THE ENIGMA OF THE SNOWSHOE HARE POPULATION CYCLE:
EXPLAINING THE LOW PHASE THROUGH STRESS AND MATERNAL
PROGRAMMING

by

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ABSTRACT

The snowshoe hare cycle has been fundamental to the development of ecological theory for more than half a century. Though these cycles have been intensively studied for over 70 years it is still unknown what mechanism causes young born in the decline to have much lower fitness than those born in the increase and peak phase and why the hare population remains low for 2-5 years after the decline phase even though the predator populations have collapsed and there is ample vegetation. My doctoral studies investigated how the risk of predation affected maternal stress and whether this would result directly in a decline in reproduction and indirectly, through maternal effects, in a decline in offspring physiology and fitness.

To study the impact of predator-induced maternal stress I used a natural monitoring study and an experimental manipulation. In the former, I examined hares throughout the cycle and found that their stress levels were directly related to predator pressure, being greatest during the decline. During the low phase stress levels remained elevated at levels similar to those found at the peak when reproduction starts to decline. Lastly, I found that the variation in the length of the low phase (2-5 years) was related to the rate of loss of hares during the decline phase. In the experimental manipulation, pregnant hares were exposed to a simulated predator for 1 min. every other day for the last third of gestation. I found that an increase in predator-induced maternal stress resulted in a decline in litter size, birth weight, and birth size. Furthermore, these offspring had a compromised stress-axis resulting in higher baseline stress levels and an enhanced stress response. This occurred both at weaning and when the offspring were adult size.
My results show that hares are highly sensitive to predation risk and that maternal stress results in a decrease in reproduction and also compromises their offspring’s stress physiology. These results support the hypothesis that the low phase of the population cycle is the result of the impact of inter-generationally inherited maternal stress caused by the high risk of predation during the decline.
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<th>Description</th>
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<tbody>
<tr>
<td>11, 17-DOA</td>
<td>11, 17-dioxoandrostanes</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>CBG</td>
<td>Corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>FCM</td>
<td>Fecal cortisol metabolites</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptors</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MCBC</td>
<td>Maximum corticosteroid-binding capacity</td>
</tr>
<tr>
<td>MI</td>
<td>Mass index</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralcorticoid receptors</td>
</tr>
<tr>
<td>N:L ratio</td>
<td>Neutrophil to lymphocyte ratio</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PCV</td>
<td>Packed-red-blood cell volume</td>
</tr>
<tr>
<td>pnd</td>
<td>Post natal day</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RHF</td>
<td>Right hind foot</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
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CO-AUTHORSHIP STATEMENT

The manuscripts presented in chapters 2 through 6 contain two to three authors in addition to myself. I devised the general outline and objectives of this thesis, and was the primary contributor to the experimental design, the collection and analysis, and the manuscript preparation. R. Boonstra and C.J. Krebs provided supervision to all components of the thesis, helped design all experiments, and provided useful criticism to all manuscripts. Additionally co-author C.O. Bosson (Chapter 2) made important secondary contributions.
1. INTRODUCTION

In this thesis I will be discussing the role of stress in the life of the snowshoe hare – how it affects them as individuals and how this translates into changes at the population level. The 10-year population cycle of snowshoe hares and their predators represents a classic problem in population ecology. We understand a great deal of what is driving this cycle, but unknowns remain and I intend to shed light on some of these unknowns related to key aspects of the cycle. I will first review the stress axis in detail, then one of the enigmas that remains in the cycle, and finally the experiments and studies I carried out to deepen our understanding of this astounding phenomenon.

1.1 Overview of stress

Exposure to direct (e.g. a severe storm) and indirect (e.g. risk of predation) environmental stressors constitute major selective forces in natural populations. Adaptations to these stressors are essential for enhancing individual fitness, and animals have evolved behavioural and physiological strategies to avoid the deleterious effects of such stressors. One of the most conserved processes in vertebrates is the ‘stress response’ which activates the hypothalamus-pituitary-adrenal (HPA) axis and subsequent secretion of glucocorticoids (GC). Throughout this thesis I will use three terms which need clarification: ‘stressors’ are any environmental disturbance that disrupts homeostasis, ‘stress response’ is the hormonal response an animal has to a stressor, and ‘stress’ is the combined basal and response level of stress hormones (e.g. an increase in maternal stress equates to an increase in both the basal GC levels and the ability to hormonally respond
to a stressor. Short-term, acute, stressors result in elevated GC levels that redirect animals into distinct emergency life history states (Wingfield et al. 1998) aimed at coping with the perturbation and recovering homeostasis (defined here as the internal constancy that an animal attempts to maintain). However, when the stressors become chronic (long-term) these responses may ultimately come at the cost of long-term survival, growth, body condition, or reproduction.

The environment experienced by a mother may also affect her offspring’s fitness. Mothers influence the development of their offspring genetically, by passing on their genes, but also through maternal effects – the effect a mother’s phenotype has on her offspring’s phenotype that cannot be solely ascribed to inherited genes. Maternal effects can cause a resemblance not just between a mother and her offspring but between grandmothers and grand-offspring (Kirkpatrick and Lande 1989; Mousseau and Fox 1998). In rats, offspring that are born to high licking-grooming mothers (i.e. dams that naturally exhibit increase pup licking-grooming in the absence of any experimental manipulation) themselves show high licking-grooming behaviour at adulthood compared with those that are born to low licking-grooming mothers (Liu et al. 1997; Meaney 2001). Cross-fostering the biological offspring of high and low licking-grooming mothers reverses the phenotype, suggesting a direct relationship between variations in maternal care and the development of these behaviours rather than an affect of genes.

The mechanisms responsible for maternal effects may vary between organisms and in mammals, stress hormones may play a vital role. The offspring born to high licking-grooming mothers show enhanced HPA axis sensitivity and more modest stress responses, whereas those born to low licking-grooming mothers show the opposite
(Caldji et al. 1998; Francis et al. 1999). However, reversing the effect on the HPA axis eliminates the influence of early life experience and modifies the offspring’s behaviour at adulthood (Weaver et al. 2004).

Maternal effects may affect not just individuals but entire populations and simple mathematical models have shown how maternal effects may be major drivers of cyclic population dynamics (Ginzburg and Taneyhill 1994; Ginzburg 1998; Inchausti and Ginzburg 1998). Thus maternal effects, through the inheritance of the effects stress, may play a substantial role in the population dynamics of free-ranging mammals.

1.2 The physiology of the stress response

The stress response is primarily mediated by the HPA axis (de Kloet et al. 2005; Owen et al. 2005). Within minutes of the onset of a stressor, the adrenal cortex begins to secrete GCs (Romero and Romero 2002). This reaction originates within the neurons of the paraventricular nucleus (PVN) of the hypothalamus that release corticotropin-releasing hormone (CRH), and arginine vasopressin (AVP). CRH and AVP stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn mediates the release of GCs (cortisol in guinea pigs, sheep, snowshoe hares, and humans; corticosterone in rats and mice) from the adrenal cortex (de Kloet et al. 1998, 2005; Wingfield and Sapolsky 2003; Owen et al. 2005; Fig. 1.1).

During a stress response, elevated GC levels (as well as catecholamines) increase catabolism including the mobilization of glucose reserves and the break-down of triglycerides and proteins into free fatty acids and amino acids. Combined with an increase in cardiovascular tone, the availability and diversion of energy to exercising
muscles is elevated. Hormones involved with the stress response also cause anxiety-likeehaviours and vigilance (Munck et al. 1984; McEwen 1998; Francis and Meaney 1999;
Sapolsky et al. 2000; Seckl 2004; Abe et al. 2007; Meaney et al. 2007). Furthermore,
these hormones suppress costly anabolism by disrupting immune and inflammatory
responses, growth, and reproduction (Munck et al. 1984; Ferin 1999; Sapolsky et al.
2000). Although these responses are highly adaptive in the face of an acute stressor,
chronic activation of the HPA axis and increases in basal cortisol can cause a variety of
illnesses ranging from hyperlipidemia, hypertension, chronic immunosuppression and
decreases in viral resistance, in states of anxiety and in depression, and finally a decrease
in reproduction (McEwen 1998; Ferin 1999; Francis and Meaney 1999).

1.3 A brief overview of reproduction and the impact of stress

At the endocrine level, reproduction is controlled by the hypothalamic-pituitary-
gonadal (HPG) axis. Hypothalamic secretion of gonadotropin-releasing hormone (GnRH)
stimulates the pituitary to release the gonadotropins luteinizing hormone (LH) and
follicle stimulation hormone (FSH). GnRH release is not tonic but rather pulsatile,
resulting in a pulsatile secretion of the gonadotropins. This has been shown to be
necessary for gonadal function and reproductive success (Belchetz et al. 1978; Veldhuis
1990). Gonadotropin stimulation of the gonads subsequently results in gametogenesis and
the synthesis of gonadal steroid and peptide hormones that then feed back to the
hypothalamus and pituitary to regulate FSH and LH secretion (Shupnik 1996). Under
non-stress conditions, hormones of the HPA axis enhance reproduction and may play
critical roles in managing energy income and use during reproduction (Sapolsky et al.
2000). However, chronic activation of the HPA axis causes physiological and behavioural suppression of the HPG axis (Ferin 1999; Sapolsky et al. 2000; Wingfield and Sapolsky 2003). In mammals, the effects of stress on reproduction differ between the sexes and here I will discuss reproduction only in females as they are the important sex in terms of maternal effects.

Activation of the HPA axis involves the release of CRH from the hypothalamus (de Kloet et al. 1998). Although the normal release of CRH activates many components of the HPG axis, when stress-induced its release causes an immediate decrease in pulsatile GnRH and LH secretion (Olster and Ferin 1987; Petraglia et al. 1987) resulting in a general inhibition of the HPG axis (Ferin 1999; Sapolsky et al. 2000). B-endorphine, released from the pituitary, also has inhibitory effects upon the release of GnRH from the hypothalamus. This results in a decline and irregularity of GnRH levels in the hypophysial-pituitary portal system within seconds (Ching 1983; Ferin 1999). In addition GCs cause a decreased sensitivity of pituitary gonadotropes to the stimulatory effects of GnRH (Suter and Schwartz 1985). Together this results in a net decline of LH released from the pituitary. GCs also decrease the number of LH receptors on the ovaries, thus decreasing their responsiveness to LH (Negro-Vilar 1993; Sapolsky et al. 2000). Overall, stress-induced activation of the HPA axis results in an extended, highly irregular reproductive cycle.

Stress-induced secretions of prolactin from the pituitary also antagonize the effects of progesterone in the uterus causing a decline in progesterone levels (Negro-Vilar 1993). Progesterone is necessary for maturation of the uterine wall for implantation during the luteal phase of the reproduction cycle.
Lastly, stress can cause a decrease in proceptive and receptive female behaviours necessary for sex (Wingfield and Sapolsky 2003). This probably reflects the fact that oestrogen promotes both these behaviours and stress-induced declines in oestrogen levels, via changes in LH levels and sensitivity, may contribute to loss of libido. Furthermore, suppression via immune-system mediated GC secretion may also dampen female libido (Wingfield and Sapolsky 2003).

1.4 Maternal effects

There is strong evidence that early life exposure to steroid hormones may be a mechanism responsible for maternal effects in mammals. An increase in maternal GC concentrations has been shown to influence fetal development and cause structural and functional changes that persist throughout life (Meaney 2001, Seckl 2004). In the biomedical literature, these maternal effects are referred to as maternal programming, and that is the way I will refer to this concept here. Programming effects reflect the influence of a specific environmental factor during the developmental period before or just after birth on the organization of target tissues and/or gene-expression patterns that affect function throughout life (Meaney et al. 2007). In the laboratory, pre-natal exposure to GC permanently alters the hippocampus and the HPA axis through changes in mineralcorticoid receptor (MR) levels and glucocorticoid receptor (GR) levels (Henry et al. 1994; Maccari et al. 1995; Liu et al. 2001; Kapoor et al. 2008). Densities of these two hippocampal receptors are critical in regulation and feedback of the HPA axis, with MRs regulating basal glucocorticoid levels and GRs regulating glucocorticoid levels in response to stressors (de Kloet et al. 2005; Owen et al. 2005). Pregnant guinea pigs
treated with a synthetic glucocorticoid resulted in offspring that had increased hippocampal MR expression and reduced basal plasma cortisol concentrations (Liu et al. 2001). Furthermore, exposure of pregnant guinea pigs to a strobe light resulted in offspring with reduced GR expression and an elevated activity of the HPA axis (Kapoor et al. 2008). Rat offspring born to mothers that were restrained during the last week of gestation had reduced GR densities (Henry et al. 1994; Maccari et al. 1995), enhanced responsiveness to stressors (Meaney 2001), and higher basal plasma corticosterone levels as adults (Liu et al. 1997; Francis et al. 1999; Welberg et al. 2001, Welberg and Seckl 2001). It has also been shown that post-natal handling of rat pups causes an increase in hippocampal GR levels, leading to a lower HPA responsiveness to stress and lower basal plasma GC levels throughout life (i.e. these individuals are better able to cope with an acute stressor than individuals with lower hippocampal GR levels) (Meaney et al. 1988; 1989; 1992; Francis and Meaney 1999; Weaver et al. 2004). These studies suggest that an increase in maternal GCs is a prime candidate for the programming of the offspring’s HPA axis.

Maternal programming of the offspring’s HPA axis may have long-term consequences not only in terms of the offspring’s stress physiology but also their reproduction. Laboratory studies of rats and guinea pigs have found that adult offspring born to stressed mothers gave birth to small litters and offspring with reduced birth weight (Drake et al. 2005; Emack et al. 2008; Götz et al. 2008). Thus, through the maternal programming of the HPA axis and the HPA-HPG link, maternal GCs are also a prime candidate for causing a decrease in the offspring’s reproduction.
1.5 The snowshoe hare cycle

Snowshoe hare populations vary cyclically in density throughout North America, with peak densities occurring every 8-11 years (MacLulich 1937; Green and Evans 1940; Keith 1963; Smith 1983; Sinclair et al. 1993). During the numerical changes many demographic and physiological changes occur as well. Survival and reproduction both decline during the late increase and decline phase (Cary and Keith 1979; Boutin et al. 1986, 1995; Stefan and Krebs 2001). Boonstra et al. (1998) found that hares had higher stress levels during the decline than during the late low phase. Many factors affecting the cycle have been investigated as a possible explanation for this shift such as food availability (Keith and Windberg 1978; Cary and Keith 1979; Hik 1995; Krebs et al. 2001), predation and the risk of predation (Krebs et al. 1986; Boutin 1995; Hik 1995), parasite loads (Keith et al. 1986; Murray et al. 1997), and social interactions (Graf et al. 1985). Although the hare cycle has been studied for over 70 years (MacLulich 1937; Elton and Nicholson 1942; Keith 1963, 1990; Krebs et al. 1986, 1995) two patterns still remain unexplained. The first is why the hare population remains low for 2-5 years after the decline phase even though predators have virtually disappeared and the vegetation is ample. The second is why the low phase varies in length.

During the low phase snowshoe hares have a significantly reduced reproductive fitness (Cary and Keith 1979; Stefan and Krebs 2001). The decrease in reproduction begins during the population peak. Reproduction continues to fall during the decline phase and does not recover until late in the low phase just prior to the next increase. To explain these changes Hik (1995) proposed a predator sensitive foraging hypothesis. He found that hares were able to assess the risk of predation in different habitats and during
the population decline limit their activity to areas of dense cover to reduce the risk of being killed (see also Wolff 1980). He proposed that the correlate of choosing areas of dense cover is being forced to eat low-quality food followed by a decline in body condition that reduced reproductive fitness. However, Hodges et al. (1999) found that body condition did not affect reproduction. Boonstra et al. (1998) proposed a complimentary hypothesis to that of Hik, the stress hypothesis. They hypothesized that hares experience higher stress during the decline due to increased predation risk and that higher stress levels caused the decline in reproduction.

1.6 Thesis objective and hypotheses

The general objective of this thesis was to investigate the effects of maternal stress on the dynamics of cyclic populations. I used both a natural monitoring study and an experimental study to test the general hypothesis that the low phase is the result of the negative impact of intergenerationally-inherited maternal stress caused by the high risk of predation during the decline phase. In the subsequent chapters, five manuscripts will be presented that test individual parts of this general hypothesis.

In Chapters 2 and 3, I validated a non-invasive enzyme immunoassay used to measure fecal cortisol metabolites (FCM). Chapter 2 (in print; Sheriff et al. 2009 Journal of Comparative Physiology – B) contains a series of experiments testing how changes in plasma cortisol levels are reflected in the feces of snowshoe hares. This was a critical first step of the study as it allowed me to measure cortisol hormones from pregnant females without having to handle and bleed the animals, which can lead to a rapid increase in hormone levels. Chapter 3 (in print, Sheriff et al. General and Comparative
Endocrinology) tests two critical assumptions that are made when using fecal metabolites to measure stress hormones.

In Chapter 4 (in print, Sheriff et al. 2009 Journal of Animal Ecology), I tested the hypothesis that elevated FCM concentrations are associated with a decline in reproduction. In the natural monitoring study the hare and lynx densities were estimated to determine the changes in the risk of predation. In the experimental study a trained dog was used to simulate predation. Maternal FCM concentrations and reproduction were measured 30 h after birth. Indices of reproduction were measured (litter size, offspring birth weight, and physical size).

In Chapter 5 (in print, Sheriff et al. Ecology), I tested the hypothesis that chronically elevated FCM concentrations in dams increased the stress hormones experienced by their offspring. The ability of the offspring to mobilize energy and their body condition in response to an increase in maternal FCM concentrations was also assessed.

In Chapter 6 (submitted, Sheriff et al.), I examined the seasonal and yearly changes in stress hormones of free-ranging hares during their population cycle. I compared stress hormone levels, energy mobilization and body condition of adult hares between early and late winter, and between the first and second litter within a breeding season from the increase (2005), the peak (2006), the decline (2007-2008), and the low phase (2009). I discuss these changes with respect to the risk of predation that occurs and build on the findings of chapters 4 and 5 to discuss the impacts of maternal stress on the low phase of snowshoe hares. Lastly, I used 10 complete hare cycles from the literature
to investigate how an index of the severity of maternal stress (the rate of decline) affects the variation in the length of the low phase.

In Chapter 7, my concluding chapter, I try to place the work into a more general context and then give an overview of the key findings of my thesis. It is my intention with this chapter to submit this as a paper to Bioscience, a more general journal targeting a wider scientific audience. It will follow on the heels of a major paper by Krebs et al. (2001) in Bioscience on the state of knowledge of the hare cycle to the point of about 8-10 years ago.

Overall, in this thesis, I examine the impact of predator-induced maternal stress on snowshoe hare demography. Specifically, I investigate how maternal stress effects reproduction and how maternally inherited stress compromises the stress physiology of offspring. I then use these results to help explain the enigma of cyclic populations; the low phase.
Fig. 1.1. The hippocampus and the hypothalamic-pituitary-adrenal (HPA) axis, the major impacts on body processes, and the glucocorticoid (GC) feedback in the mammalian brain. The hippocampus regulates the overall functioning of the HPA. A stressor causes the hypothalamic paraventricular nucleus to release corticotropin releasing hormone (CRH) and vasopressin (AVP), which causes the anterior pituitary to release adrenocorticotrophin (ACTH). ACTH initiates the synthesis and release of glucocorticoids (GCs, corticosterone in some rodents, cortisol in others) from the adrenal cortex. GCs act at multiple sites within the body to maintain homeostasis, but because of the damaging effects of extended exposure to GCs, the HPA axis is tightly regulated through feedback (inhibition indicated by -) on glucocorticoid receptors to inhibit further HPA activity. Cortisol feeds back on the hypothalamus and pituitary to cause a rapid inhibition of CRF release. Under conditions where the stressor is acute, feedback mechanisms operate efficiently and the system rapidly returns to normal, resulting in effects on body processes that are only short-term. Under conditions where the stressor is chronic, feedback signals are weak and the system remains activated for longer periods, resulting in effects on body processes that can be long term and detrimental. Short-term effects result in suppressive impacts on body processes; long-term chronic effects result in inhibitory impacts on body processes. Glucocorticoid (GR) and mineralocorticoid receptors (MR) occur in the limbic system (hippocampus and dentate gyrus) and GR occur in the PVN and anterior pituitary. In the brain, MR have a higher affinity than do GR for GCs, and at basal concentrations of cortisol, MR are occupied whereas GR remain largely unoccupied. During periods of stress, elevated plasma GCs, there is increased occupation of GR. Hippocampal MR may be primarily involved in feedback regulation during basal secretion, whereas GR become important during periods of increased GC secretion. (modified from de Kloet et al. 1999; Matthews 2002; Sapolsky 2002; Boonstra 2004).
Hippocampus

Hypothalamus

CRH  AVP

Anterior Pituitary

ACTH

Adrenal Cortex

Glucocorticoids

Feedback under chronic stress

Feedback under acute stress

Mobilization of Energy

Suppression of Growth

Suppression of Immunity

Suppression of Digestion

Suppression of Reproduction

MR

GR
1.7 References


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2. A NON-INVASIVE TECHNIQUE FOR ANALYZING FECAL CORTISOL METABOLITES IN SNOWSHOE HARES (*LEPUS AMERICANUS*)

2.1 Introduction

The adrenal cortex secretes glucocorticoids to aid in daily functions such as the regulation of energy storage and of diurnal rhythms, and in response to activities such as courtship, copulation, and hunting (Sapolsky et al. 2000). However, when presented with a stressor (defined as any environmental disturbance that disrupts homeostasis) animals respond by increasing their glucocorticoid secretion. This response is primarily mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Owen et al. 2005).

Understanding stress hormones is key to the study of natural populations. This knowledge can address questions about how stressors affect the survival and reproductive success of free-living animals and broader ones pertaining to management strategies, relocation or reintroduction, habitat disturbance, and population dynamics (Boonstra and Singleton 1993; Wasser et al. 1997; Creel et al. 2002; Cyr and Romero 2007). Since a stress response leads to an increase of blood glucocorticoids, their concentrations have been used as an index of stress in a wide range of studies (e.g., Boonstra et al. 1998; Hopster et al. 2002; Häcklander et al. 2003; Romero and Reed 2005). However, capture, handling, and bleeding can cause a rapid increase in blood glucocorticoid concentrations (Haemisch et al. 1999; Romero and Romero 2002). An alternative, non-invasive method

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1 A version of this chapter has been published: Sheriff, M.J., C.O. Bosson, C.J. Krebs, and R. Boonstra. 2009a. A non-invasive technique for analyzing fecal cortisol metabolites in snowshoe hares (*Lepus americanus*). Journal of Comparative Physiology B 179:305-313.
has been developed to monitor glucocorticoids through the use of their fecal metabolites (see review by Touma and Palme 2005; Palme et al. 2005).

Lagomorphs (hares, rabbits and pikas) have been the focus of a range of ecological research with studies on mating, dispersal, antipredator behaviours, population dynamics, among others. Since the ecology is so well known for many lagomorphs they make excellent study species for investigating endocrinology of free-living animals. However, only two previously published studies (Teskey-Gerstl et al. 2000 on European hares; Monclús et al. 2006 on rabbits) have investigated non-invasive techniques for measuring stress hormones. Teskey-Gerstl et al. (2000) found that a group-specific enzyme immunoassay (EIA) for 11, 17-dioxoandrostanes (11, 17-DOA; 11-oxoetiocholanolone -EIA) established by Palme and Möstl (1997) was best suited for hares due to its high immunoreactivity. However, Teskey-Gerstl et al. (2000) did not use the rigorous validation method of dexamethasone (DEX) suppression and adrenocorticotropic hormone (ACTH) stimulation of adrenal cortisol and its subsequent appearance in the feces. Here we extend their initial findings to assess the suitability of this EIA in the snowshoe hare, *Lepus americanus*. The primary glucocorticoid in snowshoe hares is also cortisol (Boonstra and Singleton 1993) and thus we will be monitoring fecal cortisol metabolites (FCM).

Our study had four objectives. First, to determine the proportion of FCM excreted via the urine and feces, the time course of metabolite excretion, and the sex differences we injected administration of radioactively-labelled cortisol. Second, to investigate diurnal rhythms in snowshoe hares, we monitored FCM over two days. Third, to test whether changes in adrenocortical activity can be reliably monitored in FCM using this
EIA, we carried out two tests: a DEX suppression test and an ACTH stimulation test. DEX acts as an artificial glucocorticoid agonist that mimics endogenous cortisol and reduces circulating cortisol concentrations via the negative feedback mechanism of the HPA axis (Axelrod and Reisine 1984). ACTH stimulation tests the responsiveness of the adrenals directly and acts to increase circulating blood cortisol concentrations (Miller and Tyrrell 1995). Previous work on snowshoe hares (Boonstra and Singleton 1993; Boonstra et al. 1998) has shown that this DEX-ACTH challenge causes major changes in plasma cortisol levels. Fourth, to assess whether a natural stressor resulted in an increase in FCM levels, we introduced a simulated predator (dog).

2.2 Materials and methods

2.2.1 Animals and housing

Snowshoe hares are the smallest of the hare species world wide. In the southwestern Yukon, adults weigh 1200 -1800 g. They are found throughout Canada and the northern parts of the U.S.A. They are most active at dusk and dawn when they do the majority of their feeding. However, due to the extremely long summer days in the far north hare’s active period ranges from 2000 h to 0800 h during our study. In the boreal forest, hares have a 10-year population cycle that impacts the entire ecosystem. Hares can be subject to high predation risk, especially during the decline phase of their population cycle when approximately 95% die because of predation (Krebs et al. 1995).

Eleven adult (five males and six females, >12 months old) snowshoe hares were used for these experiments. Hares were live-trapped in the Shakwak Trench east of Kluane Lake, Yukon Territory (61°N, 138° W) using Tomahawk traps (Tomahawk Live Trap Co., Tomahawk Wisconsin, U.S.A.) (see Krebs et al. 1986 for details). Hares were
placed in individual pens 5-6 days prior to the start of the experiments to allow for
habituation. Owing to space limitations, all animals were housed in the same room. Hares
were exposed to ambient conditions of both external temperature and light.

Individual pens were 60 x 60 x 120 cm wire cages. Each cage was visually
separated with a plywood wall, and partially covered on the top and front by a burlap
cloth. The floor of each cage was made with 1.30 cm reinforced wire mesh that allowed
both urine and feces to pass freely. Feces were then caught on a finer wire mesh placed
on an angle below the cage. This allowed feces to roll to the collection tray and not be
contaminated by urine. Voided urine passed through the finer mesh and was collected in
a plastic catch basin below.

Animals were fed ad libitum with standard medicated rabbit chow (Unifeed,
Okotoks, Alberta; Unifeed Ltd Cat. #19-2103, 18% protein, crude fat 2%, crude fibre
18%) supplemented daily with natural browse (small branches with leaves and bark from
Salix sp.) and watered ad libitum.

2.2.2 Experimental design

The chronology of the entire experimental procedure, including acclimation time,
time of initiation of each experiment, and exact times when feces were collected, can be
found in Table 2.1. All injections were made between 1700 and 1900 h. Free-living hares
experience intense predation pressure thus a trained dog, as a simulated predator, was
chosen as a natural stressor.

For each hare, all feces or urine produced was collected at the end of the period
and pooled; we analyzed an aliquot from each sample. Samples from all hares were
analyzed separately. The weight of each fecal sample or volume of each urine sample was determined. Results in the figures are plotted at the time of collection.

2.2.3 Administration of radioactive cortisol

\[1,2,6,7^3 \text{H}\] Cortisol (code#TRK407, batch#124) was obtained from Amersham Bioscience UK Ltd (Buckinghamshire, UK). It had a radiochemical purity of 98.9% as tested by high performance liquid chromatography (HPLC) on a Hypersil MOS column using a water:methanol gradient. Ten hares were injected with 1110 kBq of tritiated cortisol in a 450 µl solution containing 6% toluene, 10% ethanol and 84% saline; one was injected with 610.5 kBq. All injections were into an ear vein when the hare was restrained in a burlap bag. The entire procedure took 5-15 min per hare.

2.2.4 Diurnal rhythm

This experiment used data from the last 2 days of the radioinfusion experiment. Hares were not disturbed for 48 h prior to the start of this time course, and hares had recovered from the stress of handling and injection (see Fig. 2.1). We determined the amount of radioactivity excreted per gram of feces by measuring a 0.300 ± 0.05 g sample of homogenized ground feces for each sample time. We determined the total amount of radioactivity excreted at each time point by multiplying the amount of radioactivity per gram of feces by the weight of the entire fecal sample collected for that time point.
2.2.5 DEX suppression test

DEX was obtained from Sabex (Montreal, Canada). Seven hares were injected with both 0.4 mg/kg into the ear vein and 1.6 mg/kg into the thigh muscle. Four hares were used as controls and were injected with similar volumes of physiological saline. The entire procedure took 5-15 min per hare.

2.2.6 ACTH stimulation test

ACTH (Synacthen Depot) was obtained from, CIBA (Ontario, Canada). Seven hares were injected with 80 µg/kg into the thigh muscle. Four hares were used as controls and were injected with similar volumes of physiological saline. The entire procedure took 5-15 min per hare.

2.2.7 Natural stressor challenge

Hares were visually exposed to a medium sized, trained dog. The dog was allowed to smell the perimeter of each individual hare holding pen for 10-30 sec at 1800 h, 2400 h, and the following morning at 0800 h. The dog was in contact for a total of 5 min at each time. The dog whined and sniffed against each cage but did not bark or lunge at any of the hares. Since all hares were housed in the same room they served as there own control comparing FCM concentrations during and after the stressor.
2.2.8 Sample handling and extraction

Urine and feces were frozen at -80°C immediately after collection. They were transported to the University of Toronto at -20°C where fecal samples were stored at -80°C until drying and extraction, and urine samples were stored at 4°C until analyzed.

Fecal samples were first freeze dried using a Lyophilizer (LabConco, Missouri, USA) for 14-18 h to control for fibre and water content (Wasser et al. 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was either analyzed immediately if radioactive or diluted (1:10) with assay buffer and frozen at -80°C until analysis with the EIA.

2.2.9 Determination of radioactivity

After extraction a 250 µl aliquot of the fecal supernatant was counted with 5 ml ACS scintillation fluid (Amersham, USA) and its radioactivity measured in a liquid scintillation counter (Packard Tri-Carb 2900TR, Boston, Massachusetts) with quench correction. A 100 µl aliquot of each urine sample was counted with 5 ml ACS scintillation fluid with quench correction. The excretion of radioactivity in both the feces and urine was calculated by adjusting for the total weight of feces in each sample and the total volume of urine in each sample.
2.2.10 Determination of immunoreactivity

The immunoreactivity of fecal samples was determined using the 11-oxoetiocholanolone -EIA developed by Palme and Möstl (1997). Teskey-Gerstl et al. (2000) found that this EIA detected the highest amount of immunoreactive metabolites in fecal samples of the European hare (*Lepus europaeus*) as compared with cortisol- or corticosterone- EIAs. The assay had a sensitivity range from 2-500 pg per well. The inter- and intra-assay coefficients of variation were 18.6% and 6.7%, respectively.

2.2.11 Statistical analysis

All data are expressed as means ± 1 SEM, unless otherwise stated. Repeated measures ANOVA and *t*-tests were performed using the software package STATISTICA 6. The assumption of normality was tested with Shapiro-Wilks test and of homogeneity of variances with Levene’s test. If these assumptions were not met the appropriate adjustment was made (log-transformation of data or Greenhouse-Geisser adjustment; Quinn and Keough 2003). Comparisons of the means were considered significant if *P* < 0.05. Sexes were pooled as there were no significant differences between males and females for any variable examined (repeated measures ANOVA comparing sex at each variable; *P* > 0.05).

2.3 Results

2.3.1 Route and time course of steroid excretion

A total of 25 serial samples were collected after the injection of the isotope. The mean total recovery of administered radioactivity was 66.7%, with 58.30% ± 2.34 from
the urine and 8.40% ± 0.55 from the feces. The pattern of radioactive excretion in both urine and feces varied significantly over time (urine: $F_{24, 216} = 56.49, P < 0.0001$; feces: $F_{23, 230} = 26.06, P < 0.0001$, Fig. 2.1). Radioactivity in urine samples peaked at 2000 h, 3.47 h ± 0.18 after injection, and declined rapidly thereafter. The radioactivity in the peak sample was significantly higher than in the samples collected immediately before ($t_{10} = -6.78, P < 0.0001$) or after ($t_{10} = 2.91, P < 0.025$) the peak. Radioactivity in fecal samples peaked at 2400 h, 5.73 h ± 0.27 after injection, and declined rapidly thereafter. The radioactivity in the peak sample was significantly higher than in the samples collected immediately before ($t_{10} = -3.60, P < 0.005$) or after ($t_{10} = 3.21, P < 0.01$) the peak. In both the urine and the feces background levels were reached within 2 days.

2.3.2 Diurnal rhythm

Snowshoe hares exhibited a diurnal rhythm in the total amount of radioactivity excreted in the feces after injection with radioactive cortisol. However, this pattern was not reflected in the amount of radioactivity per gram of feces, but rather was due to the amount of feces defecated (Fig. 2.2). The total amount of radioactivity excreted varied significantly over 48 h ($F_{10, 100} = 5.81, P < 0.0001$), with a clear pattern of increase and decline, with peak levels occurring between 2400 h and 0400 h and minimum levels occurring at 1600 h. The amount of radioactivity excreted per gram of feces remained constant over the 48 h period. The slight decrease in radioactivity over time is due to the hares having less to excrete (the nature of a radioactive experiment). For all subsequent studies we compare the amount of FCM per gram of feces (i.e., FCM concentration).
2.3.3 DEX suppression test

DEX suppressed FCM concentrations by 61% from 1111 ng/g ± 328 (control) to 487 ± 85 (DEX) at 10 h post injection (i.e., at 0400 h, \( t_9 = -2.61, P < 0.05 \); Fig. 2.3). FCM concentrations did not increase to baseline levels until 28 h after the injection.

2.3.4 ACTH stimulation test

ACTH injections increased FCM concentrations by 1000%, from 1054 ng/g ± 151 (control) to 11130 ± 3317 (ACTH) at 10 h post injection (i.e., at 0400 h, \( t_9 = 6.09, P < 0.0005 \), data log transformed, Fig. 2.4). FCM concentrations did not decline to baseline levels until 24 h after the injection.

2.3.5 Natural stressor challenge

In the simulated predator experiment, FCM concentrations varied significantly over time (\( F_{12, 120} = 5.72, P < 0.005 \), Fig. 2.5). To assess if the exposure to the dog stressed the hares, we compared FCM levels 10 h post exposure (given that this was the lag time for both the DEX and ACTH challenges above) to FCM levels 24 h later. At 10 h post exposure to the initial dog exposure (i.e., at 0400 h), FCM concentrations were 175% higher compared with FCM concentrations at 0400 h the next day (\( t_{10} = 3.43, P < 0.01 \)). To assess the stress response of hares to repeated dog challenges we exposed them a second (at 2400 h) and third (at 0800 h) time. Due to the timing of these challenges we could only compare FCM levels 8 h post exposure to those 24 h later. At 8 h post exposure to the second and third dog exposure FCM concentrations were 151% (\( t_{10} = 2.26, P = 0.054 \)), and 173% (\( t_{10} = 2.76, P < 0.05 \)) higher than those 24 h later. FCM
concentrations did not decline to baseline values until 24 h after the final exposure to the dog.

2.4 Discussion

This study validates a non-invasive method for assessing adrenocortical activity in hares. We show that the 11-oxoetiocholanolone -EIA can be used to reliably monitor changes in cortisol concentrations via measures of FCM in snowshoe hares. Specifically, we found that: (i) the mean excretion of radioactivity peaked in the urine and feces at 3.47 h ± 0.18 and 5.73 h ± 0.27, respectively; (ii) there was a clear diurnal rhythm in the total amount of radioactivity excreted; (iii) changes in adrenal functioning via DEX suppression or ACTH stimulation were detected in FCM concentrations; and (iv) a dog stressor caused FCM concentrations to increase markedly.

2.4.1 Route and time course of steroid excretion

We recovered approximately 66% of the injected $^3$H-cortisol, with 8% being recovered in the feces. The loss of isotope is most likely due to loss in the urine collection. Copious amounts of urine were voided and thus isotope could have been absorbed into the plastic collection sheet, or dust that settled onto the sheet. As fecal pellets are large and conspicuous we do not believe that there was isotope loss from feces and that fecal recovery reflects true FCM levels. Furthermore, this value is similar to that found by Teskey-Gerstl et al. (2000) in the European hare, *Lepus europaeus*. This limited recovery does not affect the measurement itself, as the group-specific EIAs are highly
sensitive assays with an extremely low detection limit within the picogram range (Möstl et al. 2005).

The time delay between the $^3$H-cortisol injection and the peak appearance was 3-4 h in the urine and was 5-6 h in the feces (Fig. 2.1). Since we injected hares at 1800 h (the approximate start of the active phase of hares) this delay indicates the rate of cortisol metabolism and excretion during the active phase of snowshoe hares. In European hares, Teskey-Gerstl et al. (2000) found the peak appearance of radiometabolites in the feces occurred at 1000 h, 23 h after the injection. However, they injected hares during the inactive phase at 1100 h (their active phase is from 1900 h to 0700 h). Touma et al. (2003) suggested that an animal’s activity pattern and gut passage time plays an important role in the excretion of metabolites. Touma et al. (2003) found that the peak delay was 2.5 times later in mice injected at the end of their active phase compared with those injected at the beginning. Thus, if we had injected hares at the end of their active phase (i.e., 0700-0800 h), we would predict a 12-15 h delay. This delay is still not as long as Teskey-Gerstl et al. (2000) found and may be due to species specific differences in active compared with inactive phase metabolism and excretion. This difference may also be due to the difference in diet and thus metabolism and excretion of the wild-caught hares used here compared with the captive-raised hares used by Teskey-Gerstl et al. (2000).

2.4.2 Diurnal rhythm

The total amount of radioactivity excreted showed a clear diurnal rhythm but the amount of radioactivity per gram of feces did not (Fig. 2.2). Wasser et al. (1994) also
found that the amount of steroid metabolites differed between the total amount excreted versus the amount excreted per gram of feces. Diurnal patterns in FCM (measured as per gram of feces) have been shown for Columbian ground squirrels (Bosson et al. in press), mice (Touma et al. 2003), and rats (Bamberg et al. 2001). In snowshoe hares peak levels occurred between 2400 h and 0400 h, approximately 6-10 h after the beginning of their active period (Fig. 2.2). However, the diurnal rhythm seen here was not due to fluctuating concentrations of radioactivity excreted in the feces, as reflected by the per gram analysis of radioactivity (Fig. 2.2). Rather the diurnal rhythm was a function of the total amount of feces defecated (Fig. 2.2 in-set). Touma et al. (2003) suggested that an animal’s activity rhythm and gut passage time play an important role in the diurnal rhythm of FCM. As the rate of excretion increases, the rate of bile secretion and thus metabolite secretion also increases (Randall et al. 2000). Therefore, times of peak defecation should contain peak amounts of FCM. However, if the total amount of FCM increases in proportion to the increase in total feces voided, FCM concentrations may not reflect the diurnal rhythm of the total FCM. This may be important in animals that defecate copious amounts, such as snowshoe hares, as it would dilute FCM concentrations and mute the diurnal rhythm. In animals that defecate large amounts only a small portion of the total feces is collected during fecal analysis, and an even smaller portion is used. Thus a diurnal rhythm may not be detected or concentrations of FCM may be corrected for diurnal rhythms unnecessarily.

Though Wasser et al. (1994) showed that the overall rate of fecal steroid metabolite excretion differed from the concentration of fecal steroid metabolite excretion, they did not assess diurnal changes. Our study is the first to show this difference with
fecal corticosteroid metabolites and the diurnal pattern. Thus, comparisons of FCM collected at different times of the day will only be valid if researchers have intimate knowledge of the diurnal rhythm.

2.4.3 Adrenal suppression (DEX) and stimulation (ACTH)

Boonstra et al. (1998) showed that in the snowshoe hare both DEX and ACTH injections resulted in rapid changes in plasma cortisol concentrations. Using the 11-oxoetiocholanolone -EIA we were able to detect changes in FCM concentrations 10 h after either the DEX or ACTH injection compared with saline injected controls (Fig. 2.3 and 2.4). Since we collected samples every 4 h, the changes in FCM concentrations could occur as early as 8 h post injection or as late as 12 h. In other small mammals, changes in adrenal functioning have also been detected in FCM concentrations within 24 h (e.g., 4-10 h in mice [Touma et al. 2003]; 6-30 h in Belding’s ground squirrels [Mateo and Cavigelli 2005]; 12 h in European rabbits [Monclús et al. 2006]). This lag time between hormonal changes in the blood and their appearance as metabolites in the feces is likely due to the variation in gut passage time of the animal. This relationship between FCM excretion and gut passage time is well documented for a diverse array of mammalian and avian species (Wasser et al. 2000; Palme et al. 2005; Touma and Palme 2005).

2.4.4 Natural stressor challenge

The dog stressor significantly increased FCM concentrations 175% 10 h after the initial exposure compared with levels 24 h later (Fig. 2.5). Although the second and third stress events did not continue to increase FCM concentrations, levels were still higher
than the following day. The reason the latter stressors did not continue to increase cortisol levels may be due to a short-term corticoid induced feedback inhibition of the HPA axis and incomplete recovery of the HPA axis responsiveness. Other studies of changes in HPA responsiveness as a result of previous stress exposure have resulted in habituation of the HPA axis to repeated stressors of the same type (Gądek-Michalska and Bugajski 2003; Jaferi et al. 2003). In the snowshoe hare, a species subject to intense predation pressure, this temporary HPA habituation to repeated short-term stressors might function to protect the animal from the negative effects of long-term exposure to high cortisol concentrations.

Although, other studies on lagomorphs have also found significantly elevated FCM concentrations after a biological stressor, homogenized pooled samples were used and short time-scale changes in FCM concentrations could not be detected (Teskey-Gerstl et al. 2000; Monclús et al. 2006). This is the first study on lagomorphs with such a high-resolution sampling regime that allowed us to track short time-scale changes in FCM concentrations. Clearly, the EIA is able to detect changes in adrenal functioning in the snowshoe hare.
Table 2.1. Experimental design for the validation of an enzyme immunoassay measuring metabolites of cortisol in fecal extracts from *Lepus americanus*. Animals receiving a saline injection were randomized within sex.

<table>
<thead>
<tr>
<th>Date</th>
<th>Experiment</th>
<th>Treatment</th>
<th>Start of Treatment</th>
<th>Collection Times After Starting Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun 15</td>
<td>Acclimation</td>
<td>Trapped and Housed</td>
<td>0600 h</td>
<td>0, 8, 16, 24, 32, 48, 72, 96, 112, 120, 128</td>
</tr>
<tr>
<td>Jun 20</td>
<td>Radiometabolism</td>
<td>$^3$H-Cortisol</td>
<td>1800 h</td>
<td>0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92</td>
</tr>
<tr>
<td>Jun 22</td>
<td>Diurnal Variation</td>
<td>Observation within Pen</td>
<td>1200 h</td>
<td>0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48</td>
</tr>
<tr>
<td>Jun 24</td>
<td>Adrenal suppression</td>
<td>Dex or Saline</td>
<td>1800 h</td>
<td>0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48</td>
</tr>
<tr>
<td>Jun 26</td>
<td>Adrenal stimulation</td>
<td>ACTH or Saline</td>
<td>1800 h</td>
<td>0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48</td>
</tr>
<tr>
<td>Jun 28</td>
<td>Predation risk</td>
<td>Exposure to Trained Dog</td>
<td>1800, 2400 &amp; 0800 h</td>
<td>0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48</td>
</tr>
</tbody>
</table>
Fig. 2.1 Time-course of excretion of radioactivity in urine and feces in snowshoe hares (mean ± S.E.; n = 11). Note x-axis, samples were collected every 2 h until 1200 h and every 4 h thereafter.
Fig. 2.2. Excretion profile of fecal radioactivity to evaluate diurnal rhythm over a 48 h period in snowshoe hares. In-set shows the average amount of feces defecated during this time period. Mean ± S.E.; n = 11.
Fig. 2.3. FCM concentrations of snowshoe hares in response to DEX injections compared with controls (mean ± S.E.; n = 11). Asterisk denotes significant difference from controls 10 h post-injection. Arrow indicates time of injection.
Fig. 2.4. FCM concentrations of snowshoe hares in response to ACTH injections compared with controls (mean ± S.E.; n = 11). Asterisk denotes significant difference from controls 10 h post-injection. Arrow indicates time of injection.
Fig. 2.5. FCM concentrations of snowshoe hares before, during and after a simulated predator (dog; mean ± S.E.; n = 11). Asterisks denote significant differences from levels 24 h later. Arrows indicates times of stress.
2.5 References


3. ASSESSING STRESS IN ANIMAL POPULATIONS: DO FECAL AND PLASMA GLUCOCORTICOIDS TELL THE SAME STORY?  

3.1 Introduction

Understanding the effects of stress hormones is important to the study of natural populations. This knowledge can address questions about how stressors (any environmental perturbation that disrupts homeostasis) affect the survival and reproductive success of free-living animals and broader ones pertaining to management strategies, relocation or reintroduction, habitat disturbance, and population dynamics (Boonstra and Singleton 1993; Wasser et al. 1997; Creel et al. 2002; Cyr and Romero 2007; Sheriff et al. 2009a). When encountering a stressor, animals respond by increasing their glucocorticoid (GC) production, and this stress response is primarily mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Owen et al. 2005). A number of techniques have been used to measure GC concentrations and these include sampling blood and feces.

Blood GC concentrations have been used as an index of stress in a wide range of studies (e.g., Boonstra et al. 1998; Hopster et al. 2002; Hackländer et al. 2003; Romero and Reed 2005; Sheriff et al. 2009a, Kitaysky et al. 2007). Blood sampling can provide not only total GC concentrations but also the amount that is free. GCs are normally tightly bound to a carrier protein, corticosteroid-binding globulin (CBG), and only the free form (5-10% of the total) is biologically active (Rosner 1990). However, to obtain

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2 A version of this chapter has been accepted by General and Comparative Endocrinology for publication: Sheriff, M.J., C.J. Krebs, and R. Boonstra. Assessing stress in animal populations: do fecal and plasma glucocorticoids tell the same story?
free levels, it is necessary to measure both the CBG levels and to know the binding coefficients. Serial blood samples during restraint or hormone challenges allow an integrated picture of the responsiveness of an animal and thus an insight into what it has been experiencing in its recent past (Boonstra et al. 1998; Kenagy and Place 2000; Romero and Romero 2002, Kitaysky et al. 2007). However, blood sampling is invasive and capture, handling, and bleeding can cause a rapid increase in blood GC concentrations (within 3 min) and bleeding free-ranging animals within 3 minutes may not be possible (Romero and Romero 2002). Furthermore, the increase in GC concentrations due to blood sampling may be undesirable, especially in reproductive studies in which increased GC concentrations may negatively affect reproductive fitness (Sheriff et al. 2009a).

An alternative method to assess stress levels in animals is the use of fecal GC metabolite concentrations (see reviews by Wasser et al. 2000; Goymann 2005; Palme et al. 2005). This method has been used to investigate diurnal and seasonal patterns of GC levels, social and dominance interactions, the impacts of habitat degradation, transport stress, predator-prey interactions, the effects of maternal stress on reproduction, maternal effects, and population dynamics (Wasser et al. 1997; Kotrschal et al. 1998; Goymann et al. 1999; Palme et al. 2000; Touma et al. 2003; Bosson et al. 2009; Sheriff et al. 2009a). GCs are metabolized by the liver prior to excretion both through the urine and the feces via the bile (Taylor 1971; Palme et al. 2005). It is assumed that only the free GCs (i.e. not bound to CBG) are degraded by the liver (Palme et al. 2005). Thus fecal samples provide an integrated hormone profile over time with less interference from acute stressors. The interpretation of fecal assays rest on two critical, untested assumptions: first, that fecal
GC metabolites reflect free, biologically active, GC levels in the plasma; and second, that differences in fecal GC metabolite levels amongst animals are an accurate reflection of their physiological state and thus of their ability to respond to a stressor.

We tested both of these assumptions in a population of free-ranging snowshoe hares in the southwestern, Yukon, from 2006 to 2008. First, in a shot sample of hares we tested the assumption that only free cortisol (the major GC in hares - Boonstra et al. 1998) is metabolized by the liver, whereas CBG bound cortisol passes through the liver. Thus, the cortisol metabolite levels in the bile and feces should directly reflect free plasma cortisol concentrations but not total plasma cortisol concentrations. Second, in a sample of free-ranging hares we compared their fecal cortisol metabolite (FCM) concentration with their ability to respond to standardized hormone challenge (feces were collected prior to the hormone challenge). This hormone challenge consisted of a dexamethasone (Dex) suppression test followed by an adrenocorticotropic hormone (ACTH) stimulation test. Dex acts as an artificial glucocorticoid agonist that mimics endogenous cortisol through feedback inhibition of the HPA axis. In hares exhibiting greater Dex resistance, plasma cortisol levels do not fall as much as in hares where the feedback inhibition is operating normally (Axelrod and Reisine 1984). The ACTH stimulation tests the responsiveness of the adrenals directly and acts to increase circulating blood cortisol concentrations (Miller and Tyrrell 1995). Boonstra et al. (1998) has shown (in plasma free cortisol concentrations) that snowshoe hares under chronic stress are more Dex resistant and exhibit an increased response to the ACTH injection. Studies have shown that a Dex or ACTH injection leads to changes in FCM levels and this is part of the standard validation procedure for measuring FCM levels (Touma and
Palme 2005). However, it has not yet been shown that animals with greater FCM concentrations have fundamentally altered responses to these injections (Wasser et al. 2000; Mateo and Cavigelli 2005; Sheriff et al. 2009b). We tested the assumption that FCM concentrations are indicative of an animal’s responsiveness to a stressor (i.e. if an animal was compromised by chronic stress it will not respond as well as if it were not). Thus, hares with greater FCM concentrations should have a reduced response to the Dex suppression test (such that after the Dex injection cortisol levels should be greater) and a greater, prolonged, cortisol production in response to the ACTH stimulation test. To further assess the utility of measuring FCMs relative to plasma cortisol derived from the stress response to a hormonal challenge, we examined the seasonal changes in both measures in free-ranging snowshoe populations subject to different intensities of predation risk (Sheriff et al. 2009a).

3.2 Methods

3.2.1 Snowshoe hare natural history

Snowshoe hares are the smallest of the hare species world wide. In the southwestern Yukon, adults weigh 1200 -1800 g. They are found throughout Canada and the northern parts of the U.S.A. They are most active at dusk and dawn when they do the majority of their feeding. Hares do not have nests or burrows and remain active throughout the year. In the boreal forest, hares have a 10-year population cycle that impacts the entire ecosystem. Hares can be subject to high predation risk, especially during the decline phase of their population cycle when over 95% die because of predation (Krebs et al. 1995).
3.2.2 Study area and context

This study was conducted in the boreal forest near the Arctic Institute Base at Kluane Lake in the southwestern, Yukon, Canada (60°57’ N, 138°12’ W). It took place during the hare peak (2006 - 0.92 hares per ha) and the first two years of the decline (2007 – 0.79 hares per ha and 2008 – 0.35 hares per ha – Sheriff et al. 2009a). Our research was approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

3.2.3 Shot sample

To test whether free plasma cortisol levels were correlated to metabolized levels in the bile and feces we collected eleven adult snowshoe hares (9 female and 2 male, shot with a .22 caliber gun) between dusk and dawn (23:30 – 0400 h) in July and at dawn (0700-0900 h) in October, 2006 (bile could only be collected from 8 individuals). Samples of blood (0.05-0.25mL via heart puncture, within 3 min) and of bile (0.01-0.025 mL from the gall bladder) were immediately collected. Carcasses were stored in a fridge at 4° C within 1 h of being shot at the Arctic Institute Base. Fecal samples (all feces within the distal colon) were taken within 8 h of being shot. All samples were frozen at -80° C within 1 h of collection at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80 °C until analyzed.
3.2.4 Animal trapping

To assess the relationship between FCM and the stress response to a hormonal challenge 32 adult snowshoe hares (26 females and 6 males) were live-trapped using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) in autumn (October) and winter (February and March) from 2006-2008. The traps were set at 2200 h and checked at 0600 h and thus hares could only be in the traps for a maximum of 8 h. This is important as the lag between the production of cortisol in the body and the appearance of its metabolites in the feces is between 8-12 h (Sheriff et al. 2009b). Thus fecal steroid levels should not reflect the stress of live-trapping. Trapping did not occur on nights that dropped below -20 °C.

Each hare was weighed with a Pesola spring scale (± 10 g), its right hind foot (RHF) length measured (in duplicate) as an index of body size, an ear-tag was placed in its right ear (No. 3 Monel tags, National Band and Tag Co., Newport, Kentucky, USA.), and its sexual condition assessed (i.e. scrotal or not in males, no females were pregnant at this time). A fecal sample was collected from below the trap prior to the hormone challenge. Hares were then transferred in burlap sacs to a quiet, dimly lit laboratory (heated to 5-10 °C in winter) at the nearby Arctic Institute Base at Kluane Lake. Hares were kept in the burlap sacs and allowed to habituate to the laboratory conditions for 1-2 h prior to the start of the hormone challenge. They were not fed throughout the experiment.
3.2.5 Hormone challenge

Each hare was bled five times (0.3 ml per bleed) from an ear artery using 28
gauge needles (0.36 x 13 mm) and heparinised 0.5 ml syringes (Lo-Dose U-100 insulin
syringes, Becton Dickinson and Company, New Jersey, USA). The first blood sample
(base bleed) was immediately followed by an injection of 0.4 mg/kg of dexamethasone
sodium phosphate (Sabex, Quebec, Canada) into an ear vein. The second bleed (Dex
bleed) assessed the inhibition response to Dex and occurred 2 h later. It was followed
immediately by an intramuscular injection in the thigh of 40 ug/kg of synthetic ACTH
(Synacthen Depot, CIBA, Ontario, Canada). The remaining three bleeds assessed the
stimulation response to ACTH and occurred 30, 60, and 120 min post-ACTH injection
(called the P30, P60, and P120 bleeds, respectively).

Total plasma cortisol was measured in duplicate using a radioimmunoassay
(Clinical Assays GammaCoat Cortisol $^{125}$I RIA Kit, DiaSorin, Minnesota, USA) with an
intra- and inter- assay coefficient of variation of 2.4% and 12.4%. Maximum
corticosteroid-binding capacity (MCBC) levels were measured in duplicate using a
radioimmunoassay described by Boonstra and Singleton (1993), with an intra- and inter-
assay coefficient of variation of 2.6% and 4.9%. MCBC is a measure of the
corticosteroid-binding globulin and free cortisol is the portion not bound by this carrier
protein. Free cortisol concentrations were calculated using the procedures and binding
coefficients outlined in Boonstra et al. (1998).

We assessed the integrated response of each hare to the ACTH stimulation from
the time of the ACTH injection to the P120 bleed by calculating the area under the curve
over this 2 h period. This is the cumulative measure of the entire response that accounts
for both the peak levels as well as the duration, both of which are important in terms of effect on the free cortisol on the body (Dallman and Bhatnagar 2001). Some statistical packages will calculate this measure (e.g. GraphPad’s Prism), though we made our calculations with a simple geometry algorithm.

3.2.6 Bile and fecal cortisol metabolite assay

We used an enzyme immunoassay (EIA) to measure bile and fecal cortisol metabolite concentration, validated to measure FCM levels in snowshoe hares (Sheriff et al. 2009b). Within 1 h of collection, samples were stored at -80 ºC at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80 ºC until analyzed.

Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) for 14-18 h to control for fibre and water content (Wasser et al. 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-tube vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was diluted (1:10) with assay buffer and frozen at -80ºC until analysis. Bile samples were already liquid and an extraction step was not performed.

Bile and fecal cortisol metabolite concentrations were measured following the methods outlined by Sheriff et al. (2009b) using the 11-oxoaetiocholanolone-EIA developed by Palme and Möstl (1997). Briefly, 50 µl of extracted samples (in duplicate; bile samples were diluted 1:5 and fecal samples diluted 1:25 with assay buffer) were incubated with 100 µl of biotynilated steroid label (11-oxoaetiocholanolone-3-
glucosiduronate-DADOO-biotin) and 100 µl antibody (11-oxoetiocholanolone-3-HS:BSA raised in rabbits) at 4°C on a plate shaker overnight. Plates were then washed four times with 0.05% TWEEN 20 (Merck 822184) solution and blotted dry. Into each well 250 µl of streptavidin peroxidase solution (1 µl strepatvidin POD, 500 mU/µl [Boehringer 1089153] added to 30 ml assay buffer) was added and plates were incubated on plate shaker for 45 min at 4°C. Plates were washed and then developed for 45 min at 4°C on a plate shaker with 250 µl of tetramethybenzedine solution. The enzymatic color reaction was stopped using 50 µl of 2 M sulfuric acid. Absorbance was measured at a wavelength of 450 nm with an automated plate reader (VERSAmax microplate reader, Molecular Devices, Sunnyvale, California). This EIA had an intra- and inter-assay coefficient of variation of 6.3% and 10.3%, respectively.

3.2.7 Statistical analysis

General linear regressions and t-tests were performed using the software package STATISTICA 6. The assumption of normality was tested with Shapiro-Wilks test and the assumption of homogeneity of variances was tested with Levene’s test. If these assumptions were not met the data were log-transformed (x+1) (Quinn and Keough 2003). We used a t-test with a Bonferroni correction to examine the seasonal changes in hares’ responsiveness and FCM concentrations (thus significance levels are $P < 0.02$). All other comparisons of the means were considered significant if $P < 0.05$. We found no difference between males and females and the sexes were pooled. All data are expressed as means ± 1 SE.
3.3 Results

True base (sampled < 3 min) plasma free cortisol levels were directly correlated to bile cortisol metabolite levels ($r^2 = 0.53; F_{1,5} = 7.68, P < 0.05$; Fig. 1) and to FCM levels ($r^2 = 0.59; F_{1,8} = 6.63, P < 0.05$; Fig. 2). However, true base plasma total cortisol levels were not correlated to bile cortisol metabolites ($r^2 = 0.03; F_{1,5} = 1.21, P > 0.05$; Fig. 1) nor to FCM levels ($r^2 = 0.26; F_{1,8} = 0.16, P > 0.05$; Fig. 2). Nominal base (sampled > 3 min) were the first bleeds from the hormonal challenge experiment and represent samples of the hares stressed by capture, handling, and transport to the lab. Nominal base plasma free cortisol levels were not correlated to FCM levels ($r^2 = 0.03; F_{1,28} = 0.60, P > 0.05$; Fig. 3).

FCM levels directly reflected the changes in endogenous plasma free cortisol levels in response to the hormonal challenge. Hares with greater FCM concentrations exhibited a reduced suppression of free cortisol concentrations after the Dex injection ($r^2 = 0.33; F_{1,28} = 9.43, P < 0.001$; Fig. 4) and an increased, prolonged, production of free cortisol after the ACTH injection ($r^2 = 0.39; F_{1,28} = 13.60, P < 0.0001$; Fig. 5). There was a strong correlation between the Dex resistance and the ACTH stimulation tests ($r^2 = 0.26; F_{1,28} = 10.17, P < 0.003$).

Stress levels of hares increased from autumn 2006 to winter 2007, decreased in autumn 2007, and remained similar from autumn 2007 to winter 2008 as indicated by the Dex suppression test, by the ACTH stimulation test, and by FCM levels (Fig. 6). The Dex injection resulted in a greater suppression of free cortisol (i.e. hares had lower free cortisol levels after the injection) in autumn 2006 than in winter 2007 (by 3-fold; $t_{14} = -4.32, P < 0.02$), a reduced suppression in winter 2007 than in autumn 2007 (by 3-fold; $t_{11}$...
= - 4.04, \( P < 0.02 \)), and a similar suppression in autumn 2007 and in winter 2008 (\( t_{13} = 0.018, P > 0.02 \)). The ACTH injection resulted in a lower response (i.e. hares had lower free cortisol after the injection) in autumn 2006 than in winter 2007 (by 1.5-fold; \( t_{14} = - 2.58, P < 0.02 \)), greater response in winter 2007 than in autumn 2007 (by 1.5-fold; \( t_{11} = - 3.01, P < 0.02 \)), and a similar response in autumn 2007 and winter 2008 (\( t_{13} = - 2.03, P > 0.02 \)). FCM levels increased from autumn 2006 to winter 2007 (by 1.55-fold; \( t_{14} = - 3.83, P < 0.02 \)), decreased in autumn 2007 (by 1.5-fold; \( t_{11} = - 4.97, P < 0.02 \)), and remained similar in winter 2008 (\( t_{13} = - 0.732, P > 0.02 \)).

### 3.4 Discussion

We tested the assumptions that fecal glucocorticoid metabolite concentrations accurately reflect both an animal’s free cortisol in the blood and its ability to respond to a stressor and we validated both. Our results showed that bile cortisol metabolite concentrations and FCM concentrations mirrored that of true base plasma free cortisol concentrations when animals were bled within 3 min but not true base plasma total cortisol concentrations (Figs. 1 and 2). FCM concentrations did not reflect nominal base plasma free cortisol concentrations when sampling occurred more than 3 min after capture and handling (Fig. 3). However, the hormonal challenge got around the problem of capture and handling and an animal’s FCM levels were indicative of their ability to respond to a the Dex and ACTH injections (Figs. 4 and 5). Furthermore, seasonal changes in FCM concentrations in natural populations were in concordance with those in plasma free cortisol concentrations when hares were hormonally challenged (Fig. 6).
Studies have shown that fecal GC metabolites are positively correlated to total plasma GCs. Cavigelli (1999) compared fecal and plasma GC concentrations in semifree-ranging lemurs, *Lemur catta*. She found that fecal and plasma GC levels were significantly correlated. Mateo and Cavigelli (2005) found that in Belding’s ground squirrels, *Spermophilus beldingi*, fecal GC metabolites were positively correlated with plasma total GC levels (sampled within 3 min of handling) taken 24 h after the fecal samples were collected. However these studies did not determine CBG levels and could not calculate the plasma free cortisol concentrations. It is critical that FCM concentrations reflect free GC concentrations in the blood as only the free, unbound GCs are thought to be biologically active (Rosner 1990, but see Breuner and Orchinik 2001). Total GC concentrations do not necessarily reflect free GC concentrations. For example, house sparrows (*Passer domesticus*) show seasonal changes in their baseline and stress-induced total GC concentrations. However, their CBG capacity also varies seasonally, which resulted in similar free GC concentrations across seasons (Breuner and Orchinik 2001). Conversely, supplementally-fed snowshoe hares had similar total GC concentrations compared with controls. However, they had a significantly greater CBG capacity and this resulted in significantly lower free GC concentrations (Boonstra and Singleton 1993). Our results show that FCM concentrations reflected the physiological state of an animal - both the true base free GC concentrations and the responsiveness to a stressor. We found that hares with greater FCM concentrations had a greater true base free cortisol concentrations in their blood (Fig. 2) and a greater ability to respond to a stressor (Fig. 4-5). Furthermore, our findings show that FCM concentrations reflected a hare’s total response to a stressor not just the maximum response (Fig. 4). Since FCMs
are not a point sample of an animal’s state, but rather an integrated picture of the total amount of free GCs released in response to a stressor, it appears a very powerful method to assess the stress profile of an animal.

The magnitude and duration of free GC release are also equally important and this is often underappreciated in the study of stress physiology – our measure being the area under the response curve after the ACTH injection (Fig. 5). Dallman and Bhatnagar (2001) found that the biological effects of the stress response result from the hormone-receptor interactions over the entire time course of the stress response, not just at the peak of free GC release. For example, in a population of free-living baboons, Papio anubis, subordinates had a lower maximal free GC release to stressor than did dominants. However, the subordinates had a much longer duration of free GC response resulting in an overall greater total amount of free GC release (Sapolsky 1993). This greater free GC exposure was then linked to cardiovascular problems in subordinates (Sapolsky and Share 1994). Thus, the measurement of the total amount of free GC released, a function of both the maximum and duration of release, is the important variable to measure, not simply the maximum alone. Since FCM assays integrate both the baseline and total free GC release they provide a powerful indicator of the physiological state of an animal.

We have also shown that FCM concentrations can reliably track changes in plasma free cortisol concentrations. Snowshoe hares’ free cortisol concentrations fluctuated across the two years of this study and this was mirrored in their FCM concentrations (Fig. 5). These seasonal differences are likely due to the major differences in predation risk during this time (Sheriff et al. 2009a; Sheriff unpublished data). Very few studies have measured both the plasma GC concentrations and the FCM
concentrations at different time points. Wasser et al. (1997) found that the transfer of a captive owl from her usual enclosure to a novel environment at the Department of Animal Health resulted in a comparable response in both serum and fecal corticosterone levels. Mashburn and Atkinson (2004) found that Stellar sea lions exposed to an ACTH challenge had a 3-fold increase in serum cortisol concentrations and an 18-fold increase in FCM concentrations. Thus changes in FCM concentrations are a good indicator of changes in plasma GC concentrations in captive, experimental, and free-ranging animals.

In conclusion, our results support the assumptions that fecal GC metabolites reflect free, biologically active, GC concentrations in the blood and that fecal GC metabolites were an excellent predictor of the responsiveness of an animal to a stressor. Furthermore, we showed that fecal GC metabolite concentrations reliably track changes in free GC concentrations.
Fig. 3.1. Bile cortisol metabolite concentrations (ng/ml) in a sample of shot snowshoe hares \((n = 8)\) and the relationship to their plasma free cortisol concentrations (nmol/L) \(r^2 = 0.53; y = 5.40 + 0.0006x)\) and to their total cortisol concentrations (nmol/L) \(r^2 = 0.03; y = 87.29 + 0.0012x)\).
Fig. 3.2. Fecal cortisol metabolite concentrations (ng/g) in a sample of shot snowshoe hares \((n = 11)\) and the relationship to their plasma free cortisol concentrations (nmol/L) \((r^2 = 0.59; y = 3.15 + 0.026x)\) and to their plasma total cortisol concentrations (nmol/L) \((r^2 = 0.26; y = 5.06 + 0.20x)\).
Fig. 3.3. Fecal cortisol metabolite concentration (ng/g) of snowshoe hares \((n = 32)\) and the relationship to their plasma free cortisol concentration (nmol/L) at the nominal base bleed of the hormonal challenge \((\text{bled} > 3 \text{ min after capture and handling})\) \((r^2 = 0.03; y = 124.78 + 0.03x)\).
Fig. 3.4. Dexamethasone resistance in snowshoe hares. Fecal cortisol metabolite concentrations (ng/g) \((n = 32)\) at capture and their free plasma cortisol concentrations (nmol/L) 2 h after the dexamethasone injection \((r^2 = 0.33; y = -1.65 + 0.014x)\).
Fig. 3.5. ACTH stimulation in snowshoe hares. Fecal cortisol metabolite concentrations (ng/g) \((n = 32)\) at capture and their integrated plasma free cortisol response (nmol/L) from the time of the ACTH injection to 2 h later, measured cumulatively as the area under the response curve \((r^2 = 0.38; y = 19146.00 + 32.72x)\). Free cortisol concentrations were measured at 30 min, 60 min, and 120 min after the ACTH injection.
Fig. 3.6. Seasonal changes in plasma free cortisol (mean ± SE) from snowshoe hares subjected to a dexamethasone (Dex) resistance test (a) and to an adrenocorticotropic hormone (ACTH) stimulation test (b) and the changes in their fecal cortisol metabolite (FCM) concentrations at capture. The integrated response to ACTH was measured as the area under the curve. Hares were captured in the autumn ($n = 7$) and winter ($n = 9$) of 2006/07 and in the autumn ($n = 5$) and winter ($n = 11$) of 2007/08.
3.5 References


4. THE SENSITIVE HARE: SUBLETHAL EFFECTS OF PREDATOR STRESS
ON REPRODUCTION IN SNOWSHOE HARES

4.1 Introduction

Predation is a central organizing agent shaping population and community processes (Krebs et al. 2001a; Schmitz 2008). Traditionally, ecologists have focused on the direct effects of predation - the killing of prey (Paine 1966; Taylor 1984; Krebs et al. 1995). However, predators also have significant indirect effects on prey populations (see reviews by Lima 1998; Creel and Christianson 2008) and these effects can be as great as their direct effects (Schmitz et al. 1997; Nelson et al. 2004; Preisser et al. 2005; Pangle et al. 2007). Prey responses to the high risk of predation can be morphological such as changes in secondary sexual characteristics and anti-predator defences (Tollrian and Harvell 1999; Day and Young 2004; Vamosi and Schluter 2004) or behavioural such as changes in preferred habitats, in vigilance, and in foraging (Hik 1995; Lima and Bednekoff 1999; Childress and Lung 2003; Armitage 2004; Creel et al. 2005; Winnie and Creel 2007). These responses ultimately come at the cost of survival, growth, body condition, or reproduction (Hik 1995; Boonstra et al. 1998; Krebs et al. 2001a; Olaf and Halle 2004; Bian et al. 2005; Hodges et al. 2006).

The indirect effects of predators act through physiological processes. One of the most conserved processes in vertebrates is the ‘stress response’, defined here as the set of neural and endocrine responses that help restore homeostasis (Sapolsky 1987). Central to

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the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent secretion of glucocorticoids (GC), lasting several minutes to hours (Sapolsky 1992; Wingfield and Romero 2001). A stressor may be any environmental perturbation that disrupts homeostasis, such as harsh weather, habitat changes, anthropogenic disturbances, decreased food availability, and predation attempts (Sapolsky 1987). The presence of short-term elevated GC concentrations facilitates escape from life-threatening situations (Wingfield et al. 1998). However, chronic activation of the HPA axis may trade off future reproduction for present survival (Boonstra and Singleton 1993; Boonstra et al. 1998; Sapolsky et al. 2000; Romero and Wikelski 2001; Wingfield and Romero 2001).

The decline in reproduction not only has individual fitness consequences but may also have long-term population consequences (Wingfield and Sapolsky 2003). Though many studies have shown that elevated GC concentration can have negative effects on reproduction, these have been conducted on laboratory animals (e.g. Ferin 1999; Lesage et al. 2001; Hayward and Wingfield 2004; Romero 2004; Eriksen et al. 2006; Götz et al. 2008). Studies on free-ranging animals that suggest elevated GC concentrations have negative effects on reproduction often use GC or reproductive proxies without measuring GC concentration or reproduction directly (Bian et al. 2005; Saino et al. 2005; Charbonnel et al. 2008; Lidgard et al. 2008), or they correlate an increase in GC with a decline in reproduction on a population wide level without showing a direct causal link at the individual level (Boonstra et al. 1998; Hackländer et al. 2003; Lanctot et al. 2003; Young et al. 2006; but see Cyr and Romero 2007). Here we carry out a field study on
Snowshoe hares to examine the causal link between changes in GC concentrations and predator-induced stress.

Snowshoe hares (Lepus americanus) are an ideal species to study the effects of GCs on reproduction. Snowshoe hares undergo a regular cyclic fluctuation, with 8-10 years between peak densities (Keith 1963; Krebs et al. 1986). As hare populations increase so do that of their predators, but with a lag of 1-2 years. During the hare population decline, predators are the direct cause of up to 83% of hare deaths (Boutin et al. 1986; Krebs et al. 1995). Hare reproduction also cycles, with maximum rates occurring during the early increase phase (when predator numbers are lowest), but then progressively declining to a nadir during the decline (when predator numbers are at their peak), (Cary and Keith 1979; O’Donoghue and Krebs 1992; O’Donoghue et al. 1997; Stefan and Krebs 2001). Predators could be the indirect cause of this decline, with the inhibition of the gonadal axis being mediated by the stress of high predation risk through the activation of the HPA axis. Boonstra et al. (1998) showed that plasma cortisol concentrations (the major GC in snowshoe hares) fluctuated with the risk of predation, such that hares experiencing a greater risk of predation had higher plasma cortisol. They proposed that chronic stress, as measured by elevated cortisol concentrations, caused the marked deterioration of reproduction during the decline phase.

Here we test the hypothesis that elevated GC concentrations cause a decline in reproduction in free-ranging hares in two ways. First, in a natural monitoring study, we measured cortisol concentrations and reproduction 30 h after birth in natural populations of free-ranging snowshoe hares from 2006 to 2008. We estimated both the hare and the predator density during this time to determine when the population peak and the
maximum risk of predation would occur. Second, in an experimental manipulation, we increased the risk of predation during the last two-thirds of gestation in a sample of wild-caught snowshoe hares held in pens and measured cortisol concentrations and reproduction 30 h after birth. Cortisol concentrations were measured noninvasively using a fecal analysis enzyme immunoassay (EIA). Reproduction was measured as litter size, and offspring birth mass and RHF length.

In the natural monitoring study we predicted that as the risk of predation increased fecal cortisol metabolite (FCM) concentrations in dams would increase. In the experimental manipulation we predicted that FCM concentrations would be higher in the stressed group compared with the control group. In both studies we expected that an increase in FCM concentration in dams would cause a decrease in their litter size, offspring birth mass and offspring RHF length.

4.2 Methods

4.2.1 Snowshoe hare biology

Snowshoe hares are synchronous, seasonal breeders with mating occurring immediately post partum. This results in two to four distinct litter groups, depending on the phase of the population cycle (four litters during the early increase phase and these progressively decline to a nadir of only two litters during the decline phase; Stefan and Krebs 2001). Breeding begins in late April with the first litter born near the end of May, and each subsequent litter borne approximately 36-39 days later (Cary and Keith 1979; Stefan and Krebs 2001). Early litters are weaned at 24-28 days of age, but the last litter of the year may be nursed for up to 40 days (O’Donoghue and Bergman 1992). The young are born precocious and remain together for the first 3-5 days, after which they separate and only
come together once a night to nurse (O’Donoghue and Bergman 1992). Snowshoe hares do not have nests or burrows (Severaid 1942; Graf and Sinclair 1987) and are crepuscular, making it nearly impossible to monitor reproduction in the wild.

4.2.2 Animal trapping

Our research was approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care. Female snowshoe hares were live-trapped in the Shakwak Trench east of Kluane Lake, Yukon Territory (61º N, 138º W) using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.). The traps were set at 2200 h and checked at 0600 h and thus hares could only be in the traps for a maximum of 8 h. This is relevant as the lag between the production of cortisol in the body and the appearance of its metabolites in the feces is between 8-12 h. Therefore, the cortisol metabolites in the feces represent non-observer induced measures of stress.

Upon capture, each hare was weighed with a Pesola spring scale (± 10 g), its right hind foot (RHF) length measured as an index of body size, an ear-tag was placed in its right ear (No. 3 Monel tags, National Band and Tag Co., Newport, Kentucky, USA.), and its sexual condition assessed (see Krebs et al. 1986 for details). Pregnancy was determined by body mass, by the colour of the lactational tissue, and by palpating the abdomen (O’Donoghue and Krebs 1992; Stefan and Krebs 2001). Pregnant females were transferred to an outdoor enclosure constructed at the Arctic Institute Base for use in either the natural monitoring experiment or the stress manipulation experiment. The enclosure was a 3 m high game fence with a black, heavy-duty, fabric cloth surrounded by an electric bear-proof fence to protect the hares from mammalian predators such as lynx, coyotes and grizzly bears. The
ceiling of the entire enclosure was completely secured with chicken wire (2.5 cm) to prevent raptors (great horned owls and goshawks) and corvids (ravens and magpies) access to the hares in the pens. The enclosure was located in an isolated section of forest approximately 1 km from the main site of human activity.

4.2.3 Natural monitoring

Population densities of both the hares and the predators were measured, initially as part of the Kluane Boreal Forest Ecosystem Project (Krebs et al. 2001b) and thereafter as part of a monitoring study; here we present the data from 1990 to 2008 (two complete cycles). Snowshoe hare densities were estimated on two 36 ha grids. Live-traps were pre-baited with alfalfa cubes for 3-5 days before being set. Trapping sessions consisted of 2-3 nights of trapping within a 5-day period. Trapping did not occur on nights that dropped below -20 ºC. Population density was estimated with the program CAPTURE (Otis et al. 1978) and the Jolly-Seber full model, as in previous studies (e.g., Krebs et al. 1995).

Avian and mammalian predator populations fluctuate in synchrony with the hare cycle (Doyle and Smith 2001; O’Donoghue et al. 2001; Rohner et al. 2001). An index of the fluctuations in predator populations were obtained by using evidence from lynx and coyote data, as these are reflective of all other predators, including avian predators. We counted lynx and coyote tracks each winter (October through April) along a 25-km transect that traversed our study area, on days after fresh snowfalls while tracks were distinguishable. Tracks counts for lynx and coyotes are highly correlated to their population density in this valley (lynx: $r^2 = 0.95$, coyote: $r^2 = 0.88$) and thus give a reliable estimate of changes in predator density (O’Donoghue et al. 1997).
To standardize measurements across years, we monitored females’ cortisol concentration and reproduction during the first and second litters. To estimate reproductive output we live-trapped hares one-week on either side of the average parturition dates (first litter May 25; second litter June 30; mean parturition dates estimated from O’Donoghue and Krebs 1992 and Stefan and Krebs 2001). Pregnant females (n = 30) were transferred to the outdoor enclosure and placed in a 60 x 60 x 120 cm chicken wire maternity cage until parturition, (for details see O’Donoghue and Krebs 1992). Hares were held in the maternity cages for an average of 3 days and a maximum of 6 days. Hares were fed ad libitum with standard rabbit chow (Unifeed, Okotoks, Alberta; Unifeed Ltd Cat. #19-2103, 18% protein, crude fat 2%, crude fibre 18%) and apples, supplemented daily with natural browse (small branches with leaves and bark from Salix spp.) and water ad libitum.

Thirty hours after parturition dams were trapped within the maternity cages and a fecal sample obtained. We recorded litter size and each leveret was sexed, weighed (Pesola spring scales ± 1 g), measured (RHF length – mm), and ear-tagged (No. 1 Monel tags). Families were then released back at the site of capture. Neonates were placed in a litter site created at the base of a willow, under a dead fall, or at the base of a cluster of trees so that the female could easily locate them (Stefan and Krebs 2001). Before releasing the family all young were held up to the dam and she was released at the litter site after the young. Hares were transported to and from the enclosure in a burlap bag. This procedure did not affect mortality rates of dams as most released hares were re-trapped at a later date. As juveniles disperse at the time when they first enter the traps, it is difficult to assess their survival rate.
4.2.4 Experimental manipulation

A total of 26 pregnant hares (12 controls and 14 stressed) were live-trapped in the first week of May in 2006 and in 2007. Hares were transferred to the outdoor enclosure (as described above) and placed in individual 4 x 4 m chicken wire pens. Each pen was separated by a burlap covered wall to prevent hares from seeing each other. Control pens were separated from the stress pens by a black, heavy-duty, fabric cloth and a 4 m open corridor. Hares were fed as described above.

A trained dog was used to simulate a mammalian predator in the stress pens. We did this for two reasons. First, lynx and coyotes are responsible for approximately 60% of known hare predation (Krebs et al. 1995) and thus hares should have evolved to be acutely sensitive to a mammalian predator threat. Second, as it was critical that the simulated predator be under tight control, but both visually and olfactory evident, a highly trained dog (as opposed to a raptor) could be more easily handled and directed. The hares in the stress pens were separated from those in the control pens by a heavy black cloth and a 4 m corridor. The dog was taken into each stress pen for 1-2 min every other day for the last 15 days of gestation. During an exposure hares adjacent to the pen would remain hidden at the far end of their pen (4 m away). To ensure habituation did not occur (Dallman and Bhatnagar 2001), the dog was used at various times throughout the day and the order of exposure was randomized. The dog was trained not to bark or whine and did not physically contact any of the hares. The dog was not introduced once the females gave birth. Control hares had no contact, visually or physically, with the dog. Although they may have smelled it, control hares did not alter their behaviour during stress exposures. The same dog was used throughout the experiment.
Thirty hours after parturition, dams were live-trapped and a fecal sample taken. Reproductive measurements on the neonates were taken as above. Families were kept in the pens for an additional 28 days for a separate experiment. At the end of experimentation all hares were released back to the site of their capture.

4.2.5 Fecal cortisol metabolite analysis

We used an enzyme immunoassay (EIA) to measure fecal cortisol metabolite (FCM) concentration, validated specifically for snowshoe hares. Fecal samples were collected from underneath the live-trap on the morning of trapping, a maximum of 8 hours after the time of setting. Previously we showed (Sheriff et al. 2009) that there is an 8-12 h lag between cortisol production in the snowshoe hare and the appearance of its metabolites in the feces. Thus our samples provided an integrated measure of circulating cortisol prior to the stress of being captured. Samples were stored at -80°C within 1 h of collection at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80°C until analyzed.

Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) for 14-18 h to control for fibre and water content (Wasser et al. 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was diluted (1:10) with assay buffer and frozen at -80°C until analysis.
Fecal cortisol metabolite concentrations were measured following the methods outlined by Sheriff et al. (2009) using the 11-oxoaetiocholanolone-EIA developed by Palme and Mostl (1997). Briefly, 50 µl of extracted samples (in duplicate; diluted 1:25 with assay buffer) were incubated in duplicate with 100 µl of biotynilated steroid label (11-oxoaetiocholanolone-3-glucosiduronate-DADOO-biotin) and 100 µl antibody (11-oxoaetiocholanolone-3-HS:BSA raised in rabbits) at 4°C on a plate shaker overnight. Plates were then washed four times with 0.05% TWEEN 20 (Merck 822184) solution and blotted dry. 250 µl of streptavidin peroxidase solution (1 µl strepatvidin POD, 500 mU/µl [Boehringer 1089153] added to 30 ml assay buffer) was added into each well and plates were incubated on plate shaker for 45 min at 4°C. Plates were washed and then developed for 45 min at 4°C on a plate shaker with 250 µl of tetramethylbenzidine solution. The enzymatic colour reaction was stopped using 50 µl of 2 M sulfuric acid. Absorbance was measured at a wavelength of 450 nm with an automated plate reader (VERSAmax microplate reader, Molecular Devices, Sunnyvale, California). This EIA had an inter- and intra-assay coefficient of variation of 6.3% and 10.3%, respectively.

4.2.6 Statistical analysis

All data are expressed as means ± 1 SE, unless otherwise stated. ANCOVAs and ANOVAs were performed using the software package STATISTICA 6. The assumption of normality was tested with Shapiro-Wilks test and the assumption of homogeneity of variances was tested with Levene’s test. If these assumptions were not met the appropriate adjustment was made (log-transformation of data or Greenhouse-Geisser
adjustment; Quinn and Keough 2003). Comparisons of the means were considered significant if \( p < 0.05 \).

### 4.3 Results

#### 4.3.1 Natural monitoring

**4.3.1.1 Population density**

The snowshoe hare population reached a peak of 0.92 hares per ha in 2006 and declined to 0.79 and 0.35 hares per ha in 2007 and 2008, respectively (Fig. 4.1). This population peak was considerably lower than the previous peak in 1998 of 1.98 hares per ha. The track index of mammalian predator populations (lynx and coyotes) was highest in 2007 with approximately 56 predator tracks counted per track night over a 100 km transect, compared with a previous peak of 117 tracks in 1999 (Fig. 4.1). The track index prior to the peak, 2006, and just after it, 2008, was approximately 31 and 35 predator tracks counted per track night over a 100 km transect, respectively. Thus predators peaked one year after the hare peak.

**4.3.1.2 Fecal cortisol metabolite concentration**

To test for differences in FCM concentrations we ran a two-way ANOVA (litter group x year). We found an effect of litter group \( (F_{1,24} = 11.70, \quad P < 0.005) \) but no effect of year \( (F_{2,24} = 1.42, \quad P > 0.05) \) or interaction between litter group and year \( (F_{2,24} = 2.36, \quad P > 0.05; \quad \text{Fig. 4.2a}) \). Fecal cortisol metabolite concentrations in dams decreased 52% from the first litter \( (509.35 \pm 107.22 \text{ ng/g/kg, } n = 15) \) to the second litter \( (248.72 \pm 45.56 \text{ ng/g/kg, } n = 15) \). Fecal cortisol metabolite concentrations were similar in 2006 \( (383.54 \pm 84\text{ ng/g/kg, } n = 15) \).
121.15 ng/g/kg, n = 10), in 2007 (476.62 ± 107.48 ng/g/kg, n = 11), and in 2008 (263.92 ± 56.30 ng/g/kg, n = 9).

4.3.1.3 Reproduction

To test for differences in reproductive measures we ran a two-way ANCOVA (litter group x year) and included FCM concentration as a continuous covariate. For litter size we found an effect of litter group ($F_{1,23} = 8.24$, $P < 0.01$) and FCM concentration ($F_{1,23} = 4.47$, $P < 0.05$), but no effect of year ($F_{2,23} = 3.37$, $P > 0.05$) or interaction between year and litter group ($F_{2,23} = 1.62$, $P > 0.05$). Litter size was 19% smaller in the first litter (3.87 ± 0.22 young) compared with the second litter (4.73 ± 0.18 young) and litter size was negatively correlated to FCM concentrations in dams (Fig. 4.3a).

For offspring body mass we found an effect of litter group ($F_{1,23} = 13.55$, $P < 0.005$) and FCM concentration ($F_{1,23} = 4.47$, $P < 0.05$), but no effect of year ($F_{2,23} = 0.93$, $P > 0.05$) or interaction between year and litter group ($F_{2,23} = 0.56$, $P > 0.05$). Offspring had a 24% lower body mass in the first litter (52.26 ± 2.98 g) compared with the second litter (68.03 ± 2.80 g) and offspring body mass was negatively correlated to FCM concentrations in dams (Fig. 4.4a).

For offspring RHF length we found an effect of litter group ($F_{1,15} = 10.94$, $P < 0.005$), year ($F_{1,15} = 8.38$, $P < 0.05$), and FCM concentration ($F_{1,15} = 18.69$, $P < 0.001$), but no interaction between litter group and year ($F_{2,15} = 0.014$, $P > 0.05$). Offspring were 11% smaller in the first litter (33.67 ± 1.22 mm) than in the second litter (37.61 ± 0.83 mm), and 10% smaller in 2007 (34.06 ± 1.04 mm) than in 2008 (37.56 ± 1.14 mm). We
did not measure RHF length in 2006. Offspring RHF length was negatively correlated to FCM concentrations in dams (Fig. 4.5a).

4.3.2 Experimental manipulation

In the experimental manipulation 11 out of 12 control hares and 9 out of 14 stressed hares gave birth to viable young. To test for differences in birth rate we ran a Pearson Chi-square test and found that control hares had a significantly higher birth rate than stressed hares ($\chi^2 = 4.91, P < 0.05$). As the remaining five hares in the stressed group gave birth to pre-term or stillborn young, they were considered unsuccessful, and included in the analysis of FCM concentration as a separate group referred to as unsuccessful-stressed. They were not included in the reproductive analysis since they did not birth viable young. A single control hare did not give birth and she was not included in any of the analysis.

4.3.2.1 Fecal cortisol metabolite concentration

To test for differences in FCM concentration at the time of capture and 30 h after birth we ran a one-way ANOVA. We found, at the time of capture, there was no difference in FCM concentrations between control dams, stressed dams, and unsuccessful-stressed dams ($F_{2,22} = 0.91, P > 0.05$; Fig. 4.2b). At the time of birth FCM concentrations were significantly different in the three groups ($F_{2,22} = 19.20, P < 0.0001$; Fig. 4.2b). Control dams had FCM concentrations 54% and 89% lower than stressed dams (Tukey’s HSD post-hoc $P < 0.05$) and unsuccessful-stressed dams (Tukey’s HSD
Stressed dams had FCM concentrations 75% lower than unsuccessful-stressed dams (Tukey’s HSD post-hoc $P < 0.005$).

### 4.3.2.2 Reproduction

To test for differences in reproductive measures we ran a one-way ANCOVA, including FCM concentration as a continuous covariate. For litter size we found no effect of treatment ($F_{1,17} = 0.06$, $P > 0.05$) or FCM concentration ($F_{1,17} = 1.70$, $P > 0.05$). Control dams had a similar litter size (3.64 ± 0.24 young) compared with stressed dams (3.22 ± 0.28 young; Fig. 4.3b).

For offspring body mass we found an effect of treatment ($F_{1,17} = 11.68$, $P < 0.005$) and FCM concentration ($F_{1,17} = 13.96$, $P < 0.005$). Control dams gave birth to offspring with a 58% greater body mass (57.23 ± 2.69 g) compared with stressed dams (36.16 ± 3.35 g). Increasing FCM concentrations in dams were associated with a reduced offspring body mass (Fig. 4.4b).

For RHF length we found an effect of treatment ($F_{1,9} = 10.59$, $P < 0.01$) and FCM concentration ($F_{1,9} = 5.29$, $P < 0.05$). Control dams gave birth to offspring with an 18% greater RHF length (36.08 ± 0.86 mm) compared with stressed dams (30.57 ± 0.74 mm). Increasing FCM concentrations in dams were associated with a reduced offspring RHF length (Fig. 4.5b).
4.4 Discussion

The snowshoe hare population in our study area peaked in 2006 and the predators peaked in 2007 (Fig. 4.1). In the natural monitoring study we found that female FCM concentrations were higher at parturition of their first litter compared with that at their second (Fig. 4.2a). However, we did not find differences in female FCM concentrations between years. In the experimental manipulation study we found that unsuccessful-stressed dams (those that did not give birth to viable young) had the highest FCM concentrations followed by stressed dams and then control dams (Fig. 4.2b). In both studies, dams with higher FCM concentrations had lower reproductive fitness in terms of number and quality of young (Fig. 4.3-4.5).

4.4.1 Fecal cortisol metabolite concentrations in dams

Many factors have been shown to affect stress levels in wild animals including density and social status, parasitism, food, and the risk of predation (Boonstra et al. 1998; Creel 2001; Chapman et al. 2007). It has long been recognized that high population densities could disrupt spacing behaviour and increase agonistic interactions and competition leading to an increase in stress and ultimately a decline in reproduction (Christian 1980). More recently, it has been shown that not only density but social status can influence GC concentrations and disrupt breeding (Creel 2001; Young et al. 2006). However, there is little evidence of this in hares. Boonstra et al. (1998) found that hares were less stressed living in experimentally fed populations whose densities were 4 to 13 times those of controls. Although, hares have been shown to display dominance hierarchies in pens and at feeding areas in the wild (Graf 1985), they are not territorial
and have broadly overlapping home ranges (Boutin 1984). Furthermore, our results show that hares in 2006 (peak population) were not different than hares in 2008 (second year of the decline; Fig. 4.1 and 4.2a).

Parasites can also be important in shaping animal communities and have been shown to influence GC concentrations in mammals (Chapman et al. 2007). In snowshoe hares Keith et al. (1986) studied parasitism for many years in Alberta and concluded that the many parasites of hares were not a direct cause of mortality. Experimental work with antihelminthics in field populations of hares had no measureable impact on survival or reproduction (Sovell and Holmes 1996), or produced effects only in combination with predation and food (Murray et al. 1997). Thus parasitism may affect some hare populations but is likely not a direct factor affecting GC concentrations.

Food and predation are the two of the greatest factors affecting animal populations and these have been shown to have interactive synergistic affects (Krebs et al. 1995, 2001a). In snowshoe hares the change in FCM concentrations between litters (Fig. 4.2a) could be a result of first, an increase in food availability during the growing season, and second, a decrease in predation risk. The first litter occurs during the late winter-early spring when the winter snowpack is in the process of melting and prior to the flush of new vegetation, while the second litter occurs during late spring-early summer when new vegetative growth is nearing it peak (Sinclair et al. 1982). This change in quality and quantity of food could explain the differences in FCM concentrations. Reduced food intake has been found to cause an increase in cortisol levels in mammals and birds (Harris et al. 1994; Kitasisky et al. 2001; Ortiz et al. 2001). Second, the difference between the litter groups could be due to the decrease in the risk
of predation from the first litter to the second. Boutin et al. (1986) found that predation rates decreased from winter to summer. Thus, the risk of predation should also decrease at this time. Since food quantity and quality also increase from the first litter to the second litter, an increase in food availability may also allow hares to forage in a less risk-prone manner (Hik 1995; Murray 2002). Likely, the effects of the increase in food availability and decrease in predation are not mutually exclusive and a combination of the two could explain the decline in FCM concentration from the first to the second litter.

We found that FCM concentrations were similar in the hare peak (2006) and decline (2007 and 2008; Fig. 4.2a), but predicted that as the risk of predation increased FCM concentrations in dams would also increase. Part of the explanation may be that the females in our study were a high quality subset of females from the hare population, all giving birth within one week of the estimated parturition date. Our experimental results showed that females that gave birth to non-viable young, (either stillborn or aborted), had high FCM concentrations compared with females that gave birth (Fig. 4.2b). Furthermore, the number of females that give birth to non-viable young increased during the decline phase (Stefan and Krebs 2001). Had we assessed FCM concentrations from all females within the population, not just the successful ones, we may have seen yearly differences.

In our experimental manipulation stressed hares had elevated FCM concentrations compared with control hares (FCM concentrations were within the range found in the natural monitoring experiment; Fig. 4.2b). That hares did not become habituated to the repeated dog treatment shows that they are extremely sensitive to predation risk. Within treatments there was also considerable individual variation in FCM concentration (Fig.
4.2b). This suggests that individuals naturally differed in their physiological response and ability to cope with stressors. This is consistent with the results found by Pride (2005) for ringtailed lemurs, *Lemur catta*, and by Cabezas et al. (2007) for European wild rabbits, *Oryctolagus cuniculus*.

Together the natural monitoring and experimental manipulation studies argue that snowshoe hares are highly sensitive to changes in the risk of predation and that slight differences lead to measurable differences in FCM concentrations. Boonstra et al. (1998) found that plasma cortisol levels were higher during the decline phase of the hare cycle compared with the low phase and that this was due to the risk of predation that hares experience. We are currently investigating long-term, population level, seasonal and yearly changes in both plasma cortisol and FCM concentrations in response to the changes in the risk of predation during the snowshoe hare population cycle.

4.4.2 Reproduction

In both the natural monitoring and experimental manipulation studies we found that higher FCM concentrations in dams were associated with a decline in reproductive indices (Figs 4.3-4.5) and extremely high FCM concentrations were associated with non-viable births, either abortions or stillborn litters (Fig. 4.2b). Chronic exposure to elevated GC concentrations can have deleterious physiological consequences to reproduction by decreasing the amount of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) produced in the body (Ferin 1999; Owen et al. 2005). Davis and Meyer (1973) found that in snowshoe hares seasonal variation in gonadotropins paralleled seasonal changes in reproduction, and that there was a sharp decline in
gonadotropin levels which coincided with a sharp decline in reproductive rates. We suggest that predator-induced changes in GC concentrations may be responsible for the changes seen in gonadotropin levels and ultimately the changes in reproduction.

However, the question remains as to why snowshoe hares would decrease their reproductive output when their chance of survival also decreases. There are three possible answers that we will discuss here. The first is that GC concentration affects survival and reproduction in a classic trade-off scenario. The second is that GC concentrations match offspring quality with the dam’s ability for maternal investment. The third is that GC concentrations help regulate maternal programming of the offspring.

A classic trade-off of reproduction for survival is easily understood in predator-prey relationships. As the risk of predation increases, prey species alter their behaviour to increase survival (Hik 1995; Lima 1998) and these changes are modulated by the short-term release of GCs (Sapolsky et al. 2000; Wingfield and Kitaysky 2002). Although the magnitude of the stress response and a high concentration of GCs can negatively affect survival (Pride 2005; Blas et al. 2007), recent work has shown that at moderate levels chronic exposure to GCs can increase survival (Cote et al. 2006). Cabezas et al. (2007) found that in a free-ranging population of European wild-rabbits long term exposure to moderately elevated GC concentrations increased survival after the stressor was removed. Snowshoe hares are a good candidate for increasing GC concentrations in order to survive at the cost of reproduction. Hik (1995) found that snowshoe hares alter their behaviour in response to increasing predation risk, and we know that the stress response of hares is highly sensitive to changes in the risk of predation (shown above; Boonstra et al. 1998). However, the trade-off hypothesis is an
unlikely evolutionary driving force in snowshoe hares. Hares have both a limited breeding period and a very poor survival rate. Snowshoe hares breed only during the summer months from May to August, and over 70% of the current year’s breeding population is made up of hares born the previous year (Krebs et al. 2001a). Furthermore, during the decline phase, adult survival rates actually decrease and can drop as low as 65% over 30 days (Krebs et al. 2001a). Since the gestation length for snowshoe hares is 35-37 days (Cary and Keith 1979), there is only a 42% chance of living long enough to give birth to a second litter and only a 23% chance of living long enough to give birth to a third litter. Thus, it would benefit snowshoe hares to maximize reproduction at the cost of an already low chance of survival (see Wingfield and Sapolsky 2003 for review).

Love and Williams (2008) recently suggested that maternally derived GCs could act as an adaptive mechanism linking maternal quality to offspring quality. The GC-induced matching of offspring phenotype could reduce the investment in current reproduction for low-quality mothers resulting in fitness gains through increased survival and future fecundity. Although we did not test this directly, we do not believe that it is occurring in the snowshoe hare. As mentioned above, hare survival rate is extremely low and thus mothers should maximize investment in their current reproduction. Furthermore, Hodges et al. (1999) found that body condition does not directly affect reproduction in snowshoe hares.

The adaptive advantage of maternal programming is easy to conceptualize. If a pregnant female is living in an environment where the risk of predation is high, it is beneficial that she transmit anti-predator behaviours to her offspring. It is logical that this signal is transmitted through the HPA axis, as it is not only responsible for the stress
response but it is also associated with certain anti-predator behaviours such as fearfulness, anxiety, and vigilance (Meaney 2001; Seckl 2004). An increase in prenatal GC concentrations has also been shown to influence offspring dispersal and survival (Silverin 1997; Meylan et al. 2002; Meylan and Clobert 2005). Many studies have shown that the HPA axis is highly susceptible to permanent programming during early life development (e.g., Francis and Meaney 1999; Matthews 2002; Seckl 2004; Owen et al. 2005) and prenatally stressed offspring have been shown to have lower glucocorticoid and mineralocorticoid receptor expression in the hippocampus leading to elevated GC concentrations (Welberg and Seckl 2001; Welberg et al. 2001) as well as higher reactivity of the HPA axis to stressors (Hayward and Wingfield 2004). For snowshoe hares maternal programming of offspring through elevated GC concentrations could be highly adaptive. Hares give birth to precocial young without a protective nest or burrow and do not stay with them after birth, returning only briefly each night to nurse (Keith and Windberg 1978). O’Donoghue (1994) showed that the proximate cause of mortality for offspring was predation and that 70% of juvenile mortality occurred within the first 5 days after birth, and 51% of litters had no survivors after 14 days. Thus, young are highly vulnerable to predation. As the risk of predation increases, the elevated GC concentrations of the dam could prenatally program the HPA axis of the offspring such that they are born with a greater stress response, increased vigilance and anxiety-like behaviours, and increased dispersal rates. Thus, elevated GC concentrations during times of high predation risk may cause a decline in reproductive output, but may ultimately increase fitness by promoting juvenile survival.
Here we have shown that increases in GC concentrations in individual dams are associated with a decline in reproduction. For snowshoe hares these increased GC concentrations are likely explained by both food and predation; however this is not the case for all species. GC concentrations can also be influenced by density and social status, parasitism, weather, and human activity among other things (Christian 1980; Wasser et al. 1997; Creel 2001; Romero and Wikelski. 2001; Chapman et al. 2007).

Since we found that elevated GC concentrations, not simply predation, are associated with a decline in reproduction the type of stressor is essentially irrelevant. Our study has broad implications to all physiologically stressful situations.
Fig. 4.1. Snowshoe hare, lynx, and coyote population density in the southwestern Yukon, Canada from 1994 to 2008.
Fig. 4.2. Fecal cortisol metabolite (FCM) concentrations (means ± SE) of female snowshoe hares from the two studies. (a) The natural monitoring study 30 h after birth in litter 1 and 2 across three years. Dams from litter 1 (pooled years) had elevated FCM concentrations compared with those from litter 2 (pooled years) \(P < 0.005\). (b) The experimental manipulation at the time of capture and 30 h after birth. There was no difference in FCM concentrations at capture \(P > 0.05\); however at birth, control dams had reduced FCM concentrations compared with stressed successful dams and with stressed unsuccessful dams \(P < 0.0001\).
Fig. 4.3. Fecal cortisol metabolite (FCM) concentration in dams and their litter size. (a) In the natural monitoring study, first litters (n = 15, open symbols) were smaller than second litters (n = 15, closed symbols; \( P < 0.01 \)) (\( y = -0.0012x + 4.75, r^2 = 0.19; P < 0.05 \)). Each data point represents a single dam from 2006 (n = 10), 2007 (n = 11), and 2008 (n = 9). (b) In the experimental manipulation, control dams (n = 11) and stressed dams (n = 9) had similar litter sizes (\( P > 0.05 \)) (\( y = -0.0008x + 3.80, r^2 = 0.10; P < 0.05 \)). Each data point represents a single dam.
Fig. 4.4. Fecal cortisol metabolite (FCM) concentration in dams and the average body mass of their offspring 30 h after birth. (a) In the natural monitoring study, first litter offspring (n = 15, open symbols) were lighter than second litter offspring (n = 15, closed symbols; \( P < 0.005 \)) (\( y = -0.0234x + 69.14, r^2 = 0.32; P < 0.05 \)). Each data point represents a single dam, from 2006 (n = 10), 2007 (n = 11), and 2008 (n = 9). (b) In the experimental manipulation, offspring from control dams (n = 11) were heavier than those from stressed dams (n = 9; \( P < 0.005 \)) (\( y = -0.032x + 62.11, r^2 = 0.57; P < 0.005 \)). Each data point represents a single dam.
Fig. 4.5. Fecal cortisol metabolite (FCM) concentration in dams and the average right hind foot (RHF) length of their offspring 30 h after birth. (a) In the natural monitoring study, first litter offspring (n = 10, open symbols) had smaller RHF length than second litter offspring (n = 10, closed symbols; \(P < 0.005\)) (\(y = -0.0101x + 39.511, r^2 = 0.64; P < 0.001\)). Each data point represents a single dam from 2007 (n = 11), and 2008 (n = 9). (b) In the experimental manipulation, offspring from control dams (n = 5) had a smaller RHF length than those from stressed dams (n = 6; \(P < 0.01\)) (\(y = -0.007x + 36.489, r^2 = 0.52; P < 0.05\)). Each data point represents a single dam.
4.5 References


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5. THE GHOSTS OF PREDATORS PAST: POPULATION CYCLES AND THE ROLE OF MATERNAL PROGRAMMING UNDER FLUCTUATING PREDATION RISK

5.1 Introduction

Adaptations to the direct (e.g. a severe storm) and indirect (e.g. risk of predation) effects of environmental stressors are essential for ensuring individual fitness in natural populations. The environment that an individual’s mother experiences may also affect their fitness. Mothers influence the development of their offspring genetically, by passing on their genes, but also through maternal effects - the non-genetic effects of the mother on the development of her offspring. Maternal effects can cause a resemblance not just between a mother and her offspring but between grandmothers and grand-offspring, and these effects may affect not just individuals but entire populations (Kirkpatrick and Lande 1989). In the biomedical literature, these maternal effects are referred to as maternal programming, and that is the way we will refer to this concept here. Programming effects reflect the influence of a specific environmental factor during the developmental period before or just after birth on the organization of target tissues and/or gene-expression patterns that affect function throughout the life (Meaney et al. 2007). Mechanisms responsible for this maternal programming may vary between organisms and in mammals stress hormones may play an organizational role.

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4 A version of this chapter has been accepted by Ecology: Sheriff, M.J., C.J. Krebs, and R. Boonstra. The ghosts of predators past: population cycles and the role of maternal programming under fluctuating predation risk.
In mammals, numerous laboratory studies have shown that stressors acting during pregnancy and lactation can have long lasting effects on offspring (Meaney et al. 2007; Weinstock 2008; Mastorci et al. 2009). The mechanisms underlying this relationship involve an increase in glucocorticoids (GC) in either the fetus or neonate.

Glucocorticoids (corticosterone in mice and rats, cortisol in most other mammals) are produced by the hypothalamic-pituitary-adrenal (HPA) axis in response to a stressor (defined as any environmental disturbance that disrupts homeostasis; Owen et al. 2005). In laboratory animals, increased levels of maternal stress hormones or exposure to stressors has been linked to offspring depression, anxiety-like behaviors, and alterations in HPA function and brain development (Abe et al. 2007; Meaney et al. 2007; Kapoor et al. 2008).

Extrapolating from laboratory studies to natural populations is problematic in that most of the former have subjected pregnant females to highly artificial stressors (e.g. immobilization, immersion in cold water, bright lights - Henry et al. 1994; Kapoor and Matthews 2005; Meaney et al. 2007), whereas in the latter, reproductive females have a long evolutionary history of adaptation to natural stressors (predation, social interactions, disease, or severe weather). Furthermore, in the laboratory, the timing when mothers are exposed to stressors markedly affects their subsequent impact on the offspring (Kapoor and Matthews 2005). In contrast, though some stressors in nature may be very short-lived (e.g. a severe snowfall), others are chronic for the length of the pregnancy and lactation (e.g. high predation risk or high population density) and although chronic, these stressors are not static and can fluctuate (i.e. although prey may be exposed to a high risk of predation the number of predators and prey will change). As well, in nature, animals are
exposed to multiple stressors simultaneously and may have a variety of coping mechanisms that allow them to integrate all of these (Wingfield and Sapolsky 2003). For example, in the arctic, free-ranging male white-crowned sparrows abandoned their normal nesting behavior in response to a spring snow storm, but resumed nesting after it had passed. The change in behavior was associated with plasma corticosterone concentrations going from high to low, respectively, over that interval (Wingfield et al. 1983).

Here we examine a free-ranging population of snowshoe hares, *Lepus americanus*, from the Yukon to elucidate the effects of natural stressors during pregnancy on the offspring’s physiological development. Snowshoe hares undergo a regular cyclic fluctuation, with 8-10 years between peak densities (Keith 1963; Krebs et al. 1986). A major factor influencing the hare cycle is predation. As hare populations increase so do those of their predators, but with a lag of 1-2 years. During the population decline, predators are the direct cause of up to 100% of hare deaths (Hodges et al. 2001) and of these, lynx and coyote were responsible for 65-75% of predator-caused deaths, with raptors being responsible for the rest. Furthermore, snowshoe hares are highly sensitive to changes in the risk of predation. In the late winter Boonstra et al. (1998a) showed that plasma cortisol concentrations (the major GC in snowshoe hares) fluctuated with the risk of predation, such that hares experiencing a greater risk of predation had higher plasma cortisol. However, they did not link these GC changes directly to the time when hares were breeding and, in terms of maternal programming, such predation-induced stress must occur during the summer months when reproduction occurs. In addition, they did not link the maternal state with that in their offspring.
In this study we tested whether chronically elevated GC concentrations in dams increased the concentration of the stress hormones of their offspring. We also measured the ability of offspring to mobilize energy and their body condition in response to an increase in maternal GC concentrations. First, in a natural monitoring study in the Yukon we measured fecal cortisol metabolite (FCM) concentration during the breeding season in dams (within one week after parturition) and in juveniles at weaning (postnatal day [pnd] 28) and after weaning (pnd 60, 90, and 120) to investigate the long-term programming of prenatal GC exposure. As hares have up to four synchronous litter groups during the breeding season, we assessed whether litter groups differed both within a year and among years to examine the effects of seasonal changes in vegetation growth (Seccombe-Hett and Turkington 2008) and the effects of among year differences in predation risk (O’Donoghue and Boutin 1995). This study took place from 2005 (increase) to 2008 (decline). Second, in a captive study we measured FCM concentrations in dams at birth and measured their offspring’s response to a hormonal challenge at weaning. In the juveniles we obtained measures of the stress response (plasma free-cortisol and maximum corticosteroid-binding capacity [MCBC – a measure of corticosteroid-binding globulin capacity]), of energy mobilization (glucose and free-fatty acids), of condition (packed red blood cell volume), and of immunity (white blood cell counts). This study took place in 2006 (peak) and 2007 (decline).
5.2 Methods

5.2.1 Natural monitoring study

Population densities of both the hares and lynx were measured as part of a monitoring study (Krebs et al. 2001a) and densities from 2002 to 2008 are shown here. Snowshoe hare densities were estimated with the program CAPTURE and the Jolly-Seber full model from trapping records on two 36 ha grids (Krebs et al. 1986). Lynx tracks were counted along a 25-km transect that traversed our study area, on days after fresh snowfalls while tracks were distinguishable. Lynx densities were estimated from the regression \( y = 0.355 + 0.288x \), where \( x \) equals track counts (Hone et al. 2007). An index of snowshoe hare overwinter survival was estimated as the proportion of hares present in spring of that present in the autumn (i.e. density in spring/density in previous autumn).

Physiological stress measurements occurred over 4 summers from 2005 to 2008. Snowshoe hares were live-trapped in the Shakwak Trench east of Kluane Lake, Yukon Territory (61° N, 138° W) using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.). The traps were set at 2200 h and checked at 0600 h and thus hares could only be in the traps for a maximum of 8 h. This is relevant as the lag between the production of cortisol in the body and its appearance in the feces as metabolites is between 8-12 h (Sheriff et al. 2009b). Therefore, the cortisol metabolites in the feces represent non-observer induced measure of stress hormones.

We monitored FCM concentration in females one-week after the mean parturition date during the first (n = 32), second (n = 32), and third (n = 20) litter of the breeding season (May to August). FCM concentrations were monitored in juvenile hares from the first (n = 36) and second (n = 35) litter (and third (n = 5) litter in 2006) within 3 days of
weaning (pnd 28). Since the relatedness of dams and juveniles is unknown, we compared a pooled sample of the average dam FCM concentrations to the average juvenile FCM concentrations from each litter group in each year.

To examine the long-term effects of prenatal GC exposure we compared first and second litter juveniles in August (pnd 60 and 28 for first (n = 25) and second (n = 22) litter, respectively) when juveniles are still growing and in October (pnd 120 and 90 for first (n = 13) and second (n = 11) litters, respectively) when juveniles are adult size.

Upon capture, each hare was weighed with a Pesola spring scale (± 10 g), its right hind foot (RHF) length measured as an index of body size, an ear-tag was placed in its right ear (No. 3 Monel tags, National Band and Tag Co., Newport, Kentucky, USA.), and its sexual condition assessed (see Krebs et al. 1986). A fecal sample was also collected from below the trap. Lactation was determined by the color of the lactation tissue, and mating of the hair around the nipple. Juvenile age was determined by body mass. O’Donoghue and Krebs (1992) found that juveniles weigh 397 – 492 g and 905 – 1000 g within 3 days of pnd 28 and pnd 60, respectively. At pnd 90 and 120 juvenile hares are the same size as adults and indistinguishable from them. Thus, we only collected feces from juveniles of known age.

5.2.2 Captive study

A total of 8 pregnant hares were transferred to an outdoor enclosure constructed at the Arctic Institute Base, Kluane Lake (southwestern Yukon, Canada) and placed in individual 4 x 4 m chicken wire (2.5 cm) pens (see Sheriff et al. 2009a for details). Hares were held in pens for an average of 20 days before parturition and for 28 days thereafter.
Hares were fed ad libitum with standard rabbit chow (Unifeed, Okotoks, Alberta; Unifeed Ltd Cat. #19-2103, 18% protein, crude fat 2%, crude fiber 18%) and apples, supplemented daily with natural browse (small branches with leaves and bark from *Salix spp.*) and water ad libitum.

Thirty hours after parturition, dams were live-trapped and a fecal sample taken. Reproductive measurements on the neonates were taken for a separate study (Sheriff et al. 2009a). Twenty-eight days after parturition dams and juveniles were live-trapped within the enclosure. Dams were released at the site of capture. Juveniles were subject to a hormone challenge at the Arctic Institute Base before being released to the site of capture of their dams.

5.2.3 Fecal cortisol metabolite analysis

We used an enzyme immunoassay (EIA) to measure fecal cortisol metabolite (FCM) concentrations, validated specifically for snowshoe hares. Samples were stored at -80°C within 1 h of collection at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80°C until analyzed.

Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) for 14-18 h to control for fiber and water content (Wasser et al. 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was diluted (1:10) with assay buffer and frozen at -80°C until analysis. Fecal cortisol metabolite concentrations were measured
following the methods outlined by Sheriff et al. (2009b) using the 11-o xoaoetiocholanolone-EIA developed by Palme and Mostl (1997). This EIA had an inter- and intra-assay coefficient of variation of 6.3% and 10.3%, respectively.

5.2.4 Hormone challenge

Juvenile hares were captured, transferred to a burlap bag, and taken to a dimly lit laboratory and allowed to settle down and habituate for 1-2 h prior to the challenge. Each hare was bled three times (0.3 ml per bleed) from an ear artery using 28 gauge needles (0.36 x 13 mm) into heparinised 0.5 ml syringes (Lo-Dose U-100 insulin syringes, Becton Dickinson and Company, New Jersey, USA). The first blood sample (basal bleed) was immediately followed by an injection of 0.4 mg/kg of dexamethasone sodium phosphate (Sabex, Quebec, Canada) into an ear vein. The second bleed (Dex bleed) occurred 2 h later, followed immediately by an intramuscular injection in the thigh of 40 µg/kg of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada). The final bleed (P60 bleed) occur 1 h later.

Measurement of glucose concentrations (using a FreeStyle glucometers -Abbott Diabetes Care, Inc., Alameda, CA) and preparation of blood smears were completed within 5 min and measurement of packed red-blood-cell volume (PCV - measured in duplicate after a 9 min centrifugation at 13 460 g on an IEC Micro-Hematocrit Centrifuge, Model 5413) and staining of slides (using a modified Wright stain technique called Diff-Quick [Dade International Inc., Florida, USA]) were completed within 45 min of blood collection. Blood smears and PCV were conducted on the first bleed only and glucose concentrations were measured after every bleed. The remaining blood was
centrifuged at 8 800 g for 10 min in an Eppendorf Micro Centrifuge 5413. The separated plasma was then frozen at -80 °C at the Arctic Institute Base and at the University of Toronto, until analysis for plasma cortisol, MCBC and free fatty acids (FFA).

Total plasma cortisol was measured in duplicate using a radioimmunoassay (Clinical Assays GammaCoat Cortisol $^{125}$I RIA Kit, DiaSorin, Minnesota, USA) with an inter- and intra-assay coefficient of variation of 12.4% and 2.4%. MCBC levels were measured in duplicate using a radioimmunoassay described by Boonstra and Singleton (1993), with an inter- and intra-assay coefficient of variation of 2.6% and 4.9%. Free cortisol concentrations were calculated using the procedures and binding coefficients outlined in Boonstra et al. (1998a).

Free fatty acids were measured in duplicate using an in vitro enzymatic colorimetric method assay for the quantitative determination of non-esterified fatty acids (HR Series NEFA-HR [2], Wako Diagnostics, Virginia, USA). This assay had an inter- and intra-assay coefficient of variation of 5.2% and 9.9%.

White blood-cell counts from stained slides were carried out at the University of Toronto. Differential white blood-cell counts were based on counts of 100 leucocytes.

5.2.5 Statistical analysis

All data are expressed as means ± SE, unless otherwise stated. ANOVAs, general linear models and Newman-Keuls post-hoc analyses were performed using the software package STATISTICA 6. Mixed effects models were performed using the software package STATISTICA 9. The assumption of normality was tested with Shapiro-Wilks test and the assumption of homogeneity of variances was tested with Levene’s test. If
these assumptions were not met data was log transformed and the assumptions re-tested. Comparisons of the means were considered significant if $P < 0.05$.

5.3 Results

The snowshoe hare population increased 46-fold from a spring low of 2 (2-4, 95% confidence limits) hares/km$^2$ in 2002 to a spring peak of 92 (85-113) hares/km$^2$ in 2006 and then declined 2.6-fold over the next two years to 35 (34-43) hares/km$^2$ in the spring of 2008 (Fig. 1). During the increase phase over-winter survival must have been extremely high, with spring density estimates of 81% (2002-2003), 149% (2003-2004), 102% (2004-2005), and 105% (2005-2006) of those in autumn (Table 1 and Fig. 1). The biologically impossible increases in estimates from autumn to spring is likely due to the decrease in food availability in the late winter/spring which leads to an increase in movements and trappability of spring hares relative to autumn hares. However, over-winter survival must have been greatly reduced during the decline, with spring density estimates being only 68% (2006-2007) and 56% (2007-2008) of those in autumn (Table 1 and Fig. 1). Across this cycle the lynx population increased from a low of 1 (1-2, 95% confidence limits) lynx/100 km$^2$ in 2002 to a peak of 12 (9-15) lynx/100 km$^2$ in 2007 and then declined to 5 (3-7) lynx/100 km$^2$ in 2008 (Fig. 1).

5.3.1 Natural monitoring study

Dam FCM concentrations in free ranging hares varied significantly from 2005 to 2008 (ANOVA: $F_{3,28} = 5.56$, $P = 0.004$; Fig. 1), being higher in 2007 (750.21 ± 65.56 ng/g) than in 2005 (398.49 ± 39.43 ng/g; $P = 0.004$), 2006 (455.60 ± 63.48 ng/g; $P =$
0.01), and 2008 (514.15 ± 51.20 ng/g; \( P = 0.02 \)). To examine how our index of maternal stress (FCM) varied both within and among years (the first three litter groups) we carried out a two-way ANOVA (year x litter) comparing 2006, 2007 and 2008. We found a year effect (\( F_{4,66} = 5.09, P = 0.009 \)), a litter group effect (\( F_{4,66} = 19.83, P < 0.0001 \)), and no interaction effect (\( F_{4,66} = 1.76, P = 0.15 \); Fig. 1). Dams in 2007 (515.07 ± 50.51 ng/g) had significantly greater FCM concentrations than those in 2006 (352.22 ± 33.18 ng/g; \( P = 0.006 \)) and 2008 (430.31 ± 41.41 ng/g; \( P = 0.05 \)). Dams had significantly greater FCM levels at the first litter (560.68 ± 40.80 ng/g) than at the second (290.92 ± 28.42 \( P = 0.0001 \)) and third (424.45 ± 39.03 ng/g; \( P = 0.02 \)). Dams at the second litter had FCM levels that were lower than at the third litter (\( P = 0.0009 \)). Thus, dam FCM concentrations fluctuated in synchrony with predator numbers (Fig. 1), increasing from 2005 and 2006 to reach a maximum in 2007, and then declining in 2008. Within a year, dam FCM concentrations declined from the first to the second litter and then increased again at the third litter.

To examine how juvenile FCM levels varied both within and among years (first three litter groups) we carried out a mixed effects model (year x litter) with year as a fixed effect and litter group as a random effect. We found a year effect (\( F_{3,68} = 21.37, P = 0.01 \)), a litter group effect (\( F_{2,68} = 13.81, P = 0.009 \)), and no interaction effect (\( F_{2,68} = 0.45, P = 0.64 \); Fig. 1). Juvenile FCM concentrations were significantly higher in 2007 (510.74 ± 37.41 ng/g) than in 2006 (285.27 ± 34.85 ng/g; \( P = 0.0002 \)) and 2008 (288.92 ± 26.40 ng/g; \( P = 0.0002 \)), but were similar in 2007 and 2005, and in 2005, 2006 and 2008. Juveniles born in the first litter (440.10 ± 32.65 ng/g) had significantly higher FCM concentrations than those born in the second (290.84 ± 25.98 ng/g; \( P = 0.0003 \)) and the
third (292.08 ± 27.26; \( P = 0.04 \)) litter. Although juveniles born in the second and third litter had similar FCM concentrations (\( P = 1.00 \)) this is likely due to the fact that only in 2006 were we able to sample juveniles born in the third litter. Therefore we carried out a separate one-way ANOVA testing for litter differences in 2006 and found significant differences (\( F_{2,24} = 4.84, P = 0.02 \); Fig. 1). Juveniles from the first litter (379.20 ± 67.85 ng/g) had significantly greater FCM levels than those from the second (188.25 ± 34.43 ng/g; \( P = 0.05 \)) but similar to those from the third (292.07 ± 27.26; \( P = 0.66 \)). Juveniles from the second litter had FCM levels that were lower (\( P = 0.05 \)) than those from the third litter. Thus, juvenile FCM concentrations were similar in 2005 and 2006, increased in 2007, and declined in 2008. Within a year FCM concentrations declined from the first litter to the second and increased again in the third. These changes mirrored those found in the dams.

To assess the relationship between dam and juvenile FCM concentration from the same year and litter group we carried out a general least-squares regression including the first, second and third litter groups from 2005 to 2008. Dam FCM concentration just after birth was a good predictor of juvenile FCM concentration at weaning (\( r^2 = 0.73, P = 0.007 \); Fig. 2).

To determine whether the differences we observed among years and litter groups in juveniles at weaning resulted in long-term changes of their FCM levels at older ages, we carried out a two-way ANOVA (litter group x trap date) in 2006 and 2007 to compare FCM concentrations between the first and second litters in August (pnd 60 and 28 for first and second litters, respectively) when the juveniles are still growing and in October (pnd 120 and 90 for first and second litters, respectively) when juveniles had reached
adult size. We found a litter effect (2006 $F_{1,33} = 8.29$, $P = 0.007$; 2007 $F_{1,30} = 6.21$, $P = 0.02$), a season effect (2006 $F_{1,33} = 5.17$, $P = 0.03$; 2007 $F_{1,30} = 5.01$, $P = 0.03$), and no interaction effect (2006 $F_{1,33} = 0.00$, $P = 1.00$; 2007 $F_{1,30} = 0.39$, $P = 0.54$; Fig. 3). Thus, first litter juveniles maintained their greater FCM concentrations than second litter juveniles, irrespective of their age.

5.3.2 Captive study

To test whether the stress hormones of dams (as reflected by their FCM concentrations) were correlated to those of their offspring, we subjected juvenile hares to a hormone challenge at the time of weaning (pnd 28). To account for differences within the litters we averaged males and females within each litter thus each dam is compared with two offspring for the analysis, giving us a conservative estimate.

As dam FCM concentrations increased, offspring plasma cortisol decreased at the basal bleed ($r^2 = 0.71$, $F_{1,14} = 34.32$, $P < 0.0001$; Fig. 4a), and increased at the dexamethasone (Dex) bleed ($r^2 = 0.30$, $F_{1,14} = 5.91$, $P = 0.03$; Fig. 4b) and increased at the 60 minute (P60) bleed following adrenocorticotropic hormone (ACTH) administration ($r^2 = 0.23$, $F_{1,14} = 4.31$, $P = 0.05$; Fig. 4c). To determine how dam FCM concentration affected the responsiveness of the juvenile’s HPA-axis we subtracted the basal cortisol levels from the 60 minute cortisol levels. We found that as dams FCM concentrations increased offspring cortisol responsiveness also increased ($r^2 = 0.43$, $F_{1,14} = 12.46$, $P = 0.003$; Fig. 4d). Thus, young became increasingly dexamethasone resistant and ACTH responsive as their mother’s FCM concentrations increased.
Dam FCM concentration did not affect the MCBC, free-fatty acid (FFA) or glucose levels of their offspring at any time during the challenge (basal: MCBC $F_{1,14} = 0.59, P = 0.45$; FFA $F_{1,14} = 1.60, P = 0.23$; glucose $F_{1,14} = 0.43, P = 0.52$; Dex: MCBC $F_{1,14} = 0.77, P = 0.39$; FFA $F_{1,14} = 3.43, P = 0.09$; glucose $F_{1,14} = 0.26, P = 0.62$; P60: MCBC $F_{1,14} = 0.79, P = 0.38$; FFA $F_{1,14} = 0.81, P = 0.38$; glucose $F_{1,14} = 1.09, P = 0.31$).

Dam FCM concentrations significantly affected aspects of their offspring’s hematology (Fig. 5). As dam FCM concentrations increased, the packed red-blood cell volume (PCV) of their offspring decreased ($r^2 = 0.25, F_{1,14} = 4.78, P = 0.05$; Fig. 5a), the neutrophil to lymphocyte (N:L) ratio increased (N:L ratio $r^2 = 0.63, F_{1,14} = 24.04, P = 0.0002$, Fig. 5b), and the eosinophil counts decreased (eosinophil $r^2 = 0.45, F_{1,14} = 11.58, P = 0.004$, Fig. 5c). Dam FCM concentrations did not affect monocyte counts ($r^2 = 0.03, F_{1,15} = 0.49, P = 0.50$; Fig. 5d). Thus, young had worsening indices of condition (PCV) and of immunity as their mothers FCM concentrations increased.

5.4 Discussion

The 10-year snowshoe hare cycle and its attendant cycles in lynx and other furbearers have been fundamental to the development of ecological theory for more than half a century (Stenseth et al. 1999) and empirical studies have been used to examine both this theory as well as fundamental concepts such as ‘the balance-of-nature’, predator-prey fluctuations, food web dynamics, and community organization (Elton and Nicholson 1942; Keith 1963; Pimm 1981; Krebs et al. 1986; Boonstra et al. 1998a; Stenseth et al. 1998, Sinclair et al. 2000). However, critical elements of the pattern of these cycles have eluded our understanding. During the low phase virtually all of the
predators have died and the vegetation has recovered, yet the hare population remains low for 3-5 years (Krebs et al. 2001b). Reproductive rates remain low at this time (Cary and Keith 1979; Stefan and Krebs 2001). In a related paper, we report that a predator-induced increase in maternal FCM levels resulted in a decline in reproduction (Sheriff et al. 2009a). An increase in dam FCM concentrations of 250 ng/g resulted in one less baby per litter and the babies born were 24% lighter and 11% smaller. From captive studies on cyclic populations of both snowshoe hares and voles, we know that mothers with high reproductive fitness have daughters with high reproductive fitness; conversely, mothers with low reproductive fitness have daughters with low reproductive fitness. Thus, there are intrinsic differences between mothers that are perpetuated in their offspring (Mihok and Boonstra 1992; Sinclair et al. 2003). Our study took place during the increase (2005), peak (2006), and decline phase (2007-2008) of the hare cycle. We elucidate the following causal connections between the predator-induced increase in stress hormones experienced by the mothers during the population decline and its consequences for their offspring. First, that a maternal index of stress hormones (FCM concentrations) fluctuated in synchrony with predator density during the breeding season. Second, that this stress hormone index is directly echoed in their offspring, with entire litter groups reflecting the pattern of their mothers at the time the young are born. Third, that a mother’s FCM levels affect the HPA stress axis of her progeny, with higher maternal FCM levels resulting in increased dexamethasone resistance and a heightened responsiveness to ACTH in progeny. We suggest that maternal programming, linked to the experience of high stress hormones caused by high predation risk, may explain the poor recovery of reproductive rates even after the predator numbers have declined markedly. This maternal
programming caused by stress hormones may be the ultimate explanation of the low phase of the snowshoe hare cycle.

5.4.1 Maternal stress

Food and predation are the two of the greatest factors affecting animal populations and these have interactive synergistic affects (Krebs et al. 1995; Zanette et al. 2003). In our natural monitoring study dams FCM concentrations fluctuate among and within years (Table 1 and Fig. 1). Among years FCM concentrations were highest in 2007 when the number of predators was greatest. We have also found that an experimental increase in the risk of predation resulted in dams with higher FCM concentrations (Sheriff et al. 2009a). Boonstra et al. (1998a) found that outside the breeding season snowshoe hares had a greater plasma cortisol concentration and stress response and a worse body condition during the decline than the low. An increase in the number of predators has also been shown to increase GC levels in other free-ranging mammals and birds (Silverin 1997; Hik et al. 2001; Scheuerlein et al. 2001; but see Creel et al. 2009).

We also expected FCM levels in the second year of the decline (2008) to be higher than the increase phase (2005), however found that levels, although elevated, were statistically similar. Part of the explanation may be that the females in our study were a high quality subset of females from the hare population, all giving birth within one week of the estimated parturition date. Sheriff et al. (2009a) found that females that gave birth to non-viable young, (either stillborn or aborted), had high FCM concentrations compared with females that gave birth. Since the number of females that give birth to
non-viable young increase during the decline phase (Stefan & Krebs 2001), had we assessed FCM concentrations from all females within the population, not just the successful ones, we may have seen greater FCM levels persisting throughout the decline phase.

Within a year we found that dam FCM concentrations were highest in the first litter, declined in the second, and increased again in the third (Fig. 1). The combined effects of changing risk of predation and food between the first and second litter could explain the changes in FCM concentrations. Boutin et al. (1986) found that predation rates decreased from winter to summer and thus the risk of predation should also decrease at this time. Furthermore, the first litter occurs during the late winter-early spring when the winter snowpack is in the process of melting and prior to the flush of new vegetation, while the second litter occurs during late spring-early summer when new vegetative growth is nearing its peak (Sinclair et al. 1982). Reduced food intake has been found to cause an increase in GC levels in mammals and birds (Harris et al. 1994; Kitaysky et al. 1999; Ortiz et al. 2001). The increase in food availability from the first to the second litter may also allow hares to forage in a less risk-prone manner (Hik 1995; Murray 2002).

The increase in maternal FCM concentrations between the second and the third litter (Fig. 1) could be a result of allostatic load. Allostatic load is the cumulative wear and tear on an animal coping with multiple stressors over its lifetime, such as the risk of predation, the extreme temperatures and food scarcity of winter, and pregnancy (McEwen and Wingfield 2003; Romero et al. 2009). As the allostatic load increases animals become less able to respond normally and require a greater stress response to counteract the stressor. For snowshoe hares during the decline phase, the high risk of predation
during the winter followed by two litters would greatly increase their allostatic load. This may be one explanation as to why hares during the increase phase (when the risk of predation is low) may have up to 4 litters a summer, whereas those during the decline may have only 2 litters. Thus, changes in maternal FCM concentrations may be affected primarily by changes in the number of predators with food augmenting these effects. As hares allostatic load increases they begin to show an even greater stress response to the increase in the risk of predation.

In our captive study maternal FCM concentrations were within those found in the natural monitoring study. Our captive females also showed a considerable variation in FCM concentrations, consistent with what we found in the free-ranging females. This suggests that individuals naturally differed in their physiological response and ability to cope with stressors, and this was maintained in captivity. This has also been found by Pride (2005) for ringtailed lemurs, *Lemur catta*, and by Cabezas et al. (2007) for European wild rabbits, *Oryctolagus cuniculus*. Thus, our captive results are a good representation of nature.

5.4.2 Developmental impacts of maternal stress

In our natural monitoring study dams with increased FCM concentration produced offspring with increased FCM concentrations and this was maintained into adulthood (Figs 1-3). Fecal cortisol metabolite levels are an integration of blood cortisol levels over the previous 8-12 h (Sheriff et al. 2009b) and reflect both basal and stress-induced cortisol exposure experienced during this time. In our experimental study we were able to separate these two factors and found that dams with increased FCM concentration
produced offspring with decreased basal plasma cortisol levels and an increased responsiveness of the HPA axis (Fig. 4).

The decrease in basal cortisol and the increased responsiveness seen in experimental juveniles born to mothers with elevated FCM concentrations could be due to an increase in mineralcorticoid receptors (MR) and a decrease in glucocorticoid receptors (GR) in the brain. Densities of these two hippocampal receptors are critical in regulation and feedback of the HPA axis, with MRs regulating basal glucocorticoid levels and GRs regulating glucocorticoid levels in response to stressors (Owen et al. 2005). It has been shown that the treatment of pregnant guinea pigs with a synthetic glucocorticoid resulted in offspring with increased hippocampal MR expression and reduced basal plasma cortisol concentrations (Liu et al. 2001). Furthermore, exposure of pregnant guinea pigs to a strobe light at gestation day 50 resulted in offspring with reduced GR expression and an elevated activity of the HPA axis (Kapoor et al. 2008). Rat offspring born to mothers exposed that were restrained during the last week of gestation also resulted in reduced GR densities (Henry et al. 1994; Maccari et al. 1995). These differences in receptor levels persist into adulthood (Liu et al. 1997; Francis et al. 1999) and we also found that differences in FCM concentrations at weaning persisted into adulthood (Fig. 3). For snowshoe hares the differences in MR and GR receptor levels would allow them to mount an appropriate response to predation, yet allow them to have low cortisol exposure at times when the risk of predation is low, alleviating the negative effects of high cortisol exposure such as a decrease in reproduction and survival. Thus, during the low phase reproductive output could begin to recover and population density increase.
Dam FCM concentrations also affected juvenile hematology (Fig. 5). Juvenile hares born to dams with elevated FCM concentration had a lower PCV (Fig. 5a). PCV is an indicator of body condition with lower values indicating a poorer body condition (Hellgren et al. 1993; Boonstra et al. 1998a). Previously, we found that snowshoe hares with predator-induced increases in cortisol levels gave birth to lighter and smaller babies compared with controls (Sheriff et al. 2009a). This has also been found for rats and male guinea pigs (Lesage et al. 2001; Emack et al. 2008). Juvenile hares born to dams with elevated FCM concentration also had a higher neutrophil to lymphocyte (N:L) ratio (Fig. 5b). High N:L ratios generally indicate high GC levels (Davis et al. 2008, but see Boonstra et al. 1998a). However, this is not the complete picture as an infection may also create a rise in neutrophil counts sufficient to substantially increase the N:L ratio. In order to disassociate stress from an infection both eosinophil and monocyte ratios must also be measured, as an infection or parasite will result in an increase in these WBC types (Davis et al. 2008). We found that an increase in dam FCM concentration resulted in a decrease in eosinophil counts and no change in monocyte counts in juveniles (Fig. 5c). Thus an increase in maternal FCM levels resulted in a shift in leukocyte profile indicative of high GC levels. Eosinophils are important in fighting parasitic infections and a reduced numbers may also result in a decreased immunity (Bullock and Rosendahl 1984). Thus, an increase in prenatal GC exposure results not only in an increase in offspring stress hormones, but a decrease in body condition and immunity.

The increase in stress hormones in the offspring born to mothers with high FCM concentrations may trade-off the decrease in reproduction by increasing their offspring’s anti-predator behavior (Emack et al. 2008) and thus increasing survival. However, during
the low phase when predation risk is low, the same mechanism would lower fitness since the risk of predation is much lower. In order for this mechanism to be adaptive the gain in fitness during the decrease phase should at least compensate for the loss of fitness in the early low phase. Future work must be done to quantitatively assess different tactics to determine the adaptive component of maternally-programmed anti-predatory behaviour (Lambin et al. 1995).

In summary, we have shown a direct relationship between the cortisol concentrations found in mothers and that in their offspring. Furthermore, the ability of offspring to handle a stressor was directly linked to the FCM levels of its mother. Our results provide support for the hypothesis that chronically elevated GC concentrations in dams increase the stress hormones of their offspring in free-ranging animals and this occurs at a population wide level. This intergenerational inheritance of stress hormones may negatively impact the next generation’s reproductive fitness even when predators have virtually disappeared and vegetation is ample. Ultimately this may explain the low phase of cyclic populations.
Table 5.1. Yearly and seasonal changes in snowshoe hare density (hares/km$^2$) from the autumn of 2002 to the spring of 2008.

<table>
<thead>
<tr>
<th>Year</th>
<th>Autumn</th>
<th>Spring</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002/03</td>
<td>14 (13-18)</td>
<td>11 (10-20)</td>
<td>81%</td>
</tr>
<tr>
<td>2003/04</td>
<td>15 (12-31)</td>
<td>22 (20-30)</td>
<td>149%</td>
</tr>
<tr>
<td>2004/05</td>
<td>37 (32-52)</td>
<td>38 (34-53)</td>
<td>102%</td>
</tr>
<tr>
<td>2005/06</td>
<td>88 (60-110)</td>
<td>92 (85-114)</td>
<td>105%</td>
</tr>
<tr>
<td>2006/07</td>
<td>117 (105-143)</td>
<td>79 (74-100)</td>
<td>68%</td>
</tr>
<tr>
<td>2007/08</td>
<td>63 (59-83)</td>
<td>35 (35-43)</td>
<td>56%</td>
</tr>
</tbody>
</table>

Note: Values are mean (95% confidence limits). The percent change is the estimate of the spring density compared to that in autumn.
Fig. 5.1. Fecal cortisol metabolite (FCM) concentrations (means ± SE) in free-ranging dams and juveniles snowshoe hares during the breeding season in 2005 (n = 4, 5, 0 adults, 1st, 2nd, 3rd litter; n = 6 juveniles, 1st litter), in 2006 (n = 10, 11, 8 adults; n = 11, 11, 5 juveniles), in 2007 (n = 8, 8, 8 adults; n = 10, 11 juveniles), and in 2008 (10, 8, 4 adults; n = 9, 13 juveniles). Juveniles were live-trapped within one week of weaning. Inset shows snowshoe hare and lynx population population estimates (mean ± 95% confidence limits) in the southwestern Yukon, Canada from 2002 to 2008. Lynx densities were estimated during the winter (i.e. from the autumn of one year to the spring of the following).
Fig. 5.2. Fecal cortisol metabolite (FCM) concentration (means ± SE) in free-ranging snowshoe hare dams and juveniles. Each point is the average from a different litter in 2005 (litter 1 ▲), 2006 (litter 1 ●, 2 ○, 3 ◊), 2007 (litter 1 ■, 2 □), and 2008 (litter 1 ♠, 2 ◊).
Fig. 5.3. Fecal cortisol metabolite (FCM) concentration (mean ± SE) of free-ranging juvenile snowshoe hares born in the first and second litter, in August and October, 2006 and 2007. First litter juveniles are pnd 60 (2006 n = 16; 2007 n = 9) and 120 (n = 4; 9) in August and October, respectively. Second litter juveniles are pnd 28 (n = 11; 11) and 90 (n = 6; 5). Asterisks denote significant difference between first and second litters ($P < 0.05$).
Fig. 5.4. Fecal cortisol metabolite (FCM) concentrations in dams and the plasma cortisol concentrations of their offspring in response to a standardized hormone challenge at: a) basal bleed; b) 2-h after a dexamethasone injection (Dex bleed); c) 1-h after an adrenocorticotropic hormone injection (P60 bleed); d) the difference from the basal plasma concentration to the P60 bleed.
Fig. 5.5. Fecal cortisol metabolite (FCM) concentrations in dams and the hematology of their offspring at: a) Packed cell volume of red blood cells (%); b) Ratio of neutrophils to lymphocytes in 100 white blood cells (WBC); c) Number of eosinophils per 100 WBC; d) Number of monocytes per 100 WBC.
5.5 References


6. THE 10-YEAR POPULATION CYCLE AND THE IMPACT OF
FLUCTUATING PREDATION RISK ON SNOWSHOE HARE STRESS
PHYSIOLOGY AND DEMOGRAPHY\(^5\)

6.1 Introduction

The 10-year snowshoe hare cycle and its attendant cycles in lynx and other furbearers have been fundamental to the development of ecological theory for more than half a century. Empirical studies have examined both this theory as well as fundamental concepts such as ‘the balance-of-nature’, predator-prey fluctuations, food web dynamics, and community organization (Elton and Nicholson 1942; Keith 1963; Pimm 1981; Krebs et al. 1986; Royama 1992; Boonstra et al. 1998\(^b\); Stenseth et al. 1998, Sinclair et al. 2000; Korpimäki et al. 2004; Inchausti and Ginzburg 2009). These hare-lynx cycles are cited in virtually every ecological text book, and often are used as one of the few examples validating the Lotka-Volterra predator-prey equations. Although these cycles have been studied for over 30 years (Keith 1974, 1990; Krebs et al. 1986, 1995) there remain two unexplained patterns in the population dynamics of snowshoe hares. The first is why hare populations remain low for 2-5 years after the decline phase even though the predator populations have collapsed and there is ample vegetation. The second enigma is why the low phase varies in length.

During the low phase snowshoe hares have a significantly reduced reproductive fitness (Cary and Keith 1979; Stefan and Krebs 2001). The decrease in reproduction

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begins during the population peak, continues to fall during the decline phase, and does not recover until late in the low phase. To explain these changes Hik (1995) proposed a predator sensitive foraging hypothesis. He found that hares were able to assess the risk of predation in different habitats and during the population decline limit their activity to areas of dense cover to reduce the risk of being killed (see also Wolff 1980). He proposed that the correlate of choosing areas of dense cover is being forced to eat low-quality food, and a subsequent decline in body condition that reduced reproductive fitness. However, Hodges (1999) found that body condition did not affect reproduction. Boonstra et al. (1998a) proposed a complimentary hypothesis to that of Hik, the stress hypothesis. They hypothesized that hares experience higher stress during the decline due to increased predation risk and that higher stress levels caused the decline in reproduction. The hormonal and physiological evidence (Boonstra et al. 1998a) and the mass dynamics (Hodges et al. 2006) are consistent with this hypothesis.

In vertebrates, the key to responding adaptively to environmental challenges is the ‘stress response’, defined here as the set of neural and endocrine responses that help restore homeostasis (Sapolsky et al. 2000). Central to the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the resultant secretion of glucocorticoids, lasting several minutes to hours (Sapolsky 1992, Wingfield and Romero 2001). The stress response may be activated by any number of environmental perturbations that disrupt homeostasis, such as harsh weather, habitat changes, anthropogenic disturbances, decreased food availability, and predation attempts (Sapolsky 1987). It is designed to deal with acute perturbations, shutting down non-essential functions such as the immune response and reproduction, and is essentially
catabolic in nature, mobilizing energy and stimulating hepatic gluconeogenesis (Munck et al. 1984, Wingfield et al. 1998). Its short-term activation facilitates escape from life threatening situations. However, when activated chronically, the stress response can be severely deleterious affecting long-term survival and fitness, particularly through its negative effects on reproduction.

In a free-ranging population of snowshoe hares we have shown that an increase in predator-induced maternal stress results in a decline in reproductive fitness (Sheriff et al. 2009b). Furthermore, prenatally-stressed offspring had higher basal cortisol levels (the major glucocorticoid in hares - Boonstra et al. 1998a) and a greater stress response to a standardized stressor compared with those that were not prenatally stressed (Sheriff et al. unpub. data). In the laboratory, numerous studies have shown that maternal stress can have long lasting effects on offspring, being linked to offspring depression, anxiety-like behaviors, alterations in brain development, and increased glucocorticoid production in adulthood (Abe et al. 2007; Meaney et al. 2007; Emack et al. 2008; Kapoor et al. 2008; Weinstock 2008; Mastorci et al. 2009). In cyclic populations it has also been documented that there is a ‘memory’ of the past as expressed in reproductive fitness. Sinclair et al. (2003) found that when hares from different phases of the cycle were brought into the laboratory and bred under ideal conditions, they maintained the breeding performance of their field counterparts, indicating intrinsic, field derived differences among them. Similar results have been found in cyclic vole species (Mihok and Boonstra 1992). Simple mathematical models have shown how these maternal effects (the effects a mother’s phenotype has on her offspring in addition to her direct genetic contribution)
may be major drivers of cyclic population dynamics (Ginzburg and Taneyhill 1994; Ginzburg 1998; Inchausti and Ginzburg 1998).

We propose that the low phase of cyclic populations is the result of the negative impact of intergenerational, maternally-inherited stress originating during the decline caused by intense predation risk. We predict that snowshoe hares should be highly sensitive to changes in the risk of predation, and should be more stressed during the decline phase than the increase, the peak, and the low phase. Furthermore, this sensitivity should be obvious even within a winter season and a breeding season and hares should respond rapidly to increases in predation risk. Finally, if there is variation amongst declines in the severity of the predation risk experienced by hares, this ‘memory’ may affect long-term demography and explain differences among cycles in the length of the low phase, which can vary from 1-2 years to 4-5 years. We predict that greater predator-induced maternal stress during the decline phase should result in a longer low phase.

To address these questions we measured indices of stress, energy mobilization, and body condition in hares during the winter and breeding seasons of the increase (2005), the peak (2006), the decline (2007-2008) and the first year of the low (2009) phases of the population cycle. The predator density and, during the winter season, the ratio of the number of hares to the number of lynx were measured as indices of the risk of predation. We used fecal cortisol metabolites (FCM) and plasma cortisol measures from a hormonal challenge to assess changes in snowshoe hare physiology. Cortisol metabolites appear in the feces 8-12 h after production (Sheriff et al. 2009a) and thus FCM provides an integrated measure of circulating cortisol prior to the stress of being captured. The hormonal challenge involves a dexamethasone (Dex) suppression test followed by an
adrenocorticotropic hormone (ACTH) stimulation test. The Dex suppression test is used
to assess whether the brain is registering glucocorticoid levels (Dex is an artificial
glucocorticoid) correctly and making the necessary negative-feedback adjustments by
reducing cortisol production. Stress has been found to diminish the suppressive effects of
Dex (Sapolsky 1983, Boonstra 1998a). The ACTH stimulation test is used to assess the
responsiveness of the adrenals directly. Stress has been found to increase cortisol
production in response to ACTH injections (Sapolsky 1983, Boonstra 1998a).

We predict that under conditions of higher predation risk snowshoe hares should
be more stressed as indicated by the following:
1) Hares should have higher FCM concentrations and basal plasma free cortisol, and a
lower maximum corticosteroid-binding capacity (MCBC - in the blood most cortisol is
bound by corticosteroid-binding globulin with only 5-10% being in the free, active form
[Sitteri et al. 1982]). Corticosteroid-binding globulin levels are regulated by cortisol
production, with chronically high levels reducing this binding protein (Schlechte and
Hamilton 1987; Frairia et al. 1988). MCBC is a measure of corticosteroid-binding
globulin. Furthermore, hares should exhibit a greater Dex resistance (i.e. when
dexamethasone is given, endogenous cortisol levels should not fall as rapidly in resistant
animals as in normal ones) and a greater response to the ACTH injection.
2) Hares should have a greater energy mobilization. Greater glucocorticoid levels
produced by chronic stress enhance the livers capacity for gluconeogenesis, thus
increasing hepatic production and storage of glucose as glycogen. This comes at the
expense of peripheral tissues by changes at four levels: by decreasing their glucose
uptake and utilization; by the release of gluconeogenic substrate from them; by increasing
protein breakdown in several tissues such as muscle, adipose, and lymphoid; by decreasing protein synthesis; and by increasing energy substrates such as free fatty acids (FFA) (Vander et al. 1990). Thus hares under greater stress should have a greater ability to mobilize glucose but a lesser ability to mobilize free fatty acids.

3) Hares should be more compromised with respect to their immune response (as indicated by a shift in their leukocyte profile) (Davis et al. 2008) and body condition (as measured by hematocrit [packed red blood cell volume] values and hares’ mass index) (Hellgren et al. 1993).

Finally to determine if a greater maternal stress during the decline results in a longer low phase we examined how the rate of loss of hare populations affected the subsequent length of the low phase. The rate of loss may be a good index for the severity of predator-induced maternal stress as it incorporates the peak in predator numbers (the greater the hare peak the greater the number of predators [Doyle and Smith 2001; O’Donoghue et al. 2001; Rohner et al. 2001]), the severity of predation (the difference between peak and low densities of hares) and the length of the decline (the shorter the decline the more severe the impact of predation). We conducted a literature review and assessed 10 complete hare cycles in which we had reliable estimates of hare density from the peak to the beginning of the subsequent increase phase. We predict that the greater the rate of loss per year the longer the low phase.
6.2 Methods

6.2.1 Study area

This study was conducted in the boreal forest near the Arctic Institute Base at Kluane Lake in the southwestern Yukon, Canada (60°57’ N, 138°12’ W). It has a relatively high elevation (600-1100m) and is located within the rain shadow of the St. Elias mountain range. Thus it is relatively dry and cool with an average summer (June to July) temperature of 9 °C and an average winter (October to April) temperature of -18 °C. This region is dominated by a single conifer species, white spruce (*Picea glauca*), with a mixed understory of grey willow (*Salix glauca*), bog birch (*Betula glandulosa*), soapberry, (*Sherperdia canadensis*), and other herbaceous plants (Krebs et al. 2001b).

Our research was approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

6.2.2 Life history

Snowshoe hares undergo a regular cyclic fluctuation, with 8-10 years between peak densities (Keith 1963; Krebs et al. 1986). A major driver of the hare cycle is predation. As hare populations increase so do those of their predators, but with a lag of 1-2 years. During the population decline, predators are the direct cause of almost 100% of hare deaths (Hodges et al. 2001) and of these deaths, lynx and coyote were responsible for 65-75%, with raptors responsible for the rest.

Snowshoe hares are synchronous, seasonal breeders with mating occurring immediately post partum. This results in two to four distinct litter groups, depending on the phase of the population cycle (four litters during the early increase phase and these...
progressively decline to a nadir of only two litters during the decline phase; Stefan and Krebs 2001). Breeding begins in late April with the first litter born near the end of May, and each subsequent litter borne approximately 36-39 days later (Cary and Keith 1979; Stefan and Krebs 2001). Early litters are weaned at 24-28 days of age, but the last litter of the year may be nursed for up to 40 days (O’Donoghue and Bergman 1992). The young are born precocious and remain together for the first 3-5 days, after which they separate and only come together once a night to nurse (O’Donoghue and Bergman 1992). Hares first breed in the summer after their birth.

6.2.3 Population monitoring

Snowshoe hare populations have been continuously monitored since 1976 (see Krebs et al. 1986 and Hodges et al. 2001). The basic trapping protocol has been similar over this entire period. Live-traps were pre-baited with alfalfa cubes for 3-5 days before being set. Trapping sessions consisted of 2-3 nights of trapping within a 5-day period in both early spring (late March-early April) and late autumn (October – early November). Population density was estimated with the program CAPTURE (Otis et al. 1978) and the Jolly-Seber full model, as in previous studies (e.g., Krebs et al. 1995).

Avian and mammalian predator populations fluctuate in synchrony with the hare cycle (Doyle and Smith 2001; O’Donoghue et al. 2001; Rohner et al. 2001). An index of the fluctuations in predator populations was obtained by using evidence from lynx data, as these are reflective of all other predators, including avian predators. Lynx populations have been continuously monitored since 1987 (O’Donoghue et al. 2001). Each winter (October through April), on days after fresh snowfalls while tracks were distinguishable,
lynx tracks were counted along a 25-km transect that traverses our study area. Track counts for lynx are highly correlated to their population density in this valley (lynx: $r^2 = 0.95$) and we calculated density as $y = 0.355 + 0.288x$, where $y$ is lynx density (per 100 km$^2$) and $x$ is lynx track count (per track night per 100km) (Hone et al. 2007).

6.2.4 Live trapping

Snowshoe hares were live-trapped using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.). Trapping occurred during the early (October) and late (February and March) winter from 2006-2009 (thus a single winter is denoted as 2006/07 or 2007/08), and during the first (late May) and second (late June and early July) litter of the breeding season from 2005-2008. The traps were set at 2200 h and checked at 0600 h and thus hares could only be in the traps for a maximum of 8 h (the lag between the production of cortisol in the body and the appearance of its metabolites in the feces is between 8-12 h and thus fecal steroid levels reflect basal levels not affected by the stress of live-trapping; Sheriff et al. 2009a). Trapping did not occur on nights that dropped below -20 °C, and fecal samples were not collected from hares that had been trapped within the past 48 h.

Upon capture, each hare was weighed with a Pesola spring scale (± 10 g), its right hind foot (RHF) length measured as an index of body size, an ear-tag was placed in its right ear (No. 3 Monel tags, National Band and Tag Co., Newport, Kentucky, USA.), its sexual condition assessed, and a fecal sample collected from below the trap. During the winter season samples were not collected from sexually reproductive hares (this was identified by observing the male testis, which start to descend in mid-February). During
the breeding season samples were collected from females within one-week after birth. Samples from males were collected within one-week of the mean parturition date for each litter.

During two winter seasons (October 2006 and March 2007; October 2007 and February 2008), a sample of female hares was subjected to a hormone challenge (see below). Upon capture they were transferred to a burlap sack and taken to a quiet and dimly lit field laboratory heated to 5-10 °C at the Arctic Institute Base. Only females were used as these are the relevant sex in terms of reproductive fitness and maternal inheritance.

As a measure of body condition, we determined a mass index (MI) for hares, which was the deviation of the mass from that predicted by a measure of skeletal size. It was calculated as the observed mass divided by the expected mass, with the expected mass calculated from the relationship between skeletal size (RHF) and mass. The MI fluctuates around 1, with the average animal having a value of 1, a good condition animal greater than 1, and a poor condition animal less than 1. Since this is a relative rather than absolute index variation in body condition was comparable even though these equations were developed using different snowshoe hares from a previous cycle on our study area. The equations are outlined in Hodges et al. (1999) and were developed for use across an entire cycle. The MI was calculated for hares that also had another measure of condition taken - hematocrit (see below).
6.2.5 Fecal cortisol metabolite assay

We used an enzyme immunoassay (EIA) to measure fecal cortisol metabolite (FCM) concentration, validated specifically for snowshoe hares. Within 1 h of collection samples were stored at -80 °C at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80 °C until analyzed.

Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) for 14-18 h to control for fibre and water content (Wasser et al. 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 (1 SE) g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-tube vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was diluted (1:10) with assay buffer and frozen at -80°C until analysis.

Fecal cortisol metabolite concentrations were measured following the methods outlined by Sheriff et al. (2009a) using the 11-oxoaetiocholanolone-EIA developed by Palme and Möstl (1997). Briefly, 50 µl of extracted samples (diluted 1:25 with assay buffer) were incubated in duplicate with 100 µl of biotynilated steroid label (11-oxoaetiocholanolone-3-glucosiduronate-DADOO-biotin) and 100 µl antibody (11-oxoaetiocholanolone-3-HS:BSA raised in rabbits) at 4°C on a plate shaker overnight. Plates were then washed four times with 0.05% TWEEN 20 (Merck 822184) solution and blotted dry. Into each well 250 µl of streptavidin peroxidase solution (1 µl strepatvidin POD, 500 mU/µl [Boehringer 1089153] added to 30 ml assay buffer) was added and plates were incubated on plate shaker for 45 min at 4°C. Plates were washed and then developed for 45 min at 4°C on a plate shaker with 250 µl of tetramethybenzidine
solution. The enzymatic colour reaction was stopped using 50 µl of 2 M sulfuric acid. Absorbance was measured at a wavelength of 450 nm with an automated plate reader (VERSAmax microplate reader, Molecular Devices, Sunnyvale, California). This EIA had an intra- and inter-assay coefficient of variation of 6.3% and 10.3%, respectively.

6.2.6 Hormone challenge

Each adult female hare was bled five times (0.3 ml per bleed) from an ear artery using 28 gauge needles (0.36 x 13 mm) and heparinised 0.5 ml syringes (Lo-Dose U-100 insulin syringes, Becton Dickinson and Company, New Jersey, USA). The first blood sample (base bleed) was immediately followed by an injection of 0.4 mg/kg of dexamethasone sodium phosphate (Sabex, Quebec, Canada) into an ear vein. The second bleed (Dex bleed) assessed the inhibition response to Dex and occurred 2 h later. It was followed immediately by an intramuscular injection in the thigh of 40 µg/kg of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada). The remaining three bleeds assessed the stimulation response to ACTH and occurred 30, 60, and 120 min post-ACTH injection (called the P30, P60, and P120 bleeds, respectively).

Measurement of glucose concentrations (FreeStyle glucometer -Abbott Diabetes Care, Inc., Alameda, CA) and preparation of blood smears (in duplicate) were completed within 5 min of sample collection. Measurements of hematocrit – the packed red blood cell volume - (measured in duplicate after a 9 min centrifugation at 13 460 g on an IEC Micro-Hematocrit Centrifuge, Model 5413) and staining of slides (using a modified Wright stain technique called Diff-Quick [Dade International Inc., Florida, USA]) were completed within 45 min of blood collection. White blood cell (WBC) ratios were
calculated from a count of 100 WBCs in the smears. Blood smears and hematocrit measurements were conducted on the first bleed only and glucose concentrations were measured after every bleed. The remaining blood was centrifuged at 8 800 g for 10 min in an Eppendorf Micro Centrifuge 5413. The separated plasma was then frozen at -80 °C at the Arctic Institute Base and at the University of Toronto, until plasma cortisol, MCBC and free-fatty acids (FFA) were analyzed.

Total plasma cortisol was measured in duplicate using a radioimmunoassay (Clinical Assays GammaCoat Cortisol $^{125}$I RIA Kit, DiaSorin, Minnesota, USA) with an intra- and inter-assay coefficient of variation of 2.4% and 12.4%. MCBC levels were measured in duplicate using a radioimmunoassay described by Boonstra and Singleton (1993), with an intra- and inter-assay coefficient of variation of 2.6% and 4.9%. Free cortisol concentrations were calculated using the procedures and binding coefficients outlined in Boonstra et al. (1998a).

FFA were measured in duplicate using an in vitro enzymatic colorimetric method assay for the quantitative determination of non-esterified fatty acids (HR Series NEFA-HR [2], Wako Diagnostics, Virginia, USA). This assay had an intra- and inter-assay coefficient of variation of 5.2% and 9.9%.

6.2.7 Length of the low phase

We used 10 complete cycles, spanning the years from 1849 to 2009, in which we were able to obtain actual hare density estimates or robust indices of hare population change. It included five estimates from the Hudson Bay pelt records (MacLulich 1957) and five mark-recapture estimates; three from Kluane, Yukon (Krebs et al. 1986, 2001a;
Krebs unpublished data), and two from Rochester, Alberta (Meslow and Keith 1968; Keith and Windberg 1978). We did not include data from the snowshoe rabbit enquiry, 1931-1948, as this data only indicated an increase, decrease or no change in hare populations but no overall population estimates (see Chitty 1950). Since each of the three studies occurred in different regions and at different scales we used the proportion of the maximum number of hares, rather than the absolute number, within each study (note this is not the maximum of each cycle). We then calculated the rate of loss per year as the proportion of hares lost during the decline over the number of years of the decline.

\[
\frac{(\text{proportion of hares at the peak}) - (\text{proportion of hares in the first year of the low phase})}{\text{length of decline (in years)}}
\]

We determined phase changes based on the criteria of Keith (1990) who showed that the rates of population growth averaged 2.04 per year during the increase phase and 0.46 per year during the decline phase (these rates were averaged from studies across North America from 1940-1978). The length of the low phase was calculated as the number of years from the end of a population decline to the start of a population increase (i.e. when the population had population growth rates between 0.46 and 2.04 per year).

6.2.8 Statistical analysis

All data are expressed as means ± 1 SE. ANOVAs, post-hoc tests, and general linear regressions were performed using the software package STATISTICA 6. The assumption of normality was tested with Shapiro-Wilks test and the assumption of
homogeneity of variances was tested with Levene’s test. If these assumptions were not met the data was transformed with a log \((x + 1)\) (Quinn and Keough 2003). A Tukey’s-HSD post-hoc was used to examine the significance of main effects. Comparisons of the means were considered significant if \(P < 0.05\). We give \(P\) values between 0.10 and 0.05 and infer that these may be biologically, though not statistically, significant, possibly because of reduced-power sample sizes in some of our results (Yoccoz 1991). We found no difference between males and females during the winter season and the sexes were pooled. During the breeding season we found a sex effect \((P < 0.05)\) and males and females were analyzed separately.

To examine how the risk of predation affected hares we carried out a number of different analyses: 1) FCM levels were compared using a one-way or two-way ANOVA and a Tukey’s HSD post-hoc test; 2) free cortisol, MCBC, glucose and FFA levels were compared using a repeated-measures ANOVA. Since the values in a repeated-measures design are not independent of each other we used a conservative Greenhouse-Geisser epsilon to adjust the degrees of freedom prior to calculating the \(P\)-value; 3) White-blood cell ratios, hematocrit, and the MI were non-parametric and were compared using a Mann-Whitney \(U\) test.

### 6.3 Results

#### 6.3.1 Population dynamics

Hares underwent four cyclic fluctuations from 1976 to 2009 (Fig. 6.1). Density increased 13 to 49-fold from the low phase to subsequent peaks in 1980, 1990, 1998, and 2006. Early winter hare densities were almost always higher than late winter densities as
a result of juveniles entering the trappable population during the breeding season followed by mortality overwinter. The highest peak measured in late winter was 288 per km$^2$ in 1980, and the lowest peak was 92 per km$^2$ in 2006. Three low phases lasted 2, 3, and 5 years, respectively. Lynx densities were measured from the winter of 1987/88 to the winter of 2008/2009, and underwent 3 cycles (Fig. 6.1). They increased 5 to 12-fold during a cycle, reaching peak numbers in the winter following the hare peak. Their numbers tracked those of the hares with the highest lynx numbers (1998) following the highest hare numbers (1997; when densities for both were assessed).

During the time of our physiological measurements, in late winter 2005-2009, hare densities increased 2.4 times from 38 hares/km$^2$ in 2005 to a peak of 92 hares/km$^2$ in 2006, then declined 2.6 times over the next three years to reach a low of 28 hares/km$^2$ in 2009 (Fig. 6.1). Over the same years the lynx population increased from a low of 4 lynx/100 km$^2$ in 2005 to a peak of 12 lynx/100 km$^2$ in 2007, and then declined to 6 lynx/100 km$^2$ in 2009 (Fig. 6.1).

The proportion of hares to lynx also fluctuated greatly across the cycle, decreasing 37% from 1314 hares per lynx at the hare peak (late winter 2006) to 488 hares per lynx at the end of the decline phase (late winter 2008). Within a winter season the number of hares per lynx was consistently higher in the early winter compared with the late winter from 1987/88 to 2008/09 ($t_{21} = -5.26, P < 0.0001$). In the two winter seasons compared in this study, the proportion of hares to lynx decreased from early to late winter 33% (998 hares per lynx to 677 hares per lynx) in 2006/07 and 45% (1250 hares per lynx to 695 hares per lynx) in 2007/08. Hence, the risk of predation for an individual hare increase as the winter progressed.
6.3.2 Fecal cortisol metabolite levels

FCM concentrations in late winter varied significantly from 2006 to 2009 ($F_{3,73} = 6.08$, $P < 0.001$; Fig. 6.2), being significantly higher ($P < 0.05$) in 2007 than in 2006 (by 50%) and 2009 (by 30%). FCM concentrations were similar in 2007 and 2008, and in 2006, 2008, and 2009.

During the non-breeding, winter season (comparing both early and late winter of 2006/07 and 2007/08) we found a year effect ($F_{1,69} = 4.35$, $P < 0.05$), a season effect ($F_{1,69} = 11.78$, $P < 0.005$), and no interaction ($F_{1,69} = 0.22$, $P > 0.05$). FCM concentrations decreased 20% from 2006/07 to 2007/08, and increased from early to late winter by 36% in 2006/07 and 32% in 2007/08 (Fig. 6.3).

During the breeding season, FCM concentrations of adult hares decreased between the first and second litter of 2005-2008 (Fig. 6.4), but the decrease was less dramatic in some years. We found a year effect (male: $F_{3,58} = 4.13$, $P < 0.05$; female: $F_{3,57} = 5.03$, $P < 0.005$), a season effect (male: $F_{3,58} = 30.88$, $P < 0.0001$; female: $F_{3,57} = 22.03$, $P < 0.0001$), and an interaction effect for males ($F_{3,58} = 2.96$, $P < 0.05$) but not females ($F_{3,57} = 0.75$, $P > 0.05$; Fig. 6.4). For males (Fig. 6.4a), FCM concentrations were significantly lower ($P < 0.05$) in 2005 than in 2006 (by 60%), in 2007 (by 63%), and in 2008 (by 60%). There was no difference among the latter three years. FCM concentrations were significantly higher ($P < 0.05$) during the first litter than during the second litter in 2006 (by 73%), in 2007 (by 173%), and in 2008 (by 56%), but were not different between litter groups in 2005. For females (Fig. 6.4b), FCM concentrations were significantly higher ($P < 0.05$) in 2007 than in 2005 (by 70%), in 2006 (by 51%),
and in 2008 (by 42%). There was no difference among 2005, 2006, and 2008. FCM concentrations were significantly higher ($P < 0.05$) during the first litter than during the second in all years; in 2005 (by 25%), in 2006 (by 52%), in 2007 (by 75%), and in 2008 (by 96%).

6.3.3 Plasma cortisol levels

Free cortisol concentrations, averaged over the entire hormone challenge, were significantly higher ($P < 0.05$) in the winter of 2006/07 than in the winter of 2007/08 (by 25%) and in late winter than in early winter (by 45%; Table 6.1). Free cortisol varied significantly over time in response to the hormone challenge in all cases ($P < 0.0001$), and there were interaction effects between the response and year ($P < 0.05$) and the response and season ($P < 0.0001$; Table 6.1). Hares had higher free cortisol concentrations in the winter of 2006/07 than in the winter of 2007/08 at the base bleed (by 20%), at the Dex bleed (by 100%; i.e. the Dex injection had a reduced suppression of cortisol), and in response to the ACTH injection (by 26%; Fig. 6.5a). Free cortisol was higher in late winter than in early winter at the base bleed (by 35%), at the Dex bleed (by 75%), and in response to the ACTH injection (by 45%; Fig. 6.5a).

MCBC levels, averaged over the entire hormone challenge, were significantly lower ($P < 0.05$) in the winter of 2006/07 than in the winter of 2007/08 (by 32%) and in late winter than in early winter (by 35%; Table 6.1). A post-hoc analysis showed similar seasonal MCBC levels at the base bleed ($P > 0.05$) and Dex bleed ($P > 0.05$) in the winter of 2007/08. MCBC varied significantly in response to the hormone challenge in all cases ($P < 0.0001$), and there were interaction effects between the response and season ($P <$
Hares had lower MCBC levels in late winter than in early winter at the base bleed (by 41%), at the Dex bleed (by 31%), and in response to the ACTH injection (by 52%; Fig. 6.5b). Thus, hares in 2007/08 and in early winter were better able to handle the hormonal challenge than hares in 2006/07 and in late winter, respectively, as indicated by their lower free cortisol levels and higher MCBC levels.

6.3.4 Energy mobilization

Glucose levels, averaged over the entire hormone challenge, were similar in the winter of 2006/07 and 2007/08 ($P > 0.05$), but were significantly higher in the late winter than in the early winter (by 19%, $P < 0.001$; Table 6.1). Glucose levels varied significantly over time in response to the hormone challenge in all cases ($P < 0.0001$), and there were interaction effects between the response and year ($P < 0.01$) and the response and season ($P < 0.005$). Hares had higher glucose levels in the winter of 2006/07 than in the winter of 2007/08 at the base bleed (by 5%), at the Dex bleed (by 5%), and in response to the ACTH injection (by 15%; Fig. 6.5c). Glucose levels were higher in late winter than in early winter at the base bleed (by 14%), at the Dex bleed (by 20%), and in response to the ACTH injection (by 21%; Fig. 6.5c).

Free fatty acid levels, averaged over the entire hormone challenge, were similar ($P > 0.05$) in the winter of 2006/07 and 2007/08, and in the early and late winter; Table 6.1). However, free fatty acids varied significantly over time in response to the hormone challenge in all cases ($P < 0.0001$), and there were interaction effects between the response and season ($P < 0.05$). These results are complicated by an interaction effect between the response, year, and season ($P < 0.0001$). In the winter of 2006/07 FFA levels
were higher in late winter than in early winter at the base bleed (by 52%) and the Dex bleed (by 11%) but were lower in response to the ACTH injections (by 43%; Fig. 6.5d). In 2007/08 FFA levels were similar in both early and late winter at the base bleed and in response to the ACTH injection (Fig. 6.5d). Thus, hares had a greater ability to mobilize glucose in winter 2006/07 than in 2007/08 and in late winter than in early winter. FFA mobilization was similar between the winter of 2006/07 and 2007/08, and was lower in late winter than early winter 2006/07 but not 2007/08.

6.3.5 Immunology and body condition

Leukocyte profiles varied by both winter and season (Table 6.2). In the winter of 2006/07 neutrophils were higher (by 33%, $Z = 2.32, P < 0.05$), lymphocytes did not change ($Z = -0.41, P > 0.05$), and eosinophils and monocytes were lower (by 32% $Z = -2.30, P < 0.05$, and by 38% $Z = -3.26, P < 0.005$, respectively) than in the winter of 2007/08. In the late winter neutrophils were higher (by 23%, $Z = -4.15, P < 0.0001$), lymphocytes and eosinophils were lower (by 26%, $Z = 2.92, P < 0.005$, and by 39%, $Z = 2.76, P < 0.01$, respectively), and monocytes did not change ($P > 0.05$) than in the early winter.

Body condition was measured by both the hematocrit values (Fig. 6.6a) and the mass index (MI; Fig. 6.6b) of hares. Hematocrit values were lower in the winter of 2006/07 (0.44) than in the winter of 2007/08 (0.47) ($Z = -2.34, P < 0.05$) and in late winter (0.42) than in early winter (0.49) ($Z = 3.38, P < 0.001$). MI was similar in the winter of 2006/07 and 2007/08 ($Z = 0.42, P > 0.05$) and tended to be lower in late winter (0.94) than in early winter (0.98) ($Z = 1.81, P = 0.06$). Thus, hares had a more
compromised immune response and body condition during 2006/07 and late winter compared with 2007/08 and early winter, respectively.

6.3.6 Length of the low phase

The rate of loss per year was positively related to the length of the low phase ($r^2 = 0.44$, beta $= 0.66$, $P < 0.05$, Fig. 6.7) and if we removed the anomalous 1-year low (from the Hudson Bay pelt records; MacLulich 1957) this relationship became even better ($r^2 = 0.70$, beta $= 0.84$, $P < 0.005$). Thus, the greater the rate of loss in the decline phase (i.e. the larger the percentage of the population killed each year in the decline) the longer the low phase.

6.4 Discussion

This study was designed to investigate the change in the stress physiology of snowshoe hares during the increase (2005), peak (2006), decline (2007-2008), and low phase (2009) (Fig. 6.1) and its cause in the boreal forest of southwestern, Yukon. We found that hares had the greatest fecal cortisol metabolite (FCM) levels during the decline phase, in 2007, than the increase, peak, and low phases (Fig. 6.2). During the non-breeding season, we used FCM levels and a hormonal challenge to compare hares in the early and late winter of 2006/07 and 2007/08. Similar to our among year results, hares were more stressed in the winter of 2006/07, the first year of the decline, than the next winter (Figs 6.3-6.6). Within a winter hares became more stressed as it progressed (i.e. late winter hares had greater FCM levels [Fig. 6.3], greater basal cortisol levels [Fig. 6.5], a greater HPA responsiveness [Fig. 6.5], a greater ability to mobilize glucose [Fig. 6.5], a
stressful shift in their leukocyte profile [Table 6.2], and a decrease in their hematocrit and body condition index [Fig. 6.6]). Among breeding seasons, males had greater FCM levels during the peak (2006), and decline phase (2007-2008) than during the increase phase (2005) (Fig. 6.4a). Females had greater FCM levels during the decline phase than during the increase and peak phase (Fig. 6.4b). Within a breeding season, all hares had greater FCM levels during the first litter than the second (Fig. 6.4). Finally, the rate of loss per year in the decline phase was positively related to the length of the subsequent low phase (Fig. 6.7). We will argue that the changes in hares’ stress physiology are consistent with the hypothesis that changes in predation risk are causing most of the physiological changes and are associated with stress. Below we discuss each piece of evidence in turn and ultimately how this may help to explain the low phase of the hare population cycle.

6.4.1 Caveats

There are two caveats that must first be addressed before we discuss the results. First, could the seasonal differences in overnight temperature have influenced our fecal metabolite concentrations (i.e. more degradation of the feces occurred under varying temperature regimes) and thus have affected our results? We do not think so for two reasons. 1) Morrow et al. (2002) found that in dairy cattle fecal samples kept at room temperature (25-28°C) and on ice (1-2°C) for 9 h had metabolite concentrations that were approximately 150-200% greater than those that were immediately frozen (-20°C). However, Washburn and Millspaugh (2002) found that white-tailed deer fecal samples kept at room temperature (22°C), high heat (38°C), and alternating 12 h cycles between high heat and room temperature for seven days had metabolite levels comparable to those
that were immediately frozen (-20° C). In our study when overnight temperature were the
warmest (early winter compared with late winter and second litter compared with first)
hares had lower FCM concentrations. Thus, if temperature did affect FCM concentrations
our results may be a conservative estimate of the seasonal differences. 2) The hormonal
challenge data support our FCM data comparing early and late winter.

Second, could seasonal differences in food availability have influenced our FCM
concentrations and thus affected our results? We do not think so for two reasons. 1)
Wasser et al. (1993) found that an increase in dietary fibre resulted in a decrease in fecal
steroid metabolite levels. In our study when dietary fibre was the greatest due to a woody
diet (late winter compared with early winter and first litter compared with second) hares
had higher FCM concentrations. Thus, if dietary fibre did affect FCM concentrations our
results may be a conservative estimate. 2) Changes in food availability may result in a
different percentage of water in the feces and thus fecal weight. An increase in fecal
weight may affect metabolite concentrations since they are based on a per gram feces
amount. In our study we freeze dried fecal samples to remove water and thus eliminate
possible differences due to water content.

6.4.2 Stress physiology

Fecal Cortisol Metabolites –Snowshoe hares’ FCM concentrations fluctuated
both among and within years (Figs 6.2-6.4). Among years we found that FCM
concentrations were highest in the first year of the decline (2007) and greater in the
winter of 2006/07 than in the winter of 2007/08 (Figs 6.2-6.3). Thus hares were more
stressed when the number of predators was greatest (Fig. 6.1). Boonstra et al. (1998a)
also found that hares were more stressed during the decline phase when predator numbers were greatest than during the late low phase when they were the lowest. Charbonnel et al. (2008) also found that cyclic populations of water voles, *Arvicola scherman*, were more stressed during the decline phase compared with the peak. Additionally, an increase in the number of predators has been shown to increase glucocorticoid (GC) levels in a number of other non-cyclic free-ranging mammals and birds (Silverin 1997; Hik et al. 2001; Scheuerlein et al. 2001).

During winter we found that FCM concentrations were greater in the late winter than in the early winter in both years where we had data (Fig. 6.3). The increase in stress levels in the late winter could be explained by an increase in the risk of predation and a decrease in food availability as the winter progressed. We found that as the winter progressed the number of hares per lynx declined, such that there was 0.40-fold fewer hares per lynx in the late winter than in the early winter. This would increase the chances that an individual hare would be killed. Simultaneously, available food resources also may decline overwinter. Sinclair et al. (1982) found that both the quantity and quality of food decreased during this time, reaching its lowest point in late winter. Reduced food intake has been found to cause an increase in GC levels in mammals and birds (Harris et al. 1994; Kitaysky et al. 1999; Ortiz et al. 2001). A consequence of the decrease in food availability from the early to the late winter may force hares to forage in a more risk-prone manner (Hik 1995; Murray 2002). Thus both increased predation risk and reduced food resources are operating overwinter and may help to explain the FCM pattern we observe.
During the breeding season snowshoe hares had higher FCM concentration during the first litter than the second (Fig. 6.4). These changes may also be driven by both by declines in the risk of predation and increases in food availability. Boutin et al. (1986) found that predation rates on snowshoe hares decreased from winter to summer. The proportion of hares to lynx from the first to the second litter would also likely increase, due to the birth of the juvenile hares, resulting in a decrease in the risk of predation, though this may be counterbalanced by the birth of predator young. The first litter also occurs during the late winter-early spring when the winter snowpack is in the process of melting and prior to the flush of new vegetation, while the second litter occurs during late spring-early summer when new vegetative growth is nearing it peak (Sinclair et al. 1982). Thus there are changes in both the quality and quantity of food during this time.

_Hormonal Challenge_ – In snowshoe hares fecal cortisol metabolites are an integration of blood cortisol levels over the previous 8-12 h (Sheriff et al. 2009a) and reflect both basal and stress-induced cortisol experienced during this time. In our hormonal challenge we were able to separate these factors to determine how the HPA axis changed in response to changes in the risk of predation. Our results reflect what we found with FCM concentrations such that free cortisol concentrations were higher at the base bleed, the Dex bleed and in response to the ACTH injection in the winter of 2006/07 (when the number of predators was greatest – Fig. 6.1) than in that of 2007/08 and in the late winter (when the proportion of hares to lynx was lowest) than in the early winter (Fig. 6.5a). The higher basal cortisol concentrations, the disrupted negative feedback (Dex resistance), and the enhanced cortisol release in response to ACTH are all indicative
of the animals being more stressed (Wingfield et al. 1998; Sapolsky et al. 2000; Romero 2004).

The changes in free cortisol were directly tied to changes in the opposite direction of MCBC, with levels being lower in 2006/07 than in 2007/08 and in late winter than in early winter Fig. 6.5b). MCBC is a measure of the capacity of corticosteroid-binding globulin (CBG) to bind cortisol in the plasma (Boonstra et al. 1998). Though a variety of stressors that increase GC concentrations have been found to cause a decline in CBG levels (Schlechte and Hamilton 1987; Frairia et al. 1988), Boonstra and Tinnikov (1998) showed that CBG levels in hares remain relatively constant following an ACTH injection, but the binding capacity (MCBC) rapidly increases. This response was first seen by Boonstra and Singleton (1993) and seems to be unique to hares (Boonstra and Tinnikov 1998). Since it is only the unbound, free cortisol which is active, a decrease in the MCBC levels, as seen in 2006/07 and in the late winter, are indicative of hares being less able to buffer high cortisol concentrations (Sitteri et al. 1982).

Our results show that snowshoe hares are highly sensitive to the risk of predation, with the highest stress levels, as indicated by both FCMs and plasma free cortisol, occurring when the number of predators was greatest. Our seasonal effects suggest that food also affects hare’s stress levels. Food is not likely to be the primary driver of hare’s stress levels but plays a secondary role, augmenting the effects of predation. If food was the primary driver, FCM concentrations in the late winter of 2005/06 (hare peak) should have been greater than those of 2007/08 (second year of the decline). During these times the risk of predation was similar (Fig. 6.1); however, due to the greater number of hares during the peak, food would have been scarcer (Keith 1963). Furthermore, during the
time of sampling, the snowpack in 2008 was much less than in 2006 thus hares would have greater access to food (Sheriff pers. observ.). Boonstra et al. (1998a) also found that food affected hare’s stress levels but not to the extent of predation risk. They found that although fed hares were less stressed than controls, the improvement in their stress response to a hormonal challenge paralleled that of the controls as predation pressure decreased, and both converged during the low phase.

6.4.3 Energy expenditure

Glucocorticoids play a key role in sustaining energetic responses to stress by promoting gluconeogenesis (the production of glucose through the breakdown of other body tissues Miller and Tyrrell 1995), increasing the supply of gluconeogenic precursors and maintaining glycogen availability in the liver (Fujiwara et al. 1996). Snowshoe hares had a moderately elevated glucose mobilization in 2006/07 compared with 2007/08 but a much greater mobilization in late winter than in early winter (Fig. 6.5c). Boonstra et al. (1998a) found that glucose mobilization in hares was much greater during the decline than the late low phase when cortisol concentrations were also greater than the late low phase. An increase in glucose levels has also been found in a population of free-ranging arctic ground squirrels (Spermophilus parryii plesius) under duress (Hik et al. 2001). The increase in glucose mobilization in response to increased predation risk must be extremely costly to hares during the food scarce winter since a greater glucose mobilization means greater liver glycogen stores and gluconeogenesis comes at the expense of peripheral tissues (by inhibiting non-hepatic glucose utilization, by promoting substrate delivery to the liver, and by converting protein to glucose [Miller and Tyrrell
1995]). Although hares have a reduced energy expenditure in winter (Sheriff et al. 2009c) they have minimal winter body reserves (Whittaker and Thomas 1983) and would need to increase foraging to compensate for the increased glucose mobilization, further exposing them to predators.

Free fatty acids (FFA) are one of the substrates delivered to the liver during gluconeogenesis (Miller and Tyrrell 1995). We expected that as glucose mobilization increased the ability to mobilize FFA should decline, and this is what we found in 2006/07 (Fig. 6.5d). Snowshoe hares had a reduced ability to mobilize FFA in the late winter than in early winter. However, in the early to late winter in 2007/08 FFA mobilization was the same. Boonstra et al. (1998a) found that food supplementation resulted in elevated FFA levels in hares. Thus, the similar levels in 2007/08 may be due to the low snowpack level in the late winter of 2007/08, allowing hares greater access to food and an increased ability to mobilize FFA. Together these results provide evidence that the risk of predation affects snowshoe hare’s ability to mobilize energy and that this is further affected by food availability.

6.4.4 Immunology and body condition

High GC levels can act as an immunosuppressant (Munck et al. 1984) and this is normally reflected in lower counts of white blood cells (WBC). However, if an increase in cortisol is associated with an infection, WBC counts would be elevated. In an attempt to rectify this potential confusion, we measured snowshoe hares’ leukocyte profiles. Leukocyte profiles are the relative proportion of each WBC type in a count of 100 leukocytes. Leukocyte profiles are particularly useful in examining the effects of stress
because they are altered in a predictable manner (Dhabhar et al. 1996; Davis et al. 2008). In a stressful situation neutrophil counts increase (neutrophilia) and lymphocyte numbers decrease (lymphopenia) resulting in an increased N:L ratio. However, this is not the complete picture as an infection may also create a rise in neutrophil counts sufficient to substantially increase the N:L ratio. In order to disassociate stress from an infection both eosinophil and monocyte ratios must also be measured, as an infection or parasite will result in an increase in these WBC types (Jain 1986; Campbell 1996). We found that when the risk of predation was higher, in 2006/07 compared with 2007/08 and in the late compared with the early winter, snowshoe hares’ leukocyte profiles shifted into a pattern indicative of a more stressed animal (Table 6.2), specifically, neutrophil counts increased whereas those of lymphocytes, eosinophils and monocytes decreased.

Snowshoe hares’ hematocrit values were higher in 2007/08 than in 2006/07 and in early winter than in late winter (Fig. 6.6a). High hematocrit values have been linked to better nutritional status in black bears (Hellgren et al. 1993), whereas lower values have been linked to increased predation risk in snowshoe hares (Boonstra et al. 1998a) and in arctic ground squirrels (Hik et al. 2001). Hare’s mass index (MI) values were similar among years, but lower in late winter than in early winter (Fig. 6.6b). Although the values in late and early winter were not statistically different ($P = 0.07$), they may be biologically significant (Yucooz 1991). Our results show that hares have a compromised immunology and body condition when the risk of predation is high.
6.4.5 Alternative explanations

Snowshoe hares had greater stress levels, a greater ability to mobilize energy, a leukocyte profile indicative of greater stress, and a poor body condition when the number of predators was highest and, within a season, when the proportion of hares to lynx was the lowest and the food availability the worst. We have argued that an increase in the risk of predation results in hares being more stressed and that food plays a secondary role in this, augmenting or alleviating some of the effects of predation depending on availability. However, other factors (e.g. density, social status, and parasitism) could affect stress levels in hares as they have in other species. Here we will discuss each in turn and why we dismissed them as primary factors affecting snowshoe hares.

Density and Social Status - It has long been recognized that high population densities could disrupt spacing behaviour and increase agonistic interactions and competition leading to an increase in stress (Christian 1980). More recently, it has been shown that not only density but social status can influence GC concentrations (Creel 2001; Young et al. 2006). However, there is little evidence of this in hares. Boonstra et al. (1998a) found that hares were less stressed living in experimentally fed populations whose densities were 4 to 13 times those of controls. Although hares have been shown to display dominance hierarchies in pens and at feeding areas in the wild (Graf 1985), they are not territorial and have broadly overlapping home ranges (Boutin 1984). Our results show that FCM concentrations in 2006 (peak population) were similar to those in 2009 (low phase) even though densities were 3 times greater.

Parasites - Parasites can also be important in shaping animal communities and have been shown to influence GC concentrations in mammals (Chapman et al. 2007). In
snowshoe hares, Keith et al. (1986) studied parasitism for many years in Alberta and concluded that parasites were not a direct cause of mortality. Experimental work with antihelminthics in field populations of hares had no measureable impact on survival or reproduction (Sovell and Holmes 1996), or produced effects only in combination with predation and food (Murray et al. 1997). Thus parasitism may affect some hare populations but is likely not a direct factor affecting GC concentrations.

6.4.6 The low phase

Boonstra et al. (1998b) proposed two major classes of hypotheses to try to account for the low phase of cyclic populations. They suggested that the low phase may be a result of either an extrinsic or intrinsic factor affecting hares and concluded that the most likely candidates were a predation (extrinsic) and a senescence-maternal-effects (intrinsic) hypothesis. Here we merge both classes of hypotheses and suggest that the low phase of cyclic populations is caused by intense predation risk (extrinsic factor) during the decline that then results in intergenerational, maternally-inherited stress (intrinsic factor) affecting hare reproduction during the low.

In order for this hypothesis to be true three criteria must be met. First, snowshoe hares must be highly sensitive to the risk of predation, such that an increase in the risk of predation must result in an increase in hares’ stress physiology. Here we show that hares’ stress levels fluctuated across the cycle and that the highest levels were found during the decline phase. This was primarily driven by the changes in the risk of predation and these effects may have also been affected by a decrease in food availability (both absolute and relative). Second, an increase in maternal stress must result in a decline in reproductive
fitness. Sheriff et al. (2009b) found that, from the peak to the second year of the decline, free-ranging snowshoe hare mothers stress levels were directly associated with a decline in their litter size, and their offspring’s body mass and size. In an experimental manipulation, they found that mothers exposed to a simulated predator had significantly higher cortisol levels than control mothers, resulting in lighter and smaller offspring.

Third, maternal stress must be inherited from mother to daughter. On the same population of hares that maternal stress resulted in a decline in reproduction we found that maternal stress was inherited from mother to daughter (Sheriff et al. unpub. data). These effects were not only evident at weaning, but were permanent, persisting into adulthood. Clearly, each of the above criteria has been met, and we conclude that the low phase is caused by predation induced stress acting through maternal effects.

However, the above conclusion does not explain the variation in the length of the low phase among population cycles. In microtine populations the low phase can last 1-3 years (Henttonen et al. 1987; Framstad et al. 1993, 1997), whereas in snowshoe hares it can last 2-5 years (MacLulich 1957; Meslow and Keith 1968; Keith and Windberg 1978; Krebs et al. 2001a). We suggest that the length of the low phase is a result of the severity of predator-induced maternal stress during the decline. This hypothesis proposes that a greater initial maternal stress during the decline can prolong the intergenerational inheritance of stress (and the negative impacts it imposes on reproduction), and thus result in a longer low phase.

We found that the rate of loss per year during the decline phase was positively correlated to the length of the low phase (Fig. 6.7). The rate of loss may be a good index for the severity of maternal stress during the decline for a number of reasons. First, it
accounts for variation in the risk of predation, in both predator number and proportion of hares to predators. The greater number of hares at the peak, the greater the number of predators at their peak. Furthermore, predator populations typically lag behind hare populations by one year, therefore a greater loss of hares per year means a lower proportion of hares to predators and thus a greater risk of predation. Second, it accounts for variation in the severity of predation. Predator numbers not only increase with increased hare density but their functional response also increases (O’Donoghue et al. 2001). This results in a greater efficacy of predator to kill hares and may result in an increase in stress of hares. Third, the rate of loss also accounts for the generation time for hares (1 year). The greatest risk of predation occurs in the first year of the decline when the number and proportion of predators is highest. If the decline is extremely rapid, the parents of low phase hares would experience this severe risk of predation. However, if the decline is slow, lasting a number of years, then the grandparents or great-grandparents would experience this severe risk of predation and the parents of low phase hares would experience a lower risk of predation. Thus in the latter situation hares during the low phase may have a lower stress level and consequently a higher reproductive output (Sheriff et al. 2009b) compared with those in the former situation, potentially shortening the low phase.

In conclusion, this study has shown that hares are highly sensitive to the risk of predation and as the risk increases, particularly during the decline phase, hares are highly stressed. Based on these and our previous findings we propose that the low phase of population cycles is the result of the impact of intergenerational, maternally-inherited
stress. Furthermore, we suggest that the length of the low phase is a result of the severity of maternal stress during the decline phase.
Table 6.1. Repeated-measures ANOVA testing for differences in year (2006/07 vs. 2007/08) and season (early vs. late winter) in response to the hormonal challenge in snowshoe hares.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Free Cortisol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>1</td>
<td>163792</td>
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<td>409314</td>
<td>16.24</td>
<td>0.0002</td>
</tr>
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<td>Y x S</td>
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<tr>
<td>Subject (Group)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td>4</td>
<td>1339253</td>
<td>196.70</td>
<td>0.0001</td>
</tr>
<tr>
<td>Response x Year</td>
<td>4</td>
<td>18842</td>
<td>2.77</td>
<td>0.03</td>
</tr>
<tr>
<td>Response x Season</td>
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<td>44528</td>
<td>6.54</td>
<td>0.001</td>
</tr>
<tr>
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<td>4747</td>
<td>0.70</td>
<td>0.5</td>
</tr>
<tr>
<td>Error</td>
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</tr>
<tr>
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<td>0.003</td>
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<td></td>
</tr>
<tr>
<td>Response</td>
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<td>4.00</td>
<td>0.01</td>
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<tr>
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<td>1409</td>
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<tr>
<td>Error</td>
<td>184</td>
<td>830</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>9659</td>
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</tr>
<tr>
<td>Error</td>
<td>184</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Free Fatty Acids</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
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<td>0.02</td>
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<td>0.84</td>
</tr>
<tr>
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<td>0.48</td>
</tr>
<tr>
<td>Y x S</td>
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<td>13.10</td>
<td>0.0007</td>
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<tr>
<td>Subject (Group)</td>
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<td></td>
</tr>
<tr>
<td>Response</td>
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<td>30.58</td>
<td>348.72</td>
<td>0.0001</td>
</tr>
<tr>
<td>Response x Year</td>
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<td>0.13</td>
<td>1.45</td>
<td>0.22</td>
</tr>
<tr>
<td>Response x Season</td>
<td>4</td>
<td>0.35</td>
<td>4.04</td>
<td>0.02</td>
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<tr>
<td>R x Y x S</td>
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<td>1.24</td>
<td>14.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>184</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: P*-values of the within subject (response) analysis were adjusted using the Greenhouse-Geisser estimate.
Table 6.2. Number of major cell types (mean ± SE) of leucocytes in a count of 100 cells per slide (2 slides per hare) and N:L ratios from snowshoe hares from early and late winter in 2006/07 and 2007/08.

<table>
<thead>
<tr>
<th>Leucocytes</th>
<th>Season</th>
<th>2006/07</th>
<th>2007/08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Early Winter</td>
<td>55 ± 2 (14)</td>
<td>48 ± 3 (16)</td>
</tr>
<tr>
<td></td>
<td>Late Winter</td>
<td>66 ± 1 (13)</td>
<td>61 ± 2 (13)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Early Winter</td>
<td>33 ± 2</td>
<td>37 ± 3</td>
</tr>
<tr>
<td></td>
<td>Late Winter</td>
<td>26 ± 1</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Early Winter</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>Late Winter</td>
<td>2 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Early Winter</td>
<td>3 ± 0</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>Late Winter</td>
<td>4 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>N:L Ratios</td>
<td>Early Winter</td>
<td>1.8 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Late Winter</td>
<td>2.6 ± 0.2</td>
<td>2.8 ± 0.5</td>
</tr>
</tbody>
</table>

Note: Samples were obtained from the base bleed of the hormonal challenge. Sample sizes are in parentheses and are given for neutrophils only and are the same for all other cell types. Single asterisks denote a significant change from early to late winter, double asterisks denote a significant change from winter 2006/07 to winter 2007/08. N:L ratios were not statistically compared since neutrophil and lymphocyte counts were.
Fig. 6.1. Snowshoe hare and lynx population densities in the southwestern Yukon, Canada from the winter of 1976/77 to the winter of 2008/09.
Fig. 6.2. Fecal cortisol metabolite (FCM) concentrations (mean ± SE) of snowshoe hares in the late winter from 2006 (n = 20), 2007 (n = 19), 2008 (n = 20), 2009 (n = 18). Letters denote significant differences ($P < 0.05$).
Fig. 6.3. Fecal cortisol metabolite (FCM) concentrations (mean ± SE) of snowshoe hares in the winter of 2006/07 (n = 14 and 17 for early and late winter, respectively) and 2007/08 (n = 20 for both early and late winter). Asterisks denote significant differences (* \( P < 0.05 \); ** \( P < 0.005 \)).
Fig. 6.4. Fecal cortisol metabolite (FCM) concentration (mean ± SE) for (a) adult male and (b) adult female snowshoe hares during the first and second litters of the breeding season, 2005-2008. (males n = 2,5; 10,10; 10,9; 10,10 and females n = 4,5; 10,10; 8,10; 10,8 for the first and second litters, respectively; 2005-2008). See results for statistical comparisons.
Fig. 6.5. Responses over time in plasma concentrations (mean ± SE) of (a) free cortisol (b) maximum corticosteroid-binding capacity (MCBC) (c) glucose, and (d) free fatty acids (FFA) to the hormone challenge in snowshoe hares from early and late winter in 2006/07 and 2007/08. (cortisol and MCBC n = 12,12; 13,13; glucose n = 12,10; 10,11; FFA n = 14,10; 12,14 in early and late winter, respectively, from 2006/07 and 2007/08). Base indicates values at the initial bleed, Dex indicates values 2 h after the dexamethasone injection, and P30, P60, and P120 indicates values 30, 60, and 120 min, respectively, after the adrenocorticotropic hormone (ACTH) injection. See results for statistical comparisons.
Fig. 6.6. Changes (mean ± SE) in (a) hematocrit (%) measured as the packed red-blood cell volume and (b) mass index in snowshoe hares from early (n = 14, 13) and late (n = 16, 15) winter in 2006/07 and 2007/08. Hematocrit was measured at the base bleed. The mass index was obtained from measurements at the time of trapping. Hematocrit and mass index were measured on the same hares. Asterisks denotes significant differences (* $P < 0.05$, ** $P < 0.1$).
Fig. 6.7. The length of the low phase and previous rate of loss of 10 snowshoe hare population cycles (y = 8.88x + 0.65). Diamonds represent data from Hudson Bay records (see MacLulich 1957), squares represent data from Kluane (see Krebs et al. 1986, 2001b; Krebs unpublished data), and triangles represent data from Rochester (see Meslow and Keith 1968; Keith and Windberg 1978).
6.5 References


Boonstra, R., and A.A. Tinnikov. 1998. Increased corticosteroid binding capacity of plasma albumin but not of CBG caused by ACTH induced changes in free fatty acid concentrations in snowshoe hares and rabbits. Journal of Endocrinology 156:205-212.


7. GENERAL DISCUSSION AND CONCLUSIONS

Recent studies on the effects of climate change have shown that it may result in a mismatch between the timing of breeding and the timing of food availability in many animal species (Visser et al. 1998; Both et al. 2008; Post and Forchhammer 2008). Such a mismatch may occur because increases in springtime temperatures lead to advances in the timing of spring events such as plant growth and insect emergence (Visser and Holleman 2001; Walther et al. 2002; Post 2003b) whereas the onset of reproduction for many animals is cued by seasonal changes in daylength (Post 2003a), which remains constant. The consequences of this mismatch may be severe and ultimately reproductive failure or recruitment failure may occur, leading to population collapse (Both et al. 2006). The inheritance of traits, through maternal effects, may also cause animals to be mismatched with their environment (Boonstra and Boag 1987; Boonstra and Hochachka 1997).

Maternal effects represent the transmission of non-genetic developmental factors and developmental plasticity that results from the effects a mother’s phenotype has on her offsprings’ phenotype that cannot solely be ascribed to inherited genes (Uller 2008). Theoretical work on maternal effects has shown that it can cause a resemblance not just between parents and offspring but between grandparents and grandoffspring (Kirkpatrick and Lande 1989). Simple mathematical models have shown how maternal effects may be major drivers of cyclic population dynamics (Ginzburg and Taneyhill 1994; Ginzburg 1998; Inchausti and Ginzburg 1998) such as those occurring in forest insects, microtines (voles and lemmings), and snowshoe hares.
The mechanisms responsible for maternal effects may vary between organisms, but in many animals hormones play a vital role (insects - Rossiter 1994; fish - McCormick 1998; reptiles - Meylan and Clobert 2005; birds - Hayward and Wingfield 2004; Love et al. 2005; mammals - Meaney 2001; Seckl 2004). The key to responding adaptively to environmental challenges in vertebrates is the ‘stress response’, defined here as the set of neural and endocrine responses that help restore homeostasis following an exposure to a stressor (Sapolsky et al. 2000). Central to the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the resultant secretion of glucocorticoids (GC), lasting several minutes to hours (Sapolsky 1992, Wingfield and Romero 2001). When females experience an increase in GC levels during pregnancy or lactation in mammals and during yolk formation in egg-laying vertebrates, their offspring may also experience an increase in circulating GC levels (Hayward and Wingfield 2004; Owen et al. 2005). This increase in GC concentrations in the fetus can permanently program their HPA axis resulting in greater GC concentrations after birth (Henry et al. 1994; Liu et al. 1997; Kapoor et al. 2008). Exposure to increased maternal GC levels has also been linked to a decrease in offspring number, size, weight and growth rates (Hayward and Wingfield 2004; Sheriff et al. 2009a) as well as offspring depression and anxiety-like behaviors (Abe et al. 2007; Meaney et al. 2007; Emack et al. 2008).

Snowshoe hares are an excellent animal in which to investigate the impact maternal effects have on population dynamics. Snowshoe hare populations undergo a regular cyclic fluctuation with 8-10 years between peak densities (Keith 1963; Krebs et al. 1986). As hare population density increases so does that of their predators (lynx, coyotes, great horned owls, goshawks, etc…), but with a lag of 1-2 years. During the hare
population decline, predators are the direct cause of up to 100% of hare deaths (Hodges et al. 2001). Hare survival and reproduction also cycles, with maximum rates occurring during the early increase phase (when predator numbers are lowest), but they then progressively decrease to a nadir during the decline (when predator numbers are at their peak) (Cary and Keith 1979; Boutin 1986; O’Donoghue and Krebs 1992; O’Donoghue et al. 1997; Hodges et al. 2001; Stefan and Krebs 2001). In cyclic populations it has also been documented that there is a ‘memory’ of the past as expressed in reproductive fitness. Sinclair et al. (2003) found that when hares from different phases of the cycle were brought into the laboratory and bred under ideal conditions, they maintained the breeding performance of their field counterparts, indicating intrinsic, field-derived differences among them. Similar results have been found in cyclic vole species going through 3-4 year population cycles (Mihok and Boonstra 1992).

The 10-year snowshoe hare cycle and its attendant cycles in lynx and other furbearers have been fundamental to the development of ecological theory for more than half a century. Empirical studies have examined both this theory as well as fundamental concepts such as ‘the balance-of-nature’, predator-prey fluctuations, food web dynamics, and community organization (Elton and Nicholson 1942; Keith 1963; Pimm 1981; Krebs et al. 1986; Royama 1992; Boonstra et al. 1998b; Stenseth et al. 1998, Sinclair et al. 2000; Korpimäki et al. 2004; Inchausti and Ginzburg 2009). Although these cycles have been studied for over 70 years (MacLulich 1937; Keith 1963; Krebs et al. 1986, 1995) two unexplained patterns in the population dynamics of snowshoe hares remain. The first is why hare populations remain low for 2-5 years after the decline phase even though the predator populations have collapsed and there is ample vegetation. The second enigma is
why the low phase varies in length. Here I will present evidence to explain these two enigmas. I discuss the ecological advantages of maternally inherited stress, its potential to create a mismatch between the environment and the animal, and how this plays a substantial role in the low phase of cyclic populations.

7.1 Ecological advantages of maternally inherited stress

Although an increase in maternal GCs may have adverse affects on offspring development, trade-offs exist that may make these effects advantageous. In nature the two most important factors affecting animal populations are predation and food, and these factors can act separately or together in a synergistic fashion (Krebs et al. 1995; Clinchy et al. 2004). If maternal effects are advantageous they should act as an adaptive mechanistic link between the mother’s environment (high or low predation and food availability) and the offspring’s phenotype, increasing fitness.

In an environment where the risk of predation is high, it should be beneficial that mothers transmit anti-predator vigilance behaviors to their offspring. Snowshoe hares are highly sensitive to the risk of predation (Boonstra et al. 1998a) with an increase in the risk of predation resulting in an increase in maternal GC levels (Sheriff et al. 2009b). In a free-ranging population of hares we found that an increase in dam GC concentrations at birth was associated with a similar increase in juvenile concentrations at weaning and this was seen at a population wide level (Sheriff et al. 2009b see thesis Fig. 5.2). Furthermore, offspring born to stressed mothers had a greater stress-induced response than those born to less stressed mothers. Laboratory experiments show that prenatal stress is associated with certain anti-predator behaviors such as increased fearfulness, anxiety, and vigilance,
and decreased locomotor activity (Drake et al. 2005; Emack et al. 2008). Thus, an increase in maternal GCs may result in a decrease in offspring body size and weight, but ultimately may result in offspring that are much more wary and less likely to be killed by predators.

In an environment where food availability is highly variable, Love et al. (2005) and Love and Williams (2008) suggested that maternally derived GCs could act as an adaptive mechanism linking maternal quality to offspring quality. They found that under poor food conditions an increase in maternal GCs resulted in a decrease in brood quality. By allowing females to reduce the investment in their current offspring this increased the females’ survival and future fecundity. In a population of free-ranging common murres (Uria aalge) Kitaysky et al. (2007) found that GC concentrations were negatively correlated with food abundance and that food-related stress during reproduction contributed to decreased fecundity. As GC levels increased, the parental birds changed the allocation of resources away from reproductive processes and towards self maintenance. Thus an increase in maternal GC levels decreases offspring demand and allows the maintenance of maternal body condition.

Lastly, we suggest that the combined effects of predation and food play an equal role in this adaptive mechanism. As the risk of predation increases, maternal GCs increase and this results not only in offspring with a greater stress response (Sheriff et al. 2009b see thesis Fig. 5.4), but also a decrease in litter size and offspring birth weight and size (Sheriff et al. 2009a see thesis Fig. 4.3-4.5). Thus offspring would not only be born with more vigilant anti-predator behaviors, ready to deal with an environment of high predation risk, but dams would have a reduced demand placed on them. Since stressed
dams have fewer, smaller offspring they could forage in a less risky manner. This is supported by the findings of Hik (1995) who showed that snowshoe hares modified their behavior as the risk of predation increased, utilizing areas of higher cover but with less food available. Thus maternally inherited stress may be ecologically advantageous, preparing offspring to deal with the environment that the mother perceives, while also allowing the mother to increase her survival and future fecundity.

### 7.2 Environmental mismatch

Although these maternal effects may be highly adaptive in a more constant environment (e.g., song sparrows Clinchy et al. 2004; elk Creel et al. 2005), in an environment that is highly variable this intergenerational programming may create a mismatch between the offspring’s phenotype and their environment (i.e. good environmental conditions return prior to an animal being able to capitalize on them). A mismatch between the predicted and subsequent reality can cause a severe disadvantage in fitness related traits. Hayward and Wingfield (2004) found that in Japanese quail (*Coturnix coturnix japonica*) an increase in maternal GC levels resulted in offspring that had a lower growth rate compared with controls when both were fed ad libitum. Blas et al. (2007) found that in a free-ranging population of European white storks (*Ciconia ciconia*) the magnitude of the stress response during development was negatively related to survival and recruitment under elevated population densities and food availability. Even though some animals may be able to compensate for a poor start to life should conditions improve, evidence suggests that this compensation is also associated with a variety of costs later in adult life (Metcalf and Monaghan 2001).
In mammals there has been little work on maternal programming in free-ranging populations. Sheriff et al. (2009b see thesis Fig. 5.3) found that adult-sized snowshoe hares born to mothers with greater GC levels had themselves greater GC levels. Although Sheriff et al. (2009b) were unable to investigate changes in growth, survival or reproduction in the F1 generation, laboratory studies of mammals support the hypothesis that environmental mismatches reduce fitness; a number of studies have found that the F1 generation exposed to prenatal stress but raised in good environment (fed ad libitum), had reduced litter sizes and lower offspring weight compared with the controls (Drake et al. 2005; Emack et al. 2008; Götz et al. 2008).

7.3 The low phase of cyclic populations

One of the greatest examples of a mismatch between animals and their environment may be during the low phase of cyclic populations. During the low phase, the predators have virtually disappeared and the vegetation is ample, yet the reproduction of the prey species remains low and populations do not increase for a number of years (Krebs et al. 2001). Synchrony between the prey and its environment is only re-established during the increase phase. Furthermore, the low phase varies greatly in length, from 1-3 years in microtine populations (Korpimäki and Norrdahl 1991) and 2-5 years in snowshoe hares (Keith 1990; Krebs et al. 2001). Recent research has shown that maternally-inherited stress may play a substantial role in the low phase of cyclic populations, helping to explain both the low phase itself and the variation in its length.

During the decline phase in cyclic populations of both voles and hares, GC levels are significantly higher than those during the increase, peak, and low phase (Charbonnel
et al. 2007; Fig. 6.2). Furthermore, during the early low phase of the hare cycle, dams have elevated GC levels similar to those found during the population peak (when reproduction starts to decline) (Fig. 6.2). In hares, elevated maternal GC levels have also been found to decrease reproduction and to compromise the stress axis of the offspring such that offspring born to stressed mothers have a greater stress response themselves (Sheriff et al. 2009a, b see thesis Figs 4.3-4.5 and 5.4). During the late decline phase dams would be highly stressed due to a high risk of predation. This would cause them to give birth to fewer, smaller offspring, who are born with a compromised stress axis. These offspring, even though they live during the low phase when the risk of predation is low, would have elevated GC levels that would result in poor reproductive fitness. Thus, the negative impacts of maternally inherited stress may cause the low phase in cyclic populations.

Yet this does not explain how reproduction may recover and the population increase. GC receptor levels may help explain this phenomenon. Densities of the two hippocampal receptors are critical in regulation and feedback of the stress axis, with mineralcorticoid receptors (MR) regulating basal glucocorticoid levels and glucocorticoid receptors (GR) regulating glucocorticoid levels in response to stressors (de Kloet et al. 2005; Owen et al. 2005). It has been shown that the treatment of pregnant guinea pigs with a synthetic glucocorticoid resulted in offspring with increased hippocampal MR expression and reduced basal plasma cortisol concentrations (Liu et al. 2001). Furthermore, exposure of pregnant guinea pigs to a strobe light at gestation day 50 resulted in offspring with reduced GR expression and an elevated activity of the stress axis (Kapoor et al. 2008). These differences in receptor levels persist into adulthood (Liu
et al. 1997; Francis et al. 1999). In a captive study of hares, Sheriff et al. (2009b see thesis Fig. 5.4) found that offspring born to mothers with greater GC levels had a lower basal plasma GC level but a greater stress response to a standardized stressor. This difference between basal levels and stress response could be due to an increase in MR levels and a decrease GR levels. During the low phase, these differences in MR and GR receptor levels would allow hares to mount an appropriate response to predation, yet allow them to have low GC exposure at times when the risk of predation is low, alleviating the negative effects of high GC exposure such as a decrease in reproduction. Thus, during the low phase reproduction could begin to recover and population density increase.

Lastly, maternally inherited stress could help explain the variation in the length of the low phase with a greater initial maternal stress during the decline prolonging the intergenerational inheritance of stress (and the negative impacts it imposes on reproduction), and thus resulting in a longer low phase. Using data from 10 complete cycles, spanning the years from 1849 to 2009, which included five estimates from the Hudson Bay pelt records (MacLulich 1957) and five mark-recapture estimates (three from Kluane, Yukon [Krebs et al. 1986, 2001; Krebs unpublished data] and two from Rochester, Alberta [Meslow and Keith 1968; Keith and Windberg 1978]) we found that the rate of loss per year during the decline phase was positively correlated to the length of the low phase (Fig. 6.7). The rate of loss may be a good index for the severity of maternal stress during the decline for a number of reasons. First, it accounts for variation in the risk of predation, in both predator number and proportion of hares to predators. The greater number of hares at the peak, the greater the number of predators at their peak.
Furthermore, predator populations typically lag behind hare populations by one year, therefore a greater loss of hares per year means a lower proportion of hares to predators and thus a greater risk of predation. Second, it accounts for variation in the severity of predation. Predator numbers not only increase with increased hare density but their functional response also increases (i.e. there is an increase in the number of hares killed per predator, which may result in an increase in the stress experienced by hares) (O’Donoghue et al. 2001). Third, the rate of loss also accounts for the generation time for hares (1 year). The greatest risk of predation occurs in the first year of the decline when the number and proportion of predators is highest. If the decline is extremely rapid, the parents of low phase hares would have experienced this severe risk of predation. However, if the decline is slow, lasting a number of years, then it would be the grandparents or great-grandparents experiencing this severe risk of predation, whereas the parents of low phase hares would have experienced a lower risk of predation. Thus in the latter situation hares during the low phase may have a lower stress level and consequently a higher reproductive output compared with those in the former situation, potentially shortening the low phase.

7.4 Conclusion

In summary, maternally inherited stress may be highly advantageous to animals that live in a relatively stable or predictable environment. This inheritance may promote anti-predator behaviors in offspring and reduce the demand on the mother allowing her to forage in a less risk prone manner. However, in a highly variable environment, like that being created by climate change, maternal effects may cause animals to be mismatched
with their environment. This mismatch may result in severe consequences for the animal such as a decline in survival and reproduction, and ultimately may cause populations to decline. In cyclic populations, in which the environment is always changing, maternally inherited stress causes the offspring to be mismatched with their current environment. This mismatch may help explain the low phase in which the predators have virtually disappeared and the food ample, but the population remains low. During the decline phase when the risk of predation is high maternal GC levels are also high. Mothers with elevated GC levels give birth to offspring that also have elevated GC levels which then results in low reproductive output. The more severe the initial maternal stress levels the longer the inheritance and its negative impacts on reproduction and thus the longer the low phase.
7.5 References


MacLulich, D.A. 1937. Fluctuations in the numbers of the varying hare (*Lepus americanus*). University of Toronto Studies, Biological Series. The University of Toronto Press, Toronto, Can.


APPENDICES

Appendix A - Animal Care Certificates
ANIMAL CARE CERTIFICATE

Application Number: A05-1661

Investigator or Course Director: Charles J. Krebs

Department: Zoology

Animals: Hares 236

Start Date: April 25, 2005

Approval Date: September 30, 2005

Funding Sources:

Funding Agency: UBC Grants from Federal Departments

Funding Title: Energetics of snowshoe hares in the Canadian Arctic

Unfunded title: Stress and Thermal Ecophysiology of Snowshoe Hares in the Canadian Arctic

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A07-0004

Investigator or Course Director: Charles J. Krebs

Department: Zoology

Animals: Hares 236

Start Date: January 4, 2007

Approval Date: April 3, 2007

Funding Sources:

Funding Agency: UBC Grants from Federal Departments
Funding Title: Energetics of snowshoe hares in the Canadian Arctic

Unfunded title: Stress and Thermal Ecophysiology of Snowshoe Hares in the Canadian Arctic

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A07-0004

Investigator or Course Director: Charles J. Krebs

Department: Zoology

Animals: Hares 236

Start Date: January 4, 2007

Approval Date: April 17, 2008

Funding Sources:

Funding Agency: UBC Grants from Federal Departments

Funding Title: Energetics of snowshoe hares in the Canadian Arctic

Unfunded title: Stress and Thermal Ecophysiology of Snowshoe Hares in the Canadian Arctic

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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