Cyclical Variation in Predation Risk: Impacts on Snowshoe Hare (*Lepus americanus*) Offspring Through Maternal Programming

by

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Abstract

Snowshoe hare (*Lepus americanus*) populations cycle over 10-year periods, primarily driven by predation. High predation risk triggers the decline phase of the cycle through both direct mortality and non-consumptive stress effects that curtail reproduction and survival. The decline phase is followed by an enigma, the prolonged low phase, where the population fails to recover. This suggests maternal stress effects may act to program offspring across generations in this population. Through a natural monitoring study spanning the hare cycle, we investigated the impact of changing predation risk and maternal stress on offspring. Offspring in the decline phase showed a suite of changes to their behaviour, condition, physiology and gene expression compared to offspring from the increase and peak phases, suggesting maternal programming is occurring. This study was novel in documenting changes in gene expression of hippocampal glucocorticoid receptors in response to a natural increase in predation risk in a wild mammal.

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List of Abbreviations

ACTH: Adrenocorticotropic hormone

CGB: Corticosteroid-binding globulin

DEX: Dexamethasone

DNA: Deoxyribonucleic acid

EIA: Enzyme immunoassay

FCM concentration: Fecal cortisol metabolite concentration

GCs: Glucocorticoids

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GR: Glucocorticoid receptor

HPA axis: Hypothalamic-Pituitary-Adrenal axis

MR: Mineralocorticoid receptor

MCBC: Maximum corticosteroid binding capacity

PCV: Packed red blood cell volume

PPIA: Peptidylprolyl isomerase A (Cyclophilin A)

PVN: Paraventricular nucleus

qPCR: Quantitative real-time polymerase chain reaction

RHF length: Right hind foot length

RIA: Radioimmunoassay

RNA: Ribonucleic acid

YWHAZ: Tyrosine 3-Monooxygenase gene

11β-HSD: 11-Beta-Hydroxysteroid dehydrogenase

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Chapter 1
General Introduction

1 General Introduction

1.1 Population Cycles

1.1.1 Description and regulation

In 1859, Darwin published his book "On the Origin of Species by Means of Natural Selection". He recognized that species have the capacity to increase in numbers, but do not continue to do so, and therefore there must be a limiting factor or regulator to this. From his observations, Darwin (1859) believed the species' environment must be limiting population growth, specifically through extrinsic factors such as food resources, predation, climate and possibly disease. Throughout the 20th century, researchers continued to investigate the limiting factors in population regulation, often concluding that the abundance of food resources regulate population size over time (Lack, 1954). However, density-dependent intrinsic factors including intraspecific social interactions, territoriality, competition, and stress, have been found to play integral roles in population regulation in many species (Chitty, 1967; Krebs, 1978). These intrinsic factors in combination with extrinsic factors, can limit the capacity for population growth regulate population size dynamically over time.

Yet, certain species undergo fluctuations in abundance (i.e. rise and fall) over predictable and relatively consistent periods of time, deemed "cycles" (Krebs, 1996). These cycles have intrigued scientists for generations and have been the focus of extensive research to understand the drivers and mechanisms of this dynamic population regulation (Chitty, 1977; Christian, 1950; Elton, 1924; Elton and Nicholson, 1942; Krebs and Myers, 1974). Krebs and Myers (1974) identified parameters to define cyclic populations, such as: phase-related changes in reproductive rates, survival and age at sexual maturity. Additional research identified that time lags in response to environmental change or density change are also characteristic of these cycles (Batzli, 1992).

Furthermore, a significant body of research has developed and tested several hypotheses for these regular cycles in population density being caused solely by extrinsic factors, or by an interaction of extrinsic and intrinsic factors (Chitty, 1960; Chitty, 1977; Christian, 1950). For example Elton (1924) recognized these cycles to be common in Arvicoline rodents (lemmings and voles) in Northern ecosystems and postulated this was due to extrinsic climatic variations. Christian (1950) ruled out the hypothesis that disease, infections, and parasites at high densities led to a decline in population abundance and put forward the stress hypothesis. The stress hypothesis suggests that mutual interactions between individuals lead to physiological changes which reduce births and increase deaths (Christian, 1950). Next, Chitty (1967) formulated the genetic-behavioural hypothesis, stating that during low and increasing densities, mutual interference is minimal and non-aggressive genotypes are selected. Whereas, at high population densities, mutual interference is intense and aggressive genotypes are selected for their ability to withstand intraspecific competition (Chitty, 1967). Implicit in this hypothesis is the assumption that the changes which occur with changing density have a genetic basis and that these changes are correlated with changes in spacing behaviour. However, evidence from Boonstra and Boag (1987) and Boonstra and Hochachka (1997) rejected Chitty's genetic-behavioural hypothesis and suggested differential maternal condition and stress response plays a larger role and may override genetic contributions to individual differences.

The most likely cause of these cycles is maternal effects; i.e. the transmission of maternal condition to offspring, specifically when condition may be poor at peak densities, thus leading to a decline (Boonstra, 1985; evidence in voles Mihok and Boonstra, 1992; evidence in snowshoe hares Boonstra and Singleton, 1993; Boonstra et al., 1998a; Boonstra et al., 1998b; Sinclair et al., 2003). Chitty (1967) assumed that population cycles should be driven by the same mechanisms in all species. As species who undergo cyclic fluctuations in abundance are quite different in terms of life history characteristics, sociality, territoriality, trophic interactions and periodicity of their cycle, this is likely not true. For example, snowshoe hare (*Lepus americanus*) have not been found to exhibit territoriality, spacing behaviour or social mortality due to density, in contrast to other cyclic species (Krebs et al., 1986a). Rather, they cycle with their specialist predator, the Canada lynx (*Lynx canadensis*), making them a unique and interesting area of population cycle research for which to examine maternal effects, likely driven by high predation risk.

1.2 Study organism

1.2.1 Snowshoe hare biology

Snowshoe hare (*Lepus americanus* Erxleben 1777) are a small mammal in the order Lagomorpha (Fig. 1.1). Snowshoe hare populations fluctuate cyclically over a period of 8-11 years (Cary and Keith, 1979; Krebs et al., 2001a; Smith, 1983). The periodicity of the cycle remains relatively constant over an average of 9-10 years, but the amplitude can change drastically (Krebs et al., 1995), as hare density has been found to change 20- to 200-fold over the course of one cycle (Hodges et al., 2001; Keith and Windberg, 1978; Krebs et al., 2018a). The snowshoe hare cycle has four characteristic phases: the increase, the peak, the decline and the low.

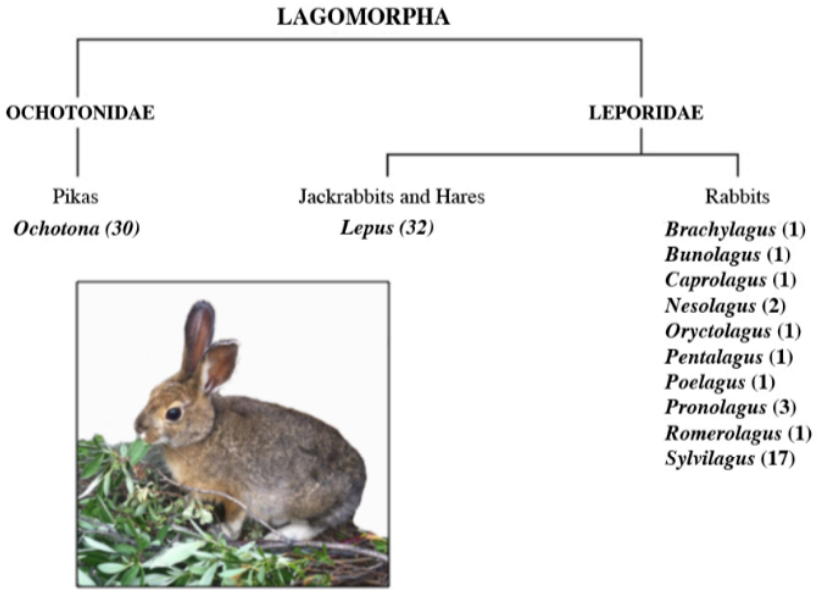

Figure 1.1: The order Lagomorpha, showing families and genera of living lagomorphs (number of species). Snowshoe hare (*Lepus americanus*; inset – summer morph) are in the Leporidae family. *Obtained from* Lavergne (2018), *originally modified from* Chapman and Flux (2008).

The snowshoe hare's range extends across Canada and Alaska, as well as some of the northern contiguous United States. Snowshoe hare are the dominant herbivore in the boreal forests of northern Canada, representing over 50% of the small herbivore biomass (Boutin et al., 1995). Snowshoe hare are central to the boreal forest food web, being consumed by a variety of predators (Boonstra et al., 2018), and living 1-5 years due to this high predation (Hodges, 1999).

The main predators of snowshoe hare are Canada lynx (*Lynx canadensis*), coyotes (*Canis latrans*), great horned owls (*Bubo virginianus*), and Northern goshawks (*Accipiter gentilis*) (Krebs et al., 2001b). Lynx are specialist predators of snowshoe hare and their populations also undergo cyclic fluctuations, closely linked to snowshoe hare, with a lag of one to two years (Boutin et al., 1995; Elton and Nicholson, 1942; Keith and Windberg, 1978).

Snowshoe hare are a multi-littered species, with synchronous breeding and post-partum estrous (i.e. get pregnant immediately after birth of a litter) (Cary and Keith, 1979; Severaid, 1942), which may serve to maximize reproductive output during the short northern summer. The breeding season begins in April each year and the first litter is born in mid to late May (Keith and Windberg, 1978; O'Donoghue and Krebs, 1992). Offspring are born in two to four distinct litters over the course of the summer breeding season, spaced by a gestation period of 35-40 days (Cary and Keith, 1979; O'Donoghue and Krebs, 1992; Stefan, 1998). Snowshoe hare give birth to precocial young, leverets, who are born fully furred, ambulatory and with their eyes open less than an hour after birth (Cary and Keith, 1979; Severaid, 1942). Litter size varies from one to nine young, with leverets weighing around 30-70 grams and birth mass being highly correlated with litter size (O'Donoghue and Krebs, 1992). Snowshoe hare display a type III survivorship curve, with up to 94% of newborns dying within the first 14 days of life (O'Donoghue, 1994). At least 82% of this leveret mortality is due to predation, including from species of their own trophic level, such as red squirrels (*Tamiasciurus hudsonicus*) and arctic ground squirrels (*Spermophilus parryii*) (O'Donoghue, 1994). However, this may no longer be true as arctic ground squirrels are now functionally extinct in certain areas of the boreal forest and red squirrel populations are at low levels (Krebs et al., 2018b; Werner et al., 2015).

1.2.2 Snowshoe hare demography

Over the course of a snowshoe hare cycle, we also see cyclical changes in demography (Hodges et al., 1999b). The occurrence of third and fourth litters of the breeding season fluctuate over the course of the cycle, and proceed changes in population density (Stefan and Krebs, 2001). In the late low and early increase phases, dams have four litters per breeding season, whereas dams only have two litters per breeding season at the peak and decline phases (Stefan, 1998; Stefan and Krebs, 2001). This leads to a huge cyclic variation in reproductive output, anywhere from

3.3 to 18.9 leverets per breeding season, depending on the phase of the cycle (Boonstra et al., 1998a; Cary and Keith, 1979; Stefan and Krebs, 2001). This loss of entire litter groups could be due to changes in female body condition, which have been found to be lowest in the decline phase, and rapidly improved during the low phase (Boonstra et al., 1998a; Stefan and Krebs, 2001). Hare survival also fluctuates over the course of the cycle based on predator numbers, and adult survival has been found to be as low as 11% in the decline phase (Keith, 1974).

The snowshoe hare cycle is not synchronous throughout Canada and Alaska's boreal forests, and appears to follow a travelling wave with peak populations varying up to 4 years across these areas (Krebs et al., 2001a; Krebs et al., 2018a; Smith, 1983). Movement of predators is likely key in driving this pattern, as lynx and raptors are capable of moving hundreds of kilometers in search of prey (Krebs et al., 2001a; Mowat et al., 2000). Keith and colleagues (1974, 1968) began research on snowshoe hare in northern Alberta in 1961. Krebs and colleagues began research on snowshoe hare in the Kluane Lake region of Yukon Territory in 1976 (Krebs et al., 2001b). In the Yukon, we have snowshoe hare density estimates from 1976 to present, and lynx density estimates from 1988 to present (Fig. 1.2). Over the course of these five past cycles, much headway has been made in investigating hypotheses into the cyclic dynamics of snowshoe hare.

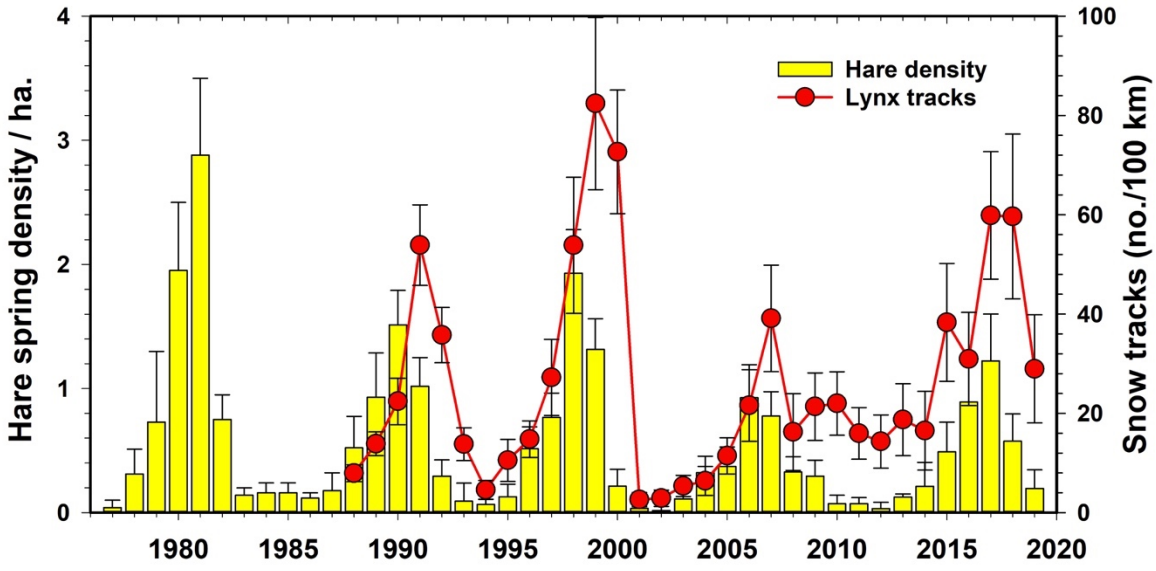

Figure 1.2: Snowshoe hare spring density estimates (means ± 95% CI) from 1976 to 2019, and lynx population estimates (means ± 1 SE) from 1988 to 2019 in the Kluane Lake region, Yukon Territory. *Obtained and adapted from* Krebs et al. (2018b).

1.2.3 The prolonged low phase

After years of research, snowshoe hare population dynamics are well understood. During the decline phase, snowshoe hare and their predators drop and reach very low densities. However, the hare populations do not recover immediately and remain low for two to five years (Boonstra and Singleton, 1993; Boonstra et al., 1998b). As the enigma of the snowshoe hare cycle, the low phase is characterized by abundant high-quality food, high survival rates, low predator numbers, yet low population growth and recovery from the decline phase (Boonstra et al., 1998b; Hodges et al., 1999b). This leads to two hypotheses: first, something is wrong with the extrinsic environment (food, predation), and/or second, something is wrong with the animals themselves (intrinsic; stress effects, maternal effects) (Boonstra et al., 1998b).

1.2.4 Extrinsic factors in snowshoe hare cyclic dynamics

Food shortage from over browsing by snowshoe hares at the peak was initially postulated to be the driver of the decline phase (Keith, 1983; Pease et al., 1979). In the Yukon, Boutin (1984) supplemented snowshoe hare populations with food during the peak and early decline, and found densities increased 2 to 3-fold in these areas, with no changes to cyclic dynamics (Boutin, 1990). Although some animals were undernourished at the peak, the addition of food did not prevent the population decline from occurring (Boutin, 1990; Krebs et al., 1986a). However, food shortage at the peak could predispose hares to die of predation, therefore representing an interaction between food and predation (Sinclair et al., 1988).

Large-scale factorial experiments were undertaken in the Yukon in attempt to tease apart the roles of food, predation and their interaction in snowshoe hare population dynamics (described in Krebs et al., 1995; Krebs et al., 2001a). Four $1km^2$ experimental areas in the boreal forest were used: control (unmanipulated), food addition, food addition + predator removal, and predator removal (Krebs et al., 1995). Predator removal consisted of exclosures constructed to exclude terrestrial predators (accessible to avian), while remaining permeable to snowshoe hare (Krebs et al., 1995). Food addition and predator removal together had additive effects on increasing the amplitude of the cycle (i.e. density), yet no treatment was sufficient in changing the periodicity

of the cycle and preventing the population decline from occurring (Krebs et al., 1995). As no food shortages have been observed in the Yukon during any point in the cycle (Hodges and Sinclair, 2003; Krebs et al., 1986b), these findings suggest that predation plays a larger role in the snowshoe hare cycle than food.

Direct predation is high in snowshoe hare, with up to 80-90% of hares being consumed (Boutin et al., 1986; Hodges et al., 2001; Keith and Windberg, 1978), suggesting predation plays a large selective role (Krebs et al., 1995). Yet there was no effect of predator exclusion in preventing the decline phase of the cycle (Krebs et al., 1995), and predation alone is not sufficient to drive the changes we see in snowshoe hare demography, condition, reproduction and physiology (Korpimaki et al., 1994). However, predation is not simply direct consumption; predators can indirectly induce fear and stress effects in populations through non-consumptive effects of risk (reviewed in Lima, 1998). These non-consumptive stress effects have been found to induce a toll of prey populations as strong, if not stronger than direct consumption (Preisser et al., 2005; Zanette et al., 2011).

1.2.5 Intrinsic factors in snowshoe hare cyclic dynamics

Predators can indirectly affect prey by leading to intrinsic changes in behaviour and stress physiology (reviewed in Lima, 1998; Preisser et al., 2005). As snowshoe hares are heavily predated upon, they must exhibit a balance in obtaining sufficient food resources and avoiding predation (Brown, 1999; Hik, 1995). In response to increased predation pressure in the decline phase, Hik (1994, 1995) found that hares were following a predation-sensitive foraging strategy, exhibiting a trade-off between predation risk and nutrition. Hares exhibited a microhabitat shift, using more closed, safer habitats with less available food resources, rather than open, riskier habitats with more available food (Hik, 1995). The consumption of less and/or poorer-quality food resources could result in the changes in reduced body condition and reproductive output which we see in the decline phase (Hik, 1995).

The effects of high predation risk can also affect snowshoe hare physiology (Boonstra et al., 1998b). The hares may sense increased predation risk through chases, failed attacks or successful attacks on nearby hares (who typically release a high-pitched scream when attacked) (Boonstra

et al., 1998a). Hares may then be forced into a state of high vigilance and alertness, which can lead to chronic stress and the associated negative connotations (described further in section 1.3; (Boonstra and Singleton, 1993; Boonstra et al., 1998a)). Chronic stress is a known inhibitor of reproduction (Munck et al., 1984), and can lead to intrinsic changes in the hares themselves (Boonstra et al., 1998a). Support for this hypothesis comes from Sinclair et al. (2003), who brought hares from the peak into the laboratory under optimal conditions and the population continued to have lower condition and reproductive output and declined to extinction. Whereas, hares from the late low/early increase phase were brought into captivity and they continued to increase to high numbers (Sinclair et al., 2003). This suggests changes in maternal quality and stress physiology in the peak carry over to the decline and low phases through maternal effects and intergenerational inheritance (discussed further in section 1.4) (Boonstra et al., 1998b).

1.3 The hypothalamic-adrenal-pituitary (HPA) axis

1.3.1 Function

The hypothalamic-pituitary-adrenal (HPA) axis is the key neuroendocrine mechanism in mammals regulating the interaction between the individual and the environment. The HPA axis is essential in the normal circadian rhythm of vertebrates, by mediating waking, locomotion, food-seeking and exploratory behaviour (McEwen et al., 1988). The HPA axis is also referred to as the “stress” axis, as its secondary action is to respond to environmental perturbations, i.e. stressors (e.g. predator attack, severe weather) in the short term, as well as long-term evolutionary responses to persistent ecological pressures (Wingfield and Romero, 2001).

1.3.2 Glucocorticoids and the stress response

The stress response is a set of internal responses to handle potentially harmful changes in the environment and allow for a rapid response in the individual (Lee and McDonald, 1985). In response to a stressor, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH and AVP then stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the blood

stream, which stimulates the adrenal cortex to synthesize and release glucocorticoids (GCs; steroid hormones, corticosterone and/or cortisol, depending on the species) (Fig. 1.3). In vertebrates, this pathway can take three to five minutes before a substantial increase in GC concentrations is seen (Sapolsky and Meaney, 1986). High concentrations of GCs lead to a suppression of digestion, reproduction, immune and inflammatory responses, as well as a stimulation of hepatic gluconeogenesis to mobilize glucose from the liver and allow for a quick response to acute stressors, i.e. the "fight or flight" response (Munck et al., 1984).

After an acute stressor, GCs travel in the blood to intracellular steroid hormone receptors (glucocorticoid and mineralocorticoid) in the hippocampus and HPA axis to shut down the stress response through negative feedback (Fig. 1.3) (reviewed in Schaaf and Meijer, 2017). Mineralocorticoid receptor (MR) is a Type I receptor, located primarily the hippocampus (Herman et al., 1989). Hippocampal MR has a higher affinity for binding GCs, and is important in maintaining homeostasis and circadian rhythms through controlling the inhibitory tone of the HPA axis (McEwen et al., 1988; Zhe et al., 2008). Glucocorticoid receptor (GR) is a Type II receptor and is widely distributed across all brain regions (Herman et al., 1989). GR becomes progressively occupied after a stressor and regulates the negative feedback of the HPA axis through reducing the levels of free GCs (de Kloet and Joels, 1996; Van Eekelen et al., 1988). This negative feedback primarily occurs through GR in the hypothalamus and pituitary and serves to bring the body back to its pre-activation state (Sapolsky and Meaney, 1986). The amplitude and duration of the stress response is often correlated with the overall health of the animal (Boonstra et al., 1998a; Sapolsky, 1993).

The functioning of the HPA axis and the stress response can be tested in what we now call a "hormone challenge" (Boonstra and Singleton, 1993). A basal bleed is taken, followed by an injection of Dexamethasone (DEX) to test the function of the pituitary-adrenal negative feedback to reduce circulating levels of free GCs, followed by an injection of ACTH to test the function of the adrenal glands to synthesize and release high amounts of circulating GCs (Boonstra and Singleton, 1993). From this hormone challenge, we are then able to assess the amplitude of the stress response that the animal can mount, as well as the functioning of the negative feedback.

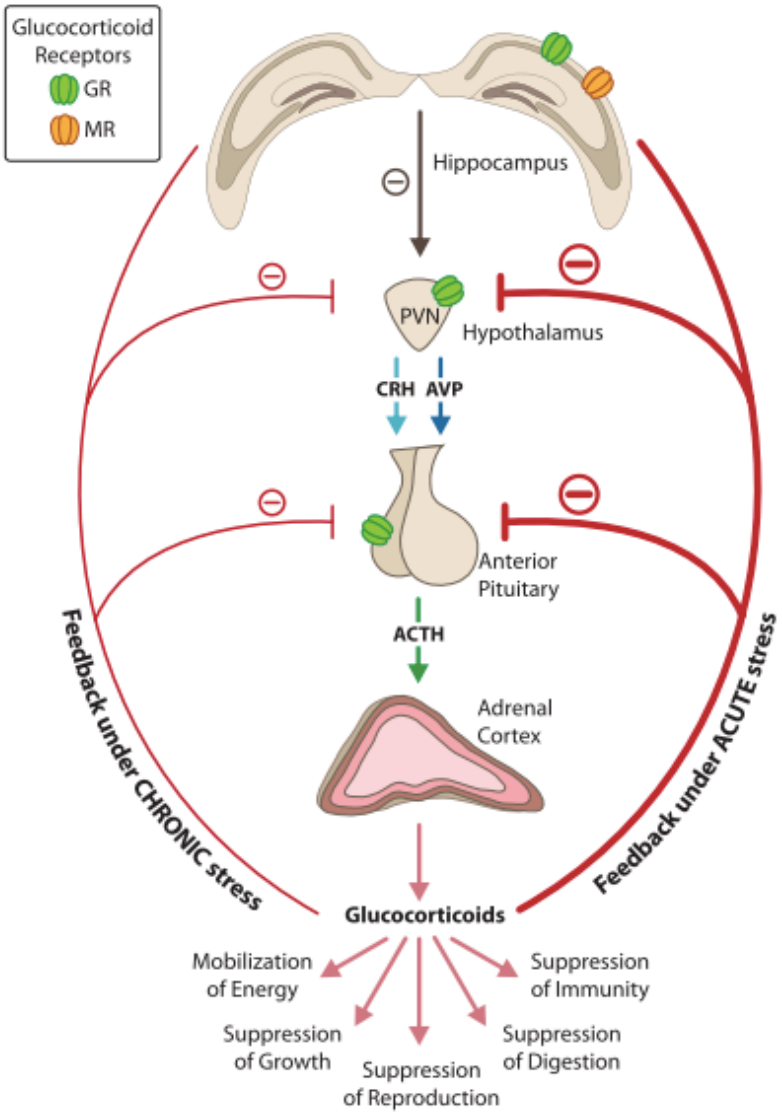

Figure 1.3: The vertebrate hypothalamic-adrenal-pituitary (HPA) axis. In response to a stressor, a signalling cascade starts at the paraventricular nucleus (PVN) of the hypothalamus, with the release of corticosteroid releasing hormone (CRH) and arginine vasopressin (AVP). These act on the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH) into the blood steam, which stimulates the adrenal cortex to synthesize and release glucocorticoids (GCs). Listed underneath are the many effects elevated circulating GCs can exert on the body. As these effects can be negative with extended exposure, the HPA axis is controlled by negative feedback of GCs on glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (inhibition is indicated by a minus (-) sign). Shown is the negative feedback in response to an acute stressor (right), compared to the reduced negative feedback and sustained activation under chronic stress (left). *Obtained from* Boonstra et al. (2014)*, originally modified from* Boonstra (2004).

1.3.3 Chronic stress

Stress can be acute or chronic in nature depending on the frequency and length of a stressor. Chronic stressors constitute of sustained activation of the HPA axis with muted termination of the stress response by negative feedback, which can lead to reduced sensitivity (Fig. 1.3) (Lee and McDonald, 1985; McEwen et al., 1988). Chronic stress also results in sustained high levels of GCs which can suppress reproduction, growth, digestion, immune and inflammatory responses, and mobilize energy at the cost of storage (Boonstra et al., 1998a; Sapolsky et al., 2000). Stressors such as high predation risk can become chronic in nature (Boonstra and Singleton, 1993). In snowshoe hare, Boonstra et al. (1998b) proposed the chronic stress hypothesis as being responsible for the marked deterioration of body condition and reproduction seen during the decline phase. Boonstra et al. (1998b) tested this hypothesis by conducting hormone challenges in the decline and low phases of the snowshoe hare cycle. In the hormone challenge, chronic stress should be reflected in a slower and less intense response to DEX and ACTH compared to individuals in better condition (Boonstra and Singleton, 1993). Hares in the decline phase exhibited these signs of chronic stress, as well as higher plasma free cortisol, reduced indices of body condition (hematocrit levels), reduced indices of immune function (leukocyte counts), increased glucose mobilization and increased overwinter mass loss compared to hares from the low phase (Boonstra et al., 1998a). The effects of chronic stress are typically thought of as pathological, however they can be adaptive in nature if they act to redirect life history, physiology and behaviour to increasing survival and fitness (Boonstra, 2013; Wingfield et al., 1998).

1.4 Inherited maternal effects

1.4.1 Maternal effects and programming

Acute and chronic stressors during pregnancy and can have major effects on developing offspring (McCormick et al., 1995). Offspring can be directly exposed to maternal circulating GCs *in utero*, which can lead to significant changes in offspring phenotypes, deemed maternal effects or maternal programming (reviewed in Love and Williams, 2008). Many studies have

demonstrated the HPA axis of offspring to be highly susceptible to permanent programming during early life developmental periods, such as gestation and lactation (Francis and Meaney, 1999; Matthews, 2002; Seckl, 2001). However, mothers have mechanisms to prevent stress and high levels of circulating GCs from programming their offspring during pregnancy and lactation. In arctic ground squirrels, mothers increased their corticosterone-binding globulin (CBG) levels by 4-fold during pregnancy and lactation to bind free cortisol and shield the developing offspring from the negative effects of high GCs (Edwards and Boonstra, 2016). Maternal GC exposure during gestation can be controlled through 11-Beta-Hydroxysteroid dehydrogenase (11β-HSD) expression in the placenta, an enzyme which converts cortisol to its inert form cortisone (Burton and Waddell, 1999). However, increases in maternal GC concentrations are not always associated with increases in 11β-HSD expression, and the developing offspring can still be exposed to high GCs (Burton and Waddell, 1999).

Overall, developing offspring exposed to high maternal GCs demonstrate changed phenotypes, through altered behaviour, physiology and gene expression (Meaney et al., 2007; Szyf et al., 2007; Weaver et al., 2004a). Offspring exposed to high maternal GCs during gestation show an increase in anxiety-related behaviours and higher GC levels in laboratory studies (Hayward and Wingfield, 2004; Meaney et al., 2007; Seckl and Meaney, 2004). Additionally, a downregulation of MR and GR, particularly in the hippocampus, has been found in these offspring in response to maternal chronic stress (Matthews, 2002; Sapolsky et al., 1984; Sapolsky et al., 1985). This lower GR and MR expression can lead to higher HPA axis reactivity to stressors, delayed and inhibited negative feedback and a prolonged elevation in GC concentrations in these offspring, which is thought to be maladaptive and pathological (Hayward and Wingfield, 2004; Matthews, 2002; Meaney et al., 2007; Welberg and Seckl, 2008). However, many of these laboratory stressors are highly artificial, whereas wild animals have a long evolutionary history of adaptation to natural stressors such as predation risk, social interactions, weather, and disease (Sheriff et al., 2010a).

1.4.2 Offspring phenotype and the environment

Maternal GC programming of offspring can be adaptive in nature if it increases the “match” between the offspring’s phenotype and their future environment (Love and Williams, 2008a).

The transmission of anti-predator behaviour (e.g. vigilance, fearfulness, anxiety-related behaviours) to offspring through maternal programming of the HPA axis can be beneficial to the mother and offspring's fitness (Seckl and Meaney, 2004; Weaver et al., 2004b). High GCs in offspring have also been found to positively influence dispersal behaviour and survival (Meylan and Clobert, 2005). Additionally, changes in their steroid receptors (GR and MR) in key brain regions associated with the HPA axis will shape their present and future responses to a stressful environment (in laboratory rodents, see reviews Gray et al., 2017; Meaney et al., 2007). However, if the offspring are programmed by maternal GCs and their phenotype does not match their future environment, then they are considered to be "mismatched" (Love and Williams, 2008a; Sheriff and Love, 2013). Nevertheless, maternal programming that appears maladaptive for the offspring can actually be adaptive for mothers if it maximizes their lifetime fitness. Love and Williams (2008) found that gestational GC-mediated matching of offspring condition to mother condition reduced the investment in current offspring of low-quality mothers, which results in fitness gains to these mothers through increased survival and future fecundity.

For example, in snowshoe hare, maternal GC programming could result in offspring exhibiting anti-predator behaviours and having higher fitness in environments with high predation risk (Boonstra, 2013). This would be an adaptive strategy in the peak and decline phases, when maternal predation risk is high, stress is high, and predation risk for offspring will also be high. Offspring are therefore "matched" to their environment and will be able to better handle the stressor of predation risk and have higher fitness. However, this may turn into a mismatch in the transition from the decline to the low phase. Dams may be experiencing the tail end of high predation risk, while the predators are also declining to a low, and subsequently their offspring will experience lower predation risk. Offspring programmed by high maternal GCs will demonstrate these same stressed, anti-predator phenotypes, which will now be mismatched and maladaptive to their environment, leading to decreased fitness (Boonstra, 2013; Sheriff and Love, 2013). If these changes and their negative connotations are passed down to offspring through maternal stress, this intergenerational programming could be responsible for the prolonged low phase of the snowshoe hare cycle.

1.5 Objective and approach

Research into the snowshoe hare cycle suggests physiological sensitivity to predation risk and predator-induced maternal stress effects are occurring in their populations. Chronic stress in female hares in response to high predation risk has been found to result in decreased reproductive fitness (lighter and smaller litters) and to be inherited by her progeny, shown by an increased responsiveness of their HPA axes in the decline phase of the snowshoe hare cycle (Sheriff et al., 2009a; Sheriff et al., 2010b; Sheriff et al., 2011). Lavergne's (2018) research built on these findings to investigate changes in offspring physiology and GR and MR expression in key brain regions in response to increased predation risk from the increase to the peak phases of the cycle. Offspring from the peak (higher natural predation risk) exhibited increased expression of GR in the amygdala and increased expression of MR in the dorsal hippocampus (Lavergne, 2018). However, during this period the dams showed no indication of experiencing chronic stress and the offspring displayed an adaptive “sensitive and neuroresilient phenotype”, likely preparatory for life in a predator abundant environment (Lavergne, 2018). Continuing this work into the decline phase is a critical test of the maternal effects hypothesis, allowing us to investigate maternal stress and offspring programming in snowshoe hare exposed to high predation risk, and how this changes with phase of the cycle.

This leads to the question: are dams experiencing chronic stress in the decline phase programming their offspring *in utero* and further altering their phenotypes? I hypothesized that fluctuating predation risk over the course of the cycle results in snowshoe hare offspring from the decline phase being inherently different in terms of condition, stress physiology and gene expression from those in the increase and peak phases. Thus, I predict the decline phase offspring to suffer the negative consequences of their mothers’ chronic stress in terms of increased cortisol, decreased GR and MR and a decline in body condition. This was investigated through a natural monitoring study spanning two years of the decline phase (2018 and 2019) in collaboration with Lavergne, who investigated the increase phase (2015) and peak phase (2017). We assessed maternal physiology during gestation, and their corresponding reproductive output, as well as offspring morphology, physiology, behaviour and patterns of gene expression in relation to natural increases in predation risk (**Chapter 2**).

Chapter 2
The 10-year snowshoe hare cycle: impact of heightened risk on maternal programming of young

2 The 10-year snowshoe hare cycle: impact of heightened risk on maternal programming of young

2.1 Introduction

2.1.1 Snowshoe hare cyclic dynamics

The 10-year snowshoe hare cycle dominates much of the ecosystem dynamics of the vertebrate community of North America's boreal forests for at least the past 200 years (Elton and Nicholson, 1942). Three intensive studies have investigated these cycles: Green and Evans (e.g. 1940a, b, c) studied snowshoe hare in Minnesota from 1932 to 1939; Keith and colleagues (e.g. Cary and Keith, 1979; Keith and Windberg, 1978) studied them in central Alberta from 1961 to 1976; and Krebs and colleagues (e.g. Krebs et al. 1986; Krebs et al. 2001a; Krebs et al. 2018a) have been studying them in southwestern Yukon from 1976 to the present. Changes in food supply and predation by their specialist predator, the Canada lynx, were initially hypothesized to be the key factors in causing these cycles (Krebs et al., 2018a). Lynx populations also cycle, but lag behind hares by one to two years (Boutin et al., 1995; Krebs et al., 2001a). Large scale factorial experiments manipulating food and restricting predation increased the amplitude of the snowshoe hare cycle, but did not affect the periodicity (Krebs et al., 1995). These studies pinpointed predation as the key process driving the cycle. During the peak and decline phase of the cycle, the majority of hares are killed by their predators (Boutin et al., 1986; Hodges et al., 2001; Keith and Windberg, 1978). Simultaneously there is a marked decline in reproduction from four to two litters per breeding season as the cycle progresses from the increase to the decline phase (Hodges et al., 2001; Stefan, 1998). At the end of the decline, both hares and their predators reach very low densities. However, hare populations fail to recover immediately and remain low for two to five years, despite abundant high-quality food resources and low predator numbers (Boonstra et al., 1998b; Hodges et al., 1999b). Thus, this low phase remains a major enigma of the cycle and poorly understood after years of research.

2.1.2 Non-consumptive predator effects

Predation on its own is not enough to drive the changes we see in snowshoe hare condition, reproduction, physiology and demography (Boonstra et al., 1998b; Korpimaki et al., 1994). Predation is made up of two components: direct consumption and non-consumptive effects (Lima, 1998). Non-consumptive, or fear effects of predators are a relatively new area of study, but have been found to induce a toll on prey demography as strong, if not stronger than direct consumption (MacLeod et al., 2018; Preisser et al., 2005; Zanette et al., 2011). However, experiments testing non-consumptive fear effects may be using unrealistic magnitudes of predator cues in the absence of any consumption effects, which does not replicate the natural environment (Peers et al., 2018). This showcases our need for further research in natural systems with real selective forces and adaptations occurring (Boonstra, 2013; Sheriff et al., 2017).

Non-consumptive fear effects can be manifested in predator sensitive foraging (Hik, 1995), and predator-induced stress effects (Boonstra et al., 1998a). Creel (2018) postulated that reactive responses by the prey in response to unpredictable or uncontrollable predation risk will generally be associated with stress mediated costs. Associated with the prey stress response to predation is activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to a hormonal cascade (described in Chapter 1), and an increase in circulating glucocorticoids (cortisol in hares). This increase in glucocorticoids has been found to lead to a decrease in reproduction, and an increase in antipredator behaviour (Boonstra et al., 1998a; Hik, 1995). Boonstra et al. (1998a) put forward the chronic stress hypothesis as an explanation for the prolonged low phase in the snowshoe hare cycle. It states that hares sense high predation risk during the decline phase and are chronically stressed by it, leading to changes in body condition and reproduction (Boonstra and Singleton, 1993; Boonstra et al., 1998a). This non-lethal effect of their predators therefore leads to intrinsic changes in the hares themselves (Boonstra et al., 1998a). Support for these intrinsic changes came from Sinclair et al. (2003) who brought wild hares from two phases (the peak and the late low phase) into the laboratory under optimal conditions. Populations from both phases echoed the performance of their wild counter parts, with hares coming from the peak continuing to have lower reproductive output and declining to extinction (Sinclair et al., 2003). This suggests maternal effects and inheritance of stress from one generation to the next are playing a role in snowshoe hare population demography.

2.1.3 Maternal stress effects

Offspring can be directly exposed to maternal circulating glucocorticoids prenatally during development, and postnatally through lactation (reviewed in Welberg and Seckl, 2001). This glucocorticoid exposure can lead to significant changes in offspring phenotype, deemed maternal effects or maternal programming (reviewed in Love and Williams, 2008). In snowshoe hare, dams experiencing higher predation risk had higher indices of stress and lower reproductive output (Sheriff et al., 2009a), suggesting offspring are exposed to this prenatal stress. This was supported, as juvenile stress indices tracked those of their mothers with a signature that lasted into adulthood (Sheriff et al., 2010b; Sheriff et al., 2011). Additionally, mothers with lower reproductive fitness had daughters with lower reproductive fitness, suggesting inheritance of these stress effects (Sheriff et al., 2010b). Due to the high predation environment and the vulnerability of young precocial snowshoe hares, it should be beneficial for mothers to "program" their offspring through maternal effects to better match their post-natal environment (Sheriff, 2015).

During development *in utero*, the HPA axis of offspring is highly susceptible to long-lasting programming effects by maternal glucocorticoid levels (Moisiadis and Matthews, 2014). High levels of glucocorticoids during gestation, such as in response to high predation risk, can significantly influence offspring through changes in morphology, behaviour and physiology, which can persist into adulthood and have long lasting population demographic effects (reviewed in Clinchy et al., 2013; Lima, 1998; Werner and Peacor, 2003). Offspring have been found to be smaller and slower growing (Zanette et al., 2011), have increased anxiety-related behaviour and reduced locomotion (St-Cyr et al., 2017), and have altered stress axis function (Love et al., 2013; Welberg and Seckl, 2001). As indices of stress axis function, offspring exposed to prenatal stress have shown higher glucocorticoid levels in fecal samples (Sheriff et al., 2010b), hair samples (Dettmer et al., 2014), and blood samples (Kapoor and Matthews, 2005; St-Cyr and McGowan, 2015). Maternal stress-induced neurophysiological changes to offspring have also been seen in the laboratory (reviewed in Champagne, 2012; Matthews, 2002; McEwen et al., 2015; Seckl and Meaney, 2004).

2.1.4 Neurophysiology

Laboratory evidence on domestic rodents indicates that during gestation, maternal glucocorticoids can lead to changes in stress hormone receptors in key brain regions of offspring (Preisser et al., 2005; Werner and Peacor, 2003). Changes in offspring's steroid hormone receptors (glucocorticoid and mineralocorticoid) can shape an individual's present and future responses to a stressful environment (reviewed in Gray et al., 2017; Meaney et al., 2007). Mineralocorticoid receptors (MR) in the hippocampus are important in controlling the inhibitory tone of the HPA axis, maintaining homeostasis and circadian rhythms, and have a higher affinity for binding glucocorticoids (reviewed in Gomez-Sanchez and Gomez-Sanchez, 2014). Whereas glucocorticoid receptors (GR) become progressively occupied after a stressor and regulate the negative feedback of the stress axis (Sapolsky et al., 1985; Zhe et al., 2008). GR is widely distributed across all brain regions, whereas MR is primarily located in the hippocampus, a key regulatory region of the HPA axis (Herman et al., 1989). Changes in GR and MR expression in different brain regions have been widely seen in laboratory rodents exposed to maternal stress *in utero* (e.g. Henry et al., 1994; Kapoor et al., 2008; Liu et al., 2001). Reduced expression of GR and MR in the hippocampus can lead to delayed and inhibited negative feedback of the HPA axis, and a prolonged stress response activation, which is thought to be negative and pathological (Matthews, 2002; Sapolsky et al., 1984). However, in nature, these developmental and organizational effects on offspring are thought to be adaptive if they lead to offspring having an altered phenotype and better "matching" their post-natal environment (Dantzer et al., 2013; Love and Williams, 2008a). However, Boonstra et al. (2014) proposed that intergenerational inheritance of maternal stress effects in snowshoe hare will vary from adaptive to negative based on the phase of the cycle; and could ultimately be responsible for the prolonged low phase, as stress effects would be maladaptive during this period of low predator density.

The likely mechanism for transmission and inheritance of changes to offspring neurophysiology and overall phenotype is epigenetics, i.e. changes in gene expression without changes in the DNA sequence itself (McClelland et al., 2011; Mychasiuk et al., 2011). Of these epigenetic changes, the most widely studied is DNA methylation, which is the posttranslational addition of a methyl group to DNA, repressing gene transcription when located in the promoter regions of genes (Kappeler and Meaney, 2010; Kinnally et al., 2011).

2.1.5 Approach, hypothesis and predictions

Lavergne et al. (2014) were the first to demonstrate changes in gene expression in the hippocampus of wild snowshoe hare exposed to different intensities of natural predation risk. Lavergne (2018) then furthered this research by investigating the link between maternal stress and gene expression changes in key limbic regulatory regions in the brains of offspring over the course of the increase and peak phases of the snowshoe hare cycle. Lavergne was unable to assess such changes during the impending decline. Thus, the female hares had not yet experienced chronic stress of high predation risk during gestation. I continued this research of Lavergne (2018) in the decline phase of the snowshoe hare cycle in 2018 and 2019. Taken together, our natural monitoring study aims to elucidate the effects of fluctuating natural predation risk on maternal physiology during gestation and on her offspring morphology, behaviour, physiology and neurophysiology.

I tested the hypothesis that dams experiencing severe predation risk during the decline phase of the snowshoe hare cycle will program their offspring through maternal effects *in utero*. In testing this hypothesis, I investigated snowshoe hares during the decline years of 2018 and 2019, when the population is undergoing high mortality due to predation, and the dams are experiencing high predation risk. I predicted that offspring from these two consecutive decline years would be fundamentally different in terms of hormone levels, stress condition signatures and gene expression from offspring in the increase and peak phases. My predictions build on the data collected during the increase (2015) and peak (2017) years of the cycle by Lavergne (2018). I predicted mothers from the decline phase to have higher fecal cortisol metabolite (FCM) concentrations, a non-invasive index of stress. I predicted offspring born to these mothers will: **a.** be lighter and smaller, **b.** show more anxiety and freezing in response to a behaviour test **c.** be in worse condition (lower hematocrit), **d.** have higher hair cortisol, plasma total cortisol, and blood glucose, and critically, **e.** show reduced expression of GR and MR in the dorsal hippocampus, relative to offspring from the increase and peak years.

2.2 Materials and Methods

2.2.1 Study area

The study area is located in the Shakwak Trench, in the Kluane Lake region of the southwestern Yukon Territory, Canada (61°N, 138°W). This area is a broad glacial valley of the boreal forest, 900m above sea level, surrounded by alpine mountainous areas to the northwest and southeast (Boutin et al., 1995). Located in the boreal cordillera ecozone; the climate is cold continental, with a growing season from mid-May to mid-August. The dominant vegetation in this area of the boreal forest is white spruce (*Picea glauca*), with a dense but patchy shrubby understory of willow (*Salix glauca* (gray willow) and *S. alaxensis* (Alaska willow)), dwarf birch (*Betula glandulosa*) and soapberry (*Shepherdia canadensis*) (Douglas, 1974). These plant species are also the primary forage of snowshoe hare (Smith et al., 1988). Our snowshoe hare study area spans a 30 km stretch of the Alaska Highway which runs through the Shakwak Trench valley from the Arctic Institute of North America's Kluane Lake Research Station (KLRS) to slightly passed the Kluane Red Squirrel Project's headquarters (Fig 2.1).

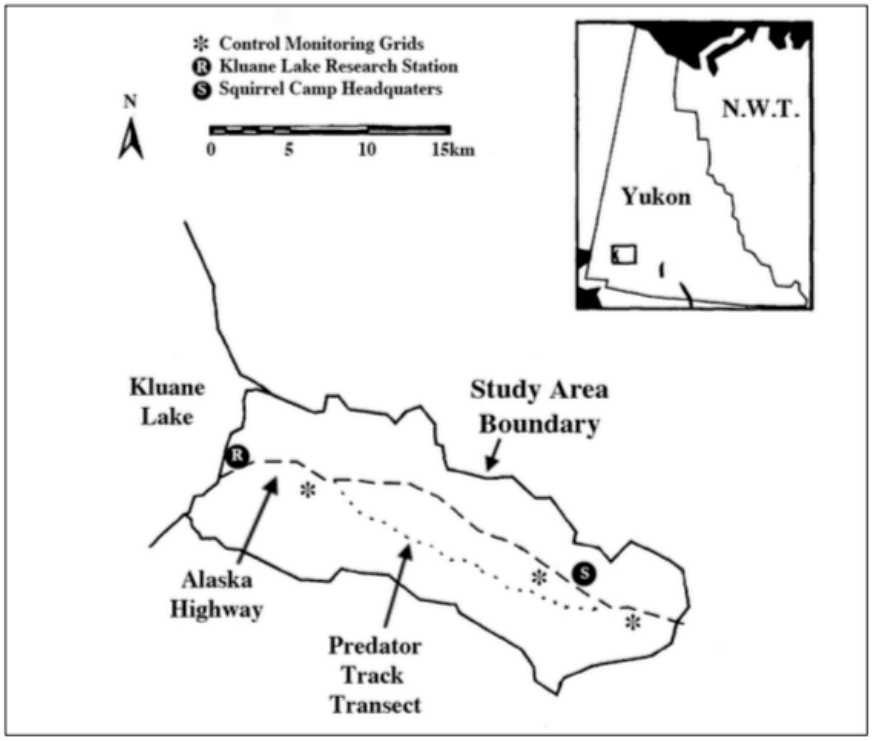

Figure 2.1: Research study area, Kluane Lake, Yukon Territory. *Obtained from* Lavergne (2018), *originally adapted from* O'Donoghue et al. (1997).

2.2.2 Natural monitoring

Natural monitoring activities have been focused extensively in the Kluane Lake area since 1976, which makes it the longest natural monitoring project in North America (Krebs et al., 2001b; Krebs et al., 2018a). Over the past 43 years, this project has achieved abundance estimates for a variety of boreal forest plant, fungi, small mammal, and predator species, among others (Krebs et al., 2018a).

2.2.2.1 Snowshoe hare density estimates

Snowshoe hare density estimates were obtained in the spring and fall of each year, through the use of mark-recapture trapping on three 36-hectare control monitoring grids (see Fig. 2.1). Hares were live-trapped during 2-3 night trapping sessions, and untagged hares were given an identifying ear-tag in their right ear (Monel No. 3 tags, National Band and Tag, Kentucky, USA). Population density estimates were calculated from the maximum likelihood spatial estimating in Efford's DENSITY 4.4 program (Efford et al., 2009).

Densities were higher in the fall, owing to recruitment from the breeding season, therefore density values presented in this thesis are spring estimates. Density estimates of snowshoe hare from 1976-2019 are presented in Fig. 1.2.

2.2.2.2 Predator density estimates

Density of lynx, coyotes and other mammalian predators was estimated through the use of a 25km winter tract transect (see Fig. 2.1). From October to April every year, this 25km transect was snowmobiled following every fresh snowfall and tracks were counted. This use of track counts has been validated, and provides a reliable index of predator density in our study area (O'Donoghue et al., 1997). Density estimates of lynx from 1988-2019 are presented in Fig. 1.2.

2.2.3 Integrating previous research

These methods follow exactly the methods of Lavergne (2018), as this is a collaborative multi-year study and consistency is important. Lavergne collected data in 2015 and 2017, and I repeated this in 2018 and 2019. The sample sizes used for this study were obtained through considerable effort, as this is a natural study using wild snowshoe hare and densities were quite low, especially in the decline years. Lavergne developed the brain protocols, as well as designed and lead all molecular work towards quantifying gene expression. I will be presenting my research with Lavergne's and interpreting the data together for the remainder of this thesis. All data presented in this thesis is from **control** dams only.

2.2.4 Snowshoe hare live-trapping

Snowshoe hares were trapped on a variety of areas to capture pregnant females for our study. Trapping was focused around the peak dates of first litter (mid-late May) and second litter (late June to early July) (Cary and Keith, 1979; O'Donoghue and Boutin, 1995; Sheriff et al., 2009a). Trapping around the birth of first litter was used to estimate parturition date of second litters, as it occurs around 35-37 days later, due to synchronous breeding and immediate post-partum estrous and pregnancy (O'Donoghue and Krebs, 1992). Second litter was chosen for this study, as dams have larger litters with lower survival rates (0.15 over 30 days), since it corresponds to the period of increasing independence of juvenile potential predators (O'Donoghue, 1994).

In 2018 and 2019, snowshoe hare were live-trapped (No. 106; Tomahawk Live Traps Co, Tomahawk, Wisconsin, USA) from May to July following the general trapping methods (Krebs et al., 2001b). I and an assistant set traps between 22h00-01h00 and checked from 06h00 onward, to limit the time hares were in the traps. Traps were baited with commercial (Hi-Pro Feeds, Texas, USA) rabbit chow (17% crude protein), alfalfa cubes, and apple slices for hydration during the summer months and to prevent trap sickness (as described in Keith et al., 1968). Once trapped, the hares were transferred to burlap bags to limit visual stimuli and the stress of handling. Hares were weighed with a Pesola spring-scale (±10g), their right-hind foot (RHF) length (±1mm) was measured as an index of skeletal size, their sex and reproductive

status were assessed (described in section 2.2.4.2), and they were ear tagged in their right ear if not previously captured. Fecal samples were collected from under the traps when hares were in traps <8 hours, before stress from trapping can be reflected in the sample (Sheriff et al., 2009b). All fecal samples were stored at -20ºC until processing at the University of Toronto Scarborough (UTSC).

2.2.4.1 Assessing female reproductive condition

The sex of the snowshoe hare was assessed through everting the genitalia. The reproductive condition of females was determined by weight, palpating the abdomen and looking at the lactation tissue and nipples (Stefan and Krebs, 2001). The stage of pregnancy was determined by the size and firmness of the embryos upon palpation, which were classified as peas, grapes or walnuts (adapted from Keith et al., 1968).

2.2.4.2 Maternity cages

Once a dam was determined to be in her last week of pregnancy, determined by the above guidelines, estimated date of first litter parturition, and with embryos the size and feel of 'walnuts', she was placed into a maternity cage. Maternity cages are wooden framed cages (60 x 60 x 120 cm), with chicken wire sides covered by burlap, a Tomahawk live-trap built in to the back right and a heavy wooden lid (Fig 2.2). The bottom of the cage was lined with straw, and the hares were fed natural forage (gray willow, white spruce and dwarf birch), rabbit chow, apple slices and water daily. During their daily feeding, we also checked if they had given birth and a fecal sample was collected from under their trap.

In 2018, the maternity cages were placed on control grids, within each dam's home range near where she was trapped. In 2019, due to disturbance by Canada lynx, black bears (*Ursus americanus*) and grizzly bears (*Ursus arctos*), the maternity cages were placed within the predator-proof enclosures in the forest at the Kluane Lake Research Station (KLRS) (as described in Sheriff et al., 2009b). The enclosure is surrounded by a high fence covered in black

felt, as well as an electric fence around the perimeter (Sheriff et al., 2009a). Each maternity cage was placed within its own pen inside the enclosure, to limit the hares from seeing each other.

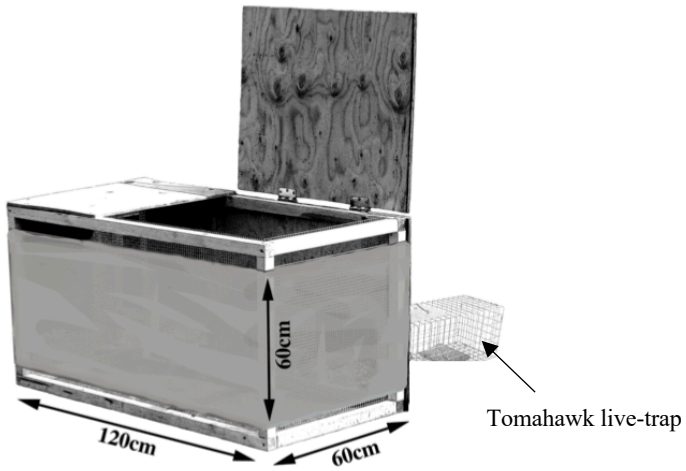

Figure 2.2: Field maternity cages used to temporarily house pregnant dams during this study. *Obtained and adapted from* Lavergne (2018).

2.2.5 Dam and litter processing

Upon parturition, a fecal sample was collected during normal maternity cage checks in the morning, and the hare was left undisturbed with her litter until 13h00. At 13h00, the female was trapped in the Tomahawk live-trap in her maternity cage and fed apple slices. Her litter was counted and gathered into a cotton bag to obtain total litter weight with a Pesola (± 1g) and the litter was processed away from the maternity cage, as to not disturb the dam.

Prior to litter processing, each individual leveret was tested in a pre-handling behaviour test. This behaviour test consisted of placing the leveret out in the open in a shaded spot on the boreal forest floor facing away from people and assessing the latency to movement over a 60 second silent period. When the leveret moved a paw, the timer was stopped, and if it reached 60 seconds, they were deemed ‘did not move’. Each leveret was then sexed, weighed, measured (RHF length), and ear tagged in the right ear (Monel No. 1 tags, National Band and Tag, Kentucky, USA). Additionally, a small (1x1 cm) patch of fur was shaved from the right rump of each leveret with pet trimmers for hair cortisol analysis. Then the behaviour test was repeated, to

assess the leverets' responses to handling. One male and one female from each litter were chosen for dissection (described in section 2.2.6).

The dam was then placed into a burlap bag, weighed, and a patch of fur (1x1 inch) was shaved from her right rump for hair cortisol analysis. Hair samples were stored in paper envelopes at room temperature until processing. Dam hair was not collected in 2018. The dam and her remaining litter were then transported back to her original location of capture. A makeshift nest was created slightly away from the trap line, under a deadfall, or at the base of a willow, to make sure the leverets are sheltered and the dam can find them easily (based on O'Donoghue and Krebs, 1992; Stefan and Krebs, 2001). As snowshoe hare do not create nests or burrows (O'Donoghue and Bergman, 1992; Severaid, 1945), we tried to keep these nest areas as natural as possible. The dam was placed in the Tomahawk live-trap at her trapping location and each leveret was held up to her to smell before being placed in the makeshift nest (as in Stefan and Krebs, 2001). Once the litter was together and huddled, the trap was opened directly in front of the 'nest' to encourage the dam to see and smell the nest and her litter before hopping away (Stefan and Krebs, 2001).

2.2.6 Tissue and brain collection

The selected male and female leveret were anesthetized with an isoflurane soaked cotton ball in a falcon tube placed over the nose and mouth (as per Lavergne, 2018). A blood sample was then obtained from the infraorbital sinus with a heparinized glass pipette (as described in Bradshaw, 2003; Kenagy and Place, 2000). The leverets were further anesthetized and the absence of reflexes was confirmed, prior to swift decapitation (as described in Lavergne, 2018). Within 4 minutes, the brain was extracted from the skull using sterile dissection tools, the cerebellum was cut from the cerebrum and both sections were frozen cut-side down on a sterile foil covered glass plate on dry ice, to ensure even freezing. The pituitary gland was also extracted from the sella turcica using needle-tipped tweezers, placed in a 0.5mL tube and frozen immediately on dry ice. Following this, the bodies were dissected, and the right lobe of the liver, both kidneys, and the stomach and intestines were collected in sterile 1.5mL tubes and bags and placed on ice. In addition, sex was confirmed, and notes were taken on body condition, stomach appearance, and fat stores. Samples were transported back to the Yukon laboratory at KLRS, brains were then

wrapped twice in parafilm and stored at -80ºC, as well as the pituitary and other organs. The bodies, stomachs and intestines were stored at -20ºC. The blood was processed as described below.

2.2.7 Blood processing

One drop of blood was used immediately for the FreeStyle glucometer (Abbott Laboratories) to obtain a value for blood glucose. Additional drops of blood were placed on glass slides to obtain blood smears for white blood cell differentials and counts. The remaining blood was put into a 2mL heparinized tube, two 75µL capillary tubes were filled, and all samples were placed on ice before transport back to the laboratory.

Once in the laboratory, the remaining whole blood was spun in an Eppendorf Micro Centrifuge (Model 5413) for 10 minutes (5500 x g) to isolate the plasma. The plasma was pipetted off into a 0.5mL tube for later use in hormone assays (section 2.2.8.2). The capillary tubes were also spun in a IEC Micro-Hematocrit Centrifuge (Model MB) for 8 minutes (13,460 x g). The tubes were then read on a hematocrit reader to obtain readings of packed red blood cell volume (PCV %, hematocrit) as an index of condition (Johnstone et al., 2017). The capillary tubes were then cut with a diamond tipped pen and the isolated plasma was added to the plasma tube and frozen at -80ºC. Blood smears were fixed in 100% methanol and stained with Diff-Quik (Dade Behring) to differentiate and count lymphocytes, neutrophils, monocytes and eosinophils in counts of 100 leukocytes using light microscopy (Lavergne, 2018). Plasma, and all other samples stored at -80ºC were transported on dry ice to UTSC for analysis.

2.2.8 Hormone assays

2.2.8.1 Fecal cortisol metabolites

Fecal samples are a non-invasive method of assessing circulating hormone levels, providing an integrated hormone profile over time with less signatures of acute stressors (Sheriff et al., 2010a). Fecal samples were stored at -20ºC prior to being freeze-dried on a FreeZone 4.5L lyophilizer (LabConco, Missouri, USA) for 14-18 hours to control for water content. Debris was

then removed from the dried sample using tweezers and the sample was crushed using liquid nitrogen and a mortar and pestle, to ensure homogenization. Following crushing, 60mg (± 5) of sample was weighed out and extracted in 1mL of 80% ethanol for 30 minutes (1500 rmp) on a Vibrax orbital shaker (IKA-Werke GmbH & Co., Germany). Following this, the sample was centrifuged for 15 minutes (5500 rmp). After centrifugation, 100µL of the extract supernatant was added to 900µL of assay buffer (1:10 dilution) and frozen at -20ºC prior to analysis. Fecal cortisol metabolite (FCM) concentrations were obtained for all samples using an 11-oxoactiocholanolone enzyme immunoassay (EIA) method (Palme and Mostl, 1997). Samples were run in duplicate with a seven-point standard curve, and with a high and low concentration quality control at the beginning and end of each plate to ensure low intra- and inter-assay coefficients of variation. The use of this 11-oxoactiocholanolone EIA for snowshoe hare was validated by Sheriff et al. (2009a).

2.2.8.2 Total plasma cortisol

Blood sampling is an invasive measure to assess circulating glucocorticoids at a precise time point (Romero and Romero, 2002). Total plasma cortisol concentrations were obtained using the Cortisol ^{125}I radioimmunoassay (RIA) kit (MP Biomedicals, New York, USA). This assay has a low cross-reactivity and was validated for snowshoe hare plasma by Boonstra and Singleton (1993) through parallelism and quantitative recovery. Following Boonstra and Singleton (1993) with slight modifications, 10µL of plasma was used in each tube, followed by 20µL ddH_2O and 60µL ammonium hydroxide, which saponifies triglycerides to minimize potential cortisol binding interference (Barkley and Goldman, 1977). Samples were run in duplicate against a 6-point standard concentration curve (0-100 ng/mL), with the addition of a high and low concentration quality control run at the beginning and end of each run to ensure low intra- and inter-assay coefficients of variation.

2.2.8.3 Hair cortisol

Hair sampling is a non-invasive method of assessing circulating levels of hormones during the period of hair growth, which provides a representative sample over a longer time period than

FCM or plasma cortisol, as cortisol is slowly deposited into the hair shaft as it grows (reviewed in Meyer and Novak, 2012). Hair samples were prepared following the methods outlined in Mastromonaco et al. (2014). Hair samples were cut into 0.5cm sections to homogenize, then weighed in a range from 10-25mg in a 7mL glass scintillation vial. Hair samples were then washed by adding 3mL of 100% methanol, vortexing for 10 seconds and pipetting off the methanol immediately. Samples were left uncapped for around 30 minutes until they were dry, then capped and stored at room temperature. Hair cortisol is then extracted in 100% methanol, by using 0.005 g/mL (ex: sample weight: 0.0025g / 0.005 g/mL = 5 mL methanol added). Samples then shake on Vibrax orbital shaker for 24 hours (~700 rmp). After 24 hours, the samples were centrifuged for 5 minutes (2400 x g) and the supernatant was pipetted off into a clean 7mL glass scintillation vial. An aliquot of this is then obtained for hair cortisol analysis; 1500µL for adult hare hair samples, and 450µL for newborn hare hair samples. These aliquots were then left for 24-72 hours uncapped in the fume hood until they are dried. The remaining samples were sealed with parafilm and stored at -20ºC for future use. Dried samples were then sent for analysis to the Endocrinology Laboratory at the Toronto Zoo (Toronto, Ontario, Canada). Cortisol concentrations are low in hair and the dried down samples are reconstituted to a 10X concentration for adults and 3X concentration for newborns, using 150µL of phosphate-buffered saline. Samples were assayed in duplicate relative to a standard concentration curve using a proprietary cortisol EIA with a minimum detection threshold of 2.5 ng/g (as described in Lavergne, 2018).

2.2.9 Brain sectioning and RNA extraction

Prior to sectioning, the brains (cerebrum only) were thawed from -80ºC to -18ºC in the cryostat chamber (Leica CM3050 cryostat, Leica BioSystems, GmbH & Co., Germany) for one hour. Each brain was then mounted, positioned and sectioned coronally at a thickness of 50µm. Tissues were microdissected from the dorsal and ventral hippocampus, amygdala and hypothalamus using landmarks from a coronal section[4] and MRI-based rabbit brain atlases (Brain Atlas of the Domestic Rabbit (*Oryctolagus cuniculus*) #73-211. University of Michigan State Comparative Mammalian Brain Collections; Muñoz-Moreno et al., 2013). RNA was then extracted from all brain tissues using Epicentre's MasterPure Complete DNA and RNA

Extraction kit (Cat. MCR85102, Illumina). As per Lavergne (2018), the manufacturer's protocol was modified slightly by adding more volume of Proteinase K and doubling the length of all centrifugation steps to increase yield. Following the protocol described in Lavergne (2018), 1µg of this extracted RNA was converted to complimentary DNA (cDNA) using a High Capacity Reverse Transcription Kit (Applied BioSystems) on a Veriti Thermal Cycler (Applied BioSystems). The cDNA was then tested for quantity and quality on a NanoDrop spectrophotometer (Thermo Scientific).

2.2.10 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) was used to amplify DNA fragments and detect selected nucleic acid sequences in samples to assess gene expression. Lavergne (2018) designed all primer sequences in Primer3 primarily using the European rabbit (*Oryctolagus cuniculus*) genome sequences (UCSC Rabbit Genome Browser, Apr 2009 oryCun2.0 assembly) or from other closely related species. Primers were selected for high target specificity and mammalian sequence conservation, as listed in Appendix I (Lavergne, 2018).

After extensive testing of six commonly used reference genes, Lavergne (2018) identified peptidylprolyl isomerase A (PPIA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tyrosine 3-monooxygenase (YWHAZ) as the most stably expressed in our samples, through the use of qbase geNorm Plus software (Biogazelle, Belgium) (sequences in Appendix I). Our main target genes for qPCR were GR and MR (sequences in Appendix 1). PCR reactions used 1µg of cDNA, 1µM of forward primer, 1µM of reverse primer and Fast SYBR Green Master Mix (as per Lavergne, 2018). Samples were run in triplicate on a 96-well plate, with two quality control samples run on each plate on a StepOnePlus Real-Time PCR System (Applied BioSystems). Standard cycling conditions were used, and Lavergne (2018) previously determined optimal annealing temperatures for each primer pair using gradient qPCR.

We used cycle threshold (Ct) values to analyze relative quantification of our qPCR samples, rather than expression (i.e. absolute quantification), and calculated gene expression by means of a fold change through the $2^{-\Delta\Delta Ct}$ method (described in Livak and Schmittgen, 2001; Rao et al., 2013). Using GenEx 7.0 (MultiD Analysis AB), we calibrated the data using two interplate

calibrators, to account for differences between plates. We then repeated reference gene selection with GenEx 7.0, through the use of NormFinder to select the most stably expressed gene(s) between PPIA, YWHAZ and GAPDH. From this analysis, we concluded that the use of the geometric mean of all three reference genes was the least variable option for data normalization. A normalization factor was applied to each target sample based on the geometric mean of the three reference genes (Vandesompele et al., 2002). The expression data was then normalized to the series mean using the $2^{-\Delta\Delta Ct}$ method. Values are thus reported as relative gene expression.

2.2.11 Statistical Analysis

All data is presented as means ± SE, unless otherwise indicated. Dams who were in a maternity cage longer than 12 days, and dams who had stillborn litters were excluded from our analyses. As there were some mixed litters of viable and stillborn offspring, only viable offspring were used in our analyses. Extreme outliers were also excluded from the data. Sexes were considered separately in all analyses of offspring physiology and gene expression, as sexes are known to differ greatly in response to maternal prenatal stress (Matthews, 2002). Data were tested for normality with the Shapiro-Wilk test, and tested for homogeneity assumptions with Levene's equality of variances test. Data was log transformed where necessary to achieve normality. Data that could not be normalized was analyzed through the use of non-parametric independent samples tests.

General Linear Models (GLM) (ANOVA or Repeated Measures) were performed using SPSS statistical software (Version 26, IBM) on dam data, with year as a fixed effect, and litter size as a covariate in some cases. Tukey's HSD (honestly significant differences) was used as the post-hoc test for GLMs. Linear Mixed Models (LMM) were performed on all offspring birth data, and dissected offspring data to account for year and sex effects. LMMs were conducted with year and sex as fixed effects and maternal ID as a random effect. Fisher's LSD (least significant difference) was used as the post-hoc test for LMMs, as it does not adjust the confidence interval. Offspring birth data that was not normally distributed was analyzed through the use of a generalized linear mixed model (GLMM), using the same fixed and random effects as above. Proportion of offspring who moved was analyzed using a GLMM binary logistic regression, also with the same fixed and random effects as above. For morphological offspring data, normal

distributions were assumed, as sample size was >30 samples, and violations of normality with large sample sizes are not considered problematic (as per Ghasemi and Zahediasl, 2012). LMMs were run on offspring morphological data, and litter size was included as a covariate. Correlations between variables were analyzed using bivariate correlations with Pearson correlation coefficient and one-tailed test of significance. Effects were considered statistically significant at $p \leq 0.05$ for all analyses.

2.3 Results

2.3.1 Population dynamics

Snowshoe hare density went through an increase phase from 2013-2016, reaching a peak in 2017 and declining through 2018-2019 (Fig. 2.3). My research took place over these decline years. Density doubled from the increase phase in 2015 to reach a peak of 1.21 hares/ha in 2017 (95% CI [1.02, 1.35]). Snowshoe hare abundance then declined 75% over two years to reach a low of 0.30 hares/ha in 2019 (95% CI [0.22, 0.40]).

Lynx population estimates increased 220% from 2013 to 2017, reaching a peak of 59.9 (±6.4) tracks/100 km, which was maintained into 2018 (59.7 ± 8.2 tracks/100km), hence a 1-year lag to snowshoe hare densities (Fig. 2.3). In 2019, they began to decline, and population estimates decreased by 51%.

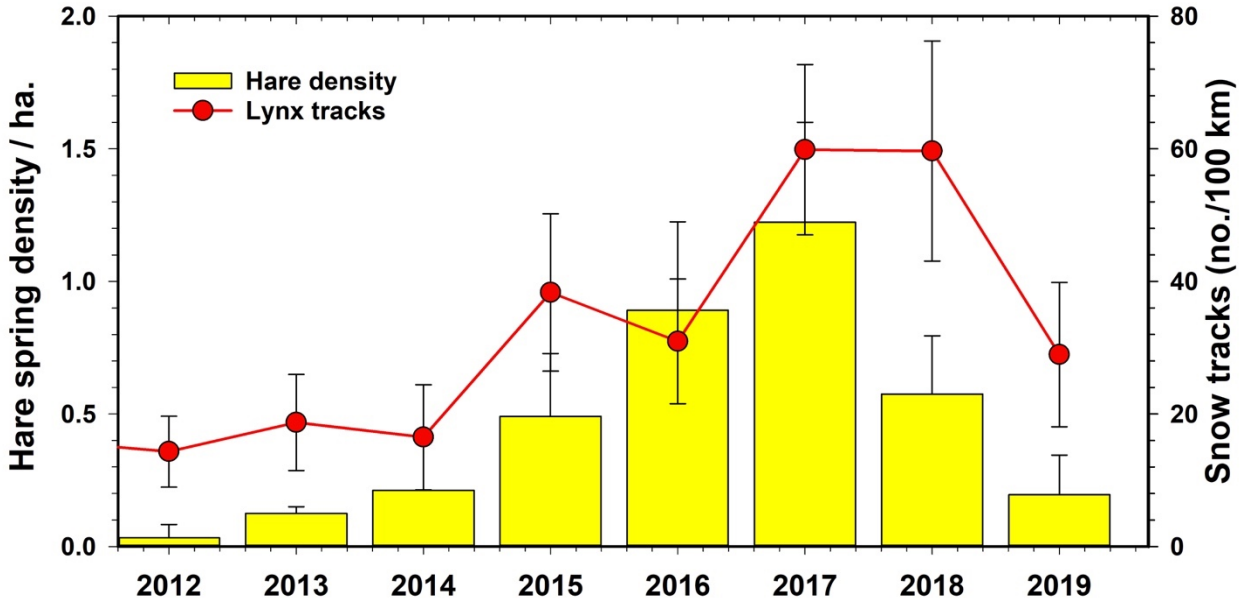

Figure 2.3: Snowshoe hare spring density estimates (means ± 95% CI) and lynx population estimates (means ± 1 SE) from 2012 to 2019 in the Kluane Lake region, Yukon Territory. *Obtained and adapted from* Krebs et al. (2018b).

2.3.2 Maternal conditions

2.3.2.1 Reproductive output

The average number of days dams were in maternity cages prior to parturition was 6.1 (± 0.85) days, which did not differ significantly among years ($F_{3,27}$= 2.6, p=0.07). The start date of second litter parturition varied from June 26th to July 2nd in all years (June 26th (± 1.00) day in 2015 to July 2nd (± 0.44) days in 2017 to July 1st (± 0.46) days in 2018 and June 28th (± 1.85) days in 2019). All dams survived and gave birth to some viable young (i.e. no stillborn litters, but some stillborn within a litter) in all years. The average litter size did not differ among years (mean 5.17 (± 0.41) leverets; $F_{3,27}$= 4.39, p=0.55; Table 2.1). The total birth mass also did not differ among years (mean 294.99 (± 21.28) g; $F_{3,26}$=0.36, p=0.79; Table 2.1). However, total birth mass varied as a function of litter size, i.e. larger litters had greater mass ($F_{1,26}$=155.68, p<0.01). The sex ratio of males to females did not differ and was similar in all years ($F_{3,27}$=0.35, p=0.79, Table 2.1).

Table 2.1: Reproductive outcomes across phase years of the snowshoe hare cycle. Reproductive output metrics are presented as means ± 1 SE.

Phase	Year	n	Proportion viable litters	Litter size	Total birth mass (g)	Sex ratio (M:F)	Mean birth mass (g)	Mean birth RHF (mm)
Increase	2015	6	1.00	5.83 (0.48)	334.83 (27.15)	0.488 (0.04)	56.59 (1.71)	33.11 (0.60)
Peak	2017	8	1.00	4.75 (0.49)	270.25 (23.15)	0.400 (0.09)	57.20 (1.69)	35.61 (0.46)
Decline	2018	8	1.00	4.75 (0.37)	273.06 (18.90)	0.462 (0.08)	57.46 (1.63)	34.56 (0.53)
Decline	2019	9	1.00	5.33 (0.29)	301.83 (15.91)	0.499 (0.07)	56.59 (1.16)	33.69 (0.38)

2.3.2.2 Maternal physiology

Maternal baseline fecal cortisol metabolites (FCM) concentrations (late pregnancy, at capture for transfer to maternity cages), did not differ among years ($F_{3,25}$= 0.20, p=0.90). Baseline levels were similar in 2015, 2018 and 2019, and were 31% higher at the peak in 2017 (Fig. 2.4). Maternal FCM at parturition did not differ among years ($F_{3,25}$=0.63, p=0.60) but was significantly higher than at baseline in all years of the cycle ($F_{1,23}$= 15.45, p<0.01, Fig. 2.4). We saw a similar trend at parturition day, with FCM levels at the peak being 56% higher than 2015, 2018 or 2019, which were similar (Fig. 2.4). As FCM could differ by date of pregnancy, we confirmed that FCM concentrations at parturition day were not affected by the number of days in maternity cages for all dams (p=0.15).

Newborn hair cortisol differed among years ($F_{3,27.21}$=12.54, p<0.01, Fig. 2.5). There were no significant differences between sexes or effect of offspring from the same dam (p=0.25). A few samples fell below the detection threshold and were given assumed values of 2.5 ng/g (assay detection threshold) or 0.01 ng/g. Statistical outcomes did not differ when assuming values were at the detection threshold or below, therefore they were assigned a value of 2.5 ng/g, as that data was normally distributed. Hair cortisol was similar in 2015 and 2017, but declined 51% in 2018 and 2019, which were also similar (p<0.05, Fig. 2.5). There was no correlation between hair cortisol and maternal FCM at capture, or on parturition date (p=0.46 and 0.31, respectively).

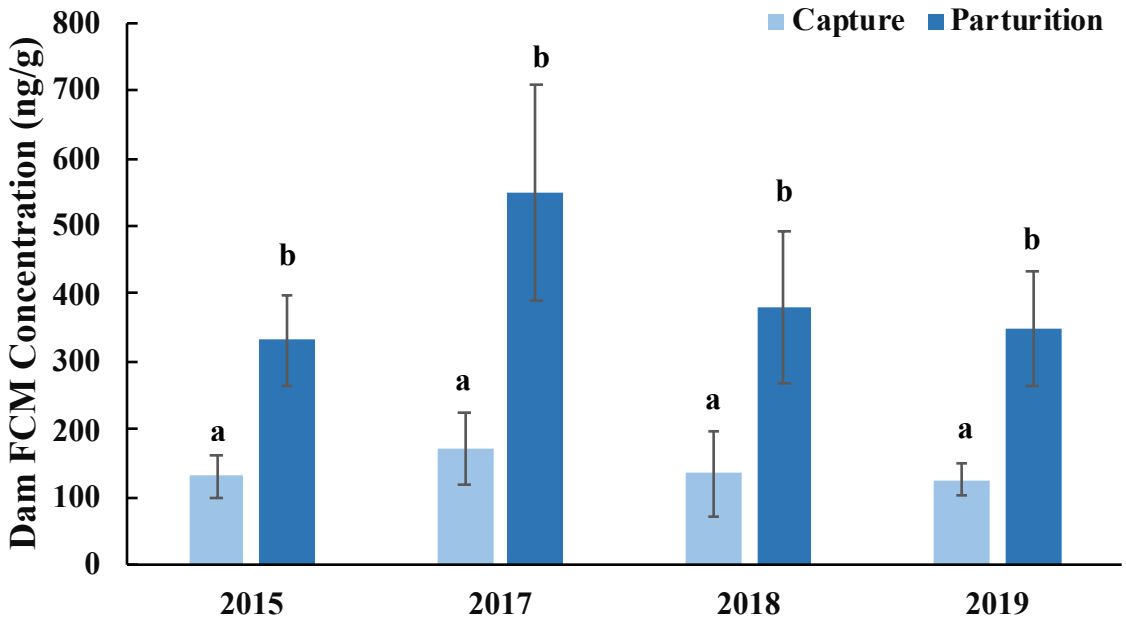

Figure 2.4: Dam fecal cortisol metabolite concentrations (means ± 1 SE) at capture and on the day of parturition in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of $p \leq 0.05$ indicated by letters.

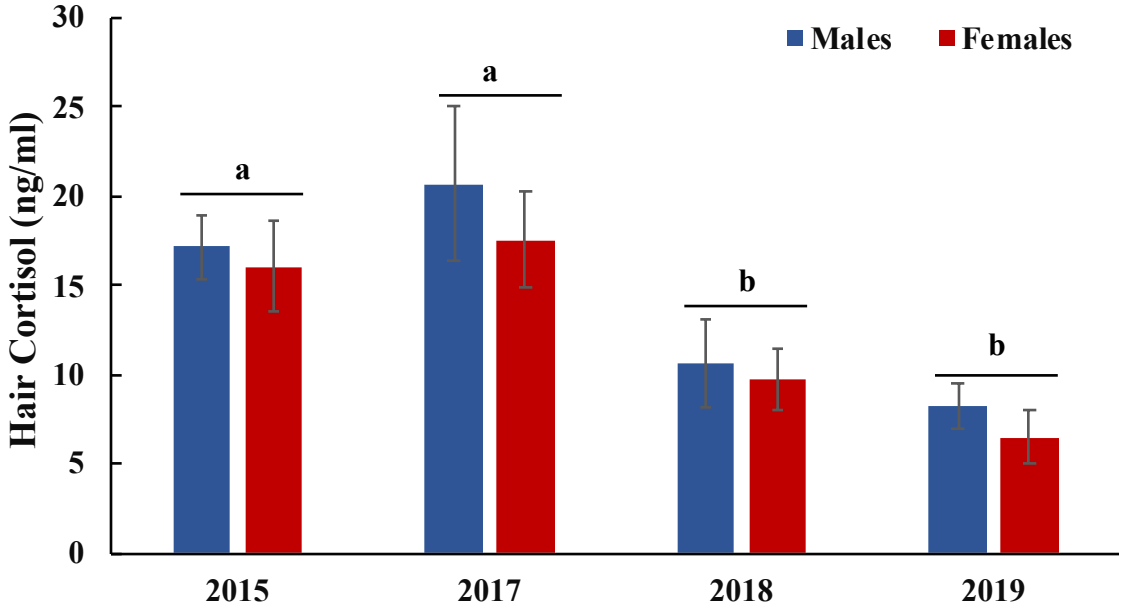

Figure 2.5: Male and female offspring hair cortisol concentrations (ng/ml, means ± 1 SE) in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of $p \leq 0.05$ indicated by letters.

2.3.3 Offspring conditions

2.3.3.1 Offspring morphometrics

Individual offspring mass did not differ among years (mean 56.96 ± 1.55 g; $F_{3,24.01}$= 0.17, N=156, p=0.91, Table 2.1). However, offspring from larger litters were smaller and there was a significant dam effect (p<0.01). Offspring right hind foot (RHF) length, an index of skeletal size, differed as a function of mother, but not as a function of year ($F_{3,25.32}$=2.16, N=156, p=0.12, Table 2.1). In a *post hoc* analysis, offspring RHF was significantly larger in 2017 (35.61 ± 0.46 mm) than in 2015 (33.11 ± 0.60 mm) (p<0.05). Newborn mass was positively correlated with newborn RHF length (R^2=0.33, p<0.05). Offspring morphometrics were not correlated with maternal FCM at capture or parturition (p>0.05).

2.3.3.2 Offspring behaviour

The proportion of the litters that moved differed among years ($F_{3,148}$= 3.04, N=156, p=0.03), with 75 (±10) % of the litter moving in 2017, compared with 44 (±10) % in 2019 (p<0.05, Fig. 2.6). Of the offspring who moved, latency to movement also differed among years ($F_{3,81}$= 2.64, N=89, p=0.05). Offspring moved later in 2018 (31.4 ± 5.0 s), than in 2017 (21.9 ± 3.1 s) or 2019 (20.0 ± 4.6 s) (p<0.05, Fig. 2.7). Of the offspring who moved and also had physiological data collected, time to movement was negatively correlated to hematocrit (R^2=0.21, N=31, p<0.05, Fig. 2.8).

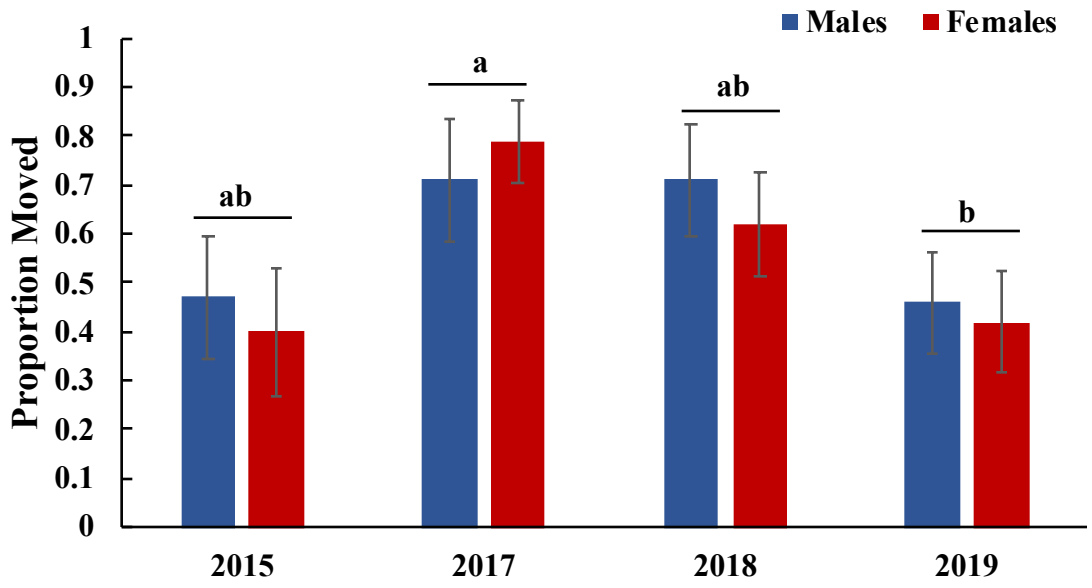

Figure 2.6: Proportion of male and female offspring who moved in the pre-handling behaviour test (means ± 1 SE) in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of $p \leq 0.05$ indicated by letters.

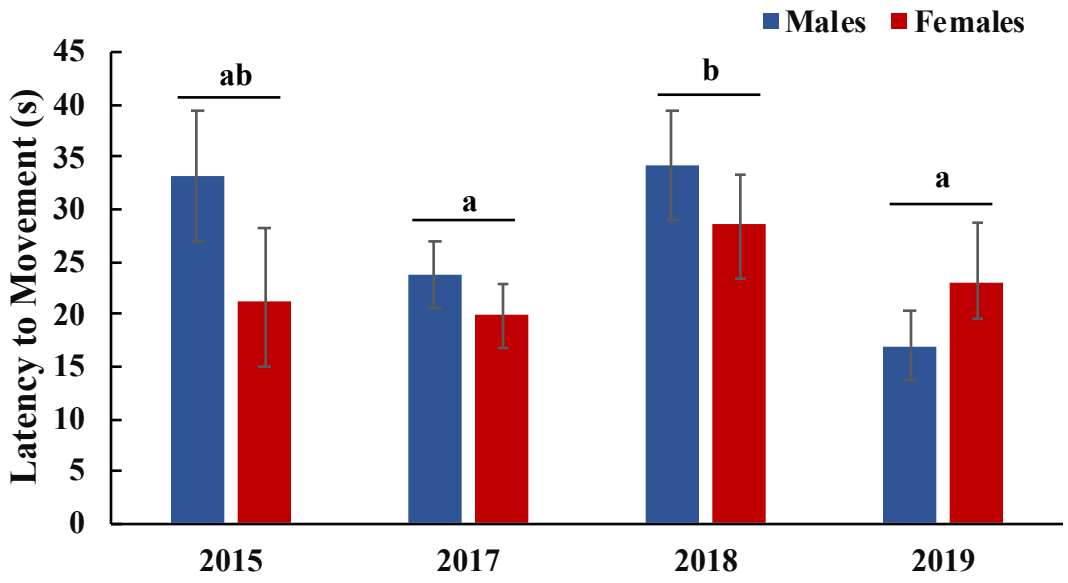

Figure 2.7: Latency to movement (in seconds) of male and female offspring who moved in the pre-handling behaviour test (means ± 1 SE) in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of $p \leq 0.05$ indicated by letters.

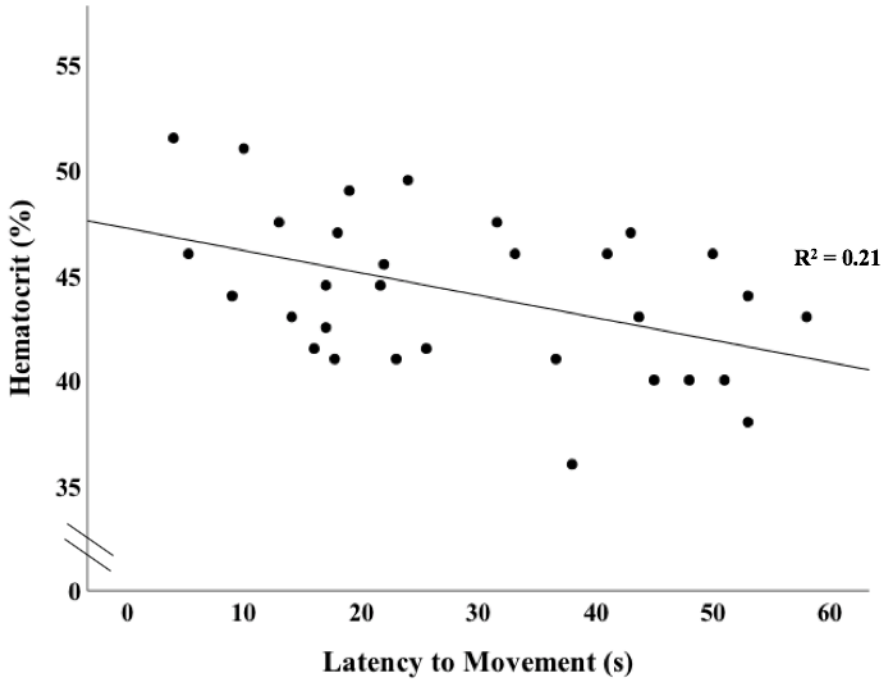

Figure 2.8: Correlation of latency to movement (in seconds) in offspring who moved in the pre-handling behaviour test with mean hematocrit (% packed red blood cell volume). R^2=0.21, p<0.05, N=31. Note the axis break from 0 to 35% in hematocrit percentage.

2.3.3.3 Offspring physiology

Mean hematocrit (% packed red blood cell volume), an index of body condition, differed among years ($F_{3,24.45}$= 8.509, p<0.01, Fig. 2.9). Offspring in 2015 had the lowest values of 39.78 (± 1.59) %, 2017 and 2018 were similar (43.79 ± 1.00 % and 42.94 ± 1.42 %, respectively) and those in 2019 had the highest values of 47.59 (± 1.02) % (p<0.05, sexes pooled).

Plasma total cortisol increased over the years ($F_{3,53}$= 4.69, p<0.01, Fig. 2.10). There was no sex difference in total cortisol (p=0.11), therefore the following results are presented as sexes pooled. Total cortisol was similar in 2015 and 2017 (52.32 ± 23.2 ng/ml and 43.15 ± 6.0 ng/ml, respectively). In 2018 (90.84 ± 17.8 ng/ml), total cortisol increased higher than 2017, and total cortisol in 2019 (121.90 ± 27.0 ng/ml), was higher than 2015 or 2017 (p<0.05, Fig. 2.10). Blood glucose did not differ among years ($F_{3,27.11}$= 0.68, p=0.57), but overall was high in all years (mean 130.88 ± 10.06 mg/dL). Individual offspring total cortisol was found to be positively correlated with offspring hematocrit (R^2=0.09, N=56, p<0.05), and negatively correlated to

maternal FCM at capture (R^2=0.05, N=57, p<0.05) . There was no correlation between offspring plasma total cortisol and hair cortisol (N=61, p=0.07).

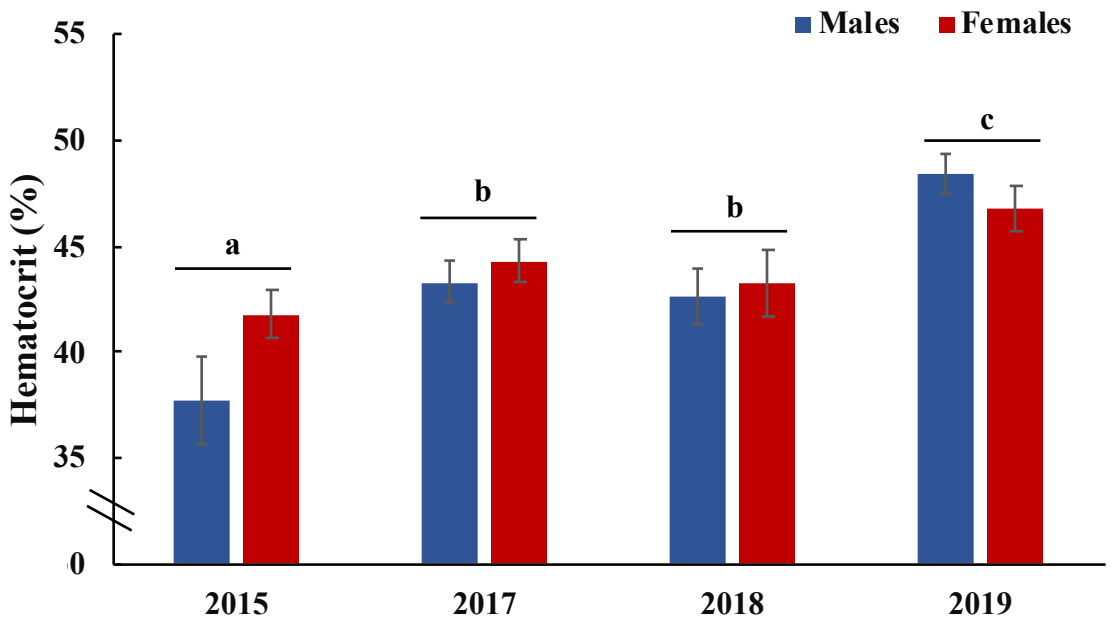

Figure 2.9: Hematocrit (% packed red blood cell volume) of male and female offspring (means ± 1 SE) in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of p≤0.05 indicated by letters. Note the axis break from 0 to 35% in hematocrit percentage.

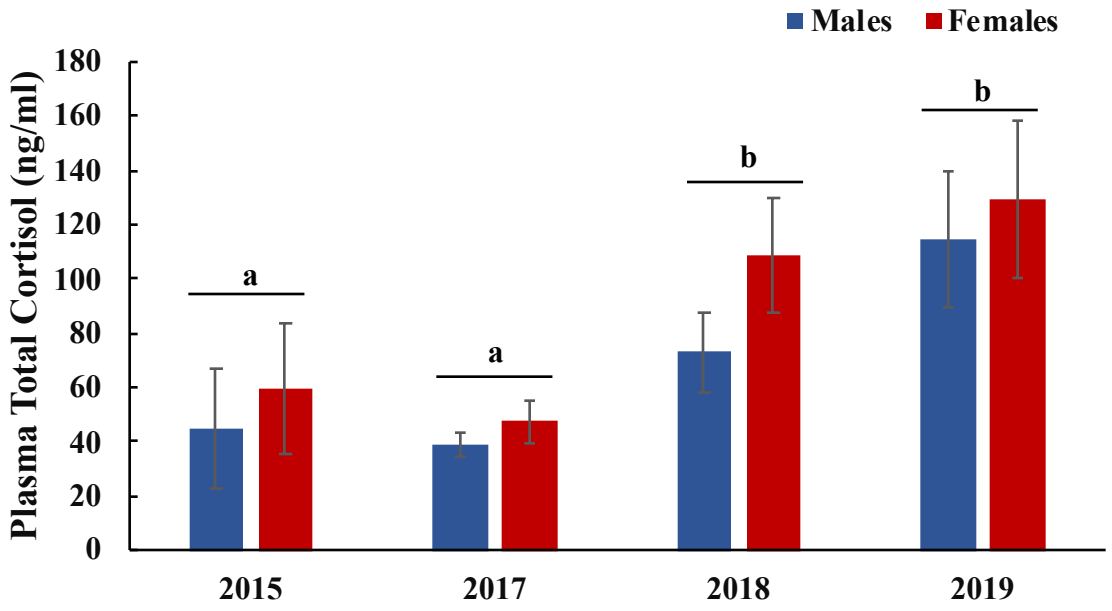

Figure 2.10: Plasma total cortisol (ng/ml) of male and female offspring (means ± 1 SE) in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Significant differences of $p \leq 0.05$ indicated by letters.

2.3.3.4 Offspring gene expression

Gene expression of GR and MR in the dorsal hippocampus differed among years, and both genes showed similar patterns of variation over time. There were no sex effects in gene expression among years ($p=0.17$), therefore data are presented as sexes pooled. Expression of GR was similar in 2015 and 2017 ($p=0.73$). However, relative to the mean value of those two years, expression in 2018 decreased by 26%, and increased by 48% in 2019 ($F_{3,27.92}= 43.99$, $p<0.01$, Fig. 2.11). GR expression also increased by 100% from 2018 to 2019 ($p<0.01$). Expression of MR followed the same pattern, with 2015 and 2017 being similar ($p=0.08$). Relative to the mean value of those two years, expression in 2018 decreased by 52%, and increased by 54% in 2019 ($F_{3,27.75}= 51.24$, $p<0.01$, Fig. 2.12). MR expression also increased by 224% from 2018 to 2019 ($p<0.01$). Gene expression values of GR and MR were positively correlated to each other ($R^2=0.79$, $N=60$, $p<0.01$, Fig. 2.13).

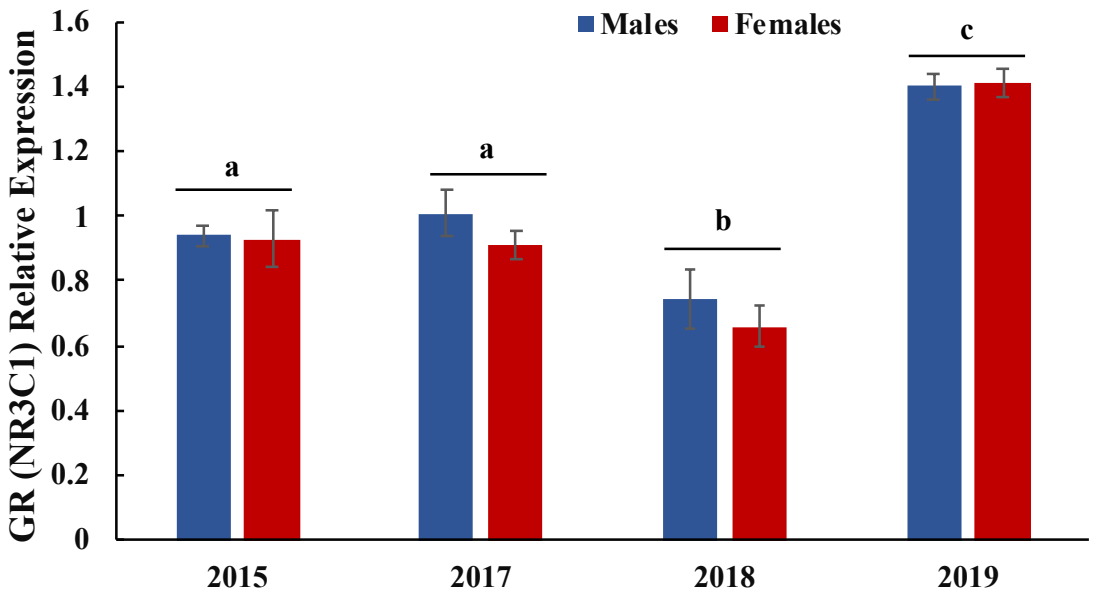

Figure 2.11: Transcript abundance in the dorsal hippocampus for the gene encoding glucocorticoid receptors (GR, NR3C1) of male and female offspring in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019 (decline phase). Values are shown as relative expression levels (means ± 1 SE), normalized to the geometric mean of reference genes GAPDH, PPIA and YWHAZ. Significant differences of $p \leq 0.05$ indicated by letters.

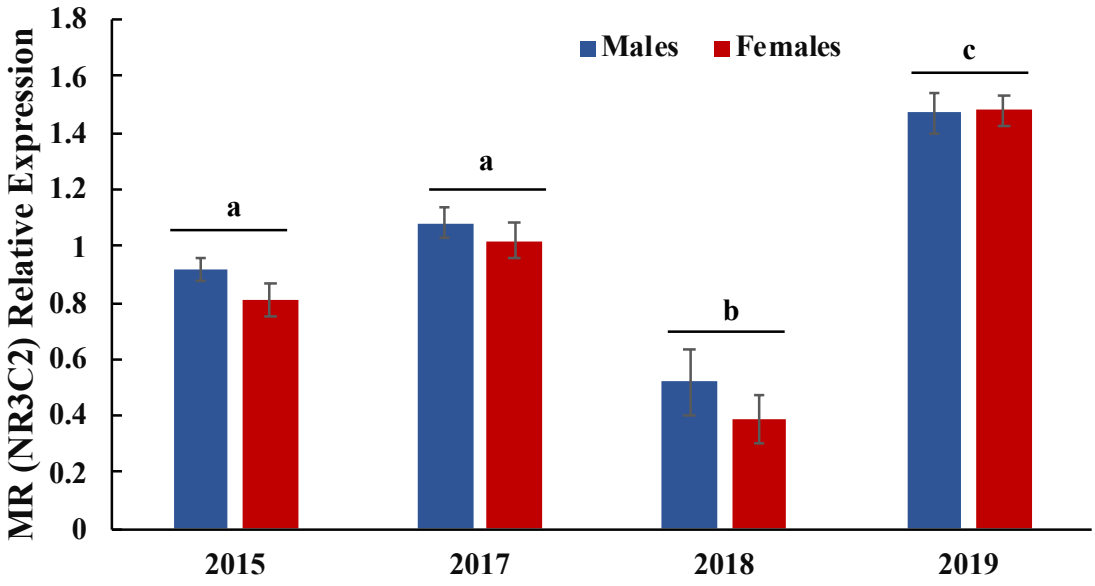

Figure 2.12: Transcript abundance in the dorsal hippocampus for the gene encoding mineralocorticoid receptors (MR, NR3C2) of male and female offspring in 2015 (increase phase), 2017 (peak phase) and 2018 and 2019

(decline phase). Values are shown as relative expression levels (means ± 1 SE), normalized to the geometric mean of reference genes GAPDH, PPIA and YWHAZ. Significant differences of $p \leq 0.05$ indicated by letters.

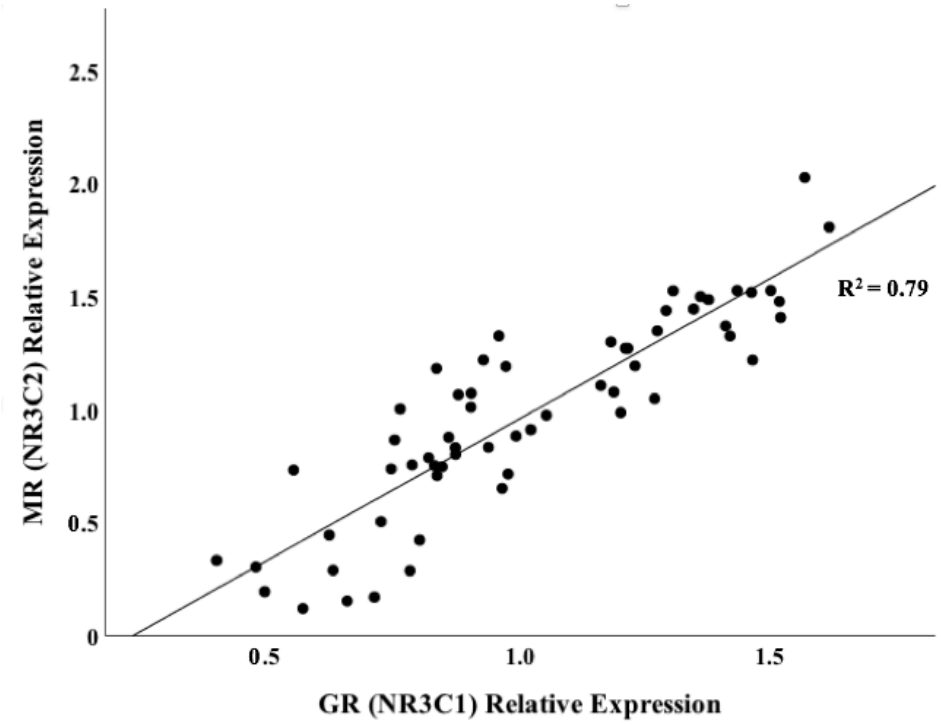

Figure 2.13: Correlation of transcript abundance of genes encoding glucocorticoid receptors (GR, NR3C1) with mineralocorticoid receptors (MR, NR3C2) in the dorsal hippocampus of offspring. Values are relative expression levels, normalized to the geometric mean of reference genes GAPDH, PPIA and YWHAZ. R^2=0.79, $p<0.01$, N=60.

2.4 Discussion

I tested the hypothesis that dams experiencing severe predation risk during the decline phase of the snowshoe hare cycle will program their offspring through maternal effects *in utero*. I relied on data collected by Lavergne (2018) for the increase (2015) and peak (2017) phases and obtained evidence from two years of the decline phase (2018 and 2019). During this time, changes in hare and lynx populations (Fig. 2.3) were monitored intensively as part of the ongoing research into their dynamics (Krebs et al., 2018b). Predation risk was highest during the peak (2017) and first year of the decline (2018) phase of the snowshoe hare cycle when lynx numbers were high. In the second year of the decline phase (2019), lynx densities have decreased

by half and predation risk has likely eased up. Our combined results over this period powerfully demonstrate major changes in offspring behaviour, physiology and gene expression. I reached five major conclusions.

First, contrary to predictions, maternal fecal cortisol metabolite (FCM) concentrations at capture or parturition did not differ among years but were higher at parturition (Fig. 2.4). Similarly, neither maternal reproductive output, nor offspring mass and size differed among years in our study (Table 2.1). Second, offspring behaviour varied among years. As predicted, a lower proportion of offspring moved (i.e. more freezing) in the second year of the decline (2019) than during the peak (2017) of the hare cycle (Fig. 2.6). Of the offspring who moved, offspring took longer to do so in the decline in 2018 compared to the peak (Fig. 2.7). However, latency to movement in 2019 was similar to that in 2015 and 2017. Third, our index of offspring condition (% packed red blood cell volume), differed among years. Contrary to predictions, offspring were in the worst condition in the increase phase in 2015, and the best condition in the second year of the decline phase in 2019 (Fig. 2.9). Offspring with higher hematocrit levels were quicker to movement in the behaviour test (Fig. 2.8). Fourth, offspring's hair cortisol and plasma total cortisol differed among years. Offspring's plasma total cortisol was higher in the decline phase (Fig. 2.10), but their hair cortisol was completely opposite and lowest in the decline phase (Fig. 2.5). Fifth, expression of GR and MR in the dorsal hippocampus differed among years, with expression being much lower in the 2018 decline year but rebounding in the 2019 decline year and being higher than all other years (Fig. 2.11 (GR); Fig. 2.12 (MR)). Expression of GR and MR were positively correlated to each other (Fig. 2.13). In general, our results affirm the hypothesis, as we see major changes in offspring behaviour, physiology and gene expression among years, suggesting maternal programming.

2.4.1 Caveats

There are four caveats that need to be addressed before I discuss our findings. First, given that this was a multi-year collaborative study, the data was collected by two people which has the potential to introduce unwanted variability to the results. We addressed this by strictly following the same methodologies in the field and laboratory. Additionally, we ran all of our laboratory samples in the same run or on the same plate wherever possible. When multiple runs were

necessary, at least two quality control samples were run on each plate to control for batch effects. We are thus confident our results are biologically true.

Second, our dam parturition fecal samples represent a 24-hour pooled sample on the date of birth, thus they are not strictly representative of post-parturition dam stress levels. As the precise timing of parturition within that time period is likely to have varied among individual dams, this is likely introducing variation to our data and swamping possible results. Our study builds off the work of Sheriff in the previous decline phase, who obtained fecal samples 30 hours after parturition for all dams and found robust results (2009a). This was not possible in our study, as dams were released as soon as possible after parturition to minimize interferences with natural maternal-litter dynamics, therefore we can only compare trends. Nevertheless, our methods were consistent across the study period and thus allow for a comparative analysis across years.

Third, the maternal age of dams used in this study was unknown and could have varied over the four years and biased the results. In long-lives species, such as the yellow-bellied marmot, age of the mother had a significant effect on FCM, reproduction and sex-ratios of offspring (Monclús et al., 2011). In contrast, snowshoe hare are a much shorter-lived species (Hodges et al., 2001). Though we do not know the age of our dams used in this study, up to 70% of the snowshoe hare breeding population is made up of yearlings each year, especially in the decline phase when mortality is high and recruitment is low (Krebs et al., 2001a). Thus, I do not expect maternal age to have played a significant part in our research.

Finally, our research into maternal and offspring physiology over the course of the snowshoe hare cycle assumes that the present cycle (and the predator-prey dynamics therein) is a replicate of those seen previously. However, in our study area, we have seen an increase in yearly temperature, a significant increase in shrub growth, and a decreased amplitude in snowshoe hare cycles from past cycles (Krebs et al., 2014; Krebs et al., 2018a; discussed further in Chapter 3). As climate is warming and the environment is changing, it is possible that our assumption may be incorrect, and this cycle may not be a perfect replicate of past ones. Nevertheless, the evolutionary susceptibility in snowshoe hare to maternal programming of predation risk should be the same and I expect that my findings are broadly indicative of what occurred in past cycles.

2.4.2 Maternal physiology and reproductive output

Predation risk was high in 2018 and 2019, yet our control dams were not showing elevated FCM concentrations, decreased body condition or any of the known signs of chronic stress that have been seen in previous decline phases (i.e. Boonstra et al., 1998a). Since we had no increased FCM levels, we also had no changes in reproductive output or offspring size/weight, which are typically associated with high maternal glucocorticoid levels in snowshoe hare (Hodges et al., 1999a). Reduced reproductive output, smaller and lighter offspring have been found in the decline phase of previous snowshoe hare cycles in our study area (Krebs et al., 2001a; Sheriff et al., 2009a; Stefan and Krebs, 2001). Increased non-consumptive predation risk has also been found to lead to increased indices of stress and reduced reproductive output in a variety of species (Clinchy et al., 2004; Dulude-de Broin et al., 2019; Zanette et al., 2011).

In this natural monitoring study, maternal FCM concentrations at capture and parturition showed no significant differences across the cycle, but followed a pattern similar to population density, with highest values occurring at the peak in 2017. Our study was conducted on the second litter of the breeding season, when fresh vegetation is abundant and predation risk is diluted by the birth of first litters. Snowshoe hare dams have been shown to have significantly lower FCM concentrations following the birth of the second litter compared to the birth of first litters (Sheriff et al., 2009a, 2011). Additionally, Sheriff et al. (2009a) found elevated maternal FCM concentrations in the first year of the decline phase, which then decreased by the second year of the decline phase, similar to what we see here. Also, similar to this study, the stress state and condition of hares in the late decline and early increase phases of Boonstra et al.'s (1998a) study were markedly better than at the peak and early decline. This suggests that maternal condition may change rapidly in response to perception of a change in predation risk.

2.4.3 Offspring behaviour

The proportion of offspring per litter that moved in the pre-handling behaviour test differed among years, as well as the latency to movement for those who moved. A lower proportion of offspring moved in the second year of the decline phase in 2019, yet of the offspring who moved, they moved sooner. Offspring moved later in the first year of the decline phase in 2018.

Newborn leverets disperse but are unlikely to outrun predators; therefore, their best strategy is to frequently remain hidden and motionless to avoid detection. Any change in behaviour phenotype which increases survival in these offspring should be adaptive, as only 30% of newborns survive the first 5 days after birth (O'Donoghue, 1994).

Mothers may transmit anti predator behaviours to her offspring through exposure to circulating glucocorticoid levels during gestation (Seckl, 2004; Welberg and Seckl, 2001). High maternal glucocorticoids have been linked to decreased locomotion and increased fearfulness, vigilance and anxiety behaviour in offspring (Fish et al., 2004; Seckl, 2004; Szyf et al., 2007). Lavergne et al. (2019) investigated juvenile snowshoe hare behaviour in an open field test during the late low/early increase of the snowshoe hare cycle. Juveniles with higher FCM concentrations were found to travel less distance, have less position transitions and spend almost 100% of their time under cover, indicating that increased glucocorticoids are associated with risk avoidance behaviours in this species (Lavergne et al., 2019). As offspring had higher plasma total cortisol and displayed overall reduced locomotion in 2019, this is consistent with the findings of Lavergne et al. (2019).

2.4.4 Offspring condition

Hematocrit values were lowest in offspring born during the increase (2015), and highest in the second year of the decline (2019), suggesting improved body condition over the course of our study (Fig. 2.9). This was unexpected as lower hematocrit and worsened body condition have been found in snowshoe hare in response to increased predation risk at the peak and early decline phases (Boonstra et al., 1998a). However, Boudreau et al. (2019) saw no effect on hematocrit in response to an experimental increase in predation risk on snowshoe hares (chased with a trained dog). Additionally, similar to our results, Boonstra et al. (1998a) saw improved condition by the late decline and early low phase.

Sheriff et al. (2010b) found that females with higher FCM levels produced offspring with lower hematocrit. We found no correlation between the two in our natural monitoring study. We did however find an interesting correlation between hematocrit and offspring latency to movement

prior to handling. Offspring in better condition moved sooner in our behaviour test than offspring with lower values.

2.4.5 Offspring physiology

Plasma total cortisol levels were higher in offspring born during the decline phase in response to handling, which was expected. This may indicate that these offspring are able to mount a greater cortisol response to an acute stressor, or that they have reduced negative feedback and ability to shut down the stress response. The former could be adaptive in nature if predation risk is high, whereas the latter might have detrimental impacts on offspring condition and health, which could be pathological and nonadaptive. Sheriff et al. (2010b) and Lavergne et al. (2019) assessed indices of juvenile stress and found them to be positively correlated to dam FCM in snowshoe hare. In this study, maternal FCM was not higher in these years when offspring total cortisol levels were higher and offspring plasma total cortisol levels were negatively correlated with dam FCM at capture and not correlated with dam FCM at parturition. Therefore, dams with lower FCM levels at capture gave birth to offspring with higher total cortisol levels in response to the stressor of handling. This suggests further research is needed, primarily to quantify the levels of corticosteroid-binding globulin (CBG) to determine the amount of cortisol in the plasma that is free and biologically active. These offspring have higher total cortisol, but it is possible that their amount of free cortisol does not differ, with more being bound to CBG.

In laboratory experiments, offspring displayed higher levels of glucocorticoids after birth when exposed to high maternal glucocorticoids *in utero* (Henry et al., 1994; Kapoor et al., 2008; Liu et al., 2001; Welberg et al., 2000). High maternal glucocorticoid levels have also been found to affect offspring based on the timing of exposure *in utero*. In guinea pigs, when females were exposed to a stressor during the period of rapid brain growth, the offspring had higher baseline plasma cortisol levels (Kapoor and Matthews, 2005). Whereas when females were exposed to a stressor later in gestation, offspring displayed a higher plasma cortisol response to a stressor and activation of the HPA axis (Kapoor and Matthews, 2005). Here we found plasma total cortisol to be higher in offspring born during the decline phase, yet no index of increased maternal stress during pregnancy. It is possible that mothers in the decline phase are experiencing chronic stress of predation risk, which leads to reduced maternal HPA axis activity and reduced ability to

mount a stress response (van der Voorn et al., 2019). This could explain why we do not see an overall signature of high maternal cortisol in our fecal or hair samples.

Offspring hair cortisol concentrations were lower during the decline phase of 2018 and 2019. Newborn hair cortisol is thought to be representative of maternal circulating glucocorticoids reaching the fetuses and being incorporated into the hair shaft during growth *in utero* (Kapoor et al., 2016; Romero-Gonzalez et al., 2018). This result was not expected, as we predicted maternal glucocorticoids would be higher in response to increased predation risk in the decline phase. This decrease in hair cortisol loosely follows the trend of decreased maternal FCM levels in these years, however the two were not significantly correlated. Hair samples represent circulating glucocorticoid levels over a longer temporal window (days to weeks) compared to FCM (hours) (Meyer and Novak, 2012), which could explain some of the differences we are seeing here. Studies in humans have shown this same result; higher levels of maternal stress during gestation leading to lower levels of cortisol in newborn hair (Romero-Gonzalez et al., 2018; Scharlau et al., 2018; van der Voorn et al., 2019). They speculated decreased maternal HPA activity under chronic stress, and changes in placental enzymes in response to maternal stress to be responsible for this pattern (van der Voorn et al., 2019).

Maternal glucocorticoids during gestation are essential for normal fetal development (Smith and Shearman, 1974), but can be buffered from reaching the developing fetuses through placental expression of 11-Beta-Hydroxysteroid dehydrogenase (11β-HSD), an enzyme which converts cortisol to its inert form cortisone (Burton and Waddell, 1999). Yet, in laboratory studies, increases in maternal stress during pregnancy were not accompanied by increases in 11β-HSD (Lesage et al., 2001; Lucassen et al., 2009), and led to decreased expression and increased exposure of the fetuses (Burton and Waddell, 1999). Although we did not examine 11β-HSD expression in this study, this represents a promising future avenue of research. In this study we saw lower hair cortisol levels suggesting females in the decline phase may be experiencing low activity of their HPA axes due to chronic stress, or be increasing their expression of 11β-HSD, to reduce the levels of circulating glucocorticoids reaching the developing fetuses during a time of high predation risk. However, we would expect maternal programming to be beneficial and adaptive for snowshoe hare living in a stressful high predation environment, as there would be a much greater cost to producing an unaltered offspring in this environment (Sheriff et al., 2018). We would not expect selection to favour mothers who utilize mechanisms to protect their

developing offspring from increased glucocorticoids, when it can provide adaptive benefits (Love and Williams, 2008a).

2.4.6 Offspring gene expression

Our most noteworthy findings in this study were the changes in offspring hippocampal GR and MR expression among the phase years. Offspring born in the decline phase in 2018 had much lower expression, followed by the steep increase in expression in the second year of the decline phase in 2019. The expression of GR and MR were positively correlated to each other in offspring, which is not often described in the laboratory literature. This suggests consistent change in the receptors regulating circadian rhythms and baseline levels (MR) and in the receptors regulating the response to stressors (GR). This is particularly interesting, as the laboratory literature tends to focus mainly on GR changes in a maternal/acute stress context, and MR typically remains unchanged (Kapoor et al., 2008; McCormick et al., 1995).

Lavergne (2018) found a significant difference between MR gene expression in snowshoe hare offspring born in the peak (2017), having 31% higher levels than those born in the increase (2015). This difference is no longer statistically significant following the addition of more years into our analysis, but we still see this increasing trend (Fig. 2.12). MR and GR are highly expressed in the hippocampus and are critical in regulating circadian rhythms, homeostasis and the activation and response to stressors (reviewed in Schaaf and Meijer, 2017). Changes in GR and MR expression demonstrate innate sensitivity to programming during gestation, as they have been seen with high exposure to glucocorticoids, as well as low exposure (Zhe et al., 2008). Since MR are responsible for maintaining homeostasis in circadian rhythms and GR are responsible for reacting to stressors and facilitating negative feedback (reviewed in de Kloet et al., 1998), changes in expression of these receptors can lead to significant changes in the manner in which an individual responds to a stressor.

In the laboratory, guinea pig offspring who were born to mothers exposed to a stressor during the period of fetal brain growth had a reduction in GR expression in the hippocampus, which reduced the functioning of the negative feedback of the HPA axis (Kapoor et al., 2008). Additionally, female rats subjected to a restraint challenge in the last week of pregnancy

produced offspring that had lower hippocampal MR and GR densities, increased basal glucocorticoids and a prolonged glucocorticoid response to a stressor (Henry et al., 1994). Sex-specificity of offspring to maternal stress has also been found, with males typically being more susceptible to programming (Love et al., 2005; St-Cyr et al., 2017). Richardson et al. (2006) also exposed rat dams to restraint stress in the last week of pregnancy; female offspring showed this prolonged glucocorticoid response to a stressor, and male offspring showed a blunted response, suggesting increased glucocorticoid receptor activity. Additionally, male guinea pig offspring showed increased hippocampus MR expression in response to prenatal exposure to synthetic glucocorticoids, but not females (Liu et al., 2001). Contrary to the laboratory evidence for sex differences in maternal programming, we saw no sex specific effects in any variables measured in this study, which could be due to the nature of the stressor. Predation risk in the wild is a strong selective stressor – you either live or you die, therefore it may be important enough that both males and females respond the same.

Findings from laboratory studies demonstrate how the type of stressor, timing of stressor and length of stressor can affect key HPA axis regulators in offspring in different ways. Nevertheless, laboratory evidence does not map on well to field evidence, as nature has true adaptive selection occurring. The changes that we observed in this study not been examined before in a wild mammal. Our results in 2018 of decreased GR and MR expression in 2018 suggest a delayed and reduced negative feedback of the HPA axis, and higher sustained levels of total cortisol in these decline-born offspring. This was consistent with our prediction that offspring in the decline phase would show reduced GR and MR expression in response to increased predation risk and maternal stress, however we saw no changes in maternal stress in this study. Additionally, in 2019, GR and MR expression increased higher than any other year, suggesting a rapid recovery from stress of the initial decline, with no consistent maternal programming effects, which was not predicted. High receptor expression should mediate rapid negative feedback of the HPA axis, thus limiting plasma cortisol concentrations in response to stressors. In contrast, we saw high levels of plasma total cortisol in these offspring, suggesting an ability to maintain heightened cortisol mobilization, possibly due to their better body condition.

2.4.7 Conclusion and future research

This study was the first to our knowledge to look at changes in GR and MR expression in response to naturally increasing predation risk. In this study, we provide evidence for maternal programming, demonstrating substantial changes in offspring physiology and gene expression in different years of the snowshoe hare cycle. As our gene expression results do not follow a consistent pattern in the two years of hare population decline, we cannot assume that intergenerational programming of prenatal stress effects is occurring in a consistent manner in this population of snowshoe hares. These changes in glucocorticoid receptors and stress phenotypes did not persist in the population from one year to the next, which is what was thought to occur based on laboratory research (reviewed in Cowan et al., 2016). However, in the decline phase we saw: 1. no change in maternal FCM, yet lower offspring hair cortisol, 2. a suite of behavioural changes, 3. increased body condition, 4. increased plasma total cortisol, and 5. decreased GR and MR expression in 2018, and increased expression in 2019, compared to the increase and peak phases. This provides evidence for short-term maternal programming effects, yet no evidence of consistent inheritance in snowshoe hare in the decline phase. We remain unsure as to the mechanisms responsible for the prolonged low phase of the snowshoe hare cycle.

It remains to be investigated if the changes we are seeing in gene expression in this study have epigenetic origins in DNA methylation. High prenatal stress has been found to have dose-dependent effects on the neurophysiology and behaviour of offspring through altered methylation patterns (Cao-Lei et al., 2013; McClelland et al., 2011; Meaney et al., 2007; Mychasiuk et al., 2011). Parental signals and pre- and post-natal glucocorticoid exposure influence DNA methylation, which leads to stable changes in gene expression (Kappeler and Meaney, 2010). With the significant gene expression changes we found in this study; investigating DNA methylation as the mechanism for these changes is the clear next step. Determining DNA methylation has been difficult in snowshoe hare, as they are a non-model species, however the snowshoe hare genome has recently been sequenced (by Dr. Sih Lok and Dr. Stephen Scherer, Hospital for Sick Children, Toronto, ON). This now allows us to investigate if these changes in gene expression have a basis in differential DNA methylation in the promoter regions of the GR and MR genes.

2.5 Authorship contributions

This project was conceptualized by Dr. Sophia Lavergne and Dr. Rudy Boonstra, with input from Dr. Patrick McGowan. Dr. Sophia Lavergne conducted the fieldwork and collected the data in 2015 and 2017, developed all protocols and methods in both the field and laboratory and developed and guided all molecular work. I carried out two summers of fieldwork to continue this project into the decline phase, conducted the corresponding laboratory work, interpreted the new combined dataset and wrote this chapter. This fieldwork highly depended on collaborations with Trent University, with the help of Dr. Melanie Boudreau, Jacob Seguin, Lee Scholl and Sam Sonnega (supervised by Dr. Dennis Murray). The success of our fieldwork is indebted to our field assistant Kat Smith, who helped in both 2015 and 2019.

Chapter 3
General Discussion

3 General Discussion

3.1 Environmental change

The boreal forest occupies almost 60% of Canada's landmass, with over 80% occurring in contiguous blocks and providing a range of ecosystem services (Nelson et al., 2014). Snowshoe hare are the keystone boreal forest herbivore species, central to the food web, and their decline or disappearance would affect many predator species, as well as the structure of the plant community (Krebs et al., 2001a; Krebs et al., 2018a). Their 10-year population cycle has proven resilient thus far to natural disturbances, fire, and short term changes in weather and climate, but the question remains; can they withstand the changes associated with human climate warming without collapsing (Krebs et al., 2001a)?

Climate change is expected to alter global precipitation, fire regimes, seasonality and temperatures, especially in northern areas (Nelson et al., 2014). From 1985 to 2016, late winter temperatures in our study area in the Yukon have increased by 2.8ºC (Krebs et al., 2018a). With this increased warming, we may see acute impacts in Canada's boreal forest. We have already seen an increase in insect outbreaks, homogenization of vegetation, and a huge increase in shrub growth, to name a few (Krebs et al., 2018a; Nelson et al., 2014). This increased warming has led to a 20-30% increase in gray willow and a 200-500% increase in dwarf birch in our study area (Grabowski, 2015), which could increase populations of herbivorous small mammals (Boonstra et al., 2018). However, reduced herbivory by snowshoe hare has actually contributed to this 'shrubification' (Krebs et al., 2014b), which could also change prey vigilance behaviour and predator hunting dynamics (Boonstra et al., 2018).

In the 1990s, an outbreak of spruce bark beetle (*Dendroctonus rufipennis*), likely facilitated by climate warming, killed around 30% of mature spruce trees in our study area (Berg et al., 2006). This can further open up the boreal forest to shrub growth, and change ecosystem dynamics (Berg et al., 2006). Another concern for the boreal forest ecosystem is fragmentation, creating

habitat patches. Snowshoe hare living in patches have been found to be extremely susceptible to predation, and do not undergo cyclic fluctuations in abundance (Keith et al., 1993).

If the boreal forest becomes fragmented in the future, as Keith et al. (1993) investigated in Alberta, this could have negative consequences for snowshoe hare populations. Coyotes were the main predator in these patch habitats, as they are cursorial and typically flush their prey (Keith et al., 1993). Dispersers from these patches had extremely low survival, with 96% of deaths in these patches owing to predation and persistence being overall very low. If forest harvesting or agriculture fragments the boreal forest, snowshoe hare cyclic fluctuations will likely cease in certain areas, greatly affecting ecosystem dynamics.

Climate models have poor agreement of future predictions for the boreal cordillera ecozone of the Yukon, as it is a small area and is likely to undergo substantial change in seasonality (Nelson et al., 2014). We may see substantial changes in temperature, rainfall patterns, growing season, and snow cover in these mountainous regions. Snowshoe hare use camouflage to limit predation and undergo a seasonal colour moult. This colour moult is likely linked to photoperiod, and shows limited plasticity to changing conditions (Zimova et al., 2014). Changes in seasonality, snow fall and snow melt patterns can lead to substantial camouflage mismatch between snowshoe hare and their surroundings, leading to increased predation (Peers, 2017; Zimova et al., 2016).

Climate change has been found to dampen and destabilize cyclically fluctuating populations (Ims et al., 2008; Kausrud et al., 2008; Millon et al., 2014). For example, the amplitude of the snowshoe hare cycle in our study area has decreased from 3-4 hares/ha at the peak in the 1970s, to 1-2 hares/ha at the peak in the 2000s (Krebs et al., 2014b). In Europe, we have seen dampening of the population cycles in voles, grouse and insects over the last two decades (reviewed in Ims et al., 2008). Also recently, lemming populations in the Arctic have ceased to cycle likely due to changing climate conditions (Ims et al., 2008; Kausrud et al., 2008), and this has resulted in huge effects on trophically linked species (Schmidt et al., 2012). Unlike snowshoe hare, voles and lemmings rely on the subnivean space for overwinter survival, and demonstrate incredible sensitivity to changing snow conditions (Kausrud et al., 2008). Nevertheless, cyclic species are often keystone species and changes to their cycles can hugely disrupt ecosystems, as well as the persistence and survival of many other species (Millon et al., 2014).

Changes in snow cover can significantly influence predator hunting efficiency on snowshoe hare. Less severe winters and more compact snow can lead to coyotes being more efficient predators of snowshoe hare (Bastille-Rousseau et al., 2017; Murray and Boutin, 1991). Coyotes have high foot loading, i.e. they cannot hunt in deep snow, whereas lynx and snowshoe hare are adapted to those conditions (Murray and Boutin, 1991; O'Donoghue et al., 1998). When conditions are favourable and there is less snow or more compact snow in the winters, the kill rate by coyotes exceeds that of lynx (Murray et al., 1994). However, with higher frequency of warming, we will have softer snow with more sinking depth, which will not benefit any of these species (Stenseth et al., 2004).

We could see drastic changes to the snowshoe hare cycle with increased coyote predation, as they are a generalist predator. At lower latitudes, snowshoe hare populations do not cycle. This is hypothesized to be due to the higher consistent predation rates by generalist predators, who tend to stabilize prey numbers (Korpimaki and Krebs, 1996; Murray, 2000). In the Yukon boreal forests, arctic ground squirrels used to undergo cyclic population dynamics similar to snowshoe hare, as they are an alternative prey for predators (Werner et al., 2015). However, their populations declined to nearly zero in 2000, and they have been locked in a predator pit (Werner et al., 2015; Werner et al., 2016). Arctic ground squirrels are now functionally extinct in the boreal forest in the Yukon, only persisting around human developments and in alpine areas (Werner et al., 2015; Werner et al., 2016). If habitat and snow conditions change greatly, we could see increased predation by generalist predators or the occurrence of a predator pit, which could limit snowshoe hare to low densities and prevent the increase phase of the cycle. The loss of cyclically fluctuating snowshoe hare populations would have dramatic effects on the entire northern boreal forest ecosystem.

3.2 Concluding remarks

Predicting the fate of populations in the face of climate and environmental change requires the integration of multiple trophic levels (Millon et al., 2014; Peers et al., 2014). Our communal effort strives for this in the Yukon. My research illuminates a key area of snowshoe hare population dynamics by investigating non-consumptive predator stress effects and maternal programming on offspring behaviour, physiology and neurophysiology. This research was the

first to our knowledge to link a natural increase in predation risk to fundamental changes in physiology and gene expression of offspring born during this period. My research during the decline phase of the cycle, combined with that of Lavergne (2018) during the increase and peak phases, has resulted in new understanding and evidence of maternal programming in natural systems. Through this research, we now have a more complete picture of the drivers and mechanisms which cause the changes we see over the course of the different phases of the 10-year snowshoe hare cycle. Our research, in combination with present lynx research, should help us gain further insight into the intricacies of multi-level trophic interactions in the boreal forest ecosystem and, by extrapolation, the potential for similar changes in other predator-prey systems.

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Appendices

Appendix I Snowshoe hare primer sequences*

Gene	Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')
NR3C1	Glucocorticoid Receptor, nuclear receptor subfamily 3, group C, member 1	GCCATTTCTGTTCATGGCGTGAGT	GACCCTTGGCAGCGATTCCAATTT
NR3C2	Mineralocorticoid Receptor, nuclear receptor subfamily 3, group C, member 2	AGCAAGACAGTAATCGTTCTGG	AAATCTTGGCTGGACTCACAC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GCTTTTAACTCTGGCAAAGTGG	GGGTGGAATCATACTGGAACAT
PPIA**	Peptidylprolyl isomerase A (cyclophilin A)	CAACACAAACGGCTCCCAGTT	CATGGCTTCCACAATGCTCAT
YWHAZ***	Tyrosine 3/tryptophan 5 - monooxygenase activation protein, zeta polypeptide	GGTCTGGCCCTTAACTTCTCTGTGTTCTA	GCGTGCTGTCTTTGTATGATTCTTCACTT

* *Obtained and adapted from* Lavergne (2018)

** *Modified from* Rai et al. (2010)

** *From* Seol et al. (2011)