

ADAPTIVE ENDOCRINE AND BEHAVIORAL RESPONSES OF FREE-LIVING RED
SQUIRRELS TO ENVIRONMENTAL VARIATION

By

Ben Dantzer

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All graduate students come to a point once or many times during their extended academic careers where they ask themselves whether they have made the right career choice. One of the most critical junctions when I asked myself this question came in the spring of 2006. During this time I was just finishing up a Master's degree in biology on the chemical ecology of red-backed salamanders. I was also furiously applying for field and laboratory jobs so that I could gain additional experience and justify to myself that this career choice was the right one. One of the most interesting jobs I applied for was as a field technician on the Kluane Red Squirrel Project working in the Yukon, Canada on red squirrels with Andrew McAdam. Although I was offered better paying positions, I was extremely intrigued and accepted this position not only because I have always been obsessed with northern latitudes and remote locations but also because of the potential to continue on as a PhD student on this project under Andrew McAdam.

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CHAPTER 1

General Introduction

Role of phenotypic plasticity in adaptive evolution

A fundamental question that permeates all of biology is how organisms adapt to changing environments. The traditional view of adaptive evolution outlined by the Modern Synthesis of evolutionary biology specifies that evolution via natural selection can only occur when genetic variation underlies phenotypic differences that covary with fitness (Mayr and Provine, 1980; Mayr, 1993). Here, only those phenotypic differences that are heritable within a genetic lineage can result in an evolutionary response to selection (“genes are leaders in evolutionary change”: West-Eberhard, 2003) and genotypes are thought to map directly to phenotypes (Pigliucci, 2010). Selection on phenotypic variation that is adaptive but environmentally induced or due to other non-genetic causes is not thought to cause an evolutionary response (Endler, 1986) and some have indicated that it could actually hinder adaptive evolution by preventing the exposure of genetic variation to selection (Falconer, 1981; Ghalambor et al., 2007).

However, an alternative view on adaptive evolution and the non-genetic mechanisms of inheritance has increasingly challenged this historical view of adaptive evolution, and some have even called for an extension or revision of the Modern Synthesis that includes the role of phenotypic plasticity in adaptive evolution (e.g., Rollo, 1995; Carroll, 2000; West-Eberhard, 2003; Pigliucci, 2007; Carroll, 2008; Pigliucci and Müller, 2010). This alternative view acknowledges that 1) much of the novel phenotypic variation available for natural selection comes from non-genetic sources such as phenotypic plasticity induced by environmental variation (“genes as followers in

evolutionary change”: Pigliucci, 2001; West-Eberhard, 2003) and 2) these phenotypic differences induced by the environment can be transmitted across generations by both genetic (Pigliucci and Murren, 2003; Räsänen and Kruuk, 2007; Crispo, 2008) and non-genetic (Jablonka and Lamb, 2005; Bossdorf et al., 2008; Bonduriansky and Day, 2009; Richards et al., 2010) mechanisms. A major focus of this alternative view is that phenotypic plasticity is thought to facilitate adaptation to changing environments (Schlichting and Pigliucci, 1998; West-Eberhard, 2003; Crispo, 2008; Pfennig et al., 2010).

Phenotypic plasticity exists when the same genotype gives rise to different phenotypes in different environments (Schlichting, 1986; West-Eberhard, 1989; Schenier, 1993; Pigliucci, 2001). The manner in which the phenotype of an individual or its genotype changes across environments is typically represented as its reaction norm (Schlichting and Pigliucci, 1998). For continuously distributed traits, reaction norms are usually visualized by plotting a line connecting the phenotypic values observed from each individual or its genotype (y-axis) when exposed to an environmental gradient (x-axis). Each line connects the phenotypic values for the individual or genotype in each environment and phenotypic plasticity exists when the slopes of these lines are different from zero. Non-parallel lines (i.e., when the rank of the phenotypic value of the genotype varies with the environment) imply that the individuals or genotypes respond differently to the environmental gradient. This among-individual or genotype variation in plasticity could be due to additive genetic variation (genotype x environment interaction: $G \times E$) or another non-genetic cause (e.g., permanent environmental effects: Nussey et al., 2007). Although there was some controversy in the past, most now agree that

phenotypic plasticity is a quantitative trait that is potentially heritable and can evolve via natural selection (Stearns, 1989; Via et al., 1995; Pigliucci, 2001; Scheiner, 2002; DeWitt and Scheiner, 2005).

The ability of an organism to alter its morphological, physiological, or life history traits in response to the prevailing environmental conditions has long been recognized as being intuitively advantageous to cope with changing environments (e.g., Gause, 1947; Bradshaw, 1965). Accordingly, theoretical models predict that when environments are heterogeneous, polymorphic phenotypes may be favorable when a single phenotype is not the most fit in all environments (Levins, 1962, 1963, 1968; Moran, 1992). Whether these polymorphisms are genetically-based or induced by phenotypic plasticity depends upon the grain of the environment (Levins, 1968). In temporally or spatially coarse-grained environments in which there is environmental heterogeneity but organisms experience only one of several environments and no phenotype achieves the highest fitness under all environments, genetically-based polymorphisms may be favored (Levine, 1953; Levins, 1962, 1968; Moran, 1992; Sinervo and Svensson, 2002). On the other hand, selection is expected to favor the evolution of adaptive phenotypic plasticity to cope with heterogeneous environments when 1) individuals experience several different environmental conditions during their lifetime (i.e., temporally or spatially fine-grained environment), 2) there is reliable environmental information, 3) different phenotypes are favored in the different environments, and 4) there is no single phenotype that is favored in all environments (Levins, 1963, 1968; Via and Lande, 1985; Lively, 1986).

Despite these cogent theoretical underpinnings, the role of phenotypic plasticity in adaptive evolution has remained contentious (Via et al., 1995; DeWitt et al., 1998; West-Eberhard, 2003; de Jong, 2005; Ghalambor et al., 2007; Pfennig et al., 2010). Phenotypic plasticity is not always adaptive, as it can also be neutral or even maladaptive (Conover and Schultz, 1995; de Jong, 2005; van Kleunen and Fischer, 2005; Ghalambor et al., 2007; Crispo, 2008). Further, not all traits or organisms are highly plastic, which is unlikely to be due to a lack of additive genetic variation (Scheiner, 2002; DeWitt and Scheiner, 2005), but may perhaps be due to costs associated with phenotypic plasticity (Auld et al., 2010) or because genotypes need to buffer themselves to some degree from the environment to produce specific phenotypes (DeWitt et al., 1998; Ghalambor et al., 2007).

Nonetheless, the potential role of phenotypic plasticity in facilitating adaptive evolution is both promising and exciting. However, it has received less attention from evolutionary biologists than understanding the genetics of adaptation. This is surprising given that theoretical (Baldwin, 1896) and empirical (Goldschmidt, 1940; Waddington, 1942; Schmalhausen, 1949) assessment of the role of phenotypic plasticity in adaptive evolution was underway before and during the formulation of the Modern Synthesis of evolutionary biology (reviewed by Pigliucci and Murren, 2003). The lack of focus is largely because research about how adaptive phenotypic plasticity can facilitate adaptive evolution has had to overcome its presumed Lamarckian overtones. How can the phenotypic variation that is induced by environmental stimuli become genetically-based?

Among those that study phenotypic plasticity, there is actually little controversy about this question. Phenotypic variation induced by the environment can become genetically fixed when the phenotypic variation is genetically accommodated (stabilized) and/or assimilated (West-Eberhard, 2003; Braendle and Flatt, 2006). Genetic accommodation is the process by which novel phenotypes that are induced by the environment (and also genetic mutations) expose cryptic genetic variation that allows the induced phenotype to be shaped into an adaptive phenotype via quantitative genetic changes (West-Eberhard, 2003; Gibson and Dworkin, 2004; Moczek, 2007). Phenotypes induced by a recurrent environmental stimulus can be genetically accommodated when quantitative genetic changes 1) increase the sensitivity of the organism to the environmental signals, 2) exaggerate the phenotypic response to the environmental signal, 3) ameliorate the negative effects, and 4) heighten the positive effects of the induced phenotype by increasing its overall integration with other phenotypic traits (Pigliucci, 2001; West-Eberhard, 2003). If the environmentally-induced phenotype no longer requires the environmental stimulus to be expressed (i.e., is no longer plastic), it becomes genetically assimilated and the induced phenotype becomes fixed or canalized (Waddington, 1961; Pigliucci and Murren, 2003; Price et al., 2003; Pigliucci et al., 2006). Nonetheless, studies of the role of genetic accommodation and assimilation in adaptive evolution are rare (but see Waddington, 1961; Yeh and Price, 2004; Suzuki and Nijhout, 2006, 2008; Badyaev, 2009).

Parental effects as a mechanism of adaptive phenotypic plasticity

Parental effects represent one potentially important mechanism of adaptive phenotypic plasticity by which organisms can cope with changing environments.

Parental effects have been broadly defined (Mousseau and Fox, 1998; Uller, 2008; Badyaev and Uller, 2009; Wolf and Wade, 2009), but here parental effects are defined as the influence of the parental genotype or phenotype on offspring phenotype in addition to their direct genomic contribution (Rossiter, 1996; Mousseau and Fox, 1998). They are potentially of extreme importance in understanding the role of phenotypic plasticity in adaptive evolution (Mousseau and Fox, 1998; Badyaev, 2008; Badyaev and Uller, 2009) because they enable organisms to induce adaptive changes in offspring phenotype according to the prevailing environmental conditions, and then transmit the mechanisms that cause these changes across generations (“transgenerational phenotypic plasticity”: Bernardo, 1996; Mousseau and Fox, 1998; Wolf et al., 1998; Agrawal et al., 1999; Galloway, 2005). For example, variation in the maternal behavior of laboratory rats can have profound effects on offspring neuroanatomy, physiology, and behavior (Meaney, 2001). These changes can then be preserved within genetic lineages via epigenetic mechanisms that facilitate the persistence of this variation in maternal behavior (Champagne, 2008). Although this now classic example still lacks information on whether ecologically relevant stimuli trigger the plasticity in maternal behavior and whether the changes in offspring phenotype are adaptive in natural populations, it demonstrates that parental effects are a heritable mechanism that enables modification of offspring phenotype.

Parental effects are widespread across taxa and they can have profound effects upon offspring phenotype (Mousseau and Fox, 1998; Groothuis et al., 2005). An important attribute is that they essentially enable parents to transmit information about the current environment to their offspring. If the parental environment can be used to

predict the environment that offspring will experience at independence, parental effects may allow parents to modify offspring phenotype adaptively for the anticipated environment (Marshall and Uller, 2007; Uller 2008). Because previous studies in natural populations have demonstrated that some parental effects are adaptive (Badyaev et al., 2002), heritable (McAdam et al., 2002; Räsänen and Kruuk, 2007), and can affect the rate of evolution (McAdam and Boutin, 2004), parental effects could not only induce adaptive phenotypic plasticity, but also enable relatively rapid evolutionary responses to changing environments (Agrawal et al. 1999; Räsänen and Kruuk, 2007; Badyaev, 2008).

Hormone-mediated maternal effects in mammals

In mammals, variation in the ecological or social environment that elicits a neuroendocrine response in breeding females can induce considerable transgenerational phenotypic plasticity via prenatal hormone exposure (Maestripieri and Mateo, 2009). For example, variation in the social environment such as an elevated frequency of interactions between conspecifics that may occur under high population density may affect concentrations of circulating androgens (testosterone: Wingfield et al., 1990; Cavigelli and Pereira, 2000; Hirschenhauser and Oliveira, 2006) and glucocorticoids (stress hormones: Christian, 1961; Rogovin et al., 2003; McCormick, 2006). These changes in the levels of circulating hormones in gestating females can then serve as a bridge between the maternal/outside environment and offspring phenotype. Variation in prenatal hormone exposure caused by the changes in maternal hormone levels can have lifelong consequences on offspring morphology, physiology, neuroanatomy, and behavior (Clark and Galef, 1995; Welberg and Seckl, 2001;

Groothuis et al., 2005; Maestriperi and Mateo, 2009). As such, hormone-mediated maternal effects may enable adaptive modification of offspring phenotype to the anticipated environment (Dufty et al., 2002; Mazuc et al., 2003; Lancaster et al., 2007). These modifications in offspring phenotype may persist across multiple generations via nongenetic mechanisms such as via epigenetic programming of neuroendocrine traits (Seckl and Meaney, 2004; Meaney et al., 2007; Champagne, 2008) or other permanent environment effects (Lindström, 1999; Descamps et al., 2008). On the other hand, if individuals differ in their hormonal response to a recurrent environmental stimulus and their sensitivity and hormonal response to the stimulus is heritable, the phenotypic variation created by prenatal hormone exposure could provide the raw material to enable evolutionary change and adaptation to changing environments.

Focus of Dissertation

The overarching goal of my dissertation research was to determine if hormone-mediated maternal effects can facilitate adaptation to changing environments by functioning as a mechanism of transgenerational phenotypic plasticity. I focused on the neuroendocrine mechanisms that generate phenotypic variation in response to ecological stimuli, and whether the changes induced in offspring phenotype via early hormone exposure were adaptive. I aimed to discover whether a major source of phenotypic plasticity generated during development in mammals (early hormone exposure) modifies offspring phenotype adaptively according to the social environment. I tested the hypothesis that information about the competitive environment (population density) acquired during pregnancy and early lactation is used by females to adaptively

modify offspring phenotype through hormone-mediated maternal effects in free-ranging North American red squirrels (*Tamiasciurus hudsonicus*).

Why Test this Hypothesis in North American Red Squirrels?

North American Red squirrels are an ideal species in which to examine whether transgenerational phenotypic plasticity induced by hormone-mediated maternal effects can facilitate adaptation to a changing environment for the following reasons. First, red squirrels in my study area in the Yukon, Canada live in a variable environment in which there are recurrent fluctuations in their major food source (seeds from conifer cones). These resource pulses create variable selection for the competitive ability of offspring over vacant territories (McAdam and Boutin, 2003; McAdam et al., in prep). Second, based upon their lifespan (up to 8 years: McAdam et al., 2007) and the frequency of resource pulses (~2-4 years: LaMontagne and Boutin, 2007), female red squirrels are likely to reproduce under a variety of environmental conditions in which selection on offspring phenotype varies. Females essentially experience a fine-grained environment because they can experience both high and low densities during reproduction. It is important to note that offspring experience a coarse-grained environment because they only experience density during recruitment once. However, I focused on how females can use adaptive plasticity in maternal effects to cope with this fine-grained environment. Third, the maternal environment during gestation is strongly correlated with the competitive environment offspring will encounter upon independence; this may potentially allow females to transfer reliable environmental information to offspring (Marshall and Uller, 2007). Fourth, there is density-dependent selection on offspring

phenotype such that different offspring phenotypes are favored in different environments (McAdam et al., in prep). When there is heightened competition for vacant territories (high population density), there is strong positive directional selection on offspring growth rate (McAdam and Boutin, 2003). However, when there is less competition for vacant territories, directional selection on growth rates is relaxed or even negative (McAdam and Boutin, 2003). Finally, the ecological cues (population density) that may induce transgenerational phenotypic plasticity in this species can be experimentally manipulated (Dantzer et al., in press). Therefore, I was able to test my hypothesis experimentally because I could manipulate population density with or without food-supplementation, which has rarely been done in natural populations (but see Svensson and Sinervo, 2000; Calsbeek and Smith, 2007).

Overview of North American Red Squirrels and Study System

Red squirrels are a relatively small sized (adult body mass averaging 200-250 g) tree squirrel that is distributed across most of the northern United States and into Canada ranging from the west to the east coasts (Steele, 1998). Red squirrels are primarily granivorous, consuming the seeds of conifers and other deciduous trees, but will also consume fungi (both mushrooms and truffles), other vegetative matter (tree buds, bark, fruits), invertebrates (Pretzlaw et al., 2006), and even animal matter such as snowshoe hare leverets and nestling birds (Layne, 1957; Smith, 1968; Krebs et al., 2001; Willson et al., 2003). In the southwest Yukon, the seed contained in white spruce (*Picea glauca*) cones are the primary food source for red squirrels (Fletcher et al., 2010; Boutin et al., unpublished data).

Red squirrels are diurnal and active year round. Although they live in areas with extreme low temperatures, they do not hibernate (Pauls, 1978; Woods, 2009). Instead, red squirrels during winter appear to rely upon their highly insulated nests in addition to behavioral strategies such as only being active during the warmest part of the day to limit energetic costs associated with thermoregulation (Woods, 2009).

Red squirrels in the southwest Yukon are seasonal breeders and, in most years, females are in behavioral estrous for 1 day and produce 1 litter after a ~35 day gestation period, and have a ~70 day lactation period. In some years, in anticipation of the autumn mast of spruce cones, females will produce 2 successful litters (Boutin et al., 2006). Red squirrels have a scramble-competition mating system in which females are receptive to mating attempts from multiple males during their periods of behavioral estrous (Lane et al., 2008). Generally, a female is in estrous for 1 day per reproductive bout. During their entire day of estrous, territoriality is relaxed and multiple male squirrels chase the females around her territory or other areas, emit distinctive vocalizations (buzzes: Smith, 1978), and attempt to copulate with her (“mating chases”: Lane et al., 2008; McFarlane et al., 2011). During these periods of estrous, females copulate with around 7 males and litters consist of offspring with multiple fathers (Lane et al., 2007, 2008; McFarlane et al., 2011). After a ~35 day gestation period (Steele, 1998; Lane et al., 2008), females produce a litter ranging from 1-7 pups (McAdam et al., 2007). Offspring generally first emerge from their natal nest at around 40 days post-parturition but are nursed by their mothers up until 70 days post-parturition (Humphries and Boutin, 1996; Steele, 1998). Females rarely produce more than one successful litter

per year except in years in which they anticipate the availability of food resources (Boutin et al., 2006).

Red squirrels in the southwest Yukon experience episodic pulses of their major food source, seeds from white spruce trees. Years of high cone production (mast years) are followed by years of no or little cone production (LaMontagne and Boutin, 2007). In the autumn of each year, red squirrels harvest spruce cones and cache them in a larder hoard (“midden”) located in the center of their territory (Fletcher et al., 2010), which they defend vigorously using territorial vocalizations called rattles (Smith, 1968, 1978).

Fluctuations in population density driven by resource pulses generate density-dependent selection on offspring phenotype (McAdam et al., in prep). As population density during the spring breeding season increases, so does the number of juveniles competing over each vacant territory. Juveniles that do not acquire a territory prior to experiencing their first winter generally do not survive (Larsen and Boutin, 1994). Previous studies in this study system have found that offspring growth rates (measured as the change in mass between birth and 25 d post-parturition) are an important predictor of successful acquisition of a territory, recruitment, and overwinter survival (McAdam and Boutin, 2003). In years of high population density, high offspring growth rates are under strong positive selection, but in other years of varying population density, offspring growth rates are under either no or even negative selection (McAdam and Boutin, 2003; McAdam et al., in prep). Other physiological or behavioral correlates of the competitive ability of emerged juveniles are unknown.

Summary of General Methods

Study area

All of my study areas were located in a forested glacial valley (Shakwak Trench) in the southwest Yukon, Canada (61° N, 138° W) near Kluane National Park. The flora and fauna in this area have been well documented through the long-term Kluane Boreal Forest Ecosystem Study (Krebs et al., 2001). The dominant vegetation in this area consists of white spruce (the only conifer in this area) with patches of willow (*Salix* spp.) and trembling aspen (*Populus tremuloides*; see Krebs and Boonstra, 2001 for a description of the area). Other common mammalian herbivores in this area are snowshoe hares (*Lepus americanus*), least chipmunks (*Tamias minimus*), and arctic ground squirrels (*Spermophilus parryii*). Common predators of red squirrels in this area are Harlan's hawks (*Buteo jamaicensis*), goshawks (*Accipiter gentilis*) and Canadian lynx (*Lynx canadensis*). The climate in this area is cold, with January being the coldest month (-22 C) and July being the warmest month (10.8 C: Burwash Landing Climate Station, Yukon). Most of the precipitation that falls in this area is snow, and typically the ground is covered in snow from October until mid-May.

As part of a long-term project in the southwest Yukon, Canada (Kluane Red Squirrel Project), the survival and reproduction of individual red squirrels has been followed from 1987 to the present (Boutin et al., 2006; McAdam et al., 2007). During my study from 2006-2011, the reproduction of individual male and female red squirrels ($n > 600$) on 6 different study areas (Agnes, Chitty, Joe, Kloo, Lloyd, and Sulphur) was followed using live-trapping and behavioral observations (see below). I also performed experimental manipulations during my dissertation research on 3 additional study areas

(Back of Agnes, Blue Trailer, and Rolo: described in Chapter 6). All of my study areas were spread out along the Alaska Highway and were located within 9 km of each other. All of the study areas were staked and/or flagged at 30 m intervals to enable the recording of spatial locations of live-trapping or behavioral observations.

Live-trapping procedures

Squirrels were live-trapped using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) baited with peanut butter from March-August in each year to assess and track reproductive condition of individual male and female squirrels, identify territory ownership, and determine recruitment of the offspring from the previous year (see McAdam et al. 2007 for details). During each capture event, the identity of squirrels was determined by reading their uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA) that were applied when they were temporarily removed from their natal nest around 25 d of age. Squirrels were weighed, sexed, and the reproductive condition of males (scrotal or abdominal testes) and females (pregnant, lactating, or neither) was determined by palpating and/or checking nipple condition in females. If available, a fecal sample was collected during every live-trapping event. Fecal samples were placed into 1.5 mL microcentrifuge tubes until they were transferred to a -20 °C freezer. Fecal samples collected in the winter months (January-April) were generally frozen upon collection. In the warmer months (May-September), fecal samples were placed into an insulated container with wet ice until they were placed in the freezer. I have previously found that the period of time between collection and placement in the freezer did not influence fecal cortisol or androgen metabolite levels (Dantzer et al., 2010; Dantzer et al., 2011).

Measuring behavior

Red squirrels are amenable to examining relationships between population density and behavior because they are diurnal, visually and acoustically conspicuous, and readily habituate to the presence of humans. All red squirrels inhabiting my study areas had small pieces of colored wire in unique combinations threaded through their ear tags so that they could be identified during behavioral observations. The behavior of red squirrels was recorded through both 1) casual or ad libitum sampling from 1989-2008 and 2) focal sampling of individually marked squirrels that were radiocollared (model PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada) over 10 years from 1994-2008. Casual observations of agonistic interactions between adult squirrels were recorded ad libitum (Altmann, 1974) whenever observers were live-trapping or specifically recording behavioral observations on study grids.

I categorized behaviors in the same way as in previous studies on red squirrels (Stuart-Smith and Boutin 1995; Humphries and Boutin, 2000; Anderson and Boutin 2002) including whether the squirrel was in or out of its nest, feeding, foraging, travelling, resting, interacting with adult conspecifics, vigilant, vocalizing (“barking” and “rattling”: Smith 1968) or out-of-sight (not visible in a tree). Barking is an alarm call where rattling is a territorial vocalization (Smith 1968). Vigilance was observed when a squirrel was alert but inactive (not mobile).

In most years, behavioral data were collected in the same manner. However, because I was interested in how population density affected the frequency of territorial behavior, I recorded supplemental information during the behavioral observations

recorded in 2008. In 2008, I recorded in a continuous fashion (all-occurrences: Altmann, 1974) the total number of rattle vocalizations emitted by the focal squirrel.

Collection of life history data

In all years, female and male reproduction were monitored through a combination of frequent live-trapping, behavioral observations, locating nests using radio telemetry, and paternity analyses. Male reproductive success was measured using paternity analysis of microsatellite markers (Lane et al. 2008), and parameters of female reproduction (litter size, offspring growth rates) were determined by accessing pups from their natal nest soon after their birth and at ~25 d post-parturition (McAdam et al., 2007). When the pups were accessed from their nest, they were sexed and weighed. In some cases, I used digital calipers to measure hind foot length, zygomatic arch width, and anogenital distance of pups when they were ~25 old. Pups were permanently marked prior to first emergence from their natal nest using uniquely numbered metal ear tags. Juvenile dispersal from the natal territory is low (often <100 m: Berteaux and Boutin, 2000), and therefore recruitment of offspring can be accurately determined. As a result, I could accurately measure the reproductive success of female (number of offspring surviving to 200 d post-parturition) and male (number of offspring sired) squirrels.

Measuring population density

Three of my six study areas were unmanipulated (“Control”) areas in which I measured population density, maternal hormones and reproduction. Two of the unmanipulated study areas (Kloo and Sulphur) have been monitored continuously since 1987, while a third unmanipulated study area (Chitty) has been monitored continuously

since 2006. These control grids have experienced natural fluctuations in food abundance and population density (Chapter 5).

Red squirrels are conspicuous, diurnal, highly territorial, and can be readily live-trapped. Therefore all individuals living on my study areas could be completely enumerated and population density could be estimated accurately. In May and August of each year, population density was determined by completely censusing the study areas using live-trapping and behavioral observations. Territory ownership was determined by repeated live-trapping of the same individual on a midden, or by observing the same individual emit territorial vocalizations on the midden. Squirrels were repeatedly live-trapped on their midden throughout each year and a change in midden ownership is thought to reflect mortality of the former owner. Unless they bequeath their midden to offspring, red squirrels generally keep the same midden throughout their lives (Berteaux and Boutin, 2000).

Completely enumerating all individual red squirrels living on my study areas allowed me to generate an estimate of study area population density for each year. There is clearly inter-annual variation in these large-scale estimates of population density that is driven by pulses of white spruce cone crops (Boutin et al., 2006). However, assigning these large-scale estimates of population density for an overall study area to each individual squirrel might overlook important local variation in population density (e.g., Garant et al. 2005, 2007). Therefore, I also used estimates of local density measured at many different spatial scales to determine how population density affected hormone levels, reproduction, the frequency of territorial intruders, and red squirrel behavior during natural fluctuations in local population density. Because the

spatial coordinates were recorded for all behavioral and trapping observations, I was able to calculate local density by determining the number of different middens that were owned by a single squirrel at many different spatial scales surrounding the midden of interest. I examined several different scales of density (e.g., 12 different spatial scales of radii from 25-300 m away from the midden of interest) and found that local population density measured at 150 m away from the center of the midden is the most appropriate measure of local density (Chapter 5; Dantzer et al., in press). This is not surprising given that the maximum distance at which rattles are heard by humans and presumably red squirrels is around 120-130 m (Smith, 1968; Shonfield, 2010).

Experimentally manipulating numerical population density

Population density on three of my study areas was experimentally increased using long-term food supplementation. This food addition has resulted in a near doubling of population density compared to the unmanipulated study grids in recent years and densities similar to the highest population densities recorded ever for these populations (Chapter 5). One of these study areas (Agnes) has been food-supplemented since 2004 while the other two, Lloyd and Joe, have been supplemented since 2005 and 2006, respectively. Almost all of the individual squirrels that own territories on the food-supplemented areas are provided with a bucket containing 1 kg of all natural peanut butter (no salt or sugar added) that is hung from a tree in the center of its midden. The peanut butter within the buckets is replenished systematically every ~6 weeks from October-May of each year while always ensuring that breeding female squirrels had access to supplemental food.

Experimentally manipulating perceived population density

Red squirrels are amenable to other experimental manipulations of density through playbacks of territorial vocalizations. Red squirrels are highly territorial and rarely physically interact with each other (Dantzer et al., in press). However, there is a strong positive relationship between population density and the frequency of territorial vocalizations ('rattles': Dantzer et al., in press). Therefore, in addition to the manipulation of population density using food-supplementation, I also manipulated perceived population density using persistent playbacks of red squirrel territorial vocalizations. I also exposed groups of female squirrels to avian vocalization playbacks or no playbacks at all.

Rattles used in these experiments were recorded from squirrels between 2005 and 2009 using a Marantz Professional Solid State Recorder (Model PMD660, Marantz Inc., Mahwah, NJ) attached to a Sennheiser shotgun microphone with K6 powering module and foam windscreen (Model ME66, Sennheiser Electronic, Wedermark, Germany). Rattles were recorded in the 0.01-22.5 kHz range and stored as uncompressed .wav files, which were each transferred to separate CDs for the playback experiments. Rattles were recorded at least 500 m away from where they were used as playbacks. Because territory fidelity is high and dispersal from the natal site is low in this species (Berteaux and Boutin, 2000), I assumed that the vocalizations used for the playbacks were at least from unfamiliar individuals and also likely from unrelated individuals.

I used songs and sounds (chips, seets) from boreal chickadees (*Poecile hudsonicus*) as heterospecific control vocalizations. In my study area in the Yukon,

boreal chickadees, ravens (*Corvus corax*), and gray jays (*Perisoreus canadensis*) are non-migratory and thus present during the red squirrels' breeding season. However, I used vocalizations from boreal chickadees because they appear to be the most abundant species during the red squirrel breeding season, they are non-predatory, and they are not known to compete with red squirrels over resources (personal observations). In contrast, due to their large size compared to chickadees, ravens and gray jays could depredate red squirrel nestlings or juveniles. I obtained 17 unique recordings of boreal chickadee songs and sounds from the Macauley Laboratory of Ornithology at Cornell University, open source recordings (www.xenocanto.com), as well as from commercially available sources (Bird Songs of North America, Monty Brigham). These sounds were recorded from boreal chickadees all across North America.

I used a total of 106 rattles from 87 different male and female squirrels that were recorded from between 2005 and 2009 to develop unique combinations of rattles from each of 4 different squirrels for each squirrel exposed to the rattle playbacks. This ensured that the rattle playback treatment squirrels were exposed to a unique combination of rattles (2 rattles recorded from males and 2 recorded from females). I developed 17 unique combinations of 4 chickadee vocalizations from the library of chickadee vocalizations I compiled. These steps were taken to avoid pseudoreplication that can occur in acoustic playback studies where the same set of playbacks are used for all animals (Kroodsma et al., 2001). To reduce the possibility of creating 'social instability' (Wingfield et al., 1990), each squirrel was exposed to the same set of rattle or chickadee playbacks during the entire experiment.

Vocalizations of 4 different red squirrels or 4 different chickadee recordings were broadcasted through 2 different portable speakers (Alec Lansing Orbit MP3-IM237) such that each speaker played 2 different recordings for approximately 12 hours per day. These speakers were placed 15 m away from the center of the midden. This distance was chosen to simulate an ambient increase in vocalization frequency rather than placing the speakers directly on the midden, which might be considered a territorial intrusion for the rattle playback squirrels. For the rattle playback squirrels, I simulated an increase of 4 additional neighboring red squirrels. I first determined that squirrels emit an average of approximately 1 rattle every 7 minutes using focal observation behavioral sampling (Chapter 5; Dantzer et al., in press). Thus, each rattle playback squirrel experienced 4 additional rattles from 4 different squirrels (2 males and 2 females) every 7 minutes. Similarly, each control squirrel experienced 4 additional boreal chickadee sounds every 7 minutes.

I used these playback experiments to manipulate perceived population density in two different years. In 2008, I performed a pilot study to assess the behavioral and hormonal consequences of these playback procedures. In this pilot study, I exposed squirrels to rattle playbacks ($n = 5$) and compared their behavioral and hormonal responses to the responses of squirrels that were not exposed to any playbacks ($n = 5$). Male and female squirrels were exposed to these playbacks for 12 h a day for ~40 continuous days. Chapter 5 uses some of these data to examine how the playbacks affected behavior.

In 2010, I conducted a large-scale playback experiment in which I manipulated the perceived population density experienced by breeding female red squirrels.

Breeding female red squirrels were exposed to rattle playbacks (n = 44 squirrels) or chickadee playbacks (n = 27 squirrels). Here, only female squirrels were exposed to the playbacks from mid-gestation until 5 d post-parturition (~34 continuous days: see Chapter 6). I chose this period of exposure because it appears to be the most sensitive period for maternal programming via prenatal hormone exposure in rodents (Welberg and Seckl, 2001). I monitored the behavior, hormones, and reproduction of these females exposed to the rattle and chickadee playbacks as well as females that were not exposed to any playbacks and those on a food-supplemented study area.

Outline and Summary of Data Chapters

Overview of chapters 2-4

A major portion of my dissertation work involved measuring maternal hormonal levels in response to variation in population density. In free-ranging animals, there are a number of advantages of measuring hormone levels in fecal samples versus repeated plasma samples (Sheriff et al., 2011). First, fecal hormone metabolite levels are not prone either to researcher-induced biases introduced by restraint/handling, or to short-term fluctuations in steroid hormone levels due to normal pulsatile changes in hormone secretion. For example, the time from the initial GC release due to a stressor until the signature appears in the feces (delay time) is much longer than the time the animal spends restrained or in a live-trap (fecal glucocorticoid metabolite levels change over a period of 6-24 hours depending upon the species - Palme et al., 2005). The second is that plasma steroid hormone levels are point of time estimates and can be heavily influenced by time of day due to circadian patterns or other short-term fluctuations due

to minor disturbances. Fecal hormone metabolite levels are thought to reflect an integrated average of plasma free steroid hormone levels that the animal has secreted, metabolized, and excreted over the course of a species-specific amount of time (depending on the frequency of defecation - Palme et al., 1996; Goymann, 2005; Palme et al., 2005). It is unclear whether fecal hormone metabolite levels represent an accurate estimate of baseline hormone levels or an integrated average of total steroid hormone levels experienced by the animal (e.g., Millspaugh and Washburn, 2004; Goymann, 2005). However a recent study definitively demonstrated that fecal hormone metabolite levels accurately reflect plasma free hormone levels (Sheriff et al., 2010).

Although there are many advantages of measuring hormone levels in fecal samples compared to plasma samples, the fecal assays require extensive validation procedures (Palme, 2005; Touma and Palme, 2005). The major focus of Chapter's 2 and 3 was to validate enzyme-immunoassays to measure fecal cortisol metabolite (FCM) and fecal androgen metabolite (FAM) levels in fecal samples collected from red squirrels. This work involved capturing red squirrels and holding them in captivity during the validation procedures as well as measuring FCM and FAM in many fecal samples collected from male and female red squirrels in the Yukon across a range of reproductive conditions.

Summary of chapter 2

In Chapter 2, I validated an enzyme-immunoassay (EIA) to measure FCM (cortisol is the major glucocorticoid in red squirrels: Boonstra and McColl, 2000) in fecal samples from red squirrels and also examined how FCM varied across the reproductive cycle in male and females. To validate the EIA, I first injected red squirrels with

radiolabeled cortisol. This radiometabolism study allowed me 1) to demonstrate that a significant amount of FCM was excreted in the feces, 2) determine the time delay until excretion of FCM, and 3) in conjunction with reverse-phase high performance chromatography (RP-HPLC), characterize the structure of the FCM and demonstrate that my EIA antibody reacted with the FCM. Secondly, I physiologically validated the EIA by demonstrating that FCM increased following either an injection of adrenocorticotrophic hormone (ACTH) that stimulates adrenal GC production (Boonstra and McColl, 2000) or a handling stressor. Finally, I found that reproductive condition affected FCM in females but not males. Similar to other studies, FCM increased during gestation, peaked around parturition, and then declined after parturition. This study was published in *General and Comparative Endocrinology* in 2010.

Summary of chapter 3

Although the major goal of Chapter 3 was to validate an enzyme-immunoassay (EIA) to measure FAM in fecal samples from red squirrels, I also examined in detail how FAM and maternal behavior varied across the reproductive cycle in females using 3 years of fecal sample data and 10 years of behavioral observations. As in Chapter 2, to validate the EIA I first injected red squirrels with radiolabeled testosterone so that I could 1) demonstrate that a significant amount of FAM was excreted in the feces, 2) identify the time delay until excretion of FAM, and 3) in conjunction with RP-HPLC, characterize the structure of the FAM and demonstrate that my EIA antibody reacted with the FAM. Secondly, I physiologically validated the EIA by demonstrating that effects of female reproductive condition on plasma androgen levels are mirrored in FAM. I further validated this EIA for male red squirrels in Chapter 4 using the same approach.

Finally, I found interesting patterns in maternal FAM and maternal behavior that suggested covariation between maternal androgens and behavior. I found that FAM increased after conception and parturition, peaked during mid-lactation around juvenile emergence from their natal nest, and then declined into lactation. Interestingly, the peak of FAM during mid-lactation coincided with a time period when nest use was lowest, but territory defense and time spent foraging were at their highest levels. In Chapter 3, I suggested that this apparent covariation between androgens and behavior supports the hypothesis that androgen concentrations drive behavioral trade-offs in breeding female red squirrels. This study was published in *Animal Behaviour* in 2011.

Summary of chapter 4

The ease of collection of fecal samples to measure hormone metabolite levels belies some of the difficulties associated with measuring fecal hormone metabolite levels in free-ranging animals (Buchanan and Goldsmith, 2004; Millspaugh and Washburn, 2004; Tuma and Palme, 2005; Sheriff et al., 2011). One important issue for studies in free-ranging animals that has received relatively little attention is how diet affects fecal hormone metabolite levels. In omnivores or herbivores, variability in consumption of plant fiber may be an important variable that affects FHM. For example, in humans, a high fiber diet can increase estrogen metabolite concentrations in the feces (Goldin et al., 1982; Pusateri et al., 1990), whereas in yellow baboons *Papio cynocephalus cynocephalus* (Wasser et al., 1993), it can decrease progesterone metabolite concentrations. In Chapter 4, I investigated how experimental changes in the diets of captive red squirrels affected FCM and FAM levels. Because I manipulated population density via long-term peanut butter supplementation, I was particularly

interested in how FCM and FAM levels compared between squirrels fed peanut butter and those eating white spruce seed. To identify how diet affect FCM and FAM levels, I initially fed a group of captive red squirrels the same diet and then switched their diets to either exclusively peanut butter or exclusively white spruce cones. I found that over time, FCM and FAM levels rapidly declined in squirrels fed peanut butter whereas those in squirrels fed white spruce cones increased over time. These differences may be due to differences in gut passage time associated with slight differences in dietary fiber content of peanut butter and white spruce seed. This study suggests caution in the interpretation of seasonal changes in FCM or FAM levels that are observed in free-ranging animals. Future studies should perform similar experiments to determine how dietary changes in free-ranging animals affect fecal hormone metabolite levels. This study was published in *General and Comparative Endocrinology* in 2011.

Summary of chapter 5

Density-dependent population regulation is a firmly rooted principle in ecological theory that permeates all biological disciplines (reviewed by Krebs and Myers, 1974; Sinclair, 1989; Krebs, 1996). The behavior of individuals has often been implicated as a key factor in density-dependent population regulation by contributing to spacing behavior or contributing to interference and exploitative competition over limiting resources (Chitty, 1967; Sinclair, 1989; Krebs, 1996; Mougeot et al., 2003). However, the fields of behavioral and population ecology have largely developed independently of one another (Sutherland, 1996), and detailed studies documenting the behavioral responses of individuals to variation in population density are actually relatively rare. In Chapter 5, I examined how the behavior of red squirrels over 10 years (from 1994 to

2008) was affected by natural variation in population density. I also examined how experimental increases in actual population density (using long-term food-supplementation) and perceived population density (using persistent audio playbacks of territorial vocalizations) affected red squirrel behavior compared to those living on control study areas with lower density, or those exposed to no playbacks, respectively. Finally, I examined how the frequency with which squirrels interacted acoustically and physically was affected by population density in behavioral and live-trapping data collected over the past 20 years. I found that red squirrels rarely physically interacted with one another, and that there was actually a significant negative relationship between population density and the frequency of territorial intrusions. However, population density was positively associated with the frequency with which squirrels emit territorial vocalizations, which suggests that they can accurately assess population density using the number of unique territorial vocalizations they hear in their local neighborhood. Red squirrels under naturally or experimentally increased actual or perceived population density spent less time in their nest and feeding, and more time being active and vigilant. Finally, this experiment demonstrated that we could successfully manipulate perceived population density using persistent audio playbacks of territorial vocalizations. This study was published in *Behavioral Ecology and Sociobiology* in 2012.

Summary of chapter 6

Chapter 6 was the product of much laboratory and field work and was only possible after completing the previous four data Chapters. In Chapter 6, I examined relationships among population density, maternal FCM and FAM levels, and offspring growth rate. I tested the hypothesis that hormonal responses of breeding female red

squirrels to social cues that reflect population density induce an adaptive hormone-mediated maternal effect on offspring growth rates. I tested this hypothesis using an observational and an experimental approach in which I manipulated actual (using long-term food-supplementation) and perceived (using persistent playbacks of territorial vocalizations) population density. I measured FCM and FAM levels in breeding female red squirrels across six years (2006-2011) during natural variation in local population density and experimental increases in population density using food-supplementation. I examined how local population density affected FCM and FAM levels, and compared FCM and FAM levels of females on control study areas to those of females on the high density food-supplemented study area. I then examined associations between maternal FCM and FAM levels and offspring postnatal growth rates. Finally, in one year (2010) I experimentally manipulated perceived population density using persistent audio playbacks of territorial vocalizations and compared the endocrine and reproductive (litter size, neonate mass, offspring growth rates) values to those of control females (exposed to no or chickadee playbacks) and those of females on the high-density food-supplemented study area. I found that there was a significant positive association between local population density and FCM and FAM levels; squirrels on the high-density food-supplemented study area had higher FCM and FAM levels than those on the control study area. I further found that squirrels exposed to heightened perceived density (rattle playbacks) had significantly higher FCM and FAM levels than did females exposed to the chickadee playbacks. These endocrine responses were adaptive because there was a significant positive interaction between FCM and FAM levels and offspring growth rates. Females exposed to heightened perceived density adaptively

increased offspring growth rates compared to those of control females (exposed to no playbacks or chickadee playbacks). Remarkably, the growth rates of offspring produced by females exposed to heightened perceived density were similar to those of females on the food supplemented high density study area. The results from this Chapter suggest that the frequency with which squirrels hear territorial vocalizations in their local neighborhood induces adaptive hormone-mediated maternal effects on offspring growth rates. Further, these results suggest that females adaptively modify offspring growth rates, not in response to increased food availability (which is generally associated with high density conditions), but in anticipation of future natural selection. The results from this study are currently in preparation for publication.

A Note about Writing Style of the Dissertation

Chapters 2-5 have already been published and the text included in this dissertation is exactly how it appears in print. However, Chapter 3 uses American English in this dissertation although it was published in British English because this is the style of the journal this Chapter was published in (*Animal Behaviour*). The title of Chapter 3 remains in the British English. All other Chapters use American English. Finally, I use “we” throughout this dissertation to recognize all of the contributions from my co-authors.

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CHAPTER 2

Dantzer, B., McAdam, A. G., Palme, R., Fletcher, Q. E., Boutin, S., Humphries, M. M., Boonstra, R. 2010. Fecal cortisol metabolite levels in free-ranging North American red squirrels: Assay validation and the effects of reproductive condition. *General and Comparative Endocrinology* 167, 279-286.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis plays a central role in enabling organisms to cope with and adapt to their external environment (e.g., Darlington et al., 1990). Unpredictable adverse stimuli that disrupt an organism from physiological homeostasis or its normal routine generally cause activation of the HPA axis (Levine and Ursin, 1991; Reeder and Kramer, 2005). Such stressors can potentiate the release of glucocorticoids (GCs) from the adrenal cortex downstream of the HPA stimulation (Sapolsky et al., 2000). Because GCs are released following HPA stimulation, levels of circulating GCs are often used as an index of the degree of stress experienced by laboratory and free-ranging animals (Möstl and Palme, 2002).

Laboratory animals have provided much insight into the functions of GCs and the effects of sex and reproductive condition on GC levels. Perhaps because laboratory studies are unable to replicate the multi-faceted contributors to abiotic and biotic seasonality in natural environments, many laboratory animals have much less or no seasonal variation in GC levels than free-ranging animals (Romero, 2002). Few studies have examined basic relationships between reproductive status and GC levels in free-ranging animals (Romero, 2002; Reeder and Kramer, 2005), which is surprising given that GCs and HPA activity can influence survival probability in the wild (e.g., Romero and Wikelski, 2001; Blas et al., 2007).

With the rapid development of non-invasive techniques to measure GC metabolites in feces and urine, determining the levels of GCs in free-ranging animals has become more feasible (Palme et al., 2005). Fecal sampling offers a number of advantages over blood sampling in natural populations. It is non-invasive as it does not

require blood-drawing and often does not require trapping and immobilization. This is especially pertinent in study populations in which repeated blood-sampling is not possible either during certain time periods (e.g., pregnancy) or because of small body size. Furthermore, fecal hormone metabolite levels represent an integrated average measure (depending on gut passage time: Palme et al., 2005) and are therefore less affected by transient increases in GC levels (Touma and Palme, 2005).

While the utility of fecal hormone metabolite assays in natural populations is clear, their use should not be indiscriminate (Touma and Palme, 2005). Only metabolites of glucocorticoids are excreted in feces and the metabolism of unbound plasma hormones in the liver and route of excretion (urine or feces) is species-specific (Palme et al., 1996; Palme et al., 2005). As such, immunoassays for measuring fecal hormone metabolite levels need to be carefully validated in a species-specific manner to ensure that the hormone of interest is being properly measured as a metabolite in the feces (Palme, 2005; Touma and Palme, 2005).

Our major aim in this study was to validate an immunoassay for measuring fecal GC (cortisol: Boonstra and McColl, 2000) metabolites in North American red squirrels (*Tamiasciurus hudsonicus*). We also documented the effects of sex and reproductive condition on fecal cortisol metabolite (FCM) levels in a completely enumerated population of free-ranging red squirrels. Red squirrels are an excellent study species to examine how reproductive condition affects FCM levels in free-ranging mammals because repeated live-trapping of individually-marked red squirrels provides both fecal samples for GC quantification and also detailed reproductive information about males

(when testes ascend and descend) and females (day of estrous, parturition, lactation, and weaning).

Materials and Methods

Capture and husbandry of squirrels for laboratory validation experiments

We captured 11 red squirrels (5 females, 6 males) from 4 to 5 January 2008 in Algonquin Provincial Park (APP: 45° 30', 78° 40') using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI, USA). Frequent trap checking prevented any squirrel from spending greater than 2 hours in a trap. Squirrels were transported to the Wildlife Research Facility at the University of Toronto Scarborough. At the facility, each squirrel was placed into its own radiometabolism cage (91.5 x 61 x 46 cm) that contained a stainless-steel nest box (with 1 x 1 cm mesh floor), cotton bedding, and provided with ad libitum food (apples, sunflower seeds, and peanut butter) and water (bottle with stainless-steel nipple). Squirrels habituated to these conditions for 5 d before we initiated any manipulations. Floors of the radiometabolism cages were slatted to allow urine and feces to fall freely to a pan underneath each cage. Pans were covered with metal screen (0.5 x 0.5 cm) to prevent feces and urine from contaminating each other. Squirrels were maintained at a temperature of ~10 °C and on a photoperiod that was changed weekly to correspond to the natural fluctuation in photoperiod in the location of capture at that time of year. All squirrels were, reproductively, quiescent upon capture, but the 6 males became scrotal within 26 days of capture. Upon completion of this study, all 11 squirrels had gained weight (mean $\pm$ SE: 15 $\pm$ 4.66 g) and were returned to the site of capture. Red squirrels were captured in APP under

permit #AP-08-0 and our protocol for the capture and housing of red squirrels from APP was approved in accordance with the guidelines of the Canadian Council on Animal Care by the University of Toronto Institutional Animal Care and Use Committee (#20006991).

Field methods for free-ranging red Squirrels

Fecal samples from red squirrels were collected during April-July 2006, January-August 2007, and February-August 2008 from a natural population near Kluane Lake in the southwest Yukon, Canada (61° N, 138° W) that has been monitored continuously since 1987 (see McAdam et al. (2007) for details). In most years, female red squirrels here are in estrous for 1 day, and produce 1 litter after a ~35 day gestation and a ~70 day lactation period (Boutin et al., unpublished data; Steele, 1998). In years of high food abundance, some females produce 2 litters (Boutin et al., 2006). Upon first capture, all squirrels were tagged with uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA). We determined the reproductive status of males (abdominal vs. scrotal testes) and females (pregnant, lactating, or neither) via palpation during live-trapping (McAdam et al., 2007). Fecal samples were collected from underneath the traps using forceps and placed individually into 1.5 mL vials. Upon parturition, we back-calculated the day of estrous to obtain estimates of days post-conception. Females were categorized as non-breeding if they did not become pregnant during that year. We only used fecal samples from squirrels that had not previously been trapped or handled within the previous 72 hours prior to capture. Our protocol for capturing and handling red squirrels in the Yukon was approved by the Michigan State University Institutional Animal Care and Use Committee (#04/08-046-00).

Radiometabolism study in captive squirrels

We injected 8 captive squirrels (4 females and 4 males) intraperitoneally with 1110 kBq of radiolabeled cortisol (1,2,6,7- ^3H ; Amersham Biosciences, Quebec, Canada; specific activity = 1.55 TBq/mmol) dissolved in 0.1 mL physiological saline containing 5% ethanol and 5% toluene at 0800 h on day 1 of this study. We attempted to collect all urine and feces every 2 hours (except from 2200 to 0800 h) until 54 h post-injection, but in some cases at the 2-hour interval there was either no urine or no fecal samples to collect (i.e., at 1000 h and 1400 h on day 1, at 1400 h and 1800 h on day 2, and at 1000 h on day 3). To collect urine, we first aspirated any urine from the surface of the pan with a 1 mL pipette and then rinsed the pan with 4 mL of 80% methanol and added this to the urine sample. Between sampling periods, we rinsed the pans twice with a radioactive decontamination solution.

Monitoring of baseline patterns and manipulation of adrenocortical activity in captive squirrels

To obtain baseline FCM levels, we collected fecal samples ($n = 57$) every 4 hours for 48 hours (except from 2000 to 0800 h) from 11 captive squirrels (5 females, 6 males) during a period in which squirrels were not manipulated. We pooled the samples collected at the same time in the 2 days into 5 sampling periods (0800, 1200, 1600, 2000, 0800 h). We used these as baseline FCM levels to compare how the two experimental manipulations (described below) affected FCM levels. To demonstrate that changes in adrenocortical activity are well reflected in FCM levels in red squirrels, we conducted two experimental manipulations on 8 squirrels (4 females, 4 males). First, we conducted a handling stress experiment (“handling stressor”) where the squirrels were

placed into a restraining bag, weighed, sexed, their reproductive status was determined, and placed back into their radiometabolism cage (~2 min/squirrel). Second, we injected squirrels with 4.0 IU/kg of synthetic adrenocorticotrophic hormone (ACTH; Synacthen Depot, CIBA, Ontario, Canada), which increases adrenal cortisol production (Boonstra and McColl, 2000). For these two manipulations, squirrels were handled or injected at 0800 h on day 1 of the manipulation, and we collected fecal samples every 4 hours (except from 2000 to 0800 h) for the following 36 hours post-manipulation. The 3 collection periods for baseline values, handling stressor, and ACTH injection were separated by >3 days.

Collection and extraction of fecal samples from captive and free-ranging Squirrels

All fecal samples from the captive squirrels were placed into 1.5 mL vials and then stored in a -20 °C freezer within 20 min of collection. To test the stability of FCM, we conducted an experiment to determine how the time from collection to freezing affects FCM levels in red squirrel fecal samples. Immediately upon defecation, individual fresh fecal samples were fully homogenized, separated into 2 equal mass aliquots (± 0.001 g), and placed into a -20 °C freezer either immediately or after storing them at room temperature (~23 °C) for 5 hours.

Fecal samples collected in the field were placed in 1.5 mL vials and then into a -20 °C freezer within 4-5 hours after collection. During the winter months (<0 °C; January-April), when air temperatures are usually <0 °C, fecal samples are generally frozen upon collection and remain so while in the field (Dantzer, personal obs.). In the warmer months (May-September), fecal samples in 1.5 mL vials were placed into an

insulated container with wet ice until they were placed into the freezer. Samples were then shipped to the University of Toronto Scarborough on dry ice and stored at -80 °C upon arrival.

All fecal samples were lyophilized (LabConco, Missouri, USA) for 14-16 hours, frozen in liquid nitrogen, and then pulverized using a mortar and pestle. We then extracted 0.05 g of the dry ground feces by adding 1 mL of 80% methanol (Touma et al., 2003). The steroid metabolites were then extracted in a multi-tube vortexer at 1450 RPM for 30 min, and then centrifuged for 15 min at 2500 g (Palme, 2005). We then took the resulting supernatant and either analyzed it immediately for the radioactive samples or stored at -80°C for analysis via enzyme-immunoassay (EIA).

*Determination of radioactivity in fecal and urine samples
for captive squirrel radiometabolism study*

Urine samples collected during the radiometabolism study were dried down under air until only ~1 mL remained. We added 4 mL of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the dried down urine or 100 mL of the fecal extract and determined its radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, USA).

Characterization of fecal ³H-cortisol metabolites

Fecal extracts with peak radioactivity from female (n = 2) and male (n = 2) squirrels at the peak radioactivity excretion, and an extract from the ACTH stimulation test from each sex were dried down under air and sent to the University of Veterinary Medicine (Vienna, Austria). These fecal extracts were then subjected to reverse-phase high performance liquid chromatography (RP-HPLC). After separation, both the

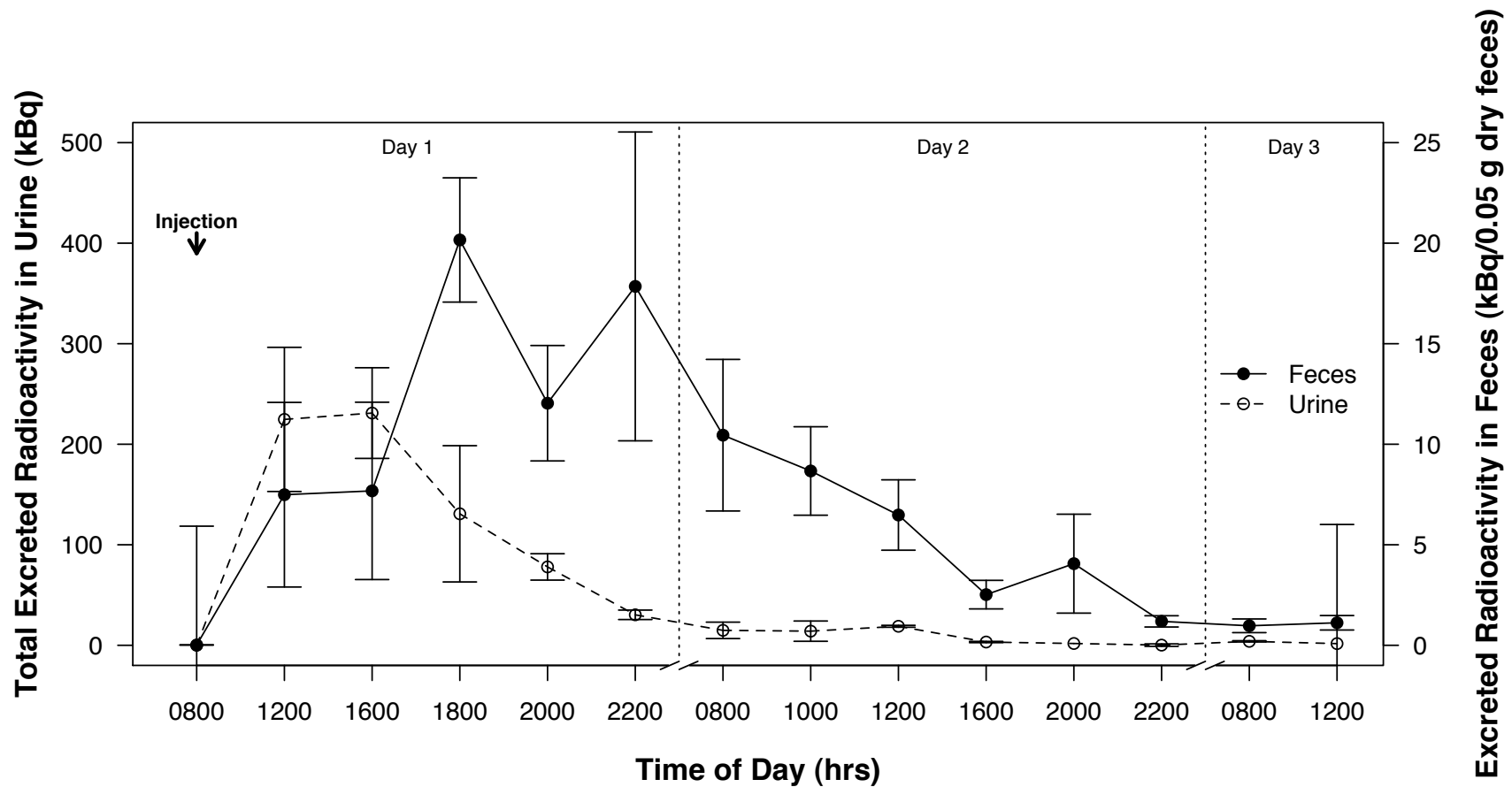


Figure 2.1. Time course of excreted radioactivity in urine (kBq/sample) and feces (kBq/0.05 g dry feces) from North American red squirrels ($n = 8$) injected with ^3H -cortisol. Background fecal and urine samples were taken at the time of injection. Data are presented as mean $\pm$ SE.

radioactivity and the immunoreactivity (see below) were measured in the fractions. Details of this method can be found in Lepschy et al. (2007) and Touma et al. (2003).

Determination of immunoreactivity

To quantify FCM levels, we used a 5 α -pregnane-3 β ,11 β ,21-triol-20-one EIA, which measures GC metabolites with 5 α -3 β ,11 β -diol structure (Touma et al., 2003), and has already been successfully validated for several species (Touma et al., 2004; Lepschy et al., 2007; Nováková et al., 2008; Bosson et al., 2009). Information regarding the cross-reactivity of the antibody used (Touma et al., 2003) and further details of the assay procedure can be found elsewhere (Palme and Möstl, 1997; Möstl et al., 2005). Intra-assay coefficient of variation (CV) was $7.6 \pm 0.2\%$ and the inter-assay CV for a high and low pooled fecal extracts were $17.9 \pm 1.3\%$ and $18.6 \pm 1.1\%$, respectively ($n = 23$ plates). FCM levels are expressed as ln-transformed ng/g dry feces.

Statistical analyses

For the captive squirrels, we used linear mixed models (LMM) to examine (1) excretion patterns of ^3H -cortisol in urine and fecal samples (fixed effects: sex and sampling period), and (2) how the two treatments (handling stressor, ACTH) affected FCM levels within 24 h post-manipulation compared to the baseline FCM levels (fixed effects: treatment, sampling period, and treatment x sampling period interaction). Sex was not included in the latter two models because the sexes did not differ in FCM levels at the different sampling periods or in their responses to the treatments (for all sex x sampling period and sex x treatment comparisons, $P > 0.09$). We used paired t -tests to examine how the time from defecation to freezing affected FCM levels. Prior to

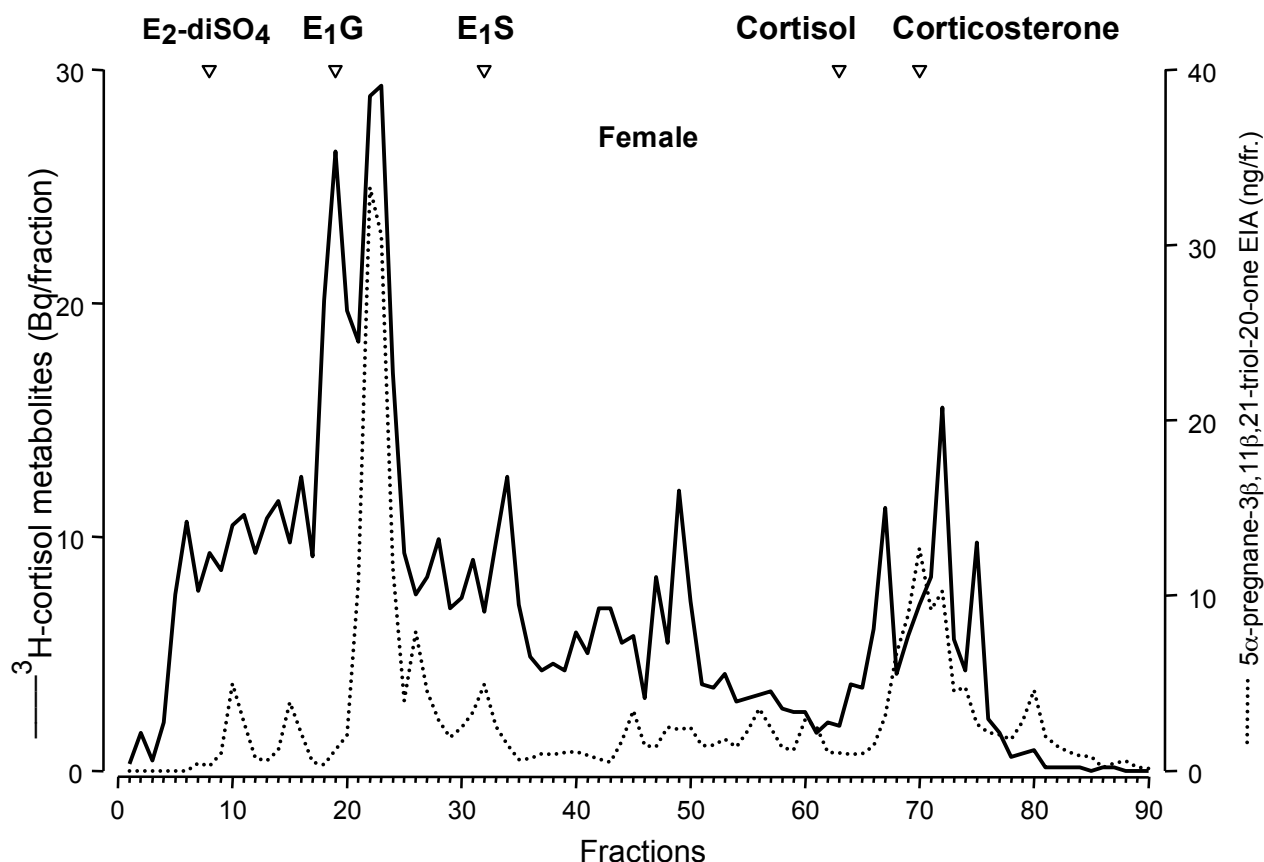


Figure 2.2. Reverse-phase high performance liquid chromatography (RP-HPLC) radioimmunoassay of peak radioactive fecal extracts from female North American red squirrels. Samples shown are representative of female squirrels injected with radiolabeled cortisol. The solid line shows the ^3H -cortisol metabolites and the dotted line shows the metabolites reacting with the 5α -pregnane- 3β , 11β , 21 -triol- 20 -one antibody. Elution times of standards are marked with open triangles for estradiol disulphate ($\text{E}_2\text{-diSO}_4$), estrone glucuronide (E_1G), estrone sulfate (E_1S), cortisol, and corticosterone.

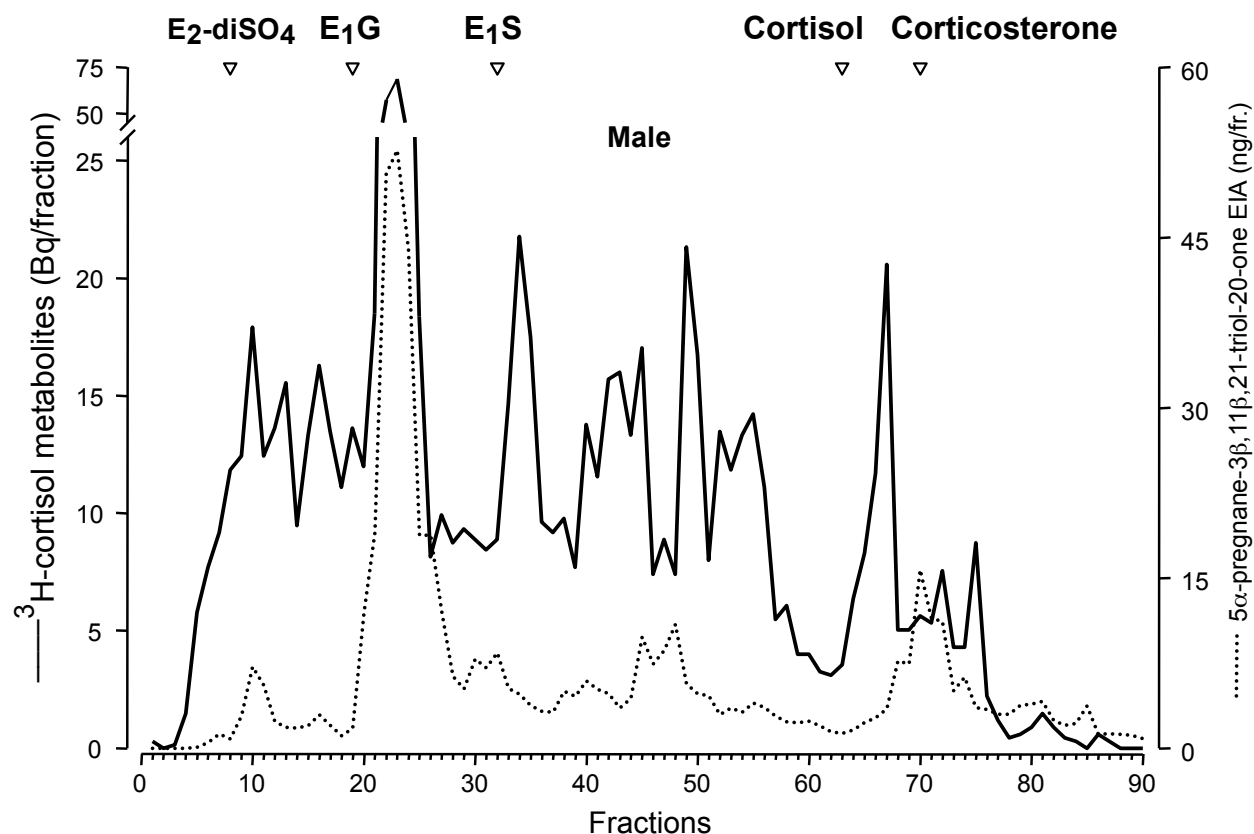


Figure 2.3. Reverse-phase high performance liquid chromatography (RP-HPLC) radioimmunogram of peak radioactive fecal extracts from male North American red squirrels. Samples shown are representative of male squirrels injected with radiolabeled cortisol. The solid line shows the ^3H -cortisol metabolites and the dotted line shows the metabolites reacting with the 5α -pregnane- 3β , 11β , 21 -triol- 20 -one antibody. Elution times of standards are marked with open triangles for estradiol disulphate ($\text{E}_2\text{-diSO}_4$), estrone glucuronide (E_1G), estrone sulfate (E_1S), cortisol, and corticosterone.

conducting this *t*-test, we used a Shapiro-Wilk normality test to determine that the data were normally distributed ($W = 0.92$, $P = 0.31$).

For the analyses of fecal samples from free-ranging squirrels, we used LMM to examine how (1) FCM levels change with reproductive condition in males and females (fixed effect: reproductive condition), and (2) how female FCM levels vary prior to and after parturition (fixed effects: day post-conception and day post-conception²). We used non-linear regression to assess non-linearities between days post-conception and FCM levels (general additive models: Hastie and Tibshirani, 1990). Following the LMM, we used Tukey's honest significant difference post hoc tests to determine differences among means of the six reproductive conditions.

We included squirrel identification (ID) as a random effect for all LMM because we had repeated measures on the same squirrels. We calculated the proportion of residual variance in the LMM that was due to the individual (i.e., repeatability: Lessels and Boag, 1987) and used likelihood ratio tests to determine if the random effects improved the fit of the models. In all the LMM that we conducted for analyzing FCM levels from captive squirrels, the random effects significantly improved the fit of the model ($P < 0.0001$ for all likelihood ratio tests).

Prior to analysis, FCM levels were ln-transformed to meet assumptions of normality. Diagnostic plots after the ln-transformation revealed that the residuals from all LMM were normally distributed, homoscedastic, and there were no outlying observations with high leverage. For all results below, we present mean $\pm$ standard error (SE) and considered differences statistically significant at $\alpha = 0.05$. All statistical analyses were conducted using R (R 2.9.0, R Development Core Team, 2009).

Results from Captive Squirrels

Route and time to peak excretion of ^3H -cortisol metabolites

We collected 97 fecal and 74 urine samples during 15 (feces) and 14 (urine) sampling periods over the 70 h following injection of ^3H -cortisol. We recovered $69.2 \pm 0.06\%$, of the 1110 kBq of ^3H -cortisol injected, of which $70.3 \pm 0.02\%$ was in the urine and $29.7 \pm 0.02\%$ in the feces. There was no sex difference in the total amount of radioactivity recovered ($t_6 = 0.63$, $P = 0.63$).

Within 4 hours of administration of ^3H -cortisol, radioactivity had significantly increased above background levels in both the urine (total kBq excreted; $t_{13} = 8.04$, $P < 0.0001$) and feces (kBq/0.05 g dry feces; $t_{14} = 6.78$, $P < 0.0001$; Fig. 2.1). The mean time to peak excretion of ^3H -cortisol metabolites in the urine and feces was 7.1 ± 0.9 and 10.9 ± 2.3 hours, respectively (Fig. 2.1). There were no sex differences in the time to peak excretion in neither the urine ($t_7 = 0.24$, $P = 0.41$) nor the feces ($t_7 = -0.93$, $P = 0.19$). Radioactivity levels gradually decreased but remained significantly higher than background levels in the urine ($t_{13} = 5.46$, $P < 0.0001$) and the feces ($t_{14} = 7.41$, $P < 0.0001$) even 54 hours post-injection of ^3H -cortisol (Fig. 2.1).

Characterization of fecal ^3H -cortisol metabolites

Cortisol was heavily metabolized with nearly no native cortisol present (Fig. 2.2 and 2.3). After the HPLC separations, there were several prominent radioactive peaks in the fecal extracts in both males ($n = 6$ peaks) and females ($n = 4$ peaks), with the

major peak eluting fraction 20 (near the estrone glucuronide standard) in both sexes. There were both polar and non-polar metabolites in the fecal extracts and few metabolites were found beyond fraction 80. Inter-individual differences in cortisol metabolites occurred in females but not males. The 5α -pregnane- $3\beta,11\beta$, 21-triol-20-one EIA detected several radioactive peaks in similar fractions (20-30, 40-50, 65-75) in both sexes (Fig. 2.2 and 2.3).

Effect of handling stressor on FCM Levels

We collected 43 fecal samples during 8 sampling periods from 0 to 36 hours after the handling stressor. Eight hours after the handling event, FCM levels had increased (7.03 ± 0.13 ln-transformed ng/g dry feces) from baseline FCM levels (6.04 ± 0.15) collected at the same time of day (1600 h), but this difference was only marginally significant ($t_4 = 2.04$, $P = 0.054$; Fig. 2.4).

Effect of ACTH on FCM levels

From 0 to 36 hours post-injection of ACTH, we collected a total of 58 fecal samples during 8 sampling periods. Eight hours after the ACTH injection, FCM levels had significantly increased (7.2 ± 0.28) compared with baseline levels (6.04 ± 0.15) collected at the same time of day (1600 h; $t_4 = -2.93$, $P = 0.021$; Fig. 2.4). FCM levels were higher in response to the ACTH injection than FCM levels following the handling stressor collected at eight hours post-manipulation, but this difference was only marginally significant ($t_7 = -1.86$, $P = 0.053$; Fig. 2.4). FCM levels at 32 hours post-injection of ACTH (7.4 ± 0.34) were significantly higher compared with those at 32 hours

after the handling stressor (6.6 ± 0.22) collected at the same time of the day (1600 h; $t_7 = -2.56$, $P = 0.019$; Fig. 2.4).

Effect of time between collection and freezing on FCM levels

Fecal subsamples stored at room temperature for 5 h tended to have higher FCM levels ($n = 6$; 6.6 ± 0.17 ; Fig. 2.5) than those paired subsamples frozen immediately ($n = 6$; 6.3 ± 0.28 ; Fig. 2.5), but this increase of $38 \pm 19\%$ in FCM levels was not significant ($t_{10} = -0.88$, $P = 0.39$).

Results from Free-ranging Squirrels

Effect of reproductive condition

Reproductive condition had a significant effect on FCM levels in male and female squirrels ($F_{5, 378} = 19.17$, $P < 0.0001$). Pregnant females ($n = 118$; 6.3 ± 0.08) had significantly higher FCM levels than those females that were lactating ($n = 198$; 5.8 ± 0.05 ; $P < 0.0001$), post-lactating ($n = 42$; 5.5 ± 0.1 ; $P < 0.0001$), or did not breed ($n = 101$; 5.1 ± 0.1 ; $P < 0.0001$; Fig. 2.6). Lactating females had significantly higher FCM levels than those that were post-lactating ($P < 0.0001$) or non-breeding females ($P = 0.001$; Fig. 2.6). Males with scrotal ($n = 19$; 5.9 ± 0.2) and abdominal ($n = 24$; 5.4 ± 0.2) testes did not differ in their FCM levels than abdominal males ($P = 0.36$; Fig. 2.6). Pregnant females had significantly higher FCM levels than abdominal males ($P = 0.025$; Fig. 2.6). The random effect for squirrel ID explained 58% of the residual variation (i.e., variation not explained by reproductive condition) and significantly improved the fit of the model ($\chi^2 = 115$, $df = 1$, $P < 0.0001$).

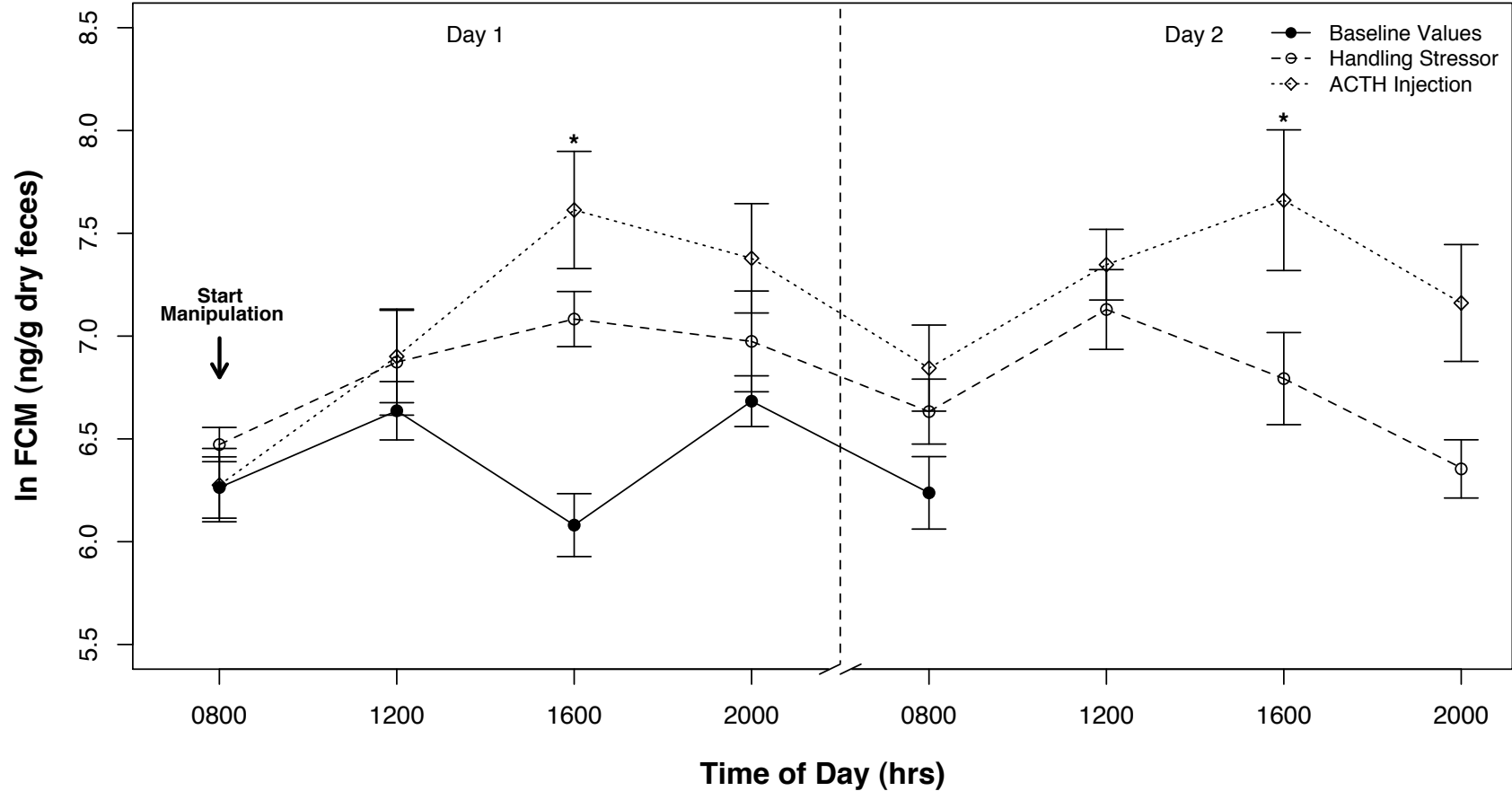


Figure 2.4. Concentrations of fecal cortisol metabolites (FCM) in North American red squirrels in which squirrels ($n = 11$) were not manipulated (“Baseline Values”), and after squirrels ($n = 8$) were subjected to handling stressor and adrenocorticotropin (ACTH) stimulation tests. Manipulations were conducted on the same squirrels but on different days separated by >72 h. Asterisks denote significant differences ($P < 0.05$) between baseline FCM levels and the two treatments from 0-24 hours post-manipulation and between FCM levels after the ACTH injection and handling stressor from 28-36 hours post-manipulation. Data are expressed as mean $\pm$ SE.

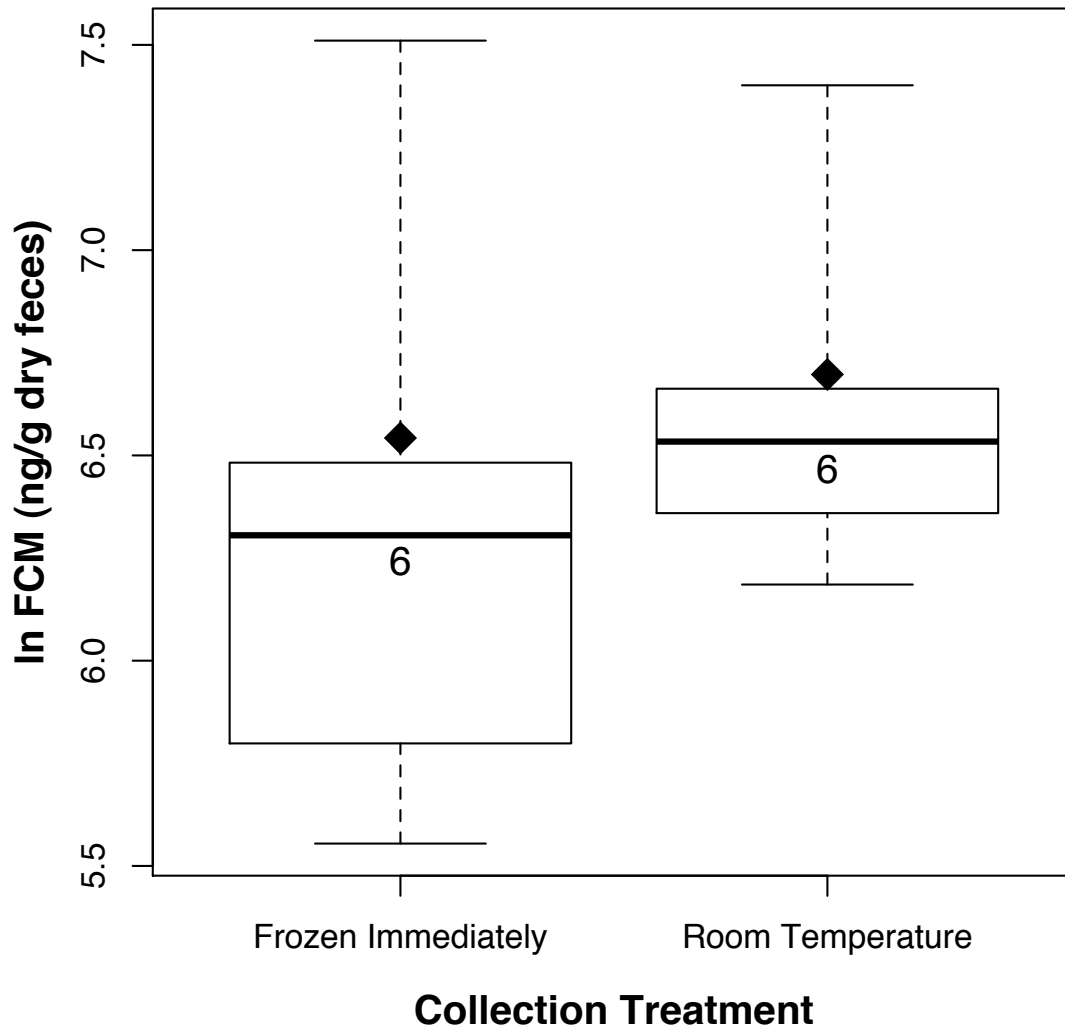


Figure 2.5. Effect of time from collection to freezing on fecal cortisol metabolite (FCM) levels in North American red squirrel feces. “Frozen Immediately” indicates that the feces were frozen immediately upon collection. “Room Temperature” indicates that the paired subsamples of feces were left at room temperature (~23°C) for 5 h and then frozen. Numbers inside boxes represent number of fecal samples. Box plots show 25-75% interquartile range (boxes), mean (filled diamonds), median (line within box), and the range (whiskers).

FCM levels during gestation and lactation

We collected fecal samples from 78 female squirrels in 2006 (n = 12 samples), 2007 (n = 74 samples), and 2008 (n = 271 samples). FCM levels significantly increased during gestation, peaked around parturition (35 days post-conception), and then significantly declined during lactation (36-104 days post-conception) and after weaning (105 days post-conception; slope on ln-scale for quadratic term for days since conception = $-6.2 \times 10^{-5} \pm 2.1 \times 10^{-5}$; $t_{356} = -2.96$, $P = 0.0017$; Fig. 2.7). The random effect for ID explained 47% of the residual variation (i.e., variation not explained by days since conception) and significantly improved the fit of the model ($\chi^2 = 153.5$, $df = 1$, $P < 0.0001$).

Discussion

Our study validates an EIA to measure FCM levels with a 5α - 3β , 11β -diol structure in North American red squirrels. We used a radiometabolism of cortisol and RP-HPLC to characterize the structure of the cortisol metabolites in the feces and showed that our antibody reacts with some of the main metabolites. Using a handling stressor and ACTH injection, we showed that adrenocortical activity is well reflected in FCM levels. Increased time from collection to freezing tended to increase FCM levels, but not significantly. Lastly, FCM levels in free-ranging female but not male red squirrels were significantly affected by reproductive condition, with pregnant squirrels just prior to parturition having the highest FCM levels overall, although there was substantial variation in FCM levels among individual free-ranging squirrels.

Radiometabolism study

While the total percent recovery of injected radiolabeled cortisol ($69.2 \pm 0.06\%$) was within the range of previous studies ($\sim 38\text{-}95\%$: Palme et al., 2005; Lepschy et al., 2007; Bosson et al., 2009), we attribute the apparent loss of radioactivity to our urine collection procedure. During urine collection, some of the urine was left on the screen that covered the pans underneath the radiometabolism cages. We rinsed the screening with 80% methanol (described above), but apparently were not able to capture all of the urine. However, because we were able to collect and extract all of the feces, we think that our estimate of the amounts of radioactivity excreted in the feces is accurate.

Radioactivity first appeared in the feces 4 hours after administration of ^3H -cortisol. The lag time from injection to peak concentration of radiolabeled cortisol (10.9 ± 2.3 h) was similar to that found in laboratory mice (8-12 h: Touma et al., 2003), but longer than that found in Columbian ground squirrels (7.03 ± 0.53 h: Bosson et al., 2009). Radiolabeled cortisol metabolites in both female and male squirrels were excreted mainly in the urine ($70.3 \pm 0.02\%$ of recovered radioactivity). In previous studies of laboratory mice (*Mus musculus*) and rats (*Rattus norvegicus*), radiolabeled corticosterone metabolites were excreted mainly in the feces (Touma et al., 2003; Lepschy et al., 2007). While the percentage of radioactive cortisol metabolites recovered in the feces in this study is low compared with these latter studies, it is greater than those reported in Columbian ground squirrels (*Spermophilus columbianus*; Bosson et al., 2009) or brown hares (*Lepus europeaus*: Teskey-Gerstl et al., 2000).

As was found in almost all species investigated so far (Bosson et al., 2009; Palme et al., 2005), HPLC indicated that cortisol was extensively metabolized. However,

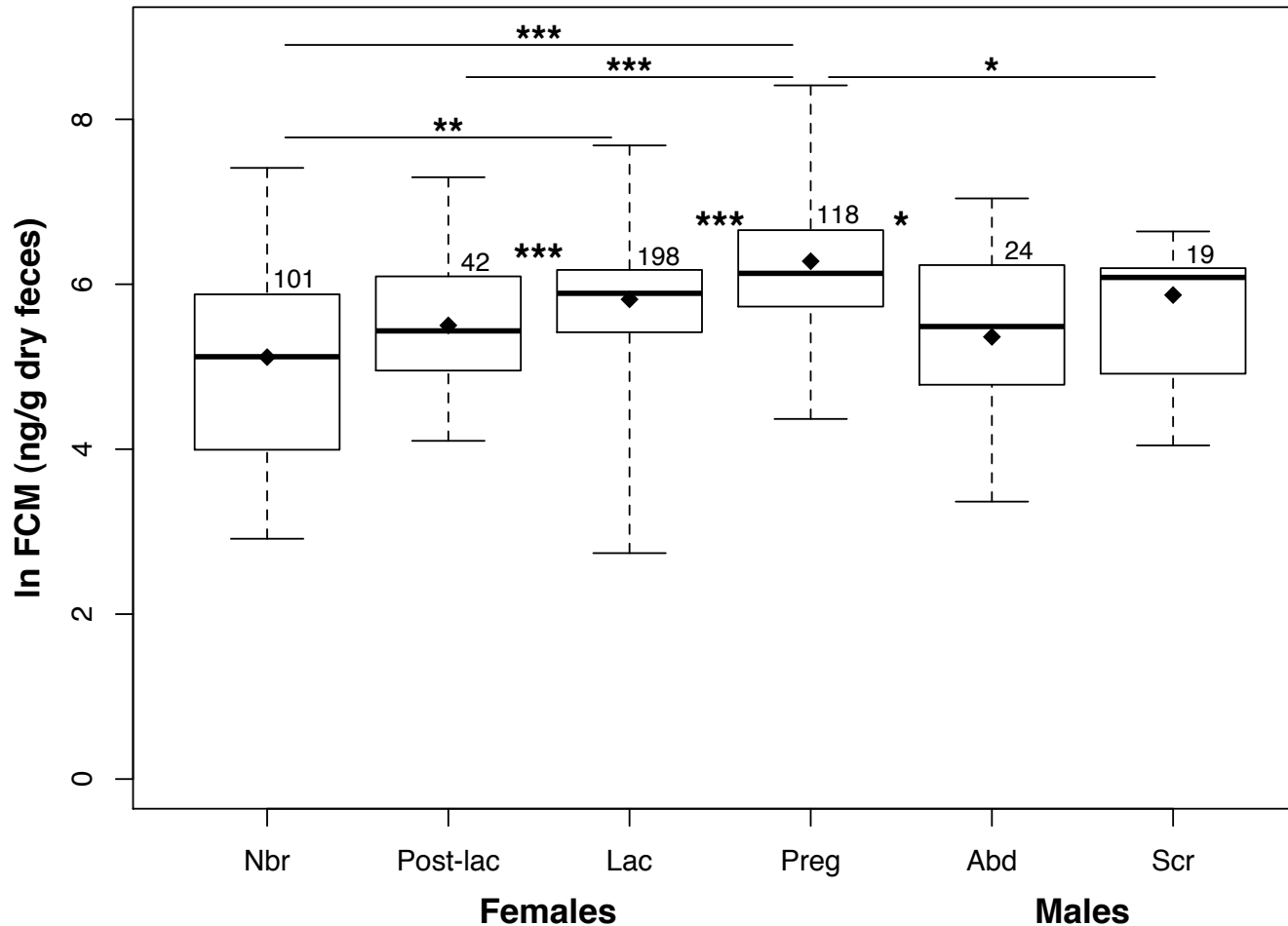


Figure 2.6. Concentrations of fecal cortisol metabolites (FCM) in North American red squirrels of different reproductive stages (“Nbr”: non-breeding females; “Post-lac”: post-lactating; “Lac”: lactating; “Preg”: pregnant; “Abd”: males with abdominal testes; “Scr”: males with scrotal testes). Ln-transformed data are shown but we used linear mixed models individual identity (random effect) to determine significant differences among reproductive conditions. Significant differences are denoted by “*” ($P < 0.05$), “**” ($P < 0.01$), and “***” ($P < 0.0001$). Asterisks between boxes indicate significant differences between the two groups. Numbers above boxes are sample size of fecal samples analyzed. Box plots show 25-75% interquartile range (boxes), mean (filled diamonds), median (line within box), and the range (whiskers).

several of the cortisol metabolites reacted with our EIA antibody. There were minor differences in cortisol metabolites among individual males. However, similarly to laboratory rats (Lepschy et al., 2007), we found large differences between the two females. Lepschy et al. (2007) hypothesized that these individual differences in formed metabolites in females might be due to the presence of differing gonadal steroids that rapidly change over the estrus cycle, which we did not measure in this study.

Physiological validation of the enzyme-immunoassay

We used a pharmacological treatment (ACTH stimulation test) and a handling stressor to demonstrate the physiological and biological validity of our EIA in red squirrels. Both the handling stressor and the ACTH stimulation test increased FCM levels 8 hours after handling/injection compared with baseline FCM levels at the same time period, but only the results from the ACTH stimulation test were significant. Because changes in adrenocortical activity were well reflected in FCM levels, we successfully validated this EIA for use in North American red squirrels.

Patterns of FCM levels in free-ranging red squirrels

Documenting the effects of reproduction on GCs in small mammals can be difficult because of low recapture rates and the rarity of known dates of conception or parturition. Although pregnancy and lactation have major effects on GC levels in free-ranging mammals (see references below), there is little consistency in the detected direction and magnitude of these effects. For example, previous studies that grouped females into reproductive categories (i.e., pregnant vs. lactating) have found that GCs

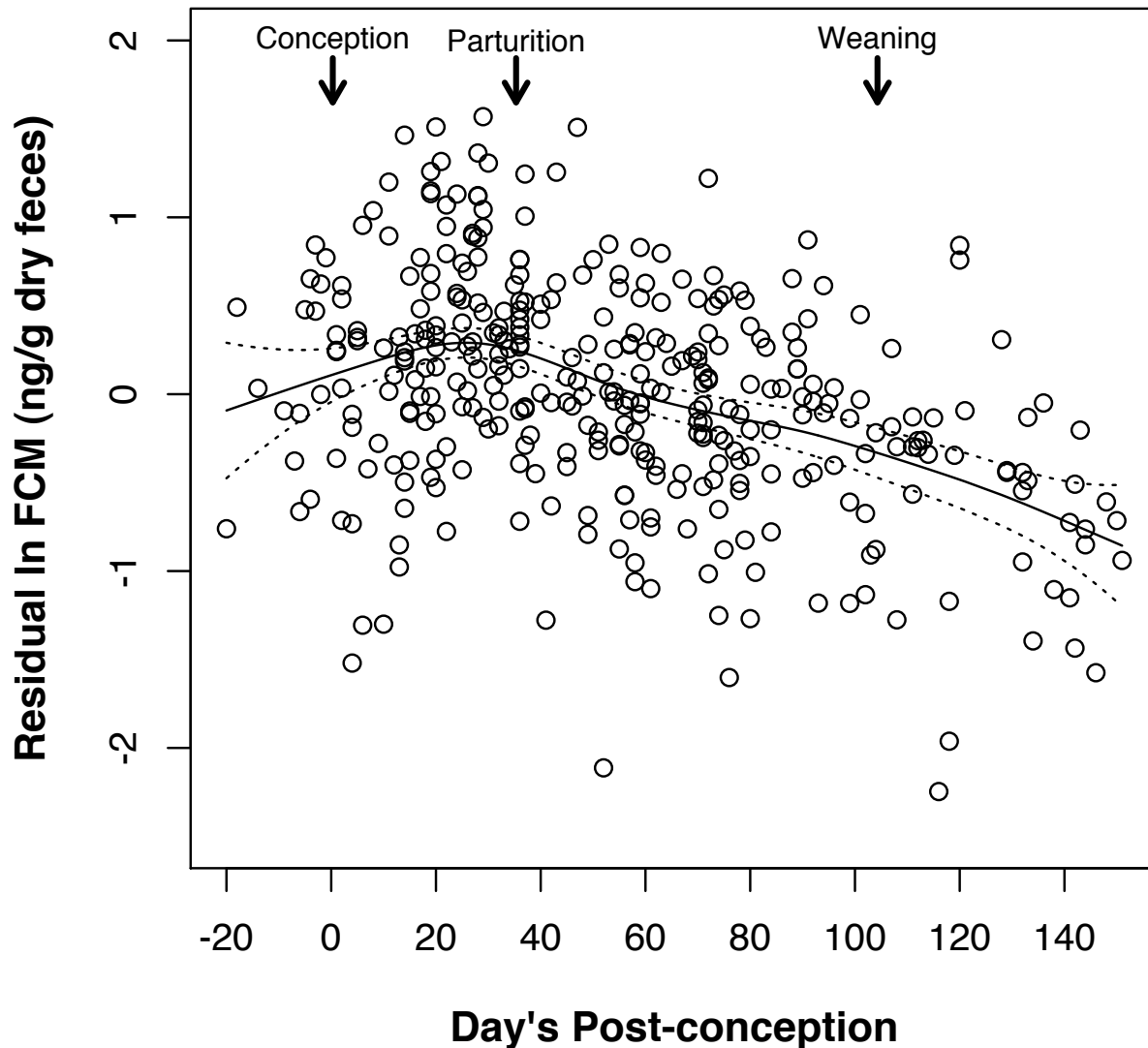


Figure 2.7. Concentrations of fecal cortisol metabolite (FCM) levels in female North American red squirrels ($n = 78$ squirrels) prior to conception, during gestation and lactation, and after weaning ($n = 387$ samples). This significant non-linear relationship between FCM level and days post-conception was fit using a cubic spline. The quadratic effect of days post-conception on ln-transformed FCM level was significant in a linear mixed model with individual (random effect). Values on y-axis represent standardized residual ln-transformed FCM levels from this latter model.

during pregnancy were either higher (Boonstra and Boag, 1992; Reeder et al., 2004; Hunt et al., 2006) or lower (Kenagy and Place, 2000) than during lactation. We found that pregnant females had significantly higher FCM levels than lactating, post-lactating, and non-breeding females. However, because we obtained repeated fecal samples from individually marked females whose day of conception was known, we were also able to examine how FCM levels varied over the course of gestation and lactation. In this analysis, we found that FCM levels increased throughout gestation, peaked around parturition, and then declined slightly throughout lactation. A similar situation was found in little brown myotis (*Myotis lucifugus*) where GC levels in lactating females were significantly lower than those in mid-to-late pregnancy females, but not females in early pregnancy (Reeder et al., 2004). Whereas the relationship between GCs and reproduction may be species-specific, available evidence suggests GC levels frequently vary during different stages of gestation and lactation. As a result, if animals are sorted into discrete categories of pregnancy and lactation, without references to days since conception or parturition, the category differences that are detected will be heavily influenced by the stage of gestation and lactation at which females happen to be sampled. We suggest that future studies exercise caution in interpreting comparisons of GCs among discrete reproductive categories

Relationship between FCM levels, energy intake and expenditure

Among most mammals, lactation is the most energetically demanding stage of the annual cycle in general and of reproduction in particular (Kenagy et al., 1989 reviewed by Speakman, 2008). In red squirrels, which do not hibernate (Pauls, 1978), daily energy expenditure (DEE) in lactating female red squirrels is significantly higher

than in non-breeding females in the summer and non-breeding females in the winter (Fletcher et al., unpublished data; Humphries et al., 2005). According to the Energy Mobilization Hypothesis, GCs should be elevated during lactation because it is such an energetically demanding state (Romero, 2002). In partial support of this hypothesis, we found that FCM levels were significantly higher in lactating than in non-breeding female squirrels. However, we also observed significantly higher GC levels during late-pregnancy than the more energetically costly lactation period and this could be explained by a preparatory rather than responsive function of GCs. High GCs prior to parturition may motivate foraging (Wingfield and Romero, 2001; Dallman et al., 2007), which could allow pregnant females to accumulate energy reserves in preparation for the energetic demands during lactation (see Romero, 2002). Some but not all studies of small mammals have documented accumulation of energy reserves during pregnancy (Millar, 1975; Randolph et al., 1977; Gittleman and Thompson, 1988). Lactating red squirrels increase body fat levels during the early stages of lactation to match the increased reproductive demands in late lactation (Humphries and Boutin, 1996), but we do not know if the same occurs during pregnancy. Future studies will examine relationships between FCM levels, DEE, and food consumption in pregnant and lactating red squirrels.

Documenting inter-individual variation in FCM levels

Documenting the presence of individual variation in the hormonal traits of free-ranging organisms and how it affects fitness is critical to understand how natural selection acts upon hormonal traits. Recently, there has been growing interest in inter-individual variation in hormonal traits (reviewed by Williams (2008)). However, in many

examinations of hormonal traits in free-ranging organisms, inter-individual differences are rarely well-documented. For example, trappability can reflect animal temperament on a shy-bold axis (Réale et al., 2007; Boon et al., 2008), which likely has a neuroendocrine basis (Koolhaas et al., 1999; Sih and Bell, 2008). Because those animals that have low capture probability are often not included in a repeated measures ANOVA due to the requirement of a balanced design (Gotelli and Ellison, 2004), estimates of both the central tendency and degree of variation of hormonal traits may be frequently biased in field studies.

In this study, we used linear mixed models (LMM) with identity as a random effect to deal with both an unbalanced design and repeated measures on the same individuals (Pinheiro and Bates, 2009). With this statistical approach, we were able to analyze all of the fecal samples collected from individual squirrels and to document that a large proportion of the residual variation in FCM levels in free-ranging squirrels was due to repeatable inter-individual differences (i.e., range 47-58%). If these inter-individual differences have a genetic basis, they could provide the raw material for natural selection. In order to make progress in understanding how natural selection acts upon hormonal traits, we suggest that future studies in free-ranging animals use similar statistical approaches to deal with unbalanced designs and report the presence of repeatable inter-individual variation in hormonal traits.

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LITERATURE CITED

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CHAPTER 3

Dantzer, B., McAdam, A.G., Palme, R., Humphries, M.M., Boutin, S., Boonstra, R.
2011. Maternal androgens and behaviour in free-ranging North American red
squirrels. *Animal Behaviour* 81, 469-479.

Introduction

The extended period of maternal care is one of the more interesting and unique features of mammalian reproduction. The proper development and survival of mammalian offspring depend upon the presence of mothers and adequate provisioning of maternal care. Because of the relatively prolonged period of postnatal interaction between mothers and their offspring, variation in maternal care can have profound and long-lasting consequences on offspring phenotype, survival, and reproductive success as well as influence maternal lifetime fitness (Clutton-Brock, 1991). For example, the time that females spend nursing their offspring can have long-term effects on body mass and sexual ornamentation (horn size) in male offspring of a sexually-dimorphic ungulate species in which body and horn size are likely to be major determinants of reproductive success (Festa-Bianchet et al., 1994). Variation in the amount of maternal care provided by laboratory rats in the form of arched-backed nursing and licking/grooming of offspring can also have life-long consequences on neural development, physiological responsiveness to stressors, and behavior (reviewed in: Meaney, 2001; Champagne, 2008; see also Maestriperi et al., 2007). This variation in maternal behavior can then be preserved within genetic lineages across multiple generations via epigenetic mechanisms (reviewed by Champagne, 2008; Champagne and Curley, 2009). Despite its potential ecological and evolutionary importance (Mousseau and Fox, 1998), maternal behavior has not been well documented in many species of free-ranging mammals, and the physiological mechanisms that may underlie variation in maternal care are relatively unexplored.

Variation in steroid hormone concentrations such as androgens may be one major source of differences in parental behavior. The activational effects of testosterone clearly influence paternal behavior (reviewed in: Lonstein and De Vries, 2000). For example in mammals, testosterone can reduce paternal care (reviewed in: Lonstein and De Vries, 2000; Nunes et al., 2000a, 2001; Schradin et al., 2009; for an opposite result, see Trainor and Marler, 2001, 2002) but increase mating effort (Clark et al., 1997). Similarly, in male birds, elevated plasma testosterone simultaneously decreases levels of paternal care and increases aggression and mating effort (Hegner and Wingfield, 1987; Wingfield et al., 1990; Ketterson and Nolan, 1999; Hau, 2007; McGlothlin et al., 2007). However, few studies have examined whether androgens influence maternal behavior, which is surprising for two reasons. First, females also produce testosterone and its androgen precursors in the gonads, the adrenals (Staub and De Beer, 1997), and potentially in the brain (Baulieu, 1991; Soma et al., 2008; Pradhan et al., 2010). Second, androgens may play an important role in basic female reproductive physiology (reviewed in: Walters et al., 2008) and behavior (Sandell, 2007).

Recent studies in natural populations have suggested an important but equivocal role for androgens in mediating maternal behavior. For example, experimental elevation of testosterone decreased the time that female dark-eyed juncos, *Junco hyemalis*, spent brooding eggs (O'Neal et al., 2008; but see Clotfelter et al., 2004 for an opposite result), but did not affect nest defense or nestling provisioning rates (Clotfelter et al., 2004; O'Neal et al., 2008). In captive mammals, correlative studies suggest that high concentrations of testosterone inhibit or perhaps lessen the quality of maternal care (Bridges et al., 1982; Lonstein and De Vries, 2000; Fite et al., 2005), which could

increase the probability of infant death (Altmann et al., 2004). As such, variation in maternal androgens could have important effects on maternal behavior and perhaps reproductive success, but these relationships have been rarely examined in free-ranging mammals. In one exception, Dloniak et al. (2006) demonstrated that maternal androgen concentrations during gestation in female spotted hyenas, *Crocuta crocuta*, influenced the aggressive behavior of their offspring, which is known to have important consequences for offspring fitness.

In this study, we investigated patterns of maternal behavior and androgen concentrations in free-ranging North American red squirrels, *Tamiasciurus hudsonicus*, using behavioral observations collected in 10 years between 1994 and 2008 and using fecal samples collected over 3 years (2006-2008). We tested the hypothesis that androgen concentrations drive behavioral trade-offs in breeding female red squirrels. Specifically, we assessed the relationship between fecal androgen metabolites (FAM) and behavioral measures of territory defense, foraging and maternal behavior (nest use) during reproduction (time from conception to weaning). Based on previous studies (Fite et al., 2005), we predicted that periods of heightened maternal androgens would be associated with increased time spent foraging and engaged in territory defense and decreased time allocated towards maternal behavior.

Materials and Methods

Study area and species

We studied a free-ranging population of red squirrels near Kluane Lake in the southwest Yukon, Canada (61° N, 138° W) that has been monitored continuously since

1987 (see McAdam et al., 2007 for details). The study area is dominated by white spruce (*Picea glauca*) trees whose seeds are the major food source for red squirrels in this region. In the autumn of each year, red squirrels collect and cache newly matured spruce cones in a larger hoard in the center of their territory (Fletcher et al., 2010). Both male and female red squirrels individually defend a cache of cones from all male or female conspecifics year-round except during periods of estrous when females tolerate males on their territories (Smith, 1968).

Most red squirrels in our study populations were permanently marked with uniquely numbered metal eartags (National Band and Tag, Newport, KY, USA) after being temporarily removed from their natal nest when they were around 25 days old. As part of a larger study, squirrels in our study population were frequently live-trapped using Tomahawk live traps (48 x 13 x 13 or 48 x 15 x 15 cm, Tomahawk Live Trap Co., Tomahawk, WI, USA). During behavioral observations, we determined the identities of squirrels from a distance during based on unique combinations of colored electrical wire that was threaded through the metal eartags.

Red squirrels in the Yukon are seasonal breeders and, in most years, females are in behavioral estrous for 1 day and produce one litter after a ~35 day gestation period followed by a ~70 day lactation period (Steele, 1998; McAdam et al., 2007; S. Boutin, personal communication). Litter size ranges from one to seven pups per litter but is generally three or four pups per litter (mean $\pm$ SE: 3.10 ± 0.043 pups per litter: McAdam et al., 2007). Juveniles typically emerge from their natal nest around 77 days postconception (range 71-86 days post-conception), but they continue to nurse until ~106 days postconception (Humphries and Boutin, 1996). During each capture event,

we determined the reproductive status of males (abdominal versus scrotal testes) and females (pregnant, lactating, or neither). We determined the dates of parturition by frequent trapping (every 3 days), weighing and palpation of pregnant females, or by assigning an age to neonates based upon their mass when they were accessed in their natal nest soon after birth (for calculation of parturition dates, see: Becker, 1993 and Boutin and Larsen, 1993). Dates of conception were estimated by subtracting 35 days from known dates of parturition.

Measuring maternal behavior in free-ranging red squirrels

We recorded the behavior of radiocollared (model PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada) breeding females with instantaneous sampling at 30 s intervals (Altmann, 1974). We conducted a total of 903 7-minute observation sessions on 125 female red squirrels that were radiocollared over a 10-year period between 1994 and 2008 (1994-1997, 1999, 2001-2004, 2008). This corresponded to an average of 7.2 sessions per female squirrel (range 1-48 sessions) and the interval between behavioral observation sessions on the same individual was at least 28 min. Behavioral observations on each female were conducted opportunistically during the entire reproductive period with the goal of maximizing the spread of observations for each female across different reproductive conditions and dates. We performed a total of 41 sessions during gestation, 588 during lactation, and 274 after weaning. Behavioral focal sessions occurred from 0600 to 2300 hours, but the majority of these observations occurred from 0700 to 1200 hours (743 of 903), which generally corresponded to the period of highest activity of red squirrels in this region (B. Dantzer, personal observations).

We categorized and analyzed female behavior in a similar way as previous studies of red squirrels (Stuart-Smith and Boutin, 1995; Humphries and Boutin, 2000; Anderson and Boutin, 2002) including whether the squirrel was in or out of its nest, feeding, foraging, travelling, resting, vigilant, vocalizing ('barking' and 'rattling': Smith, 1968) or out of sight. In lactating squirrels, we interpret time spent in the nest with offspring as an indirect measure of maternal behavior as mothers are ensuring proper thermoregulation of offspring, grooming, nursing and interacting with their dependent offspring. Foraging and feeding observations were identified as separate behaviors in the field but were grouped together to calculate the proportion of time spent self-provisioning and we refer to these two behaviors simply as foraging. Rattling is the territorial vocalization of red squirrels, whereas barking is an alarm call (Smith, 1968).

The same observer (B.D.) collected all behavioral observations in 2008, but 39 observers collected the data between 1994 and 2004. Although collection of observations was similar from 1994 to 2004 and in 2008, there were some important differences that affected our analysis of the data. First, rattling was recorded continuously (all-occurrences) only in 2008 and not from 1994-2004. Second, we only recorded whether the focal squirrel was on or off its midden (i.e. its hoard of cached white spruce cones) during observations recorded in 2008 but not in those from 1994 to 2004. As a result of these differences, we treated these two data collection periods as distinct datasets and analyzed them separately (see Statistical Analyses section below).

Validation of an enzyme immunoassay to measure fecal androgen metabolites

We validated an enzyme immunoassay (EIA) to measure fecal androgen metabolites (FAM) in this species as follows. (1) We conducted a radiometabolism study

to determine the route of excretion (feces or urine) and time delay of excretion of testosterone metabolites (sensu Palme et al., 2005) by injecting captive female red squirrels with radiolabeled testosterone and collecting urine and feces over the next 72 and 120 h, respectively (see Appendix). (2) We performed reverse-phase high performance liquid chromatography (RP-HPLC) to characterize the structure of the fecal testosterone metabolites and demonstrate that our EIA antibody detects the testosterone metabolites (see Appendix). (3) We demonstrated that in female red squirrels, FAM mirrored plasma androgen concentrations with lactating females having significantly higher FAM and plasma androgen levels than nonbreeding females (see Appendix). More details of these procedures and their results can be found in the Appendix.

Collection and extraction of fecal samples

Between 2006 and 2008, we collected a total of 384 fecal samples from 88 female squirrels prior to conception (n = 16), during gestation (range 1-37 days post-conception: n = 123), lactation (range 35-106 days post-conception: n = 196), and post-weaning (after 106 days post-conception: n = 49). Fecal samples were collected from underneath live-traps and placed in individual 1.5 ml vials in a -20 °C freezer within 5 h of collection. Squirrels were in traps for less than 2 h prior to handling, which is not long enough for FAM concentrations to be affected by trap-induced stress from the current capture event (Appendix; Dantzer et al., 2010; see also Harper and Austad, 2001). Furthermore, we only used fecal samples from squirrels that had not been trapped or handled within the previous 72 h to avoid confounding effects of previous trapping and handling events. Fecal samples collected from January to April were generally already

frozen at the time of collection and remained so while in the field (B. D., personal observation). In the warmer months (May-September), fecal samples were placed into insulated containers filled with wet ice until they were transferred to a -20 °C freezer. FAM in fecal subsamples left at room temperature for 5 h (4.28 ± 0.27 ng/g dry feces) did not differ from the FAM in subsamples from the same fecal sample that were frozen immediately (4.29 ± 0.13 ; paired t-test: $t_{10} = 0.02$, $P = 0.98$). As such, there should be no systematic bias in FAM in feces collected in the field and kept under cold conditions until frozen.

All fecal samples were lyophilized (LabConco, MO, USA) for 14-16 h, frozen in liquid nitrogen, and then pulverized using a mortar and pestle. Between samples, we rinsed the mortar and pestle with 5 ml of 80% methanol. We then extracted 0.05 g of the dry ground feces by adding 1 ml of 80% methanol. This solution was then shaken with a multivortexer at 1450 RPM for 30 min, and finally centrifuged for 15 min at 2500g ($24\,516.625\text{ m/s}^2$; Touma et al., 2003; Palme, 2005). The resulting supernatant was stored at -80 °C until analysis via EIA.

Determination of immunoreactivity in fecal samples

To quantify FAM, we used a previously developed testosterone EIA that measures 17β -OH androgens (Palme and Möstl, 1994), which has been successfully validated in some primate species (Möhle et al., 2002) and guinea pigs (Bauer et al., 2008). Details of this procedure (Möhle et al., 2002) and cross-reactivities of the antibody can be found elsewhere (Palme and Möstl, 1994). We are confident that our EIA antibody detected FAM from testosterone because it showed a high affinity for 17α -

hydroxyandrogens compared to other androgens (cross-reactivity with 17-oxo- or 17 α -hydroxyandrostanes was below 0.1%: Palme and Möstl, 1994). Samples were run in duplicate and the intra- and interassay coefficients of variation were 6.3 and 16.5%, (n = 30 plates). The assay had a sensitivity of 0.3 pg per well.

Statistical analyses

To determine how behavior varied among breeding female red squirrels, we used a principal components analysis (PCA) to reduce our multiple behavioral response variables (see Table 3.1 for list of behaviors) into a fewer number of synthetic variables. We conducted separate PCAs for behavioral observations conducted from 1994 to 2004 (n = 627 trials) and those conducted in 2008 (n = 276 trials) for the two reasons described above (see also Table 3.1 for differences in PCA loadings). We used the unrotated first principal component (PC1) of a PCA of the behavioral correlation matrix (Table 3.1), which explained 22 and 19.9% of the total variation for the 1994-2004 and 2008 behavioral observations, respectively. While there is no general agreement about how strongly an individual variable should load onto a principal component to warrant interpretation (Budaev, 2010), we selected behaviors with loadings greater than 0.30. All PC1 loadings were greater than 0.37 except for rattling from the PCA for the 1994-2004 behavioral observations (Table 3.1).

We examined changes in maternal behavior and FAM during reproduction by modelling changes in PC1 scores and FAM concentrations as a function of days since conception. We first used three separate linear mixed models (LMM) with the same fixed effects to examine the variation across reproduction (time from conception to weaning) in (1) PC1 scores from the 1994-2004 behavioral observations, (2) PC1

scores from the 2008 behavioral observations and (3) FAM concentrations.. We then examined changes in specific maternal behaviors during reproduction by modelling the proportion of time spent (1) in the nest, (2) foraging and (3) rattling as a function of days since conception using three separate generalized linear mixed models (GLMM) with binomial errors (logit link, models fit with Laplace approximation). Because we recorded rattling continuously in 2008, we only analyzed the behavioral focal data from 1994-2004 to determine how the proportion of time rattling varied among breeding females. Because maternal behavior and androgens may vary non-linearly across the reproductive period, we included both a linear and a quadratic term for days post-conception (hereafter, days post-conception²) in our statistical models when preliminary analyses using nonlinear regression showed that there were significant nonlinearities between our response and predictor variables (general additive models: Hastie and Tibshirani, 1990).

In all of these mixed effects models, we accounted for the repeated sampling of behavior or FAM from the same squirrels or by the same observers by including a random intercept term for squirrel ID and a random intercept term for observer using the lme4 package (Bates et al., 2008; Pinheiro and Bates, 2009) in R (version 2.9.2, R Development Core Team 2009). We calculated the proportion of residual variance in the LMMs that was due to the individual squirrel (i.e. repeatability: Lessels and Boag, 1987) by dividing the proportion of variance explained by the random effect (among-individual variance) by the total residual variance (among-individual variance + within-individual variance) and used likelihood ratio tests to determine whether the random effects improved the fit of each of the models (Pinheiro and Bates, 2009). We used

Table 3.1. Loadings of the first axis from principal components analysis using correlation matrices from 7 min behavioral observations conducted on breeding female red squirrels in 1994–2004 and 2008. Behaviors in boldface font indicate loadings that were used in our interpretations for principal component 1. No. observations were made ‘On midden’ in the 1994-2004 focals.

* These models are based on 627 observation sessions on 44 squirrels.

† These models are based on 276 observation sessions on 81 squirrels.

Behavior	Year (s) of behavioral observations	
	1994–2004 [*]	2008 [†]
Barking	-0.08	-0.1
Feeding	-0.37	-0.38
Foraging	-0.42	-0.42
In nest	0.63	0.41
On midden	-	0.14
Out of sight	-0.16	0.22
Rattling	-0.23	-0.45
Resting	0.04	0.11
Travelling	-0.43	-0.45
Vigilant	-0.07	-0.02

Wald t -tests to test the significance of the fixed effects of the GLMMs (Bolker et al., 2008).

To meet assumptions of normality, we ln-transformed FAM and PC1 values prior to analysis. Diagnostic plots after the transformations revealed that the residuals from the models described above were normally distributed and homoscedastic, and there were no outlying observations with high leverage. FAM concentrations are expressed as ln-transformed ng/g dry feces. For all of the LMM and GLMMs described above, days post-conception was standardized, but we present the raw days post-conception in the figures.

We also used segmented regression (segmented package in R: Muggeo, 2008) to determine whether changes in maternal behavior and FAM occurred around the same time period, which would suggest covariation between maternal androgens and behavior. Segmented or broken-line regression is typically used in data sets where the relationship between independent and dependent variables exhibits an abrupt change past some threshold (breakpoint) of the independent variable. In this approach, many separate regressions are performed on different intervals of the independent variable to identify whether there are differences in the magnitude or sign of the linear relationship between the independent and dependent variables. Here, segmented regression identified where the change in relationship between FAM or the measures of maternal behavior and days post-conception occurred (breakpoint) with some estimate of uncertainty (SE) about the location of this breakpoint in the regression lines. This approach allowed us to identify the points at which androgens and allocation towards specific behaviors peaked during lactation.

We present all results as means $\pm$ SE and considered differences statistically significant at $\alpha = 0.05$. All statistical analyses were conducted using R (version 2.9.2, R Development Core Team 2009).

Ethical note

All animal care protocols complied with both the *Canadian Council on Animal Care* and the *ASAB/ABS Guidelines for the Use of Animals in Research and Teaching*, and were approved by the Institutional Animal Care and Use Committees of the University of Toronto (no. 20006991) and Michigan State University (no. 04/08-046-00).

Results

Maternal behavior

We interpreted PC1 as an index of time allocation of breeding females as the loadings reflected a trade-off between maternal behavior (time in the nest interacting with offspring) and time engaged in other behaviors (foraging, rattling, travelling; Table 3.1). In both data sets larger values of PC1 represented sessions in which females spent more time in their nest with their pups and less time foraging, rattling and travelling (Table 3.1).

Maternal behavior varied significantly before and after parturition in the analyses of PC1 for both the 1994-2004 and 2008 data sets. For the 1994-2004 behavioral observations, PC1 scores decreased linearly with increasing days since conception (slope on ln-scale for days post-conception = -0.068 ± 0.015 ; $t_{626} = -4.49$, $P < 0.0001$; Table 3.2) indicating that nest use decreased whereas foraging, rattling and travelling increased. Maternal behavior in 2008 showed a similar pattern (slope on ln-

Table 3.2 Effects of days since conception on the behavior of breeding female red squirrels. Maternal behavior is represented by the first axis of a principal components analysis (PC1) of all behaviors recorded during behavioral observations of breeding female red squirrels collected over 10 years from 1994 to 2008. A nonlinear term for days post-conception (days post-conception²) was included to examine the nonlinear relationship between days since conception and maternal behavior (PC1). Results are from separate generalized linear mixed effects models conducted for data collected from 1994 to 2004 and those collected in 2008. Random intercept terms were included to account for repeated observations of individual squirrels (ID) and by different observers (Observer).

* These models are based on 627 observation sessions on 44 squirrels by 39 observers.

† These models are based on 276 observation sessions on 81 squirrels by 1 observer.

Model				
	Random effect	Variance	χ^2_1	P
PC1 (1994–2004 focals) *	ID	0.0076	22.36	< 0.0001
	Observer	0.0019	1.3	0.30
PC1 (2008 Focals)†	ID	0.05	13.32	0.0002
	Fixed effect	Parameter ± SE	t	P
PC1 (1994–2004 Focals) *	Intercept	1.04 ± 0.02	48.34	< 0.0001
	Days post-conception	-0.068 ± 0.015	-4.49	< 0.0001
PC1 (2008 Focals)†	Intercept	0.15 ± 0.2	0.682	0.25
	Days post-conception	-0.015 ± 0.005	-2.81	0.0027
	Days post-conception ²	0.00007 ± 0.00003	2.2	0.014

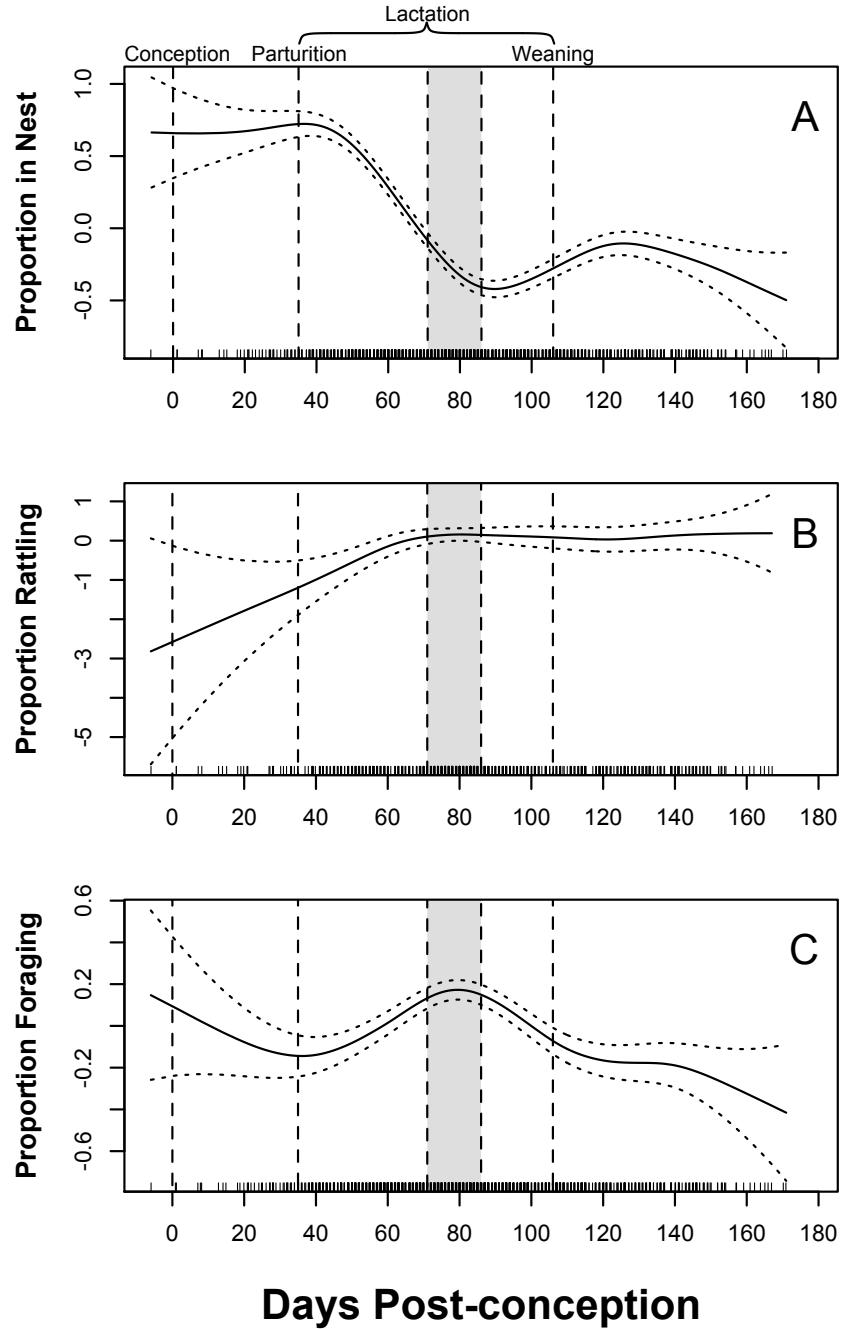


Figure 3.1. Maternal behavior of female North American red squirrels across the reproductive cycle. Behavioral variables represent the proportion of time females spent (A) in the nest, (B) rattling (territorial vocalization) and (C) foraging during 7-min behavioral observations. Values on y-axes represent standardized residual values from generalized additive models to visualize all nonlinearities, but the significant nonlinear relationships were analyzed using generalized linear mixed models. Dashed lines represent the standard errors and the grey box represents the range of juvenile emergence from the natal nest (71-86 days post-conception). Dashes on the x-axis represent each behavioral session performed. $n = 903$ for (A, C), $n = 627$ (B).

scale for days post-conception = -0.015 ± 0.005 ; $t_{275} = -2.81$, $P < 0.0001$; Table 3.2)

except that there was also a significant nonlinear relationship between PC1 scores and days post-conception (slope on ln scale for quadratic term for days post-conception = 0.00007 ± 0.00003 ; $t_{275} = 2.20$, $P = 0.014$; Table 3.2).

Changes in the three important female behaviors (proportion of time in the nest, foraging and rattling) corroborated the observed changes in PC1 scores. Soon after parturition (35 days post-conception), maternal behavior was highest ($55.9 \pm 8.5\%$ of time in nest), declined into early lactation, was lowest during mid-lactation when juveniles were first emerging from their natal nest ($15.2 \pm 2.9\%$ of time in nest), and then slightly increased following juvenile emergence (slope on ln-scale for quadratic term for days post-conception = 0.11 ± 0.02 ; $t_{902} = 5.29$, $P < 0.0001$; Fig. 3.1A; Table 3.3). The proportion of time females spent rattling was low soon after parturition ($0.4 \pm 0.28\%$ of time spent rattling), increased into early lactation and peaked during mid-lactation during the period of juvenile emergence ($2.3 \pm 0.54\%$ of time), and then remained fairly constant throughout the rest of lactation and postweaning (slope on ln-scale for quadratic term for days post-conception = -0.21 ± 0.079 ; $t_{626} = -2.65$, $P = 0.008$; Fig. 3.1B; Table 3.2). The proportion of time females spent foraging was lowest soon after parturition ($12.9 \pm 0.3\%$ of time spent foraging), increased into early lactation, was highest during mid-lactation during the period of first juvenile emergence from their natal nest ($17.8 \pm 1.4\%$ of time), and then declined following juvenile emergence and throughout the rest of lactation and postweaning (slope on ln-scale for quadratic term for days post-conception = -0.053 ± 0.022 ; $t_{902} = -2.44$, $P = 0.015$; Fig. 3.1C; Table 3.3).

Inclusion of individual squirrel identity as a random intercept term significantly improved the fit of all of our models for maternal behavior (Tables 3.2 and 3.3). From the analyses of PC1 for the 1994-2004 and 2008 behavioral data, individual identity explained 7.1 and 16.4% of the residual variance in maternal behavior not accounted by the fixed effects, respectively. This suggests that there were repeatable interindividual differences among females in the amount of time they allocated towards interacting in the nest with offspring, foraging, rattling and travelling.

Maternal androgens

Maternal androgen concentrations were significantly higher during lactation (3.57 ± 0.007 ln ng/g dry feces; $n = 196$) than during pregnancy (3.01 ± 0.008 ; $n = 123$; $F_{1,317} = -8.52$, $P < 0.0001$). Our more detailed analyses, however, showed that FAMs were lowest around conception, increased throughout gestation and after parturition, peaked during mid-lactation around juvenile emergence, and then declined during the latter part of lactation and after weaning (slope on ln-scale for quadratic term for days post-conception = -0.21 ± 0.024 ; $t_{383} = -8.75$, $P < 0.0001$; Fig. 3.2; Table 3.4).

Similar to maternal behavior, maternal FAM exhibited significant repeatable interindividual variation as inclusion of individual identity as a random intercept term significantly improved the fit of the detailed model using days post-conception ($\chi^2 = 46.1$, $df = 1$, $P < 0.0001$; Table 3.4) and explained 14.6% of the variance in FAM not accounted by the fixed effects.

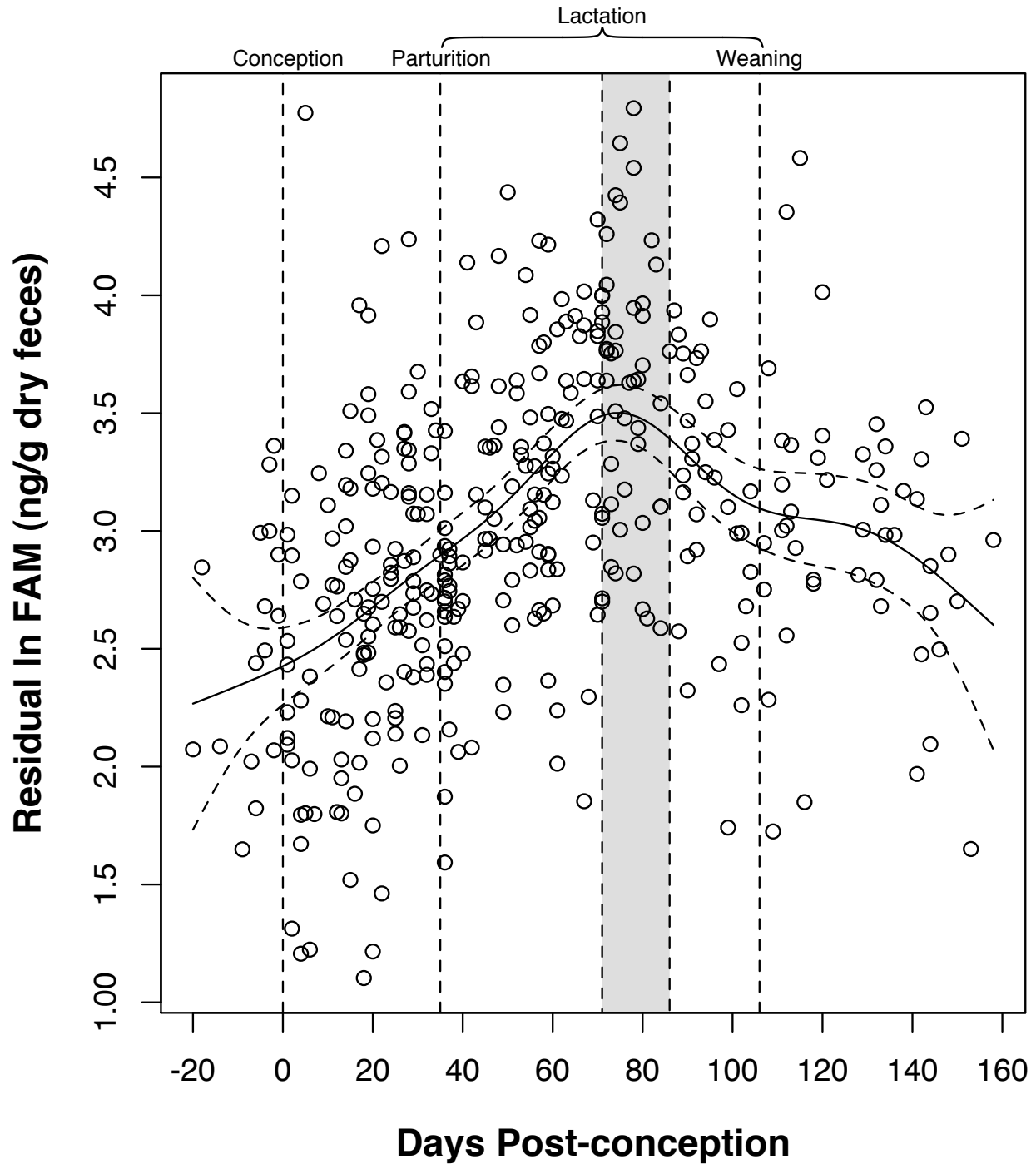


Figure 3.2. Fecal androgen metabolites (FAM) concentrations in female North American red squirrels ($n = 88$ squirrels) prior to conception ($n = 16$ samples), during gestation ($n = 123$ samples) and lactation ($n = 196$ samples), and after weaning ($n = 49$ samples). See text and Table 3.4 for results from these models. Dashed line represents the standard error and the gray box represents the range of juvenile emergence from the natal nest (71-86 days post-conception).

Relationship between maternal androgens and behavior

Using segmented regression, we found that maternal androgens peaked (breakpoint at 73 ± 4 days post-conception; Fig. 3.2) during mid-lactation around the period when juveniles were first emerging from their natal nest (71-86 days post-conception). During the same time period, the proportion of time that females spent in the nest declined to its lowest level (breakpoint at 66 ± 15 days post-conception: Fig. 3.1A) and the proportion of time that females spent rattling (breakpoint at 74 ± 16 days post-conception) and foraging (breakpoint at 79 ± 13 days post-conception) increased to their highest levels (Figs. 3.1B and 3.1C).

Discussion

Our observations are consistent with the hypothesis that androgens influence behavioral trade-offs in breeding females, whereby periods of heightened androgens are associated with a reduction in time allocated towards maternal behavior and an increase in time spent engaged in resource acquisition and territory defense. We found that FAM in breeding female red squirrels increased after conception and parturition, peaked during mid-lactation around juvenile emergence from the natal nest, and then declined during the remainder of lactation and after weaning. Around the same period when maternal androgens were highest, breeding female squirrels spent the least amount of time in the nest, but the highest amount of time rattling and foraging. Previous studies have also found evidence for this hypothesis (Fite et al., 2005). For example, the highest plasma testosterone concentrations during reproduction in female Belding's ground squirrels, *Spermophilus beldingi*, occur during peak aggressive and

Table 3.3. Effects of days since conception on the proportion of time breeding female red squirrels spent in the nest, uttering rattle vocalizations and foraging during behavioral observations collected over 10 years from 1994 to 2008. A nonlinear term for days postconception (days postconception²) was included to examine the nonlinear relationship between days since conception and the specific maternal behaviours.

* These models are based on 903 observation sessions on 125 squirrels by 40 observers.

† These models are based on 627 observation sessions on 44 squirrels by 39 observers.

Model				
	Random effect	Variance	χ^2_1	P
In nest [*]	ID	4.85	2230	< 0.0001
	Observer	1.46	413	< 0.0001
Rattle vocalizations [†]	ID	0.56	331	< 0.0001
	Observer	0.17	4.2	0.041
Foraging [*]	ID	1.98	2589	< 0.0001
	Observer	1.25	281	< 0.0001
	Fixed effect	Parameter ± SE	Wald t	P
In nest [*]	Intercept	-1.8 ± 0.38	-4.76	< 0.0001
	Days postconception	-0.43 ± 0.035	-12.09	< 0.0001
	Days postconception ²	0.11 ± 0.02	5.29	< 0.0001
Rattle vocalizations [†]	Intercept	-3.99 ± 0.18	-22.14	< 0.0001
	Days postconception	0.44 ± 0.12	3.69	0.0002
	Days postconception ²	-0.21 ± 0.079	-2.65	0.008
Foraging [*]	Intercept	-1.03 ± 0.28	-3.71	0.0002
	Days postconception	-0.075 ± 0.035	-2.11	0.035
	Days postconception ²	-0.053 ± 0.022	-2.44	0.015

nest maintenance behavior (Nunes et al., 2000*b*). Additionally, experimental elevation of testosterone in breeding female birds can inhibit some behavioral estimates of maternal investment in current offspring (time spent brooding: O'Neal et al., 2008).

In red squirrels, the observed changes in maternal androgens during reproduction may optimize parental investment in current offspring by decreasing costly maternal behavior (nursing) and increasing time investment in territory defense and self-maintenance (foraging) to maximize maternal longevity. Costs of reproduction were previously found to be absent in prime-aged females in this population (survival costs of reproduction were only documented for young and very old females: Descamps et al., 2009). It is possible that changes in maternal androgens and adjustments to maternal behavior during lactation are important for minimizing these costs of reproduction. During years of high food availability that occur every 4 to 6 years (LaMontagne and Boutin, 2007), females have greater reproductive success due to increased juvenile survival (McAdam and Boutin, 2003), and lifetime female fitness is heavily influenced by longevity (McAdam et al., 2007). The patterns of maternal androgens that we found in this study may be the result of natural selection for maternal behavior that decreases the costs associated with reproduction and increases longevity to maximize female reproductive success by increasing the chance of encountering years of high food availability in which reproductive success is increased.

Changes in maternal behavior are unlikely to be driven by seasonal changes in resource abundance or temperature. Red squirrels breed seasonally, so changes in reproductive status were associated with advancing Julian date. This caused collinearity between days post-conception and Julian date that precluded the inclusion of both

Table 3.4. Effect of days since conception on fecal androgen metabolite (FAM) concentrations of breeding female red squirrels. A nonlinear term for days postconception (days postconception²) was included to examine the nonlinear relationship between days since conception and FAM. Results are from linear mixed effects models conducted for data collected from 2006 to 2008 that included a random intercept term for repeated samples of individual squirrels (ID).

Random effect	Variance	χ^2_1	<i>P</i>
ID	0.10	46.1	< 0.0001
Fixed effect	Parameter ± SE	<i>t</i>	<i>P</i>
Intercept	3.55 ± 0.052	68.53	< 0.0001
Days	0.31 ± 0.03	9.9	< 0.0001
Days	-0.21± 0.024	-8.75	< 0.0001

terms in our statistical models. However, mean parturition dates and hence dates of gestation and lactation vary by over 1 month from one year to the next (Boutin et al., 2006). In addition, breeding by red squirrels is asynchronous, such that parturition dates within a given year also span over 1 month or more (Lane et al., 2008; Lane et al., 2009). For example, 35 days post-conception corresponded to the beginning of March for some females in some years, but late June for other females. In our study area, there are marked differences in snow pack, temperature, and seasonal food resources between the months of March and June. This inter- and intra-annual asynchrony in breeding by red squirrels would probably eliminate the possibility that the consistent nonlinear relationships between maternal behavior and days post-conception from 1994 to 2008 that we observed were due to seasonal changes (e.g. food or temperature).

Maternal androgens in mammals

We had expected that maternal androgens might be highest around weaning to reflect the absence of suckling. In many mammalian species, suckling by offspring inhibits gonadal activity, and consequently suppresses production of testosterone and estradiol is suppressed (Taya and Greenwald, 1982; reviewed in McNeilly, 2001). Prior to weaning, as offspring grow and become more independent, suckling stimulation declines and gonadal activity may increase and enable androgen production (Taya and Greenwald, 1982). However, we found that FAM peaked during mid-lactation coinciding with juvenile emergence. Juvenile emergence occurs 25-35 days prior to average weaning date (Humphries and Boutin, 1996) and does not represent a time when suckling stimulation by offspring is attenuated. In fact, suckling stimuli are probably highest at this time because of the high energetic demands of offspring due to their

large size and activity, and because they are likely to be inefficient at foraging on their own. We also found that FAM did not decline after parturition when suckling was initiated, and it increased from parturition until mid-lactation. Lastly, from first emergence until weaning, the proportion of time that juvenile red squirrels spend feeding outside of the nest significantly increases (M. C. Andruskiw, personal communication) and consequently suckling presumably decreases, but we found that FAM actually decreased. Therefore, we do not think that patterns of FAM reflect suckling stimuli by offspring in this species.

Although ovulation can occur during the later stages of lactation in red squirrels (Boutin et al., 2006; McAdam et al., 2007), it is unlikely that the observed peak in FAM around juvenile emergence reflected a surge in estradiol associated with ovulation (Shaikh, 1971). The cross-reactivity of estradiol in the testosterone EIA is very low ($<0.1\%$; Palme and Möstl, 1994). Additionally, if FAM reflects changes in estradiol concentrations during ovulation cycles, we would expect a peak in FAM around conception (the first ovulation), which we did not see (Fig. 3.2).

The peak in FAM around juvenile emergence may have also promoted the increase in female territorial vocalizations that we observed around juvenile emergence. Protecting altricial offspring from infanticide may be a major driver in the evolution of female territoriality in mammals (Wolff 1993, 1997). In many species, male testosterone concentrations are positively correlated with aggressive or territorial behavior (reviewed in Wingfield et al., 1990; Demas et al., 2007). As such, heightened concentrations of circulating androgens in females could increase antagonistic behavior, which could reduce the risk of infanticide during the period of offspring dependence (*sensu* Ostner et

al., 2002). Infanticide has been documented in this population (S. Boutin, unpublished data), but we know little about factors affecting rates of infanticide in red squirrels.

Repeatable interindividual variation in maternal behavior and androgens

Recent studies in the burgeoning field of animal personality research have demonstrated the potential for consistent interindividual differences among individuals in behavior to have important ecological and evolutionary consequences (Sih et al., 2004; Réale et al., 2007). We found that there were repeatable interindividual differences among breeding female red squirrels in how they allocated their time during the breeding season, including when they were interacting with their dependent offspring. Previous studies have demonstrated repeatable differences in maternal behavior of mammals (e.g. ‘mothering styles’: Altmann, 1980; Maestripieri 1993, 1994; Albers et al., 1999; Bardi et al., 2001). While our ability to observe how lactating red squirrels interact with their dependent offspring was limited because of their secretive behavior, we did demonstrate that there were repeatable differences among females in the amount of time they spent in the nest with their offspring as opposed to the time spent foraging and defending their territory via vocalizations. Whether these individual differences in the amount of time spent in the nest with offspring are associated with variation in the style of care provided within the nest will require creative methods for observing behavior within nests under natural conditions.

The organizational effects of prenatal exposure to androgens can have acute effects on offspring phenotype in oviparous (reviewed in Groothuis et al., 2005) and mammalian species (Seale et al., 2005; Dloniak et al., 2006). As such, the repeatable interindividual differences in maternal androgen concentrations that we found in this

study could generate significant variation in offspring phenotype, which could have important ecological and evolutionary implications. Future studies in this species will estimate the heritability of FAM and could elucidate how maternal androgens influence offspring phenotype and survival, as well as relationships between maternal androgens, survival and reproductive success.

There is now growing evidence that androgens may play a major role in shaping maternal behavior (Nunes et al., 2000*b*; Fite et al., 2005; Sandell, 2007; O'Neal et al., 2008). Although our study is correlational, our data are consistent with the hypothesis that androgens may play a similar role in shaping behavioral trade-offs between resource acquisition and parental care in breeding females as has been found in males of some species (Hegner and Wingfield, 1987; Wingfield et al., 1990; Ketterson and Nolan, 1999; Hau, 2007; McGlothlin et al., 2007). In red squirrels, androgens could be used to optimize maternal behavior by reducing maternal care and enhancing the probability of survival and the future reproduction of breeding females. Definitive tests of these hypotheses, however, will depend on long-term experimental manipulations of maternal androgen concentrations.

APPENDIX

Appendix 1 for Chapter 3: Radiometabolism Study and RP-HPLC

Methods

Radiometabolism studies are a vital part of the validation of an assay to measure hormone metabolites in fecal samples because they identify the route of excretion (urine or feces) of the metabolites as well as the time delay of excretion (Touma and Palme, 2005). From January to March 2008, we performed a radiometabolism study on red squirrels in captivity (for details about capture and husbandry of squirrels see Dantzer et al., 2010). We injected four captive female red squirrels intraperitoneally with 1110 kBq of radiolabeled testosterone (1, 2, 6, 7- ^3H ; Amersham Biosciences, Quebec, Canada; specific activity = 1.55 TBq/mmol) dissolved in 0.1 ml physiological saline containing 5% ethanol and 5% toluene at 0800 hours on day 1 of this study. We collected urine (0-52 h postinjection) and feces (0-120 h postinjection) every ~4 h (except from 2000 to 0800 hours) from pans underneath the cages that were covered with metal screening (0.5 x 0.5 cm mesh) to prevent feces and urine from mixing. Between sampling periods, we rinsed the pans twice with a radioactive decontamination solution (Decon 75, Fisher Scientific, Pittsburgh, PA, USA). Fecal and urine samples were placed into a -20 °C freezer within 20 min of collection. We extracted fecal samples as described within the text above except that we also rinsed the mortar and pestle twice with a decontamination solution (Decon 75) between samples.

We dried down urine samples under air until only about 1 ml remained. To determine radioactivity in the urine and fecal extracts, we added 4 ml of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the concentrated urine

or 100 ml of the fecal extract and quantified radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, USA).

Fecal extracts of samples containing peak radioactivity from female ($n = 2$) squirrels were dried under air and then subjected to reverse-phase high performance liquid chromatography (RP-HPLC). After separation, we measured both the radioactivity and immunoreactivity (see below) in the collected fractions. Details of this method can be found in Lepschy et al. (2007) and Touma et al. (2003). All of the procedures listed above for the captive red squirrels were approved by the Institutional Animal Care and Use Committees at the University of Toronto (no. 20006991).

Results

Route of excretion and time to peak excretion of radiolabeled testosterone

We collected a total of 135 fecal and 84 urine samples from 10 squirrels (4 females and 6 males) over the 120 h radiometabolism study. Of the 1110 kBq of ^3H -testosterone we administered to the squirrels, we recovered $37.7 \pm 0.04\%$ of which $43.7 \pm 0.1\%$ was in the urine and $56.3 \pm 10.4\%$ was in the feces. The mean time to peak excretion of ^3H -testosterone was 7.0 ± 0.9 h in the urine and 10.3 ± 1.3 h in the feces. Therefore, trapping-induced stress could not have influenced FAM as traps were checked every 2 h and the peak in radioactive FAM occurred over 10 h after injection.

Structure and polarity of testosterone metabolites from RP-HPLC analysis

Injected ^3H -testosterone was heavily metabolized and polar metabolites, resembling conjugated steroids, dominated (Fig. 3.3). Several radioactive peaks beyond fraction 60 were found and two of these peaks (eluting around fraction 83 and 87) yielded the highest immunoreactivity in the testosterone EIA. No radioactivity with corresponding immunoreactivity (as determined by the testosterone EIA) at the elution position of testosterone (around fraction 80) was present. Thus, testosterone was entirely metabolized prior to excretion as has been found in previous studies (Möhle et al., 2002).

The results from the radiometabolism study and RP-HPLC demonstrate that testosterone metabolites were excreted in the feces of North American red squirrels and that our EIA antibody reacted with testosterone metabolites around fractions 83 and 87 with a 17β -hydroxy-group.

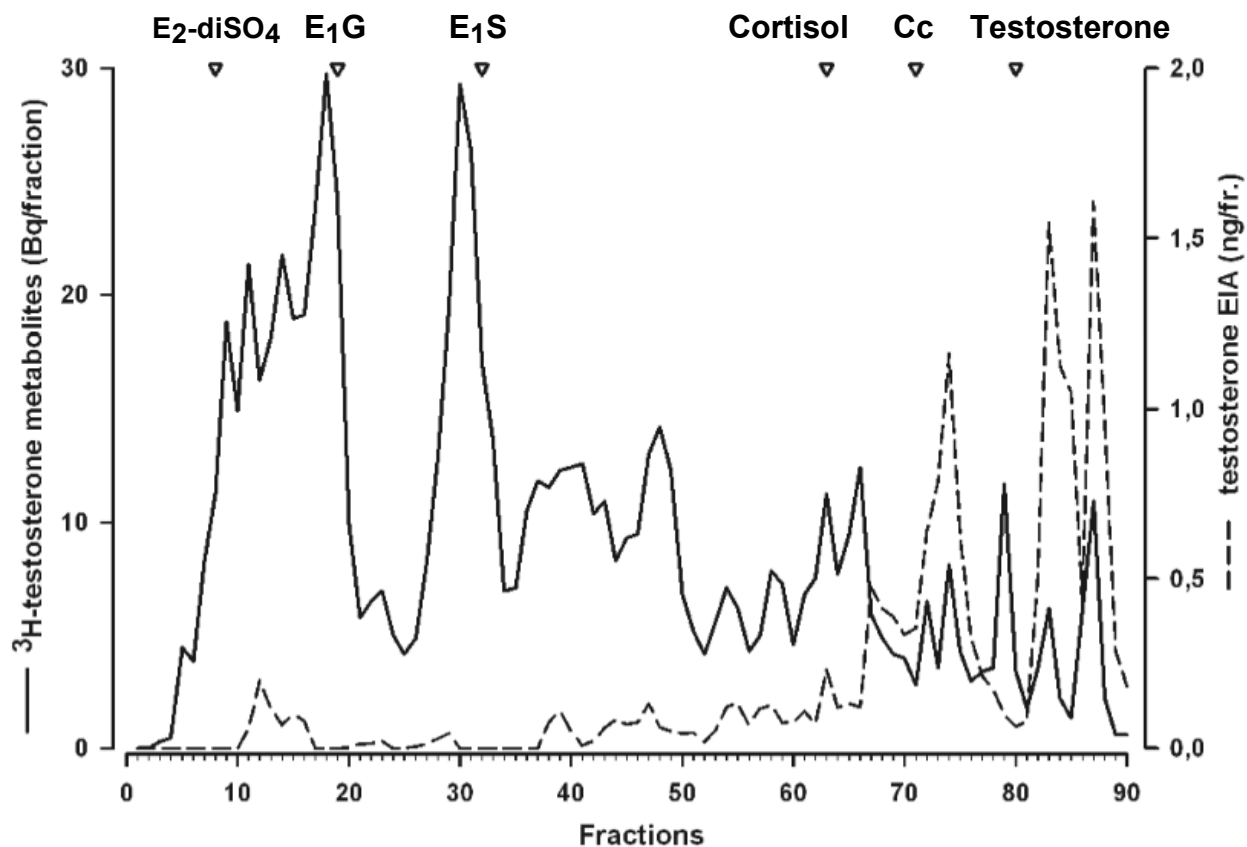


Figure 3.3. Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of fecal ^3H -testosterone metabolites (peak sample) in the feces of female North American red squirrels. Open triangles mark the approximate elution positions of respective standards ($\text{E}_2\text{-diSO}_4$ = 17β -estradiol-disulphate, E_1G = estrone-glucuronide, E_1S = estrone-sulphate, Cc = corticosterone).

Appendix 2 for Chapter 3: Physiological Validation

Methods

A second step in validating an assay to measure FAM concentrations is to demonstrate that the effects of reproductive condition on FAM mirror those found in plasma androgen concentrations. We collected fecal and plasma samples from nonbreeding (not pregnant or lactating) and lactating females. Fecal samples were collected from 2006 to 2008, while plasma samples were collected in 6 years between 1996 and 2010. We obtained plasma samples either from cutting a small piece of the toenail or from the suborbital sinus. For the latter, we first anaesthetized squirrels using isoflurane ISP (3.5% in air) and bled them from the suborbital sinus using a heparinized glass pipette (Boonstra et al. 2008). Squirrels were completely anaesthetized within 15-30 s and blood samples were collected within 1 min. All plasma samples were collected within 2 h of live-trapping and squirrels were released following collection of the blood samples. These procedures were approved by the University of Toronto Animal Care Committee (no. 20006991).

We measured plasma testosterone + dihydrotestosterone (DHT) via radioimmunoassay using a protocol based upon that of Abraham et al. (1971) that we have used previously (Boonstra and Boag, 1992; Boonstra et al., 2008). Plasma samples (25 μ l) were treated with 20 μ L NH_4OH to saponify triglycerides. The antibody (P43/11) was produced by Croze and Etches (1980) and has relatively high cross-reactivity to 5α -dihydrotestosterone (62%) and low cross-reactivity to dehydroepiandrosterone (<0.8%: Boonstra et al., 2008). Assay sensitivity was 10 pg/25 μ l plasma and non-detectable samples were given a value of 10 pg. The intra- and

interassay coefficients of variation were 5 and 6%, respectively ($n = 8$ independent assays).

To determine how the reproductive condition of female (nonbreeding or lactating) squirrels affected (1) plasma testosterone + DHT and (2) FAM concentrations, we conducted a general linear model (fixed effect: reproductive condition) and LMM (fixed effect: reproductive condition), respectively. To meet assumptions of normality, we ln-transformed plasma androgen ($x + 1$) and FAM concentrations. However, raw plasma androgen concentrations are presented below and in Figure 3.4.

Results

The effects of reproductive condition on plasma testosterone + DHT concentrations were mirrored in FAM (Fig. 3.4). Female reproductive condition had a significant effect on plasma testosterone + DHT ($F_{1, 35} = 9.064$, $P = 0.0048$), with lactating females having significantly higher plasma testosterone + DHT concentrations ($n = 18$; 0.98 ± 0.15 ng/ml) than nonbreeding females ($n = 19$; 0.53 ± 0.05 ng/ml; $t_{35} = -3.01$, $P = 0.0048$; Fig. 3.4). Reproductive condition also had the same significant effect on FAM ($F_{1, 65} = 36.86$, $P < 0.0001$; Fig. 3.4), with lactating females having significantly higher FAM ($n = 36$; 3.86 ± 0.09 ln ng/g dry feces) than nonbreeding females ($n = 94$; 2.81 ± 0.04 ln ng/g dry feces; $t_{129} = -8.09$, $P < 0.001$). These results demonstrate that this assay is fully capable of detecting variation in FAM in female red squirrels that is directly related to plasma androgen concentrations.

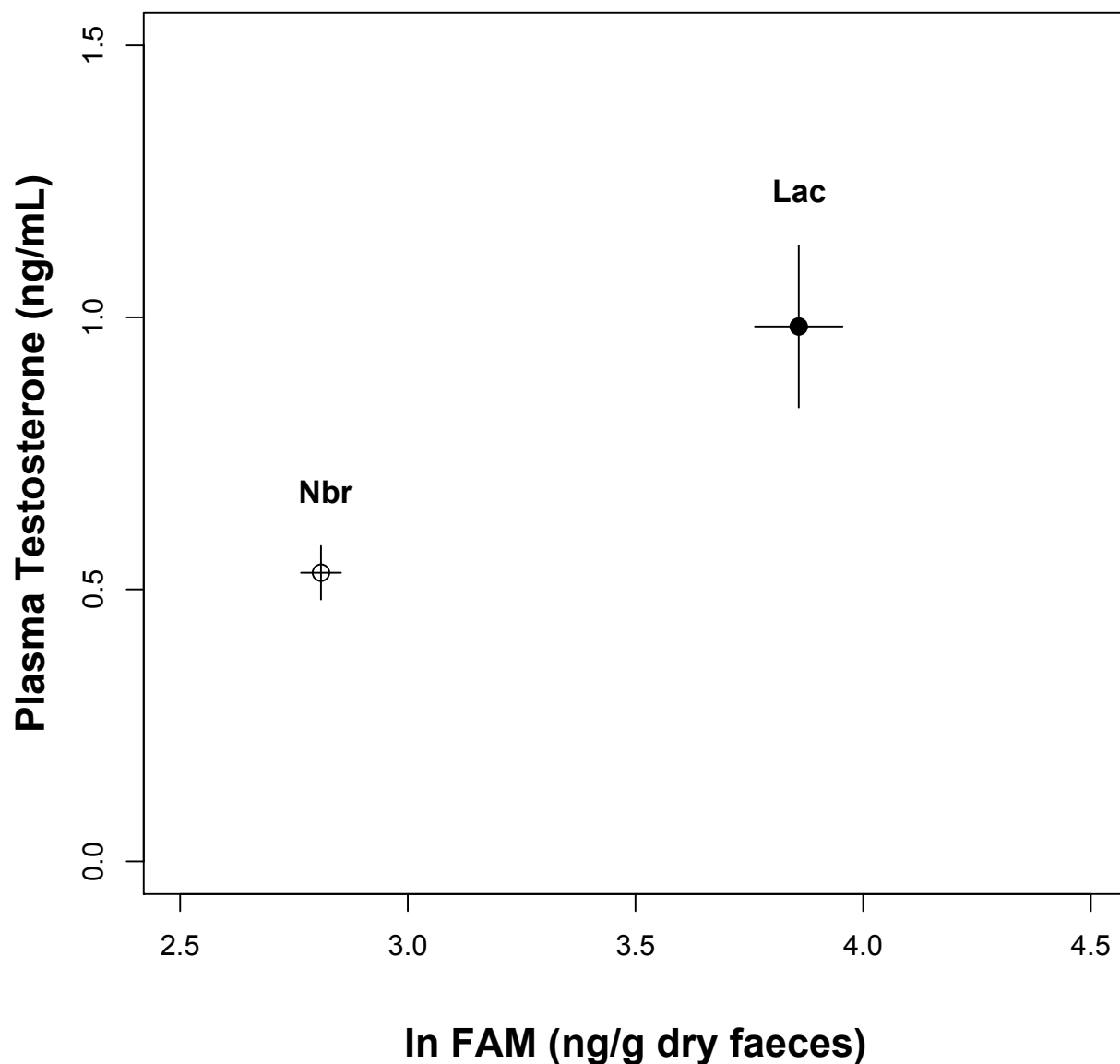


Figure 3.4. Effects of reproductive condition on plasma testosterone + dihydrotestosterone (DHT: 'Plasma Testosterone') and ln-transformed fecal androgen metabolite (FAM) concentrations in nonbreeding ('Nbr') and lactating ('Lac') female North American red squirrels (March-August). Raw plasma androgen concentrations are presented but ln-transformed ($x + 1$) values were used for the statistical analysis. Data presented are means $\pm$ SE.

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CHAPTER 4

Dantzer, B., McAdam, A. G., Palme, R., Boutin, S., Boonstra, R. 2011. How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. *General and Comparative Endocrinology* 174, 124-131.

Introduction

The neuroendocrine system can enable a prompt, adaptive, and multifaceted response to environmental variation. Responses of the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes to environmental information have been particularly well studied (Mooradian et al., 1987; Silver et al., 2002). Adrenal (glucocorticoids: GCs) or gonadal (androgens) steroid hormones are produced as a downstream response to activation of the HPA and HPG axes, respectively (Mooradian et al., 1987; Sapolsky et al., 2000). GCs and androgens are intrinsically linked to metabolism, energy allocation, and reproduction (Mooradian et al., 1987; Staub and De Beer, 1997; Sapolsky et al., 2000). There is a growing interest in measuring associations among hormones, physiological, behavioral, and life history characteristics in free-ranging animals (Ketterson and Nolan, 1992; Sinervo and Svensson, 1998; Ricklefs and Wikelski, 2002; Boonstra, 2005; Reeder and Kramer, 2005; McGlothlin and Ketterson, 2008; Bonier et al., 2009; Ganswindt et al., 2010).

Measuring hormone concentrations in free-ranging animals has traditionally required obtaining plasma samples, which can be difficult and potentially harmful to study animals because it generally requires live-trapping and withdrawal of blood from temporarily restrained animals (Sheriff et al., 2011). Unfortunately this approach may not be feasible for large or rare and endangered species. Additionally, trapping or temporary restraint can also introduce systematic biases in plasma GC and androgen concentrations (Romero and Romero, 2002; Fletcher and Boonstra, 2006; Delehanty and Boonstra, 2009).

Recent studies have increasingly measured steroid hormone metabolite concentrations in fecal samples collected from free-ranging animals. Fecal steroid hormone metabolite concentrations (FHM) are thought to reflect an integrated average of circulating unbound hormone levels over some time period rather than point estimates that are obtained from plasma samples (Palme et al., 1996; Goymann, 2005; Palme et al., 2005; Sheriff et al., 2010). FHM will typically be unaffected by trapping- or restraint-induced stress as long as the time of temporary captivity is less than the total time it takes for food to pass from the duodenum to the rectum (Palme et al., 1996; Goymann et al., 1999; Harper and Austad, 2001; Palme, 2005). For these reasons and because of the technique's relative non-invasiveness, the diversity of animals in which FHM have been measured is growing rapidly (Millspaugh and Washburn, 2004; Palme et al., 2005).

However, the ease of collection belies some of the difficulties associated with measuring FHM in free-ranging animals (Buchanan and Goldsmith, 2004; Millspaugh and Washburn, 2004; Tuma and Palme, 2005; Sheriff et al., 2011). A particularly relevant issue for studies in free-ranging animals that has received little attention is the potential effects of diet on FHM (Mooradian et al., 1987; Wasser et al., 1993; von der Ohe et al., 2004). In omnivores or herbivores, variability in consumption of plant fiber may have an important effect on FHM. For example, a high fiber diet can increase estrogen metabolite concentrations in the feces of humans (Goldin et al., 1982; Pusateri et al., 1990) and can decrease progesterone metabolite concentrations in the feces of yellow baboons *Papio cynocephalus cynocephalus* (Wasser et al., 1993). The mechanisms by which diet induces differences in FHM are largely speculative but may

be due to the effects of dietary fiber on fecal mass, gut passage time, biliary excretion of hormones, and enterohepatic circulation, or due to changes in microbial activity that alters steroid metabolite structure (Eriksson and Gustafsson, 1970; Goldin et al., 1982; MacDonald et al., 1983; von der Ohe and Servheen, 2002). For example, increased dietary fiber consumption may decrease reabsorption of steroid hormones from the intestine, which increases FHM and decreases plasma steroid hormone concentrations (Goldin et al., 1982).

Diets of free-ranging animals are rarely uniform and therefore longitudinal monitoring of FHM could be influenced by seasonal changes in their diet. For example, in herbivores that live in environments with major seasonal shifts in food availability, observed increases in fecal glucocorticoid metabolite concentrations during periods of low food availability could represent true seasonal endocrine changes, indirect effects of malnourishment, dietary influences that affect the recovery of the metabolites such as changes in fiber consumption, or a combination of all three. However, examinations of the role of diet on FHM in free-ranging or non-traditional study animals are rare (but see Wasser et al., 1993; von der Ohe et al., 2004; Goymann, 2005).

Here, we experimentally examined how diet affects fecal cortisol (FCM) and androgen (FAM) metabolite concentrations in captive North American red squirrels (*Tamiasciurus hudsonicus*). We measured FCM using an assay that we have previously validated for use in this species (Dantzer et al., 2010) and measured FAM using an enzyme immunoassay we have previously validated for use in female red squirrels (Dantzer et al., 2011) and validate for males herein. We validated the EIA to measure FAM in male squirrels by determining the route (urine or feces) and time course of

excretion of radiolabeled testosterone metabolites, using reverse-phase high performance liquid chromatography (RP-HPLC) to characterize the structure of the testosterone metabolites and identify that our EIA antibody detects these metabolites, and finally showing that our EIA accurately reflects the reproductive condition (gonads active or quiescent) of free-ranging male squirrels. We then measured the changes in FCM and FAM as we manipulated the diets of female and male squirrels in captivity. All squirrels were initially fed the same diet (sunflower seeds, peanut butter, apples), but then we switched one group of squirrels to a diet consisting of conifer seed and apples whereas the other group was fed peanut butter and apple. We then measured any changes in FCM and FAM for 94 h after the manipulation started.

Materials and Methods

Capture and husbandry of captive red squirrels

We captured 11 red squirrels (5 females, 6 males) in January 2008 in Algonquin Provincial Park (APP: 45° 30', 78° 40') using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI). Squirrels were transported to the Wildlife Research Facility at the University of Toronto Scarborough where they were maintained in captivity until their rerelease at their place of capture in March 2008. Each squirrel was placed into its own radiometabolism cage (91.5 x 61 x 46 cm) that contained a stainless-steel nest box (with 1 x 1 cm mesh floor) and cotton bedding. Squirrels were maintained at a temperature of ~10 °C and on a photoperiod that was changed weekly to correspond to the seasonal change in photoperiod in the location of capture at that time of year. All squirrels were reproductively quiescent upon capture but within 26 days after capture,

male squirrels developed scrotal testes reflecting gonadal recrudescence. Squirrels habituated to these conditions before we performed any procedures that we describe below and elsewhere (Dantzer et al., 2010, 2011). Red squirrels were captured in APP under permit #AP-08-0 and our housing protocol was approved in accordance with the guidelines of the Canadian Council on Animal Care by the University of Toronto Institutional Animal Care and Use Committee (#20006991).

Radiometabolism of testosterone in males and RP-HPLC

The first step we took to validate our assay to measure FAM in male red squirrels was to identify the route of excretion (urine or feces) and the time delay of excretion of testosterone metabolites using a radiometabolism study (Touma and Palme, 2005), which we have previously performed in female red squirrels (Dantzer et al., 2011). We injected 6 captive male red squirrels intraperitoneally with 1110 kBq of radiolabeled testosterone (1,2,6,7- ^3H ; Amersham Biosciences, Quebec, Canada; specific activity = 1.55 TBq/mmol) dissolved in 0.1 mL physiological saline containing 5% ethanol and 5% toluene. We collected urine (0-52 h post-injection; n = 50 samples) and feces (0-120 h post-injection; n = 75 samples) every ~4 h (except from 2000-0800 h: Table 4.1) from pans underneath the cages that were covered with metal screening (0.5 x 0.5 cm mesh) to prevent feces and urine from mixing. The floors of the radiometabolism cages were slatted (as well as the nest boxes) and therefore all excreta fell onto the pan (urine) or mesh screening (feces). All urine that was present at each sampling period was aspirated off of the surface of the pan using a pipette. The surface of the pans were then rinsed with 4 mL of 80% methanol and added to the urine sample. We rinsed the pans twice with a radioactive decontamination solution between sampling periods

(Decon 75, Fisher Scientific, Pittsburgh, PA, USA). Fecal and urine samples were placed into a -20 °C freezer within 20 min of collection.

The second step we took to validate this assay to measure FAM in male red squirrels was to use reverse-phase high performance liquid chromatography (RP-HPLC) to characterize fecal ^3H -testosterone metabolites and to demonstrate that our enzyme-immunoassay (EIA: see below) antibody detects the fecal testosterone metabolites. Fecal extracts of samples containing peak radioactivity (see below) from male ($n = 2$) squirrels were dried under air and then subjected to RP-HPLC. After separation, we measured both the radioactivity and immunoreactivity in the collected fractions. Details of this method can be found elsewhere (Touma et al., 2003; Lepschy et al., 2007).

Diet manipulation in captive squirrels

All captive squirrels were initially fed the same diet of ad libitum unroasted hulled sunflower seeds and all natural peanut butter (Kraft All Natural), as well as 1 apple every 48 h from 1 to 57 days post-capture. Squirrels readily drank water from a water bottle with a stainless steel nipple that was provided throughout captivity. All squirrels gained weight on this diet compared to their initial weight at capture (mean $\pm$ SE: 15 ± 4.66 g gained during entire period of captivity: Fig. 4.1).

On the first day of the diet manipulation experiment (58 days post-capture), we removed all remaining food from the radiometabolism cages. On this day and every 24 h thereafter, we provided squirrels in the “Peanut Butter treatment” (3 females and 4 males) with ~6 tablespoons of all natural peanut butter. Squirrels in the “Cones treatment” (2 females and 2 males) were provided with 40 white spruce (*Picea glauca*)

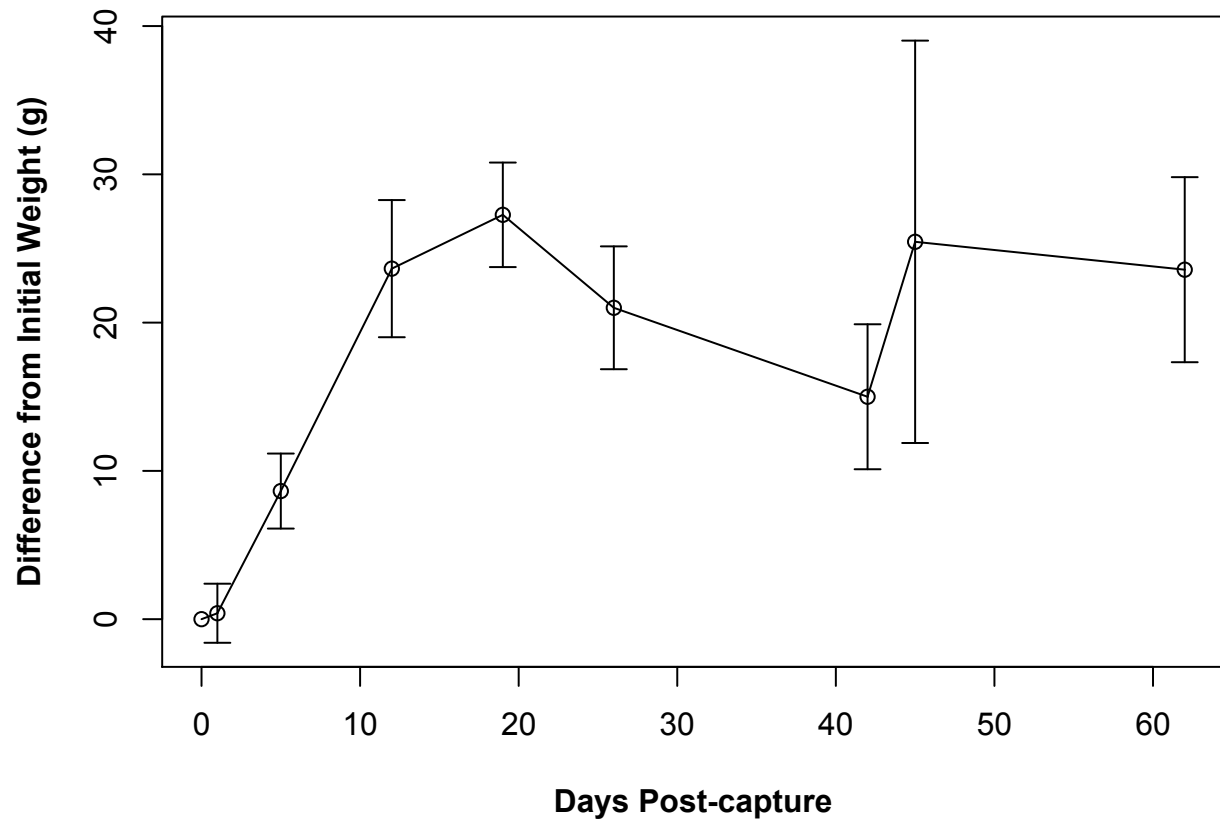


Figure 4.1. Difference in weight (g) of captive red squirrels between initial capture (0 d post-capture) and up to 62 d post-capture. Squirrels were weighed at 0, 1, 5, 12, 19, 26, 42, 45, and 62 d post-capture.

cones per day. We considered this to be ad libitum provisioning of peanut butter and spruce seed as there was always peanut butter or whole cones remaining 24 h after providing them with the food. Squirrels in both treatments received access to ad libitum water and we also provided each squirrel with one apple every 48 h. We collected fecal samples from the screens of the radiometabolism cages every 4-8 h from 0 to 94 h after the diet manipulations started (Table 4.1). Fecal samples were placed into a -20 °C freezer within 20 min of collection.

Fecal sample collection from free-ranging male squirrels

We studied a free-ranging population of red squirrels in the Yukon, Canada (61°N, 138°W) that has been monitored continuously since 1987 (McAdam et al., 2007). Male red squirrels were routinely trapped on their territories using Tomahawk live-traps and handled using a canvas and mesh bag. All squirrels on these study areas were individually marked with uniquely numbered ear tags (National Band and Tag, Newport, KY, USA). During each capture, squirrels were identified by reading their ear tags, weighed, and their reproductive status (testes scrotal or abdominal) was determined by palpation. We collected fecal samples from free-ranging male red squirrels to demonstrate that our assay to measure FAM reliably distinguished between fecal samples from males with scrotal testes versus those with abdominal testes. Fecal samples were collected during capture from underneath the live-traps using forceps, placed individually into 1.5 mL vials, and then frozen at -20 °C within 4-5 h after collection. Fecal samples were generally frozen upon collection during winter trapping (February-April). During the warmer months (May-July), fecal samples were placed into

an insulated container containing wet ice. This period of time when fecal samples are not completely frozen does not systematically affect FCM (Dantzer et al., 2010) or FAM (Dantzer et al., 2011). All fecal samples were collected within 2 h of initial capture, which is not long enough for trapping-induced stress from the current capture to affect FCM (Dantzer et al., 2010) or FAM (Dantzer et al., 2011). We also did not analyze any fecal samples from squirrels that were trapped within the previous 72 h. All of our live-trapping and handling procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (# 04/08-046-00).

Extraction of hormone metabolites from feces

All fecal samples were stored at -80°C until analysis except those collected in the field were stored at -20°C until they were shipped to the University of Toronto Scarborough and thereafter stored at -80°C . Fecal samples were first lyophilized (LabConco, MO, USA) for 14-16 h to remove any potential differences in water content [50]. Next, samples were placed into liquid nitrogen and pulverized using a mortar and pestle. Between samples, we thoroughly rinsed and cleaned the mortar and pestle with 5 mL of 80% methanol. We then extracted 0.05 g of the dry ground feces by adding 1 mL of 80% methanol, shaking this solution on a multivortexer at 1450 rpm for 30 min, and then centrifuging for 15 min at 2500 g (Touma et al., 2003). The resulting supernatant containing the metabolites was stored at -80°C until analysis via EIA.

Determination of radioactivity in fecal and urine samples from radiometabolism study

Metabolites were extracted from the fecal samples collected during the radiometabolism study as described above except that the mortar and pestle was also

rinsed twice with a decontamination solution (Decon 75) between samples. Urine samples were dried down under air until only ~1 mL remained. To determine radioactivity in the urine and fecal extracts, we added 4 mL of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the concentrated urine or 100 mL of the fecal extract and quantified radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, USA).

Determination of immunoreactivity of fecal cortisol and androgen metabolites

To quantify FCM in the fecal samples collected during the diet study from captive squirrels, we used a 5α -pregnane- 3β , 11β , 21 -triol- 20 -one EIA, which measures GC metabolites with 5α - 3β , 11β -diol structure (Touma et al., 2003). We have previously validated this antibody for use in this species (Dantzer et al., 2010). Information regarding the cross-reactivity of the antibody used (Touma et al., 2003) and further details of the assay procedure can be found elsewhere (Palme and Möstl, 1997; Möstl et al., 2005). Samples were run in duplicate and the intra- and inter-assay coefficients of variation were 6.2 and 9.1%, respectively ($n = 3$ plates). The assay had a sensitivity of 0.82 pg per well.

To quantify FAM in fecal samples collected from captive males and females and free-ranging males, we used a testosterone EIA that measures 17β -OH androgen metabolites (Palme and Möstl, 1997), which we have already validated for use in female red squirrels (Dantzer et al., 2011). Details of this procedure (Möhle et al., 2002) and cross-reactivity of the antibody can be found elsewhere (Palme and Möstl, 1994). It is likely that our EIA antibody detected FAM from testosterone because it shows a high

Table 4.1. Outline of radiometabolism (males) and diet manipulation (males and females) experiments in captive red squirrels.

Date	Experiment	Treatment	n ^a	Collection Periods for Feces (h) ^b	Collection Periods for Urine (h)
16-21 February	Radiometabolism	Injected with ³ H-testosterone	6 m	0, 4, 8, 12, 24, 28, 32, 36, 48, 52, 60, 72, 80, 96, 104, 108, 120	4, 8, 12, 24, 28, 32, 36, 48, 52, 60, 72
3-7 March	Diet Manipulation	Fed Peanut Butter	3 f, 4 m	2, 8, 10, 22, 26, 34, 46, 50, 58, 70, 74, 82 94	Not collected ^c
		Fed Spruce Cones	2 f, 2 m	2, 8, 10, 22, 26, 34, 46, 50, 58, 70, 74, 82 94	Not collected ^c

^am and f indicate males and females, respectively.

^bCollection periods shown are for hours post-injection (radiometabolism) or post-manipulation of diet.

^cUrine samples were not collected for the diet manipulation experiment.

affinity to 17β -hydroxyandrogens and the cross-reactivity with 17-oxo- or 17α -hydroxyandrostanes androgen metabolites is below 0.1% (Palme and Möstl, 1997). Samples were run in duplicate and the intra- and inter-assay coefficients of variation were 5.5% and 16.2%, (n = 14 plates). The assay had a sensitivity of 0.3 pg per well.

Statistical analyses

We used two separate linear mixed-effects models (LMMs) to determine how the diet manipulation in the captive male and female squirrels affected FCM and FAM. In both of these models, the fixed effects were diet treatment (Cones versus Peanut Butter), hours post-manipulation, and an interaction term between treatment and hours post-manipulation. We determined how male reproductive condition affected FAM in free-ranging squirrels using a linear mixed-effects model. The fixed effect in this model was male reproductive condition (scrotal or abdominal testes).

For both of our LMMs described above, we had repeated measures on the same squirrels so we included a random intercept term for the individual squirrel (Pinheiro and Bates, 2009). FCM and FAM were $\ln$ -transformed prior to analysis in all our models. We used graphical inspection to ensure that the residuals from our LMMs were normally distributed, homoscedastic, and that there were linear relationships between our predictor and response variables. We calculated Cook's distances for all our observations to determine that there were no observations with high leverage in our LMMs. Below we describe mean $\pm$ SE and all FCM and FAM are given as $\ln$ ng/g dry feces.

Results

Route of excretion and time to peak excretion of radiolabeled testosterone in males

We recovered $42.6 \pm 0.07\%$ of the 1110 kBq of ^3H -testosterone we administered to the captive male squirrels. Of the total radioactivity excreted, $55.5 \pm 0.05\%$ was recovered in the urine and $44.5 \pm 0.05\%$ in the feces. The time to peak excretion of ^3H -testosterone was 6.6 ± 0.6 h in the urine and 19.8 ± 3.5 h in the feces (Fig. 4.2).

Characterization of ^3H -testosterone metabolites by RP-HPLC analysis

Injected ^3H -testosterone was heavily metabolized and polar metabolites that resembled conjugated steroids were the most common (Fig. 4.3). Radioactive peaks beyond fraction 60 were found and two of these peaks (eluting around fraction 83 and 87) yielded the highest immunoreactivity in the testosterone EIA. No radioactivity with corresponding testosterone immunoreactivity at the elution position of testosterone (near fraction 80) was present.

Biological validation of the EIA

We collected a total of 108 fecal samples from 65 males from 2007 to 2008. Male squirrels with scrotal testes had significantly higher FAM levels ($n = 46$; 3.02 ± 0.06 ng/g dry feces) than those with abdominal testes ($n = 62$; 2.73 ± 0.06 ; $t_{107} = 2.80$, $P = 0.003$; Fig. 4.4). This suggests that our EIA can reliably distinguish the gonadal status of male red squirrels.

Effects of diet manipulation on FCM and FAM in captive squirrels

In our diet manipulation experiment, whether captive squirrels were fed conifer seed or peanut butter had significant and opposing effects on FCM as the diet

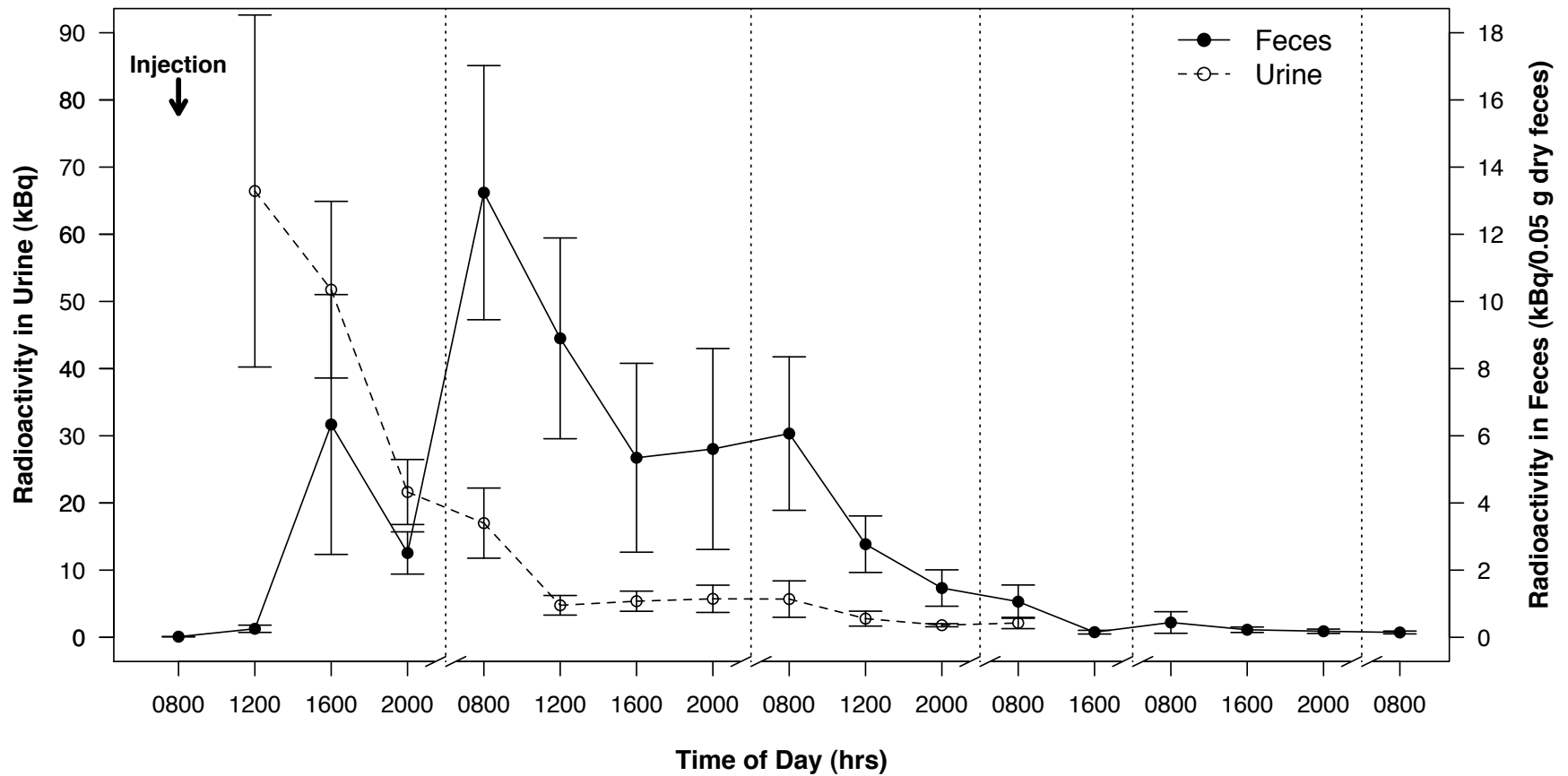


Figure 4.2. Excretion of injected radiolabeled testosterone by captive male red squirrels ($n = 6$) in urine (kBq/sample) and feces (kBq/0.05 g dry feces) over 72 and 120 h post-injection, respectively. Dashed vertical lines represent different days of study. Data shown are mean $\pm$ SE.

manipulation experiment proceeded. The initial FCM for squirrels fed conifer seed (6.28 ± 0.19 ln FCM; $n = 4$) and peanut butter (7.06 ± 0.68 ln FCM; $n = 3$) were similar ($t_{106} = 1.25$, $P = 0.11$), which is what we expected given they were initially fed the same diet. However, as the diet manipulation experiment proceeded, FCM in squirrels fed conifer seed significantly increased (slope for effects of hours post-manipulation on ln FCM = 0.01 ± 0.003 ; $t_{106} = 3.75$, $P = 0.0001$; Fig. 4.5A) whereas those in squirrels fed peanut butter significantly declined (slope for hours post-manipulation = -0.015 ± 0.003 ; $t_{106} = -4.39$, $P < 0.0001$; Fig. 4.5A). Mean FCM in samples taken 2 h after the start of the treatment (1200 h) compared to those taken at the same time of day (1200 h) but 74 h post-manipulation increased by 11% in squirrels fed conifer seed whereas they decreased by 14% in those squirrels fed peanut butter.

The diet manipulation had a similar effect on FAM. The initial FAM for squirrels fed conifer seed (4.04 ± 0.24 ln FAM; $n = 3$) and peanut butter (4.33 ± 0.22 ln FAM; $n = 3$) were also similar ($t_{84} = 0.99$, $P = 0.16$), which again is what we expected because they were initially fed the same diet. However, as the diet manipulation experiment proceeded, FAM in squirrels fed conifer seed significantly increased (slope for hours post-manipulation = 0.005 ± 0.002 ; $t_{84} = 2.41$, $P = 0.009$; Fig. 4.5B) whereas those in squirrels fed peanut butter declined but not significantly (slope for hours post-manipulation = -0.003 ± 0.003 ; $t_{84} = -1.21$, $P = 0.11$; Fig. 4.5B). Mean FAM in samples taken 2 h after the start of the treatment (1200 h) compared to those taken at the same

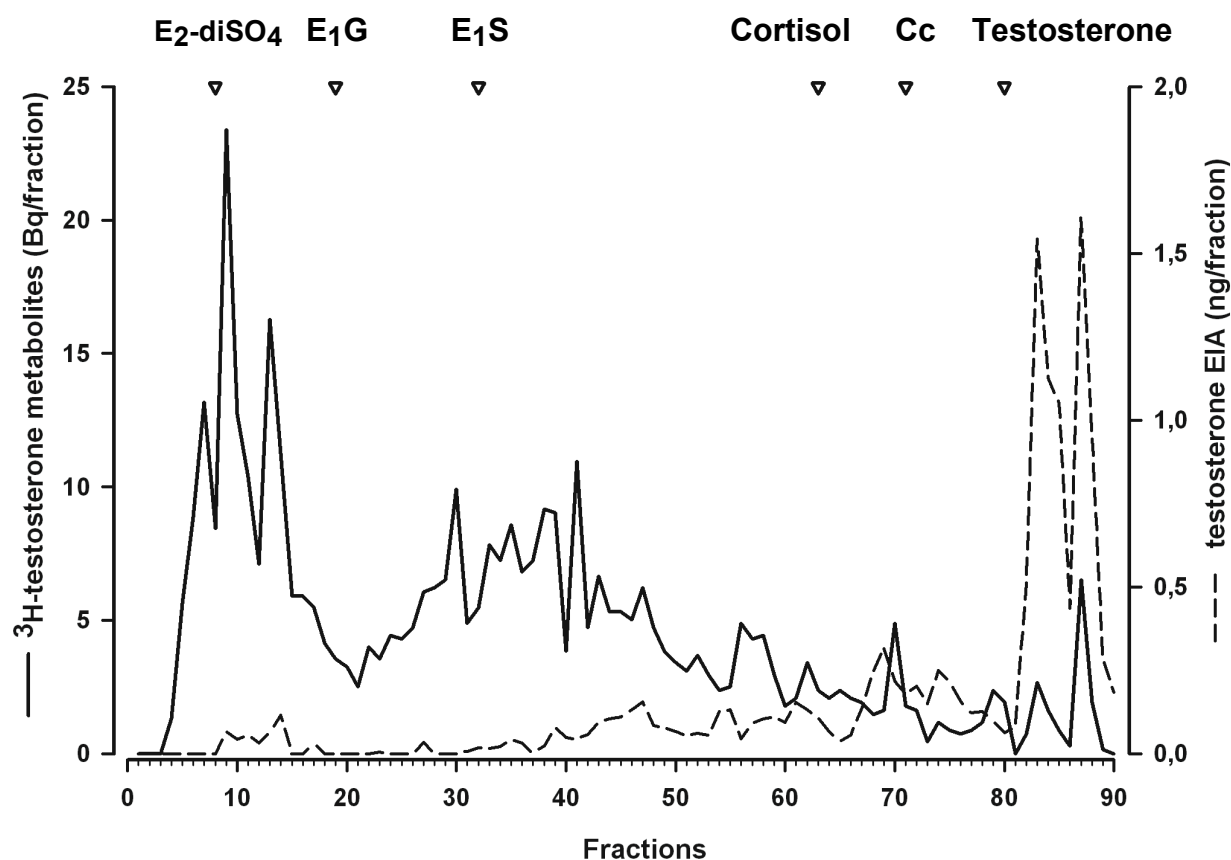


Figure 4.3. Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of fecal ^3H -testosterone metabolites (peak sample) in the feces of captive male red squirrels. Open triangles mark the approximate elution positions of respective standards ($\text{E}_2\text{-diSO}_4$ = 17β -estradiol-disulphate, E_1G = estrone-glucuronide, E_1S = estrone-sulphate, Cc = corticosterone).

time of day (1200 h) but 74 h post-manipulation increased by 12% in squirrels fed conifer seed whereas they decreased by 9% in those squirrels fed peanut butter.

Discussion

Endocrine responses to environmental variation and their fitness consequences are increasingly being studied in free-ranging animals by longitudinal monitoring of FHM. However, seasonal changes in diet could systematically bias FHM across the monitoring period. In this study, we first validated an assay to measure FAM in male red squirrels. We initially fed all the captive squirrels the same diet and their FCM and FAM were similar immediately prior to when we switched their diets. We found that FCM and FAM in captive female and male squirrels fed spruce conifer seed increased over the following 94 h after the manipulation started whereas those fed peanut butter declined over the same period. This study demonstrates that future studies monitoring FHM should carefully consider how seasonal changes in diet can influence FHM.

Validation of the EIA to measure FAM in male red squirrels

To demonstrate that this EIA provides biologically relevant measurements of androgen levels, we determined that (1) testosterone is heavily metabolized in male red squirrels, but that several testosterone metabolites were detected using our EIA and (2) FAM reflected gonadal status (scrotal or abdominal testes). We found that males with scrotal testes had significantly higher FAM than males with abdominal testes, which is typically a hallmark of physiological validation of an assay to detect FAM (Möhle et al., 2002; Beehner et al., 2009). Although these differences were statistically significant, we expect that they are a conservative estimate of the difference in FAM between males

with scrotal and abdominal testes. In a previous study (Boonstra et al., 2008), plasma androgen (testosterone and dihydrotestosterone) concentrations in male red squirrels with scrotal testes sampled in the early winter (February) prior to the start of the breeding season were significantly higher than those in males with abdominal testes sampled soon after the breeding season ended (June) and those in non-breeding condition (August). However, plasma androgen concentrations in males with scrotal testes sampled later in the breeding season (May) were not different than those sampled from males soon after the end of the breeding season and in non-breeding condition. We found that FAM in males with scrotal testes that were mostly sampled later in the breeding season rather than prior to the start of the breeding season were significantly higher than those sampled from males soon after breeding and in non-breeding condition (June-July). We only had one fecal sample from a male with scrotal testes in the early breeding season (February). We predict that FAM sampled from males in the early breeding season would be even higher than what we found here for FAM in males with scrotal testes. However, this does not detract from our finding that our assay for FAM can reliably distinguish between males with scrotal and abdominal testes.

Excretion and characterization of radiolabeled testosterone metabolites

The metabolism and route of excretion of FHM is generally species-specific (Palme et al., 1996, 2005) and may also be sex-specific (Touma et al., 2003; Goymann, 2005; Palme et al., 2005). In female red squirrels, we have previously found (Dantzer et al., 2011) that the percentage of radiolabeled testosterone recovered in the feces ($56.3 \pm 10.4\%$) was greater than what we found in this study for males. However, post hoc

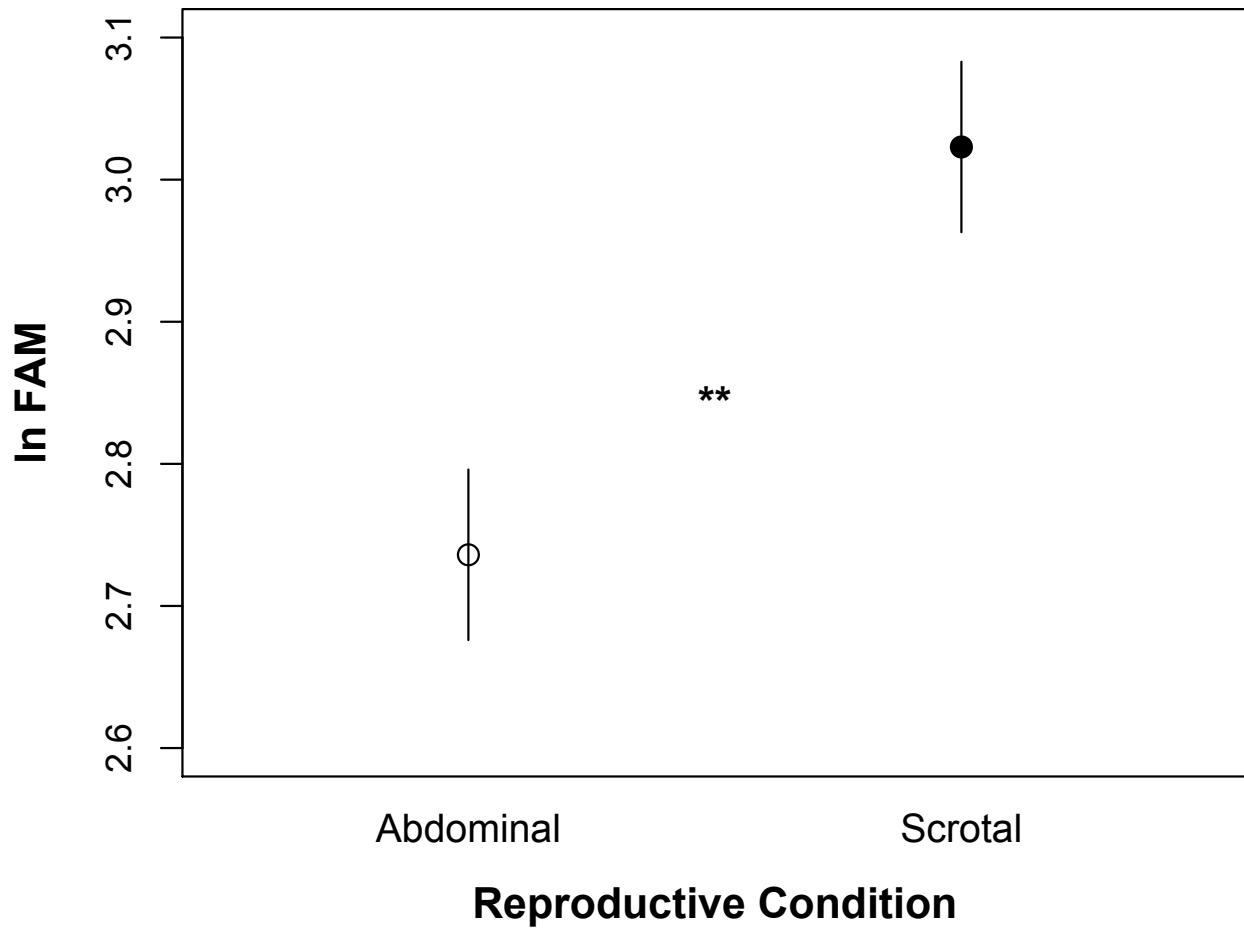


Figure 4.4. Effect of reproductive condition (abdominal or scrotal testes) on fecal androgen metabolite (FAM) concentrations in free-ranging male red squirrels. Data shown are raw mean $\pm$ SE. Asterisks represent significant differences from a linear mixed-effects model (see text) at $P < 0.01$ (“**”).

analyses indicate that these differences between the sexes for the route of excretion were not significantly different (paired t -test: urine: $t_8 = 0.02$, $P = 0.98$; feces: $t_8 = 0.85$, $P = 0.41$). Thus, there do not appear to be any sex-specific differences in the route of excretion of testosterone metabolites in red squirrels.

The time to peak excretion of radiolabeled hormone metabolites may also be sex-specific (Chelini et al., 2010). We found that the time to peak excretion of radiolabeled testosterone in the feces of male red squirrels (19.8 ± 2.7 h) was longer than what we have previously reported (Dantzer et al., 2011) in females (10.3 ± 0.8 h). However, a post hoc analysis indicates that this difference was not significantly different (paired t -test: $t_8 = 2.14$, $P = 0.065$). Nonetheless, trapping-induced stress could not have influenced FAM from feces collected in the field as traps were checked every 2 h, whereas the peak in radioactive testosterone metabolites in males occurred nearly ~20 h after injection, which is similar to what we have found previously for excretion of radiolabeled cortisol in females and males (Dantzer et al., 2010) and testosterone in females (Dantzer et al., 2011).

Finally, the structure and type of steroid hormone metabolites excreted in the feces may also be sex-specific. For example, Goymann (2005) found sex-differences in the testosterone metabolites in the excreta of European stonechats (*Saxicola torquata rubicola*) and suggested caution in comparing FAM between males and females. The type of testosterone metabolites excreted in the feces of red squirrels may also be sex-specific. In a previous study, we found three peaks of immunoreactive testosterone metabolites in the feces of female red squirrels (Dantzer et al., 2011) but in the present study in male squirrels, we only found two peaks (Fig. 4.2). Although these results urge

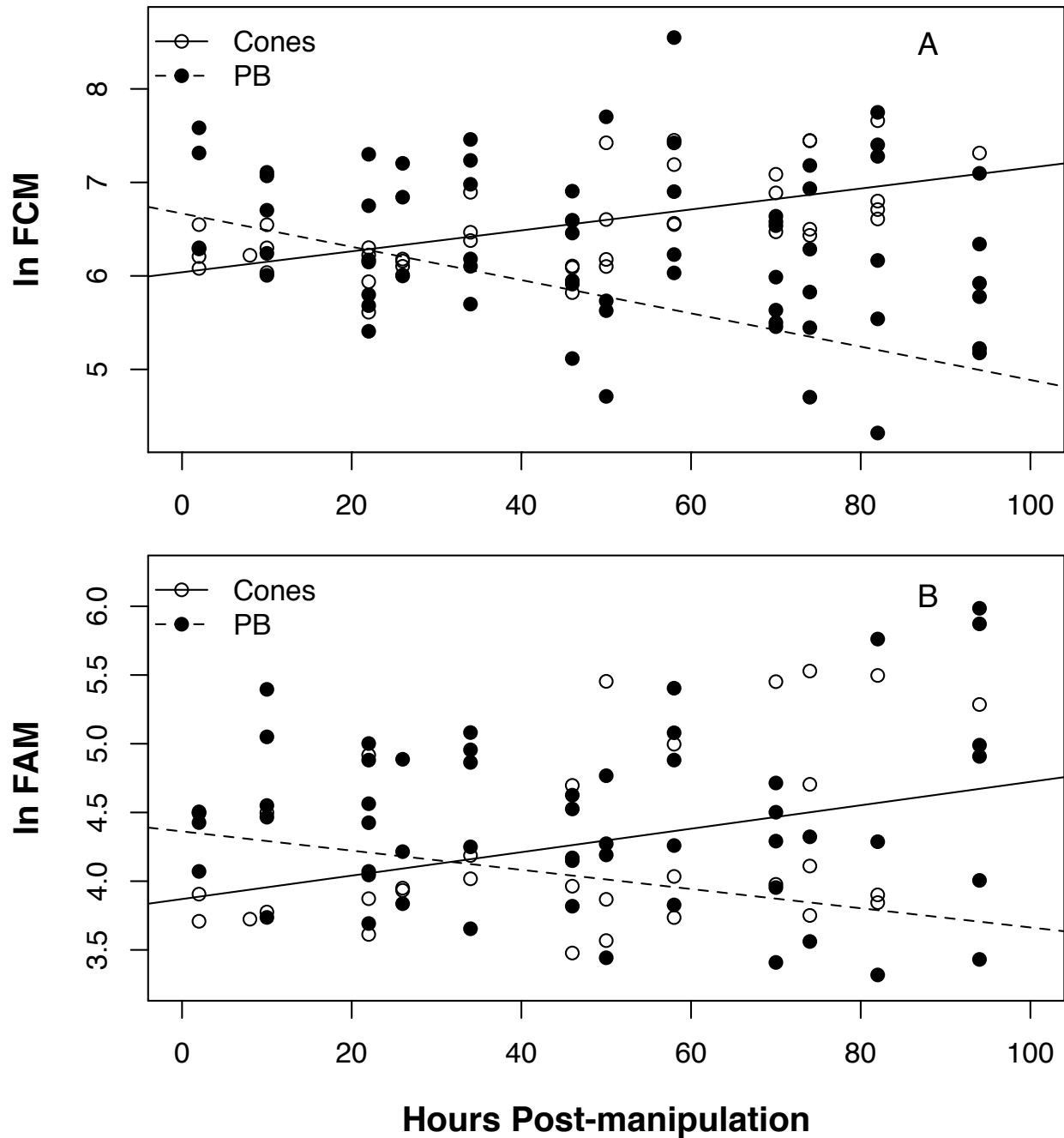


Figure 4.5. Effects of diet on fecal A) cortisol (FCM) and B) androgen (FAM) metabolite concentrations in captive male ($n = 6$) and female ($n = 5$) red squirrels. Squirrels were all initially fed the same diet and then switched (0 h post-manipulation) to a diet of apple and either 1) peanut butter ("PB"; $n = 7$) or 2) spruce seed ("Cones"; $n = 4$). Ln-transformed FCM and FAM are shown on y-axes. Regression lines shown are from general linear models but statistical inferences were made from linear mixed-effects models (see text).

caution in comparing FAM between females and males, they do not contradict the overall validations of our EIA to measure FAM in both females and male red squirrels.

Effects of diet manipulation on FCM and FAM in captive squirrels

After the diets of the squirrels were switched from the same diet of sunflower seeds, peanut butter, and apple, we found that FCM and FAM in squirrels fed spruce seed increased over the course of the diet manipulation, whereas those fed peanut butter declined during the same time period. The observed differences in FCM and FAM could have been caused by differences in dietary fiber content of sunflower seeds (21.5% acid detergent fiber content: Sarrazin et al., 2004), spruce seeds (19.8% acid detergent fiber content: Lobo and Millar, 2011), and peanut butter (6.6% crude fiber). Compared to squirrels fed peanut butter, those fed sunflower or spruce seeds consumed higher quantities of dietary fiber and also increased excretion of FCM and FAM. Previous studies have found that increased consumption of fiber can cause the excretion of FHM to increase (Goldin et al., 1982; Pusateri et al., 1990), decrease (Wasser et al., 1993; von der Ohe et al., 2004; Goymann, 2005), or have no effect (Rabiee et al., 2002; von der Ohe et al., 2004; Goymann, 2005). Our results agree with previous studies (Goldin et al., 1982; Pusateri et al., 1990) in which higher fiber consumption increased the excretion of FHM.

The mechanisms by which dietary fiber consumption affects FHM are unknown (Eriksson and Gustafsson, 1970; Goldin et al., 1982; MacDonald et al., 1983; von der Ohe and Servheen, 2002). Changes in FHM caused by increased dietary fiber consumption could be attributed to increased transition time of ingested materials from the duodenum to the rectum. Unbound hormones in the plasma are metabolized by the

liver and excreted into the gut via the bile ducts (Taylor, 1971). Some of these hormone metabolites are reabsorbed via enterohepatic circulation (Taylor, 1971; MacDonald et al., 1983). Previous studies have speculated that an increase in the frequency of defecation due to increased consumption of dietary fiber could decrease reabsorption of hormone metabolites in the small intestine and therefore cause an increase in FHM excretion (Goldin et al., 1982). Although we did find that squirrels fed spruce seed (10 ± 2.3 fecal samples collected per individual over 94 h) defecated more frequently than those fed peanut butter (8 ± 1.4), it is unknown whether this caused the observed differences in FHM.

We found that a change from sunflower seeds to spruce seeds caused a significant increase in the excretion of FCM and FAM from 0-94 h after the diets were switched. This is surprising given that the fiber content of sunflower (21.5%) and spruce (19.8%) seeds were similar. This suggests that even the type of seeds (sunflower or spruce) squirrels were fed significantly influenced FHM. As a result, in addition to changes in consumption of dietary fiber, even subtle differences in the diets of free-ranging animals can influence FHM.

Previous studies have concluded that differences in FHM caused by variation in dietary fiber consumption are primarily due to the water content of feces. Increased fiber consumption can increase the water content of feces and therefore differences in FHM caused by changes in dietary fiber can be eliminated by removing the water via lyophilization (Wasser et al., 1993). Although we do not know if there were differences in the water content of feces of squirrels fed seeds or peanut butter, we lyophilized all of our fecal samples and yet we still found persistent effects of diet on FCM and FAM.

Future studies measuring FHM should consider monitoring the diets of their study animals in addition to lyophilization of fecal samples to eliminate any differences in FHM that are observed due to dietary changes.

Free-ranging animals often exhibit major seasonal shifts in the type and quantity of foods that they consume. As previous studies have found in humans and other animals, these changes in diet can have a major influence on patterns of FHM. We found that the consumption of dietary fiber and even consumption of similar types of food (seeds) with similar fiber content significantly altered the excretion of FCM and FAM. Future studies should perform careful experiments that manipulate the diets of their study animals to determine whether seasonal changes in FHM are due to ecological variation, reproductive condition, or changes in diet.

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LITERATURE CITED

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CHAPTER 5

Dantzer, B., Boutin, S., Humphries, M. M., McAdam, A. G. Behavioral responses of territorial red squirrels to natural and experimental variation in population density. *Behavioral Ecology and Sociobiology in press*.

Introduction

Density-dependent population regulation is a key paradigm in ecology. The behavior of individuals is often implicated as a key contributor to population density-dependence by altering spacing behavior or contributing to interference and exploitative competition over limited resources (Chitty, 1967; Krebs, 1978; Sinclair, 1989; Krebs, 1996; Mougeot et al., 2003). Unfortunately, the fields of behavioral and population ecology have developed largely independently of one another (Sutherland, 1996) and the behavioral responses of individuals to variation in population density are actually relatively unknown.

Studies documenting the density-dependence of behavior are rare, which may reflect the difficulty and effort required to monitor the behavior of multiple individuals across a gradient of population density. In one exception, Bretagnolle et al. (2008) documented a positive correlation between population density and antagonistic interaction rates between neighboring ospreys (*Pandion haliaetus*; see also Watson et al., 1994; Amsalem and Hefetz, 2011; Innocent et al., 2011). Several other studies have experimentally manipulated population densities and monitored subsequent changes in feeding and foraging (e.g., Dobbs et al., 2007; Rutten et al., 2010) or offspring provisioning rates (e.g., Sillett et al., 2004; Bretagnolle et al., 2008). Although the results of these studies suggest behavioral responses of individuals to variation in population density could yield insights into density-dependent population regulation as well as how individual behavioral variation affects population-level processes (Kokko and López-Supulcre, 2007), this intersection between behavioral and population ecology remains

understudied given the centrality of density-dependent behavior to the concepts of density-dependent population growth.

Resource availability has the potential to be a major confounding influence in the assessment of density-dependent behavior based on natural variation in population density. For example, density-dependent changes in time spent foraging may be caused by concurrent changes in the frequency of antagonistic interactions (interference competition: Creswell 1997; Rutten et al. 2010), per capita resource abundance (Kuhn and Vander Wall 2008), the degree of heterogeneity in the distribution of resources (Monaghan and Metcalfe 1985), or perhaps due to increased heterospecific attraction that leads to increased predation risk (Lima et al. 1985; Hik 1995). These potentially confounding influences can be explored by experimentally manipulating the density of free-ranging animals using food-supplementation (Boutin 1990) and/or predator exclusion (Karels and Boonstra 2000), or by adding (Svensson and Sinervo 2000; Calsbeek and Smith 2007; Rutten et al. 2010) or removing (Sillett et al. 2004; Meylan et al. 2007) conspecifics to a study area. Although the results of these experimental manipulations of population density have been illuminating, they often cannot distinguish whether their effects were caused by changes in intraspecific competition, resource abundance, or predation risk. As a result, it can be extremely difficult to disentangle the unique effects of specific environmental or social variables that affect behavioral patterns even with well-designed field manipulations.

In this study, we used both observational and experimental approaches to examine whether North American red squirrels (*Tamiasciurus hudsonicus*) adjust their behavior in response to local population density and whether they use rates of territorial

vocalizations in their local neighborhood to assess density. We predicted that increases in population density of red squirrels would lead to increases in the frequency of antagonistic physical and acoustical interactions, territory intrusions and territorial behavior (frequency with which squirrels emitted territorial vocalizations and vigilance for conspecifics). Second, we predicted that squirrels experiencing heightened population density would spend more time defending their territory against actual intruders or the perceived threat of intruders (vigilance) and therefore would spend less time engaged in behavior associated with offspring provisioning or self-maintenance (proportion of time spent in the nest and feeding/foraging).

We examined these relationships first through an observational approach by analyzing 18 years of live-trapping data and 20 years of detailed behavioral observations, during which population density underwent natural fluctuations due to variation in food abundance (Fig. 5.1). We then tested the effects of population density on overall behavioral patterns using two experimental manipulations of actual and perceived (acoustic) population density. First, we compared the behavior of squirrels on a high-density food-supplemented study area to the behavior of squirrels on two low density control study areas. This five-year winter food-supplementation experiment involved the addition of peanut butter, which cannot be cached by the squirrels, and behavior was recorded only after the food-supplementation ended for the season. Despite the absence of the supplemented food at the time our data were collected, spring cone hoard sizes were larger on the food supplemented study area than on the control study areas (Donald and Boutin, 2011) perhaps because of reduced reliance on the hoard prior to the seasonal end of the supplement. Given this partial confounding of

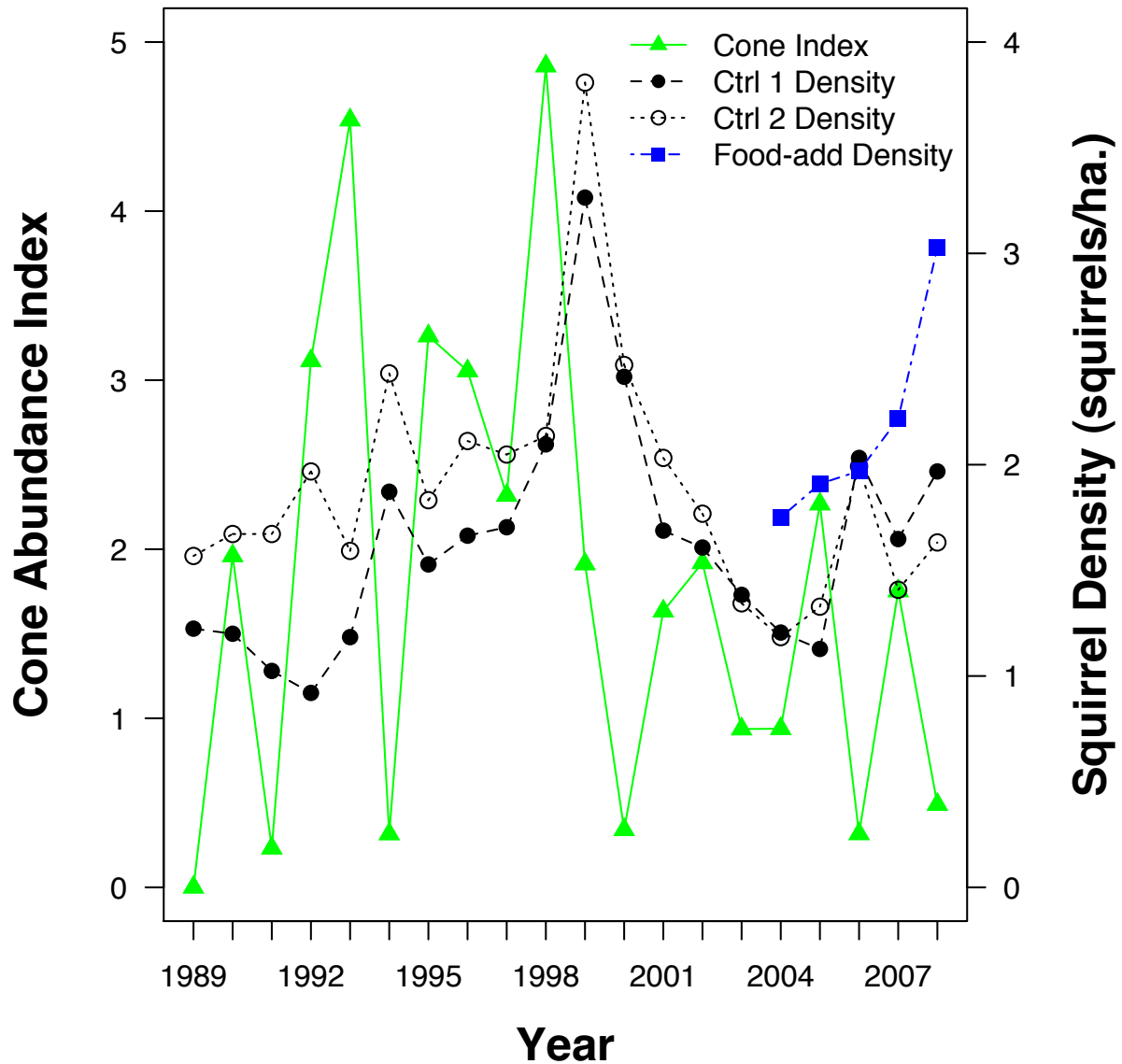


Figure 5.1. Annual variation in white spruce (*Picea glauca*) cone production and density of red squirrels on two unmanipulated study areas and one food-supplemented study area. Cone production index represents an index of the average number of cones produced on two study areas and one food-addition area (LaMontagne and Boutin 2007). Squirrel density represents the number of squirrels defending a territory per hectare (ha.) on two control study areas (39.7 hectares each; “Ctrl 1” and “Ctrl 2”) and 1 food-supplemented study area (“Food-add” 45.4 hectares).

density and resource availability, we directly tested the effects of perceived population density using persistent audio playbacks of territorial vocalizations throughout one breeding season. In this experiment, we compared the behavior of squirrels experiencing heightened perceived population density to those not exposed to playbacks.

Materials and Methods

Study area

A population of free-ranging red squirrels located in the southwest Yukon, Canada (61° N, 138° W) has been monitored continuously since 1987 (McAdam *et al.* 2007). We studied red squirrels on four study areas that were all staked and flagged at 30 m intervals, which allowed us to record the specific position of all squirrel territories and all behavioral observations to the nearest 3 m.

We analyzed live-trapping (1991-2008) and behavioral data (1989-2008) collected on two control study areas (“control study areas”: 40 hectares each) separated by the Alaska Highway that experienced natural variation in food abundance and population density (Fig. 5.1). We measured how population density affected the frequency of 1) territorial intrusions, 2) antagonistic physical interactions, and 3) overall behavioral time budgets.

In one year (2008), we monitored the behavioral responses of red squirrels to experimental increases in population density using long-term food supplementation (supplemented since 2004) or perceived (acoustical) population density on two separate

study areas (described below). We used data collected from these study areas to determine how population density affected 1) the frequency of antagonistic physical interactions, 2) the frequency with which squirrels emitted territorial vocalizations, and 3) behavioral time budgets of squirrels experiencing the experimental manipulations.

Red squirrels

Red squirrels in our study area harvest mature cones from white spruce (*Picea glauca*) trees in the late summer and early autumn of each year (Fletcher *et al.* 2010). Both male and female red squirrels are territorial and defend a larger hoard of cached spruce cones located on the center of their territory (“midden”). Territories are defended from all conspecifics year-round except for reproductive females which tolerate their own young prior to weaning, and estrous females which tolerate males on their territory for their day of estrous (typically one day each year: Smith 1968). White spruce trees produce seed in highly synchronous pulses. Years of high cone production (mast years) are followed by years of no or little cone production (Fig. 5.1: LaMontagne and Boutin 2007). In mast years, juvenile overwinter survival is increased (McAdam and Boutin 2003) and population density is generally higher the following spring (Fig. 5.1: Boutin *et al.* 2006; Descamps *et al.* 2008).

Measuring population density

In each year we completely enumerated all squirrels living on all of our study areas by determining the owners of all middens using live-trapping and behavioral observations beginning in March and ending in August in each year. Squirrels were individually marked with uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA), which they received in their natal nest around 25 days old. Small

pieces of colored wire in unique combinations were threaded through the ear tags of each squirrel so they could be identified during behavioral observations.

Squirrels were live-trapped using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) to assess and track reproductive condition, identify territory ownership, and determine recruitment of the offspring from the previous year (McAdam et al. 2007). Territory ownership was determined by combination of repeated live-trapping of the same individual on the same midden, the locations of territorial vocalizations called “rattles” (Smith, 1978), and other behavioral observations. Middens were easily identifiable by the accumulation of bracts and spines from spruce cones that had been consumed as well as the presence of hoarded cones.

Assigning estimates of population density for an overall study area to each individual squirrel might overlook important local variation in population density (Garant et al. 2005; Wilkin et al. 2006; Garant et al. 2007). For each midden that was defended by a single squirrel, we estimated the local density at scales ranging from 25-300 m away from the center of the midden. Local density was calculated by determining the number of unique squirrels defending middens located within 25-300 m from the center of the midden divided by the total area considered (squirrels/hectares). This approach essentially involved placing a circle with a radius ranging from 25-300 m (in 25 m intervals) over each midden and counting the number of unique squirrels that defended a territory within that circle.

We then calculated the Pearson correlations between all of our response variables and local density estimated at all of these spatial scales (all distances from 25-300 m away from midden of interest at 25 m intervals). We used this approach to

determine the scale at which local population density exhibited the highest Pearson correlation with our response variables including the frequency of territorial intrusions, rattles, and behavioral response variables. We found that local population density was correlated with our territorial intrusion and behavioral response variables at most scales of density but estimating local density at a scale of 150 m from the midden of interest exhibited the highest or near the highest Pearson correlation (Fig. 5.2). Although there were some spatial scales at which local density exhibited a higher Pearson correlation with a response variable (e.g., PC2 for density estimated at 50 m: Fig. 5.2), 150 m generally represented a threshold at which analyses using estimates of local density at greater scales would result in the same inferences. Therefore for all analyses discussed below, we estimated local population density as the number of squirrels within 150 m of the midden under consideration.

Although our study areas are bordered by habitat that is not suitable for red squirrels (large meadows or willow swamps) and we also censused middens just off of our study areas, we may have underestimated local population density for those squirrels owning middens on the edges of the study areas. However, our results were not affected by whether or not we separately considered squirrels in the core (>150 m from edge of study area) and those on the edge of our study areas.

Frequency of territorial intrusions

From 1991-2008, we recorded the number of trapping events for midden owners as well as intruders on 2277 middens (436 unique middens across 18 years) on the two control study areas. Territorial intrusions occurred when an adult or juvenile squirrel was

captured on a midden that was owned by another squirrel, excluding juveniles captured on their mother's territory prior to weaning.

Measuring behavior

Red squirrels are amenable to behavioral studies because they are diurnal and habituate to the presence of humans. The behavior of red squirrels on control study areas was recorded through both *ad libitum* sampling from 1989-2008 and focal sampling of radiocollared (model PD-2C, 4 g, Holohil Systems Limited, Carp, Ontario, Canada) squirrels from 1994-2008. *Ad libitum* observations (Altmann 1974) of agonistic interactions between adult male or female squirrels were recorded whenever observers were live-trapping or specifically recording behavioral observations on the study areas. These observations of interactions generally consisted of one adult squirrel chasing another adult away from its midden. All mating-related observations and interactions between mothers and their offspring on the natal territory (prior to weaning) were excluded.

Focal animal sampling of radiocollared squirrels was done by instantaneous sampling at 30-second intervals for 7 continuous minutes (Altmann 1974). These behavioral observations were collected opportunistically in 1994 (n = 96 sessions), 1995 (n = 47), 1996 (n = 10), 1997 (n = 25), 1999 (n = 34), 2001 (n = 85), 2002 (n = 119), 2003 (n = 124), 2004 (n = 92), and 2008 (n = 474). Behaviors were recorded and categorized in a similar way as previous studies on red squirrels (Humphries and Boutin 2000; Anderson and Boutin 2002; Dantzer et al. 2011) including whether the squirrel was in or out of its nest, feeding, foraging, travelling, resting, caching food items, grooming, scentmarking, interacting with adult conspecifics, vigilant, vocalizing

(“squeaking”, “barking” and “rattling”: Smith 1978) or out-of-sight (not visible). Vigilance was observed when a squirrel was alert but inactive (not mobile) as opposed to resting, which was used to describe squirrels that were inactive and not alert. Rattling is the territorial vocalization of red squirrels, whereas barking is an alarm call (Smith 1978).

We recorded additional information during the focal animal observation sessions recorded in 2008 in the form of all-occurrences of the total number of rattle vocalizations emitted by the focal squirrel. We used all of these data including observations collected during the food-supplementation period to determine how local population density affected the frequency of territorial vocalizations. The same observer (BD) collected all behavioral observations in 2008, but 39 different observers collected the data between 1994 and 2004.

Experimental manipulations of population density

One study area was provided with supplemental food beginning in winter 2004 (“food-supplemented area”: 45 hectares). Each individual squirrel that owned a midden on the food-supplemented area was provided with a bucket that was hung from a tree in the center of its midden. One kg of all natural peanut butter (no salt or sugar added) was added to each bucket approximately every six weeks between October and May of each year. In each year, the peanut butter was removed at the end of May but females that were pregnant or lactating at the removal date continued to receive supplemental food. Squirrels cannot hoard or cache peanut butter and food supplementation does not appear to have persistent effects on adult body mass as squirrels on control study areas ($n = 8939$ squirrels trapped, 246.2 ± 0.32 g) weigh similar to those on food-supplemented areas ($n = 9399$, 244.2 ± 0.32 g).

Our food supplementation procedure has led to an increase in population density compared to the average population density on control study areas (Fig. 5.1). Overall population density on the food-supplementation area (3.28 squirrels/ha) was nearly double the average density (1.75 squirrels/ha) of the two control study areas (control study area 1: 1.96 squirrels/ha; control study area 2: 1.54 squirrels/ha) in 2008 when we collected these focal animal behavioral data. Because squirrels exhibit extreme site fidelity and continue to defend their middens after the peanut butter is removed, we were able to document the effects of heightened population density without the confounding increase in food availability due directly to supplemental food by recording the behavior of squirrels after the food was removed. We only present data below for behavioral observations recorded after the food was removed. These data were used to determine how an experimental increase in population density via food-supplementation affected behavioral time budgets.

In addition to manipulating density with food-supplementation, we were also able to manipulate perceived population density without supplemental food. In 2008, we used playbacks of the territorial vocalizations of red squirrels to manipulate perceived (acoustic) population density on an additional study area (11 hectares). We compared the behavior of squirrels exposed to the playbacks ($n = 5$) to those of squirrels that were not exposed to the playbacks ($n = 5$). Because red squirrels broadcast territorial vocalizations containing individually distinct signature signals (Goble, 2008; Smith 1978), perceived density can be manipulated using long-term playbacks of these territorial vocalizations. We used a total of 20 rattles from 20 different squirrels that were recorded from 2005-2006 using a Marantz Professional Solid State Recorder (Model PMD660,

Marantz Inc., Mahwah, NJ) attached to a Sennheiser shotgun microphone with K6 powering module and foam windscreen (Model ME66, Sennheiser Electronic, Wedermark, Germany). Rattles were recorded in the 0.01-22.5 kHz range and stored as uncompressed .wav files, which were each transferred to separate CDs for the playback experiments. Rattles were recorded at least 500 m away from our study area. Because territory fidelity is high and dispersal from the natal site is low in this species (Berteaux and Boutin 2000), we assumed that the vocalizations used for the playbacks were at least from unfamiliar individuals and likely from unrelated individuals. For each manipulated squirrel, territorial vocalizations of 4 different red squirrels were broadcasted through 4 different portable speakers (Coby Electronics Corp., Lake Success, NY) such that each speaker played the rattle of only one squirrel. Each rattle was emitted from one speaker at approximately 7-min intervals for approximately 12 hours per day for 35 consecutive days. Therefore, squirrels experienced an additional 4 rattles from 4 different squirrels every 7 min or an additional ~411 rattles per day. Playback treatments (playback or control) were randomly allocated to the 10 squirrels. The behavior of the squirrels exposed to the playbacks (n = 37 sessions on 5 squirrels) and those not exposed to playbacks (n = 53 sessions on 5 squirrels) was recorded over 20 d preceding the initiation of the playbacks. During the playbacks, the behavior of squirrels exposed to the playbacks (n = 50 sessions on 5 squirrels) and control squirrels (n = 31 sessions on 5 squirrels) was recorded over the course of the 35 d playback period. These data were used to determine how an experimental increase in perceived population density affected the frequency of territorial vocalizations and behavioral time budgets.

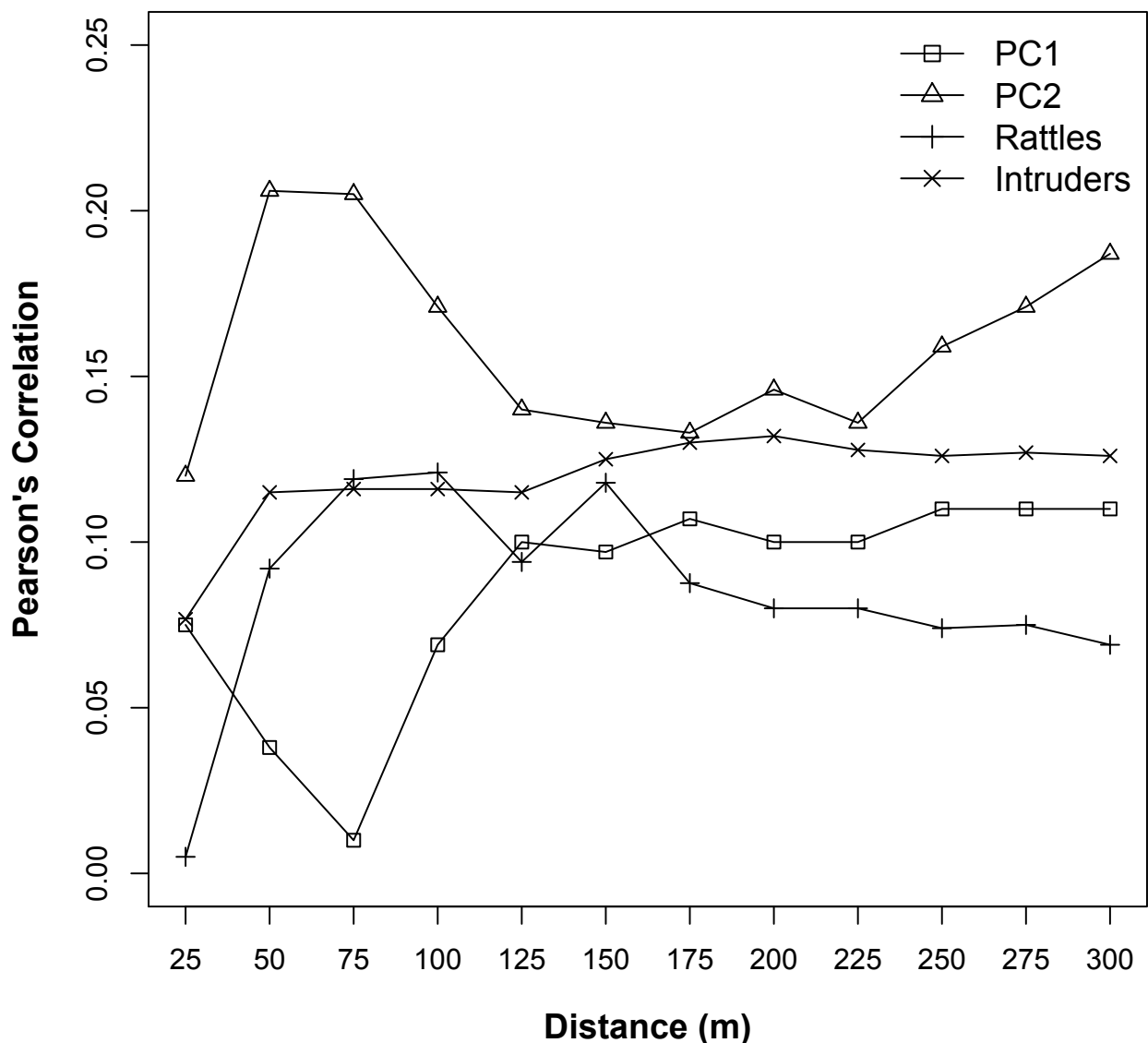


Figure 5.2. Pearson's correlations between local squirrel density (squirrels/hectare) measured from 25-300 m away from the midden of interest and the frequency of territorial intruders, territorial vocalizations, and behavioral response variables (PC1 and PC2). We calculated local density by considering a circle with a radius ranging from 25-300 m around the midden of interest and counting the number of squirrels defending a territory within these areas. Values shown on the y-axis are absolute values of Pearson's correlations. We used these data to determine that 150 m is an appropriate scale at which to measure local population density. At this scale, the Pearson correlation between local population density and three of the response variables (intruders, rattles, and PC1) is near the highest relative to the other scales. While the Pearson correlation between local population density measured at this scale and PC2 is not near the highest relative to the other scales, we are still likely to gain similar inferences measuring density at this scale compared to others because the Pearson correlation remains either weakly or strongly positive at all scales.

Statistical analyses

We were specifically interested in how population density affected the frequency of antagonistic interactions using trapping (frequency of territorial intrusions) and behavioral data (antagonistic physical interactions observed during *ad libitum* or focal-animal behavioral observations). We first determined how local population density affected the frequency of territorial intrusions from 1991-2008 on control study areas. We used a generalized linear mixed-effects model (GLMM) with Poisson error distribution (log link) that included our estimate of local population density as a fixed effect as well as a fixed effect for the number of times the owner of the midden was captured as an estimate for trapping effort. We did not statistically analyze the effects of local population density on the frequency of physical interactions observed in the behavioral data sets because these events were so rare statistical analyses were inappropriate and therefore we only present the raw data below.

We used data collected in 2008 during the behavioral observation sessions (n = 474) from the two control and food-supplemented study areas to determine how local population density affected the frequency with which squirrels emitted territorial vocalizations. The effect of local population density (fixed effect) on the frequency with which squirrels emitted rattles was determined statistically by conducting a GLMM (Poisson error distribution, log link) for the number of rattles emitted by territory owners during the 7-min behavioral observation sessions.

To determine if the perceived density manipulation experiment had an effect on the frequency with which squirrels emitted rattles, a GLMM (Poisson errors, log link) was conducted containing the fixed effect terms for the period when the behavioral

Table 5.1. Loadings of first (PC1) and second (PC2) axes from principle components analysis using a correlation matrix from 7-min behavioral observation sessions (n = 1277) conducted on red squirrels from 1994-2008. Loadings in boldface font indicate loadings that were used in our interpretation for principal component 1 and 2.

Behavior	PC1	PC2
Barking	-0.03	0.02
Caching	-0.007	-0.003
Feeding	-0.26	-0.67
Foraging	-0.08	-0.07
Grooming	-0.01	-0.0002
Nest	0.92	-0.04
Out-of-sight	-0.07	-0.04
Rattling	-0.01	-0.009
Resting	-0.09	0.14
Scentmarking	-0.0004	-0.0001
Squeaking	-0.005	0.003
Traveling	-0.17	-0.04
Vigilance	-0.19	0.72
% Variance Explained	42	17.9

observations were collected (before playbacks initiated and during playbacks), treatment (rattle playbacks or no playbacks), and an interaction term between period and treatment.

In order to determine how local population density and our experimental manipulations of actual or perceived population density affected behavioral time budgets, we first used a principal components analysis (PCA) with a correlation matrix with no factor rotation that reduced the entire behavioral dataset (all focal sampling from 1994-2008, $n = 1277$ behavioral observation sessions) into 2 principal components that explained 42 and 17.9% respectively of the total variation (Table 5.1). These PC1 and PC2 scores were then used to determine how 1) natural variation in local population density, 2) experimentally increased population density, and 3) experimentally increased perceived (acoustical) population density affected red squirrel behavior.

Secondly, for our analyses of how natural variation in population density affected red squirrel behavior that was recorded from 1994-2004, separate linear mixed-effects models (LMM) for PC1 and PC2 were conducted. For both of these models, we used estimates of local density for the midden that the focal squirrel owned. The LMM for PC1 contained a fixed effect term for local density. Because PC1 generally described patterns of nest use (in or out of the nest), the distribution of PC1 scores may be more binary than binomial. However, our results are robust to the alternative statistical approach of using a GLMM with a binary response variable to only analyze those sessions in which squirrels spent none or all of the time in the nest (results not reported here). The LMM for PC2 contained the fixed effect terms for local density and a quadratic term for local density to account for a slight nonlinear relationship between the

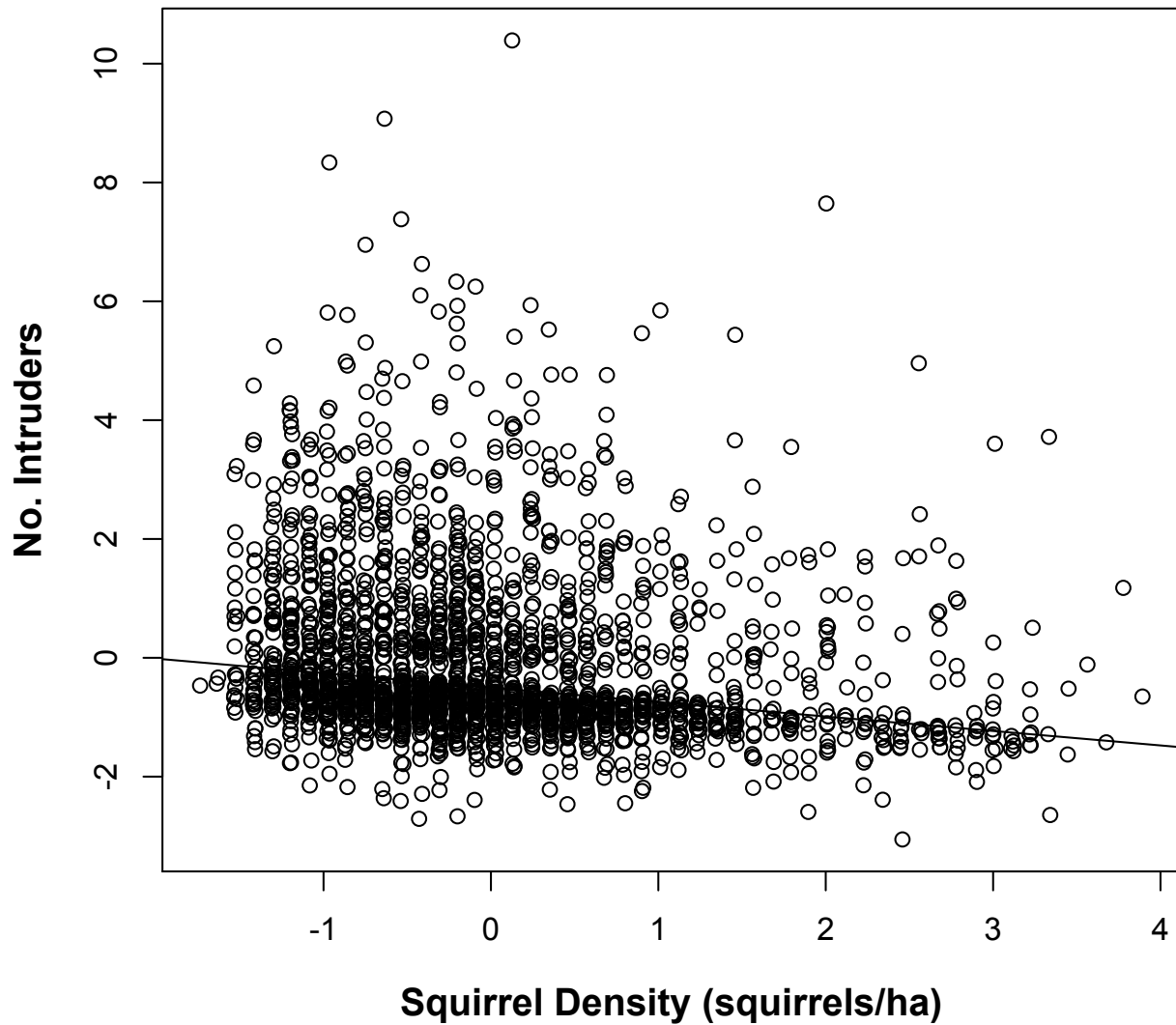


Figure 5.3. Effect of local population density on the number of territorial intruders caught on red squirrel territories ($n = 2277$) measured from 1991-2008 on two unmanipulated study areas. Values on the x-axis are standardized measures of local population density (squirrels/hectare within 150 m). Partial residuals from a linear mixed-effects model are shown on the y-axis (Table 5.2).

response and predictor variable. To determine how increased population density on the food-supplementation area affected squirrel behavior compared to the control study areas, separate LMMs for PC1 and PC2 were conducted containing the fixed effect for study area (“food-supplementation” or “control”) in which population densities differed. Thirdly, LMMs were used to determine how the playback experiment affected the behavior (PC1 and PC2) of squirrels exposed to the rattle playbacks compared to those not exposed to playbacks. Each of these models contained fixed effect terms for the period when the behavioral observations were collected (before playbacks initiated and during playbacks), treatment (rattle playbacks or no playbacks), and an interaction term between period and treatment.

For all of the mixed-effects models described above that we used to analyze red squirrel behavior or the frequency of territorial vocalizations, we included a random intercept term for individual identity of the squirrel (“Squirrel ID”) because we had repeated samples on the same squirrels (Pinheiro and Bates 2009). In the GLMM for the frequency of territorial intrusions, we included a random intercept term for midden because we had repeated estimates for middens across years. We included a random intercept term for observer in the LMMs for PC1 and PC2 to account for repeated measures by the same observer for the behavioral data collected from 1994-2004. The dispersion parameters for the GLMMs we conducted for the frequency of territorial intrusions (1.48) and territorial vocalizations (1.11) indicated that there was some overdispersion in these models (dispersion parameter for Poisson GLMMs is expected to equal 1). We therefore used Wald *t*-tests to test the significance of the fixed effects of the GLMMs (Bolker et al. 2009). We estimated the degrees of freedom to determine the

significance of our fixed effects in the LMMs as the number of levels of the random effect in the LMM. When there were two random effects in the LMM, we estimated the degrees of freedom using the random effect with the fewest number of levels. All of these mixed-effects models were conducted using the lme4 (version 0.999375-35: Bates et al. 2008) package in R (version 2.11.1, R Development Core Team 2008).

Diagnostic plots indicated that the residuals from the LMM described above were normally distributed, homoscedastic, and that there were no outlying observations with high leverage. We also calculated the Cook's Distance for all observations in all of our regression models to ensure the lack of high leverage observations. We ln-transformed some of our response variables prior to analysis to improve the normality of the residuals. Preliminary analyses using generalized additive models (Hastie and Tibshirani 1990) were used to confirm that there were no significant non-linearities between our response and predictor variables. In one model (see above) we included a quadratic effect for local density because of a non-linear relationship. Estimates of local density were standardized to a mean of zero and unit variance prior to analysis. For results below, we present mean $\pm$ SE and considered differences statistically significant at $\alpha = 0.05$.

Results

Natural variation in local population density

Among all the years from which we analyzed live-trapping data (1991-2008), local population density ranged from 0 to 7.21 squirrels/hectare ($n = 2277$ middens, mean $\pm$ SE: 2.35 ± 0.02 squirrels/hectare). Local population density was also highly

Table 5.2. Effects of local population density (squirrels/hectare) on the frequency of 1) territorial intrusions estimated from live-trapping data and 2) frequency with which squirrels emitted territorial vocalizations during 7-min behavioral observations. Results are from generalized linear mixed-effects models (Poisson response, log link). Regression coefficients are standardized.

Model	Fixed Effects	Parameter $\pm$ SE	Wald <i>t</i>	df	<i>P</i>
Territorial Intrusions ^A	No. owner caught	0.08 $\pm$ 0.003	26.77	433	<0.0001
	Local population density	-0.24 $\pm$ 0.02	-10.28	433	<0.0001
Territorial Vocalizations ^B	Local population density	0.17 $\pm$ 0.07	2.47	151	0.014

^A This model is based on 2277 observations on 436 unique middens on two study areas collected from 1991-2008.

^B This model is based on 474 observation sessions on 153 squirrels by 1 observer in 2008.

variable within a year and nearly matched the total variation in local population density observed across all years. For example, the range in local population density in 1999 was 0.71 to 7.21 squirrels/ha, but within 2005, local population density ranged only from 0.28 to 1.56 squirrels/ha. Thus, in addition to the considerable among-year variation in local population density, there was also a nearly equivalent amount of within-year variation.

Frequency of territorial intrusions

The number of intruding squirrels in a given year ranged from 0-30 squirrels but on average intruder pressure was low (1.5 ± 0.05 intruders captured over 6 months). Compared to intruders, midden owners were caught much more frequently during the course of annual trapping (7.6 ± 0.13 ; range = 0-37).

Local population density had a negative effect on the frequency of territorial intrusions, which was in the opposite direction as we had predicted. The number of intruders caught on defended middens significantly declined as local population density increased (slope = -0.24 ± 0.02 ; $t_{433} = -10.28$, $P < 0.0001$; Table 5.2; Fig. 5.3). The number of intruders captured was positively influenced by trapping effort; the number of intruders captured significantly increased when the owner of the midden was caught more frequently (slope = 0.08 ± 0.003 ; $t_{433} = 26.77$, $P < 0.0001$; Table 5.2).

Frequency of antagonistic physical interactions

Similarly to the frequency of territorial intrusions, behavioral observations of antagonistic physical interactions between adult squirrels were rare (Table 5.3). From 1989-2008, we recorded 60844 observations of red squirrel behaviors and only 373 (0.61%) of these were of antagonistic physical interactions between adult red squirrels.

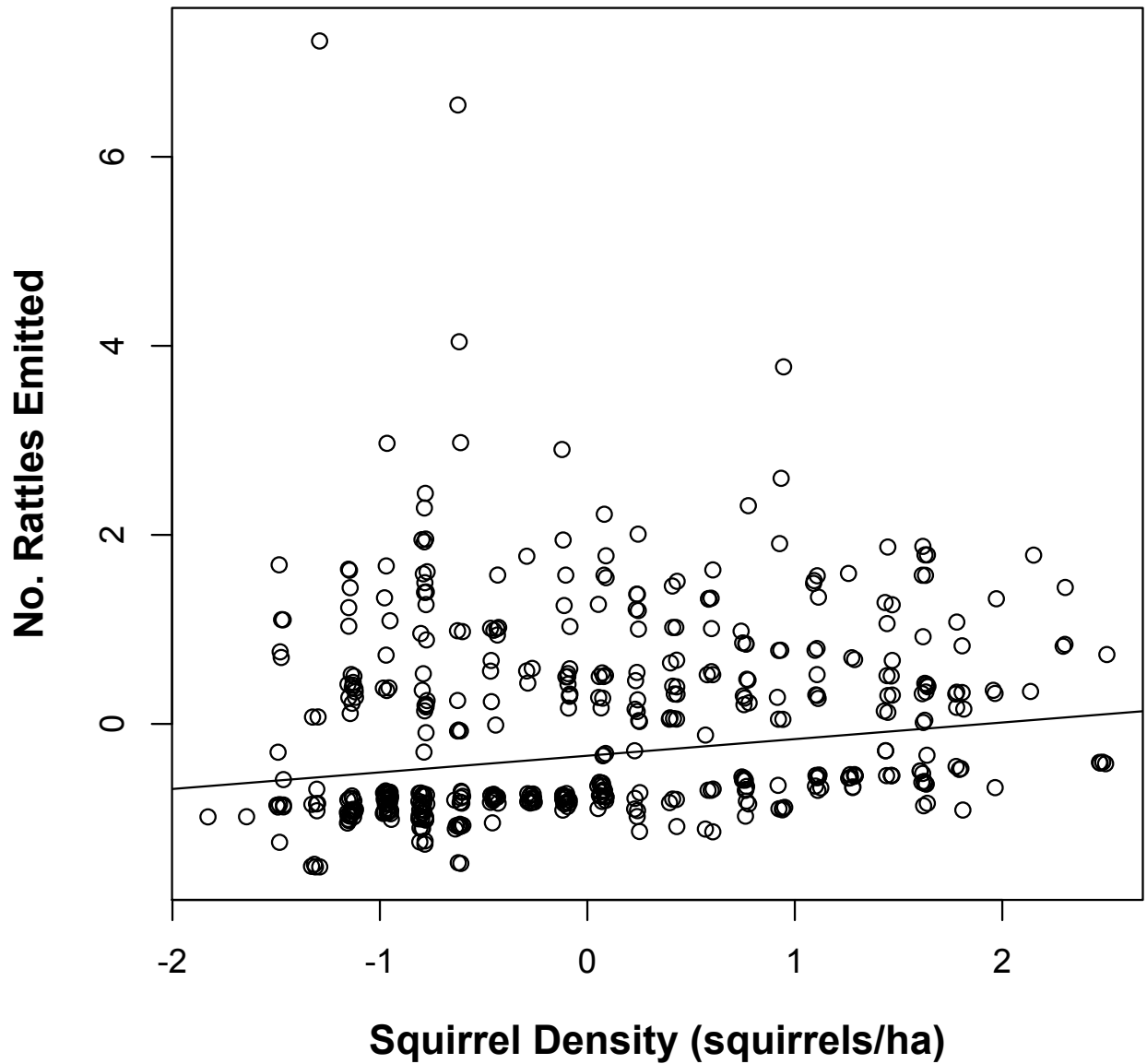


Figure 5.4. Effect of local population density on the frequency with which squirrels emitted territorial vocalizations (rattles) during 7-min behavioral observation sessions of squirrels on their territories ($n = 474$). Values on the x-axis are standardized measures of local population density (squirrels/hectare within 150 m). Partial residuals from a linear mixed-effects model are shown on the y-axis.

During 10 years from 1994-2008, we performed 1275 7-min focal animal behavioral sessions on 272 different squirrels (230 females and 42 males) that totaled 148.5 hours of observation and recorded only 11 antagonistic physical interactions between adult red squirrels. This corresponds to approximately 1.9 antagonistic physical interactions between adult red squirrels per 24 h. During all of our behavioral observations recorded in 2008, we observed a similar number of antagonistic physical interactions on the food-supplemented study area, which had experimentally heightened population density ($n = 200$ 7-min sessions; 3 interactions) compared to the control areas ($n = 274$ sessions, 2 interactions).

Frequency with which squirrels emitted territorial vocalizations

During the behavioral observation sessions in which we recorded every territorial vocalization by the focal squirrel ($n = 474$), red squirrels emitted 0.85 ± 0.05 rattles per 7-min observation session (range 0-11). Red squirrels responded to increasing local population density by emitting significantly more rattles (slope = 0.17 ± 0.07 ; $t_{151} = 2.47$, $P = 0.014$; Table 5.2; Fig. 5.4).

In contrast, squirrels did not emit more territorial vocalizations when exposed to playbacks of territorial vocalizations of conspecifics. Prior to starting the playbacks, there was no significant difference in the number of rattles emitted by control squirrels ($n = 53$ 7-min observation sessions, 0.44 ± 0.10 rattles per session) and those that would be exposed to the playbacks ($n = 37$, 0.46 ± 0.14 ; $Z = 0.13$, $P = 0.89$). During the playbacks the number of rattles emitted by squirrels exposed to the rattle playbacks ($n = 50$, 0.74 ± 0.14) was greater than control squirrels not exposed to the playbacks ($n = 31$,

Table 5.3. Number of antagonistic interactions observed during casual or *ad libitum* observation data collected from 1989-2008 and 7-min behavioral observation sessions collected from 1994-1997, 1999, 2001-2004, and 2008.

Observation Type	Years	Number	No. Interactions Observed
Casual observations	1989-2008	60844	373
7-min behavioral sessions	1994-1997, 1999, 2001-2004, 2008	1277	11

0.64 $\pm$ 0.14), but this difference was not significant (treatment x playback period interaction term: $Z = 0.26$, $P = 0.79$).

Describing behavioral patterns of red squirrels

We interpreted PC1 as a reflection of time allocated towards nest use versus other behaviors and PC2 as a reflection of time allocated towards vigilance versus feeding (Table 5.1). Larger values of PC1 represented behavioral observation sessions in which squirrels spent more time in their nest and less time performing other behaviors. Larger values of PC2 represented behavioral observation sessions in which squirrels spent more time vigilant and less time feeding (Table 5.1).

Effect of natural variation in local population density on behavior

Local population density strongly influenced the amount of time red squirrels allocated towards nest use (PC1) and feeding versus vigilance (PC2). As local population density increased, the amount of time spent in the nest significantly declined (PC1: slope on ln scale = -0.086 ± 0.05 , $t_{38} = -1.85$, $P = 0.036$; Table 5.4; Fig. 5.5A) and the amount of time allocated towards feeding significantly declined while time spent vigilant increased (PC2: slope on ln scale = 0.13 ± 0.07 , $t_{37} = 1.86$, $P = 0.035$; Table 5.4; Fig. 5.5B). Squirrels experiencing the highest local densities observed from 1994-2004 (>3 squirrels/ha) spent much less time in the nest ($n = 26$ sessions, $2.8 \pm 2\%$) and feeding ($4.3 \pm 4.3\%$) than did those squirrels experiencing the lowest local densities (<0.7 squirrels/ha) during the same time period ($n = 31$ sessions; nest: $36.8 \pm 8.5\%$; feeding: $21.2 \pm 5.9\%$). In contrast, squirrels experiencing the highest local densities spent more time vigilant ($26.7 \pm 5.5\%$) than did those experiencing the lowest local densities ($1 \pm 1\%$).

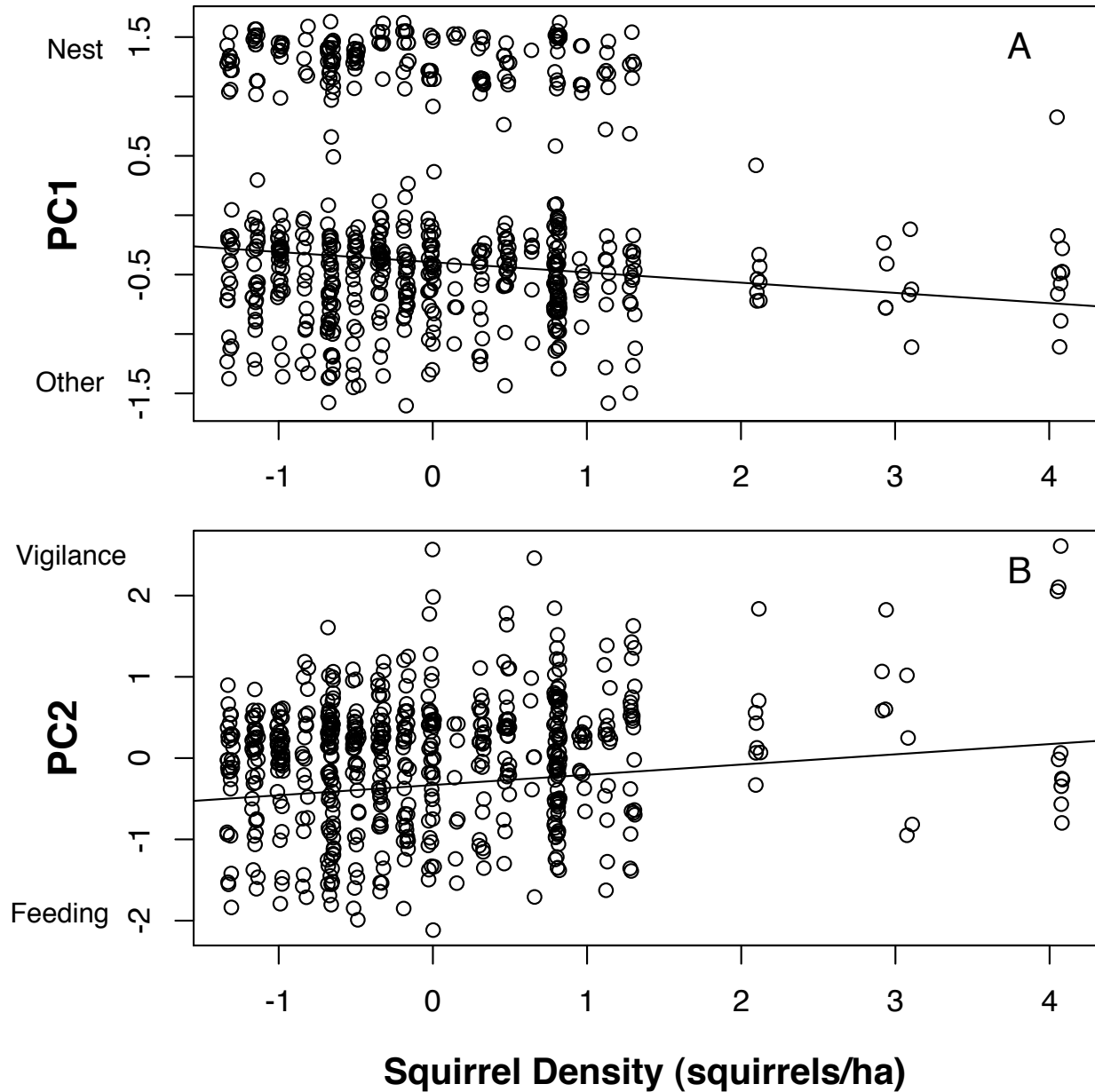


Figure 5.5. Effect of local population density on the behavior of red squirrels during 7-min behavioral sessions ($n = 631$) over 10 years from 1994-2004. Red squirrel behavior was decomposed using a principal components analysis into two principal components (PC1 and PC2). A) High PC1 scores correspond to decreased allocation towards nest use whereas B) high PC2 scores correspond to increased vigilance behavior and decreased feeding behavior. Values on the x-axis are standardized measures of local population density (squirrels/hectare within 150 m). Partial residuals from separate linear mixed-effects models for PC1 and PC2 are shown on the y-axis.

Effect of experimentally increased population density on behavior

Squirrels on the study area in which population density was experimentally increased using food-supplementation altered the amount of time they allocated towards nest use (PC1) and feeding and vigilance (PC2) in the same direction we found for our behavioral observations collected from 1994-2004 during natural variation in population density. During behavioral observations after all supplemental food was removed, squirrels on the study area with experimentally heightened population density had significantly lower PC1 ($t_{94} = -2.44$, $P = 0.008$; Table 5.4; Fig. 5.6A) and significantly higher PC2 ($t_{94} = 3.62$, $P = 0.0002$; Table 5.4; Fig. 6.6B) scores than those squirrels on the control study areas. On the study area with experimentally heightened population density, squirrels spent less time in the nest ($n = 93$ 7-min observation sessions, $8.5 \pm 2.7\%$ of total observations in nest) and more time vigilant ($43.3 \pm 3.6\%$) than those on the control study areas ($n = 142$ sessions, nest use: $19.6 \pm 3.1\%$; vigilance: $18.3 \pm 2.3\%$) Squirrels on the both study areas spent similar amount of time feeding (high density: $12.5 \pm 2\%$; control: $12.1 \pm 1.9\%$).

Effect of experimentally increased perceived population density on behavior

Compared to control squirrels not exposed to playbacks, squirrels exposed to the rattle playbacks altered the amount of time they allocated towards nest use (PC1) and feeding and vigilance (PC2) in the same direction we found for our behavioral observations collected from the other two datasets presented above. Squirrels experiencing heightened perceived population density had significantly lower PC1 scores than those control squirrels that were not exposed to the rattle playbacks ($t_6 = -$

Table 5.4. Behavioral responses of red squirrels to natural variation in local population density and experimental increases in 1) actual squirrel density (via long-term food-supplementation) and 2) perceived population density (via playbacks: “Rattle PBs”). Results are from linear mixed-effects models for principal components 1 (PC1: nest use) and 2 (PC2: feeding versus vigilance). Regression coefficients for local population density are standardized.

^AThis model is based on 631 7-min observation sessions on 46 squirrels by 40 observers from 1994-2004

^BThis model is based on 235 7-min observation sessions on 96 squirrels by 1 observer in 2008

^CThis model is based on 171 7-min observation sessions on 10 squirrels by 1 observer in 2008

Density Manipulation	Model	Fixed Effects	Parameter $\pm$ SE	<i>t</i>	df	<i>P</i>
Natural variation ^A	PC1	Local Population Density	-0.086 $\pm$ 0.05	-1.85	38	0.036
Natural variation ^A	PC2	Local Population Density	0.13 $\pm$ 0.07	1.86	37	0.035
		Local Population Density ²	0.01 $\pm$ 0.03	0.39	37	0.35
Actual density ^B	PC1	High Density Study Area	-0.28 $\pm$ 0.12	-2.44	94	0.008
Actual density ^B	PC2	High Density Study Area	0.59 $\pm$ 0.16	3.62	94	0.0002
Perceived density ^C	PC1	During PBs	0.19 $\pm$ 0.19	0.99	6	0.18
		Rattle PBs Treatment	0.18 $\pm$ 0.28	0.64	6	0.27
		Period (During PBs) x Treatment (Rattle PBs)	-0.66 $\pm$ 0.27	-2.42	6	0.026
Perceived density ^C	PC2	During PBs	-0.22 $\pm$ 0.11	-1.95	6	0.049
		Rattle PBs Treatment	-0.016 $\pm$ 0.13	-0.12	6	0.45
		Period (During PBs) x Treatment (Rattle PBs)	0.25 $\pm$ 0.15	1.63	6	0.077

2.42, $P = 0.026$; Table 5.4; Fig. 5.7A). During the playbacks, squirrels exposed to the rattle playbacks spent less than half the amount of time in the nest ($21.2 \pm 5.5\%$) than did those control squirrels not exposed to the playbacks ($46.2 \pm 8.8\%$). Although squirrels that were exposed to the rattle playbacks had higher raw PC2 scores (0.37 ± 0.14) and spent more time vigilant ($24.7 \pm 4.5\%$) than control squirrels (raw PC2: 0.023 ± 0.066 ; vigilance: $9.2 \pm 2.5\%$), this difference was not significant ($t_6 = 1.63$, $P = 0.077$; Table 5.4; Fig. 6.7B). Squirrels exposed to the playbacks spent a similar amount of time feeding ($8.7 \pm 2.4\%$) compared to those control squirrels not exposed to the playbacks ($9.2 \pm 2.5\%$).

Discussion

Frequency of antagonistic physical interactions

Some models of density-dependent population regulation assume a positive relationship between the frequency of antagonistic physical interactions and population density (Krebs 1978; Heske et al. 1988; Krebs 1996). However, long-term behavioral studies spanning extensive variation in population density are rare and the evidence for this assumption is actually quite ambiguous (Heske et al. 1988). Red squirrels were rarely observed to engage in antagonistic physical interactions with other squirrels over 18 years of natural variation in population density. Similar data on the frequency of physical interactions in asocial animals across variation in population density are rare (but see Watson et al., 1994). Recent technological advances (proximity-logging radiocollars) have enabled greater study of contact frequencies in free-ranging animals (Prange et al. 2006). Our behavioral results support a recent study that used proximity-

logging radiocollars that also documented a similarly low frequency of daily contacts between members within social groups in free-ranging European rabbits (*Oryctolagus cuniculus*: Marsh et al., 2011). This is surprising because rabbits were considered to be fairly social yet Marsh et al. (2011) show that intra-group interactions between members were nearly as rare as we found in this study in red squirrels that exhibit territoriality year-round.

Our live-trapping data suggest that the frequency of territorial intrusions may actually be weakly negatively associated with population density. Squirrels were rarely trapped on middens they did not own compared to the frequency that we caught the owners of these middens, which is not surprising given that they vigorously defend exclusive resource-based territories. As population density increased, however, the frequency of territorial intrusions declined. This pattern could suggest that the smaller territories that occur at higher densities (LaMontagne 2007) are easier to defend than larger territories that occur at lower densities. On the other hand, this pattern could suggest that the benefits of defending a territory declined with decreased resource availability (Brown 1964; Ewald and Carpenter 1978; Schoener 1983). Years of low population density are generally preceded by several years of low spruce cone production (Fig. 5.1). Therefore, intruder pressure may increase during periods of low population density and resource availability because the benefits of territoriality are minimal (Ewald and Carpenter 1978).

Territory size in red squirrels is variable and appears to be associated with population density (Steury and Murray 2003; LaMontagne 2007). The smallest territory

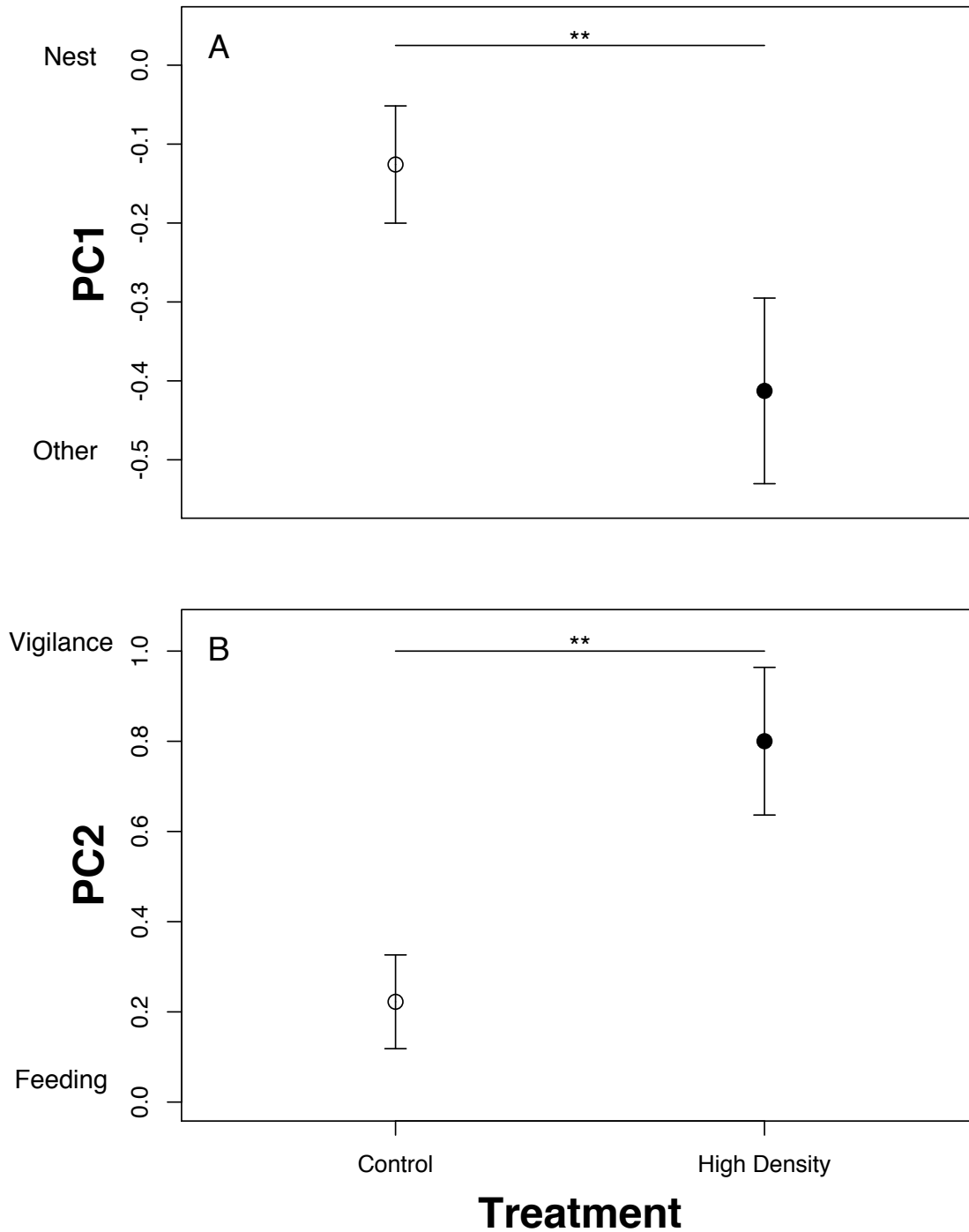


Figure 5.6. Effects of an increase in numerical population density on the food-supplemented study area (“High Density”; n = 93 sessions) compared to the unmanipulated study areas with lower density (“Control”; n = 142) on red squirrel behavior as measured during 7-min behavioral observation sessions that were collected after supplemental food was removed. A) High PC1 scores correspond to decreased nest use and B) high PC2 scores correspond to increased vigilance behavior and less feeding. Values on y-axis represent residual A) PC1 or B) PC2 scores from linear mixed-effects models. Significant differences are represented by “***” at P < 0.01.

sizes measured during our study occurred in 1994 and 1999 (LaMontagne 2007), which corresponded to years of the highest (1999) or near the highest (1994) population densities that we have observed (Fig. 5.1). During the year in which we collected behavioral observations on the high-density food-supplemented and control study areas (2008), territory sizes on the food-supplemented study area were significantly smaller compared to the control study area (Donald and Boutin, 2011). Therefore, we expected a positive association between the frequency of territory intrusions and antagonistic interactions due to an increasing probability of interacting with a heightened number of neighboring squirrels when population density was higher and territories were smaller. Although we did not find support for this prediction, animals may use other cues such as ritualized acoustical signals to space themselves and respond to variation in population density.

Frequency of antagonistic acoustical interactions

As we had predicted, the frequency of acoustical interactions (territorial vocalizations) significantly increased as local population density increased (see also Shonfield, 2010). A recent study in this population found that territory intrusions occurred more frequently following experimental removals of midden owners compared to observations when the owner of the midden was present (Donald and Boutin, 2011). Thus, when territorial vocalizations and defense by midden owners ceased during experimental removals, the frequency of intruders increased.

The effects of natural variation in local population density and the playbacks of territorial vocalizations on the frequency with which squirrels emitted rattles were similar and in the same direction (effect sizes: 0.17 and 0.26, respectively). However, the effect

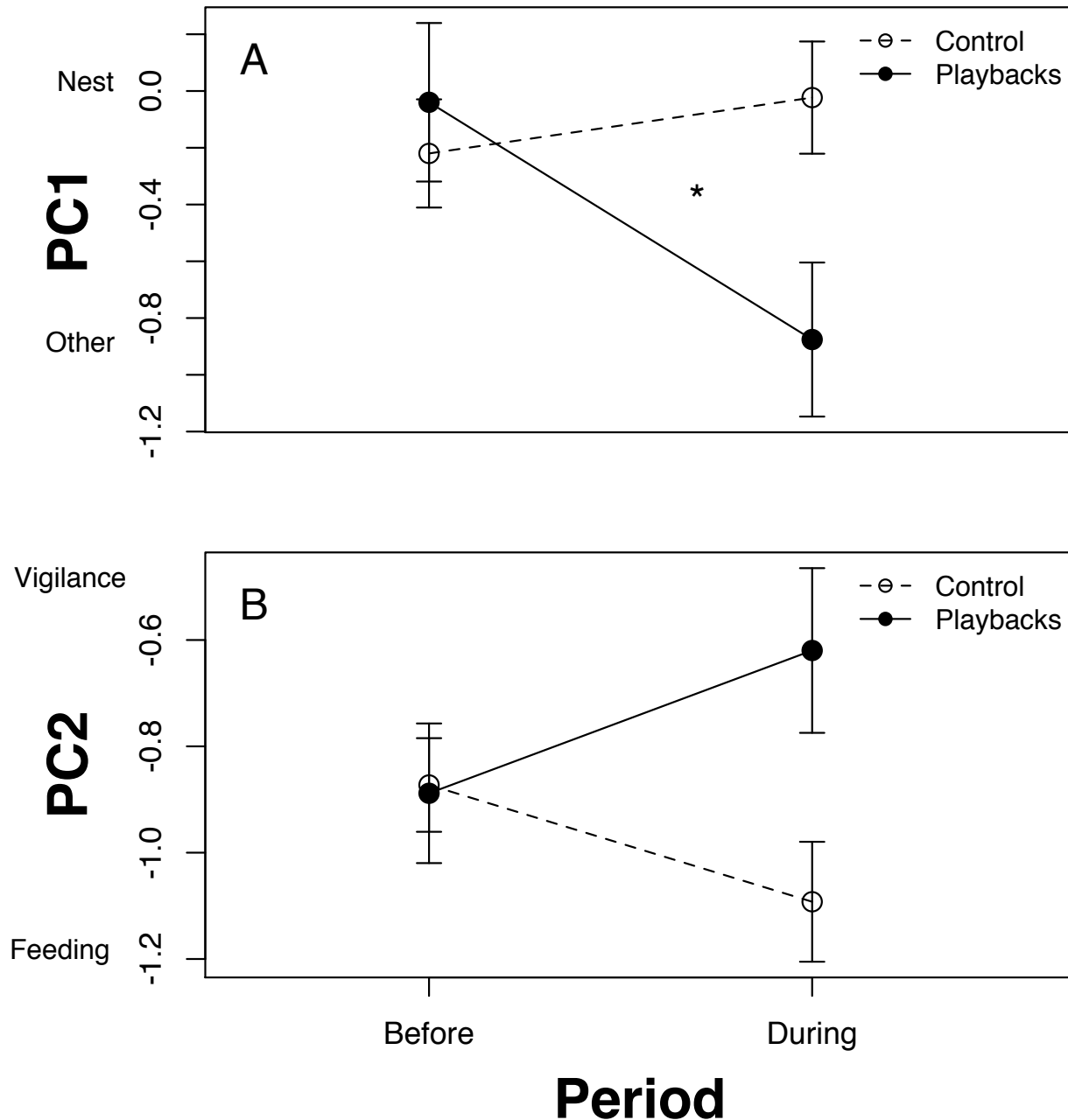


Figure 5.7. Effects of increased perceived (acoustical) population density on red squirrel behavior as measured during 7-min behavioral observation sessions conducted on squirrels exposed to territorial vocalizations (“Playbacks”) before exposure to the playbacks (“Before”; $n = 37$ sessions) and during the playbacks (“During”; $n = 50$) and squirrels not exposed to territorial vocalizations (“Control”) before ($n = 53$) and during ($n = 31$) the period when the experimental group of squirrels were exposed to the vocalizations. A) High PC1 scores correspond to decreased allocation towards nest use while B) high PC2 scores correspond to increased allocation towards vigilance behavior and decreased allocation towards feeding behavior. Values on y-axis represent residual A) PC1 or B) PC2 scores from linear mixed-effects models. Significant differences are represented by “*” at $P < 0.05$.

of the rattle playbacks on the frequency with which squirrels emitted rattles was not statistically significant. We attribute the lack of the statistical effect of the playbacks on the frequency with which squirrels emitted territorial vocalizations to low statistical power to detect statistical significance due to the small sample sizes ($n = 5$ squirrels/treatment group). Future studies using this method to increase perceived population density will use larger sample sizes to increase our power to assess detect statistical differences and to examine additional factors contributing to variation in responses to playbacks.

Our results from the playback experiments indicated that red squirrels can assess population density via the number of territorial vocalizations heard in their local neighborhood and adjust their behavioral patterns accordingly. In support of this conclusion, the approximate maximum distance red squirrels are able to detect territorial vocalizations (150 m: Smith 1968; Shonfield, 2010) corresponded to a spatial scale at which the correlations between local population density and the majority of our response variables were highest or nearly highest (Fig. 5.2). Cumulatively, our results suggest that red squirrels adjust the frequency with which they emit territorial vocalizations as well as their nest attendance and feeding and foraging behavior in response to local population density. The frequency with which territorial vocalizations are heard also appears to represent a source of social information (Dall et al. 2005) that red squirrels exploit to make behavioral decisions in response to local population density and which could also potentially be used to adaptively adjust reproductive investments in response to local density.

Effects of population density on overall behavioral patterns

Population density had unequivocal effects on how red squirrels allocated the amount of time spent in the nest, feeding, and vigilant. As population density increased, either naturally or due to experimental manipulations, squirrels allocated less time towards self-maintenance (nest use and feeding) and, for females, offspring provisioning (nest use) and more time spent vigilant. These behavioral adjustments to population density may have been sufficient to cause the inverse relationship we observed between population density and the frequency of territorial intrusions. For example, under high-density conditions, the increase in territorial vocalizations emitted and time spent vigilant may have deterred territorial intrusions.

The results from our experimental manipulation of actual density using food-supplementation paralleled our conclusions from the long-term behavioral observations. Squirrels experiencing experimentally increased actual density exhibited similar shifts in behavioral patterns as we found when squirrel density was naturally high. Because we only analyzed behavioral observations after the removal of the supplemental food, it is likely that these behavioral differences were due to differences in population density and not the supplemental food. It is also unlikely that there was spatial variation in food abundance among the study areas because the two study areas are located within 5 km from each other and spruce cone production in the region where we conducted this study is largely synchronous (LaMontagne and Boutin 2007). However, differences in hoard sizes between the food-supplemented and control study areas may have confounded our ability to disentangle the effects of food from density. In support of this possibility, the number of hoarded cones on the food-supplemented study was higher

than the number of hoarded cones on one of our control study areas (Donald and Boutin, 2011).

Experimental manipulation of cues reflective of population density using long-term playbacks of territorial vocalizations avoids any potential confounding influence of variation in resource availability. Using our audio playback approach, we were able to show directly that the frequency of territorial vocalizations was the mechanism by which red squirrel behavior was altered by population density. We observed that squirrels exposed to heightened perceived population density exhibited similar shifts in behavior as we found when actual squirrel density was naturally high or experimentally increased using food-supplementation. This suggests that high density conditions can be simulated in red squirrels using this playback protocol and that the behavioral changes that are induced under high density conditions are directly caused by the frequency of territorial vocalizations and not food abundance.

Summary

We found that the association between population density and the frequency of antagonistic physical interactions from spring until summer was either negative (territory intrusions) or so rare that they were nonsignificant (behavioral data). However, we found a significant positive relationship between population density and the frequency with which squirrels emitted territorial vocalizations. This indicates that a reliable mechanism by which squirrels can assess density is the frequency with which territorial vocalizations are emitted. During periods of naturally high population density and when density was increased with food-supplementation, squirrels spent less time in the nest, less time feeding, and more time vigilant. Our experimental manipulation of perceived

population density induced similar changes in behavior, confirming that behavioral responses were driven by differences in population density rather than variation in resources. Our results indicate that acoustical interactions in red squirrels facilitate spacing behavior and affect overall behavioral patterns. Similarly to the indirect effects predators can have on prey by altering their behavior (Lima 1998; Werner and Peacor 2003; Preisser et al. 2005; Creel and Christianson 2008), population density may have subtle effects on conspecifics by affecting overall behavioral patterns rather than just the frequency of antagonistic interactions. The population-level consequences of density-dependent behavioral changes could be significant due to their effects on individual fitness but they remain to be examined (Owens, 2006; Kokko and López-Supulcre, 2007).

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CHAPTER 6

Dantzer, B., Newman, A. E. M., Boonstra, R., Boutin, S., Palme, R., Humphries, M. M., McAdam, A. G. Experimental induction of adaptive endocrine-mediated maternal effects on offspring phenotype in red squirrels. *To be submitted*

Introduction

How organisms adapt to changing environmental conditions is a central question that pervades all biological disciplines. The increasing pace of anthropogenic environmental change and heightened rate of phenotypic evolution in such environments has magnified the importance of understanding how organisms adapt to these changing environments (Hendry et al., 2008). The Modern Evolutionary Synthesis specifies that adaptation will occur when genetic variation underlies phenotypic differences that co-vary with fitness (Mayr and Provine, 1980; Mayr, 1993). However, much of the phenotypic variation available for natural selection to act upon during contemporary adaptation can be generated by non-genetic or environmental sources through phenotypic plasticity, which is the environmentally contingent induction of phenotypic variation (Pigliucci, 2001; West-Eberhard, 2003; Bonduriansky and Day, 2009). This complementary view of adaptation focusing on the role of phenotypic plasticity has lengthy historical precedence (Baldwin, 1896; Goldschmidt, 1940; Waddington, 1942; Schmalhausen, 1949) and more recently has increasingly challenged the traditional view of adaptation outlined by the Modern Synthesis (West-Eberhard, 2003; Pigliucci and Müller, 2010).

Maternal effects are a form of transgenerational phenotypic plasticity that may enable females to induce adaptive changes in offspring phenotype in anticipation of future environmental conditions (Mousseau and Fox, 1998; Uller, 2008). Maternal effects are the influence of the maternal genotype, phenotype, or the interaction between maternal genotype and phenotype, on offspring phenotype in addition to their direct genomic contribution (Rossiter, 1996; Mousseau and Fox, 1998; Wolf and Wade,

2009). Maternal effects have been documented in many taxa and can have profound effects upon offspring phenotype (Mousseau and Fox, 1998; Groothuis et al., 2005). If the maternal and offspring environmental conditions are matched, maternal effects may allow females to modify offspring phenotype adaptively for the environment they will experience at independence (Marshall and Uller, 2007; Uller 2008). Because some maternal effects are heritable (McAdam et al., 2002; Wilson et al., 2005; Räsänen and Kruuk, 2007) and can affect the rate of evolution (McAdam and Boutin, 2004), they may enable relatively rapid evolutionary responses to changing environments (Agrawal et al. 1999; Räsänen and Kruuk, 2007; Badyaev, 2008).

Although the mechanisms underlying maternal effects in wild animals are poorly understood, endocrine mechanisms may mediate many maternal effects. Variation in the ecological or social environment that elicits a neuroendocrine response in breeding female mammals can induce considerable developmental variation in offspring phenotype through early hormone exposure (Dloniak et al., 2006; Maestriperi and Mateo, 2009). For example, variation in the social environment such as the frequency of interactions between conspecifics affects the concentrations of circulating androgens (testosterone: Wingfield et al., 1990; Cavigelli and Pereira, 2000) and stress hormones (glucocorticoids Christian, 1961; Rogovin et al., 2003; McCormick, 2006; Creel et al., submitted). These changes in the concentrations of circulating hormones in breeding females can then serve as a bridge between the external environment and offspring phenotype. Variation in early hormone exposure can have lifelong consequences for offspring morphology, physiology, neuroanatomy, and behavior (Clark and Galef, 1995; Welberg and Seckl, 2001; Groothuis et al., 2005; Maestriperi and Mateo, 2009). As

such, hormone-mediated maternal effects may enable adaptive modification of offspring phenotype to the anticipated environment (Dufty et al., 2002; Mazuc et al., 2003; Lancaster et al., 2007).

The focus of this study was to determine whether hormone-mediated maternal effects could enable female North American red squirrels (*Tamiasciurus hudsonicus*) to adjust offspring phenotype adaptively to fluctuating environmental conditions. Red squirrels at our study site in the Yukon, Canada experience pronounced fluctuations in the availability of their major food source (LaMontagne and Boutin, 2007; Fletcher et al., 2010), which generates inter-annual fluctuations in population density (Fig. 6.1). These changes in density are associated with density-dependent competition for vacant territories among juveniles. In high-density years, which follow pulses of food availability, many juveniles compete for each territory with a smaller number competing for each vacant territory in lower density years (McAdam et al., in prep). Red squirrels exhibit year-round resource defense territoriality (Smith, 1968; Dantzer et al., in press), and juveniles that fail to acquire a territory before their first winter do not survive (Larsen and Boutin, 1994).

A major predictor of whether a juvenile red squirrel acquires a territory before its first winter is its rate of postnatal growth (the rate of linear growth from 0-25 d post-parturition: McAdam et al., 2003). Over the past 20 years (1989-2008), we have found that offspring growth rates experience large, genetically-based maternal effects (McAdam et al., 2002), and that there is density-dependent natural selection on offspring growth rates (McAdam et al., in prep). Natural selection favors those females

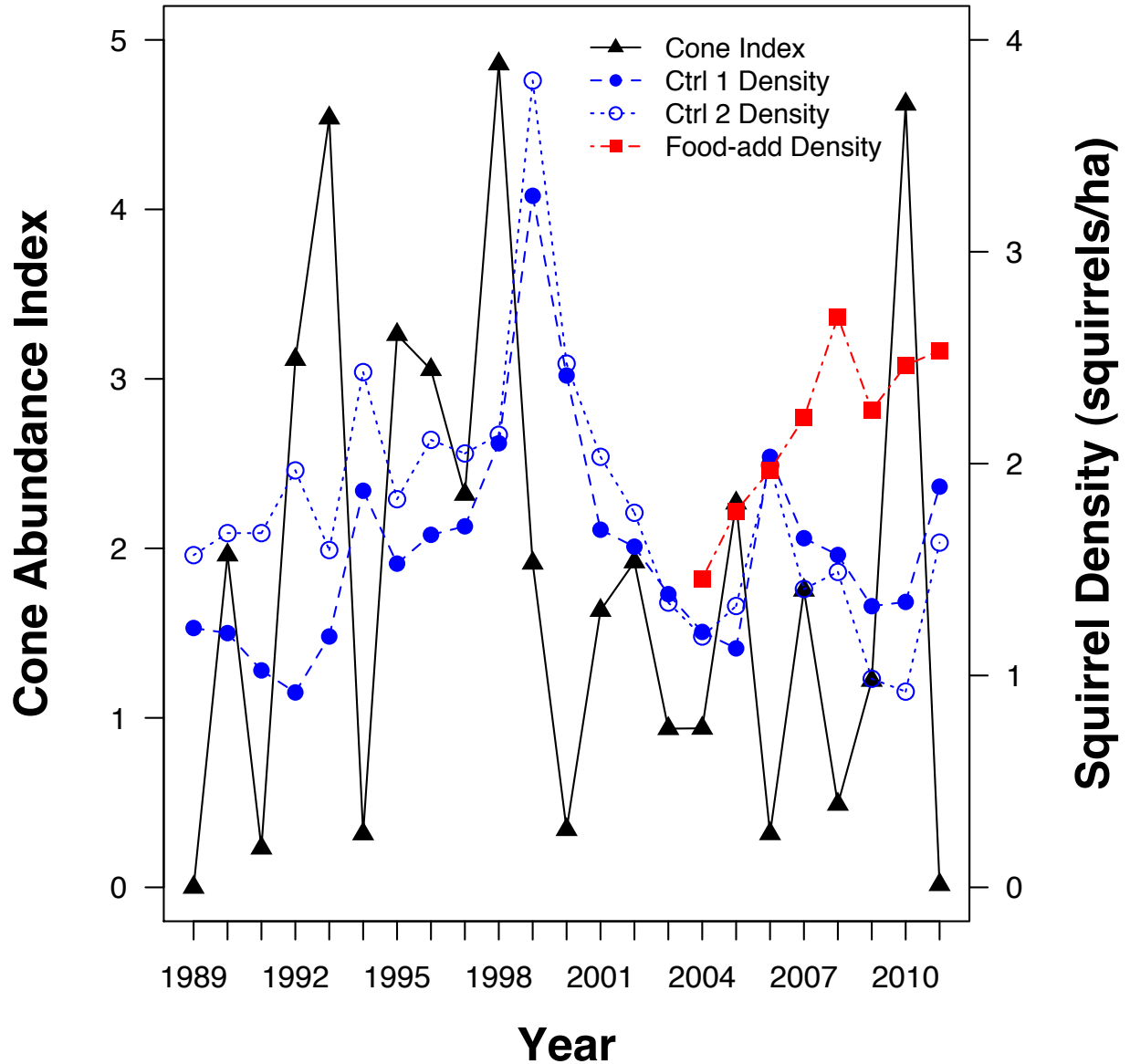


Figure 6.1. Annual production of white spruce (*Picea glauca*) cones and spring density of red squirrels on two unmanipulated study areas and one food-addition study area. Cone production is an index of the average number of cones produced on up to three study areas as measured in the autumn of each year (LaMontagne and Boutin, 2007). Squirrel density is shown for two control study areas (40 ha each: Ctrl 1 and Ctrl 2) and one study area (45.4 ha: Food-add) that has been provided with supplemental food since the fall of 2004. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation

that produce the fastest growing offspring in years of high density, whereas selection is relaxed in low-density years (Fig. 6.2; McAdam et al., in prep). Because females are relatively long-lived (1-8 years: McAdam et al., 2007), they are likely to experience both high- and low-density years. Consequently, we predicted that natural selection would favor the evolution of adaptive plasticity in maternal effects on offspring growth rates where females exert growth-enhancing maternal effects on offspring growth rates in high-density years.

Across six years of study (2006-2011), we investigated the potential endocrine mechanisms mediating these maternal effects on offspring growth rates. First, we examined relationships between population density and fecal cortisol (FCM) and androgen (FAM) metabolite concentrations in breeding females. Second, we compared FCM and FAM concentrations in breeding females inhabiting unmanipulated control study areas to those of breeding females on another study area in which we experimentally increased population density using long-term food-supplementation (Fig. 6.1). We then examined associations between FCM and FAM concentrations in breeding females and offspring growth rates to determine whether endocrine responses to variation in population density might mediate adaptive maternal effects on offspring growth rate.

Population density experienced by females during pregnancy and lactation predicts the degree of competition their offspring will encounter, and therefore social information reflecting population density may be used by females to modify offspring growth rates adaptively (McAdam et al., in prep). We experimentally manipulated the social cues that females can use to assess population density using audio playbacks of

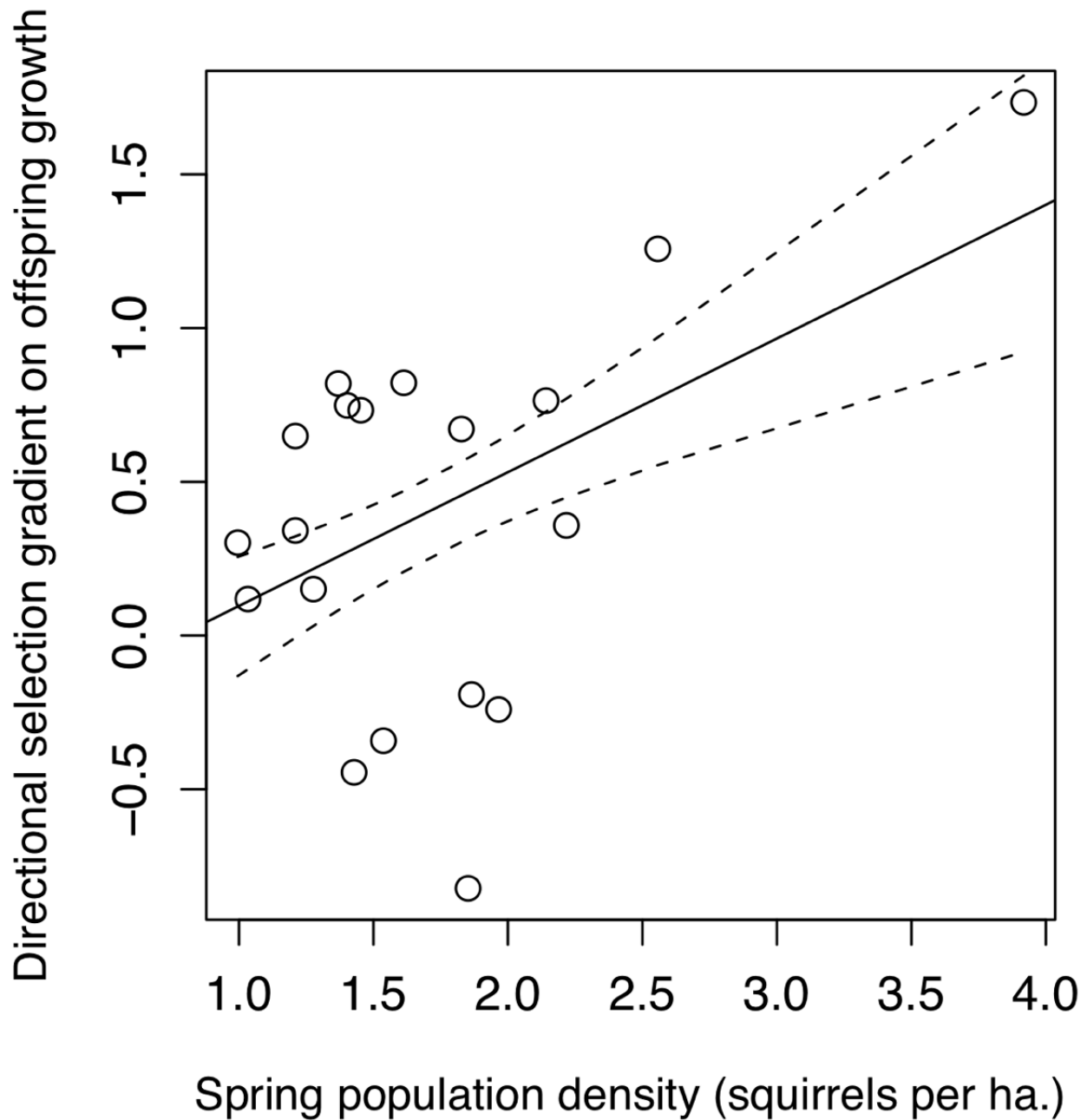


Figure 6.2. Red squirrels in the Yukon, Canada experience density-dependent natural selection on offspring postnatal growth rate (change in mass from ~1-25 d post-parturition). Each circle corresponds to a different year from 1989-2008. Figure recreated from McAdam et al. (in prep).

red squirrel territorial vocalizations, which we successfully used to simulate high perceived population density in a previous study (Dantzer et al., in press). We compared the FCM and FAM concentrations of females exposed to heightened perceived density to those of females exposed to control playbacks (avian vocalizations). We also compared the growth rates in offspring of females exposed to heightened perceived density with those of females on a study area in which population density was experimentally increased via long-term food-addition study area (Fig. 6.1) and to those of control females (exposed to control or no playbacks).

Material and Methods

Study area

A natural population of red squirrels on up to 6 different study areas in the southwest Yukon, Canada (61° N 138° W) has been monitored continuously since 1987. We documented the effects of population density on FCM and FAM concentrations and their effects on female reproductive traits on two unmanipulated study areas (40 hectares each) and one food-addition study area (45 hectares, described below). We also performed manipulations of perceived population density using audio playbacks on 4 different study areas in the same region. All of these study areas are located within 8 km of one another.

Red squirrels

Red squirrels in the southwestern Yukon rely primarily on the seeds produced by white spruce (*Picea glauca*) trees (Fletcher et al., in prep). White spruce is a mast seeding species that produces episodic, and highly synchronous, pulses of conifer cones such

that in some years there are many cones produced whereas in other years there are virtually no cones produced (Fig. 6.1; Boutin et al., 2006; LaMontagne and Boutin, 2007). In the autumn of each year, red squirrels clip the cones from the tops of the trees and cache them underground in a larder hoard located at the center of their territory called a “midden” (Smith, 1968; Fletcher et al., 2010). Because red squirrels do not hibernate or undergo torpor (Pauls, 1978), these cached cones likely provide the bulk of calories necessary for overwinter survival and reproduction, which begins during the winter months (copulations occur as early as January: Boutin et al., unpub. data).

Red squirrels exhibit resource defense territoriality and defend their territories containing the cached cones vigorously with territorial vocalizations called rattles (Smith, 1968; Dantzer et al., in press). Male and female red squirrels defend territories from both intra- and inter-sexual intruders year-round (Smith, 1968). However, females allow their offspring to remain on their territory after first emergence from their natal nest (~40 days post-parturition) and until after weaning (~70 days post-parturition), and they also tolerate males on their territory while they are in estrous (Smith, 1968; Humphries and Boutin, 1996; Steele, 1998).

The extreme inter-annual variability in white spruce cone production generates variation in red squirrel population density. In years of high cone production, overwinter survival improves among juvenile red squirrels, and consequently population densities are higher the following spring (McAdam and Boutin, 2003; Boutin et al., 2006; McAdam et al., in prep). This increase in population density in years after large cone crops in turn generates density-dependent competition for vacant territories among juvenile red

squirrels; in years of high density there is greater competition among juvenile red squirrels for vacant territories (McAdam et al., in prep).

Yukon red squirrels can breed in the winter, spring, and summer depending upon spruce cone production during the previous autumn (Boutin et al., 2006). Females are generally in behavioral estrous for one day per reproductive bout, followed by a ~35 day gestation period (Steele, 1998; Lane et al., 2008; McFarlane et al., 2011). Litter size ranges from 1-7 and females rarely produce more than one successful litter per year except in mast years (Boutin et al., 2006; McAdam et al., 2007).

General methods

All squirrels on our study areas were individually marked with uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA) as well as a unique combination of colored electrical wire pieces or pipe cleaners threaded through their ear tags that enables individual identification from afar. Most squirrels were individually marked in their natal nest around 25 d post-parturition and others received marks upon first capture. In each year from between February and August, squirrels were live-trapped on their middens every 3-14 days using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA). Upon capture, squirrels were identified (by reading ear tags), weighed, and their reproductive condition was determined via palpation of the abdomen and assessing nipple condition. Females were classified as non-breeding, pregnant (fetus palpable in abdomen), or lactating (milk expressed from teats). Parturition was confirmed by documenting a sudden weight loss, the expression of milk from teats, or by assigning an age to neonates based upon their mass when pups were accessed in their natal nest soon after birth (see Becker, 1993; Boutin and Larsen,

1993). Conception dates were estimated by subtracting 35 days from known dates of parturition (Dantzer et al., 2011a).

Females were radiocollared (model PD-2C, 4g, Holohil Systems Limited, Carp, Ontario, Canada) shortly before or after parturition so we could use radio telemetry to locate the nests containing their pups. Pups were temporarily removed from their natal nests once around 0 d post-parturition and a second time around 25 d post-parturition. During the first nest entry, pups were weighed (using a calibrated electronic portable balance to 0.1 g), sexed, and individually marked (ear notches). During the second nest entry, pups were weighed and ear tagged with uniquely numbered metal ear tags. We used the rate of change in mass from birth to 25 d post-parturition as our estimate of offspring growth rate. This is a linear period of growth (McAdam et al., 2002) during which offspring are entirely dependent upon their mothers for nutrition, as it is before their first emergence from the natal nest (Humphries and Boutin, 1996) and has been used in previous analyses (McAdam et al., 2002; McAdam and Boutin, 2003, 2004).

Measuring fecal cortisol and androgen metabolite concentrations

During each live-trapping event, we collected fecal samples from underneath the live-traps, placed them individually into 1.5 mL vials using forceps, and then placed them in a -20 °C freezer within 5 h of collection. Fecal samples collected in the winter months (February-April) were generally frozen upon collection and remained so until placed into the freezer. In the warmer months (May-August), we placed fecal samples into an insulated container containing wet ice until they were placed in the -20 °C freezer. The period of time between collection and freezing of the fecal samples does not systematically affect FCM or FAM concentrations (Dantzer et al., 2010, 2011a).

Squirrels were in traps for less than 2 h before collection of fecal samples, which is not long enough for FCM and FAM concentrations to be affected by trap-induced stress from the current capture event (Dantzer et al., 2010, 2011a). In addition, we only analyzed fecal samples from squirrels that had not been handled within the previous 72 h.

Briefly, the entire fecal sample collected was lyophilized (LabConco, MO, USA) for 14-16 h and then completely homogenized using a mortar and pestle. Fecal cortisol and androgen metabolites were extracted from 0.05 g of the dry ground feces by adding 1 mL of 80% methanol and this solution was then shaken with a multivortexer at 1450 revolutions per minute for 30 min, and then centrifuged for 15 min at 2500g (Palme, 2005; Dantzer et al., 2010, 2011a, 2011b). The resulting supernatant was stored at -80 C until analysis via enzyme-immunoassays (EIA), which we have previously validated to measure fecal cortisol (Dantzer et al., 2010) and androgen (Dantzer et al., 2011a, 2011b) metabolite concentrations in this species.

FCM concentrations were quantified using a 5α -pregnane- 3β , 11β , 21-triol-20-one EIA, which measures glucocorticoid metabolites with a 5α - 3β , 11β -diol structure (Touma et al., 2003; Dantzer et al., 2010). Details on this antibody (Touma et al., 2003) and further details of the assay procedure (Palme and Möstl, 1997; Dantzer et al., 2010) can be found elsewhere. Samples were run in duplicate and samples from different years and treatments were randomly allocated to different assays. The intra- and interassay coefficients of variation were 7.6 and 14.9% ($n = 71$ plates of 35 samples/plate). The assay had a sensitivity of 0.82 pg per well.

FAM concentrations were quantified using a testosterone EIA that measures 17 β -OH androgens (Palme and Möstl, 1994; Dantzer et al., 2011a, 2011b). Details on this antibody (Palme and Möstl, 1994) and further details of the assay procedure (Möhle et al., 2002; Dantzer et al., 2011a, 2011b) can be found elsewhere. Samples were run in duplicate and samples from different years and treatments were randomly allocated to different assays. The intra- and interassay coefficients of variation were 10.7 and 17.1% (n = 70 plates of 35 samples/plate). The assay had a sensitivity of 0.3 pg per well.

Estimating population density

In each year of study, we completely enumerated all squirrels defending territories on each of our study areas. Territory ownership was confirmed by a combination of repeated live-trapping of the same squirrel on the same midden and behavioral observations of squirrels emitting territorial vocalizations (rattles) from the same midden (Smith, 1968; Dantzer et al., in press). We estimated squirrel density on each study area in each year as the number of unique squirrels defending a territory on the study area over the total area (squirrels/hectare: Fig. 6.1). However, a uniform estimate of population density for each squirrel on a study area could overlook important local variation in population density (Garant et al., 2005, 2007; Dantzer et al., in press). Consequently, we also estimated local population density for each female we followed as the number of unique squirrels defending a territory within 150 m of her midden (see Dantzer et al., in press for a more thorough description). Although our study area is bordered by unsuitable habitat (meadows or wetlands), and although we also census middens that are on the edges of our study areas, local population density may have been underestimated for squirrels living at the edges of our study areas.

However, the inferences from our analyses of how local population density affects fecal cortisol and androgen metabolite concentrations (see below) were not affected by whether or not we separately considered squirrels in the core and those on the edge of any study area (see also Dantzer et al., in press).

Manipulation of actual population density

Since the winter of 2004, we have provided supplemental peanut butter (no sugar or salt added) on 100-120 middens on one study area. Squirrels that owned these middens were provided with a bucket that was hung between two trees near the center of the midden. One kg of peanut butter was placed into these buckets approximately every 6 weeks from October to May of each year. Peanut butter was removed at the end of May of each year but females that were pregnant or lactating on the removal date continued to receive supplemental peanut butter. Squirrels cannot hoard or cache peanut butter and it does not appear to influence female body mass (Dantzer et al., in press). This food supplementation procedure led to an increase in population density compared to the two nearby control study areas (Fig. 6.1). From 2007 to 2011, the average squirrel densities on the food-addition study area were 36-64% higher than the average squirrel densities on the two control study areas but were only 1% higher in 2006.

Manipulation of perceived population density

In one year (2010), we experimentally increased perceived population density without supplemental food using audio playbacks of territorial vocalizations (rattle playbacks). We have previously shown that this method simulates high-density conditions and induces density-dependent changes in behavior patterns that are similar

to those observed under naturally high-density conditions (Dantzer et al., in press). We exposed a group of females to rattles to simulate high-density conditions and compared their endocrine and reproductive responses to those of a separate group of control females exposed to the vocalizations of boreal chickadees (*Poecile hudsonicus*), which is a non-predatory year-round resident species of our study area. We collected fecal samples from female squirrels exposed to the rattle and chickadee playbacks before, during, and after the playbacks started.

In total, we exposed 44 females on 3 different study areas to rattle playbacks and 27 females on 4 different study areas to chickadee playbacks. Two of these study areas had both treatments with the qualification that females that were to be exposed to chickadee playbacks had to be >150 m away from a female that was exposed to rattle playbacks (i.e., out of hearing distance: Smith, 1968; Shonfield, 2010). During the course of the experiment, some of the females exposed to chickadee (n = 4) and rattle (n = 9) playbacks died from natural causes. Females in both playback treatment groups experienced litter loss from unknown but natural causes during the experiment either before or after parturition such that our final sample sizes were reduced for the number of females exposed to chickadee (n = 19 females) and rattle (n = 20 females) playbacks for which we had complete data (FCM, FAM, and offspring growth rates).

During the active part of their day, red squirrels emit approximately 1 rattle every 7-min (Dantzer et al., in press). The territorial vocalizations of red squirrels, rattles, contain individually distinct signature signals (Smith, 1979; Goble, 2008), which enabled us to manipulate the number of unique rattles that females heard during reproduction. We sought to simulate an increase of 4 additional neighboring squirrels around each

female's territory by exposing them to audio playbacks of rattles recorded from 4 different squirrels. Similarly, females in the chickadee playback treatment group were exposed to 4 different chickadee vocalizations every 7-min. Squirrel or chickadee playbacks were broadcast from an MP3 player (Coby MP-300, Lake Success, NY, USA) through two separate speakers (2 different alternating vocalizations were broadcast through each speaker: Altec Lansing Orbit, San Diego, CA, USA). We placed each speaker in a random cardinal direction ~15 m away from the center of the midden so that the vocalizations did not simulate a territory intrusion on the midden. We programmed each of the two MP3 player and speaker units around the squirrel's midden so that one rattle or chickadee playback would be broadcast from each speaker every 3.5 min. This ensured that a squirrel was exposed to 4 different rattle or chickadee playbacks every 7-min. Squirrels were exposed to the rattle or chickadee playbacks as soon as pregnancy was confirmed and until around 5 days post-parturition (~ 34 continuous days) for approximately 12 hours/day (800-2000 h). This same playback protocol has been previously used to simulate high-density conditions (Dantzer et al., in press), except in the present study we used chickadee playbacks as a control treatment in addition to control females exposed to no playbacks.

We used a total of 106 rattles from 87 different male and female squirrels that were recorded from 2005-2009 to develop unique combinations of rattles from each of 4 different squirrels for each squirrel exposed to the rattle playbacks. This ensured that each female in the rattle playback treatment was exposed to a unique combination of rattles (2 rattles recorded from males and 2 recorded from females). The methods for recording rattles and handling the sound files are discussed elsewhere (Goble, 2008;

Shonfield, 2010; Dantzer et al., in press). We only used rattle playbacks that were recorded at least 500 m away from our study area such that females likely only experienced rattles from unrelated or at least unfamiliar squirrels (dispersal from natal territory is often <100 m: Berteaux and Boutin, 2000). We used publicly available recordings of Boreal Chickadee vocalizations from various commercial (bird CDs) and other sources (www.xenocanto.org, The Cornell Lab of Ornithology Macauley Library) to develop 17 unique combinations of 4 chickadee vocalizations. Sound pressure levels of the chickadee and rattle vocalizations from each speaker were standardized to 100 dB from 1 m from the speaker using a digital sound pressure meter (Radio Shack Digital Sound Level Meter #33-2055).

Statistical analyses

We determined how variation in local population density affected FCM and FAM concentrations in data collected from 2006-2011 using three different methods. First, we conducted separate linear mixed-effects models (LMMs) to determine how local population density affected FCM or FAM concentrations using all the data we had collected. In each of these models, we included a fixed effect term for local population density (number of squirrels living within 150 m/total area). Second, we further investigated how local population density affected FCM and FAM concentrations in breeding female red squirrels at 10-day intervals from 0-100 days post-conception. We first calculated the Pearson's correlations between FCM or FAM concentrations and local population density within each of these 10-day intervals. After identifying the 10-day interval that exhibited the highest or near the highest Pearson correlation, we conducted two separate LMMs to determine how local population density affected FCM

and FAM concentrations during these two periods during reproduction. Third, in two separate LMMs, we determined how FCM and FAM concentrations differed between squirrels on the two control study areas and those on the food-addition study area in data collected from 2006-2011 by including a fixed effect term for treatment (food-addition or control). In all of these LMMs, we also included a linear term for days post-conception and, where necessary, a quadratic term for days post-conception (days post-conception²) as covariates to control for the effects of reproductive status because we have previously found that both FCM and FAM concentrations vary non-linearly from before and after conception, through pregnancy, and until weaning (Dantzer et al., 2010, 2011a).

We used LMMs to determine how FCM and FAM concentrations were affected by the rattle and chickadee playbacks in the perceived density manipulation experiment conducted in 2010. First, we determined whether there were any differences in FCM and FAM concentrations in females in both treatment groups prior to us starting the playbacks on their middens using two separate LMMs. In each model we included the fixed effects treatment (rattle or chickadee playbacks) and reproductive condition (3-level categorical variable: non-breeding, pregnant, or lactating). Next, we assessed how the playback treatments affected FCM and FAM concentrations during the period of exposure to the playbacks using two separate LMMs. In each of these models, we included fixed effect terms for the number of days after the playbacks started on which fecal samples were collected as well as a quadratic term for the number of days after the playbacks started (days after the playbacks started²) because of a significant non-linear relationship between days after the playbacks started and FCM and FAM

concentrations. In these four LMMs, we included reproductive status as a 3-level categorical variable (non-breeding, pregnant, lactating). We used this as a covariate to control for the effects of reproductive condition on FCM and FAM concentrations rather than days post-conception (as in the above LMMs) because days post-conception and days after playbacks started are collinear and also some females exposed to the rattle or chickadee playbacks died or experienced litter loss prior to parturition (from natural causes). The latter eliminated our ability to estimate their conception dates and thereby would have excluded them in our analyses if we used days post-conception. We included a treatment x days after playbacks started² interaction term to determine if the FCM and FAM concentrations were affected by the playback treatments as the period of exposure to the playbacks increased.

We first determined whether FCM and FAM concentrations differed between females exposed to the chickadee playbacks (n = 14 playbacks) and those exposed to no playbacks on the control study areas (n = 7 females) prior to determining how the rattle and chickadee playbacks and food-supplementation affected neonate mass, litter size, and offspring growth rates in the year (2010) in which we performed the playback experiments. We conducted two separate LMMs with ln-transformed FCM or FAM as the response variable and treatment (chickadee playbacks or no playbacks), days post-conception, and days post-conception² as fixed effect terms. Because we found that there were no statistically significant differences for either FCM or FAM concentrations between the chickadee and no playback treatment groups (see below), we lumped chickadee and no playback females into a control group of females (hereafter “control females”).

We used three separate LMMs to determine how neonate mass, litter size, and offspring growth rates varied among control females, those living on the food-addition study area, and those exposed to the rattle playbacks. In each LMM, we included a fixed effect term for treatment (3 level categorical variable: control, rattle playbacks, or food-addition) and Julian date of birth. In the LMM for the treatment effects on neonate mass, we included covariates for sex, litter size, an interaction term between sex and treatment (sex x treatment), and age of pups (age of neonates determined based upon their mass when they were accessed in their natal nest: Becker, 1993; Boutin and Larsen, 1993) as fixed effects. In the LMM for the treatment effects on offspring postnatal growth rates, we included fixed effect terms for sex of the pup, an interaction term between sex and treatment (sex x treatment), and an interaction term between litter size and treatment (litter size x treatment). We used a generalized linear mixed-effects model (GLMM; binomial errors, logit link) to determine if litter sex ratio varied among control females, those living on the food-addition study area, and those exposed to the rattle playbacks. In this GLMM, we included the fixed effects parturition date, litter size, treatment, and an interaction term between treatment and litter size. The dispersion parameter for this GLMM was 1.18.

We determined the association between FCM and FAM concentrations and offspring growth rates for females on the control and food-addition study areas in data collected from 2006-2011. In this model, the ln-transformed offspring postnatal growth rate was the response variable and we included fixed effect terms for FCM and FAM concentration as well as an interaction term between FCM and FAM (FCM x FAM). FCM and FAM concentrations were standardized to a mean of zero and unit variance

prior to analysis. In both models, we included covariates for white spruce cone production in the current and previous year, Julian parturition date, litter size at our first nest entry (~0 d post-parturition), days post-conception, and days post-conception².

All of our statistical analyses were conducted in R (version 2.14.1: R Development Core Team, 2008) and we used lme4 (version 0.999375-42: Bates et al., 2008) to conduct our LMMs and GLMMs. In these models, we included random intercept terms for squirrel identification because we either had repeated samples for our response variables on the same squirrels either within or across years or we had repeated estimates of litter parameters (e.g., litter size) for some females that produced a second litter after their first litter failed between 0-25 days post-parturition (Pinheiro and Bates, 2009). When we had repeated measures across years, we also included a random intercept term for year. We used diagnostic plots to confirm that the residuals from our statistical models were normally distributed, homoscedastic, and that there were no outlying observations with high leverage. We conducted preliminary analyses using generalized additive models (Hastie and Tibshirani, 1990) to investigate whether nonlinearities existed between our response and predictor variables. Quadratic terms were included when significant nonlinearities were documented (see above for which models included a quadratic term). For our LMMs, we used Markov Chain Monte Carlo methods (mcmcscamp function in lme4) to estimate the 95% credible intervals around the parameter estimates. Below we present mean $\pm$ standard error (SE) for all coefficients and present FCM and FAM concentrations as ln-transformed ng/g dry feces. Differences were considered to be statistically significant at $\alpha = 0.05$.

Results

Effects of local variation in population density on FCM and FAM concentrations

From 2006-2011 on both the control study areas and food-addition study area, there was considerable variation in local population density. Across these 6 years, the average local population density was 1.62 squirrels/ha but ranged from 0 to 4.25 squirrels/ha, with the latter being nearly the highest squirrel density ever observed in this region (Fig. 6.1). This within- and among-year variation in local population density had considerable effects on FCM and FAM concentrations. Overall, as local population density increased, both FCM (slope on ln scale = 0.14 ± 0.04 , 95% CI = 0.077 – 0.21, $t_{1301} = 3.7$, $P = 0.0001$, Table 6.1) and FAM concentrations significantly increased (slope on ln scale = 0.066 ± 0.03 , 95% CI = 0.0099 – 0.12, $t_{1311} = 2.05$, $P = 0.02$, Table 6.1). As we have found previously, both FCM and FAM varied non-linearly from before conception to weaning, peaking around parturition (FCM: slope on ln scale for days since conception² = $-6.2 \times 10^{-5} \pm 9.5 \times 10^{-6}$, 95% CI = -7.9×10^{-5} - -4.3×10^{-5} , $t_{1301} = -6.5$, $P < 0.0001$, Table 6.1) or juvenile emergence (FAM: slope on ln scale for days since conception² = $-4.2 \times 10^{-5} \pm 7.3 \times 10^{-6}$, 95% CI = -5.5×10^{-5} - -2.6×10^{-5} , $t_{1311} = -5.7$, $P < 0.0001$, Table 6.1).

We identified that the 10-day interval during the entire reproductive period of breeding females (from 0-100 days post-conception) that exhibited the highest or near the highest Pearson correlation between local population density and either FCM and FAM concentrations corresponded to around parturition for FCM (31-40 days post-conception, Fig. 6.3) or around the period of time when juveniles are first emerging from

Table 6.1. Results from linear mixed-effects models to determine how natural variation in population density affected fecal cortisol (FCM) and androgen metabolite (FAM) concentrations in breeding female red squirrels. 95% CI refers to 95% credible intervals around the parameter estimates.

Fecal Hormone Metabolite	Fixed Effect	Parameter $\pm$ SE	95% CI	<i>t</i>	df	<i>P</i>
FCM ^a	Intercept	6.85 $\pm$ 0.3	6.31 - 7.26	22.4 8	1301	<0.0001
	Days Post-conception	0.0008 $\pm$ 0.001	-0.001 - 0.0029	0.84	1301	0.2
	Days Post-conception ²	-6.2 $\times 10^{-5}$ $\pm$ 9.5 $\times 10^{-6}$	-7.9 $\times 10^{-5}$ - 4.3 $\times 10^{-5}$	-6.5	1301	<0.0001
	Population Density	0.014 $\pm$ 0.04	0.077 - 0.21	3.7	1301	0.0001
FAM ^a	Intercept	3.43 $\pm$ 0.17	3.09 - 3.76	20.1 5	1311	<0.0001
	Days Post-conception	0.008 $\pm$ 0.0008	0.006 - 0.009	10.6	1311	<0.0001
	Days Post-conception ²	-4.2 $\times 10^{-5}$ $\pm$ 7.3 $\times 10^{-6}$	-5.5 $\times 10^{-5}$ - 2.6 $\times 10^{-5}$	-5.7	1311	<0.0001
	Population Density	0.066 $\pm$ 0.03	0.0099 - 0.12	2.05	1311	0.02
FCM ^b	Intercept	8.57 $\pm$ 0.66	7 - 9.76	13.0 1	183	<0.0001
	Days Post-conception	-0.06 $\pm$ 0.017	-0.091 - -0.018	- 3.58	183	0.0002
	Population Density	0.32 $\pm$ 0.088	0.14 - 0.46	3.63	183	0.0002
FAM ^c	Intercept	2.69 $\pm$ 1.69	-0.63 - 5.97	1.59	95	0.057
	Days Post-conception	0.016 $\pm$ 0.023	-0.028 - 0.061	0.68	95	0.25
	Population Density	0.17 $\pm$ 0.076	0.0061 - 0.31	2.24	95	0.013

^aThese analyses are based upon fecal samples collected during all reproductive periods.

^bThese analyses are based upon only fecal samples collected around parturition.

^cThese analyses are based upon only fecal samples collected around juvenile emergence from their natal nest.

their natal nest for FAM (71-80 days post-conception, Fig. 6.3). We chose these periods of time because they also correspond to when FCM and FAM concentrations are at their highest during the entire reproductive period (Dantzer et al., 2010; Dantzer et al., 2011). Similarly to when we analyzed all of the data we collected, around these time periods we found that as local population density increased, both FCM (slope on ln scale = 0.32 ± 0.088 , 95% CI = 0.14 – 0.46, $t_{183} = 3.63$, $P = 0.0002$, Table 6.1, Fig. 6.4A) and FAM concentrations significantly increased (slope on ln scale = 0.17 ± 0.076 , 95% CI = 0.0061 – 0.31, $t_{95} = 2.24$, $P = 0.013$, Table 6.1, Fig. 6.4B). For example, around parturition, squirrels living under high-density conditions (>2 squirrels/ha; n = 55) had FAM concentrations that were 32.4% higher than those living under low-density conditions (<2 squirrels/ha; n = 129). Around the period of juvenile emergence, squirrels living under high-density conditions (n = 29) had FAM concentrations that were 27.9% higher than those living under low density conditions (n = 67).

Effects of experimentally increased actual population density on FCM and FAM concentrations

Population density (study area wide estimate) on the food-addition study area was generally higher than on the control study areas from 2006-2011 (Fig. 6.1). Females on the food-addition study area experiencing higher density had significantly higher FCM (n = 807, 6.8 ± 0.03 ng/g dry feces) and FAM concentrations (n = 814, 3.8 ± 0.03 ng/g dry feces) compared to the FCM (n = 577, 6.5 ± 0.03 ng/g dry feces $t_{1383} = 4.4$, $P < 0.0001$, Table 6.2, Fig. 6.5A) and FAM (n = 580, 3.63 ± 0.15 ng/g dry feces, $t_{1393} = 2.5$, $P = 0.007$, Table 6.2, Fig. 6.5B) concentrations from females on the lower density control study areas. Similar to the above, we found that in the population with

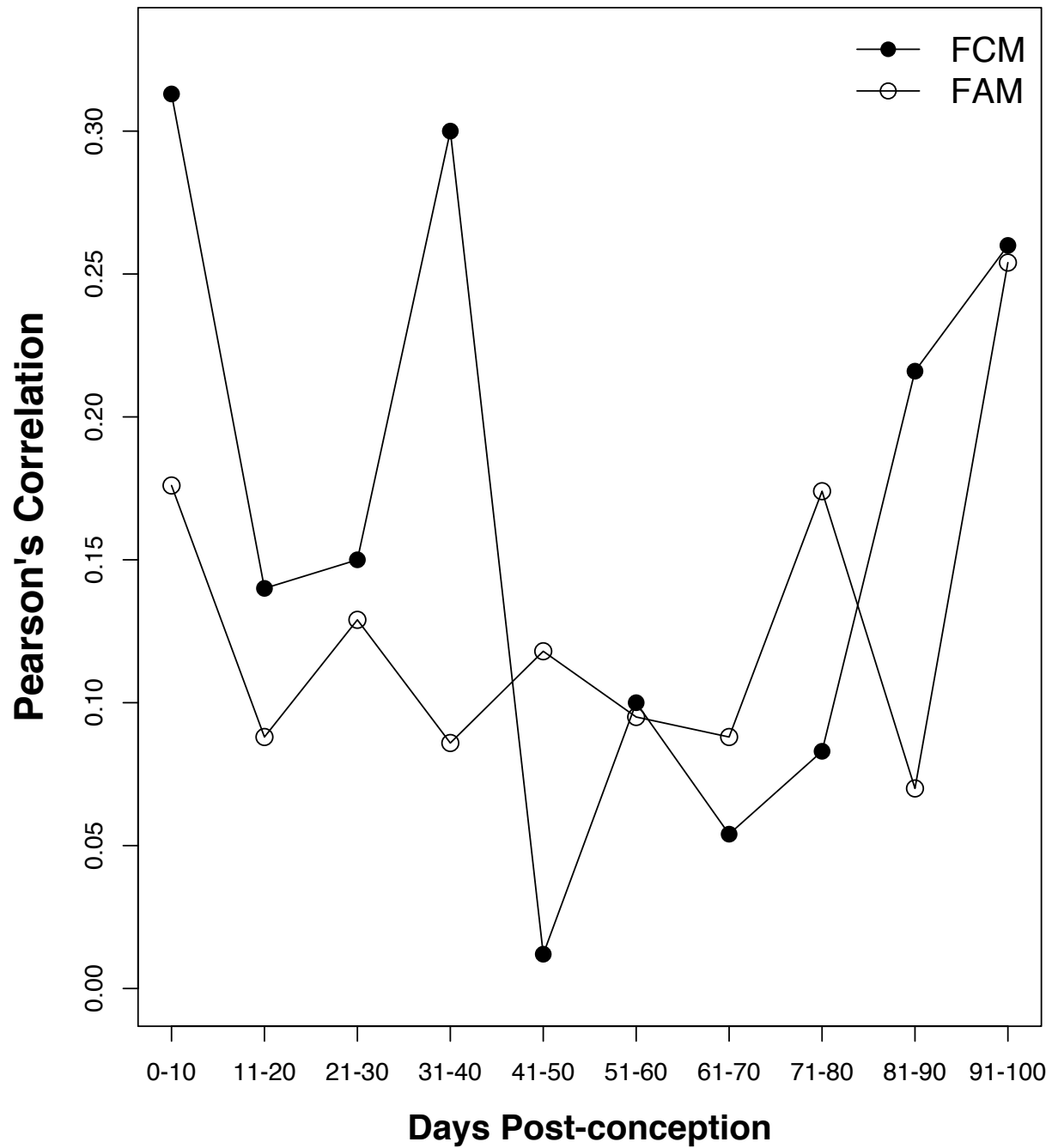


Figure 6.3. Pearson's correlations between local population density and either fecal cortisol (FCM) or fecal androgen (FAM) metabolite concentrations in pregnant and lactating female red squirrels at each of ten different 10-day intervals post-conception. Values on y-axis are absolute values of Pearson's correlations.

experimentally increased population density, both FCM (slope on ln scale for days since conception² = $-6.3 \times 10^{-5} \pm 9.3 \times 10^{-6}$, 95% CI = $-8.1 \times 10^{-5} - -4.4 \times 10^{-5}$, $t_{1383} = -6.8$, $P < 0.0001$, Table 6.2) and FAM (slope on ln scale for days since conception² = $-4.2 \times 10^{-5} \pm 7.2 \times 10^{-6}$, 95% CI = $-5.4 \times 10^{-5} - -2.6 \times 10^{-5}$, $t_{1393} = -5.9$, $P < 0.0001$, Table 6.2) varied non-linearly from before conception to weaning, peaking around parturition for FCM or juvenile emergence for FAM.

Effects of experimentally increased perceived population density on FCM and FAM concentrations

Our manipulations of perceived density had a similar effect on FCM and FAM concentrations in breeding female squirrels to that observed when actual population density was heightened either naturally or experimentally. Prior to starting the playbacks, females in the chickadee playback treatment group had FCM ($t_{186} = -1.21$, $P = 0.11$) and FAM ($t_{187} = 1.41$, $P = 0.08$) concentrations that were statistically indistinguishable from those females in the rattle playback treatment group (Fig. 6.6). After the playbacks were initiated on a squirrel's midden and as the period of exposure to the playbacks increased, FCM concentrations in pregnant and lactating females exposed to rattle playbacks declined at a slower rate than females exposed to chickadee playbacks (playback treatment x days after playbacks started², $t_{250} = 2.30$, $P = 0.011$, Table 6.3, Fig. 6.7A) such that FCM concentrations were higher in females exposed to the rattle playbacks and the magnitude of this effect increased with the number of days since the start of the playbacks (Fig. 6.7A). FAM concentrations during this period of exposure to

Table 6.2. Results from a linear mixed-effects model to determine how experimental increases in actual population density (using long-term food-addition) affected fecal cortisol and androgen metabolite concentrations in breeding female red squirrels on control and food-addition study areas. 95% CI refers to 95% credible intervals around the parameter estimates.

Fecal Hormone Metabolite	Fixed Effect	Parameter $\pm$ SE	95% CI	<i>t</i>	df	<i>P</i>
Fecal Cortisol Metabolites	Intercept	6.91 $\pm$ 0.3	6.42 - 7.35	23.01	1383	<0.0001
	Days Post-conception	0.0011 $\pm$ 0.0009	-0.0008 - 0.003	1.17	1383	0.12
	Days Post-conception ²	-6.3 $\times 10^{-5}$ $\pm$ 9.3 $\times 10^{-6}$	-8.1 $\times 10^{-5}$ - -4.4 $\times 10^{-5}$	-6.8	1383	<0.0001
	Food-add Study Area	0.29 $\pm$ 0.06	0.17 - 0.39	4.4	1383	<0.0001
Fecal Androgen Metabolites	Intercept	3.45 $\pm$ 0.16	3.14 - 3.74	21.84	1393	<0.0001
	Days Post-conception	0.008 $\pm$ 0.0007	0.006 - 0.009	11.1	1393	<0.0001
	Days Post-conception ²	-4.2 $\times 10^{-5}$ $\pm$ 7.2 $\times 10^{-6}$	-5.4 $\times 10^{-5}$ - -2.6 $\times 10^{-5}$	-5.8	1393	<0.0001
	Food-add Study Area	0.14 $\pm$ 0.05	0.05 - 0.23	2.5	1393	0.007

the playbacks in those females exposed to rattle playbacks increased, whereas those in females exposed to the chickadee playbacks declined (playback treatment x days after playbacks started², $t_{251} = 3.98$, $P < 0.0001$, Table 6.3, Fig. 6.7B). Consequently, the perceived density manipulation had persistent effects on FCM and FAM concentrations, as females that were exposed to rattle playbacks continued to have higher FCM and FAM concentrations than those exposed to the chickadee playbacks even after the playbacks ended on their midden (~34 days after playbacks started: Fig. 6.7A and 6.7B).

Breeding females exposed to chickadee playbacks ($n = 20$) had FCM ($t_{184} = -0.57$, $P = 0.15$) and FAM ($t_{188} = 1.35$, $P = 0.09$) concentrations that were statistically indistinguishable from those exposed to no playbacks ($n = 8$ females). In addition, females exposed to chickadee playbacks and those exposed to no playbacks exhibited a statistically similar pattern throughout the reproductive period from prior to conception and until weaning for both FCM (playback treatment x days since conception², $t_{184} = 0.4$, $P = 0.28$) and FAM (playback treatment x days since conception², $t_{188} = -0.08$, $P = 0.53$) concentrations. Therefore, in the analyses below for the effects of the rattle and chickadee playbacks on female reproductive traits and offspring postnatal growth rates, we considered females exposed to the chickadee playbacks and those exposed to no playbacks as a single control group ("control females" hereafter).

Effects of experimentally increased perceived density on neonate mass, litter size, and litter sex ratio

We recorded neonate mass, litter size, and litter sex ratio in litters produced by control females (i.e., females exposed to no or chickadee playbacks, $n = 29$ litters from

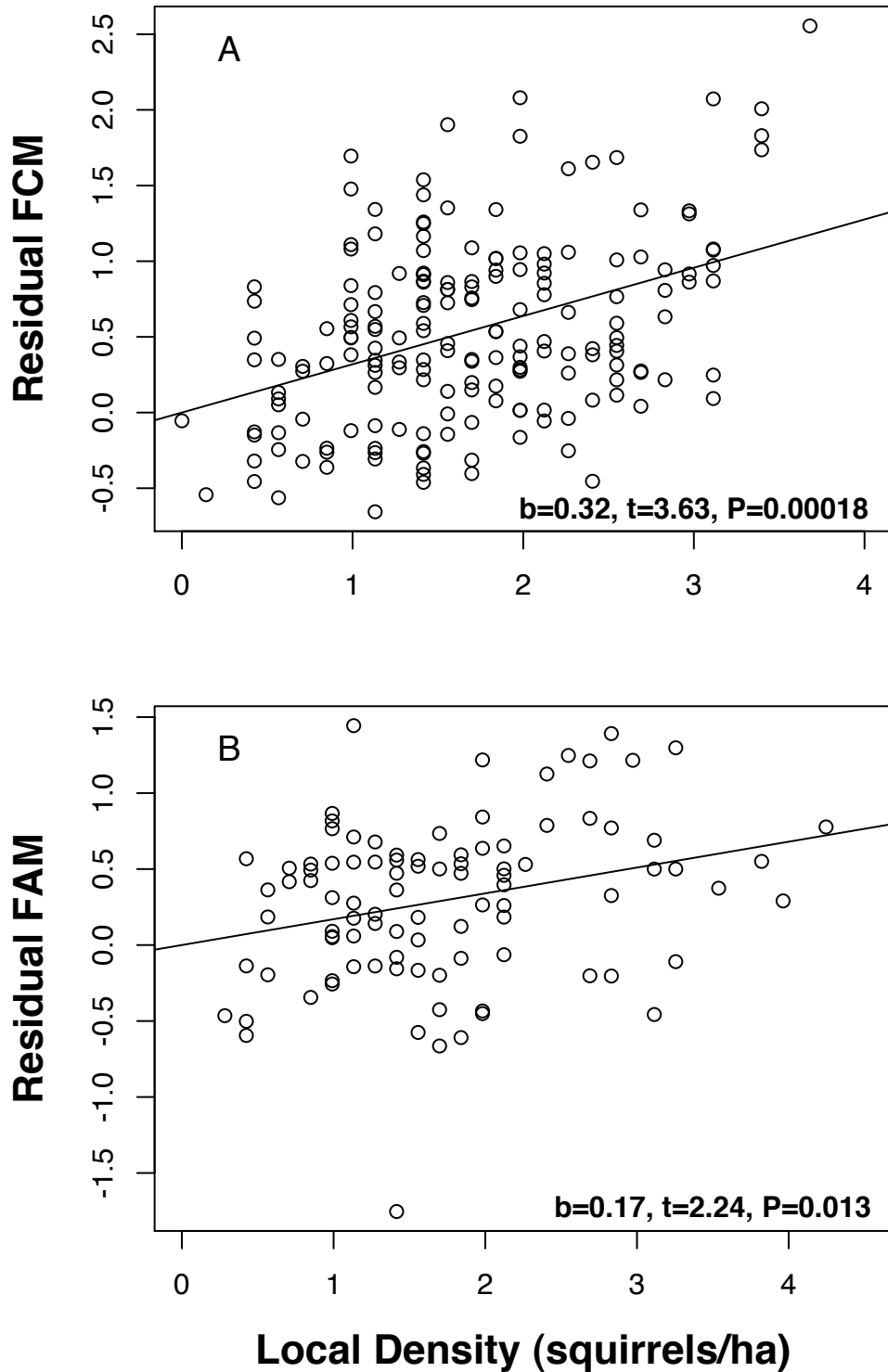


Figure 6.4. A) Fecal cortisol metabolite concentrations (FCM) measured in samples collected around parturition and B) fecal androgen metabolite concentrations (FAM) measured around the period of time when juveniles first emerge from their natal nest were significantly positively associated with local population density (squirrels/hectare) as measured from 2006-2011 on four different study areas. Values on y-axis represent standardized residual FCM and FAM from linear mixed-effects models.

28 females), females exposed to rattle playbacks ($n = 37$ litters produced by 36 females), and females on the food-addition study area ($n = 41$ litters produced by 36 females). In the year in which we performed the manipulations of perceived population density, neonate mass declined significantly for pups born later in the year (slope for advancing Julian parturition date on $\ln$ scale = -0.001 ± 0.00037 , 95% CI = $-0.0012 - 0.00022$, $t_{408} = -2.69$, $P = 0.0037$, Table 6.4) and males tended to be larger than females ($t_{408} = 1.72$, $P = 0.043$, Table 6.4). Neonate mass of pups produced by control females did not differ from those whose mothers were exposed to the rattle playbacks ($t_{408} = -0.47$, $P = 0.32$) or those whose mothers lived on the food-addition study area ($t_{408} = -1.19$, $P = 0.11$, Table 6.4). Neonate mass of pups produced by females exposed to the rattle playbacks did not differ from those whose mothers were on the food-addition study area ($t_{408} = -0.75$, $P = 0.77$). Litter size at the first nest entry (~ 0 days post-parturition) among control females, rattle playback females, or food-addition females was similar ($P > 0.19$ for all comparisons, Table 6.4). There were no significant differences in the sex ratio of litters produced by females on the food-addition study area compared to those litters produced by control females or those exposed to the rattle playbacks (Table 6.5). Overall, litters produced by rattle playback females tended to have more male offspring than those produced by control females, but this effect was not significant ($z = 1.89$, $P = 0.058$, Table 6.5). As litter size increased, litters produced by rattle playback females also tended to have fewer male offspring than those produced

Table 6.3. Results from linear mixed-effects models to determine how playback treatment (rattle or chickadee playbacks) and reproductive condition (non-breeding, pregnant, or lactating) affected fecal cortisol (FCM) and fecal androgen (FAM) metabolite concentrations in pregnant and lactating female red squirrels. Non-breeding and pregnant females refers to a 3-level categorical variable (non-breeding, pregnant, lactating). A 2-level categorical variable was included for playback treatment (Rattle and chickadee playbacks). Days after playbacks started refers to either a linear or quadratic term for the number of days after playbacks were initiated. 95% CI refers to 95% credible intervals around parameter estimates.

Fecal Hormone Metabolite	Fixed Effect	Parameter $\pm$ SE	95% CI	<i>t</i>	df	<i>P</i>
FCM	Intercept	6.5 $\pm$ 0.17	6.2 - 6.84	38.8	250	<0.0001
	Non-breeding Females	0.42 $\pm$ 0.32	-0.15 - 1.06	1.3	250	0.097
	Pregnant Females	0.44 $\pm$ 0.12	0.2 - 0.67	3.6	250	0.0001
	Days after Playbacks Started	0.015 $\pm$ 0.007	0.0001 - 0.029	2.08	250	0.019
	Days after Playbacks Started ²	-0.0003 $\pm$ 0.0001	-0.0005 - -0.00009	-2.96	250	0.0016
	Rattle Playbacks	-0.06 $\pm$ 0.11	-0.27 - 0.15	-0.53	250	0.29
	Rattle Treatment x Days after Playbacks Started ²	0.00015 $\pm$ 0.00006	0.00002 - 0.00027	2.3	250	0.011
FAM	Intercept	3.26 $\pm$ 0.13	3.39 - 3.88	28.23	251	<0.0001
	Non-breeding Females	-0.18 $\pm$ 0.25	-0.6 - 0.33	-0.47	251	0.32
	Pregnant Females	0.2 $\pm$ 0.093	0.017 - 0.38	2.19	251	0.014
	Days after Playbacks Started	0.01 $\pm$ 0.0056	-0.0017 - 0.021	1.81	251	0.036
	Days after Playbacks Started ²	-0.00017 $\pm$ 0.00008	-0.00034 - -4.3 $\times 10^{-8}$	-2.01	251	0.023
	Rattle Playbacks	0.073 $\pm$ 0.09	-0.09 - 0.23	0.81	251	0.21
	Rattle Playbacks x Days after Playbacks Started ²	0.00019 $\pm$ 0.00004	9.3 $\times 10^{-5}$ - 0.00028	3.98	251	<0.0001

by control females, but this interaction was not statistically significant ($z = -1.71$, $P = 0.086$, Table 6.5).

Effects of perceived density on offspring postnatal growth rates

In this year, 35% of all the litters produced by control, rattle playback, or food-addition females that we were following experienced complete litter loss sometime between ~0-25 days post-parturition. Consequently, we recorded the rate of postnatal growth of offspring produced by control females ($n = 65$ pups from 19 females), females exposed to rattle playbacks ($n = 71$ pups produced by 20 females), and females on the food-addition study area ($n = 75$ pups produced by 20 females).

Offspring growth rates declined significantly for pups that were born later in the year (slope for advancing Julian parturition date = -0.0048 ± 0.0016 , 95% CI = $-0.0066 - -0.0029$, $t_{210} = -3.02$, $P = 0.0014$). There were no sex differences in growth rates of offspring produced by control females ($t_{210} = -0.25$, $P = 0.4$, Table 6.4), food-addition females (food-supplementation x sex of pup, $t_{210} = 0.76$, $P = 0.22$, Table 6.4), and females exposed to rattle playbacks (rattle playbacks x sex of pup, $t_{210} = 0.63$, $P = 0.26$, Table 6.4). The growth rates of individual offspring declined significantly as litter size increased for those pups produced by control females (slope for effect of litter size on $\ln$ scale = -0.19 ± 0.049 , 95% CI = $-0.25 - -0.13$, $t_{210} = -3.97$, $P < 0.0001$, Table 6.4, Fig. 6.8). However, the negative effect of increasing litter size on the growth rates of individual offspring was attenuated for those pups produced by females experiencing high food and density conditions, although this effect was not statistically significant (food-supplementation x litter size, $t_{210} = 1.43$, $P = 0.077$, Table 6.4, Fig. 6.8). For

offspring produced by females exposed to the rattle playbacks there was a similar, but significant attenuating effect of high perceived density on the negative effects of larger litters on offspring growth rates (playback x litter size, $t_{210} = 1.86$, $P = 0.032$, Table 6.4, Fig. 6.8). As such, pups produced by females experiencing experimentally heightened actual (food-addition study area) or perceived (rattle playbacks) density grew faster than those produced by control females (Fig. 6.8).

Effects of FCM and FAM on Offspring Postnatal Growth Rates

Across 6 years of study (2006-2011), high cone production in the autumn of the previous year did not influence offspring growth rate (slope on ln scale = 0.01 ± 0.02 , 95% CI = $-0.013 - 0.045$, $t_{864} = 0.55$, $P = 0.29$, Table 6.6) but high cone production in the autumn of the current year was associated with significantly lower offspring growth rates (slope on ln scale = -0.13 ± 0.02 , 95% CI = $-0.077 - -0.0066$, $t_{864} = -5.8$, $P < 0.0001$, Table 6.6). Increasing litter size was associated with significantly lower offspring growth rates (slope on ln scale = -0.11 ± 0.007 , 95% CI = $-0.13 - -0.097$, $t_{864} = -14.2$, $P < 0.0001$, Table 6.6) whereas females that bred later in the year had significantly higher offspring growth rates (slope for advancing Julian parturition date on ln scale = 0.0008 ± 0.0002 , 95% CI = $-0.00006 - 0.0001$, $t_{864} = 3.2$, $P = 0.0004$, Table 6.6).

From 2006-2011, there was a significant interaction between FCM and FAM concentrations and postnatal growth rates (FCM x FAM slope on ln scale = 0.0075 ± 0.003 , 95% CI = $0.0014 - 0.018$, $t_{864} = 2.17$, $P = 0.015$, Table 6.6, Fig. 6.9). This

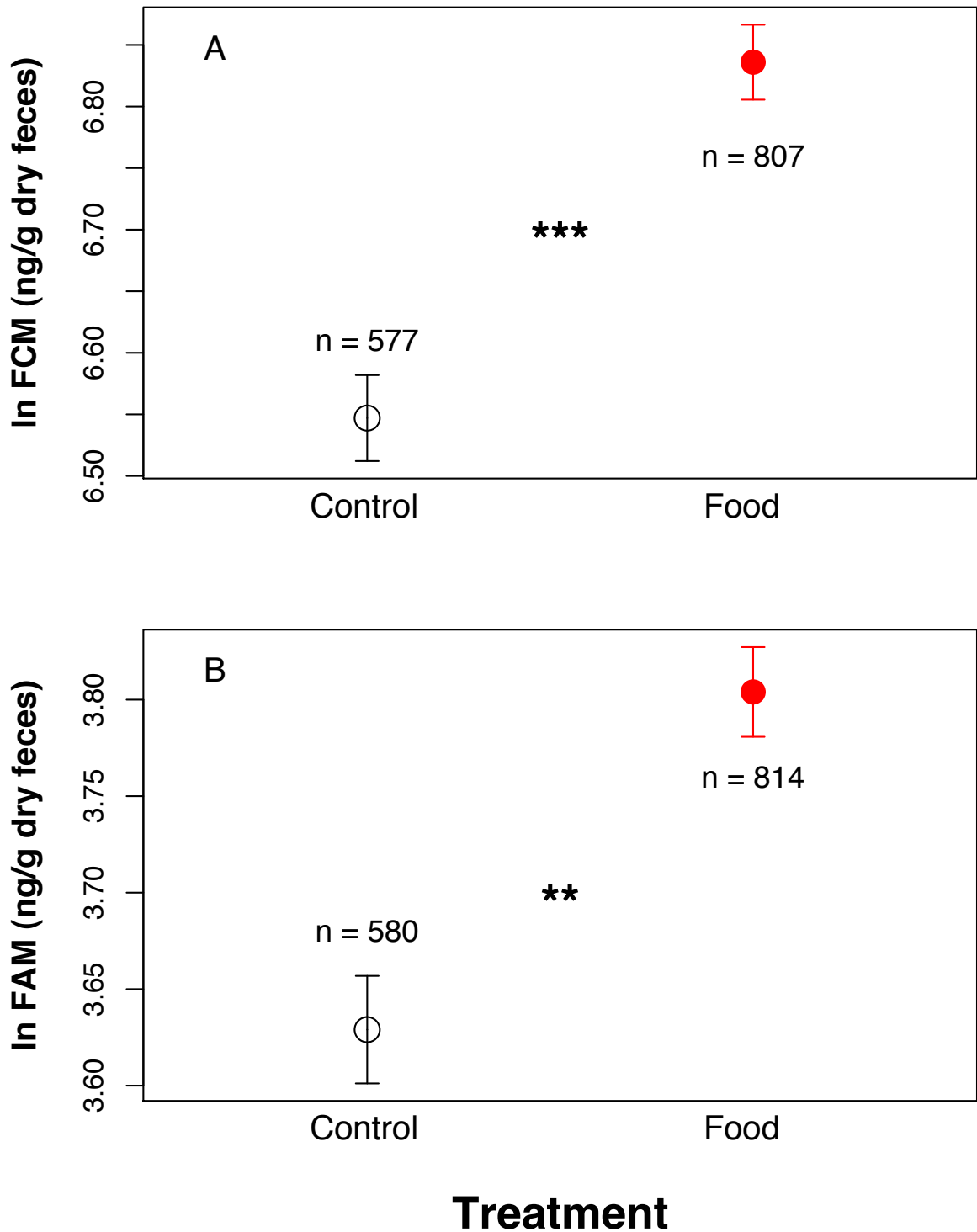


Figure 6.5. Female squirrels on the two food-addition study areas with significantly higher population density had significantly higher fecal cortisol (A) and androgen (B) metabolite concentrations than those on two control study areas as measured from 2006-2011. Sample sizes refer to the number of fecal samples analyzed. Significant differences are noted by “***” ($P < 0.001$) and “**” ($P < 0.01$). Raw values are shown on y-axis on a ln scale.

positive interaction meant that the positive effects of either FCM (slope on ln scale = 0.0015 ± 0.006 , 95% CI = $-0.003 - 0.026$, $t_{864} = 0.25$, $P = 0.40$, Table 6.6, Fig. 6.9) or FAM (slope on ln scale = 0.007 ± 0.005 , 95% CI = $-0.0024 - 0.026$, $t_{864} = 1.34$, $P = 0.09$, Table 6.6, Fig. 6.9) were accentuated by higher values of the other hormone. Mothers with high concentrations of both FCM and FAM, therefore, raised offspring with the highest postnatal growth rates (Table 6.6, Fig. 6.9).

Discussion

We investigated whether high-density conditions induced adaptive maternal effects on offspring growth rates, and also whether these maternal effects are mediated by an endocrine mechanism. We found that female red squirrels adaptively increased offspring postnatal growth rates in response to the perception of increased competitive density. Breeding females experiencing naturally or experimentally heightened density, as well as those experiencing heightened perceived density, had higher FCM and FAM concentrations than females experiencing lower density conditions. These endocrine responses to population density induced adaptive maternal effects on offspring growth rates, as we found that heightened FCM and FAM concentrations synergistically elevated offspring postnatal growth rates. This study suggests that the endocrine responses of breeding females to perceived competitive intensity (number of territorial vocalizations heard) induces an adaptive endocrine-mediated maternal effect on offspring phenotype.

Table 6.4. Results from linear mixed-effects models to determine how increased perceived (rattle playbacks) or actual (using long-term food-addition) population density affected neonate mass, litter size, and offspring growth rates in breeding female red squirrels compared to control females. A 2-level categorical variable was included for sex of offspring (male, female), food-addition (on or off food-addition study area), and playback treatment (Rattle and chickadee playbacks). 95% CI refers to 95% credible intervals around the parameter estimates.

Response Variable	Fixed Effect	Parameter $\pm$ SE	95% CI	<i>t</i>	df	<i>P</i>
Neonate Mass	Intercept	2.44 $\pm$ 0.08	2.29 - 2.57	29.1	408	<0.0001
	Parturition Date	-0.0009 $\pm$ 0.00037	-0.0012 - 0.00026	-2.53	408	0.0058
	Male Pup	0.026 $\pm$ 0.015	-0.02 - 0.063	1.72	408	0.043
	Estimated Age	0.097 $\pm$ 0.0039	0.09 - 0.1	24.5	408	<0.0001
	Litter Size	0.009 $\pm$ 0.014	-0.019 - 0.018	0.68	408	0.24
	Food-add	-0.045 $\pm$ 0.04	-0.093 - 0.016	-1.14	408	0.13
	Rattle Playbacks	-0.021 $\pm$ 0.039	-0.087 - 0.022	-0.51	408	0.3
	Male Pup x Food-add	-0.0075 $\pm$ 0.02	-0.051 - 0.059	-0.37	408	0.35
	Male Pup x Rattle Playbacks	-0.0002 $\pm$ 0.021	-0.045 - 0.068	-0.01	408	0.49
Litter Size	Intercept	1.32 $\pm$ 0.16	1.32 - 1.93	7.95	105	<0.0001
	Parturition Date	0.0003 $\pm$ 0.001	-0.0023 - 0.0018	0.28	105	0.39
	Food-add	-0.044 $\pm$ 0.076	-0.15 - 0.071	-0.58	105	0.28
	Rattle Playbacks	-0.066 $\pm$ 0.076	-0.15 - 0.052	-0.88	105	0.19
Offspring Growth Rate	Intercept	1.88 $\pm$ 0.27	1.52 - 2.19	6.96	210	<0.0001
	Parturition Date	-0.0048 $\pm$ 0.0016	-0.0066 - -0.0029	-3.02	210	0.0014
	Male Pup	-0.0072 $\pm$ 0.028	-0.073 - 0.092	-0.25	210	0.4
	Litter Size	-0.19 $\pm$ 0.049	-0.25 - -0.13	-3.97	210	<0.0001
	Male Pup x Food-add	0.032 $\pm$ 0.042	-0.11 - 0.12	0.76	210	0.22
	Male Pup x Rattle Playbacks	0.024 $\pm$ 0.039	-0.087 - 0.14	0.63	210	0.26
	Lit. Size x Food-add	0.097 $\pm$ 0.068	0.0073 - 0.18	1.43	210	0.076
	Lit. Size x Rattle Playbacks	0.15 $\pm$ 0.079	0.051 - 0.24	1.86	210	0.032

Effects of population density and food abundance on FCM and FAM concentrations

Similar to many other organisms, red squirrels experience fluctuations in food availability that generates variation in population density. This coupling of food abundance and population density can make it difficult to disentangle the effects of food abundance on hormone concentrations from those of population density. Previous studies in vertebrates have focused on understanding how food abundance or population density independently of one another is associated with circulating concentrations of plasma glucocorticoids (GCs: Christian, 1961; Kitaysky et al., 1999; Rogovin et al., 2003; McCormick, 2006) and androgens (Wingfield et al., 1990; Muller and Wrangham, 1994; Wikelski et al., 1999; Cavigelli and Pereira, 2000; Demas et al., 2007). We found a significant positive relationship between local population density and FCM and FAM concentrations. However, because food availability tends to co-vary with population density (Fig. 6.1), these changes could also be attributed to inter-annual variation in food abundance. As a result, we performed two manipulations of population density to determine how variation in population density and food abundance affect FCM and FAM concentrations.

Since 2004, we have provided red squirrels on up to three different study areas in the Yukon with supplemental food to simulate high food conditions, which has caused an increase in population density (Fig. 6.1). Previous studies performing temporary food-supplementation experiments in wild animals have found that experimental increases in food abundance are associated with increases (Schoech et al., 2004; Ruiz et al., 2010) or no significant effects (Nunes et al., 2000, 2002; Jackson and Bernard,

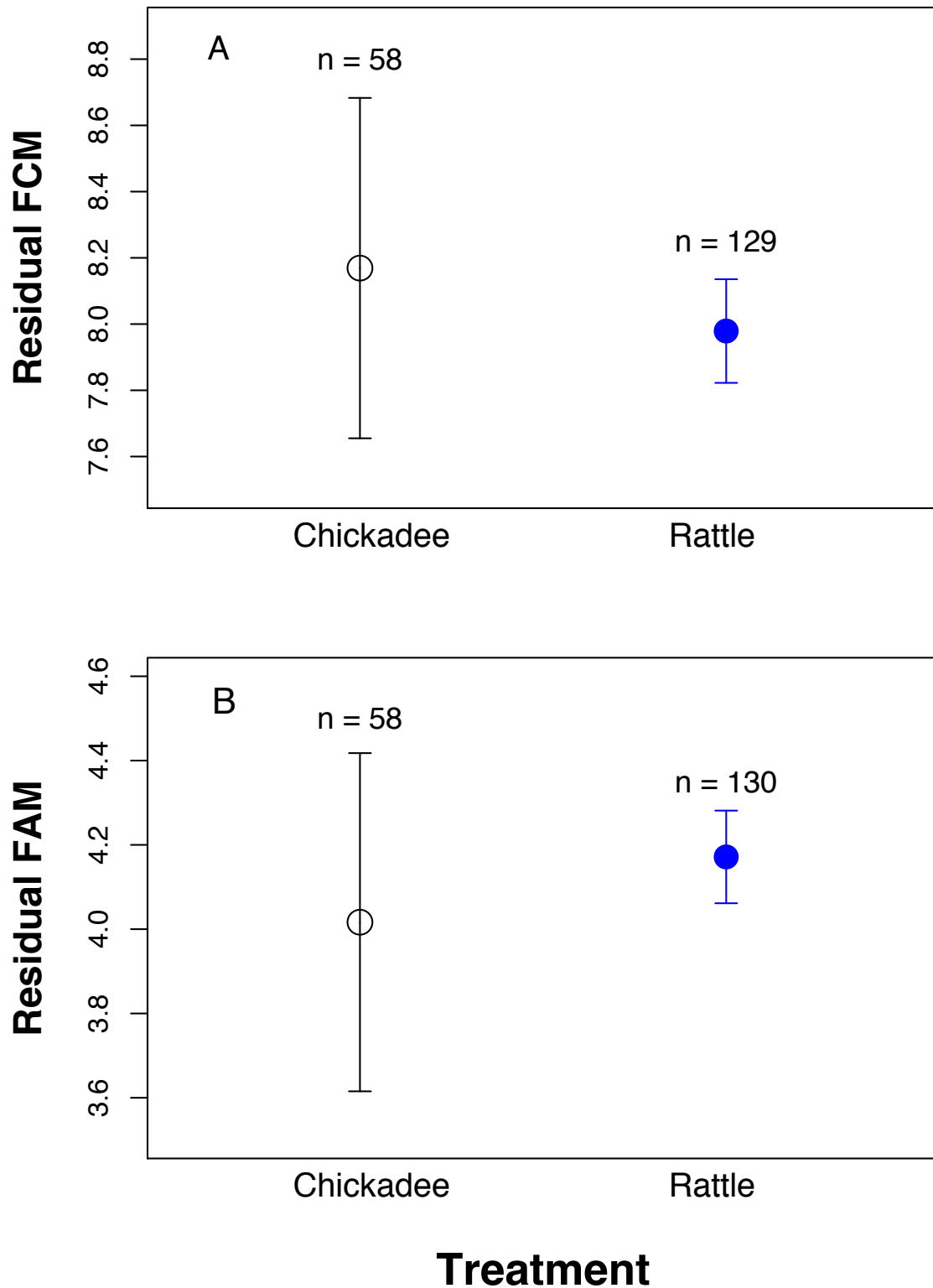


Figure 6.6. Prior to exposure to the playbacks, fecal cortisol (FCM) and fecal androgen (FAM) levels did not differ between those females that were exposed to rattle or chickadee playbacks. Sample sizes represent the number of fecal samples analyzed. Values on y-axis correspond to residuals from linear mixed-effects models.

2005) on plasma glucocorticoid (GC) or androgen concentrations. In this study, we found that squirrels receiving supplemental food during reproduction had significantly higher FCM and FAM concentrations. Although diet may have a considerable effect on fecal hormone metabolite concentrations (Wasser et al., 1993; von der Ohe and Servheen, 2002), we have previously documented that the differences in FCM and FAM concentrations between squirrels on unmanipulated study areas and those provided with supplemental peanut butter were not due to the effects of their different diets (Dantzer et al., 2011*b*). In fact, we have found previously that squirrels fed peanut butter have progressively lower FCM and FAM concentrations over time than those fed white spruce cones (Dantzer et al., 2011*b*). This suggests that the elevated FCM and FAM concentrations of those squirrels on the food-addition study area are actually biased downwardly due to their consumption of peanut butter.

Squirrels on the food-addition study area also experienced much higher density conditions than did those on the unmanipulated control study areas (Fig. 6.1). Increased population density can lead to an increased frequency of antagonistic interactions, which can lead to increased production of gonadal androgens (Wingfield et al., 1990; Muller and Wrangham, 2004; Demas et al., 2007) or increased GCs (Christian, 1961; McCormick, 2006). We have previously found that the frequency with which squirrels physically interact with one another is not affected by population density, but the frequency with which they emit territorial vocalizations, or interact acoustically, is positively associated with population density (Dantzer et al., in press). In this study, we found that breeding females experiencing heightened perceived density (rattle playbacks) had significantly higher FCM and FAM concentrations than those exposed to

Table 6.5. Results from a generalized linear mixed-effects model to determine how experimental increases in perceived (using rattle playbacks) or actual population density affected litter sex ratio compared to control females.

Fixed Effect	Parameter $\pm$ SE	<i>z</i>	<i>P</i>
Intercept	-1.16 $\pm$ 0.99	-1.17	0.24
Parturition Date	-0.0017 $\pm$ 0.0048	-0.36	0.72
Litter Size	0.29 $\pm$ 0.18	1.55	0.12
Food-add	1.42 $\pm$ 1.13	1.25	0.21
Rattle Playbacks	2.35 $\pm$ 1.24	1.89	0.058
Lit. Size x Food-add	-0.23 $\pm$ 0.26	-0.86	0.39
Lit. Size x Rattle Playbacks	-0.51 $\pm$ 0.29	-1.71	0.086

control (chickadee) playbacks. This suggests that the frequency with which squirrels hear territorial vocalizations induces changes in circulating concentrations of GCs and androgens. Importantly, our experiment manipulated population density without the confounding effects of food abundance, which indicates that population density and not food abundance induced these hormonal changes.

Effects of FCM and FAM on offspring growth rates

Increased circulating concentrations of GCs and androgens can generate variation in early hormone exposure, which can have profound effects on offspring phenotype. We found that the heightened FCM and FAM concentrations in pregnant and lactating females exhibited under high density conditions were together associated with increased offspring growth rates. Because high offspring growth rates are favored by natural selection under high-density conditions (McAdam et al., in prep), the hormonal responses of females to variation in population density we observed are associated with adaptive changes in offspring growth rates. This indicates that variation in population density induces an adaptive endocrine-mediated maternal effect on offspring growth rates.

The mechanism by which these elevated maternal FCM or FAM concentrations affected offspring growth rates is unknown and could be through an effect exerted either during the pre- or postnatal period. Elevated prenatal exposure to GCs (Lesage et al., 2001; Welberg and Seckl, 2001; Drake et al., 2004) and androgens (Schwabl, 1996; Mannikkam et al., 2004; Groothuis et al., 2005; Crespi et al., 2006) is associated with intrauterine growth restriction but an increased rate of postnatal or catch-up growth. One mechanism by which elevated prenatal exposure to GCs and androgens increases

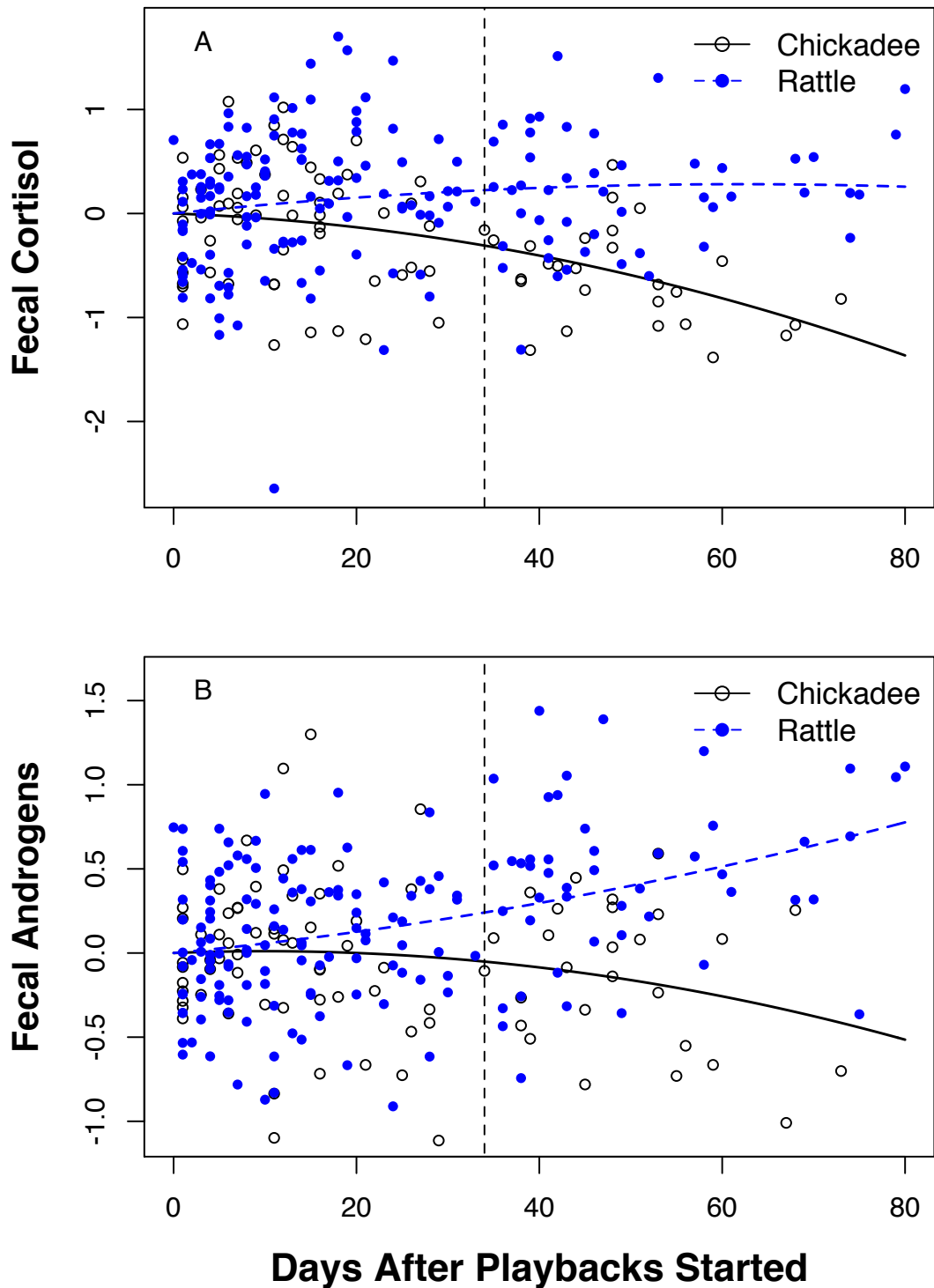


Figure 6.7. Breeding female squirrels exposed to rattle playbacks had significantly higher fecal cortisol (A) and androgen (B) metabolite concentrations than those exposed to chickadee (control) playbacks during the period of exposure to the playbacks. Individual squirrels were exposed to playbacks on their territories for an average of 34 days (indicated by two vertical dashed lines). Values on y-axis represent standardized residuals from linear mixed-effects models (see text).

offspring postnatal growth rates is their effects on offspring behavior and specifically the type and amount of maternal care and nursing they solicit (e.g., Moore and Power, 1986). For example, offspring exposed to heightened GCs early in life often exhibit altered hypothalamic-pituitary-adrenal axis functioning such that they have elevated concentrations of baseline or stress-induced GCs (Welberg and Seckl, 2001). GCs have an appetitive effect that can motivate foraging or feeding (Dallman et al., 2007), which could alter the begging or nursing intensity of offspring and cause them to grow at higher rate after birth. For example, in nestling birds, experimental increases in plasma GCs were associated with increased begging behavior thereby increasing the rate of postnatal growth (Kitaysky et al., 2001). Similarly, experimental increases in nestling testosterone concentrations are associated with increased begging (Goodship and Buchanan, 2007). Unfortunately we are not able to observe mother-offspring interactions and cannot measure relationships between maternal FCM and FAM concentrations and offspring begging behavior.

These changes in offspring postnatal growth rates could be due to the organizational effects of early exposure to androgens on the somatotrophic axis. The somatotrophic axis releases the polypeptide metabolic hormone insulin-like growth factor-1 (IGF-1), which can increase rates of cell growth, proliferation, and survival in structural tissues (reviewed by Dantzer and Swanson, 2012). A previous study found that elevated early exposure to androgens is associated with increased IGF-1 concentrations and postnatal growth (Crespi et al., 2006). Consequently, one hypothesis by which elevated FAM concentrations in females were positively associated with offspring growth rates is through their enhancing effects on IGF-1 concentrations in

Table 6.6. Results from a linear mixed-effects model to determine how food availability (previous year cones and current year cones), litter parameters (litter size, parturition date), and fecal cortisol (FCM) and fecal androgen metabolite (FAM) concentrations in breeding female red squirrels affected offspring postnatal growth rates. Days after playbacks started refers to either a linear or quadratic term for the number of days after playbacks were initiated. 95% CI refers to 95% credible intervals around the parameter estimates.

Fixed Effect	Parameter $\pm$ SE	95% CI	<i>t</i>	df	<i>P</i>
Intercept	1.01 $\pm$ 0.103	0.76 - 1.04	9.82	864	<0.0001
Previous Year Cones	0.01 $\pm$ 0.02	-0.013 - 0.045	0.55	864	0.29
Current Year Cones	-0.13 $\pm$ 0.02	-0.077 - -0.0066	-5.8	864	<0.0001
Litter Size	-0.11 $\pm$ 0.007	-0.13 - -0.097	-14.2	864	<0.0001
Parturition Date	0.0008 $\pm$ 0.0002	-0.00006 - 0.0001	3.2	864	0.0007
Days Post-conception	-0.0003 $\pm$ 0.0002	-0.0014 - -0.0001	-1.46	864	0.072
Days Post-conception ²	3.2 $\times 10^{-7}$ $\pm$ 2.2 $\times 10^{-6}$	-2.1 $\times 10^{-6}$ - 8.8 $\times 10^{-6}$	0.14	864	0.56
FCM	0.0015 $\pm$ 0.006	-0.003 - 0.026	0.25	864	0.4
FAM	0.007 $\pm$ 0.005	-0.0024 - 0.026	1.34	864	0.09
FCM x FAM	0.0075 $\pm$ 0.003	0.0014 - 0.018	2.17	864	0.015

offspring during this period of postnatal growth, but this hypothesis has not yet been tested.

Alternatively, the positive association between maternal FCM and FAM concentrations and offspring postnatal growth rates could be due to how elevated GCs and androgens affect maternal behavior. We found that the average mass of neonates produced by females exposed to the rattle playbacks was no different than the neonate mass of females exposed to the chickadee playbacks. Laboratory studies show that elevated prenatal exposure to GCs can decrease neonate mass (see above). Our lack of confirmation of this finding could be because the elevated levels of maternal androgens compensated for any negative effects of elevated maternal GCs on neonate mass or it could suggest that the elevated maternal GCs and androgens only affect offspring growth rates via a postnatal effect. Elevated maternal GCs and androgens could also influence offspring postnatal growth rates through their effects on maternal behavior. For example, lactating female laboratory rats with experimentally elevated plasma GCs lick, groom, and nurse their offspring more than control females during the early period after birth (2-11 days post-parturition: Rees et al., 2004; Casolini et al., 2007; but see Casolini et al., 1997; Brummelte et al., 2006). This increased nursing and licking and grooming could increase offspring postnatal growth rates through the enhancing effects maternal presence has on growth hormone secretion in offspring (Kuhn et al., 1990). However, we cannot distinguish the exact mechanism by which these elevated maternal GCs and androgens affected offspring postnatal growth rates.

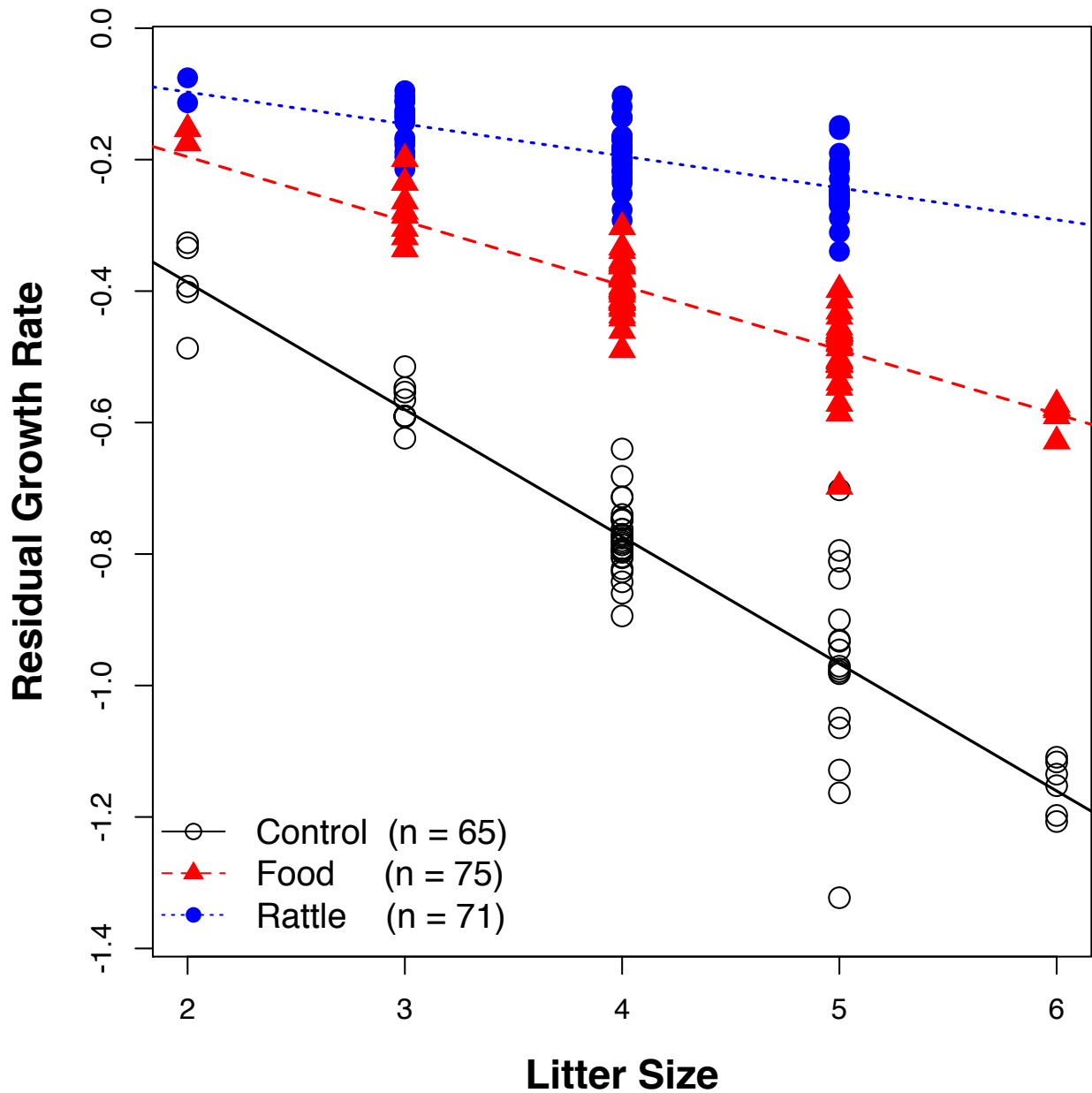


Figure 6.8. Breeding female squirrels exposed to rattle playbacks ($n = 20$ females) produced offspring that grew significantly faster than those whose mothers were exposed to chickadee or no playbacks ($n = 19$). Females on the food-addition study area ($n = 20$) produced offspring that grew faster than those exposed to chickadee or no playbacks but at a similar rate as those exposed to the rattle playbacks. Each point represents an individual pup. Values on y-axis represent standardized residuals from a linear mixed-effects model (see text).

Experimental induction of adaptive endocrine-mediated maternal effects

The growth rates of juvenile red squirrels experience large heritable maternal effects and have been found to be under density-dependent natural selection (McAdam et al., 2002, 2003, in prep). In this study, we found that female red squirrels exposed to heightened perceived population density adaptively increased offspring growth rates in anticipation of increased competitive density that their offspring were to encounter. This suggests that the number of territorial vocalizations breeding female red squirrels hear in their local neighborhood induces adaptive maternal effects on offspring growth rates. In addition, our results from our manipulation of perceived population density in which we experimentally increased maternal FCM and FAM concentrations confirms our finding that increased FCM and FAM concentrations are associated with adaptive increases in offspring growth rates. This also suggests that the maternal effects on offspring growth rates that we document here and elsewhere (McAdam et al., 2002, 2004) may be mediated in part by endocrine mechanisms.

Our study suggests natural selection may have favored the evolution of plasticity in maternal effects on offspring growth rates where females adaptively increase offspring growth rates under high-density conditions. However, offspring are not passive recipients of maternal effects and natural selection should also act on offspring to buffer themselves from potentially harmful (or 'selfish') maternal effects (Marshall and Uller, 2007). Although the growth enhancing influence of these endocrine-mediated maternal effects may be adaptive for both mothers and their offspring in the short-term by increasing maternal relative fitness and the probability that offspring will survive their first winter, they may have costly long-term effects on offspring. For example, we have

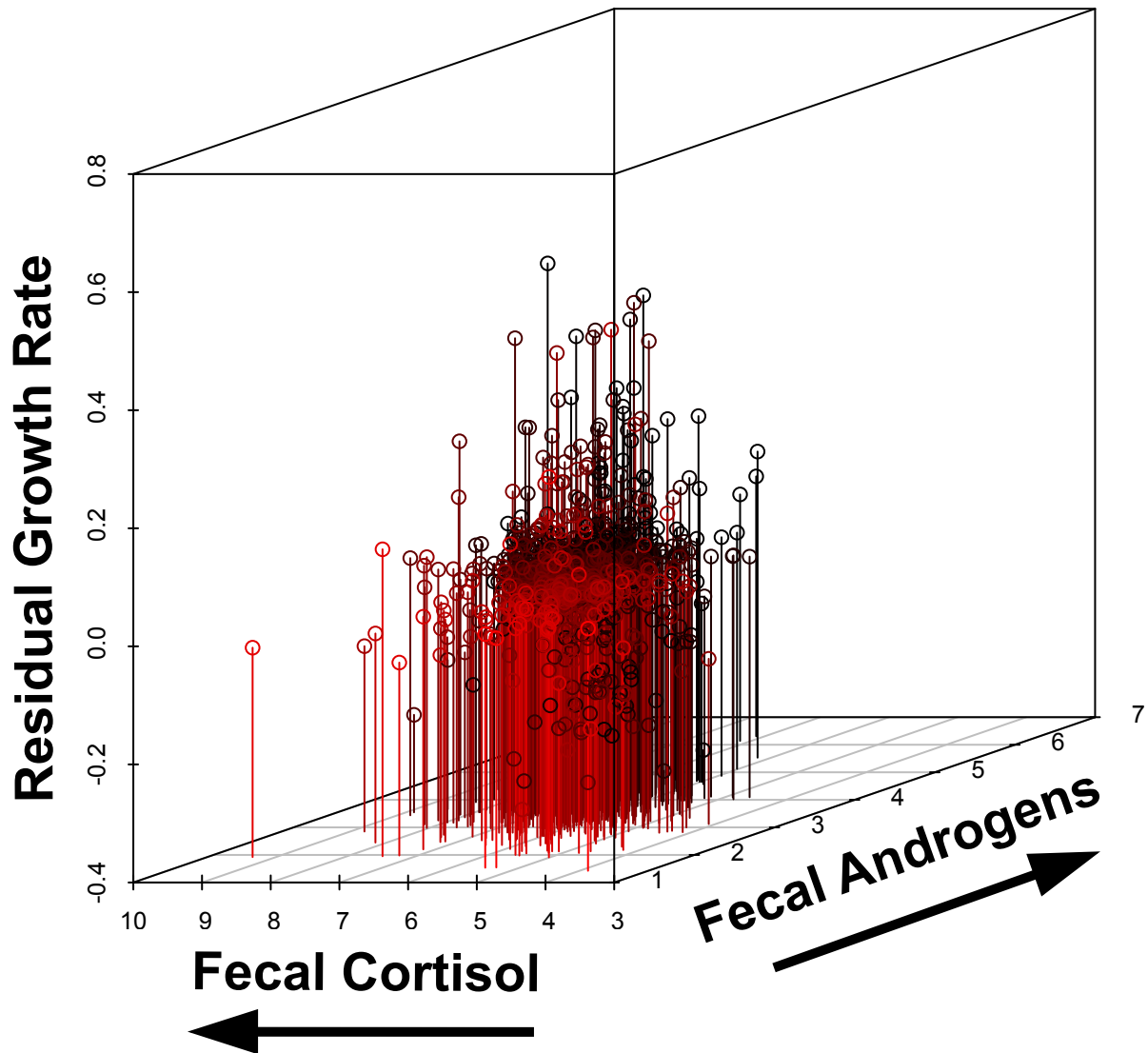


Figure 6.9. Across six years of study, female squirrels ($n = 151$) with heightened concentrations of both fecal androgen (FAM) and cortisol (FCM) metabolites during pregnancy and lactation produced offspring with significantly higher postnatal growth rates. Values on y-axis are standardized residuals from a linear-mixed effects model (see text) and FCM and FAM concentrations are on a $\ln$ scale (but model was run with centered variables). Different colored points are used to emphasize the 3-dimensional relationship and do not have any other significance.

previously found that offspring born in high-density years have a shortened lifespan (Descamps et al., 2008). This suggests that an adaptive maternal effect on offspring growth rates that is mediated through heightened early exposure to GCs and androgens may be costly for offspring in the long-term. Future studies investigating maternal effects in wild animals should focus on examining their adaptive value for mothers and their offspring in both the short- and long-term.

Finally, our study further clarifies how females can use ecological and social information to make adaptive reproductive decisions. In the year that we performed the playback experiments, females exposed to heightened perceived density produced offspring that grew at rates similar to those of offspring of females on the food-addition high density study area. This suggests that the perception of heightened competitive density is what induces growth-enhancing maternal effects on offspring growth rates and not necessarily variation in food abundance. However, this is not terribly surprising given that we have previously found in Yukon red squirrels that food abundance does not limit litter size (Humphries and Boutin, 2000) and that squirrels anticipate upcoming food pulses by adaptively increasing total offspring production (Boutin et al., 2006). Our study further emphasizes that variation in reproductive output by females is not determined purely by resource availability, but instead reflects adaptive adjustments to the changes in natural selection that result from fluctuations in food resources.

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CHAPTER 7

Concluding Remarks and Future Directions

In this dissertation, I have validated enzyme-immunoassays to measure fecal cortisol (FCM) and androgen (FAM) metabolite concentrations (Chapters 2-3), examined how reproductive condition affected FCM and FAM concentrations (Chapters 2-3), and examined associations between maternal behavior and FAM concentrations (Chapter 3). I have also shown that slight differences in the diets of red squirrels can induce substantial and important changes in FCM and FAM concentrations (Chapter 4). I showed that natural and experimental variation in population density can induce changes in red squirrel behavioral patterns and the frequency with which they emit territorial vocalizations (Chapter 5). Finally, I have shown that endocrine responses of female red squirrels to natural and experimental variation in actual or perceived population density may induce adaptive endocrine-mediated maternal effects on offspring growth rates (Chapter 6). Below I outline a few topics for future research.

Are maternal effects on offspring growth rates plastic and is this plasticity heritable?

Variable environments can favor the evolution of adaptive phenotypic plasticity (Levins, 1968) and maternal effects are a form of transgenerational phenotypic plasticity that may allow mothers to induce adaptive changes in offspring phenotype according to the prevailing environmental conditions (Bernardo, 1996; Mousseau and Fox, 1998; Wolf et al., 1998; Agrawal et al., 1999; Galloway, 2005). For example, maternal effects that influence body size or post-natal growth have been frequently documented in natural populations. During a resource pulse in one year, a maternal effect could produce many smaller offspring. However, in a second year in which there was a lack of

resources, the same maternal effect could produce fewer but larger offspring. Thus, the maternal effect on body size is plastic.

A major focus of recent studies on maternal effects parallels the above hypothetical example. Plasticity in maternal effects can adaptively modify offspring phenotype to the environment the offspring will encounter. In order for this transgenerational phenotypic plasticity to evolve via natural selection, it must be genetically based. However, few studies have actually documented the presence of additive genetic variation underlying maternal effects (Räsänen and Kruuk, 2007) and even fewer have examined whether there is a genetic component to maternal effect plasticity (Wilson et al., 2006; Räsänen and Kruuk, 2007). As a result, our understanding of how maternal effects and transgenerational phenotypic plasticity can affect evolutionary dynamics is limited.

We have previously documented that offspring growth rates in red squirrels are plastic in response to environmental variation (McAdam and Boutin, 2003*b*; Boutin et al., 2006), and experience genetically based maternal effects (McAdam et al., 2002) that accelerate the response to selection (McAdam and Boutin, 2004). Because they live in a variable environment in which there are annual fluctuations in food abundance and population density and selection on offspring growth rate is density-dependent (McAdam and Boutin, 2003*a*), selection may favor the evolution of plasticity in the maternal effects that influence offspring growth rates.

In Chapter 6 of this dissertation, I showed that female red squirrels could adaptively increase offspring growth rates in response to increased perceived population density. However, a complementary study to my own would be to

experimentally decrease actual or perceived density to examine if females adaptively decrease offspring growth rates. Using this approach, the actual level of plasticity in maternal effects on offspring growth rates could be investigated.

Are Maternal Hormone Levels Heritable and Is there Plasticity in Maternal Hormone Levels across a Gradient of Population Density?

A major focus of recent studies in evolutionary endocrinology (Zera et al., 2007) has been the role of the neuroendocrine system in facilitating and constraining adaptation to novel environments (Adkins-Regan, 2008). Hormones have pleiotropic effects on morphological and behavioral characters and the effects of hormones on correlated traits have been widely discussed (Ketterson and Nolan, 1992; Sinervo and Svensson, 1998; Ricklefs and Wikelski, 2002). Because the neuroendocrine system is a highly integrated suite of physiological traits, it is thought to play a major role in enabling or restricting multi-trait phenotypic responses to selection such as when organisms colonize new environments or face novel environmental variation (Ketterson and Nolan, 1999; Hau, 2007; McGlothlin and Ketterson, 2008; Ketterson et al., 2009). Selection on one independent endocrine trait (e.g., hormone level) can cause a multifaceted correlated response in other endocrine traits such that the whole endocrine system evolves as one integrated unit (Adkins-Regan, 2008; McGlothlin and Ketterson, 2008). In order for the endocrine system to affect adaptive evolution, however, there must be inter-individual variation in endocrine traits and some of this variation must be genetically based. Most evidence in laboratory or captive populations suggests the presence of additive genetic variation in endocrine traits (Satterlee and Johnson, 1988; Odeh et al., 2003; King et al., 2004) and that the endocrine system is responsive to

artificial selection (Carere et al., 2003; Evans et al., 2006). However, in natural populations in which natural selection can be documented, we know surprisingly little about inter-individual variation in neuroendocrine responses to environmental variation and the genetic basis of endocrine traits themselves or plasticity in endocrine traits (Kempenaers et al., 2008; Lessells, 2008; Williams, 2008).

The neuroendocrine response of breeding females to environmental variation in particular could be important for adaptation to new environments through the programming effects of early hormone exposure (Dufty et al., 2002; Groothuis et al., 2005; Lancaster et al., 2007). Variation in early hormone exposure caused by the neuroendocrine response to environmental stimuli can induce a highly integrated and complex suite of neural, physiological, morphological, and behavioral changes in offspring (Clark and Galef, 1995; Welberg and Seckl, 2001; Groothuis et al., 2005; Maestripieri and Mateo, 2009). Variation in early hormone exposure through plasticity in the neuroendocrine response to recurrent environmental stimuli could enable transgenerational adaptive phenotypic plasticity. For example, in oviparous species, prenatal exposure to androgens is associated with heightened growth and overall competitive ability (Groothuis et al., 2005). Plasticity in the androgen response of females to environmental variation would be favored if highly competitive phenotypes produced by prenatal exposure to androgens are favored in the environmental conditions that elicit increased levels in androgens but not in other environments. The plastic hormonal response of females to environmental variation could, therefore, allow them to match offspring phenotype to their environment.

If changes in offspring phenotype induced by early exposure to glucocorticoids or androgens are adaptive, selection could operate on inter-individual variation in the maternal hormonal response to the environment or in the sensitivity of offspring to the hormonal signals. Although I will not be able to address how selection acts on the sensitivity of offspring to hormonal signals, if some of the inter-individual variation in hormonal responses to environmental variation is genetically based, selection can generate an evolutionary response. However, in natural populations, we know very little about inter-individual variation in the plasticity of hormonal responses to the environment and whether any of this variation in plasticity is genetically based (Lessells, 2008).

In red squirrels, we know that there are genetically based maternal effects that influence offspring growth rates (McAdam et al., 2002; McAdam and Boutin, 2003a). My dissertation research suggests that these maternal effects on growth rates may be mediated by an endocrine mechanism that is induced by variation in local population density. Future studies should investigate whether maternal hormone levels exhibit plasticity in response to variation in population density and whether this plasticity is heritable.

How do Females Manipulate Offspring Growth Rates?

In Chapter 6, I found that the endocrine responses of female red squirrels to increased population density were associated with adaptive increases in offspring postnatal growth rates. Increased early exposure to glucocorticoids (GCs) or androgens is often associated with decreased intrauterine growth but increased postnatal growth rates. This could be due to the enhancing effects of heightened early exposure to GCs

or androgens on offspring begging behavior (see Chapter 6). As a result, the positive association between maternal FCM and FAM and offspring postnatal growth rates that I found in Chapter 6 could be due to the effects that these hormones have on the begging or nursing intensity of juvenile red squirrels. Alternatively, these effects of early exposure to GCs and androgens could influence the somatotrophic axis such that rates of cell growth, proliferation, and survival of structural tissues such as bone and muscle are elevated in those offspring whose mothers had elevated FCM and FAM concentrations.

In Chapter 6, I focused on some of the presumed endocrine mechanisms that mediate these increased offspring growth rates. However, in Chapter 5, I found that population density also affected the behavior of both male and female red squirrels. Squirrels experiencing naturally or experimentally increased actual or perceived density conditions spent less time in the nest and feeding but more time being active and vigilant. As a result, these alterations in maternal behavior that we observed under high-density conditions may contribute directly to the increased offspring growth rates.

In this dissertation, I focused on manipulating the actual ecological agents (vocal cues reflecting population density) that induce hormonal changes associated with adaptive increases in offspring growth rates. This approach is in contrast to many laboratory studies that manipulate the putative endocrine agents that may mediate these maternal effects rather. However, as Chapters 5 and 6 demonstrate, my manipulations of actual and perceived population density were associated with both changes in maternal behavior and FCM and FAM concentrations. Consequently, it is difficult to definitively state that the maternal effects on offspring growth rates are

mediated by either hormone individually. Future studies should experimentally manipulate androgen and GC levels individually to determine how they affect maternal behavior as well as female life history characteristics (neonate mass, litter size, offspring growth rates). These studies will help to determine if heightened maternal GCs or androgens are causally associated with these adaptive changes in offspring growth rates.

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