Understanding Behavioral and Physiological Outcomes of Variation in Maternal Care and Glucocorticoids

by

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Dedication

This dissertation is dedicated to Kerrlita Mitchell Westrick, Mary Belle Mitchell, and Joan Westrick.

"But behind all your stories is your mother's story, for hers is where yours begins."

– Mitch Albom, *For One More Day*

"To describe my mother would be to write about a hurricane in its perfect power. Or the climbing, falling colors of a rainbow."

– Maya Angelou, *I Know Why the Caged Bird Sings*

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Preface

A dissertation in behavioral ecology is never a solo endeavor – it takes a proverbial village to ask these questions. I conducted all of the research in this dissertation in collaboration with the Kluane Red Squirrel Project, under the guidance of Dr. Ben Dantzer. As is the inherent nature of a collaborative long-term research project, all of my research is a cooperative effort and would not be possible without significant contributions from my co-authors, who are listed at the beginning of each chapter. Additionally, the work of numerous field technicians was vital to collecting all of this field data – either directly or indirectly through supporting other crew members and myself.

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Abstract

Behavior is one of the most immediate and effective ways to respond to and cope with environmental and social stressors. Our behavioral response to stressors is proposed to be intimately linked to our hormonal stress response in a bidirectional relationship. Understanding the relationship between these two stress responses in adults and their ontogeny in juveniles helps us understand how animals will respond to rapidly changing environmental pressures and provide more context for understanding the role of individual variation in behavior in natural selection. To investigate this question, I collected hormonal and behavioral data from a wild population of North American red squirrels (*Tamiasciurus hudsonicus*) in Yukon Territory, Canada. Red squirrels in this region have proven to be amenable to large scale experimental hormone manipulations and offspring growth, hormones, and behavior can be tracked for the entire life of hundreds of individuals each year. Using long-term data on behavior and fecal cortisol, I found no relationship between the hormonal and behavioral stress response as was measured. To better understand the ontogeny of this lack of relationship between the stress responses, I examined the role of the maternal early life environment. Maternal behavior and physiology influence the development of phenotypes, many of which are closely related to fitness. However, maternal behavior is often difficult to observe and measure in wild animals, particularly small mammals. To tackle this problem, I measured maternal motivation by recording the time until mothers return to their pups following researchers removing and returning the pups in the nest, or a "simulated predator intrusion". I found wide variation in the behavioral response of mothers to this nest intrusion. Some mothers were very vocal and

aggressively attempt to protect their pups, other moms hung out in a nearby tree and eat quietly waited for us to return their pups. I found a mother’s maternal style, as measured by latency to return to pups following the intrusion, was repeatable within individuals and played a role in increasing the survival and growth rate of offspring. To further explore the impact of the maternal environment on offspring, I tested hypotheses about the impact of maternal glucocorticoid levels on offspring behavior and physiology by conducting a manipulation with mothers across three years to experimentally elevate circulating glucocorticoids in pregnant or lactating mothers. In offspring from these mothers, I found the behavioral traits of activity and aggression in these juveniles were linked to their hormonal stress reactivity, unlike the adults. Through these studies, I expanded on our understanding of the relationship between behavior and physiology, with a particular focus on maternal effects, in a wild small mammal. I added to the growing body of evidence showing a lack of relationship between behavioral and physiological stress responses in wild animals, suggesting the need to develop a more generalizable model of the relationship between the glucocorticoids and animal personality. Furthermore, I leveraged our ability to closely track reproduction in red squirrels to empirically assess the fitness consequences of individual variation in maternal behavior and conduct a unique field experiment to branch across developmental biology, behavioral ecology, and behavioral endocrinology.

Chapter 1 Introduction

Individual variation in phenotypes provides the raw material for natural selection, and by extension adaptive evolution or drift, to act upon. One mechanism underlying individual variation is developmental plasticity, or modification of phenotypes, without genetic change, by inputs during development (West-Eberhard, 2003). Maternal environments can promote phenotypic plasticity as mothers contribute to the development of their offspring's phenotypes above and beyond their genetic impact through maternal effects (Agrawal, 2001; Maestripieri & Mateo, 2009; Marshall & Uller, 2007; Wolf & Wade, 2009). Two important mechanisms of maternal effects are maternal behavior and the exchange of hormones. Studying the transgenerational impacts of maternal behavior and hormones improves our understanding of the potential for rapid change in phenotypes in response to quickly changing and unpredictable environments. As Dr. Hrdy concisely summarized, "…individualization begins as a maternal effect" (Hrdy, 1999).

One major cause of developmental plasticity is maternal stress. While the response of offspring to maternal stress or adversity is thought to be evolutionarily ancient and conserved across species with both fast and slow life history strategies (Thayer et al., 2018), maternal effects are of particular interest when studying mammals. An extended period of maternal care and offspring dependence during gestation and lactation is ubiquitous and critical in mammals (Maestripieri & Mateo, 2009; Reinhold, 2002; Roff, 1998). This prolonged exposure to maternal physiology and behavior provides many opportunities for offspring to gather information and cues from their mothers. Through maternal effects, mothers may "transmit" information about environmental conditions, often stressors (triggers of a ‘stress response’ mediated by catecholamines or glucocorticoids), to their offspring allowing offspring to adjust their behavior to prepare themselves for those environments (e.g. Storm & Lima, 2010).

Individual consistency in behavior

Individual consistency in behavior across environmental or social contexts and time is referred to by many different terms in the literature such as temperament, coping styles, personality, behavioral styles, and behavioral syndromes (Réale et al., 2007). Measurement of personality or behavioral syndromes can be challenging in natural populations as it requires repeated measurements of the same individual across different context or times (Sih, Bell, Johnson, et al., 2004; Stamps & Groothuis, 2010). It is important to keep in mind that quantification of personality traits is not an absolute measurement, but actually a measure of the trait relative to other individuals, or consistent individual differences.

Among-individual differences in behavioral syndromes, or combinations of behavioral traits, are theorized to be shaped by individual differences in hormones that exert pleiotropic actions, such as cortisol (McGlothlin & Ketterson, 2008). ‘Coping style’ is often used to describe a continuum of the stress response from proactive to reactive individuals (Koolhaas et al., 1999, 2010). In the ‘unidimensional model’, this behavioral response co-occurs with the physiological stress response and therefore ‘coping styles’ is sometimes used in reference to both the behavioral and physiological stress responses (Koolhaas et al., 1999). In this instance, proactive individuals are more active and aggressive than their reactive counterparts, and have lower hypothalamic-pituitary-adrenal (HPA) axis reactivity (Koolhaas et al., 1999). This model was subsequently updated to separate the behavior and physiology but retained the term ‘coping styles’ as a reference to the behavioral response (Koolhaas et al., 2010). In this dissertation, I use the term ‘behavioral trait’ when referring to a specific aspect of an individual’s consistent behavioral repertoire (e.g. aggression) as defined in Carter et al. (2013).

Hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis has historically been referred to as the ‘stress hormone’ axis due to its role in regulating glucocorticoids in response to external and internal challenges . Briefly, the HPA axis acts by producing corticotropin-releasing factor (CRF) in response to neural inputs to the paraventricular nucleus in the hypothalamus (Packard et al., 2016; Spencer & Deak, 2017). CRF acts in the anterior pituitary to release adrenocorticotropic hormone (ACTH) (Packard et al., 2016; Spencer & Deak, 2017). ACTH activates the adrenal cortex to release GCs (Packard et al., 2016; Spencer & Deak, 2017). High levels of GCs trigger negative feedback by the HPA axis which returns expression of CRF, ACTH, and GC to basal levels (Sapolsky et al., 1985; Spencer & Deak, 2017).

Glucocorticoids are not the only physiological response to stressful conditions and responding to stressful conditions is not the primary function of systemic GCs (MacDougall-Shackleton et al., 2019). Due to their role as metabolic hormones, GCs are essential for mobilizing energy and have many pleiotropic effects throughout the body (MacDougall-Shackleton et al., 2019). In response to acute stressors, GCs are important for survival and may promote resiliency (Spencer-Segal & Akil, 2019). In this dissertation, I focused on the HPA axis and GCs as the main physiological mechanism of interest due to how vital they are for survival and development. The pleiotropic nature of GCs means any transgenerational impacts on the development of the HPA axis can have profound widespread consequences for offspring. Additionally, the role of the HPA axis in responding to acute and chronic stressors and maintaining homeostasis makes it a strong contender for a cue pups use to gain information about the environment *in utero* and while nursing. Mothers would respond to environmental and social stressors by modulations in their HPA axis and production of GCs which are transferred between mothers and offspring.

Maternal effects

Parental effects are the impacts of the parental phenotype on the phenotype of the offspring beyond what is inherited genetically and can vary with unique combinations of parental and offspring phenotypes (Maestripieri & Mateo, 2009; Marshall & Uller, 2007; Wolf & Wade, 2009). In mammals, biparental care is rare but maternal care is universal and critical due to the extended period of care during gestation and lactation (Gubernick & Klopfer, 1981). Maternal effects in mammals have the potential to alter offspring phenotypes in an adaptive manner in response to the changing environment (Bernardo, 1996; Mousseau & Fox, 1998; Wolf & Wade, 2009), but maternal effects are not always adaptive. Maternal effects can also lead to a decrease in offspring fitness (non-adaptive maternal effects; Bernardo, 1996; Kofman, 2002) increase maternal fitness at the expense of offspring fitness (selfish maternal effects, e.g. McCormick, 2006), reduction of variance in maternal fitness (bet-hedging maternal effects, e.g. Laaksonen, 2004), or a reduction of both maternal and offspring fitness (transmissive maternal effects, e.g. Wiklund & Sundelin, 2001). Maternal effects can have profound effects on the strength and direction of natural selection (Kirkpatrick & Lande, 1989; Maestripieri & Mateo, 2009; Qvarnström & Price, 2001) and often explain a large amount of variation in offspring traits (Boonstra & Boag, 1987; Boonstra & Hochachka, 1997; Hansen & Boonstra, 2000; McAdam et al., 2002).

Maternal effects on behavior

Many aspects of behavior balance between individual consistency and plasticity, responding to internal and external cues such as the early life environment (Briffa et al., 2008; Dingemanse et al., 2010; Sih, Bell, & Johnson, 2004; Sih, Bell, Johnson, et al., 2004). One route by which maternal effects may impact behavioral traits or coping styles is through altering phenotypic correlations between behavioral and physiological stress responses (McGlothlin & Ketterson, 2008). From studies in many systems, we know that the early life environment, particularly the maternal environment (Levine, 1994), can impact the development of the hypothalamic-pituitary-adrenal axis (HPA), which is a critical component of the physiological stress response. If the behavioral and physiological stress responses are mediated by the hormones of the HPA axis, developmental plasticity in the HPA axis would result in changes in the behavioral responses. Additionally, the early life environment could also induce adaptive combinations of traits (Lancaster et al., 2007) or cause adaptive shifts in the phenotypic covariance by either strengthening or lessening the overall degree of covariance (Careau et al., 2014; Merrill & Grindstaff, 2015).

Maternal glucocorticoids

Two routes through which mothers can impact their offspring are hormones, exchanged through the placenta and milk, and post-natal parental care behavior. The role of glucocorticoid (GC) steroid hormones in exchanging information from mothers to offspring is of particular interest due to their responsiveness to environmental and social stressors and their widespread functions in metabolism and development (Sapolsky et al., 2000). Maternal GCs, and even grandmaternal GCs, can have a significant life-long impact on the development of the HPA axis in laboratory rodents, such as mice (*Mus musculus*), rats (*Rattus norvegicus domestica*), and guinea pigs (*Cavia porcellus*: Matthews, 2002; Matthews et al., 2004). For example, prenatal exposure to elevated GCs changes the physiological stress response and behavior in guinea pigs across 3 generations (Moisiadis et al., 2017). Beyond the role of the HPA axis relationship with behavior, differences in growth and development are also theorized to relate to personality traits (Réale et al., 2010; Stamps, 2007; Stamps & Groothuis, 2010) establishing theoretically two different routes by which maternal GCs may impact offspring behavior—through changes to the HPA axis of offspring or by metabolic/developmental changes such as accelerating growth rate.

Much of our current knowledge about how hormones and maternal behavior vary and their impact on offspring stems from laboratory studies of small mammals, mainly rats, mice, hamsters (*Mesocricetus auratus*), and guinea pigs. Controlled laboratory studies are invaluable in identifying specific mechanisms of how mothers impact offspring; nevertheless, integrative studies using natural populations are instrumental in identifying the natural levels of variation in hormones and behavior, and the adaptive nature of maternal effects. However, studies of maternal behavior and hormones and the impact on offspring can be challenging to track with free-ranging animals, particularly with small mammals due to their secretive nature.

North American red squirrels: "the steward who sits in the shadow of his tail"

In my dissertation, I address the consequences of the maternal environment on offspring characteristics in a natural population of North American red squirrel, *Tamiasciurus hudsonicus* Erxleben. Red squirrels are an ideal model system for understanding the drivers and consequences behind maternal effects in wild animals as the adaptive value and evolutionary consequences of maternal effects on offspring can be directly measured. The red squirrel system provides an opportunity to study the interaction of two proposed pathways of maternal effects –

hormones and maternal care behavior – as well as the fitness consequences and potential physiological and developmental trade-offs triggered by these maternal effects.

North American red squirrels in the Kluane region of Yukon Territory, Canada rely on white spruce seeds as their primary food source (Fletcher et al., 2013). Both male and female red squirrels in this area are larder hoarders of white spruce cones and other food resources and defend their cache ('midden') year-round (Dantzer et al., 2012; Siracusa et al., 2017). White spruce cones are mature and ready to be cached beginning mid-August until mid-September (Fletcher et al., 2010). During that time, red squirrels spend almost all of the day clipping and caching cones (Studd, unpublished). Red squirrels do not hibernate or enter torpor (Brigham & Geiser, 2012; Pauls, 1978) but do reduce their activity during winter and rely on the food cached in their midden to survive to breed the following year. Female red squirrels are typically in estrus for one day (with the exception of mast years; Boutin et al., 2006) and mate with many male squirrels in 'mating chases' (Lane et al., 2007, 2008). Mothers provide all the parental care in this species. Researchers on the Kluane Red Squirrel Project (KRSP) have studied this population of red squirrels continuously since 1987 and have developed methods to track individuals through their entire life (McAdam et al., 2007).

One of the logistical advantages of working with red squirrels on questions surrounding reproduction is their arboreal nature and strong territoriality. This allows researchers to closely track mothers' reproductive state and collect data on pups by accessing the nests in trees. This access to offspring throughout their development is not common in wild study systems and provides a great opportunity to study questions related to offspring development in a natural environment.

Chapter 2: Relationship between behavioral traits and HPA axis dynamics

In order to make informed predictions about the route by which mothers may impact their offspring's phenotypes, we first need to identify any relationships between hormones and behavior within individuals. In Chapter 2, I focused on characterizing the relationship between the HPA axis and personality traits of 'aggression', 'activity', and 'docility' within and among individuals.

In this chapter, I used Bayesian multivariate analysis techniques to test two competing models describing the relationship between behavior and HPA axis physiology: the unidimensional model (Koolhaas et al., 1999) and the two-tier model (Koolhaas et al., 2010). From non-invasively collected fecal samples, I used cortisol metabolite concentrations (FCM) as a measurement of HPA axis activity that is unaffected by trapping and handling-induced stress (Dantzer et al., 2010). The behavioral traits of 'activity', 'aggression', and 'docility' are often phenotypically linked in a behavioral syndrome such that squirrels fall along the proactive (more aggressive, more active, less docile) to reactive (less aggressive, less active, more docile) continuum (Taylor et al., 2012). I tested for among-individual (co)variance in activity/aggression and FCM. Our dataset contained docility measurements on the same individual squirrel over multiple trapping events which allowed me to test for within and among-individual (co)variance in docility and FCM. Overall, I found these two stress responses (behavior and HPA axis activity) were not related to one another in adult red squirrels (Westrick et al., 2019). This result suggests there are few phenotypic constraints on how the early life environment may shape behavior and hormones. In other words, the early life environment may affect either or both behavior and hormones in independent adaptive ways.

Chapter 3: Fitness consequences of maternal care

Parental investment theory would predict mothers are limited in the amount of care/resources they can give current offspring and this trade-off between investing in current and future offspring is what drives parent-offspring conflict (Trivers, 1972, 1974). However, there is not much empirical evidence that higher levels of parental investment, specifically through maternal *behavior*, results in increased survival. In this chapter, I aimed to identify how the early life care provided by mothers impacts offspring growth and survival.

In Chapter 3**,** I investigated the consequences of individual variation in maternal behavior. In the red squirrels, we see wide variance among how attentive mothers are to their pups following a nest intrusion by a researcher. I found this measurement of maternal attentiveness was repeatable and indicative of an individual's 'maternal style'. A mother's maternal style was predictive of her lifetime reproductive success – more attentive mothers had more pups survive to recruit into the breeding population across their lifetime. I found more attentive mothers produced faster-growing large litters than less attentive mothers, who experienced a trade-off between litter size and pup growth rate. Due to the importance of growth rate in predicting survival and subsequent recruitment, the mitigation of this trade-off through maternal behavior appears to be one route to increase a mother's overall reproductive success. Despite the fitness benefit of high latency, I hypothesize that variation in this maternal behavior is maintained by fluctuating selection or behavioral plasticity in response to factors such as age, environmental cues, or litter characteristics.

In a study currently in progress, I examined potential contributors to variation in maternal behavior, including internal factors, litter characteristics, and environmental cues. Another extension of this study will include the relationship between maternal GCs and maternal behavior. In Dantzer *et al.* (2013), we see perceived and actual increases in conspecific density result in a reduction in the tradeoff between growth rate and litter size which is adaptive in high density years. This study proposes maternal GCs as one route linking high-density and fast growth rates of pups (Dantzer et al., 2013). However, it is still unclear if this environmental cue is being translated into adaptive changes in maternal behavior in addition to changes in maternal GCs. This next study will be crucial for identifying whether maternal behavior is another mechanism by which mothers convey environmental cues to offspring.

Chapter 4: Impacts of maternal GCs on offspring HPA axis dynamics and behavioral traits

From Dantzer *et al.* (2013), we know increased maternal GCs during pregnancy can increase the early growth rate of pups, which is associated with increased survival in high density years. In contrast, increased maternal GCs during early lactation decreases the early growth rate of pups (Dantzer et al., 2020). To expand on our understanding of the consequences of elevated maternal GCs, in Chapter 4, I experimentally tested how maternal GCs impacted the development of her offspring's HPA axis dynamics and behavioral traits of 'activity' and 'aggression'. In this population of red squirrels, we see fluctuating selection pressures on these two behavioral traits due to the highly variable environment this population experiences (Boon et al., 2007; Taylor et al., 2014). This prompts the question of whether maternal cues interpreting the variable environment (such as changes in GCs) induce developmental plasticity such that an offspring's behavior is better aligned to conditions they will experience when they are breeding in the following year. To test this, in collaboration with other researchers, I conducted a 3-year individual GC supplementation experiment with breeding female squirrels and tested the behavior and HPA axis dynamics in weaned offspring. I found offspring from mothers supplemented with GCs did not show differences in activity, aggression, or HPA axis dynamics.

Summary

Understanding the mechanisms by which mothers influence their offspring in natural study systems can give us insights into how populations may adapt to rapidly changing environments through phenotypic plasticity, rather than genetic shifts in the population (Pigliucci et al., 2006; Scoville & Pfrender, 2010; West-Eberhard, 2003). A common thread through these chapters is the relationship between behavior and physiology (Figure 1.1). In Chapter 2, I addressed the topic through measuring the (co)variance of behavioral traits and HPA axis activity. In Chapter 3, I investigated the impact of maternal behavior on offspring physiology through changes in growth rate. In Chapter 4, I conclude by tying together maternal effects and the relationship between behavior and HPA axis physiology to better understand the ontogeny of personality. This research will provide a broader perspective of how mothers influence their offspring in a potentially adaptive manner, which is important in identifying the causes of phenotypic variation. Understanding a mechanism behind plasticity due to ecologically relevant stressors and subsequent maternal effects will provide insight into life history variation. Studies on how maternal effects may increase the potential for organisms to adapt to environmental stressors will provide a more complete understanding of rapid adaptation and plasticity.

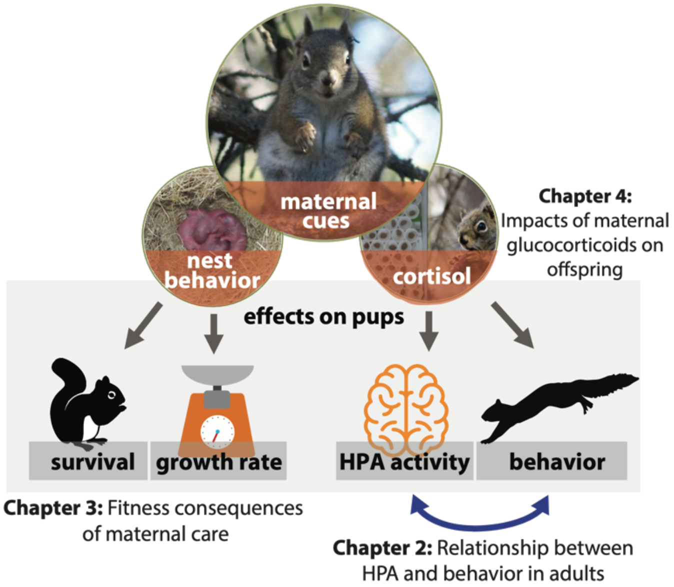

Figure 1.1 Schematic representation of the relationships between chapters

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Chapter 2 Stress Activity is Not Predictive of Coping Style in North American Red Squirrels

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Abstract

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, but not always, 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 many 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.

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 (but see Steimer and Driscoll 2003; Koolhaas et al. 2007). 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 (Gosling 2001; Réale et al. 2007; Koolhaas et al. 2010; Stamps and Groothuis 2010). Furthermore, the suite of behaviors that make up an individual's coping style is theorized to be mediated by hormones that exert pleiotropic actions (Koolhaas et al. 1999; McGlothlin and Ketterson 2008).

This unidimensional model has been repeatedly supported by studies describing how the hypothalamic-pituitary-adrenal (HPA) axis mediates coping styles (Koolhaas et al. 1999). Many of these studies have used selected lines or have been done 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 and activity compared to ‘reactive’ individuals (Koolhaas et al. 1999; Cockrem 2007; Carere et al. 2010). 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 (Baugh et al. 2012), and lower baseline corticosterone metabolites (Stöwe et al. 2010).

As more empirical studies are testing these models, the results from studies in the wild have been equivocal. Whereas there is some support for the unidimensional model in wild animals (see Table 2.1), recent studies that have used this coping style paradigm to test the relationship between behavior and HPA axis reactivity or activity in free-living animals have found that the proactive-reactive continuum is not predictive of the physiological stress response (Garamszegi et al. 2012; Ferrari et al. 2013; Dosmann et al. 2015). For example, though laboratory selection line results are consistent with predictions of the unidimensional model, when testing Great Tits in the laboratory with natural, non-selected variation in exploratory behavior, the relationship no longer holds (Baugh et al. 2012).

Discrepancies in the lab between recent observed relationships (Koolhaas et al. 2007) and the simple unidimensional model (Koolhaas et al. 1999), have 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 (Koolhaas et al. 2010). This model of coping styles reframed the original model to establish behavioral coping strategies on a continuum independent of physiological coping strategies (Koolhaas et al. 2010). 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 (Sih et al. 2004; Dantzer and Swanson 2017). 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 (McGlothlin and Ketterson 2008; Ketterson et al. 2009). 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 (Réale et al. 2007; Ferrari et al. 2013).

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*, hereafter, ‘red squirrels’). 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 (Boon et al. 2007; Taylor et al. 2012). 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 (Boon et al. 2007, 2008; Taylor et al. 2014), and variation in heritable coping styles among individuals (Taylor et al. 2012) in this population may be maintained through fluctuating selection caused by changing environmental conditions (Taylor et al. 2014).

We used fecal samples as a non-invasive proxy for HPA axis activity and reactivity, which is unaffected by trapping-induced stress (Dantzer et al. 2010). In red squirrels, FCM is representative of the circulating plasma cortisol over the past ~12 hours, with a 10.9 ± 2.3 hours lag time to peak excretion following experimental administration of cortisol (Dantzer et al. 2010). Influences of the circadian rhythm on circulating cortisol are not detected in fecal samples collected throughout the day (Dantzer et al. 2010). Additionally, glucocorticoid concentrations in fecal samples have been shown to be representative of HPA activity and reactivity (Sheriff et al. 2011; Palme 2019).

It is important to note that glucocorticoids are metabolic hormones and only one mediator of the reactive physiological stress response of an individual (Romero et al. 2009). However, evidence for both the unidimensional and two-tier models of coping styles specifically connect glucocorticoids with the behavioral response (Table 2.1), in addition to catecholamines (reviewed in Koolhaas et al. 1999, 2010). While this is not a perfect measure of the overall physiological stress response of an individual, glucocorticoids are an important physiological mediator of the multifaceted stress response (Sapolsky et al. 2000; Romero et al. 2009). Glucocorticoids are secreted to mobilize energy in response to a stressor in the environment, but also exert pleiotropic effects (Sapolsky et al. 2000). For example, fluctuating baseline glucocorticoids act as a mediator of future reproductive investment in European Starlings (*Sturnus vulgaris*) by preparing individuals for energetically expensive reproductive seasons (Love et al. 2014).

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 (Boutin and Larsen 1993). Females and males are both territorial of their food-cache (located on the center of their territory) year-round (Dantzer et al. 2012; Siracusa et al. 2017). Red squirrels in the region of our study rely on seeds produced by white spruce (*Picea glauca*) trees as their primary food source (Fletcher et al. 2010). Squirrel population density is closely associated with mast seeding of the white spruce, or episodes of booms and busts in food availability (McAdam and Boutin 2003; Fletcher et al. 2010; Dantzer et al. 2013). Red squirrels have one litter per year, with the exception of mast years when autumn spruce seed is superabundant (Boutin et al. 2006; McAdam et al. 2007).

Our 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. Male 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 (Dantzer et al. 2013; 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 (Dantzer et al. 2012). We included these squirrels to increase our sample size and included study grid type as a covariate in all models to control for variation between the grids. Additionally, high conspecific competition is a significant environmental factor influencing the physiological stress response of red squirrels (Dantzer et al. 2013) and so was important to include as a covariate in our statistical models (Table 2.2). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Animal ethics approvals were received from Michigan State University (AUF#04/08-046-00) and University of Guelph (AUP#09R006).

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 (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, 2008, 2009, 2010, and 2012 (see Table 2.2 for a breakdown of sample sizes). During these years, additional trials were conducted on squirrels in this population for multiple studies (see Boon et al. 2007, 2008; Taylor et al. 2012, 2014; Kelley et al. 2015), but for the purposes of this analyses, we only included trials for which we also had FCM concentration data for that individual. 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 (Archer 1973; Boon et al. 2008; Martin and Réale 2008), but the behavior of individual squirrels over time is known to be repeatable (Boon et al. 2007, 2008; Taylor et al. 2014). 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 (Taylor 2012). 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 (Boon et al. 2007, 2008; Taylor et al. 2012, 2014; Kelley et al. 2015; Supplemental Table 2.4). Because these videos were collected and scored across multiple years, observers used different software programs depending on what program was available the year they were scored. 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 (Blumstein and Daniel 2007). Trials conducted in 2012 were scored using Cowlog software (Hänninen and Pastell 2009). Regardless of the software used, the ethogram and the overall method of scoring the videos remained consistent. Because this is a manual process and the software simply records keystrokes indicating behaviors observed, it is not likely that the software used impacted the score. Furthermore, a previous study using some of our dataset showed high inter-observer reliability for the behavioral measures we recorded from these videos (Taylor et al. 2012), so it again seems unlikely that the software used would influence the behavioral data we extracted from the videos. The videos were scored prior to analyzing the fecal samples thus observers were blind to the animal's FCM concentrations. 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 et al. (2008). Following Taylor et al. (2012), behaviors with an inter-observer reliability of greater than 0.7 were used in analyses (see Supplemental Table 2.4 and Supplemental Table 2.5 for a list of behaviors used in the analyses).

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 Boon et al. 2007; Taylor et al. 2012), 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 Carere and van Oers 2004; Martin and Réale 2008; Montiglio et al. 2012). 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 (Boon et al. 2007; Taylor et al. 2012) and heritable (Taylor et al. 2012) in this population. 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 Table 2 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 with peanut butter for measurement of FCM concentrations corresponding to individuals with behavioral data (see Dantzer et al. 2010). 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. 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 (Dantzer et al. 2010; Palme et al. 2013). 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 Touma et al. 2003). 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 Table 2.2 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 (R Core Team 2016). 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 (Boon et al. 2007, 2008; Taylor et al. 2012, 2014; Kelley et al. 2015). To conduct the principal components analyses with correlation matrices, we used the R package 'ade4' version 1.7-10 (Dray and Dufour 2007). 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 (Supplemental Table 2.5) 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 (Boon et al. 2007, 2008; Taylor et al. 2012, 2014). 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 (Boon et al. 2007, 2008; Taylor et al. 2012, 2014). In our data set, the first component explained 60% of the variation in behavior across MIS trials.

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 and Wilson (2017). In this study, we were interested in how FCM concentrations and behavioral traits co-varied among individuals. To investigate this in a multivariate framework, we used a Bayesian generalized linear mixed effects multivariate model based on a Markov chain Monte Carlo algorithm with the R package 'MCMCglmm' version 2.25 (Hadfield 2010) 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, breeding status (breeding/non-breeding/lactating), and a variable to indicate where the fecal sample was processed (UT Scarborough/UM). Docility measurements were taken across multiple trapping events for a squirrel, therefore we included trial number to control for any variation caused by habituation to the process of being trapped and restrained in the bag (Boon et al. 2007; Taylor et al. 2012). 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 (Dantzer et al. 2010, 2013), and thus were included to control for variation among these variables. Although the correlation between UT Scarborough and UM samples was high (0.88), we included location of fecal sample processing to account for any minor variation between the locations.

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, so there is no variance in novelty of the arena across individuals. 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 Houslay and Wilson 2017) 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 (Cowles and Carlin 1996), we ran all MCMCglmm models three times for comparison using the Gelman-Rubin statistic (Gelman and Rubin 1992), and we also ran the Geweke diagnostic (Geweke 1992). All models passed both diagnostics for convergence.

Data availability

The datasets analyzed during the current study available from the corresponding author on reasonable request.

Results

Our results indicate 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 also indicated that among individuals, FCM concentrations did not correlate with docility ($r = 0.14$, CI = [-0.64, 0.83], Figure 2.1), activity ($r = 0.15$, CI = [-0.17, 0.47], Figure 2.2) or aggression ($r = 0.29$, CI = [-0.098, 0.56], Figure 2.2) (Table 2.3). Regardless of the statistical significance of these relationships, the direction of the observed effect was opposite to the predicted relationship between behavior and FCM concentrations. The direction of these correlations suggest more active and more aggressive squirrels may have higher FCM concentrations, but this is not conclusive due to confidence intervals overlapping zero.

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, which can range from a low to a high HPA axis activity. Contrary to many studies (Raulo and Dantzer 2018), proactive, or highly active/aggressive red squirrels did not always exhibit lower HPA axis activity than reactive individuals. In fact, the parameter estimates were in the opposite direction from those predicted by the unidimensional model. Specifically, the unidimensional model predicts that a more active behavioral stress response and HPA axis activity should be negatively correlated and we found that they were instead positively correlated, though again these confidence intervals overlapped zero. Although we did find that the three behavioral measures were phenotypically correlated (see Supplementary Material), physiological stress, as measured by FCM concentration, does not appear to be the pleiotropic mechanism causing this covariation.

Previous studies that have found behavior and HPA axis reactivity are linked have used a different statistical framework than our study. Thus, it is possible our results are simply the outcome of using a more conservative statistical test. However, our results were robust across statistical techniques as we also ran the same models using a different statistical technique (linear mixed effects models) that has been used in previous studies (e.g. Lendvai et al. 2011; Montiglio et al. 2012). These results from the linear mixed effects models (Supplemental Table 2.7) and those from the bivariate models (presented above) both support the hypothesis that behavior and physiology are independent in our study.

Our study contributes to an emerging trend of a lack of a strong relationship between behavioral and physiological stress responses in wild and laboratory animals (reviewed by Raulo and Dantzer 2018). 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 (*Ochotona curzoniae*: Qu et al. 2018). Additional studies measuring fecal glucocorticoid metabolites demonstrate that HPA axis activity does not correlate with shy-bold behavioral types in wild flycatchers (*Ficedula albicollis*; Garamszegi et al. 2012), or with exploration/activity in Belding's ground squirrels (*Spermophilus beldingi*; Dosmann et al. 2015). In captivity, Holstein Friesian heifer calves (*Bos taurus*) HPA axis reactivity to ACTH is not correlated with their response to novelty (van Reenen et al. 2005).

The unidimensional model posits that both HPA axis activity and reactivity should be lower in proactive animals (Koolhaas et al. 1999). However, it should be noted that measurements of fecal glucocorticoid metabolites in red squirrels may not allow for direct measurement of the reactivity of the HPA axis, which may correlate more strongly with behavioral stress responses compared to basal regulation (Baugh et al. 2012). 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 (Montiglio et al. 2012). 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 (Houslay and Wilson 2017), 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 contributes to the equivocal nature of support for these models in wild animals. Though we focused only on wild animals in our brief review (Table 2.1), empirical studies using laboratory animals also include variation in measurements. Due to the large variability across studies 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 (McAdam et al. 2007). 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. For example, perhaps juveniles with high covariance of the physiological and behavioral stress response were unable to adaptively respond to environmental conditions, whereas juveniles with low covariance were able to adaptively respond to conditions with the two stress responses decoupled. Additionally, these selective pressures fluctuate 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 (McAdam and Boutin 2003; Fletcher et al. 2010). Following the masting of spruce trees, squirrel populations increase in density, which may generate density-dependent selection on juvenile traits (Dantzer et al. 2013; Fisher et al. 2017). These fluctuations in selection may maintain genetic variation in behavioral traits in this population (Taylor et al. 2014), 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 (Ketterson and Nolan 1999; McGlothlin and Ketterson 2008). For example, if a high activity is beneficial in one environmental condition but high HPA axis reactivity is not, a strong correlation between the two traits would constrain an individual's ability to show an adaptive behavioral response to the current environmental conditions. Recent work, however, suggests a hormonal pleiotropic relationship is likely not powerful enough to constrain independent evolution of two traits (Dantzer and Swanson 2017). 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 (Ketterson et al. 2009).

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 (Kelley et al. 2015). 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 both Great Tits and rainbow trout (*Oncorhynchus mykiss*), studies using exploration selection lines have found evidence to support a correlation between behavioral and physiological stress responses (Øverli et al. 2007; Baugh et al. 2012). 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.

Our study helps establish a foundation to use in exploring the fitness consequences of variability across two axes of the stress response, behavioral and physiological. Building upon this current work, we have an opportunity to 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 Meaney 2001). Furthermore, our study provides additional evidence supporting the lack of direct phenotypic correlation between behavioral and physiological stress responses in wild animals exhibiting natural variation in stress responses. Our study, in conjunction with previous studies on these models in wild animals (Table 2.1), suggests a need for a more generalizable model of the relationship between the behavioral and physiological stress responses, perhaps taking into account the environmental conditions experienced by the species. 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.

Studies on the relationship between the behavioral and physiological stress phenotypes in wild animals in variable environments provide insight into the pleiotropic constraints on the evolutionary paths these populations may take. Our study contributes to a growing body of work in support of the two-tier model of coping styles and physiological stress reactivity and activity in wild and laboratory populations. Specifically, our study demonstrated that the FCM concentration of wild red squirrels is independent of an individual's activity, aggression, and docility. Given that red squirrels in this region experience a fluctuating environment in terms of competitors (Dantzer et al. 2013), food (Boutin et al. 2006), and predators (O'Donoghue et al.

1998; Studd et al. 2014) and also fluctuating selection on behavioral traits (Boon et al. 2007; Taylor et al. 2014), having behavioral and physiological responses that are uncorrelated may be beneficial for adapting to this environmental variability. If similar results are found in other species, the lack of a phenotypic relationship between the behavioral and physiological stress responses could have important evolutionary implications, particularly for those species living in fluctuating environments.

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Supplemental Materials

Estimating repeatability of behavioral traits and phenotypic correlations among behavioral traits

We used the R package ‘rptR’ version 0.9.21 (Stoffel et al. 2017) to estimate the within-individual repeatability of docility in a model lacking any fixed or random effects. 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.

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 (Taylor et al. 2012). To confirm that these phenotypic correlations remained in our subset of the behavioral data used in this previous study, 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 Carlo algorithm with the R package 'MCMCglmm' version 2.25 (Hadfield 2010). 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).

Results

Docility scores were significantly repeatable within-individuals (R = 0.33, CI = [0.17, 0.49]). Estimates of the phenotypic correlations between our three behavioral traits were in the same direction as previous studies in this population, with active squirrels being more aggressive and less docile (Supplemental Table 2.6), 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 (n = 23). These findings, combined with results from previous studies in red squirrels with much larger sample sizes (Boon et al. 2007; Taylor et al. 2012), support the idea that these behaviors are phenotypically correlated, and may be shaped by an underlying pleiotropic mechanism.

Assessing the association between behavioral and physiological stress responses using linear mixed-effects models

Previous studies that have assessed the association between behavioral and physiological stress responses did so using linear models (e.g. Baugh et al. 2013; Clary et al. 2014). The recently developed bivariate MCMCglmm model approach that we used here is arguably more conservative than this previously used approach (Houslay and Wilson, 2017). We wanted to compare results from this previously used linear model approach to the recently developed and perhaps more appropriate Bayesian statistical approach to this type of multivariate question (Houslay and Wilson, 2017). As a comparison to our bivariate MCMCglmm model approach presented in the main text of the Results, we also used R package 'lme4' version 1.1-15 (Bates et al. 2015) to create general linear models to test for relationships between each behavioral trait and average FCM concentrations with one measure per individual. 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 (Kuznetsova et al. 2016). 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

Consistent with our bivariate model framework (results shown in main text), 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) (Supplemental Table 2.7).

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Table 2.1 Literature review of studies testing coping styles models

This non-comprehensive table includes studies conducted with natural populations testing the unidimensional (Koolhaas et al. 1999b) and two-tier models (Koolhaas et al. 2010) of the among individual relationship between behavioral coping styles (‘Behavioral Trait’) and HPA axis activity (‘Physiological Measurement’). The first section includes studies that support the main prediction from the unidimensional model that more proactive individuals have lower HPA axis activity. The second 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 with proactive individuals having 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 in either direction. 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. Non-true baseline samples were the first sample collected but involved some handling or trapping stress. (n = sample size of individuals, NS = non-significant ($p > 0.05$), FCM = fecal cortisol metabolites, DEX = dexamethasone (synthetic corticosteroid that exerts negative feedback on the HPA axis), ACTH = adrenocorticotropic hormone that increases adrenocortical activity)

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
Evidence supporting the predictions of the unidimensional model that behavioral and physiological traits are negatively correlated				
Great Tits (*Parus major*) (Baugh et al. 2017)	Exploration	Blood corticosterone *(ACTH challenge induced)*	85	$R^2 = 0.051$, $p = 0.04$
Great Tits (*Parus major*)	Exploration	Blood corticosterone *(90-min handling-restraint stress-induced)*	16	b = -0.417, $p = 0.015$
(Baugh et al. 2013)	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)	Vigilance	FCM *(nominal baseline)*	12	b = 2.109 [0.17, 4.05]
Brook charr (*Salvelinus fontinalis*) (Farwell et al. 2014)	Activity	Whole-body cortisol *(baseline and handling/novel object stress-induced samples)*	66	NA*

Species	Behavioral Trait	Physiological Measurement	n	Correlation or Estimate [CI]
House Sparrows (*Passer domesticus*)	Hovering	Blood corticosterone *(30 min handling-restraint stress-induced)*	18	Pearson's r = -0.58, *p* = 0.017
(Lendvai et al. 2011)				
Eastern chipmunks (*Tamias striatus*)	Exploration	FCM *(coefficient of variation)*	58	b = -13.68 [-27.62, -4.45]
(Montiglio et al. 2012)				
Plateau pika (*Ochotona curzoniae*)	Shyness	Blood cortisol *(true baseline)*	292	posterior R = 0.45 [0.09, 0.66]
(Qu et al. 2018)				
Evidence in the opposite direction of predictions of the unidimensional model				
Poeciliid fish (*Brachyrhaphis episcopi*)	Exploration, Activity	Water-borne cortisol *(open-field stress-induced)*	96	Pearson's r = -0.29, *p* = 0.005
(Archard et al. 2012)				
Great Tits (*Parus major*)	Exploration	Blood corticosterone *(true baseline)*	82	b = 0.536, *p* = 0.003
(Baugh et al. 2013)				
Alpine marmots (*Marmota marmota*)	Exploration, Activity	Blood cortisol *(non-true baseline)*	28	b = 0.54, Fischer *C*-statistic model selection
(Costantini et al. 2012)				
Graylag Geese (*Anser anser*)	Aggression	FCM *(handling stress-induced)*	10	Spearman's r = 0.782, *p* = 0.008
(Kralj-Fišer et al. 2009)				
Evidence supporting the predictions of the two-tier model that behavioral and physiological traits are not significantly correlated				
Great Tits (*Parus major*)	Exploration	Blood corticosterone *(handling-restrain stress-induced)*	85	$R^2 = 0.03$, *p* = 0.14
(Baugh et al. 2017)	Exploration	Blood corticosterone *(DEX challenge response)*	85	$R^2 = 0.02$, *p* = 0.20
Belding's ground squirrels (*Urocitellus belingi*)	Exploration	FCM *(non-true baseline)*	12	b = -0.76 [-1.12, 0.98]
(Clary et al. 2014)				
Alpine marmots (*Marmota marmota*)	Exploration, Activity	Blood cortisol *(pre-restraint to post-restraint response)*	28	NA**

Species	**Behavioral Trait**	**Physiological Measurement**	**n**	**Correlation or Estimate [CI]**
(Costantini et al. 2012)				
Belding's ground squirrels (*Urocitellus belingi*)	Activity	FCM *(non-true baseline)*	157	posterior R = -0.007, [-0.123, 0.095]
(Dosmann et al. 2015)	Exploration	FCM *(non-true baseline)*	157	posterior R = -0.046, [-0.175, 0.036]
	Docility	FCM *(non-true baseline)*	157	posterior R = 0.016, [-0.077, 0.139]
Alpine marmots (*Marmota marmota*)	Activity	Blood cortisol *(non-true baseline)*	146	posterior R = 0.04, [-0.56, 0.71]
(Ferrari et al. 2013)	Impulsivity	Blood cortisol *(non-true baseline)*	146	posterior R = 0.08, [-0.68, 0.62]
	Docility	Blood cortisol *(non-true baseline)*	146	posterior R = 0.14, [-0.64, 0.63]
Collard Flycatchers (*Ficedula albicollis*)	Novel object avoidance	FCM *(non-true baseline)*	51	Pearson's r = -0.017, [-0.291, 0.260]
(Garamszegi et al. 2012)	Aggression	FCM *(non-true baseline)*	56	Pearson's r = -0.076, [-0.332, 0.191]
	Risk-taking	FCM *(non-true baseline)*	54	Pearson's r = 0.074, [-0.198, 0.335]
Nazca Boobies (*Sula granti*)	Gardening (non-social)	Blood corticosterone *(true baseline)*	222	b = 0.26, [-0.09, 0.61]
(Grace and Anderson 2014)	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

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]
	Gardening (social)	Blood corticosterone *(area under the curve across 4 time points during capture-restraint)*	222	b = 0.02 [-0.15, 0.19]
	Shaking (social)	Blood corticosterone *(area under the curve across 4 time points during capture-restraint)*	222	NA, CI in figure includes 0
	Aggression (social)	Blood corticosterone *(area under the curve across 4 time points during capture-restraint)*	222	NA, CI in figure includes 0
Graylag Geese (*Anser anser*)	Aggression	FCM *(non-true baseline)*	10	Spearman's r = -0.503, NS
(Kralj-Fišer et al. 2009)	Sociability	FCM *(non-true baseline)*	10	Spearman's r = -0.212, NS
	Sociability	FCM *(handling stress-induced)*	10	Spearman's r = -0.127, NS
House Sparrows (*Passer domesticus*)	Exploration	Blood corticosterone *(30 min handling-restraint stress-induced)*	18	Pearson's r = -0.07, *p* = 0.797
(Lendvai et al. 2011)	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*)	Docility, in cage	Blood cortisol *(true baseline)*	292	posterior R = 0.25 [-0.02, 0.63]
(Qu et al. 2018)	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]

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

* Used AICc model selection approach. All top models included activity as predictor of cortisol. Based on figure in text, the top model indicates more active individuals have less whole-body cortisol.

** Used nested path model selection approach. Based on figure in text, the top model does not include a relationship between change in cortisol and coping style

Table 2.2 Bivariate model structures and sample sizes

This table breaks down the sample sizes and the variables (fixed and random effects) considered in each bivariate mixed-effects model. Response variables shown are for the bivariate models that tested the association between behavioral traits (docility, activity, or aggression) and a measure of the physiological stress response (fecal cortisol metabolites or FCM). Italics indicate fixed effects estimated for FCM concentration only

Model	Response Variables	Fixed Effects	Random Effect	Year	# of individuals	# of fecal samples
1	docility, FCM	study grid (control or high-density) sex docility trial number breeding, non-breeding, or lactating *fecal processing lab* *Julian date of trapping event*	squirrel ID	2008:	32	36
				2009:	56	132
				total:	**79***	**168**
2	activity, FCM	study grid (control or high-density) sex breeding, non-breeding, or lactating *fecal processing lab* *Julian date of trapping event*	squirrel ID	2005:	2	9
				2008:	27	139
				2009:	15	117
				2010:	1	10
				2012:	28	260
				total:	**72***	**484**
3	aggression, FCM	study grid (control or high-density) sex breeding, non-breeding, or lactating *fecal processing lab* *Julian date of trapping event*	squirrel ID	2005:	2	9
				2008:	27	139
				2009:	15	117
				2010:	1	10
				2012:	28	260
				total:	**72***	**484**

*Some squirrels were sampled in multiple years

Table 2.3 Multivariate results for relationships between FCM and behaviors

Results from our three bivariate generalized linear mixed-effects model 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)

	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]

Supplemental Table 2.4 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.

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

(E) indicates an event behavior and (S) indicates a state behavior.

Supplemental Table 2.5 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.

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*

Supplemental Table 2.6 Multivariate results for relationships between all behaviors

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).

	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]

Supplemental Table 2.7 Full results of 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).

Response Variable	Fixed Effect	b	SE	t	*p*-value
Average FCM (ln transformed)					
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 FCM (ln transformed)					
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 FCM (ln transformed)					
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

Figure 2.1 Relationship between FCM concentration and docility

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

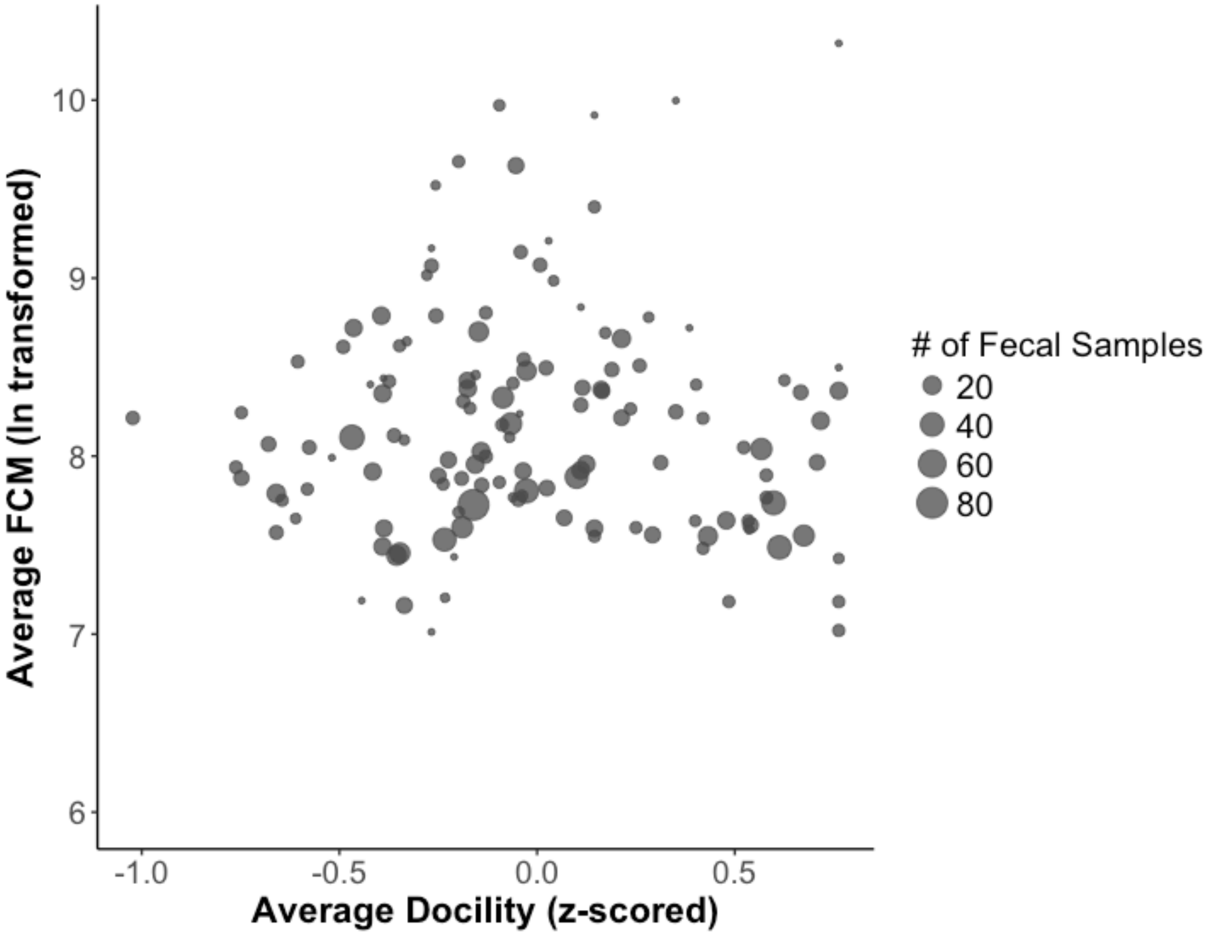
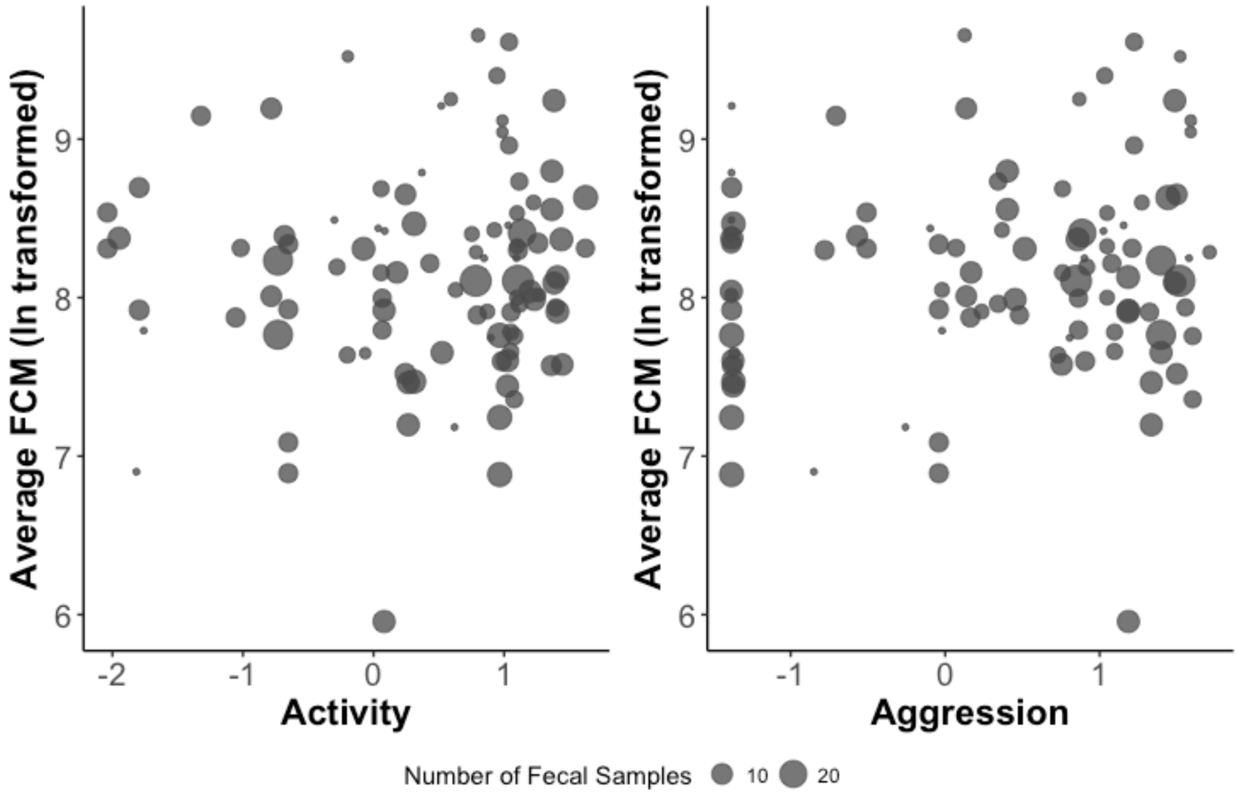


Figure 2.2 Relationship between FCM concentration and activity/aggression

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



Chapter 3 Attentive Red Squirrel Mothers Have Faster-Growing Pups and Higher Lifetime Reproductive Success

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Abstract

Parental investment theory predicts that observed levels of parental care afforded to offspring are set by the benefits (to offspring quality and survival) relative to the costs (to parental survival or future reproduction). Although difficult to document in mammals, there is often substantial individual-variation in the amount of parental care within species. We measured the impact of individual variation in maternal care ("attentiveness" towards offspring or maternal motivation) on offspring growth and survival in a wild population of North American red squirrels (*Tamiasciurus hudsonicus*). We used latency to return to pups following a nest intrusion as a measure of maternal attentiveness to pups. We found this behavior to be repeatable within individuals suggesting this behavior is a personality trait or a "maternal style". In this population, postnatal growth rate is important for pup overwinter survival. Pups from large litters grew faster if they had a highly attentive mother, indicating that maternal care can mitigate the trade-off between litter size and offspring growth and potentially improve survival of pups. Additionally, more attentive mothers had slightly higher lifetime reproductive success than less attentive mothers. These results highlight important fitness effects of having a highly attentive mother and

show that maternal care can alter a fundamental life history trade-off between offspring quantity and quality.

Introduction

Theory predicts that elevated parental investment will produce more, and/or higher quality, offspring, but this may come at a cost of decreased future fecundity and/or survival of parents (Trivers 1974; Drent and Daan 1980; Stearns 1989; Clutton-Brock 1991). In this context, parental care has been defined as parental behavior that increases fitness of offspring and is one aspect of parental investment (Clutton-Brock 1991; Royle et al. 2012). The energetic costs of reproduction, which may limit future parental investment (Drent and Daan 1980), may be especially high in mammals as offspring are highly dependent on their mother for survival until weaning (Roff 1998; Reinhold 2002; Maestripieri and Mateo 2009). Under the assumption of fixed energy budgets, these energetic costs to parents of parental care likely contribute to the fundamental life history trade-off between offspring number and size (Smith and Fretwell 1974; Charnov and Ernest 2006). This trade-off is reflected in the frequent observation that parents can rarely raise many large offspring (Rogowitz 1996), except when they have a large amount of resources to do so (van Noordwijk and de Jong 1986; Reznick et al. 2000). The trade-off for parents between offspring quality and quantity could impact the lifetime trajectory of offspring via differences in developmental rates of individuals (Clutton-Brock 1991; Royle et al. 2012; Klug and Bonsall 2014). For example, offspring growing up in large litters may exhibit slower development and be smaller at the age of independence than those from smaller litters which could in turn influence lifetime reproductive success (Lindström 1999).

In mammals, we might expect to see directional positive selection for high maternal care as long as the benefits afforded to offspring consistently exceed the costs to the mother, yet there is often much individual variation in this behavior (Bales et al. 2002; Champagne et al. 2007; Fairbanks and Ackles 2012). For example, in primates, individual variation in maternal care, or “maternal style”, can include protective, rejecting, restrictive, or laissez-faire mothers (Fairbanks and Ackles 2012). Maternal styles can have important consequences for a female’s fitness and may be a mechanism for transmitting individual differences across generations (Fairbanks and Ackles 2012). Many studies in mammals have investigated the proximate mechanisms behind individual variation in maternal care (Numan and Insel 2006), yet the ultimate mechanisms (i.e. fitness consequences) are rarely addressed, especially in wild rodents, due to the logistical challenges of accurately documenting parental behaviors.

In this study, we used latency to return to pups, a measurement commonly used with laboratory rodents (Numan and Insel 2006; Champagne et al. 2007), as a measurement of maternal attentiveness following a nest intrusion in wild North American red squirrels (*Tamiasciurus hudsonicus*). Most other common laboratory methods of measuring maternal care in rodents, measuring arched-back nursing, grooming, and grouping of pups (Mann 1993), are not possible in a wild rodent due to these behaviors being done in the nest. Any intrusion upon the nest results in mothers moving their pups to a new nest (Westrick, personal observation), therefore we are unable to record or observe any behaviors inside the nest. In laboratory studies of rodents, latency to retrieve pups after they are moved to a different location in the cage is interpreted as how motivated an individual is to attend to pups (Mann 1993; Olazábal et al. 2013). For example, virgin female laboratory rats can develop maternal motivation to retrieve pups by prolonged exposure to pups (Seip and Morrell 2008). Latency to return to a nest box after manipulation of the brood has also been used as a measure of willingness to provide parental effort in Great Tits (*Parus major*) (David et al. 2015). Many neuroendocrine studies measure retrieval behavior when manipulating brain regions to identify neural structures involved in maternal behavior (Numan and Woodside 2010). In wild animals, returning to the nest could represent how motivated a mother is to defend pups or how motivated she is to exhibit infant-directed behaviors, both important components of motivated maternal care (Olazábal et al. 2013). In sum, the latency of returning to the pups represents a measure of attentiveness towards the offspring. By measuring latency to return, our goal was to capture individual variation in how motivated a mother was to retrieve her pups to move them to a safer nest following a nest intrusion.

We conducted this study as part of a long-term project on red squirrels in Yukon, Canada. Red squirrel pups are altricial and dependent upon their mother until weaning whereupon they typically disperse to nearby territories (mean = 92 m – 102 m from natal territory; Berteaux and Boutin 2000; Cooper et al. 2017). Weaned pups experience strong selective pressures with a majority of juvenile mortality (68%, on average though this is highly variable among years) occurring over the summer before mid-August and few surviving through their first winter (61% of those alive in August, on average), depending on food availability and adult density (McAdam et al. 2007; Hendrix et al. 2019). Pups that grow faster early in life are typically more likely to survive their first winter (McAdam and Boutin 2003a; Fisher et al. 2017; Hendrix et al. 2019), particularly in years with high conspecific density (Dantzer et al. 2013). Despite the link between increased survival and fast growth in red squirrels, there is substantial variation in pup growth rates among and within litters. Some of this variation in pup growth rate is explained by large maternal effects including variation in litter size and the birth dates of litters (McAdam et al. 2002) or levels of maternal glucocorticoids during pregnancy (Dantzer et al. 2013), although much variation among females in the growth rates of their pups remains unexplained (McAdam et al. 2002). Here, we tested the hypothesis that maternal behavior predicts offspring growth rate and survival. We also tested the within-individual repeatability of this behavior to identify if red squirrels exhibit a "maternal style" and tested whether more attentive mothers had more pups that recruited into the breeding population over their lifetime. If we find that maternal attentiveness is a consistent individual phenotype (repeatable), this suggests that selection can act upon this behavior. We predicted that mothers who exhibited higher maternal attentiveness (i.e., faster return to the nest) would produce faster growing offspring and the pups would be more likely to survive to autumn. Consistent with life history theory, individual growth rates of red squirrel pups are typically lower in larger litters (Humphries and Boutin 2000). However previous research has shown pups from mothers in a high conspecific density environment or those with supplemental food show a reduction in the trade-off between litter size and pup growth rates (Dantzer et al. 2013) though the route by which the increase density results in a reduced tradeoff is still unclear. Therefore, we also hypothesized that an increase in maternal care could be one way mothers contribute to the reduction in this tradeoff.

Methods

Study population

North American red squirrels are arboreal, solitary, and sexually monomorphic (Boutin and Larsen 1993). Both sexes defend individual year-round territories and have many nests on their territory (Dantzer et al. 2012; Siracusa et al. 2017). Our study was conducted within Champagne and Aishihik First Nations traditional territory (with their explicit agreement) in Yukon, Canada (61°N, 138°W). Squirrels in our study population rely mainly on the seeds of white spruce for food (Fletcher et al. 2010, 2013), which they cache in an underground larder hoard ("midden") within their territory. Mothers provide all parental care in this species and typically produce one successful litter per year (renesting if necessary after a failed litter; Williams et al. 2014), with the exception of producing two litters during a white spruce (*Picea glauca*) mast year (Boutin et al. 2006), a synchronously large production of cones that occurs every 4-6 years (Nienstaedt and Zasada 1990; LaMontagne and Boutin 2007). Due to this boost in reproduction during mast years, the population density of red squirrels increases following mast seed events (Dantzer et al. 2013; McAdam et al. 2019). Because we have previously documented that spruce cone availability impacts growth and survival of offspring (Humphries and Boutin 1996; Boutin et al. 2000; McAdam and Boutin 2003a; Fletcher et al. 2010, 2013), we estimated the number of cones available in the study area by using visual cone counts to determine cone index (LaMontagne et al. 2005). All work was conducted under animal ethics approvals from Michigan State University (AUF#04/08-046-00), University of Guelph (AUP#09R006), and University of Michigan (PRO00005866).

Maternal behavior observations

In 2008, 2009, 2016 and 2017, we live-trapped (Tomahawk Live Trap, Tomahawk, WI, USA) breeding female squirrels (n = 288 unique breeding squirrels across 4 non-mast years) at regular intervals to determine reproductive status (see McAdam et al., 2007 for more details). Squirrels in this study were from either a control study area (n = 209 squirrels) or a study area that was provided with supplemental *ad libitum* peanut butter from 2004 to 2017, resulting in a higher density of squirrels (n = 79 squirrels) (Dantzer et al. 2013). Squirrels in the high-density study area typically have higher levels of glucocorticoids (Dantzer et al. 2013) and spend less time in the nest based on focal observations of male and female squirrels (Dantzer et al. 2012), though we note that we do not currently know whether lactating females in high-density areas spend less time with their pups. We included these squirrels from the high-density study area to increase our sample size by 27%. However, in our present study, study area did not predict growth rate (Table 3.1). Nonetheless, to control for any variation due to this difference in conspecific density and food availability, we included study area as a covariate in all models predicting pup survival or growth rate.

We estimated maternal latency to return to the nest (attentiveness) following two nest entries where we accessed the pups soon after birth (first nest entry) and when the pups were about 25 d of age (second nest entry). We chose these time periods to access the pups as this is when we measure offspring postnatal growth rate, which is a linear period of growth while the pups have never exited the nest on their own and are exclusively fed by their mother. To conduct two nest entries as pups were developing, we used VHF radio collars (Holohil PD-2C, 4-g, Holohil Systems Limited, Carp, ON, Canada) fitted on mothers to locate mothers when they were in the nest with their pups using either an R-1000 radio telemetry receiver (Communications Specialist, Inc., Orange, CA, USA) or a TR-4 radio telemetry receiver (Telonics, Inc., Mesa, AZ, USA). We performed the first nest entry as soon as lactation was detected through trapping (mean pup age ± SD: 2.75 ± 3.33 days old). We estimated parturition date based on mass of pups as well as palpation and lactation history of the mother (McAdam et al. 2007). We entered the nest for the second time when pups were ~25 days old (mean pup age ± SD: 25.8 ± 2.47 days old). Mothers wore the collar for variable lengths of time depending on the trappability of the individual but were typically removed as soon as possible after the nest entry was completed.

At each nest entry, we removed pups from the nest to weigh (to nearest 0.1 g), mark, and sex individuals. During the second nest entry, we additionally assigned unique colored disk combinations and unique alphanumeric stamped ear tags (National Band and Tag Company, Newport, KY, USA) to each pup for identification after emergence. In a sample of nests from 2016, the average time pups spent out of the nest during the first nest entry was 11:07 min:sec [range: 06:33-28:53] (n = 41 litters) and the average time pups spent out of the nest during the second nest entry was 43:05 [range: 14:47-01:33:01] (n = 36 litters). The second nest entry was longer due to the time spent ear-tagging individuals, which is not performed at the first nest entry. In general linear models, we found no relationship between the time pups spent out of the nest for processing and the latency of mothers to return to pups ($\beta = 11.98$, SE = 178.37, $t_{76} = -0.06$, $p = 0.94$) or the number of pups in a litter ($\beta = -113.4$, SE = 214, $t_{76} = -0.53$, $p = 0.60$). This suggests that the time pups spent outside of the nest for processing or the number of pups in a litter did not influence the latency for a mother to return to her nest. Data on time pups spent out of the nest were not collected in other years, but the above data should be representative of all years as data collection protocols were uniform across all years.

After processing the litter, we returned all pups to the original nest. While the pups are being processed, there is high variation in maternal behavior. Some mothers explore the empty nest and stay nearby or approach the researchers. Some vocalize during the entire process and never approach. Mothers may even enter the nest before the researcher has left the nest tree. Alternatively, some mothers will move further away from the researchers or immediately leave and only return once the researchers have left (Westrick, personal observation). Not every litter was observed for both first and second nest entries because some of the litters did not survive from the first to second nest entry (n = 47 litters), some first nest entries were missed, and maternal behavior observation was skipped at some nest entries due to logistics.

After each nest entry, we performed focal behavioral observations on mothers. An observer (n = 31 different observers) moved >5 m away from the nest tree and watched the mother's behavior for 7 minutes following returning the pups to the nest to record the time pups were replaced in the nest, the time mother returned to the nest, and the time mother began moving the pups. Because we were tracking many litters and often performed multiple nest entries on the same day, we were constrained logistically in the length of time we could watch the nest after placing the pups back in the nest. We chose 7 minutes to be consistent with previous behavioral focal observation protocols completed with this study population (Dantzer et al., 2012; Siracusa et al. 2019). Observers were blind to the previous return latencies of the focal squirrel and were blind to the specific growth rates of pups. Because the observers processed the litter prior to behavioral observations, it was impossible to keep them blind to the litter size. Five observations were at underground nests, with the remaining in trees. We determined latency to return to pups as the time between pup replacement in their original nest and the mother's return to the nest and censored any observations where the mother did not return within 7 minutes ($n = 218$ censored observations). Mothers typically moved their pups to a different nest immediately following their return to the nest after our intrusion as indicated by a strong relationship between the uncensored latency to return and latency to begin moving pups (linear model: adjusted $R^2 = 0.81$, $ß = 0.87$, $SE = 0.032$, $t = 27.43$, $p < 0.0001$). Among trials where the mother returned, 83% of mothers moved their pups within 7 minutes.

While many studies in the lab measure the time to retrieve all pups, the spatial scale at which wild female red squirrels move their offspring makes this problematic. Females move their pups individually to a new nest that is meters to tens of meters away. As a result, variation in the length of time between initiation and completion of moving pups is likely to be caused mostly by the distance between nests and the number of pups to be moved rather than by maternal motivation. Recording the latency to return to her pups following a standardized nest disturbance allows us to quickly capture the responsivity of a mother to her pups needs in a wild animal.

Offspring measurements: growth rate and survival

Growth between the two nest entries is approximately linear (McAdam and Boutin 2003b) so we calculated growth rate (g/day) of pups (n = 763 pups) as the change in mass from first to second nest entry divided by number of days between nest entries. We monitored survival of juveniles (n = 541 juveniles from 206 litters) for the remainder of the year and following spring. We recorded survival to autumn of the birth year as a binary measure (alive or dead on August 15th). As part of our long-term data collection, we censused the entire study population yearly to confirm territory ownership by August 15th and again by May 15th (McAdam et al. 2007). Survival to August 15th captures a key life-history event. In this population, caching of spruce cones typically begins mid-August and ends in September (Fletcher et al. 2010). Territory ownership before this period allows individuals to take advantage of that year's cone crop by providing them with a physical space to cache cones (cones must be cached in a midden for the seed to remain a viable food source; Streubel 1968). Because offspring disperse from their natal territory around 70-80 days old to compete for their own territory (Nice et al. 1956; McAdam et al. 2007), we limited survival data to litters born ≥ 70 days prior to August 15th. Because squirrels are diurnal and their activity (territorial defense behavior and presence) is conspicuous, we were able to completely enumerate all squirrels inhabiting the study areas through a combination of repeated live trapping and behavioral observations.

From our biannual population censuses and behavioral observations, we tracked lifetime reproductive success (LRS) of mothers. We defined LRS as the number of pups borne over the entire lifetime of a dam that recruited into the breeding population (i.e., alive for more than 199 days or roughly to the spring following their year of birth). To accurately calculate LRS, we only included mothers with known birth years before 2011 (n = 40 females) to ensure we captured the number of pups produced over their entire lifespan. We excluded mothers that died of unnatural causes. It is possible that there may be biases in offspring survival estimates, but our past work suggests that this is unlikely. First, our study areas are surrounded by unsuitable habitat for red squirrels and natal dispersal is usually about 100 m away from the site of birth (Berteaux and Boutin 2000; Cooper et al. 2017). Second, if our estimates of juvenile survival (and therefore maternal LRS) were biased due to offspring dispersal, we would expect that females on the edge of the study area have lower LRS than those on the center of the study area. However, we have not found this (Kerr et al. 2007; McAdam et al. 2007; Martinig et al. 2020) suggesting that our measures of maternal LRS are not affected by juvenile dispersal.

Statistical analyses

We conducted all statistical analyses in R version 3.4.3 (R Core Team, 2016). With censored data, the mean event time is an underestimation of the true mean (Datta 2005; Zhong and Hess 2009). With 30% or more censoring, the Kaplan-Meier estimator of the median is less biased than the mean (Datta 2005; Zhong and Hess 2009). Therefore, instead of comparing means to test for a difference between the first and second nest entries, we compared Kaplan-Meier survival curves using the R package 'survival' version 2.43-3. To estimate within-individual repeatability of maternal attentiveness, we used the R package 'rptR' version 0.9.21 (Stoffel et al. 2017). In our linear mixed effects model for repeatability, we included squirrel identity (ID; n = 104 mothers observed twice, n = 24 mothers observed three times, n = 20 mothers observed four times, and n = 1 mother observed five times [three litters across two years]) as a random intercept term, no fixed effects, and used parametric bootstrapping (n = 1000) to estimate the confidence interval.

In our models to assess how maternal nest attentiveness affected offspring growth, we included the following predictors: return latency, number of pups in litter, parity of mother (first time mother or not), parturition date of the litter, cone index of the previous autumn, sex of pup, birth year (as a factor), and study area (control or high-density). 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 (Kuznetsova et al. 2016). To detect any collinearity in the predictors included in our model, we used R package ‘car’ version 3.0-2 (Fox and Weisberg 2011) to assess the variance inflation factors. $GVIF^{(1/(2xDF))}$ for all predictors was < 3, except cone index of the previous autumn ($GVIF^{(1/(2xDF))} = 11$) which is colinear with birth year. We decided to still include spruce cone abundance (cone index: LaMontagne et al. 2005) in these models as it is a major influence on offspring survival and growth rate in this study system (McAdam and Boutin 2003a, b; Boutin et al. 2006; Dantzer et al. 2013) and we wanted to control for its influence on these traits. We included birth year as a fixed effect to control for any additional year effects, e.g. variation in predator abundance or weather effects. In this population, female squirrels typically breed their first year and each subsequent year thus maternal age and parity are closely correlated across our long-term database ($R = 0.89$, CI = [0.89-0.90], $t = 170.68$, $df = 7571$, $p < 0.001$). We included parity as a binary variable to account of the initial experience of a nest disturbance by researchers. Cone index of the previous year is also predictive of conspecific density which also influences growth rate (Dantzer et al. 2013). To assess if maternal nest attentiveness behavior could mitigate effects of increasing litter size on offspring growth rates, we included the interaction between return latency and litter size. We standardized pup growth rates, litter size, and birthdate within each study area-year combination and standardized all other continuous variables across all data (latency to return and cone index). In our data set, we had 13 one-pup litters, 46 two-pup litters, 128 three-pup litters, 28 four-pup litters, and 1 five-pup litter. Since multiple pups were measured per litter, we included litter ID as a random effect.

To model the relationship between maternal care and offspring survival, a binary value for offspring survival to autumn was predicted by the following fixed effects: return latency, pup growth rate, sex of pup, parity of mother (first time mother or not), birth date, cone index of birth year, cone index of previous year, and study area (control or high-density). We standardized pup growth rate across study area-year combinations and standardized all other continuous variables (latency to return to pups and cone indices) across all data. Again, we assessed variance inflation factors and found $\mathrm{GVIF}^{(1/(2\mathrm{xDF}))}$ for all predictors was < 2.

Due to the count nature of LRS and the high variance of LRS relative to the mean, we used a negative binomial generalized linear model to estimate the relationship between maternal attentiveness and LRS. For each squirrel, we averaged latency to return to pups across all observations of that individual. In addition to latency to return to the pups, the model included fixed effects for lifespan (in days) since lifespan is highly correlated with LRS (McAdam et al. 2007) and study area type (control or high-density). While mothers who experience a spruce cone mast in their lifetime have higher LRS on average (Haines et al. unpublished), in our dataset experiencing a mast year is highly correlated with lifespan (Pearson's correlation R: 0.87, $t = 11.78$, $df = 43$, $p < 0.00001$), therefore we left this out of the model. We also fit the same model reducing the dataset to only observed return latencies, excluding any censored data, as a comparison. To fit these models, we used the R package 'MASS' version 7.3-51.1 (Venables and

Ripley 2002). We standardized all continuous fixed effects to allow for comparison of effect size. $GVIF^{(1/(2xDF))}$ for all predictors was < 2.

We ran all models for growth rate and survival with observations from the two nest entries separately due to the potential for different levels of maternal investment at different times in the breeding season. Specifically, squirrels born earlier in the year generally are more likely to survive until the following year so mothers that lose their litter earlier in the season (e.g. right after birth) have the potential to successfully breed again (McAdam et al. 2007; Williams et al. 2014), whereas mothers that lose their litter later in the season (e.g. a month after birth of the first litter) may not have the same potential for a successful second litter in a non-mast year. Additionally, newborn pups are hairless and more dependent on their mother for temperature regulation than ~25-day old pups with fur. Consequently, we might expect behavior observations at the two nest entries to vary due to this difference in maternal investment and pup developmental stage, thus the measurements at the two nest entries may not be equivalent. Additionally, due to natural litter failures and missed observations, not every litter was observed at both the first and second nest entries.

Results

Variation and repeatability in maternal nest attentiveness

Mothers varied in the time it took them to return to the nest following our temporary removal of their pups (median: 394 s, CI = [335, 420]). Observations were censored at 7 minutes (49% of observations) and the latency to return ranged from 0 s to the maximum observation of 420 s. During 16% of observations, mothers returned within 2 minutes. Using Kaplan-Meier survival curves, the median latency to return was slightly faster during the second nest entry compared to the first nest entry, but the survival curves do not significantly differ (nest entry 1 median = 420 s; nest entry 2 median = 350 s; difference between curves: $\chi^2 = 0.7$, p = 0.4; Supplemental Figure 3.3).

In our models for within-individual repeatability of latency to return to pups, we found mothers were consistent across observations of maternal nest attentiveness behavior whether we included censored observations (R = 0.25, SE = 0.061, CI = [0.13, 0.36], $p < 0.0001$), or excluded them (R = 0.32, SE = 0.10, CI = [0.11, 0.51], p = 0.0045).

Maternal nest attentiveness and pup growth rate

We found that the apparent cost for an individual pup of being in a litter with many siblings, in terms of a reduced growth rate, was lessened by having a highly attentive mother. As predicted by life history theory, in both our models of growth rate, pups from larger litters grew more slowly than pups in small litters (nest 1: ß = -0.14, SE = 0.025, $t_{192.53} = -5.87$, $p < 0.00001$; nest 2: ß = -0.11, SE = 0.024, $t_{175.09} = -4.60$, $p < 0.00001$; Table 3.1). However, pups in large litters with mothers that returned soon after the pups were returned (i.e., more attentive mothers) grew faster than those in large litters with mothers that took longer to return to the nest, particularly early in pup development (latency x litter size interaction - nest entry 1: ß = -0.051, SE = 0.023, $t_{196.22} = -2.22$, $p = 0.028$; nest entry 2: ß = -0.021, SE = 0.023, $t_{170.77} = -0.91$, $p = 0.37$; Figure 3.1; Table 3.1).

In both models for the first and second nest entry, male pups grew faster than female pups (nest entry 1: ß = 0.040, SE = 0.018, $t_{370.67} = 2.22$, $p = 0.027$; nest entry 2: ß = 0.054, SE = 0.020, $t_{338.66} = 2.71$, $p = 0.0071$; Table 3.1) and pups born later in the year also grew faster than pups born earlier (nest entry 1: ß = -0.10, SE = 0.033 $t_{180.18} = -3.07$, $p = 0.0025$; nest entry 2: ß = -0.087, SE = 0.037, $t_{164.44} = -2.37$, $p = 0.019$; Table 3.1).

Maternal nest attentiveness and survival

Faster growing pups in both models were more likely to survive their first summer to autumn (nest entry 1: ß = 0.24, SE = 0.11, z = 2.12, $p = 0.034$; nest entry 2: ß = 0.29, SE = 0.12, z = 2.44, $p = 0.015$; Table 3.2). There was no further effect of maternal attentiveness once the direct effect of growth was accounted for (nest entry 1: ß = -0.11, SE = 0.11, z = -1.00, $p = 0.32$; nest entry 2: ß = -0.12, SE = 0.11, z = -1.05, $p = 0.29$; Table 3.2). Overall, females were more likely than males to survive until autumn (nest entry 1: ß = 0.71, SE = 0.22, z = -3.30, $p <$ 0.0001; nest entry 2: ß = -0.54, SE = 0.23, z = -2.34, $p = 0.019$; Table 3.2). Pups born earlier in the year (nest entry 1: ß = -0.27, SE = 0.12; z = -2.17, $p = 0.00098$; nest 2: ß = -0.19, SE = 0.13, z = -1.47, $p = 0.14$; Table 3.2) and pups born during years where there was high autumn spruce cone production were more likely to survive (nest entry 1: ß = 0.25, SE = 0.11, z = 2.21, $p =$ 0.027; nest 2: ß = 0.36, SE = 0.14, z = 2.64, $p = 0.0084$; Table 3.2).

Maternal nest attentiveness and lifetime reproductive success

Mothers with a more attentive maternal style had more pups that recruited into the population during their lifetime (ß = -0.31, SE = 0.15, z = -2.09, $p = 0.037$; Figure 3.2; Table 3.3). Given the mean LRS for females who survive to potentially breed is 1.54 pups (McAdam et al. 2007), this effect size is substantial. On average, mothers who returned immediately after pups were replaced in the nest had ~1 more pup that recruited than mothers who returned at the end of the 7 min observation period. Female squirrels who lived longer had higher LRS (ß = 0.69, SE = 0.14, z = 4.91, $p < 0.0001$; Table 3.3). Female squirrels from the high-density food addition study area did not have more pups that recruited than control mothers (ß = -0.22, SE = 0.41, z = -0.54, p =0.59; Table 3.3).

Discussion

Maternal styles

We found that maternal nest attentiveness following a nest intrusion is a repeatable behavior among female red squirrels, which suggests individuals exhibit maternal styles with some mothers being more attentive to the pups and other mothers adopting a more laissez-faire approach. The repeatability of this maternal behavior is near the average repeatability of other behavior studies (R = 0.37; Bell et al. 2009), but lower than the repeatability of other behavioral traits in red squirrels (docility R = 0.41, aggression R = 0.44, activity R = 0.51; Taylor et al. 2012). Individual repeatability in parental behavior has been documented in other species as well. For example, the average amount of time a Goeldi's monkey (*Callimico goeldii*) spent carrying infants from their first litter was positively correlated with the amount of time infant carrying for subsequent litters (Schradin and Anzenberger 2001). Research in wild deer mice (*Peromyscus maniculatus*) also demonstrates low, but significant, repeatability of multiple maternal behaviors with a high degree of seasonal plasticity (Stewart and McAdam 2014). Although we and these other studies document significant repeatability in maternal behaviors measured in different ways, maternal attentiveness is not entirely repeatable, and it is likely that some degree of variation in maternal attentiveness in red squirrels is impacted by environmental factors. For example, maternal attentiveness in red squirrels may be plastic in response to variation in spruce cone availability, but our dataset does not include observations during a white spruce mast year. Future studies should not only attempt to identify whether parental behaviors exhibit repeatability but also document the environmental drivers of plasticity in these behaviors.

Our study interprets the latency to return to the nest following a nest disturbance as a measure of maternal attentiveness. However, maternal attentiveness could simply reflect “boldness” or be one trait in a behavioral syndrome that includes boldness. For example, in Great tits, fast exploring parents return faster to the nest box following a nest disturbance by researchers (David et al. 2015). We may predict that more bold squirrels are more likely to return to the nest quickly despite recent human intrusion. The latency to return to the pups following an intrusion may be a similar measurement to the latency to resume normal behavior after a startling event which is often used to describe ‘boldness’ of an individual (e.g. van Oers et al. 2004; Martins et al. 2007; Wilson and Krause 2012). In the lab, rodent mothers show increased boldness or decreased ‘fear’ after giving birth (Hard and Hansen 1985; Wartella et al. 2003). Thus, it is possible that mothers with higher levels of maternal attentiveness are also bolder and that bold and attentive mothers have higher lifetime reproductive success, but we unfortunately do not have adequate data to address this.

Maternal behavior and pup growth rate

Red squirrel mothers that were highly attentive at the first nest entry had faster growing pups and their litters exhibited a reduction in the trade-off between litter size and pup growth rates. This amelioration of the negative impact that siblings can have on the growth of each offspring in the litter could be one way that maternal behavior alters offspring lifetime fitness trajectories (Klug and Bonsall 2014). This indicates that there could be large fitness benefits for highly attentive mothers as offspring that grow up in large litters may grow nearly as fast as offspring in smaller litters if they have a highly attentive mother. However, there was substantial variation in the attentiveness of mothers and the ecological causes of this variation in maternal behavior is yet to be determined.

Maternal behavior and pup survival

In our study population of red squirrels, faster early life growth rate is associated with an increased probability of pup survival into adulthood, especially when population density is high (McAdam and Boutin 2003a; Dantzer et al. 2013; Hendrix et al. 2019). Because growth rate is predicted by maternal behavior, including these two measurements as predictors in the same model may mask the indirect impact of maternal care on survival. These results suggest growth rate may be the mechanism by which maternal behavior increases survival of pups. Additionally, we found that, over their lifetime, mothers that exhibited a more attentive maternal style had more offspring that recruited into the breeding population. Latency to return to pups and subsequently move them to a new nest following a nest disturbance may affect offspring growth and survival through a variety of pathways.

It is also likely that maternal attentiveness, as we measured it here, does not have any direct effects on growth and survival but is simply representative of a suite of maternal behaviors representing maternal style. For example, lactating female red squirrels are known to move their pups between nests on their territory as the ambient temperature fluctuates to maintain an optimum temperature for offspring growth (Guillemette et al. 2009). Since latency to return to pups is highly correlated with latency to move pups (see Methods), highly attentive mothers may be better able to move offspring from one nest to another that puts offspring in the optimal thermal environment that maximizes growth. Since our current data set is limited to one observed behavior, future work should dissect our findings further to address the specific mechanism by which maternal attentiveness increases growth rate and reproductive success.

Maintaining individual variation in maternal behavior

The fact that highly attentive mothers had higher lifetime reproductive success begs the question of why substantial individual variation in this highly beneficial behavior is maintained in the population. There are likely costs experienced by highly attentive mothers which may contribute to the persistence of alternative maternal styles in the population. For example, if the nest was intruded upon by predators, highly attentive mothers that quickly return to the nest could face the cost of potentially being preyed upon themselves. Additionally, there are likely substantial energetic costs associated with moving pups to a new nest; on average, one ~25-day old pup weighs ~18% of the body mass of an adult female. We have not yet documented the costs of maternal attentiveness but there are three possible explanations for why there is substantial individual-variation in maternal attentiveness despite the clear fitness benefits we documented in this study. First, high maternal nest attentiveness could be exhibited by high quality mothers who can afford higher investment in current reproduction, and variation we see in maternal behavior is due to limitations on the mother and current environmental conditions, rather than fitness consequences (van Noordwijk and de Jong 1986). However, individual 'quality' is not easily defined (Wilson and Nussey 2010; Bergeron et al. 2011) and can even be considered dynamic across contexts, rather than a static trait (Lailvaux and Kasumovic 2011). If we consider high quality squirrels as those with the most food resources, maternal attentiveness may be one mechanism by which higher quality female squirrels covert their larger energy stores into increased fitness. Though this explanation seems unlikely to explain the entirety of our results given we do not see a significant benefit, in terms of pup growth rate or LRS, of increased food availability for squirrels living on the food-addition study area, although this result for LRS is expected given that food-supplementation increases conspecific density which should lower the number of offspring that can recruit into the population.

Secondly, it is possible that the survival costs to females of increased attentiveness are underrepresented in our data due to the 'invisible fraction', or individuals that do not survive to reproduce and express this behavior (Grafen 1988; Hadfield 2008). We were not able to measure maternal attentiveness on all squirrels and many squirrels die prior to even reproducing so we are unable to collect data on them. We are more likely to have sampled older individuals for maternal attentiveness which means the survival costs to females with increased attentiveness are likely to be underrepresented in our data. Essentially, a cost associated with increased attentiveness or a related trait of boldness could have already been paid prior to us being able to measure attentiveness.

Lastly, there may also be years when the fitness benefits associated with maternal attentiveness are reduced. Red squirrels in Yukon experience large fluctuations in their major food resource (white spruce seeds) and population density and therefore substantial inter-annual variation in directional selection on offspring growth rate (Humphries and Boutin 2000; McAdam and Boutin 2003a; Dantzer et al. 2013). In some years, there is strong positive selection favoring fast growth whereas it is reduced or non-significant in other years (Dantzer et al. 2013). Consequently, it may be an unreliable strategy for mothers to invest in faster growing offspring if it can result in a high energetic cost with little fitness benefit (McAdam and Boutin 2003a; Dantzer et al. 2013). This should result in balancing selection on maternal style where less attentive mothers with slow growing pups have an advantage in years when fast growth is not under positive selection, thereby maintaining individual-variation in maternal style. While we are limited in testing these predictions with our current dataset, we have already observed balancing selection for other repeatable behavioral traits in female red squirrels such as the aggression and activity of mothers (Taylor et al. 2014). It is possible that the fitness benefits associated with maternal care depending upon ecological conditions is likely to maintain variation in this important behavior that is closely linked to fitness. The relatively small effect size of maternal behavior predicting lifetime reproductive success suggests environmental shifts may change this relationship, thus maintaining variation in this behavior through fluctuation in fitness benefits.

Conclusions

Here we provide empirical evidence that maternal styles with increased attentiveness correspond to increased offspring growth rate and higher lifetime reproductive success. We propose future studies investigate the causes of individual variation maternal styles in more detail, including the effects of age or breeding experience, heritability, and plasticity of maternal style. Our current study highlights how individual variation in maternal behavior can be linked to variation in developmental trajectories in a wild animal.

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Table 3.1 Full results for linear mixed-effects models on the relationship between growth rate and maternal attentiveness

We ran two distinct models for observations from the (a) first and (b) second nest entries (at ~1 day post-parturition and ~25 days post-parturition, respectively). We standardized litter size by study area-year each combination and standardized all other continuous variables (latency to return to pups and cone indices) across all data. Bold font indicates statistical significance of $p < 0.05$.

a) Latency observations at first nest entry (pups ~1 day old):

Response Variable	Fixed Effect	ß	SE	df	t	*p*-value
Growth rate (g/day)						
n = 524 individuals	Intercept	1.15	0.94	180.78	1.23	0.22
n = 193 litters	Latency to return to pups	-0.036	0.024	259.71	-1.49	0.14
n = 167 mothers	**Litter size**	**-0.14**	**0.025**	**192.53**	**-5.87**	***p* < 0.0001**
	Parity of mother					
	primiparous	-0.14	0.10	181.42	-0.86	0.39
	Year					
	2009	0.075	0.61	179.87	0.12	0.90
	2016	0.46	0.94	180.72	0.49	0.63
	2017	0.22	0.77	180.78	0.29	0.77
	Birth date	**-0.10**	**0.033**	**180.18**	**-3.07**	**0.0025**
	Cone index of previous year	0.34	0.72	180.62	0.72	0.47
	Study area					
	Food addition + high-density	0.081	0.071	180.97	1.14	0.26
	Sex of pup					
	male	**0.040**	**0.018**	**370.67**	**2.22**	**0.027**
	Latency to return x litter size	**-0.051**	**0.023**	**196.22**	**-2.22**	**0.028**
	Random effect:	**Variance**	**SD**			
	litter ID	0.10	0.32			
	residual	0.027	0.16			

b) Latency observations at second nest entry (pups ~25 days old):

Response Variable	Fixed Effect	ß	SE	df	t	*p*-value
Growth rate (g/day)						
n = 480 pups	Intercept	0.82	1.00	164.21	0.83	0.41
n = 178 litters	**Latency to return to pups**	**-0.06**	**0.028**	**160.96**	**-2.19**	**0.030**
n = 152 mothers	**Litter size**	**-0.11**	**0.024**	**175.09**	**-4.60**	***p* < 0.0001**

Parity of mother					
primiparous	-0.061	0.11	164.15	-0.57	0.57
Year					
2009	0.32	0.65	163.47	0.49	0.62
2016	0.81	1.00	164.14	0.81	0.42
2017	0.45	0.82	164.23	0.56	0.58
Birth date	**-0.087**	**0.037**	**164.44**	**-2.37**	**0.019**
Cone index of previous year	0.45	0.51	164.08	0.89	0.37
Study area					
Food addition + high-density	0.10	0.078	165.31	1.35	0.18
Sex of pup					
male	**0.054**	**0.020**	**338.66**	**2.71**	**0.0071**
Latency to return x litter size	-0.021	0.023	170.77	-0.91	0.37
Random effect:	**Variance**	**SD**			
litter ID	0.11	0.34			
residual	0.029	0.17			

Table 3.2 Full results for binomial linear models on the relationship between survival to autumn and maternal attentiveness

We ran two distinct models for observations from the (a) first and (b) second nest entries (at ~1 day post-parturition and ~25 days post-parturition, respectively). We standardized growth rate (g/day) and birth date within study area-year combination. We standardized all other continuous variables (latency to return to pups and cone indexes) across all data. Bold font indicates statistical significance of $p < 0.05$.

a) Latency observations at first nest entry (pups ~1 day old):

Response Variable	Fixed Effect	ß	SE	z	*p*-value
Alive in August (Y/N)					
n = 489 pups	**Intercept**	**-0.74**	**0.17**	**-4.42**	**< 0.0001**
n = 183 litters	Latency to return to pups	-0.11	0.11	-1.00	0.32
n = 160 mothers	**Growth rate**	**0.24**	**0.11**	**2.12**	**0.034**
	Parity of mother				
	primiparous	0.040	0.48	0.080	0.94
	Sex of pup				
	male	**-0.71**	**0.22**	**-3.30**	**0.00098**
	Birth date	**-0.27**	**0.12**	**-2.17**	**0.030**
	Cone index of current year	**0.25**	**0.11**	**2.21**	**0.027**
	Cone index of previous year	0.096	0.13	0.75	0.45
	Study area				
	Food-addition + high-density	-0.090	0.23	-0.39	0.70

b) Latency observations at second nest entry (pups ~25 days old):

Response Variable	Fixed Effect	ß	SE	z	*p*-value
Alive in August (Y/N)					
n = 446 pups	**Intercept**	**-0.99**	**0.19**	**-5.19**	**< 0.0001**
n = 168 litters	Latency to return to pups	-0.12	0.11	-1.05	0.29
n = 145 mothers	**Growth rate**	**0.29**	**0.12**	**2.44**	**0.015**
	Parity of mother				
	primiparous	-0.18	0.49	-0.36	0.72
	Sex of pup				
	male	**-0.54**	**0.23**	**-2.34**	**0.019**
	Birth date	**-0.19**	**0.13**	**-1.47**	**0.14**
	Cone index of current year	**0.36**	**0.14**	**2.64**	**0.0084**
	Cone index of previous year	0.22	0.13	1.67	0.095
	Study area				
	Food-addition + high-density	0.0085	0.25	-2.34	0.97

Table 3.3 Full results for negative binomial linear models on the relationship between lifetime reproductive success of females and maternal motivation

We defined lifetime reproductive success as the number of pups born surviving over winter to recruit into the breeding population. For each squirrel, we averaged latency to return to pups across all observations of that individual, including censored observations as 420 s. We standardized both continuous fixed effects. We included squirrels with known birth years prior to 2011 (range: 2004 to 2010). Bold font indicates statistical significance of $p < 0.05$.

Response Variable	**Fixed Effect**	**ß**	**SE**	**z**	***p*-value**
Lifetime reproductive success (# of pups)					
n = 40 mothers	**Intercept**	**1.04**	**0.14**	**7.51**	**< 0.0001**
	Average latency to return to pups	**-0.31**	**0.15**	**-2.09**	**0.037**
	Lifespan (days)	**0.69**	**0.14**	**4.91**	**< 0.0001**
	Study area				
	Food-addition + high-density	-0.22	0.41	-0.54	0.59

Figure 3.1 Interaction between the effects of litter size and maternal behavior on pup growth rate

Pups from larger litters typically grew slower than pups from smaller litters. This relationship was mitigated by highly attentive mothers, at both the (A) first nest entry and (B) second nest entry, though the relationship was not statistically significant for the second nest entry (Table 3.1). Pups in large litters grow faster if their mother was highly attentive for latency to return to the nest at the first nest entry. More saturated, darker colors indicate a faster growth rate. Partial residuals of growth rate are plotted. Both litter size and latency to return to pups are standardized.

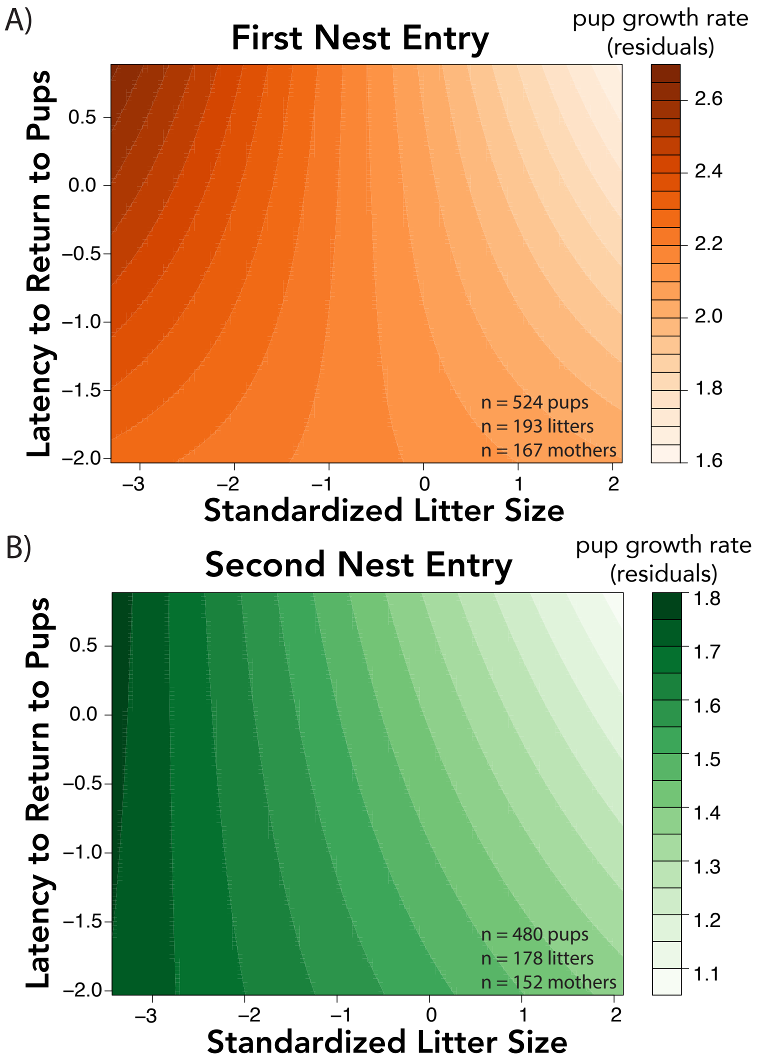
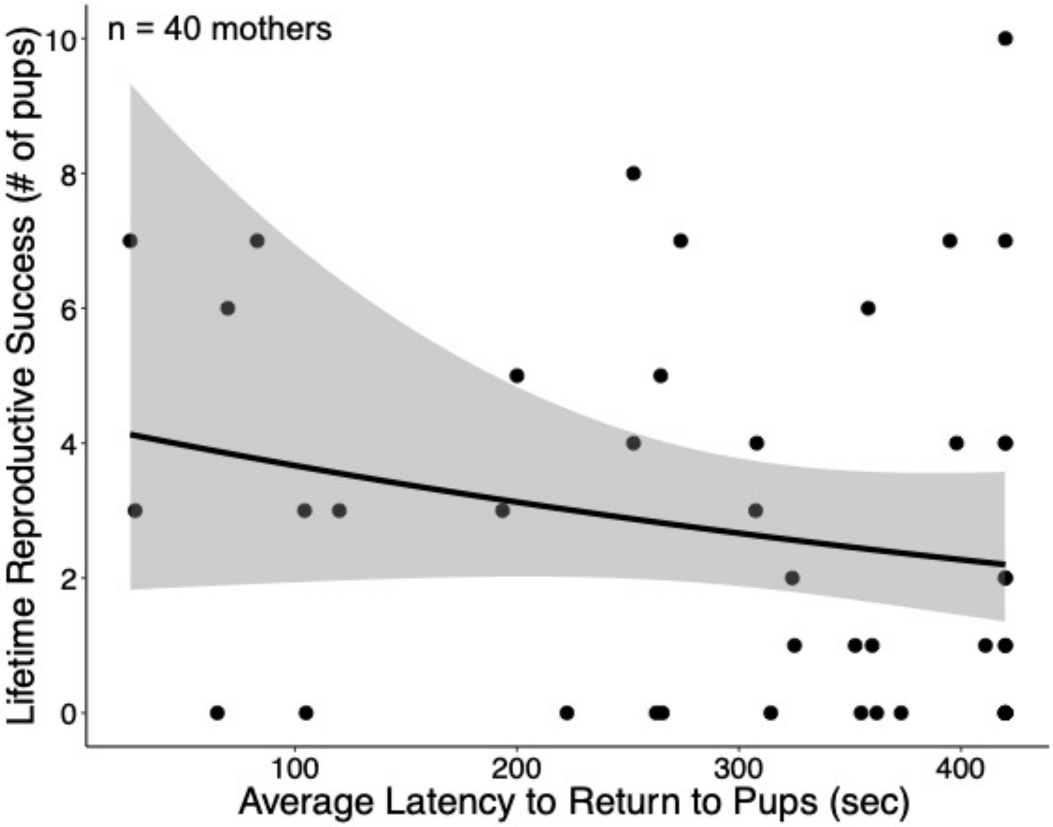


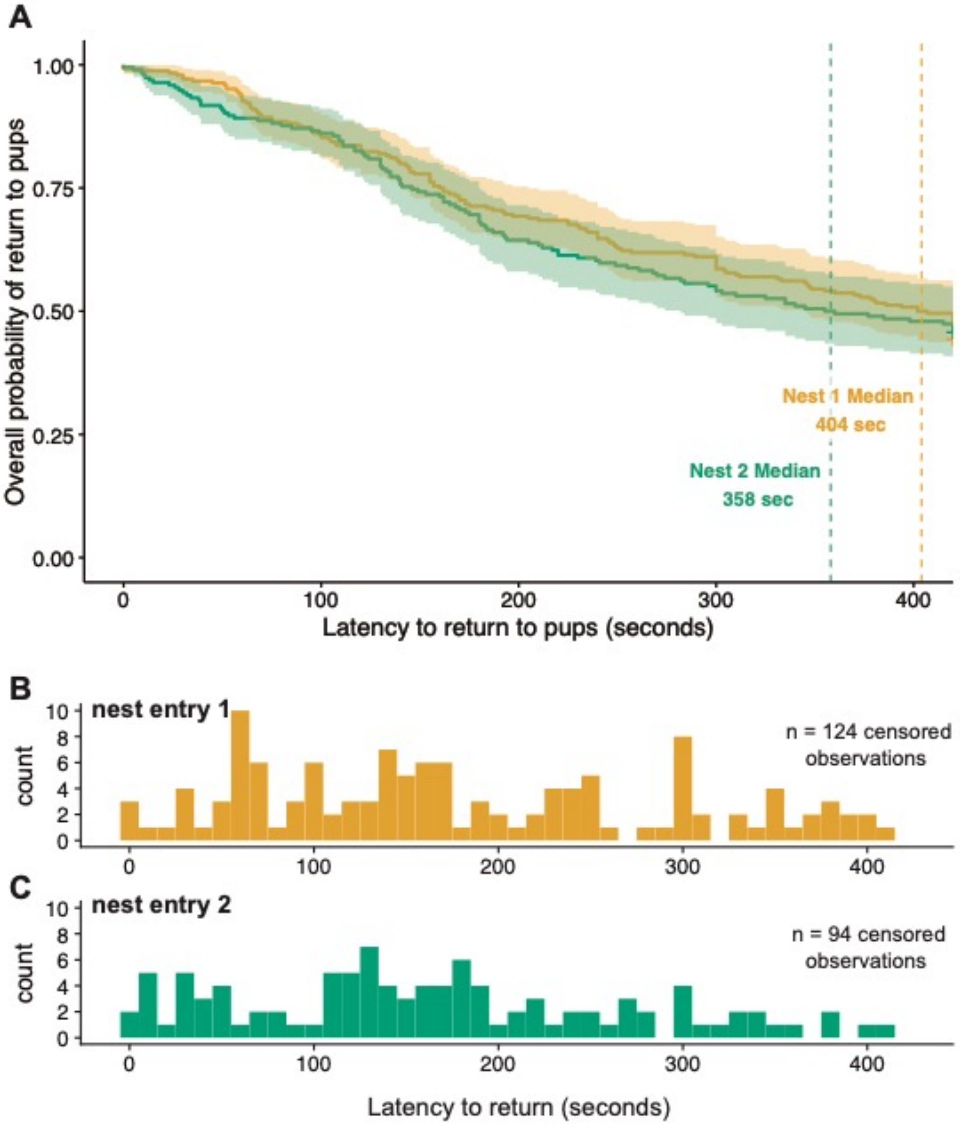
Figure 3.2 Relationship between nest attentiveness and lifetime reproductive success

Highly attentive mothers have more pups survive to recruit into the breeding population than less attentive mothers.



Supplemental Figure 3.3 Survival curves and histograms of observed latency to return

We recorded the amount of time (sec) until mothers returned to their nest following a nest intrusion by researchers. We censored the observations at 7 min (420 sec). Due to this censoring, we used **(A)** Kaplan-Meier survival curves to compare the median latency to return at the first nest entry to the median latency at the second nest entry. The survival curves did not significantly differ between the first and second nest entry observations (nest entry 1 median = 404 s; nest entry 2 median = 358 s; difference between curves: $\chi^2 = 0.0$, p = 0.9). Observations at the **(B)** first and **(C)** second nest entry varied across the whole 7 min observation period (censored observations not included in histogram).



Chapter 4 Maternal Glucocorticoids Do Not Impact HPA Axis Activity or Behavior of Juvenile Wild North American Red Squirrels

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Abstract

Environmental factors experienced during development can affect the physiology and behavior of offspring. In mammals, the maternal environment is particularly impactful, specifically the transfer of maternally-derived hormones to the offspring across the placenta or through milk. Maternal glucocorticoids (GCs) may convert environmental cues experienced by the mother into a cue triggering adaptive developmental plasticity in offspring. In a wild population of North American red squirrels, behavioral traits are under fluctuating selection in response to conspecific density, a highly salient environmental stressor. We experimentally elevated maternal GCs during gestation or early lactation to assess the role of GCs in mediating maternal effects on offspring behavior and physiology. When offspring reached the age of weaning, we measured two behavioral traits (activity and aggression), two aspects of hypothalamic-pituitary-adrenal (HPA) axis activity (adrenal reactivity and negative feedback), and the interaction between behavior and physiology of offspring in response to this manipulation. We found that an increase in maternal GCs during lactation, but not gestation, only slightly elevated activity levels in offspring. Aggression, adrenal reactivity, and negative

feedback did not differ between GC-treated and control groups for either gestation or lactation treatments. Juvenile squirrels with higher adrenal reactivity did exhibit lower aggression, but the relationship between adrenal reactivity and aggression was not impacted by treatment with maternal GCs. These results suggest maternal GCs during gestation or early lactation alone may not be a sufficient cue to produce changes in behavioral and physiological stress responses in offspring in natural populations.

Introduction

Maternal effects, or the influence of a mother's phenotype on those of her offspring, are a significant factor in developmental plasticity which contributes to among-individual variation (Rossiter 1991; Stamps and Groothuis 2010; Reddon 2012). Maternal glucocorticoids (GCs) have been proposed as one proximate mechanism by which maternal effects shape offspring phenotypes (Meaney 2001; Kapoor et al. 2008), particularly in 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 (Sih 2011; Del Giudice et al. 2011). 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). Similarly, an increase of maternal corticosterone in pregnant female *Lacerta vivipara* lizards improved odds of survival for male offspring (Meylan and Clobert 2005). Understanding the proximate role of maternal GCs in the development of behavior can provide valuable information into how phenotypic variation persists in populations.

One way in which maternal GCs could lead to adaptive shifts in offspring behavior is through changes in 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 due to their release in response to stressful situations and adverse environmental conditions (Sapolsky et al. 2000; Tsigos and Chrousos 2002; Charmandari et al. 2005; Spencer and Deak 2017). High levels of systemic GCs induce negative feedback by binding to receptors in the hypothalamus and pituitary to return expression of CRF, ACTH, and GC to basal levels after a response to an acute stressor (Sapolsky et al. 1985; Spencer and Deak 2017).

Two prevailing models have linked the activity of the HPA axis, or the physiological stress response, to the behavioral stress response of coping styles. The term ‘coping styles’ is often used to describe the behavioral stress response along a continuum from ‘proactive’ to ‘reactive’ (Koolhaas et al. 1999). In this paradigm, proactive individuals are typically more active and aggressive, while reactive individuals are more docile and less active. The unidimensional model posits the behavioral response is tightly linked with the physiological stress response such that more proactive individuals exhibit lower HPA axis activity by producing less GCs in response to a stressor than reactive individuals (Koolhaas et al. 1999). This model is consistent with hypotheses on the pleiotropic effects of hormones on behavior in the sense that the specific suites of correlated behaviors exhibited by proactive and reactive individuals may be driven pleiotropically by a hormonal response (Koolhaas et al. 1999; McGlothlin and Ketterson 2008). Conversely, the two-tier model proposes the behavioral and physiological stress responses are distinct and not predictive of one another (Koolhaas et al. 2010).

There are many terms in the literature used when discussing among-individual differences in behavior that persist over contexts and time, including temperament, coping styles, personality, and behavioral syndromes (Réale et al. 2007). Each has a slightly different meaning though they are often used interchangeably in the literature. In this study, we will use the term 'behavioral trait' when discussing a specific aspect of an individual's consistent behavioral repertoire (e.g. activity or aggression) as defined in (Carter et al. 2013).

There are now many laboratory and field studies demonstrating a strong effect of the early life environment on offspring behavior and physiology (Caldji et al. 2011). In particular, the maternal environment experienced during an individual's development has been associated with the development of the physiological stress responses (reviewed in Meaney 2001). It is important to note that many studies investigating the impact of prenatal stress do not directly manipulate GCs (cortisol or corticosterone), but rather induce an overall stress response through techniques such as physical restraint, foot shocks, cold exposure, food deprivation, or noise and flashing lights (Weinstock 2008). Prenatal stressors are going to impact the HPA axis differently when applied at different time periods relative to the development of the HPA axis (Weinstock 2008). In an experiment using maternal adrenalectomies and administration of exogenous GCs, Barbazanges *et al.* (1996) showed the role of excess maternal GCs in mediating the impairment in 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). In addition, GC exposure via milk during lactation has been shown to impact behavioral traits of offspring. In laboratory rats, increasing GCs of mothers during lactation improves learning and reduces fearfulness of offspring (Catalani et al. 2000), whereas higher GCs in milk of rhesus macaques (*Macaca mulatta*) produces more 'nervous' and less 'confident' offspring (Hinde et al. 2015). Maternal effects could also induce adaptive combinations of traits combinations of traits (Lancaster et al. 2007) or cause adaptive shifts in phenotypic covariance by either strengthening the degree of covariation among particular traits (Merrill and Grindstaff 2015) or perhaps lessening the overall degree of phenotypic covariance (Careau et al. 2014).

North American red squirrels (*Tamiasciurus hudsonicus*) in Yukon Territory, Canada experience among-year fluctuations in the availability of their major food source, seeds from white spruce trees (Fletcher et al. 2010, 2013; Ren et al. 2017). Red squirrels defend a territory year-round that contains a hoard of 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 in turn 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 who were more aggressive and perhaps less active (in standardized behavioral assays: Taylor et al., 2014) were 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 those elevated GCs during pregnancy produce faster growing pups (Dantzer et al. 2013, 2020). This suggests that elevations in maternal GCs during pregnancy may induce adaptive increases in offspring growth but whether or not elevations in maternal GCs cause adaptive increases in offspring aggressiveness is not clear.

In this study, we link knowledge of the developmental effects of maternal stress on the HPA axis to the ontogeny of behavioral traits. We asked if 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. In mammals, the transfer of maternally-derived hormones to the offspring across the placenta or through milk (Kulski and Hartmann 1981; O'Donnell et al. 2009; Grey et al. 2013) can induce developmental plasticity. Previous studies in our study population identified that elevations in maternal GCs during pregnancy causes increases in offspring postnatal growth but elevations in maternal GCs during lactation reduced offspring postnatal growth (Dantzer et al. 2013, 2020). We therefore investigated how elevations in maternal GCs during pregnancy or lactation, via ingestion of GCs mixed in peanut butter, 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 (open field trials) and aggression towards conspecifics (mirror image stimulation tests). We quantified the impact of experimentally elevated maternal GCs on offspring HPA axis activity by conducting 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 following administration of ACTH: van Kesteren et al. 2019). Finally, because maternal GCs may cause simultaneous changes in offspring behavior and stress physiology or the association between the two behavioral traits (activity and aggression), we also assessed how the treatments impacted the association between the physiological stress response (negative feedback, ability to mount a physiological stress response) and offspring behavior (activity, aggression) as well as the phenotypic correlation between activity and aggression.

Methods

Study population

We conducted the field work for this study in Yukon Territory, Canada on the traditional lands of the Champagne and Aishihik First Nations. Both sexes of North American red squirrels are highly territorial and defend their food cache year-round (Dantzer et al. 2012; Siracusa et al. 2017). To identify adult individuals in this study, we used unique alphanumeric stamped ear tags (National Band And Tag Company, Newport, KY, USA) and unique combinations of colored wire in both ears of the squirrel. We used live trapping (Tomahawk Live Trap, Tomahawk, WI, USA) to trap squirrels for monitoring reproductive status by abdominal palpation to detect fetuses and manual milk expression to detect lactation (McAdam et al. 2007). Upon identifying lactation in female squirrels, we fit a VHF radio collar (Holohil PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada) on the female and used telemetry to locate her nest with pups. We briefly removed the pups from the nest soon after birth and again ~25 days post-parturition in order to identify sexes, record weights to calculate post-natal growth, and ear-tag pups with alphanumeric tags and unique combinations of colored disks 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 University of Michigan (PRO00005866).

Glucocorticoid supplementation experiment

To stimulate chronic increases in GCs, we treated pregnant or lactating females with exogenous GCs mixed in 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 individual squirrels with GCs daily, we provisioned individuals with small amounts of peanut butter (~8-g) and wheat germ (~2-g) mixed with dissolved hydrocortisone [H4001, Sigma Aldrich]. To make these individual treatments, we first dissolved the hydrocortisone 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 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 kept doses at -20°C until needed for provisioning the squirrels. Control treatments were made in exactly the same manner but did not include hydrocortisone in the peanut oil.

On the territory of each squirrel in our experiment, we hung a 10.5-L bucket with two holes cut into the sides of the bucket. Each bucket was covered with a lid and hung ~7-10 m off the ground in the center of the squirrel's territory. We placed individual peanut butter treatments in the buckets for provisioning each day. Squirrels were randomly assigned to either the control treatment (8-g all-natural peanut butter, 2-g wheat germ, no cortisol) or GC treatment (8-g all-natural peanut butter, 2-g wheat germ, 8 or 12-mg of cortisol). We fed all pregnancy treated squirrels 8-mg of cortisol/day and lactation treated squirrels either 8 or 12-mg of cortisol/day. We selected these dosages of cortisol 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). Wilcoxon Rank Sum tests on the four response variables (two HPA axis measurements and two behavioral traits) show no significant differences between 8 and 12-mg of cortisol/day in the lactation treatment group (Supplemental Table 4.6). Therefore, we combined the 8 and 12-mg treatments into one GC treatment group to increase power for our statistical analyses (Dantzer et al. 2020).

To examine whether the timing of an increase in maternal GCs produces 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. Due to variation in detecting the precise stage of pregnancy via palpation, we actually treated mothers from 10.8±0.7 (mean±SD) days 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: lac control, lac GC, preg control, and preg GC (see Table 4.1 for sample sizes). In van Kesteren et al. (2019), we found squirrels that were fed GC treatments showed higher baseline plasma cortisol concentrations in the first 9 hours after consuming the peanut butter, so we are confident that GC treatments are increasing circulating GCs. Additionally, corticosteroid-binding globulin concentrations in plasma were reduced in squirrels fed GC treatments (van Kesteren et al. 2019). For more detailed information about this manipulation and how it impacts circulating levels of cortisol in plasma, see van Kesteren *et al*. (2019).

Behavioral trials

We live-trapped offspring from our experimental females around the age of weaning (mean ± SD = 67.79 days old ± 3.76; weaning age is ~70 days old: Boutin and Larsen 1993). Using a canvas handling bag, we weighed the juvenile squirrel before transferring them to our behavioral assay arena. For our open-field and mirror image stimulation trials, we used the same white corrugated plastic arena (60 x 80 x 50 cm) with a clear acrylic lid as described in previous studies in this study system (Boon et al. 2007, 2008; Taylor et al. 2012, 2014; Kelley et al. 2015). 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 served as the acclimation period for the following mirror image stimulation trial which lasted 5-min after the mirror (45 x 30 cm) on one side of the arena was exposed.

We used JWatcher (Blumstein and Daniel 2007) to manually score the videos using the same ethogram used in previous red squirrel personality papers (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

With offspring from our experimental females, we performed HPA axis hormone challenges by administering dexamethasone (DEX; a GC receptor antagonist) and ACTH as previously described (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 cortisol levels, while ACTH acts upon the adrenals to upregulate the production of cortisol. We began by collecting a blood sample from a rear toenail using heparinized microcapillary tubes (described in van Kesteren et al. 2019; Dantzer et al. 2020) for measurement of initial levels 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. Due to this substantial amount of handling and disturbance, we consider the initial sample levels of cortisol as stress-induced though the length of time between trapping and the first bleed was variable. We then injected 3.2-mg/kg of dexamethasone intramuscularly in the squirrel's upper rear leg. We released the squirrel back into the live-trap and waited 1-hr before taking another blood sample (DEX bleed). Next, we injected 4-IU/kg of ACTH intramuscularly in the alternate upper rear leg. We kept the squirrel in the live-trap before taking blood samples 30-mins (ACTH 30) and 1-hr post-injection (ACTH 60). We have previously shown that these dosages of dexamethasone and ACTH are sufficient to decrease or increase circulating plasma cortisol levels in adult red squirrels (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 -20 C.

To quantify total plasma cortisol concentration, we used an ImmuChem coated tube cortisol 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-ul of sample and 500-ul 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, DEX bleed n = 3, ACTH 30 min n = 6, ACTH 60 min n = 4). To maximize the number of animals included in our study, we used the 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 all four assays, our average standard and sample intra-assay CVs were 9.5%, our average intra-assay CVs for red squirrel plasma samples was 9.28%, and our average inter-assay CVs for the five standards provided (10, 30, 100, 300 and 1000 ng/ml cortisol) was 14%. The experimenters conducting the HPA axis challenges were not blind to maternal treatments due to the same researchers provisioning the mothers and trapping the squirrels but 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

All statistical analyses were run in R version 3.5.2 (R Core Team 2016). Using the R package 'ade4' version 1.7-10 (Dray and Dufour 2007), we ran two distinct principal components analyses with correlation matrices (one on open-field behaviors and one on mirror-image stimulation behaviors) to reduce behavioral variables down to one major component for each assay. Based on the loadings (Table 4.2), we interpreted the first component of the open-field trial as 'activity', explaining 30% of variation in open-field behaviors in our data set. We interpreted the first component of the mirror image stimulation trial as 'aggression', explaining 50% of variation in mirror image stimulation behaviors in our data set. Previous studies in this system have used the same methods to analyze 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; Taylor et al. 2012, 2014; Kelley et al. 2015; Cooper et al. 2017; Westrick et al. 2019). All subsequent analyses used the individual scores calculated from the principal component loadings for each trial (Table 4.2). Higher 'activity' scores mean the squirrel spends 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 low scoring squirrels (Table 4.2).

We calculated the adrenal responsivity, or the net integrated response of cortisol over the 60-mins 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 (Janssens et al. 1994; Ingram et al. 1997; Saltzman et al. 2000; Rich and Romero 2005; Heidinger et al. 2008). Based on a recent review about calculating HPA 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).

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 (Kuznetsova et al. 2016). We used the R package 'multcomp' version 1.4-8 (Hothorn et al. 2008) to run a Tukey 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 fit separate models for pregnancy and lactation treatment groups. To control for the variability in the number of doses mothers in the pregnancy treatment group received (16 days±3.59), we included maternal treatment length in all pregnancy models. Treatment length among mothers in the lactation groups did not vary considerably (10 days±0.49) therefore we did not include this in the lactation models. We compared the HPA response variables (ACTH AUC and DEX response) between juveniles in GC-treated groups to the appropriate control groups using linear mixed-effects models. In the pregnancy models to predict ACTH AUC, we included treatment group (GC or control), sex, post-DEX injection plasma total cortisol concentration, maternal treatment length (standardized across all data for all analyses), treatment year (categorical), and age of the juvenile (standardized across all data for all analyses) as fixed effects. In the pregnancy models to predict the response to DEX, we included treatment group, maternal treatment length, sex, and age. We removed year of treatment from this model due to singular fit when it was included. Since we included multiple pups from the same litter, we included a litter identity (ID) as a random effect. For the lactation model predicting ACTH AUC, we included treatment group, sex, post-DEX injection plasma total cortisol concentration, treatment year, and age as fixed effects and excluded litter ID as a random effect. In the lactation model predicting response to DEX, we included treatment group, sex, treatment year, and age as fixed effects. We excluded litter ID as a random effect due to singular fit, indicating the model was overfit, when it was included.

To assess the impact of maternal GCs on behavior and the relationship between HPA and behavior in our juveniles, we fit separate linear mixed-effects models for activity and aggression. In our pregnancy treatment activity model, we included ACTH AUC, response to DEX, treatment group, sex, maternal treatment length, treatment year, and age as fixed effects. In our lactation treatment activity model, we included ACTH AUC, response to DEX, treatment group, treatment year, and age as fixed effects, but did not include sex due to singular fit when it was included. We included litter ID in both models as a random effect. For our pregnancy treatment aggression model, we fit a linear model predicting aggression including ACTH AUC, response to DEX, treatment group, sex, maternal treatment length, treatment year, and age as fixed effects. Due to overfitting, we were unable to fit a linear mixed-effects model including litter ID as a random effect for this model. For the lactation treatment aggression linear mixed-effects model, we included ACTH AUC, response to DEX, treatment group, sex, treatment year, and age as fixed effects and litter ID as a random effect.

To assess the impact of maternal treatment on the relationship between activity and aggression, we fit linear mixed-effects models with activity as the response variable and fixed effects including the interaction between aggression and maternal treatment group, treatment year, and sex with litter ID as a random effect. For the pregnancy model, we included an additional fixed effect of maternal treatment length. We excluded age as a variable in both models due to singular fit. 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 $GVIF^{(1/(2xDF))} < 2$ for all predictors across all models. Due to small sample sizes, we did not include an interaction between sex and treatment in any models.

Results

On average, all treatment groups responded to DEX as expected with a decrease in plasma cortisol and responded to ACTH as expected with an increase in plasma cortisol (Figure 4.1). A simple linear mixed model with Tukey post-hoc comparisons showed plasma cortisol did not differ between the initial handling-stress induced sample and ACTH 30 min bleed (ACTH 30 - initial sample: ß = -4.77, z = -2.03, p = 0.18) and was lowest at the DEX bleed, 1-hr after the injection of DEX (DEX bleed - initial sample: ß = -36.78, z = -15.67, *p* < 0.001; ACTH 30 – DEX: ß = -32.01, z = 13.66, p < 0.001; ACTH 60 - DEX: ß = 27.08, z = 11.55, p < 0.001). Plasma cortisol did not differ significantly between ACTH 30 min and ACTH 60 min bleeds (ACTH 60 – ACTH 30: ß = -4.93, z = -2.10, p = 0.15). One individual (out of 57 total individuals) from the preg control treatment group did not respond to DEX and was excluded from further analyses.

Effect of maternal glucocorticoids on HPA axis activity

After controlling for starting values of total cortisol concentration (cortisol at the DEX bleed), juveniles from mothers treated with exogenous glucocorticoids during pregnancy or lactation did not differ in their adrenal response to ACTH, as measured by AUC, compared to controls (pregnancy treatment: ß = 8.54, $p = 0.23$; lactation treatment: ß = -8.45, $p = 0.32$; Table 4.3; Figure 4.2). There were no sex differences in the response to ACTH (Table 4.3). Older pups in the lactation treatment group (not in the pregnancy group) had smaller responses to ACTH (ß = -7.72, $p = 0.015$). Likewise, treatment length for pregnancy treatments did not predict variation in ACTH AUC (ß = -1.85, $p = 0.74$; Table 4.3A). We found no differences in ACTH AUC across the three years of this experiment (Table 4.3).

Juveniles from mothers treated with exogenous glucocorticoids during pregnancy or lactation did not differ in the magnitude of their negative feedback response to DEX compared to controls (pregnancy treatment - ß = 9.77, $p = 0.14$; lactation treatment - ß = -0.11, $p = 0.99$; Table 4.3; Figure 4.2). The sex and age of the juvenile did not contribute significantly to variation in negative feedback and neither did treatment length for pregnancy treatments (Table 4.3). We found no differences in negative feedback among years of the experiment (Table 4.3).

Effect of maternal glucocorticoids on behavioral traits

Among juveniles from both preg GC and preg control mothers, more active individuals exhibited a lower adrenal response to ACTH (ACTH AUC) than less active individuals (ß = -0.02, $p = 0.0058$; Table 4.4A; Table 4.3). However, juveniles from lac GC and lac control mothers did not exhibit any relationship between ACTH AUC and activity (ß = 0.01, $p = 0.66$; Table 4.4B; Figure 4.3). We saw a non-significant trend for juveniles from lac GC mothers having slightly higher activity levels than control individuals (ß = 1.17, $p = 0.07$; Table 4.4B;

Figure 4), however preg GC offspring were not significantly different than preg control offspring in activity levels (Table 4.4A; Figure 4.4). Again, sex and age of the juvenile did not contribute to variation in activity (Table 4.4). We found the negative feedback response to DEX did not predict activity in either pregnancy or lactation treatments (Table 4.4; Figure 4.3). We found no differences in activity among years of the experiment (Table 4.4).

Among all juveniles, more aggressive individuals had lower adrenal response to ACTH than less aggressive individuals (pregnancy treatment – ß = -0.02, *p* = 0.02; lactation treatment – ß = -0.03, *p* = 0.04; Table 4.4; Figure 4.3). In the lactation treatment group, males were slightly more aggressive than females, though this sex difference was not statistically significant (ß = 0.71, *p* = 0.06; Table 4.4) No other factors in the models predicted variation in aggression (Table 4.4).

Relationship between behavioral traits and HPA axis activity

Treatment with exogenous GCs during pregnancy or lactation did not impact the relationship between activity and aggression (pregnancy treatment – ß = 0.06, *p* = 0.91; lactation treatment – ß = -0.34, *p* = 0.50; Table 4.5). Aggression was not predictive of activity in either pregnancy or lactation treated individuals, though the effect was in the expected direction, of more aggressive individuals exhibiting more active behavior based on previous studies with much larger sample sizes (pregnancy treatment – ß = 0.49, *p* = 0.21; lactation treatment – ß = 0.56, *p* = 0.15; Table 4.5; Figure 4.5). Treatment group, treatment length, juvenile's age, juvenile's sex, and year of experiment all showed no relationship with activity among pregnancy treated juveniles (Table 4.5). In this model structure, lac GC juveniles showed higher activity levels than lac control juveniles (ß = 1.12, *p* = 0.046; Table 4.5).

Discussion

Overall, our experimental increase of maternal GCs during pregnancy or lactation had only a few subtle impacts on behavioral traits of juvenile offspring. Activity was slightly higher in the lac GC treatment group compared to controls, but there were no statistically significant differences between the pregnancy treatment groups and aggression was not impacted by the GC treatments. Exogenous GCs provided to pregnant or lactating females did not impact their offspring's HPA axis response to dexamethasone (negative feedback) and ACTH (adrenal reactivity). However, we did find a relationship between one measure of HPA axis activity and both behavioral traits of the juvenile squirrels we tested. Less active squirrels from the pregnancy treatment groups were more responsive to ACTH, but we did not find this relationship among squirrels in the lactation treatment groups. Similarly, less aggressive squirrels were more responsive to ACTH among all juveniles from all of the pregnancy and lactation treatment groups. The negative feedback response of the HPA axis was unrelated to activity and aggression.

Contrary to our findings, a recent meta-analysis with 39 studies across 14 vertebrate species found an overall positive relationship between prenatal stress and offspring GC levels with a particularly strong effect for experimental studies compared to observational ones (Thayer et al. 2018). This meta-analysis also found a stronger effect of prenatal stress on the negative feedback of the HPA axis than baseline or peak GC response to a stressor (Thayer et al. 2018). It is important to note this meta-analysis includes studies with either administration of exogenous GCs or general prenatal stress exposure, so it does not directly address the impact of exogenous GCs in particular (Thayer et al. 2018). There are multiple non-mutually exclusive potential explanations for why our experiment did not follow these trends. For example, inevitably not all offspring from the pre- and post-natal GC supplementation experiment survived to weaning (van Kesteren et al. 2019), and potentially not all offspring from the experiment stayed within our study range (though unlikely, see: Berteaux and Boutin 2000; Kerr et al. 2007; Cooper et al. 2017), both of which could have resulted in a survivor bias in our results. Alternatively, we may have had selection biases in our trapping success rate. Despite extensive trapping efforts, differences in behavior among offspring may have resulted in a reduction in our ability to trap less aggressive, less active squirrels and led to underestimation of effect sizes and reduce our power to detect any correlations and treatment group effects (Carter et al. 2012; Kelley et al. 2015). However, based on the number of juveniles alive at weaning (determined via yearly population census, trapping, and behavioral observations), we actually included a considerable majority (68%) in this study.

One proximate mechanism potentially limiting our detection of effects of maternal GCs in our experiment could be the ongoing neural pruning of important brain structures responsible for consistent behavioral traits and reactions to external stressors between early life and adolescence (Spear 2000; Groothuis and Trillmich 2011). In other words, perhaps the effects of the GC manipulation were not long-lasting into adolescence due to this neural pruning or our manipulation was not sufficiently long enough to cover this critical developmental window. In this environment, it may be adaptive to adjust behavioral phenotypes throughout development (Kelley et al. 2015) and by testing these offspring at the stage of weaning, their behavior and HPA axis may have been shaped more by their own phenotypes and personal experiences than their early-life environment (Nettle and Bateson 2015). Additionally, too much sensitivity to the early life environment may be maladaptive for a species in a highly variable environment. Red squirrels in this region live in an environment that can fluctuate dramatically in predation risk, resource availability, and conspecific competition between an individual's birth and first breeding season (McAdam and Boutin 2003; Dantzer et al. 2013; Taylor et al. 2014; Hendrix et al. 2019). In red squirrels, the short developmental window could be impacted by short-term stochastic processes that result in a mismatch between the parental environment and offspring environment and therefore it would be maladaptive for offspring to be attuned to cues subject to short-term stochastic processes (Langenhof and Komdeur 2018). In essence, the world the developing juveniles experienced may have contradicted the environmental information that was conveyed by the cue of maternal GCs and therefore the maternal cues were "overwritten" by the immediate cues (Leimar and McNamara 2015).

In addition to testing maternal GC treatment effects, our results also provide evidence supporting the original unidimensional coping styles model (Koolhaas et al. 1999) which posits more active, aggressive individuals should exhibit a decreased physiological stress response, specifically decreased HPA axis activation. Previously, we tested the unidimensional (Koolhaas et al. 1999) and two-tier coping style (Koolhaas et al. 2010) models in adult red squirrels and found no relationship between the physiological and behavioral stress responses, thus supporting the two-tier model (Westrick et al. 2019). While the conclusions of our two studies are different, we believe the studies are not directly comparable. There are two important ways in which our two studies differ.

First, in our previous study, we used the same behavioral trial methods, however 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 of scale, whereas measuring plasma cortisol in response to a standardized challenge could provide more relevant measurement of the acute HPA axis response, though research on snowshoe hares (*Lepus americanus*) suggests higher fecal glucocorticoid metabolite concentrations correspond to a smaller decrease in plasma cortisol in response to DEX and a larger response ACTH (Sheriff et al. 2010).

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). From previous work, we know these behavioral traits "regress to the mean" in this study system, meaning there is less variation and a lack of individuals at the extremes of activity and aggression in the adult population compared to the juvenile population (Kelley et al. 2015). This could mean the wide range in behavioral stress responses in the juvenile population of red squirrels is more consistent with the lab populations and selection lines for individuals at the extreme ends of the proactive-reactive spectrum (which often find support for the unidimensional model: Koolhaas et al. 1999; Westrick et al. 2019) compared to the adult population of red squirrels. It is also possible that only individual juveniles with low covariance between the behavioral and physiological stress responses were able to adaptively respond to conditions due to the two stress responses being decoupled and therefore the surviving adult population is less likely to exhibit high covariance between HPA axis activity and behavioral traits. Additionally, young animals need to be adapted to the experience of that life stage to survive to adulthood which may require a different set of adaptations (Groothuis and Trillmich 2011). We believe 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 study is one of the first to use an experimental manipulation to understand the effects of the maternal environment, as encoded through GCs, on the ontogeny of 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; Taylor et al. 2012, 2014; Kelley et al. 2015; Cooper et al. 2017) and our knowledge of how maternal GCs shape specific aspects of early development in red squirrels (Dantzer et al. 2013, 2020), this study system provided the ideal opportunity to test the role of maternal GCs on the ontogeny of behavioral traits and personality in a natural environment (Groothuis and Trillmich 2011; Langenhof and Komdeur 2018). The fact that we did not find a substantial effect of pre- or post-natal GCs may indicate maternal GCs are not an ecologically relevant cue for a short-lived animal in a highly-variable environment and therefore not a cue that offspring are particularly sensitive too, 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 this species.

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Table 4.1 Sample Sizes

Number of mothers supplemented, litters produced, and pups tested for each condition. Two mothers were treated with both GC and control during pregnancy on different years. Not all squirrels underwent an OF/MIS trial, so the sample sizes for analyses of HPA axis dynamics without any behavioral variables (A) included a few more squirrels than the analyses of HPA axis dynamics and behavior (‘activity’ and ‘aggression’) (B).

A) HPA axis dynamics by treatment group

	Pregnancy Treatments	**Lactation Treatments**
GC peanut butter (8 or 12 mg hydrocortisone)	13 mothers supplemented 13 litters produced 20 pups tested	8 mothers supplemented 8 litters produced 13 pups tested
control peanut butter (0 mg hydrocortisone)	13 mothers supplemented 13 litters produced 21 pups tested	9 mothers supplemented 9 litters produced 13 pups tested

B) HPA axis dynamics and behavior by treatment group

	Pregnancy Treatments	**Lactation Treatments**
GC peanut butter (8 or 12 mg hydrocortisone)	12 mothers supplemented 12 litters produced 16 pups tested	7 mothers supplemented 7 litters produced 12 pups tested
control peanut butter (0 mg hydrocortisone)	12 mothers supplemented 12 litters produced 18 pups tested	9 mothers supplemented 9 litters produced 11 pups tested

Table 4.2 PCA loadings for behaviors scored in the open-field trials (OF) and mirror image stimulation trials (MIS)

PCA loadings for 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.

OF Behavior	**Loading**
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

MIS Behavior	**Loading**
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

Table 4.3 Full results of HPA axis dynamics models

We ran four distinct models testing the effect of maternal GCs on the HPA axis dynamics. We ran separate models for the (a) pregnancy and (b) lactation treatment groups and the different aspects of the HPA axis dynamics. We standardized treatment length across all data for the pregnancy group. The comparison group for categorical variables is control treatment females from 2015. Bold font indicates statistical significance of $p < 0.05$.

A) Pregnancy treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Area under the curve (DEX to ACTH 60)					
	Intercept	**75.25**	**10.20**	**7.38**	**< 0.0001**
	Treatment				
	preg GC	8.54	6.82	1.25	0.23
	Sex				
	males	-3.00	6.68	-0.45	0.66
	DEX cortisol (ug/dL)	**1.13**	**0.24**	**4.67**	**< 0.0001**
	Treatment length	-2.22	5.33	-0.42	0.68
	Year				
	2016	-23.77	12.40	-1.92	0.07
	2017	-22.05	12.50	-1.76	0.09
	Age of pup	-2.07	4.48	-0.46	0.65
	Random effect:	*Variance*	*SD*		
	Litter ID	84.3	9.18		
Relative decrease from initial handling-stressed sample to DEX (%)					
	Intercept	**67.51**	**5.40**	**12.50**	**< 0.0001**
	Treatment				
	preg GC	9.77	6.48	1.51	0.14
	Sex				
	males	-9.09	6.65	-1.37	0.18
	Treatment length	6.65	3.89	1.71	0.10
	Age of pup	-0.57	3.77	-0.15	0.88
	Random effect:	*Variance*	*SD*		
	Litter ID	21.45	4.63		

B) Lactation treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Area under the curve (DEX to ACTH 60)					
	Intercept	**63.11**	**9.50**	**6.64**	**< 0.0001**
	Treatment				
	lac GC	-8.45	8.13	-1.04	0.32
	Sex				
	males	4.39	5.41	0.81	0.43
	DEX cortisol (ug/dL)	**1.59**	**0.20**	**7.80**	**0.00035**
	Year				
	2016	-8.04	13.55	-0.59	0.56
	2017	0.93	8.63	0.11	0.92

	Age of pup	**-7.72**	**2.60**	**-2.96**	**0.015**
	Random effect:	*Variance*	*SD*		
	Litter ID	193.91	13.93		
Relative decrease from initial handling-stressed sample to DEX (%)					
	Intercept	**64.35**	**16.51**	**3.90**	**< 0.001**
	Treatment				
	lac GC	-0.11	9.10	-0.01	0.99
	Sex				
	males	-0.49	8.20	-0.06	0.95
	Year				
	2016	-8.37	13.43	-0.62	0.55
	2017	-4.41	8.53	-0.52	0.62
	Age of pup	-6.46	4.04	-1.60	0.13

Table 4.4 Full results of behavioral trait models

We ran four distinct models testing the effect of maternal GCs on activity and aggression. We ran separate models for the (a) pregnancy and (b) lactation treatment groups and the two behavioral traits. We standardized treatment length across all data for the pregnancy models. The comparison group for categorical variables is control treatment females from 2015. Bold font indicates statistical significance of $p < 0.05$.

A) Pregnancy treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Activity					
	Intercept	1.69	1.07	1.59	0.12
	Adrenal response (AUC)	**-0.02**	**0.01**	**-3.02**	**0.0058**
	Negative feedback (%)	0.00	0.01	0.45	0.65
	Treatment				
	preg GC	-0.03	0.32	-0.11	0.92
	Sex				
	male	0.09	0.28	0.33	0.75
	Treatment length	-0.14	0.25	-0.57	0.57
	Year				
	2016	-0.53	0.60	-0.90	0.48
	2017	-0.72	0.60	-1.28	0.22
	Age of pup	-0.28	0.18	-1.48	0.15
	Random effect:	*Variance*	*SD*		
	Litter ID	0.19	0.44		
Aggression					
	Intercept	1.56	1.03	1.52	0.14
	Adrenal response (AUC)	**-0.02**	**0.01**	**-2.48**	**0.02**
	Negative feedback (%)	0.00	0.01	0.18	0.86
	Treatment				
	preg GC	-0.13	0.28	-0.46	0.65
	Sex				
	males	-0.37	0.28	-1.31	0.20
	Treatment length	-0.07	0.23	-0.32	0.75
	Year				
	2016	-0.27	0.52	-0.51	0.61
	2017	-0.54	0.50	-1.07	0.29
	Age of pup	-0.14	0.18	-0.79	0.44

B) Lactation treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Activity					
	Intercept	-1.86	2.47	-0.76	0.46
	Adrenal response (AUC)	0.01	0.02	0.45	0.66
	Negative feedback (%)	0.00	0.02	0.03	0.98
	Treatment				
	lac GC	1.17	0.55	2.11	0.07
	Year				
	2016	1.29	0.86	1.50	0.17
	2017	0.12	0.55	0.23	0.83

	Age of pup	0.28	0.27	1.03	0.32
	Random effect:	*Variance*	*SD*		
	Litter ID	0.16	0.40		
Aggression					
	Intercept	3.75	1.88	2.00	0.07
	Adrenal response (AUC)	**-0.03**	**0.01**	**-2.16**	**0.04**
	Negative feedback (%)	-0.02	0.01	-1. 47	0.12
	Treatment				
	lac GC	-0.52	0.57	-0.91	0.38
	Sex				
	males	0.71	0.31	2.28	0.06
	Year				
	2016	-0.30	0.90	-0.33	0.74
	2017	0.04	0.59	0.06	0.95
	Age of pup	-0.31	0.21	-1.49	0.16
	Random effect:	*Variance*	*SD*		
	Litter ID	0.88	0.94		

Table 4.5 Full results of the relationship between behavioral traits models

We ran two distinct models testing the effect of maternal GCs on the relationship between activity and aggression among individuals. Again, we ran separate models for the (a) pregnancy and (b) lactation treatment groups. We standardized treatment length across all data for the pregnancy model. The comparison group for categorical variables is control treatment females from 2015. Bold font indicates statistical significance of $p < 0.05$.

A) Pregnancy treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Activity					
	Intercept	-0.05	0.44	-0.13	0.90
	Aggression	0.49	0.39	1.29	0.21
	Treatment				
	preg GC	-0.02	0.35	-0.06	0.95
	Sex				
	male	0.34	0.35	0.97	0.34
	Treatment length	0.04	0.28	0.14	0.89
	Year				
	2016	-0.08	0.52	-0.16	0.88
	2017	-0.34	0.61	-0.56	0.58
	Aggression*Treatment	0.06	0.49	-0.12	0.91
	Random effect:	*Variance*	*SD*		
	Litter ID	0.09	0.30		

B) Lactation treatments

Response Variable	Fixed Effect	ß	SE	t	*p*-value
Activity					
	Intercept	-0.97	0.57	-1.70	0.11
	Aggression	0.56	0.37	1.52	0.15
	Treatment				
	lac GC	**1.12**	**0.49**	**2.27**	**0.046**
	Sex				
	male	0.29	0.54	0.54	0.60
	Year				
	2016	0.59	0.73	0.81	0.45
	2017	-0.09	0.51	-0.17	0.87
	Aggression*Treatment	-0.34	0.49	-0.70	0.50
	Random effect:	*Variance*	*SD*		
	Litter ID	0.04	0.19		

Supplemental Table 4.6 Comparison of lactation treatment dosages

We ran a Wilcoxon Rank Sum Test for each of the four response variables measured to test for an effect of GC treatment dosage (8-mg or 12-mg) in the lactation treatment group.

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

Figure 4.1 HPA axis hormone challenge curves by treatment group

Plasma cortisol concentrations (ng/mL) plotted across the hormone challenge time series. Blue lines and points indicate lactation treatments and green lines and points indicate pregnancy treatments. Data are staggered at each timepoint by mother's treatment group for ease of visualizing. Black bars indicate the standard error around the mean for each group at each timepoint.

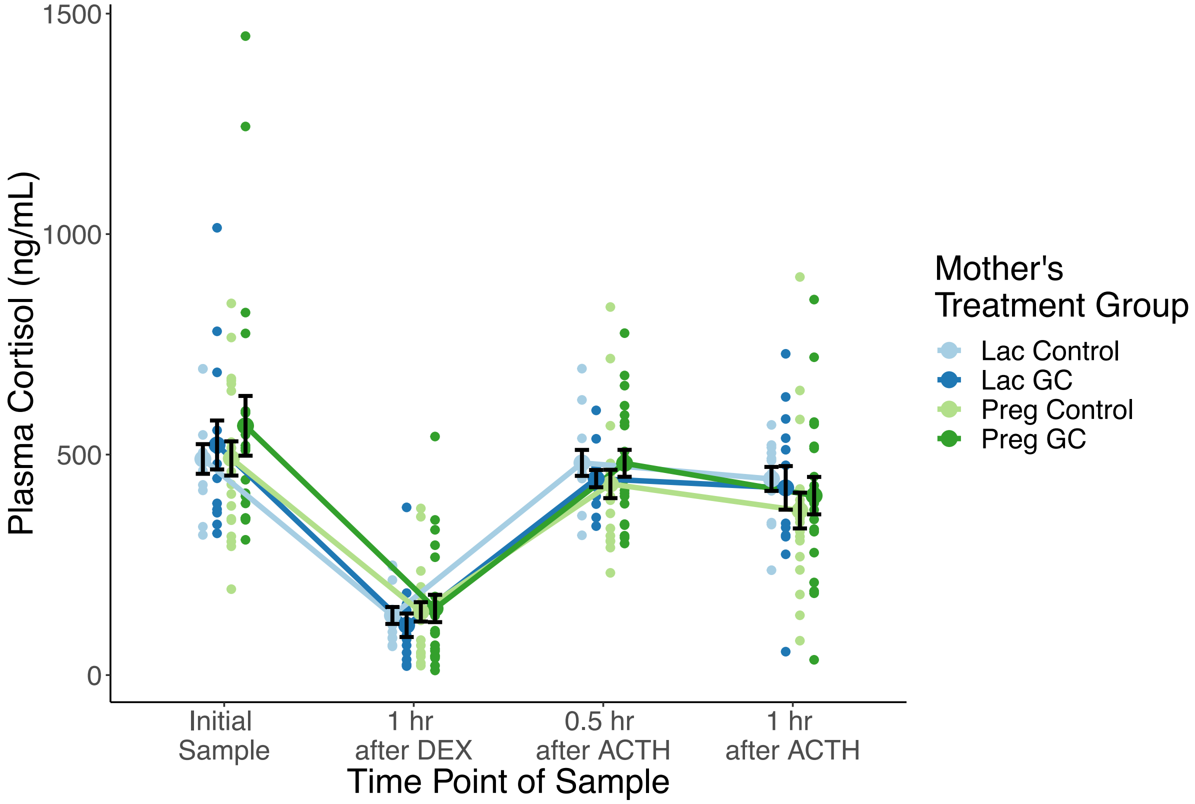


Figure 4.2 Effect of treatment group on HPA axis dynamics

A) Adrenal reactivity (response to ACTH) and B) negative feedback (response to dexamethasone) were not impacted by maternal treatment with exogenous GCs.

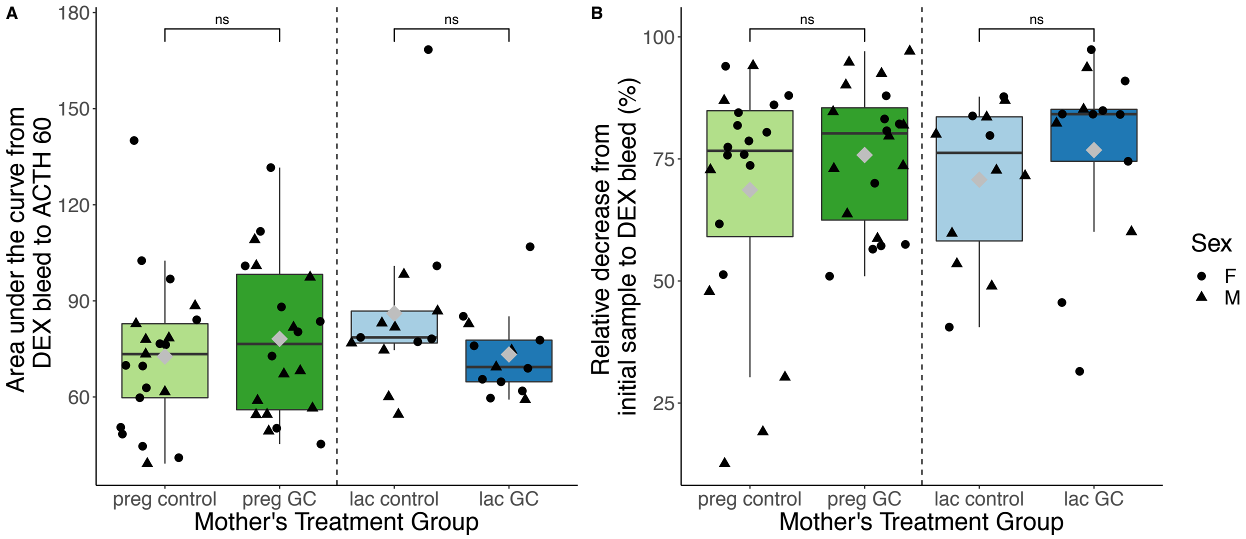


Figure 4.3 Relationships between behavioral traits and HPA axis dynamics

More active (A) and aggressive (B) juveniles 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). More aggressive juveniles from the lactation treatment groups (both GC-treated and controls) also exhibited smaller ACTH AUC (F). Panels A-D include raw data from pregnancy treated juveniles. Panels E-H include raw data from lactation treated juveniles. Separate regression lines for each treatment group (GC-treated and controls) are not shown because there was no significant interaction between treatment and the independent variable (Table 5). Panels A, C, E, and G show the relationship between activity and our two measures of HPA axis dynamics. Panels B, D, F, and H show the relationship between aggression and HPA axis dynamics.

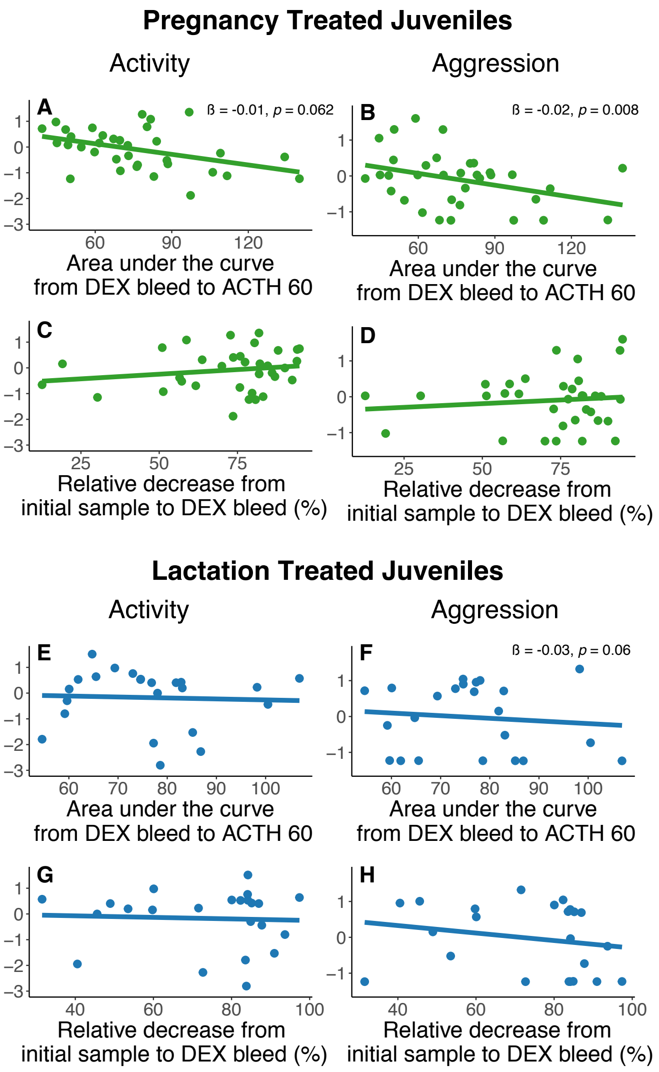


Figure 4.4 Effects of treatment group on activity and aggression

A) Activity and B) aggression were not significantly impacted by maternal treatment with GCs, though lac GC juveniles showed slightly higher levels of activity than controls.

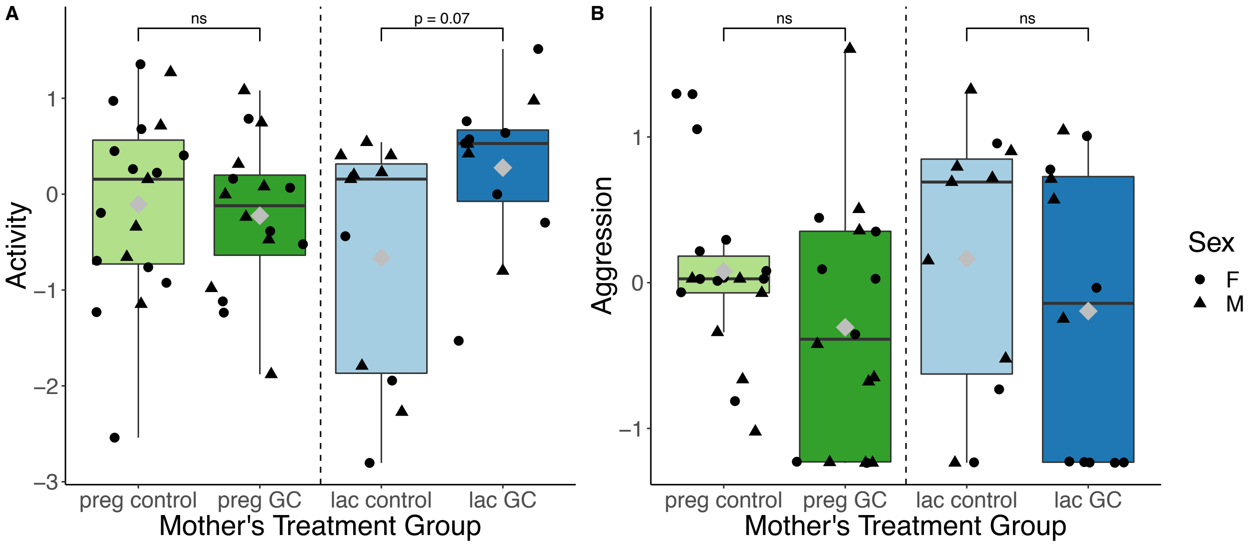
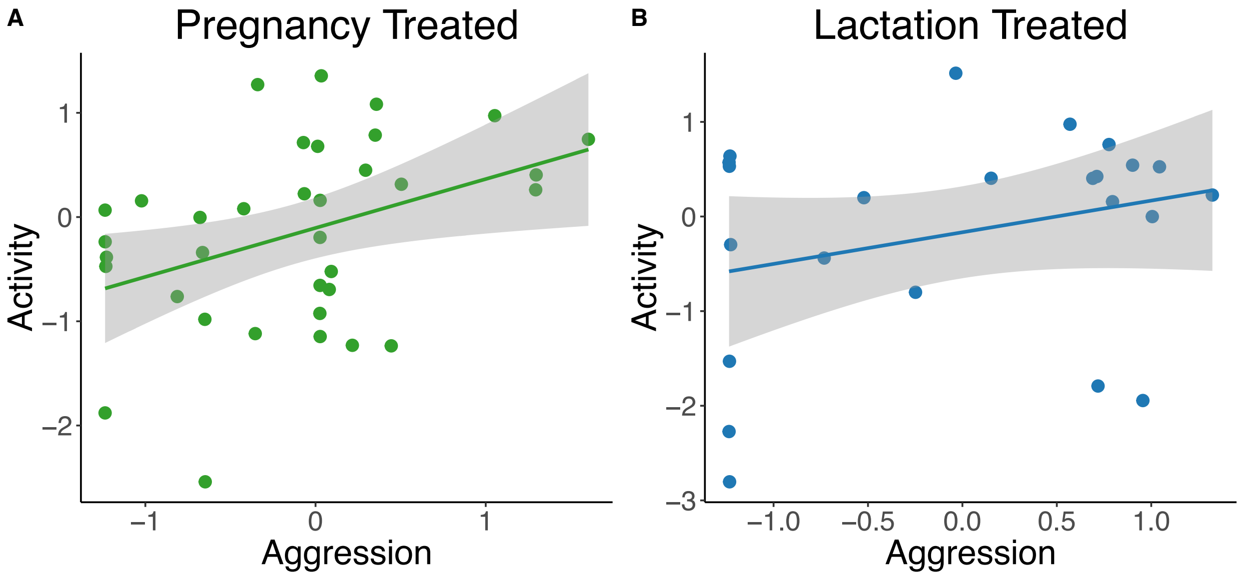


Figure 4.5 Relationship between 'activity' and 'aggression'

The relationship between behavioral traits (activity and aggression) for the two treatment groups, pregnancy (A) and lactation (B) treated individuals, was positive, though not statistically significant (Table 5). There was no significant interaction between treatment and aggression on activity levels (Table 5), and so GC and control individuals for each period (pregnancy or lactation) were combined and only the main effect of aggression was plotted. Grey shaded areas indicate 95% confidence intervals of the fitted regression line.



Chapter 5 Discussion

In this dissertation, I examined the relationship between the HPA axis and behavior in North American red squirrels. In Chapter 2, I investigated the relationship between the HPA axis and three standardized behavioral trait measurements in this study system. The results of this study suggest that one measure of the HPA axis and these behavioral traits are unlinked, despite evidence in other systems for these specific behavioral traits to be linked by the pleiotropic nature of hormones like cortisol. This surprising disconnect between personality traits and HPA axis dynamics could leave the door open for the maternal environment to impact both the HPA axis and personality traits separately. In laboratory rodents and humans HPA axis dynamics are known to be shaped by early life experiences, particularly maternal grooming and nursing. This developmental "programming" in turn impacts behavioral traits, such as anxiety-like behavior. In a species with no clear relationship between the HPA axis dynamic and personality traits, how would the maternal environment impact either phenotype or the relationship between these traits? In Chapter 4, I examined this question using a GC manipulation experiment of breeding females. By supplementing pregnant or lactating mothers with GCs, I investigated the role of GCs as a specific mechanism offspring may respond to in shaping their HPA axis and behavior during development. From eloquent and thorough laboratory experiments, we understand mothers may impact offspring through multiple mechanisms. In addition to hormones, one critical mechanism is her maternal behavior and how attentive she is to her offspring. To test how maternal behavior shapes life history, in Chapter 3, I asked how maternal attentiveness relates to growth rate and survival of offspring.

I found slightly conflicting results about the behavioral and physiological stress responses in adult and juvenile red squirrels. While the results of my analyses of adult behavioral trials and HPA axis activity suggest the two are not linked, the results from the juvenile HPA axis hormone challenges and behavioral trials provide support for the unidimensional model where the physiological and behavioral stress responses are connected. Two of the major differences between these two studies are the life history stage of the focal individuals (adult vs. juvenile) and the methods used to measure the HPA axis dynamics (fecal glucocorticoid metabolites vs. DEX/ACTH challenge). In the conclusions of Chapter 4, I address how these differences may shape our results to produce these contradictory findings. Red squirrels face strong natural selection pressures between these two life history stages. These selection pressures may shape our results by selecting for individuals that are not constrained by the two stress responses being linked or correlational selection favors juveniles with linked stress responses. Additionally, red squirrel personality traits are less extreme in adults than they were as juveniles—they regress to the population mean. Perhaps these behavioral traits are more plastic through life history stages than the HPA axis dynamics. Additionally, while these methods of measuring HPA axis activity are theoretically comparable, perhaps the acute HPA axis response relates more closely to the behavioral response than the ‘baseline’ HPA axis response. Fecal glucocorticoids may not have the appropriate resolution to detect these relationships. I am beginning to test these ideas using data from HPA axis challenge dynamics and behavioral traits in adult squirrels.

As with all science, this dissertation opens more questions than it answers. Some of these questions I have had the opportunity to pursue during my time as a PhD student, but others are for future students to answer. For example, while we see no functional differences in the dynamics of the HPA axis in offspring from GC-treated mothers compared to control (Chapter 4), the extensive literature about the impact of maternal GCs on the development of the HPA axis suggests we may find still differences at the molecular level in the neuroendocrine mechanisms. To this end, I collected brains from the GC experiment offspring to probe for gene expression differences in the neural transcriptome between the treatment groups. From these brains, I dissected the hypothalamus and hippocampus for RNA-seq analyses. This will be the first study to use the red squirrel neural transcriptome.

The differences in the growth rates between pregnancy-treated offspring and lactation-treated offspring led me to wonder whether mothers are buffering the concentration of GCs they transfer via milk or whether the exogenous increase in circulating GCs translates directly to an increase in GCs in the milk. To start to answer this question, I first need to develop methods to collect milk from red squirrels and determine how to reliably detect concentrations of cortisol in the milk. This project is still underway but could open the door for future maternal investment studies to measure differences between mothers in the energy they provide through milk.

These are only two of the many questions left lingering after these studies. We still have yet to determine who pays the cost for faster growth rate—do fast-growing offspring live shorter lives or does mom pay with a reduced investment in future litters? Dantzer *et al.* (2020) begins to explore this from a proximate lens where we find increased maternal GCs surprisingly do not induce oxidative stress or telomere shortening in offspring. The costs paid by the mother are still relatively unexplored. We also have few insights into the role of *paternal* stress on red squirrel offspring. Despite providing no parental care, fathers can still impact offspring development through molecular mechanisms in sperm (e.g. Dias & Ressler, 2014).

Measuring latency to retrieve pups after a nest intrusion is only scratching the surface at quantifying maternal care behavior. While I was able to collect preliminary accelerometer data on the behaviors and overall activity levels of lactating mothers, logistical and technical challenges throughout my PhD tenure limited my measurement of in-nest maternal behavior, specifically in using accelerometers and temperature loggers, but this is an avenue of research that should be explore more in the future. It would also be useful to measure other behavioral traits in mothers, in addition to retrieval latency, to determine how maternal styles correspond with other aspects of personality. I am also interested in understanding how motherhood shapes the personality traits we measure in red squirrels. For instance, mothers actively caring for pups often have a reduce fear response, how does this translate into maternal aggression or boldness when a mother is lactating vs. non-breeding? Essentially, how plastic is personality in response to infant caretaking?

One important limitation of the studies included in this dissertation is that none of the datasets included white spruce mast years. Red squirrels are able to anticipate when white spruce will mast in the upcoming fall. In anticipation, mothers produce larger litters and multiple litters through the summer, even nursing into late fall. To produce these multiple litters, breeding female squirrels must mate very soon after the birth of their first litter, meaning they are pregnant and lactating at the same time. In other mammals, lactation often suppresses ovulation leaving open the question of how, behaviorally and physiologically, red squirrels mate while lactating considering the duration of lactation is longer than gestation. Also, anticipation of the mast alters many normal behaviors, for example males commit infanticide, mothers nest off-territory more often, territory boundaries break down, and squirrels become more difficult to capture in traps. Monitoring maternal behaviors during this process could produce fascinating insights into behavioral plasticity in response to an increased risk to pups. Do we never see infanticide occur during non-mast years because mothers are better at protecting their pups on their territory or does moving pups off-territory reduce the risk of infanticide during mast years? Additionally, mast years are typically years of low food availability during breeding, due to the extreme reduction in spruce seed production in the years between masts. How does this food availability shape the energetic resources mothers can invest in pups? The work in this dissertation is only a stepping-stone towards more discovery of how the natural world works. There are still many avenues of maternal behavior left to investigate (in this species and beyond) both proximate and ultimate.

Through my research and teaching, I have considered the role of mothers (and fathers) more deeply. Though most of my results center around offspring, through the process of collecting these data and reading the literature, I have expanded my appreciation of motherhood. Traditionally, research about mothers, including the work in this dissertation, is offspring-centered, rather than focused on the mother herself. As Hinde (2015) argues, “The focus on motherhood and the importance to infants, however, risks depriving mothers of personhood. Emphasizing the maternal ‘exalts women as mothers and not women qua women’ (Waggoner, 2013).” To advance our understanding of the process and impacts of motherhood, I believe we need to incorporate more diverse viewpoints that consider the individual mother in addition to the implications for the offspring. As more underrepresented genders and racialized groups enter academia, I hope the conversation around motherhood shifts from “mom-blaming” for adverse outcomes in offspring to a more comprehensive view of the costs and benefits of motherhood for the mother herself.

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