped ear tags (National Band And Tag Company, Newport, KY, USA). At each live-trapping (Tomahawk Live Trap, Tomahawk, WI, USA) event, body mass and reproductive status of the squirrel were recorded. Female reproductive status was determined through changes in body mass, by nipple condition, and by abdominal palpations of developing fetuses in females. Males reproductive status was determined by palpating for the presence of testicles either in the scrotum (breeding) or abdomen (non-breeding). A more detailed description of the population and general methods can be found in McAdam et al. (2007).

The local population of red squirrels was broken down into three study populations in different locations: two were control populations (referred to hereafter as "control grids") and one was provided with supplemental food between 2004 and 2017, such that squirrel density was increased (36) (hereafter referred to as "high-density grid"). Squirrels on the high-density grid were provided with 1 kg of peanut butter (no sugar or salt added) approximately every six weeks from October to May (see (41) for more information). All work was conducted under the animal ethics approvals from Michigan State University (AUF#04/08-046-00) and University of Guelph (AUP#09R006).

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Behavioral trials. Squirrels were subjected to two behavioral trials to measure 'activity' and 'aggression': an open-field (OF) trial, and a mirror image stimulation (MIS) trial (28, 29)(Boon et al. 2007; Taylor et al. 2012). These behavioral trials were conducted for other projects and were not evenly distributed across years. We performed trials in 2005 (n = 2), 2008 (n = 26), 2009 (n = 15), 2010 (n = 1), and 2012 (n = 28). During these years, additional trials were conducted on squirrels in this population for multiple studies (see (28–31, 46)), but for the purposes of this analyses, we only included trials for which we also had FCM concentration data for that individual (n = 9 male and 48 female control grid squirrels, n = 2 male and 13 female high-density grid squirrels). All squirrels were mature adults (>1 year old) at the time of the trial.

To measure an individual's coping style, we conducted OF and MIS trials during the same trapping event, with the OF trial completed first followed by the MIS trial. Squirrels habituate to these tests (30, 47, 48), but the behavior of individual squirrels over time is known to be repeatable (28, 30, 31). For simplicity, we thus used only the results of each individual's first test as a measure of its activity and aggression. We transferred focal squirrels from a live trap into the arena using a canvas handling bag. The same portable testing arena was used for both trials, and consisted of a 60 x 80 x 50 cm white corrugated plastic box with a clear acrylic lid (49). Four blind holes made with black PVC caps in the bottom of arena allowed the squirrel to explore possible 'escape routes'. We exposed a 45 x 30 cm mirror fixed to one end of the arena after the OF trial to begin the MIS trial. A digital video camera recorded behavior in the arena. We performed all behavioral trials on the territory of the focal individual. Between trials, we cleaned the arena using 70% isopropyl alcohol.

To quantify behavior from the videos, we used manual scoring methods with an ethogram developed and used in previous red squirrel studies (28–31, 46) (table 2), using different software programs depending on the year they were conducted. Trials conducted in 2005 were scored using The Observer Video-Pro 5.0 (Noldus Information Technology, Wageningen, The Netherlands). Trials conducted from 2008 - 2010 were scored using JWatcher Video 1.0 (50). Trials conducted in 2012 were scored using Cowlog software (51). Regardless of the software used, the ethogram remained consistent. During the OF trial, we recorded the mutually exclusive behaviors of time spent walking, sniffing, chewing, rearing, grooming, and being still. Additionally, we recorded the number of jumps and head-dips in the false holes. During the MIS trial, we recorded the amount of time spent in the third of the arena closest to the mirror, and the amount of time spent in the third of the arena farthest from the mirror. We also recorded the number of aggressive contacts with the mirror (attacks), the latency until the first attack, and the latency until the first approach towards the mirror. A detailed description of the video scoring methods can be found in Boon, Réale, & Boutin (2008). Following Taylor et al. (2012), behaviors with an inter-observer reliability of greater than 0.7 were used in analyses (see table 2 for a list of behaviors used in the analyses).

OF Behavior	Description
jump (E)	Jumping.
hang (S)	Hanging from the top of the arena. Behaviors like chew, or scan can be performed while hanging.
chew or scratch (S)	Scratching at or chewing the OF arena.
groom (S)	Paw or mouth grooming.
hole (E)	Interactions with one of the 4 blind holes.
still (S)	When the squirrel is still for 2 seconds.
walk (S)	When the squirrel is moving around the arena.
MIS Behavior	**Description**
attack mirror (E)	Each time the squirrel aggressively contacts the mirror
approach mirror (S)	When the squirrel moves in the direction of the mirror.
front (S)	When the squirrel enters the front of the arena.
back (S)	When the squirrel enters the back of the arena.

Table 2. Ethogram of open-field and mirror image stimulation trials Ethogram used to score behaviors in the open-field and mirror image stimulation trials, modified from Boon et al. (2007). All state behaviors for the open-field trial are mutually exclusive unless noted. In the mirror image stimulation trial, approaching the mirror is non-mutually exclusive with location in the arena.

As an additional behavioral measurement, we also measured 'docility' of individual squirrels. In 2008 and 2009, docility measurements were collected on many squirrels for other studies (see (28, 29)), but for the purposes of this study, we focused only on trials that were conducted during a trapping event where fecal samples were also collected and subsequently analyzed (n = 168 trapping events). We quantified docility as the squirrel's response to handling (for examples in other species, see (22, 48, 52)). We transferred squirrels from the trap into a canvas handling bag and placed the squirrel on a flat surface. We measured docility during handling by counting the number of seconds out of 30 seconds in which the squirrel was not struggling. A squirrel that spent most of the time immobile during the test was considered docile, a trait previously demonstrated to be repeatable in this population (28). This test was conducted an average of 8 (min = 1, max = 42) times on 79 individual squirrels caught between 2008 and 2009. Docility scores were z-scored for analyses. See Fig. 1 for detailed sample sizes.

Fecal cortisol metabolites. From 2005 to 2014, we opportunistically collected a total of 703 fecal samples during routine trapping of squirrels for measurement of FCM concentrations corresponding to individuals with behavioral data (see (32)). Fecal samples were collected from under live-traps within two hours of trapping and placed in 1.5 mL vials stored in a -20 °C freezer within five hours of collection.

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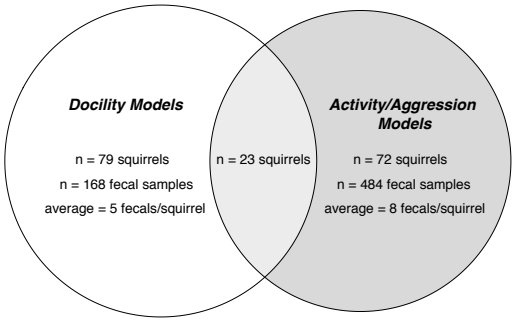

Fig. 1. Sample sizes Our study relies on two overlapping datasets. One dataset is comprised of squirrels with data on docility and FCM concentrations. The other dataset is comprised of squirrels with data on activity/aggression and FCM concentrations. There are 23 squirrels with data on both docility and activity/aggression. The model including both docility and activity/aggression data does not include FCM concentrations.

Urine contaminated feces were excluded. All fecal samples were lyophilized for 14-16 h before being pulverized in liquid nitrogen using a mortar and pestle. Using 0.05 g of dry ground feces, steroid metabolites were extracted by adding 1 mL of 80% methanol and vortexing samples at 1450 RPM for 30 min, and then centrifuging for 15 min at 2500 g (32, 53). The resulting supernatant was stored at -20 °C for analysis via glucocorticoid metabolite assay using a 5α-pregnane-3β,11β,21-triol-20-one antibody enzyme immunoassay (EIA; see (54) for more details). A detailed validation and description of steroid extraction and EIA with red squirrel fecal samples can be found in Dantzer et al. (2010).

Fecal samples were analyzed across multiple assays and in two different labs (n = 355 at University of Toronto Scarborough and n = 348 at University of Michigan) but using the same protocol. We confirmed that our measures of FCM concentrations were highly repeatable across assays or labs through the following. First, a separate group of fecal samples (n = 128 samples) were analyzed in both labs and the optical density of these samples were closely correlated (Pearson correlation = 0.88). This indicates that the data were comparable but we also included a covariate in our statistical models for where the data were analyzed (see below). Second, using pooled samples that were run repeatedly on different plates (n = 115), we found that the estimates of optical density for these pool samples were highly repeatable (R = 0.85, 95% CI = 0.54-0.93). Finally, using a linear mixed-effects model, we partitioned the variance in the optical density recorded for the pooled samples that were run across these different plates. We found that most of the variance was due to the sample itself (85.1%) with relatively little of it being explained by intra-assay variation as all samples were run in duplicate (4.9%) or by inter-assay variation (9.9%). Together, this indicates that our measures of FCM concentrations should be comparable across assays and across labs. See Fig. 1 for a representation of how sample sizes were broken down in each dataset.

Statistical methods. All statistical analyses were conducted in R version 3.4.3 (55). We used the R package 'rptR' version 0.9.21 (56) to estimate the within-individual repeatability of docility. Due to the count nature of the data, we performed repeatability estimation using a Poisson distribution generalized linear mixed-effects model with individual identity (ID) as a random intercept effect. We used parametric bootstrapping (n = 1000) to estimate the confidence interval for repeatability of docility.

For the OF and MIS trials, we used two principal components analyses to reduce the redundancy among behavioral measurements and calculate composite behavioral scores for each trial, as we have done previously in this system (28–31, 46). To conduct the principal components analyses with correlation matrices, we used the R package 'ade4' version 1.7-10 (57). By reducing the multiple behaviors observed down to one metric for each trial, we were able to assess the primary variation among individuals along those axes. All further analyses used the scores calculated from the principal component loadings (table 3) for each trial. From the OF trial, we interpreted the first principal component as a measure of overall 'activity', as it has previously been interpreted in this population (28–31). In our data set, the first component explained 64% of the variation in behavior across OF trials. From the MIS trial, we interpreted the first principal component as a measure of 'aggression', as it has also previously been interpreted (28–31). In our data set, the first component explained 60% of the variation in behavior across MIS trials.

OF Behavior	**Loading**
time spent walking	*0.327*
time spent hanging	*0.260*
chewing or scratching	0.134
number of jumps	0.128
hole head dips	0.019
time spent grooming	0.005
not moving	-0.888
MIS Behavior	**Loading**
time spent in front of arena	*0.422*
number of attacks	0.142
time spent in back of arena	*-0.434*
latency to attack	*-0.499*
latency to approach	*-0.603*

Table 3. Principal component analyses loadings Principal component analysis results from the open-field and mirror image stimulation trials. Loadings greater than 0.2 were used to interpret the components and are italicized.

A previous study on red squirrel personality found phenotypic and genetic correlations among activity, aggression, and docility, such that more active squirrels were more likely to be aggressive and less docile (29). To confirm that these phenotypic relationships remained in our subset of behavioral data, we tested for pairwise correlations between activity, aggression, and docility among individuals. To do this while preserving the multiple measurements of docility per individual, we used a Bayesian generalized linear mixed effects multivariate model based on a Markov chain Monte

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Carlo algorithm with the R package 'MCMCglmm' version 2.25 (58). The MCMCglmm framework allowed us to test for an among-individual correlation between a trait with repeated measures (in this case, docility) and variables with only one observation per individual (in this case, activity and aggression) by controlling for the lack of within-individual variation of the behavioral trait with only one observation. This model was limited to data from individuals tested for all three behavioral traits (n = 23; Fig. 1).

Because we were interested in an estimate of the covariance of FCM concentrations and personality, we used a multivariate framework to conservatively address how the two types of stress responses interact, as explained in Houslay & Wilson (2017)(59). In this study, we were interested in how FCM concentrations and behavioral traits covaried among individuals. To investigate this in a multivariate framework, we used Bayesian generalized linear mixed-effects bivariate models based on a Markov chain Monte Carlo algorithm to assess the relationship between FCM concentrations and behavior. All fecal cortisol metabolite concentration data were ln-transformed to improve normality of residuals.

For docility analyses, we used measurements of docility paired with the FCM concentrations of that trapping event. Using a bivariate generalized linear mixed-effects model, we asked whether individuals with higher mean FCM concentrations have higher mean docility scores (among-individual covariance), and whether individual observations of FCM concentration and docility relative to the individual's mean concentrations were correlated (within-individual covariance). Within-individual covariance indicates how the FCM concentrations and docility scores of one individual covary across multiple observations for that individual; in essence if we have multiple unique measurements of FCM concentration and docility from one individual, does the docility score predict FCM concentrations on that day? In contrast, among-individual covariance measures the relationship between FCM concentration and docility across individuals in the population; in other words, does an individual's average docility score predict its average FCM concentration? The bivariate model for docility and FCM concentration included fixed effects of study grid (control or high-density), sex, Julian date of trapping event (continuous), trial number (to control for possible habituation across trials(28)), breeding status (breeding/non-breeding/lactating), and a variable to indicate where the fecal sample was processed (UTScarborough/UM). We specified in the model to estimate the fixed effects of Julian date of trapping event and the location of fecal sample processing for only FCM concentration. These fixed effects were included because they have previously been shown to influence FCM concentration in red squirrels (32, 36), and thus were included to control for variation among these variables.

Because we only had one measurement of aggression and activity per individual, we were unable to estimate the within-individual covariance between FCM concentration and the activity/aggression of that individual. Thus, the bivariate models for activity and aggression were structured to control for only one individual activity and aggression score per individual, and only estimate among-individual covariance. The models for activity and aggression included the same fixed effects as the model for docility, except trial number was not included (all trials were the first trial in the lifetime of that individual). Again, we estimated fixed effects of the Julian date of trapping event and location of fecal sample processing for only FCM concentration. With this model structure, we were able to more precisely control for variation in FCM concentration due to reproductive condition and time of year.

We fit all bivariate MCMCglmm models with uninformative priors (as in (59)) for 2,100,000 iterations with the first 100,000 discarded, and 1 out of every 1,000 of the remaining iterations used for parameter estimations. Credible intervals (95%) around the correlation were based on the MCMC chain iterations. To confirm convergence using a combination of methods, as suggested in a comparative review (60), we ran all MCMCglmm models three times for comparison using the Gelman-Rubin statistic (61) and we also ran the Geweke diagnostic (62). All models passed both diagnostics for convergence.

As a comparison to our bivariate MCMCglmm model approach, we also used R package 'lme4' version 1.1-15 (63) to create general linear models to test for relationships between each behavioral trait and average FCM concentrations with one measure per individual. This linear model approach has been used previously for these types of questions (e.g. (21, 22)), and we wanted to compare our linear model results to the recently developed and perhaps more appropriate Bayesian statistical approach to this type of multivariate question (59). For our linear models, we used the average of all ln-transformed FCM concentrations from an individual as a response variable. The relationship between behavior and hormones can be bidirectional and we do not propose any causality with these models. However, to keep our linear models consistent with our bivariate models with respect to fixed effects we structured the linear models with average ln(FCM concentration) as the response variable in order to identify the relationship between FCM concentration and variation in behavior, while controlling for variation due to study area and sex. To estimate P-values, we used the R package 'lmerTest' version 2.0-36 (64). Separate models were used for each behavioral trait. Linear models for docility tested for the relationship between average docility across all trials of an individual and average FCM concentration of that individual. Fixed effects in these models predicting average FCM concentration included the behavioral trait of interest (docility, activity, or aggression), study area (control or high-density), and sex. Normality and homoscedasticity of residuals of all linear models were confirmed visually.

Results

Docility scores were significantly repeatable within-individuals (R = 0.33, CI = [0.17, 0.49]). Estimates of the correlations between our three behavioral traits were in

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	Docility	Activity	Aggression
Docility	0.68 [0.30, 1.26]	-0.42 [-0.77, -0.006]	-0.22 [-0.60, 0.14]
Activity	-0.33 [-0.74, 0.13]	0.91 [0.42, 1.47]	0.12 [-0.30, 0.50]
Aggression	-0.23 [-0.65, 0.20]	0.14 [-0.45, 0.63]	1.41 [0.83, 2.16]

Table 4. Results from our multivariate model using a subset of data to examine the relationships between docility, activity, and aggression (n = 23 squirrels). Among-individual variances are listed on the diagonal, covariances below and correlations above (with the lower and upper bounds of 95% CIs in brackets).

the same direction as previous studies in this population, with active squirrels being more aggressive and less docile (table 4), though the credible intervals for the estimates were near zero or overlapped zero, likely due to the smaller sample size of individuals with data for all three behavioral traits (Fig. 1). These findings, combined with results from previous studies in red squirrels with much larger sample sizes (see (28, 29)), support the idea that these behaviors are phenotypically correlated, and may be shaped by an underlying pleiotropic mechanism.

Both the bivariate framework and the linear model framework indicated that docility, activity, and aggression did not co-vary with FCM concentrations among individuals. Using a bivariate generalized linear mixed effects model approach, the within-individual covariance indicated that an individual's FCM concentrations did not correlate with docility (r = 0.020, CI = [-0.14, 0.28]). Our models indicated that among individuals, FCM concentrations did not correlate with docility (r = 0.14, CI = [-0.64, 0.83], Fig. 2), activity (r = 0.15, CI = [-0.17, 0.47], Fig. 3) or aggression (r = 0.29, CI = [-0.098, 0.56], Fig. 3) (table 5). Regardless of the significance of these relationships, the direction of the observed effect was opposite to the predicted relationship between behavior and FCM concentrations (Fig. 4). The direction of these correlations suggest more active and more aggressive squirrels may have higher FCM concentrations.

Likewise, our linear models did not indicate a relationship between the behavioral coping style and physiological stress response. The models did not detect a significant relationship between average FCM concentrations and activity (b = 0.08, t = 1.18, p = 0.24), aggression (b = 0.11, t = 0.1.66, p = 0.099), or average docility (b = -0.0029, t = -0.38 p = 0.71) (table 6).

Discussion

We demonstrated that the behavioral coping style (represented by three behaviors) and one measure of the physiological stress response (FCM concentrations) did not co-vary in a free-ranging mammal. Independent variation between the behavioral and physiological stress responses supports the two-tier model of coping strategies proposed by Koolhaas et al. (2010). This model proposes that within a species, individuals can exhibit a consistent behavioral response anywhere along the proactive-reactive continuum but independent of their physiological stress response, that can range from a low to a high HPA axis activity. Contrary to many laboratory studies, proactive, or highly active/aggressive red squirrels did not always exhibit lower HPA activity than reactive individuals. In fact, the parameter estimates were in the opposite direction from those predicted by the unidimensional model. Although we did find that the three behavioral measures were phenotypically correlated, physiological stress, as measured by FCM concentration, does not appear to be the pleiotropic mechanism causing this covariation. Additionally, our findings were robust across statistical techniques with both the bivariate framework and linear model framework providing support for independence between the behavioral traits measured and FCM concentration.

	ln(FCM)	Docility
ln(FCM)	0.049 [1.86-8, 0.13]	0.14 [-0.63, 0.83]
Docility	0.012 [-0.074, 0.11]	0.40 [0.15, 0.71]
	ln(FCM)	**Activity**
ln(FCM)	0.075 [0.018, 0.14]	0.15 [-0.17, 0.47]
Activity	0.04 [-0.034, 0.11]	0.55 [0.38, 0.73]
	ln(FCM)	**Aggression**
ln(FCM)	0.077 [0.016, 0.14]	0.29 [-0.098, 0.56]
Aggression	0.061 [-0.03, 0.16]	1.00 [0.68, 1.33]

Table 5. Multivariate results for relationships between FCM and behaviors Results from our three multivariate models to examine the relationships between FCM and each of three behaviors individually (activity and aggression models: n = 484 fecal samples; docility model: n = 168 fecal samples). Among-individual variances are listed on the diagonal, covariances below and correlations above (with the lower and upper bounds of 95% CIs in parentheses).

Our study contributes to an emerging trend of a lack of a strong relationship between behavioral and physiological stress responses in wild animals or along natural levels of continuous variation in coping styles of lab animals (reviewed by (65)). For example, wild alpine marmots (*Marmota marmota*) exhibit a lack of among-individual correlation between activity and plasma cortisol concentrations, as well as between docility and plasma cortisol (Ferrari et al. 2013). Likewise, docility and exploration were not correlated with a change in plasma cortisol in response to a stressor in plateau pika(23) (*Ochotona curzoniae*). Additional studies measuring fecal glucocorticoid metabolites demonstrate that HPA activity does not correlate with shy-bold behavioral types in wild flycatchers(13) (*Ficedula albicollis*, n = 51-56 individuals), or with exploration/activity in Belding's ground squirrels(11) (*Spermophilus beldingi*, n = 157 individuals). However, it should be noted that measurements of basal glucocorticoids in red squirrel fecal samples may not allow for direct measurement of the reactivity of the HPA axis, which

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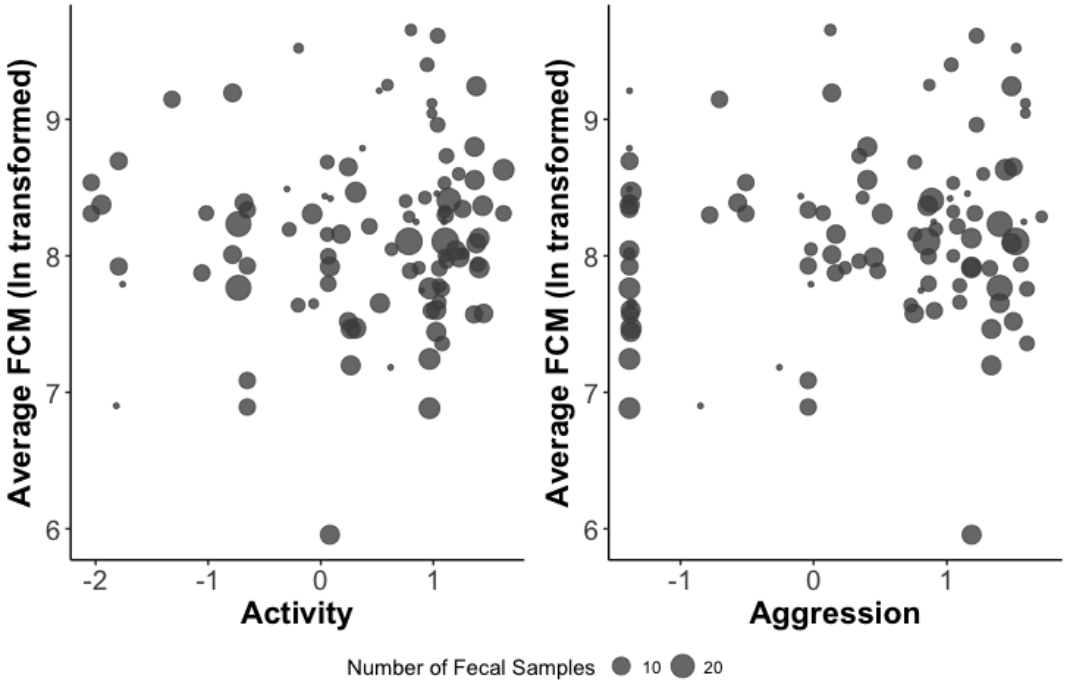

Fig. 2. Stress reactivity, as measured by average FCM concentrations, is not predicted by activity or aggression in the North American red squirrel. Activity and aggression are from scores determined by the principle component analyses. Size of the points represents number of fecal samples included for that individual.

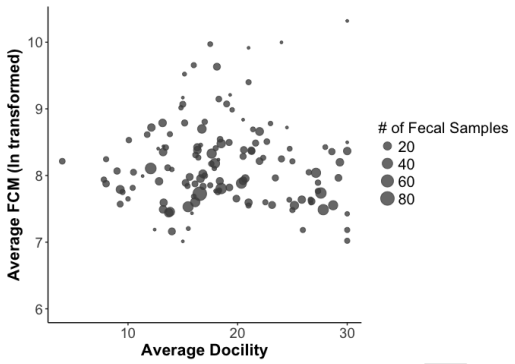

Fig. 3. Stress reactivity, as measured by average FCM concentration, is not predicted by docility in the North American red squirrel. Size of the points represents number of fecal samples included for that individual.

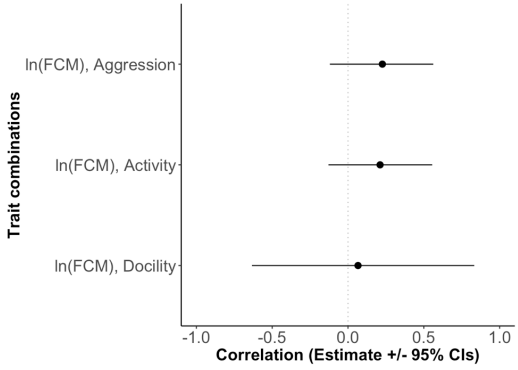

Fig. 4. Correlation estimates between traits from generalized linear mixed effects bivariate models based on a Markov chain Monte Carlo. There was no significant correlation between FCM concentration and aggression, activity, or docility (z-scored), as all the estimates overlap zero.

may correlate more strongly with behavioral stress responses compared to basal regulation (e.g. (9)). Although a study on free-ranging eastern chipmunks (*Tamias striatus*) showed evidence supporting covariance of behavioral response and physiological stress response from fecal samples, the study used only one metric of physiological stress (coefficient of variation of fecal glucocorticoid metabolites) per individual (22). This statistical method was limiting in that it did not consider the uncertainty around each individual's measure of HPA axis activity. Our research expands upon the chipmunk study by using more conservative statistical methods, which were not widely used until recently, in addition to linear models and multiple behavioral assays to establish coping styles. Using both of these approaches, we showed that the behavioral coping style (comprised of three correlated behaviors) does not covary with one measure of physiological stress. We acknowledge that the studies included in table 1 are across multiple taxa, behaviors, and HPA axis activity measurements. This likely contribute to the equivocal nature of support for these models in wild animals. Due to the large variability across wild species in measurements of HPA axis activity and behavior, perhaps a less generalized model of the relationship between behavior and physiological stress may be more predictive for future studies than our current models.

Our study was conducted using adult red squirrels. In this population, high juvenile mortality results in a high opportunity for selection during the first year of life (45). Due to these strong selective pressures, we must consider the possibility that selection may have already eroded the (co)variance of physiological and behavioral stress responses in surviving adults. Additionally, these selective pressures fluctuate

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across years because red squirrels rely on a masting food source (white spruce) that goes through episodes of booms and busts in production of reproductive cones (43, 44). Following the masting of spruce trees, squirrel populations increase in density, which may generate density-dependent selection on juvenile traits (36, 66). These fluctuations in selection may maintain genetic variation in behavioral traits in this population (31), and, if a pleiotropic hormone was the mechanism underlying these behavioral correlations, it could limit an adaptive behavioral response to this fluctuating selection if the selective forces on hormone levels and the behavior push in opposite directions (6, 67). Recent work, however, suggests a hormonal pleiotropic relationship is likely not powerful enough to constrain independent evolution of two traits (26). Alternatively, if the hormone does not show a pleiotropic relationship with behavior and selection for both is working in the same direction then the absence of a correlation could slow their adaptive response relative to a situation with a positive pleiotropic relationship between the two traits (27).

We also must consider the possibility that different behavioral traits are favored at different life stages. Additional work in this study system has shown wider variation in these behavioral traits among juvenile squirrels, with individuals at both extremes of the proactive-reactive continuum, and individuals regress to the mean as they age (46). This is a potential limitation of our study. Studies using selection lines, and therefore individuals at extremes in behavioral response, may therefore be more appropriate for making predictions about juvenile red squirrels, though perhaps not appropriate for predictions about adults. In great tits and rainbow trout (*Oncorhynchus mykiss*), studies using exploration selection lines have found evidence to support a correlation between behavioral and physiological stress response (9). This suggests the potential to detect a relationship between behavioral and physiological stress responses in juvenile red squirrels, when individuals are more widely dispersed along the proactive-reactive continuum. Future work on the relationship between coping styles and physiological stress responses should investigate the ontogeny of the relationship, and how it may change across life stages.

This study helps establish a foundation for exploring the fitness consequences of variability across two axes of the stress response, behavioral and physiological. Building upon this work, we can explore the mechanisms contributing to each axis of variation independently. For instance, the maternal environment during ontogeny may influence the development of the physiological stress axis, or contribute to the behavioral coping style (reviewed in (68)). Furthermore, our study provides additional evidence supporting the lack of direct correlation between behavioral and physiological stress responses in wild animals exhibiting natural variation in stress responses, as opposed to lab animals or selection lines restricted to the extremes of the possible variation. Our study, in conjunction with previous studies on these models in wild animals (table 1), suggests a need for a more generalizable model of the relationship between the behavioral and physiological stress responses. Further studies in wild animals are needed to explore the mechanisms underlying this variation along the phenotypic landscape of the stress response and the adaptive value of such variation.

Our study contributes to a growing body of work in support of the two-tier model of coping styles and physiological stress reactivity in wild populations. The lack of a phenotypic relationship between the two stress responses could have important evolutionary implications, particularly for species living in fluctuating environment environments.

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Response Variable	Fixed Effect	b	SE	t	p-value
Average ln(FCM)					
n = 72 individuals	**Intercept**	**8.04**	**0.081**	**99.28**	**< 0.0001**
	Activity	0.081	0.069	1.18	0.24
	Grid type:				
	high density	**0.44**	**0.18**	**2.43**	**0.017**
	Sex:				
	males	0.086	0.20	0.43	0.67
Average ln(FCM)					
n = 72 individuals	**Intercept**	**8.03**	**0.079**	**102.18**	**< 0.0001**
	Aggression	0.11	0.063	1.66	0.099
	Grid type:				
	high density	**0.44**	**0.18**	**2.50**	**0.014**
	Sex:				
	males	0.14	0.20	0.70	0.49
Average ln(FCM)					
n = 79 individuals	**Intercept**	**8.11**	**0.16**	**50.70**	**< 0.0001**
	Average Docility	-0.0029	0.0077	-0.38	0.71
	Grid type:				
	high density	0.18	0.10	1.75	0.082
	Sex:				
	males	0.18	0.11	1.56	0.12

Table 6. Full Results – Linear Models Full results from three linear models between average FCM concentration and each behavioral trait independently. Significant fixed effects ($p < 0.05$) are in bold. Grid type compares the high-density grid to the control (intercept) grid. Sex shows how males differ from females (intercept).

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