

IN FOCUS

The physiology of predator stress in free-ranging prey



A 12-hours-old snowshoe hare born during the stress manipulation experiments. Photo by Jeffery R. Warner.

M.J. Sheriff, C.J. Krebs & R. Boonstra (2009) The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *Journal of Animal Ecology*, **78**, 1249–1258.

Ecologists have only begun to understand the physiological mechanisms underlying individual- and population-level responses of prey- to predator-related stress. Sheriff, Krebs and Boonstra advance this field by providing evidence that predator-induced increases in glucocorticoid concentrations in wild female snowshoe hares (*Lepus americanus*) impact both litter size and offspring condition. They hypothesize that the glucocorticoid-mediated effects on reproduction provides an adaptive benefit: mothers ‘programming’ their offspring to be timid and risk-averse in high-risk environments should increase their survival probability. This research illuminates the connection between stress physiology and population-level changes and demonstrates the surprisingly far-reaching impact of predation risk.

Although a predator’s most obvious community impacts involve their consumption of prey, great progress has also been made in understanding the sublethal consequences of predation. Across a wide array of taxa, predation risk can induce changes in prey behavior and/or morphology that alter an organism’s activity and habitat use, foraging rate, growth, reproduction and other traits (reviewed in Preisser & Bolnick 2008). Although sometimes subtle on an individual level, these changes (‘nonconsumptive effects’) typically affect many more individuals than are directly consumed by predators (‘consumptive effects’). As a result, the population- and community-level consequences of nonconsumptive

effects can equal or exceed that of consumptive effects (Pangle, Peacor & Johannsson 2007; Creel & Christianson 2008; Schmitz 2008). Research in this area has proceeded along a variety of fronts and involved organisms ranging from *Daphnia* (Riessen 1999) to tadpoles (Fraker *et al.* 2009) to elk (Creel, Winnie & Christianson 2009). In particular, increased recognition of the large-scale importance of nonconsumptive effects has dovetailed with rapid progress in understanding the physiological mechanism(s) underlying an organism’s response to predation risk or other stressors. In vertebrates, this ‘stress response’ involves the activation of the hypothalamic–pituitary–adrenal axis and leads to increased glucocorticoid production. As glucocorticoid concentrations rise, organisms enter an ‘emergency state’ involving both

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behavioral and physiological changes (Wingfield *et al.* 1998; Blas *et al.* 2007). These changes increase the organism's chance of survival but might involve a tradeoff between current reproduction and mortality risk (Wingfield & Sapolsky 2003). Interestingly, increased glucocorticoid concentrations may not always act as a causal mechanism: recent research into elk (*Cervus elaphus* L.) failed to detect a similar relationship between glucocorticoids and reduced reproduction (Creel *et al.* 2009). Although glucocorticoid-related changes in reproduction have been explored in a variety of laboratory experiments, Sheriff, Krebs & Boonstra (2009) take this work a step further by demonstrating that predator-related stress in free-ranging snowshoe hares (*Lepus americanus*) increases glucocorticoid concentrations and affects reproduction. Because snowshoe hares have been intensively studied as a model system for mammalian population cycles (Krebs *et al.* 2001a), their research thus provides evidence critical to linking individual-level physiology with community- and ecosystem-level processes (Boonstra *et al.* 1998; Krebs, Boutin & Boonstra 2001b).

Sheriff *et al.*'s findings emerge from two separate avenues, the first a large-scale monitoring survey and the second an experimental manipulation. Over a 3-year period starting at peak hare density and ending in the decline phase, they used a combination of live traps and track counts on two 36-ha grids to assess both predator (lynx and coyote) and hare densities in a boreal forest (for details of the system, see Krebs *et al.* 2001b). During the first and second snowshoe hare litters of each year, they also live-trapped 30 pregnant females and placed them in small individual outdoor pens (in order to protect the mothers from predators) within a large outdoor enclosure until they gave birth. In addition to recording the litter size and each leveret's condition, they also analysed maternal fecal samples taken 30 h following the birth for fecal cortisol metabolites, an indicator of maternal glucocorticoid concentrations. They found that metabolite concentrations decreased sharply between the first and second litters in each of the 3 years, and were inversely correlated with increases in both litter size and leveret condition. Predation risk decreased and food supply increased over this same period, suggesting that the waning of one or both of these potential stressors reduced metabolite concentrations and increased reproductive output. The second, experimental, part of their research involved capturing near-birth females, taking baseline measurements of the fecal cortisol metabolites, and then assigning them to large pens in the presence or absence of predator risk (a trained dog) until they gave birth. Litter size and leveret condition was then assessed, along with post-birth metabolite concentrations. Although litter size did not differ between the two groups, they found that stressed dams gave birth to lighter and smaller offspring and that maternal fecal cortisol metabolites were negatively correlated with offspring condition. In combination with the results from the large-scale survey, they conclude that predation risk (or another stressor) that increases glucocorticoid concentrations will reduce reproductive output.

In addition to making a solid case for the linkage between predator risk, increased glucocorticoid concentrations and reduced reproduction in free-ranging hares, Sheriff *et al.* evaluate several hypotheses for why such an apparently maladaptive stress response might occur. From a fitness perspective, trading off current reproduction for increased survival is adaptive only if organisms that do so are likely to survive to the next breeding period (Lima 1998). This is not the case for snowshoe hares, however; over 70% of each breeding population is yearlings, and females have only a 42% chance of surviving the period between their first and second litters (Sheriff *et al.* 2009). Hares that delay reproduction are thus unlikely to survive long enough to benefit fitness-wise from their choice. As a result, a tradeoff between reproduction and survival seems unlikely to explain the observed results. The short life and hard times of adult hares also argues against the recently suggested 'maternal matching' hypothesis (Love & Williams 2008). This hypothesis emerges from research in which stressed female European starlings raising glucocorticoid-injected eggs had both higher survival rates and fledged higher-quality (but fewer) offspring than did stressed mothers with control eggs. Although ingenious, this hypothesis' invocation of future reproductive events as a means of compensating for the stressed mother's reduced brood size makes it unlikely to occur in snowshoe hares.

Assuming that the observed hormonal changes are in fact adaptive, Sheriff *et al.* ultimately favor the 'maternal programming' hypothesis. This posits that mothers can increase the likelihood of their offspring responding appropriately to stressor(s) present in their natal environment. Leverets born to predator-stressed mothers would thus be more likely to exhibit anti-predator behavior and survive to adulthood. This explanation draws on several lines of evidence regarding the hypothalamic-pituitary-adrenal axis, including the fact that it can be permanently altered in the pre- and perinatal life stages (Meaney, Szyf & Seckl 2007), that glucocorticoid concentrations and stress responses are higher in the offspring of stressed mothers (Hayward & Wingfield 2004) and that its activation can increase fearfulness, vigilance, dispersal ability and, ultimately, survival (Meylan & Clobert 2005; Cabezas *et al.* 2007). Snowshoe hares do not shelter their young in burrows, and juvenile mortality rates are extremely high (O'Donoghue 1994); in such a situation, females that respond to stress by producing a smaller number of more fearful (and thus risk-averse) offspring might actually increase their fitness. This provides a plausible explanation for the paper's results, and an adaptive rationale for why maternal stress in snowshoe hares might yield fewer, smaller and more timid offspring.

The recent advances in snowshoe hare research highlight the fact that our understanding of the link between stress physiology and population-level processes is far more developed for vertebrates than for many other groups. At present, similar research on invertebrates has largely addressed the physiological underpinnings of growth-predation risk tradeoffs (Slos & Stoks 2008; Slos, Meester & Stoks 2009). While many invertebrates show a wide range of both

behavioral and physiological responses to predation risk (e.g. Beckerman, Wieski & Baird 2007), ecologists have just begun to explore the hormonal/chemical pathways that underlay morphological and life-history changes. Understanding the reproductive consequences of such changes is made even more challenging by the fact that many well-studied invertebrate taxa (e.g. insects such as damselflies, mayflies, butterflies and beetles) possess complex life cycles in which the immature stages are relatively sessile and occupy different habitats than do the wide-ranging adults. By laying out the protocols necessary to link individual- and population-level processes in a vertebrate system, Sheriff *et al.*'s paper should also spur ecologists to begin thinking about and testing similar hypotheses in non-vertebrate communities.

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The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares

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Summary

1. Prey responses to high predation risk can be morphological or behavioural and ultimately come at the cost of survival, growth, body condition, or reproduction. These sub-lethal predator effects have been shown to be mediated by physiological stress. We tested the hypothesis that elevated glucocorticoid concentrations directly cause a decline in reproduction in individual free-ranging female snowshoe hares, *Lepus americanus*. We measured the cortisol concentration from each dam (using a faecal analysis enzyme immunoassay) and her reproductive output (litter size, offspring birth mass, offspring right hind foot (RHF) length) 30 h after birth.

2. In a natural monitoring study, we monitored hares during the first and second litter from the population peak (2006) to the second year of the decline (2008). We found that faecal cortisol metabolite (FCM) concentration in dams decreased 52% from the first to the second litter. From the first to the second litter, litter size increased 122%, offspring body mass increased 130%, and offspring RHF length increased 112%. Dam FCM concentrations were inversely related to litter size ($r^2 = 0.19$), to offspring birth mass ($r^2 = 0.32$), and to offspring RHF length ($r^2 = 0.64$).

3. In an experimental manipulation, we assigned wild-caught, pregnant hares to a control and a stressed group and held them in pens. Hares in the stressed group were exposed to a dog 1–2 min every other day before parturition to simulate high predation risk. At parturition, unsuccessful-stressed dams (those that failed to give birth to live young) and stressed dams had 837% and 214%, respectively, higher FCM concentrations than control dams. Of those females that gave birth, litter size was similar between control and stressed dams. However, offspring from stressed dams were 37% lighter and 16% smaller than offspring from control dams. Increasing FCM concentration in dams caused the decline of offspring body mass ($r^2 = 0.57$) and RHF ($r^2 = 0.52$).

4. This is the first study in a free-ranging population of mammals to show that elevated, predator-induced, glucocorticoid concentrations in individual dams caused a decline in their reproductive output measured both by number and quality of offspring. Thus, we provide evidence that any stressor, not just predation, which increases glucocorticoid concentrations will result in a decrease in reproductive output.

Key-words: 10-year snowshoe hare cycle, glucocorticoid metabolites, maternal effects, natural populations, predation risk

Introduction

Predation is a central organizing agent shaping population and community processes (Krebs *et al.* 2001a; Schmitz 2008). Traditionally, ecologists have focused on the direct effects of predation – the killing of prey (Paine 1966; Taylor 1984; Krebs *et al.* 1995). However, predators also have significant

indirect effects on prey populations (see reviews by Lima 1998; Creel & Christianson 2008) and these effects can be as great as their direct effects (Schmitz, Beckerman & O'Brien 1997; Nelson, Matthews & Rosenheim 2004; Preisser, Bolnick & Benard 2005; Pangle, Peacor & Johannsson 2007). Prey responses to the high risk of predation can be morphological such as changes in secondary sexual characteristics and anti-predator defences (Tollrian & Harvell 1999; Day & Young 2004; Vamosi & Schluter 2004) or behavioural such as

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changes in preferred habitats, in vigilance, and in foraging (Hik 1995; Lima & Bednekoff 1999; Childress & Lung 2003; Armitage 2004; Creel *et al.* 2005; Winnie & Creel 2007). These responses ultimately come at the cost of survival, growth, body condition, or reproduction (Hik 1995; Boonstra *et al.* 1998; Krebs *et al.* 2001a; Olaf & Halle 2004; Bian, Wu & Liu 2005; Hodges, Stefan & Gillis 1999).

The indirect effects of predators act through physiological processes. One of the most conserved processes in vertebrates is the 'stress response', defined here as the set of neural and endocrine responses that help restore homeostasis (Sapolsky 1987). Central to the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent secretion of glucocorticoids (GC), lasting several minutes to hours (Sapolsky 1992; Wingfield & Romero 2001). A stressor may be any environmental perturbation that disrupts homeostasis, such as harsh weather, habitat changes, anthropogenic disturbances, decreased food availability, and predation attempts (Sapolsky 1987). The presence of short-term elevated GC concentrations facilitates escape from life-threatening situations (Wingfield *et al.* 1998). However, chronic activation of the HPA axis may trade off future reproduction for present survival (Boonstra & Singleton 1993; Boonstra *et al.* 1998; Sapolsky, Romero & Munck 2000; Romero & Wikelski 2001; Wingfield & Romero 2001).

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

Snowshoe hares (*Lepus americanus*) are an ideal species to study the effects of GCs on reproduction. Snowshoe hares undergo a regular cyclic fluctuation, with 8–10 years between peak densities (Keith 1963; Krebs *et al.* 1986). As hare populations increase, so do that of their predators, but with a lag of 1–2 years. During the hare population decline, predators are the direct cause of up to 83% of hare deaths (Boutin *et al.* 1986; Krebs *et al.* 1995). Hare reproduction also cycles, with maximum rates occurring during the early increase phase (when predator numbers are lowest), but then progressively declining to a nadir during the decline (when predator numbers are at their peak), (Cary & Keith 1979; O'Donoghue

& Krebs 1992; O'Donoghue *et al.* 1997; Stefan & Krebs 2001). Predators could be the indirect cause of this decline, with the inhibition of the gonadal axis being mediated by the stress of high predation risk through the activation of the HPA axis. Boonstra *et al.* (1998) showed that plasma cortisol concentrations (the major GC in snowshoe hares) fluctuated with the risk of predation, such that hares experiencing a greater risk of predation had higher plasma cortisol. They proposed that chronic stress, as measured by elevated cortisol concentrations, caused the marked deterioration of reproduction during the decline phase.

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

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

Materials and methods

SNOWSHOE HARE BIOLOGY

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

ANIMAL TRAPPING

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

Canadian Council for Animal Care. Female snowshoe hares were live-trapped in the Shakwak Trench east of Kluane Lake, Yukon Territory (61°N, 138°W) using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI, USA). The traps were set at 22.00 h and checked at 06.00 h and thus hares could only be in the traps for a maximum of 8 h. This is relevant as the lag between the production of cortisol in the body and the appearance of its metabolites in the faeces is between 8–12 h (Sheriff *et al.* 2009). Therefore, the cortisol metabolites in the faeces represent non-observer induced measures of stress.

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

NATURAL MONITORING

Population densities of both the hares and the predators were measured, initially as part of the Kluane Boreal Forest Ecosystem Project (Krebs, Boutin & Boonstra 2001b) and thereafter as part of a monitoring study; here we present the data from 1994 to 2008 (two complete cycles). Snowshoe hare densities were estimated on two 36-ha grids. Live-traps were pre-baited with alfalfa cubes for 3–5 days before being set. Trapping sessions consisted of 2–3 nights of trapping within a 5-day period. Trapping did not occur on nights that dropped below -20 °C. Population density was estimated with the program CAPTURE (Otis *et al.* 1978) and the Jolly-Seber full model, as in previous studies (e.g. Krebs *et al.* 1995).

Avian and mammalian predator populations fluctuate in synchrony with the hare cycle (Doyle & Smith 2001; O'Donoghue *et al.* 2001; Rohner, Doyle & Smith 2001). An index of the fluctuations in predator populations were obtained by using evidence from lynx and coyote data, as these are reflective of all other predators, including avian predators. We counted lynx and coyote tracks each winter (October through April) along a 25-km transect that traversed our study area, on days after fresh snowfalls while tracks were distinguishable. Track counts for lynx and coyotes are highly correlated to their population density in this valley (lynx: $r^2 = 0.95$, coyote: $r^2 = 0.88$) and thus give a reliable estimate of changes in predator density (O'Donoghue *et al.* 1997).

To standardize measurements across years, we monitored females' cortisol concentration and reproduction during the first and second litters. To estimate reproductive output, we live-trapped hares one-week on either side of the average parturition dates (first litter May 25; second litter June 30; mean parturition dates estimated from O'Donoghue & Krebs 1992 and Stefan & Krebs 2001). Pregnant females ($n = 30$) were transferred to the outdoor enclosure and placed in a $60 \times 60 \times 120$ cm chicken wire maternity cage until parturition (for details see O'Donoghue & Krebs 1992). Hares were

held in the maternity cages for an average of 3 days and a maximum of 6 days. Hares were fed *ad libitum* with standard rabbit chow (Unifeed, Okotoks, AB, Canada; Unifeed Ltd Cat. #19-2103, 18% protein, crude fat 2%, crude fibre 18%) and apples, supplemented daily with natural browse (small branches with leaves and bark from *Salix spp.*) and water *ad libitum*.

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

EXPERIMENTAL MANIPULATION

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

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

Thirty hours after parturition, dams were live-trapped and a faecal sample taken. Reproductive measurements on the neonates were taken as above. Families were kept in the pens for an additional 28 days for a separate experiment. At the end of experimentation, all hares were released back to the site of their capture.

FAECAL CORTISOL METABOLITE ANALYSIS

We used an enzyme immunoassay (EIA) to measure faecal cortisol metabolite (FCM) concentration, validated specifically for snowshoe

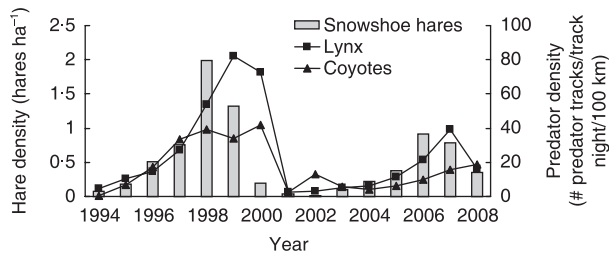


Fig. 1. Snowshoe hare, lynx, and coyote population density in the south-western Yukon, Canada from 1994 to 2008.

hares. Faecal samples were collected from underneath the live-trap on the morning of trapping, a maximum of 8 h after the time of setting. Previously we showed (Sheriff *et al.* 2009) that there is an 8–12 h lag between cortisol production in the snowshoe hare and the appearance of its metabolites in the faeces. Thus, our samples provided an integrated measure of circulating cortisol before the stress of being captured. Samples were stored at -80°C within 1 h of collection at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at -80°C until analysed.

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

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

STATISTICAL ANALYSIS

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

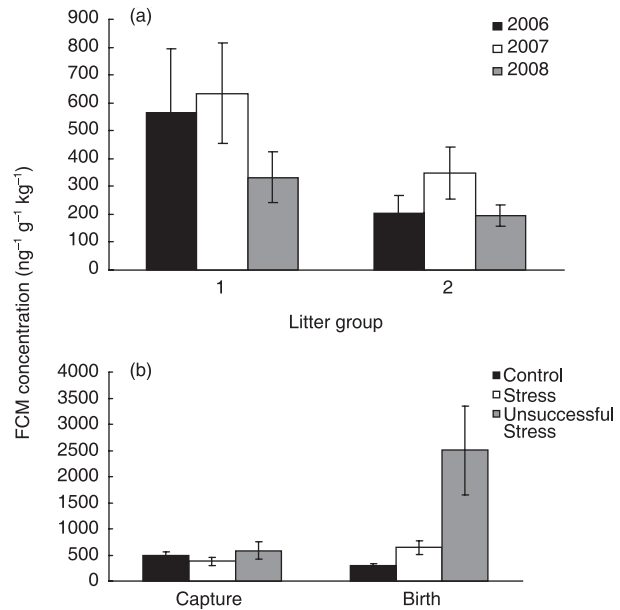


Fig. 2. FCM concentrations (means \pm SE) of female snowshoe hares from the two studies. (a) The natural monitoring study 30 h after birth in litter 1 and 2 across 3 years. Dams from litter 1 (pooled years) had elevated FCM concentrations compared with those from litter 2 (pooled years) ($P < 0.005$). (b) The experimental manipulation at the time of field capture and 30 h after birth. There was no difference in FCM concentrations at capture ($P > 0.05$); however at birth, control dams had reduced FCM concentrations compared with stressed successful dams and with stressed unsuccessful dams ($P < 0.0001$).

Results

NATURAL MONITORING

Population density

The snowshoe hare population reached a peak of 0.92 hares ha^{-1} in 2006 and declined to 0.79 and 0.35 hares ha^{-1} in 2007 and 2008, respectively (Fig. 1). This population peak was considerably lower than the previous peak in 1998 of 1.98 hares ha^{-1} . The track index of mammalian predator populations (lynx and coyotes) was highest in 2007 with approximately 56 predator tracks counted per track night over a 100-km transect, compared with a previous peak of 117 tracks in 1999 (Fig. 1). The track index before the peak, 2006, and just after it, 2008, was approximately 31 and 35 predator tracks counted per track night over a 100-km transect, respectively. Thus, predators peaked 1 year after the hare peak.

Faecal cortisol metabolite concentration

To test for differences in FCM concentrations, we ran a two-way ANOVA (litter group \times year). We found an effect of litter group ($F_{1,24} = 11.70$, $P < 0.005$) but no effect of year ($F_{2,24} = 1.42$, $P > 0.05$) or interaction between litter group and year ($F_{2,24} = 2.36$, $P > 0.05$; Fig. 2a). Faecal cortisol metabolite concentrations in dams decreased 52% from the first litter (509.35 ± 107.22 ng g^{-1} kg^{-1} , $n = 15$) to the second

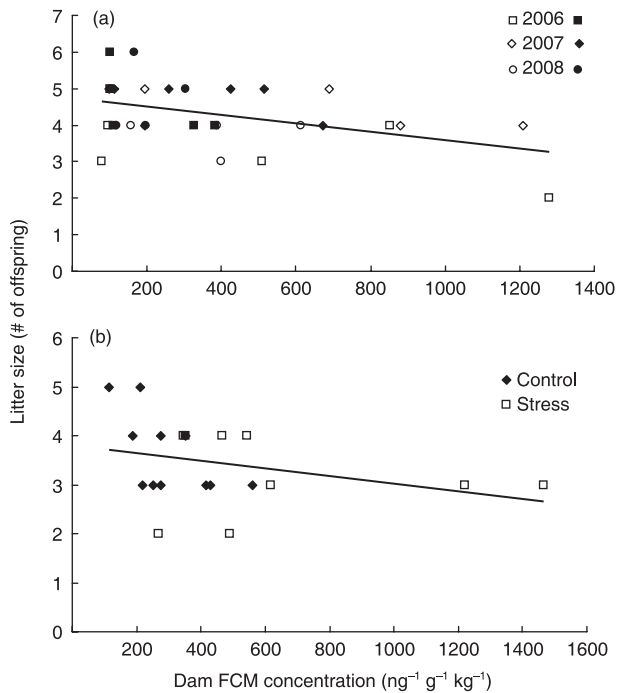


Fig. 3. FCM concentration in dams and their litter size. (a) In the natural monitoring study, first litters ($n = 15$, open symbols) were smaller than second litters ($n = 15$, closed symbols; $P < 0.01$) ($y = -0.0012x + 4.75$, $r^2 = 0.19$; $P < 0.05$). Each data point represents a single dam from 2006 ($n = 10$), 2007 ($n = 11$), and 2008 ($n = 9$). (b) In the experimental manipulation, control dams ($n = 11$) and stressed dams ($n = 9$) had similar litter sizes ($P > 0.05$) ($y = -0.0008x + 3.80$, $r^2 = 0.10$; $P < 0.05$). Each data point represents a single dam.

litter (248.72 ± 45.56 ng g⁻¹ kg⁻¹, $n = 15$). Faecal cortisol metabolite concentrations were similar in 2006 (383.54 ± 121.15 ng g⁻¹ kg⁻¹, $n = 10$), in 2007 (476.62 ± 107.48 ng g⁻¹ kg⁻¹, $n = 11$), and in 2008 (263.92 ± 56.30 ng g⁻¹ kg⁻¹, $n = 9$).

Reproduction

To test for differences in reproductive measures, we ran a two-way ANCOVA (litter group \times year) and included FCM concentration as a continuous covariate. For litter size, we found an effect of litter group ($F_{1,23} = 8.24$, $P < 0.01$) and FCM concentration ($F_{1,23} = 4.47$, $P < 0.05$), but no effect of year ($F_{2,23} = 3.37$, $P > 0.05$) or interaction between year and litter group ($F_{2,23} = 1.62$, $P > 0.05$). Litter size was 19% smaller in the first litter (3.87 ± 0.22 young) compared with the second litter (4.73 ± 0.18 young) and litter size was negatively correlated to FCM concentrations in dams (Fig. 3a).

For offspring body mass, we found an effect of litter group ($F_{1,23} = 13.55$, $P < 0.005$) and FCM concentration ($F_{1,23} = 4.47$, $P < 0.05$), but no effect of year ($F_{2,23} = 0.93$, $P > 0.05$) or interaction between year and litter group ($F_{2,23} = 0.56$, $P > 0.05$). Offspring had a 24% lower body mass in the first litter (52.26 ± 2.98 g) compared with the second litter (68.03 ± 2.80 g) and offspring body mass was negatively correlated to FCM concentrations in dams (Fig. 4a).

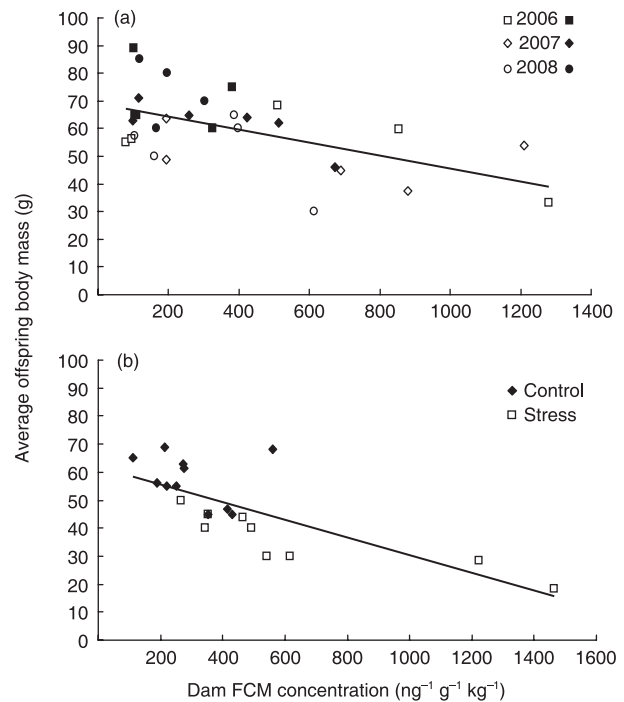


Fig. 4. FCM concentration in dams and the average body mass of their offspring 30 h after birth. (a) In the natural monitoring study, first litter offspring ($n = 15$, open symbols) were lighter than second litter offspring ($n = 15$, closed symbols; $P < 0.005$) ($y = -0.0234x + 69.14$, $r^2 = 0.32$; $P < 0.05$). Each data point represents a single dam, from 2006 ($n = 10$), 2007 ($n = 11$), and 2008 ($n = 9$). (b) In the experimental manipulation, offspring from control dams ($n = 11$) were heavier than those from stressed dams ($n = 9$; $P < 0.005$) ($y = -0.032x + 62.11$, $r^2 = 0.57$; $P < 0.005$). Each data point represents a single dam.

For offspring RHF length, we found an effect of litter group ($F_{1,15} = 10.94$, $P < 0.005$), year ($F_{1,15} = 8.38$, $P < 0.05$), and FCM concentration ($F_{1,15} = 18.69$, $P < 0.001$), but no interaction between litter group and year ($F_{2,15} = 0.014$, $P > 0.05$). Offspring were 11% smaller in the first litter (33.67 ± 1.22 mm) than in the second litter (37.61 ± 0.83 mm), and 10% smaller in 2007 (34.06 ± 1.04 mm) than in 2008 (37.56 ± 1.14 mm). We did not measure RHF length in 2006. Offspring RHF length was negatively correlated to FCM concentrations in dams (Fig. 5a).

EXPERIMENTAL MANIPULATION

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

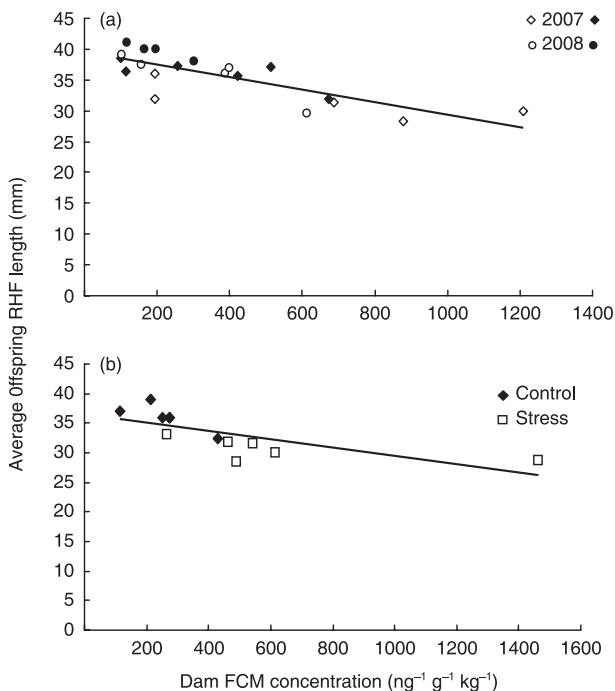


Fig. 5. FCM concentration in dams and the average RHF length of their offspring 30 h after birth. (a) In the natural monitoring study, first litter offspring ($n = 10$, open symbols) had smaller RHF length than second litter offspring ($n = 10$, closed symbols; $P < 0.005$) ($y = -0.0101x + 39.511$, $r^2 = 0.64$; $P < 0.001$). Each data point represents a single dam from 2007 ($n = 11$), and 2008 ($n = 9$). (b) In the experimental manipulation, offspring from control dams ($n = 5$) had a smaller RHF length than those from stressed dams ($n = 6$; $P < 0.01$) ($y = -0.007x + 36.489$, $r^2 = 0.52$; $P < 0.05$). Each data point represents a single dam.

give birth to viable young. A single control hare did not give birth and she was not included in any of the analysis.

Faecal cortisol metabolite concentration

To test for differences in FCM concentration at the time of capture and 30 h after birth we ran a one-way ANOVA. We found, at the time of capture, there was no difference in FCM concentrations between control dams, stressed dams, and unsuccessful stressed dams ($F_{2,22} = 0.91$, $P > 0.05$; Fig. 2b). At the time of birth, FCM concentrations were significantly different in the three groups ($F_{2,22} = 19.20$, $P < 0.0001$; Fig. 2b). Control dams had FCM concentrations 54% and 89% lower than stressed dams (Tukey's HSD post-hoc $P < 0.05$) and unsuccessful stressed dams (Tukey's HSD post-hoc $P < 0.0005$), respectively. Stressed dams had FCM concentrations 75% lower than unsuccessful stressed dams (Tukey's HSD post-hoc $P < 0.005$).

Reproduction

To test for differences in reproductive measures we ran a one-way ANCOVA, including FCM concentration as a continuous covariate. For litter size we found no effect of

treatment ($F_{1,17} = 0.06$, $P > 0.05$) or FCM concentration ($F_{1,17} = 1.70$, $P > 0.05$). Control dams had a similar litter size (3.64 ± 0.24 young) compared with stressed dams (3.22 ± 0.28 young; Fig. 3b).

For offspring body mass we found an effect of treatment ($F_{1,17} = 11.68$, $P < 0.005$) and FCM concentration ($F_{1,17} = 13.96$, $P < 0.005$). Control dams gave birth to offspring with a 58% greater body mass (57.23 ± 2.69 g) compared with stressed dams (36.16 ± 3.35 g). Increasing FCM concentrations in dams were associated with a reduced offspring body mass (Fig. 4b).

For RHF length, we found an effect of treatment ($F_{1,9} = 10.59$, $P < 0.01$) and FCM concentration ($F_{1,9} = 5.29$, $P < 0.05$). Control dams gave birth to offspring with an 18% greater RHF length (36.08 ± 0.86 mm) compared with stressed dams (30.57 ± 0.74 mm). Increasing FCM concentrations in dams were associated with a reduced offspring RHF length (Fig. 5b).

Discussion

The snowshoe hare population in our study area peaked in 2006 and the predators peaked in 2007 (Fig. 1). In the natural monitoring study, we found that female FCM concentrations were higher at parturition of their first litter compared with that at their second (Fig. 2a). However, we did not find differences in female FCM concentrations between years. In the experimental manipulation study, we found that unsuccessful stressed dams (those that did not give birth to viable young) had the highest FCM concentrations followed by stressed dams and then control dams (Fig. 2b). In both studies, dams with higher FCM concentrations had lower reproductive fitness in terms of the number and quality of young (Fig. 3–5).

FAECAL CORTISOL METABOLITE CONCENTRATIONS IN DAMS

Many factors have been shown to affect stress levels in wild animals including density and social status, parasitism, food, and the risk of predation (Boonstra *et al.* 1998; Creel 2001; Chapman, Saj & Snaith 2007). It has long been recognized that high population densities could disrupt spacing behaviour and increase agonistic interactions and competition leading to an increase in stress and ultimately a decline in reproduction (Christian 1980). More recently, it has been shown that not only density but social status can influence GC concentrations and disrupt breeding (Creel 2001; Young *et al.* 2006). However, there is little evidence of this in hares. Boonstra *et al.* (1998) found that hares were less stressed living in experimentally fed populations whose densities were 4 to 13 times those of controls. Although, hares have been shown to display dominance hierarchies in pens and at feeding areas in the wild (Graf 1985), they are not territorial and have broadly overlapping home ranges (Boutin 1984). Furthermore, our results show that hares in 2006 (peak population) were not different from hares in 2008 (second year of the decline; Fig. 1 and 2a).

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

Food and predation are two of the greatest factors affecting animal populations and these have been shown to have interactive synergistic effects (Krebs *et al.* 1995, 2001a). In snowshoe hares, the change in FCM concentrations between litters (Fig. 2a) could be a result of first, an increase in food availability during the growing season, and second, a decrease in predation risk. The first litter occurs during the late winter–early spring when the winter snowpack is in the process of melting and before the flush of new vegetation, while the second litter occurs during late spring–early summer when new vegetative growth is nearing its peak (Sinclair, Krebs & Smith 1982). This change in quality and quantity of food could explain the differences in FCM concentrations. Reduced food intake has been found to cause an increase in cortisol levels in mammals and birds (Harris *et al.* 1994; Kitasysky *et al.* 1999; Ortiz, Wade & Ortiz 2001). Second, the difference between the litter groups could be due to the decrease in the risk of predation from the first litter to the second. Boutin *et al.* (1986) found that predation rates decreased from winter to summer. Thus, the risk of predation should also decrease at this time. Since food quantity and quality also increase from the first litter to the second litter, an increase in food availability may also allow hares to forage in a less risk-prone manner (Hik 1995; Murray 2002). Likely, the effects of the increase in food availability and decrease in predation are not mutually exclusive and a combination of the two could explain the decline in FCM concentration from the first to the second litter.

We found that FCM concentrations were similar in the hare peak (2006) and decline (2007 and 2008; Fig. 2a), but predicted that as the risk of predation increased FCM concentrations in dams would also increase. Part of the explanation may be that the females in our study were a high-quality subset of females from the hare population, all giving birth within one week of the estimated parturition date. Our experimental results showed that females that gave birth to non-viable young, (either stillborn or aborted), had high FCM concentrations compared with females that gave birth (Fig. 2b). Furthermore, the number of females that give birth to non-viable young increased during the decline phase (Stefan & Krebs 2001). Had we assessed FCM concentrations from all females within the population, not just the successful ones, we may have seen yearly differences.

In our experimental manipulation, stressed hares had elevated FCM concentrations compared with control hares

(FCM concentrations were within the range found in the natural monitoring experiment; Fig. 2b). The extreme sensitivity of hares to predation risk is shown by the lack of habituation to repeated dog exposures. Within treatments, there was also considerable individual variation in FCM concentration (Fig. 2b). This suggests that individuals naturally differed in their physiological response and ability to cope with stressors. This is consistent with the results found by Pride (2005) for ringtailed lemurs, *Lemur catta*, and by Cabezas *et al.* (2007) for European wild rabbits, *Oryctolagus cuniculus*.

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

REPRODUCTION

In both the natural monitoring and experimental manipulation studies, we found that higher FCM concentrations in dams were associated with a decline in reproductive indices (Figs 3–5) and extremely high FCM concentrations were associated with non-viable births, either abortions or stillborn litters (Fig. 2b). Chronic exposure to elevated GC concentrations can have deleterious physiological consequences to reproduction by decreasing the amount of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) produced in the body (Ferin 1999; Owen, Andrews & Matthews 2005). Davis & Meyer (1973) found that in snowshoe hares, seasonal variation in gonadotropins paralleled seasonal changes in reproduction, and that there was a sharp decline in gonadotropin levels which coincided with a sharp decline in reproductive rates. We suggest that predator-induced changes in GC concentrations may be responsible for the changes seen in gonadotropin levels and ultimately the changes in reproduction.

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

A classic trade-off of reproduction for survival is easily understood in predator–prey relationships. As the risk of predation increases, prey species alter their behaviour to increase survival (Hik 1995; Lima 1998) and these changes

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

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

The adaptive advantage of maternal programming is easy to conceptualize. If a pregnant female is living in an environment where the risk of predation is high, it is beneficial that she transmit anti-predator behaviours to her offspring. It is logical that this signal is transmitted through the HPA axis, as it is not only responsible for the stress response but it is also associated with certain anti-predator behaviours such as fearfulness, anxiety, and vigilance (Meaney 2001; Seckl 2004). An increase in prenatal GC concentrations has also been shown to influence offspring dispersal and survival (Silverin 1997; Meylan *et al.* 2002; Meylan & Clobert 2005). Many studies have shown that the HPA axis is highly susceptible to permanent programming during early life development (e.g. Francis & Meaney 1999; Matthews 2002; Seckl 2004; Owen *et al.* 2005) and prenatally stressed offspring have been shown

to have lower glucocorticoid and mineralocorticoid receptor expression in the hippocampus leading to elevated GC concentrations (Welberg & Seckl 2001; Welberg, Seckl & Holmes 2001) as well as higher reactivity of the HPA axis to stressors (Hayward & Wingfield 2004). For snowshoe hares, maternal programming of offspring through elevated GC concentrations could be highly adaptive. Hares give birth to precocial young without a protective nest or burrow and do not stay with them after birth, returning only briefly each night to nurse (Keith & Windberg 1978). O'Donoghue (1994) showed that the proximate cause of mortality for offspring was predation and that 70% of juvenile mortality occurred within the first 5 days after birth, and 51% of litters had no survivors after 14 days. Thus, young are highly vulnerable to predation. As the risk of predation increases, the elevated GC concentrations of the dam could prenatally program the HPA axis of the offspring such that they are born with a greater stress response, increased vigilance and anxiety-like behaviours, and increased dispersal rates. Thus, elevated GC concentrations during times of high predation risk may cause a decline in reproductive output, but may ultimately increase fitness by promoting juvenile survival.

Here we have shown that increases in GC concentrations in individual dams are associated with a decline in reproduction. For snowshoe hares, these increased GC concentrations are likely explained by both food and predation; however, this is not the case for all species. GC concentrations can also be influenced by density and social status, parasitism, weather, and human activity among other things (Christian 1980; Wasser *et al.* 1997; Creel 2001; Romero & Wikelski 2001; Chapman *et al.* 2007). Since we found that elevated GC concentrations, not simply predation, are associated with a decline in reproduction, the type of stressor is essentially irrelevant. Our study has broad implications to all physiologically stressful situations.

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