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Michael J. Sheriff, Charles J. Krebs & Rudy Boonstra

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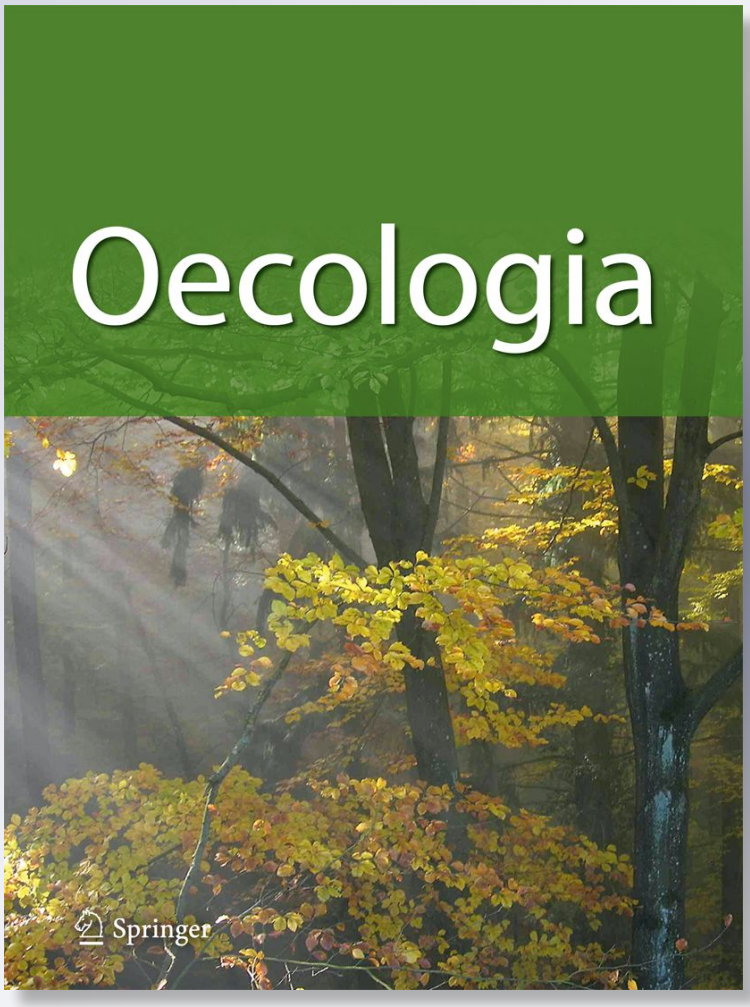

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POPULATION ECOLOGY - ORIGINAL PAPER

From process to pattern: how fluctuating predation risk impacts the stress axis of snowshoe hares during the 10-year cycle

Michael J. Sheriff · Charles J. Krebs · Rudy Boonstra

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Abstract Predation is a central organizing process affecting populations and communities. Traditionally, ecologists have focused on the direct effects of predation—the killing of prey. However, predators also have significant sublethal effects on prey populations. We investigated how fluctuating predation risk affected the stress physiology of a cyclic population of snowshoe hares (*Lepus americanus*) in the Yukon, finding that they are extremely sensitive to the fluctuating risk of predation. In years of high predator numbers, hares had greater plasma cortisol levels at capture, greater fecal cortisol metabolite levels, a greater plasma cortisol response to a hormone challenge, a greater ability to mobilize energy and poorer body condition. These indices of stress had the same pattern within years, during the winter and over the breeding season when the hare:lynx ratio was lowest and the food availability the worst. Previously we have shown that predator-induced maternal stress lowers reproduction and compromises offspring's stress axis. We propose that predator-induced changes in hare stress physiology affect their demography through negative impacts on reproduction and that the low phase of cyclic populations may be the result of predator-induced maternal stress reducing the fitness of progeny. The hare population cycle has far reaching ramifications on predators, alternate prey, and vegetation. Thus, predation is the predominant organizing process for much of the North American boreal forest community, with its indirect signature—stress in hares—producing a pattern of hormonal changes that provides a sensitive reflection of fluctuating predator pressure that may have long-term demographic consequences.

Keywords Cortisol · Population cycles · Low phase · Maternal effects · Sublethal effects

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M. J. Sheriff · R. Boonstra
Centre for the Neurobiology of Stress,
University of Toronto Scarborough,
1265 Military Trail, Toronto, ON M1C 1A4, Canada
e-mail: boonstra@utsc.utoronto.ca

M. J. Sheriff (✉) · C. J. Krebs
Department of Zoology,
University of British Columbia,
6270 University Boulevard,
Vancouver, BC V6T 1Z4, Canada
e-mail: mjsheriff@alaska.edu

C. J. Krebs
e-mail: krebs@zoology.ubc.ca

Introduction

Predators can limit animal populations, a fact that can have profound implications not only for their prey populations but for entire ecosystems (Krebs et al. 2001a; Schmitz 2008). Predators can affect prey populations both directly, through the killing of prey (Paine 1966; Taylor 1984; Krebs et al. 1995), and through sublethal changes in behavior, morphology, and physiology (Hik 1995; Lima 1998; Lima and Bednekoff 1999; Tollrian and Harvell 1999; Childress and Lung 2003; Armitage 2004; Vamosi and Schluter 2004; Creel et al. 2005; Winnie and Creel 2007). The sublethal effects of predation can be as great as the direct effects (Schmitz et al. 1997; Nelson et al. 2004; Preisser et al. 2005; Pangle et al. 2007) and prey responses ultimately come at the cost of survival, growth, body condition, or reproduction (Boonstra et al. 1998a, b; Hodges et al. 1999, 2006; Olaf and Halle 2004; Bian et al. 2005; Sheriff et al. 2009a).

The physiological response of prey to the immediate threat of predation is the 'stress response', defined here as the set of neural and endocrine responses that help the prey to respond to the threat and then restore homeostasis. It is a highly conserved response among vertebrates (Sapolsky et al. 2000). Central to this response is the activation of the hypothalamic–pituitary–adrenal (HPA) axis and the resultant secretion of glucocorticoids (GC), lasting several minutes to hours (Sapolsky 1992; Wingfield and Romero 2001). It is designed to deal with acute perturbations, temporarily minimizing or shutting down non-essential functions such as the immune response and reproduction, and is essentially catabolic in nature, mobilizing energy and stimulating hepatic gluconeogenesis (Munck et al. 1984; Miller and Tyrrell 1995; Wingfield et al. 1998). Its short-term activation facilitates escape from life-threatening situations. However, when activated chronically, the stress response can be severely deleterious, affecting long-term survival and fitness. Here, we carry out a field study to examine how fluctuating predation risk affects GC levels in snowshoe hares.

Snowshoe hares (*Lepus americanus*) are an ideal species to investigate the impact of predation on GC levels. They undergo a regular cyclic fluctuation, with 8–10 years between peak densities (Keith 1963; Krebs et al. 1986). As hare populations increase so do those of their predators, but with a lag of 1–2 years. During the hare population decline, predators are the direct cause of nearly all hare deaths (Hodges et al. 2001). Following the decline phase, hare populations remain low for 2–5 years even though predator numbers are low and vegetation ample (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 highest) (Cary and Keith 1979; O'Donoghue and Krebs 1992; O'Donoghue et al. 1997; Stefan and Krebs 2001). Hare populations do not increase until the late low phase when their reproduction has recovered.

Predators could be ultimately responsible for this reproductive decline and the lag in its recovery. However, correlation is not causation and the main alternative explanations could involve the direct effects of fluctuating hare density acting through intraspecific competition or, alternatively, that food quantity or quality fluctuate with hare density and drive changes in the latter. Neither explanation is sufficient given previous experimental evidence (Boonstra et al. 1998a; Hodges et al. 2006) based on a large scale factorial design (Krebs et al. 2001b). Hares in winter show no significant spacing behavior that is likely to be affected by density (they are not territorial and their home ranges overlap broadly; Boutin 1984). In addition, when hare density was experimentally increased by 4–13 times above control levels through food addition and predator exclusion, hares on the experimental grids were less, not more, stressed than those on controls (Boonstra et al. 1998a). Food addition by itself did not prevent evidence of stress (Boonstra et al. 1998a) nor of weight loss (Hodges et al. 2006) during the decline, with both indices improving markedly only after predator density had declined drastically. Only in areas where hare populations were both fed and from which predators were excluded was weight loss during the decline prevented. In addition, Sinclair et al. (1988) found that some hares were nutritionally stressed during the late winters of the increase and peak phase, but not the decline phase. Furthermore, they found that no hares were nutritionally stressed during the summer at any time during the cycle. Thus, we focus on fluctuating predation risk as the most plausible explanation for the expected changes in GC levels in snowshoe hares. We have recently shown that an increase in predator-induced maternal stress results in a decline in reproductive fitness (Sheriff et al. 2009a). Furthermore, prenatally stressed offspring had higher nominal base cortisol levels (the initial blood sample taken after trapping and handling) and a greater stress response to a standardized stressor compared with those offspring that were not prenatally stressed. The signature of these effects lasted into adulthood (Sheriff et al. 2010a).

We test two hypotheses on the sensitivity of snowshoe hares to fluctuating predation risk:

Hypothesis 1 That hares will be more stressed during the decline phase of the cycle when the number of predators is higher than at other times in the cycle. The alternative hypothesis is that there is no evidence that levels of stress change during the cycle.

Hypothesis 2 That predation risk is the key factor causing stress in hares, and hare stress levels will change over short time frames (within a season) in response to changing predation risk. The alternative hypotheses are that hare stress levels will be constant or that levels will change for other reasons, such as changes in the quantity or quality of food.

To address these two hypotheses we measured the predator density and, during the winter season, the ratio of the number of hares to the number of lynx (*Lynx canadensis*) as indices of the risk of predation. We used fecal assays and a hormone challenge to measure indices of stress [fecal cortisol metabolite (FCM) and plasma cortisol levels], of energy mobilization [glucose and free fatty acid (FFA) levels], of leukocyte profiles (white blood cell ratios) and body condition [hematocrit levels and mass index (MI)] in hares during the winter non-breeding and summer breeding seasons of the increase (2005). This study occurred during the peak (2006), the decline (2007–2008) and the first year of the low (2009) phases of their population cycle.

Materials and methods

This study was conducted in the boreal forest near the Arctic Institute Base at Kluane Lake in the southwestern Yukon, Canada (60°57′N, 138°12′W). The study area is 600–1,100 m above sea level and is located within the rain shadow of the St. Elias Mountains. It is relatively dry and cool with an average summer (June–July) temperature of 9°C and an average winter (October–April) temperature of −18°C. This region is dominated by a single conifer species, white spruce (*Picea glauca*), with a mixed understory of grey willow (*Salix glauca*), bog birch (*Betula glandulosa*), soapberry (*Sherperdia canadensis*), and other herbaceous plants (Krebs et al. 2001b).

Population monitoring

Snowshoe hare populations have been monitored continuously since 1976 (see Krebs et al. 2001b for a discussion of the area) and thus changes in hare abundance have been followed through almost four cycles. In our study, we focus on the period of the last cycle from 2005 to 2009. The basic trapping protocol has been similar over this entire period. Live-traps were pre-baited with alfalfa cubes for 3–5 days before being set. Trapping sessions consisted of 2–3 nights of trapping within a 5-day period in both early spring (late March–early April) and late autumn (October–early November). Population density was estimated with the program CAPTURE (Otis et al. 1978) and the Jolly–Seber full model, as in previous studies (e.g., Krebs et al. 1995).

Avian and mammalian predator populations fluctuate in delayed synchrony with the hare cycle, peaking about 1–2 years after the hare peak (Doyle and Smith 2001; O'Donoghue et al. 2001; Rohner et al. 2001). An index of the fluctuations in predator populations was obtained using evidence from lynx data, as these are reflective of the fluctuations in other major predators (correlation between lynx densities and other predators:coyotes $r^2 = 0.95$; marten $r^2 = 0.30$; wolves $r^2 = 0.47$; great horned owls, *Bubo virginanus*, $r^2 = 0.49$; Doyle and Smith 2001; O'Donoghue et al. 2001; Rohner et al. 2001; Krebs, unpublished data). Lynx populations have been continuously monitored since 1987 (O'Donoghue et al. 2001). Each winter (October through April), 1–3 days after fresh snowfalls while tracks were distinguishable, lynx tracks were counted along transects that traverse our study area. On average, 402 km of transect were covered each winter. Track counts for lynx are highly correlated to their population density in this valley ($r^2 = 0.95$) and we calculated density as $y = 0.355 + 0.288x$, where y is lynx density and x is lynx track count (Hone et al. 2007).

Live trapping

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

Upon capture, each hare was weighed with a Pesola spring scale (±10 g), its right hind foot (RHF) length was 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), its sexual condition assessed, and a fecal sample collected from below the trap. During the winter season, samples were not collected from sexually reproductive hares (this was assessed by palpating the testes, which start to descend in mid-February). During the breeding season, samples were collected from adult females within 1 week after birth. Samples from adult males were collected within 1 week of the mean birth date for each litter.

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

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

from which hematocrit levels were also obtained; both are measures of condition (see below).

Fecal cortisol metabolite assay

Within 1 h of collection, fecal samples were stored between −20°C and −80°C at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at −80°C until analyzed. Fecal samples were freeze-dried using a lyophilizer (LabConco, 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 (1 SE) of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15,000 rpm on a multi-tube vortexer. After centrifugation (15 min at 2,500*g*), an aliquot of the supernatant was stored at −80°C until analysis.

Fecal cortisol metabolite concentrations were measured using the 11-oxoaetiocholanolone-EIA method developed by Palme and Möstl (1997) and validated specifically for snowshoe hares by Sheriff et al. (2009b). This EIA had an intra- and inter-assay coefficient of variation of 6.3 and 10.3%, respectively.

Hormone challenge

We used a hormone challenge to get an integrated picture of the hare's recent past while overriding the immediate stress response the hare was experiencing because of the effects of capture and handling. This protocol involved two steps: the dexamethasone suppression test (Kalin et al. 1981) followed by the adrenocorticotropic hormone (ACTH) stimulation test (Boonstra et al. 1998a). The dexamethasone suppression test is a method to assess whether the brain is registering glucocorticoid levels correctly, and making the necessary negative feedback adjustment by reducing ACTH and cortisol production. Dexamethasone is an artificial glucocorticoid which the brain registers as a mimic for the normal endogenous glucocorticoid. The ACTH stimulation test is a method to probe the responsiveness of adrenals directly.

Each adult female hare was bled five times (0.3 ml per bleed) from an ear artery using 28-gauge needles (0.36 × 13 mm) and heparinised 0.5 ml syringes (Lo-Dose U-100 insulin syringes; Becton–Dickinson and Company, NJ, USA). The first blood sample (nominal base bleed) was immediately followed by an injection of 0.4 mg/kg of dexamethasone sodium phosphate (DEX-Sabex, QC, Canada) into an ear vein. The second bleed (DEX bleed) assessed the inhibition of cortisol in response to DEX and occurred 2 h later. It was followed immediately by an intramuscular injection in the thigh of 40 μg/kg of synthetic adrenocorticotropic hormone (ACTH-Synacthen Depot; CIBA, ON, Canada). The remaining three bleeds assessed the stimulation response to ACTH and occurred 30, 60, and 120 min post-ACTH injection (called the P30, P60, and P120 bleeds, respectively). Blood samples were centrifuged at 8,800*g* for 10 min in an Eppendorf Micro Centrifuge 5413. The separated plasma was then frozen at −80°C at the Arctic Institute Base and at the University of Toronto until analysis. For each of these bleeds, we measured the levels of total cortisol, MCBC, glucose, and FFA.

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

Glucose was measured within 5 min of sample collection (FreeStyle glucometer; Abbott Diabetes Care, Alameda, CA, USA). Excessive levels of cortisol produced under chronic stress should increase liver production and storage of glucose as glycogen (Fujiwara et al. 1996) by enhancing the liver's capacity for gluconeogenesis (Miller and Tyrrell 1995, and this should come at the expense of peripheral tissues. Thus, if hares are more stressed at certain times, they should have a greater ability to mobilize glucose from larger liver stores.

Free fatty acids (FFA) were measured in duplicate using an in vitro enzymatic colorimetric method assay for the quantitative determination of non-esterified fatty acids (HR Series NEFA-HR [2]; Wako Diagnostics, VA, USA). During gluconeogenesis, FFA are one of the substrates delivered to the liver because of gluconeogenesis (Miller and Tyrrell 1995) and should decline under chronic stress. This assay had an intra- and inter-assay coefficient of variation of 5.2 and 9.9%.

For the first blood sample (prior to centrifugation of the blood), we also measured hematocrit levels and made blood smears within 30 min of blood collection. Measurements of hematocrit—the packed red blood cell volume—were made in duplicate after a 9-min centrifugation (13,460*g* on an IEC Micro-Hematocrit Centrifuge, Model MB). Hematocrit (packed red blood cell volume) is an integrative index of body condition in which higher values have been linked to better condition (see references in Boonstra et al. (1998a).

To obtain leukocycte profiles (white blood cells, WBC) slides were stained using a modified Wright stain technique called Diff-Quick (Dade International, FL, USA). These profiles provide the relative proportion of each WBC type in a count of 100 leukocytes. Leukocyte profiles are particularly useful in examining the effects of chronic stress

Table 1 Overview of the tests and statistical analyses used to compare the stress physiology of snowshoe hares (*Lepus americanus*) among years and within seasons during the increase (2005), peak (2006), decline (2007–2008) and low (2009) phases of the hare cycle

Tests performed	Variables	Comparisons	Statistical analysis	Implications
Non-breeding (winter)				
FCM analysis	FCM	Late winter 2006–2009	One-way ANOVA (years) Tukey's post hoc	Cortisol levels comparison
		Early–late winter 2006/2007–2007/2008	Two-way ANOVA (years × winter season)	Cortisol levels comparison
Hormonal challenge consisting of five sequential bleeds	Plasma cortisol	Early–late winter 2006/2007–2007/2008	Two-way repeated measures ANOVA (years × winter season)	Cortisol levels comparison
	MCBC	Early–late winter 2006/2007–2007/2008	Two-way repeated measures ANOVA (years × winter season)	Cortisol levels comparison
	Glucose	Early–late winter 2006/2007–2007/2008	Two-way repeated measures ANOVA (years × winter season)	Energy mobilization ability
	FFA	Early–late winter 2006/2007–2007/2008	Two-way repeated measures ANOVA (years × winter season)	Energy mobilization ability
	WBC ratios[a]	Early–late winter 2006/2007–2007/2008	Mann–Whitney *U* test	Body condition indicator
	Hematocrit[a]	Early–late winter 2006/2007–2007/2008	Mann–Whitney *U* test	Body condition indicator
Mass index measure		Early–late winter 2006/2007–2007/2008	Mann–Whitney *U* test	Body condition indicator
Breeding (spring/summer)				
FCM analysis	FCM	First–second litter 2005–2008	Two-way ANOVA (litter × years) Tukey's post hoc	Cortisol levels comparison

[a] Measured only at the first bleed of the hormonal challenge

because they are altered in a predictable manner (Dhabhar et al. 1996; Davis et al. 2008). In a chronically stressful situation neutrophil (*N*) numbers increase (neutrophilia) and lymphocyte (*L*) numbers decrease (lymphopenia) resulting in a greater *N*:*L* ratio. However, this is not the complete picture as an infection may also increase neutrophil counts and the *N*:*L* ratio. In order to disassociate chronic stress from an infection, both eosinophil and monocyte ratios must also be measured, as an infection or parasite will result in an increase in these WBC types (Jain 1986; Campbell 1996). WBC ratios were calculated from a count of 100 WBCs in the smears.

Data analysis

To examine how the risk of predation affected hares we carried out a number of different analyses (see Table 1 for details). First, FCM levels were compared using a one-way (year) or two-way (year × season) ANOVA and a Tukey's HSD post hoc test. Second, in response to the hormonal challenge, we measured four response variables (free cortisol, MCBC, glucose and FFA levels) using a repeated-measures ANOVA. The main effects in the analysis were year and season, with the time factor being the response of each variable during each of the five bleeds. Since the values in a repeated-measures design are not independent of each other, we used a conservative Greenhouse-Geisser epsilon to adjust the degrees of freedom prior to calculating the *P* value. And third, white blood cell ratios, hematocrit, and the MI were non-parametric and were compared using a Mann–Whitney *U* test. The unit of measurement was the individual as no individuals contributed to two different time points (year or season); hence, each mean was independent. We give *P* values between 0.10 and 0.05 and infer that these may be biologically, though not statistically, significant (Yoccoz 1991). We found no difference between males and females during the winter season and the sexes were pooled for analysis of FCM levels (females only were used during the hormone challenge). During the breeding season, we found a sex effect ($P < 0.05$) and males and females were analyzed separately.

Results

Population dynamics

Between 2005 and 2009, hare densities in the late winter increased 2.4 times from 38 hares/km^2 in 2005 to a peak of 92 hares/km^2 in 2006, then declined 2.6 times over the next 3 years to reach a low of 28 hares/km^2 in 2009 (Fig. 1a). Over the same years, the lynx population increased from a low of 4 lynx/100 km^2 in 2005 to a peak of 12 lynx/100 km^2 in 2007, and then declined to 6 lynx/100 km^2 in 2009 (Fig. 1a).

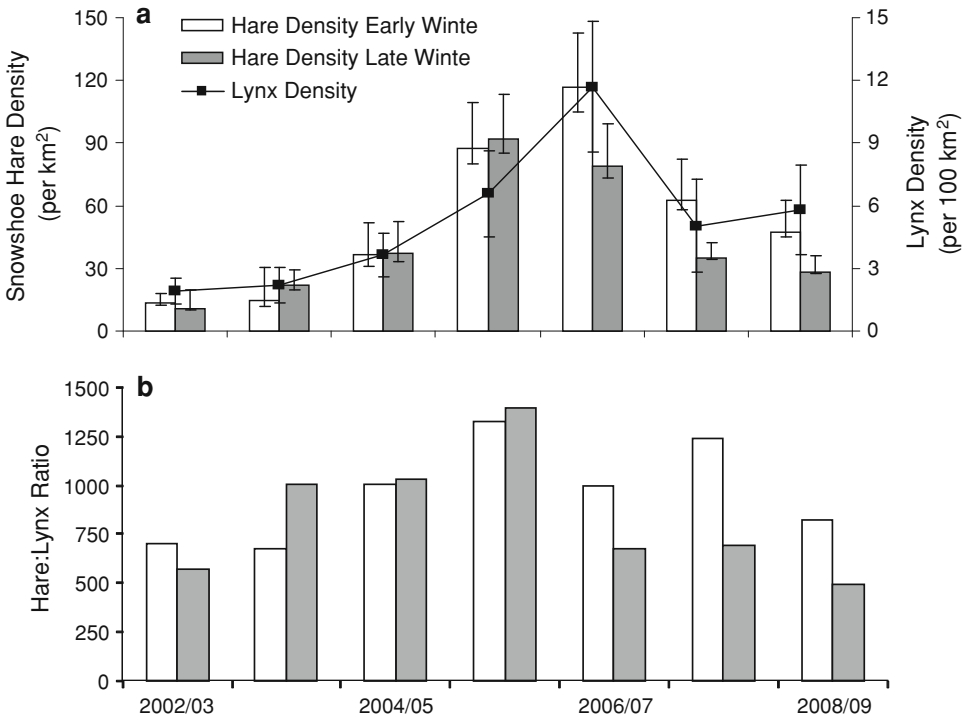

Fig. 1 **a** Snowshoe hares (*Lepus americanus*) and lynx (*Lynx canadensis*) population densities (mean ± 95% CL) in the southwestern Yukon, Canada from the winter of 2002/2003 to the winter of 2008/2009. **b** Snowshoe hare:lynx ratio from the winter of 2002/2003 to the winter of 2008/2009

The hare:lynx ratio fluctuated greatly across the cycle (Fig. 1b), decreasing 50% from 1,394 hares per lynx at the hare peak (late winter 2006) to 695 hares per lynx at the end of the decline phase (late winter 2008). Within a winter season, the hare:lynx ratio decreased on average 39% from early to late winter in 2006/2007–2007/2008. Hence, the exposure of an individual hare to predators increased as the winter progressed.

Fecal cortisol metabolite levels

During the non-breeding season, FCM concentrations in late winter varied significantly from 2006 to 2009 ($F_{3,73} = 6.08$, $P < 0.001$; Fig. 2). FCM concentration averaged 40% higher in 2007 than in 2006 and 2009 ($P < 0.05$); there was no significant difference between 2007 and 2008, or between 2006, 2008, and 2009. Comparing early and late winters of 2006/2007 and 2007/2008, we found a season effect ($F_{1,69} = 11.78$, $P < 0.005$), a year effect ($F_{1,69} = 4.35$, $P < 0.05$), and no interaction effect ($F_{1,69} = 0.22$, $P > 0.1$). FCM concentrations increased from early to late winter by an average of 34% and decreased from 2006/2007 to 2007/2008 by 20% (Fig. 3).

During the breeding season, FCM concentrations in adult hares decreased between the first and second litter groups of 2005–2008 (Fig. 4). We found a litter effect (male $F_{3,58} = 30.88$, $P < 0.0001$; female $F_{3,57} = 22.03$, $P < 0.0001$), a year effect (male $F_{3,58} = 4.13$, $P < 0.05$; female $F_{3,57} = 5.03$, $P < 0.005$), and an interaction effect in

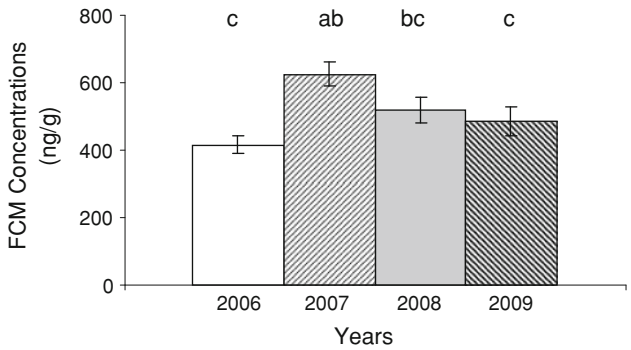

Fig. 2 Fecal cortisol metabolite (FCM) concentrations (mean ± SE) of snowshoe hares in the late winter from 2006 ($n = 20$), 2007 ($n = 19$), 2008 ($n = 20$), 2009 ($n = 18$). *Letters* denote significant differences ($P < 0.05$)

males ($F_{3,58} = 2.96$, $P < 0.05$) but not females ($F_{3,57} = 0.75$, $P > 0.1$; Fig. 4). In adult males, FCM concentrations at the time of the first litter averaged 101% higher than at the time of the second litter ($P < 0.05$) in 2006–2008; there was no difference between litters in 2005. FCM concentrations averaged 61% lower in 2005 than in 2006–2008 ($P < 0.05$); there was no difference among 2006–2008. In adult females, FCM concentrations at the time of the first litter averaged 62% higher than at the time of the second litter ($P < 0.05$) in all years. FCM concentrations averaged 54% higher in 2007 than in 2005, 2006, and 2008 ($P < 0.05$); there was no difference among 2005, 2006, and 2008.

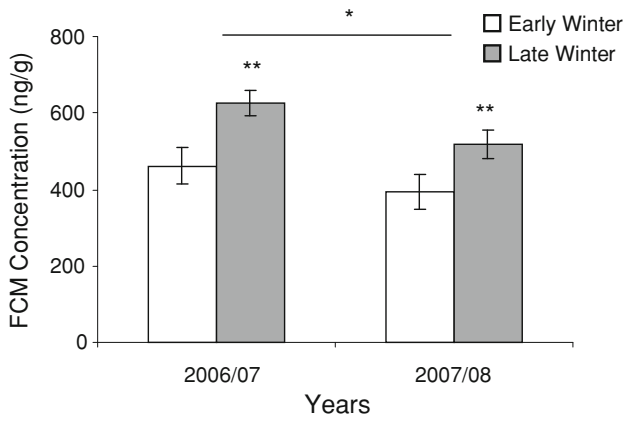

Fig. 3 Fecal cortisol metabolite (FCM) concentrations (mean ± SE) of snowshoe hares in the winter of 2006/2007 (n = 14 and 17 for early and late winter, respectively) and 2007/2008 (n = 20 for both early and late winter). Significant differences: *$P < 0.05$, **$P < 0.005$

Plasma cortisol levels during hormone challenge

Free cortisol concentrations (Fig. 5a), averaged over the entire hormone challenge, were significantly higher in the winter of 2006/2007 than in the winter of 2007/2008 (by 25%; $F_{1,46} = 6.50$, $P < 0.01$) and in late winter than in early winter (by 45%; $F_{1,46} = 16.24$, $P = 0.0002$). Free cortisol varied significantly over time in response to the hormone challenge in all cases ($F_{4,184} = 196.70$, $P < 0.0001$), and there were interaction effects between time and year ($F_{4,184} = 2.77$, $P < 0.03$) and time and season ($F_{4,184} = 6.54$, $P < 0.001$). Hares averaged 49% higher free cortisol concentrations in the winter of 2006/07 than in the winter of 2007/2008 and had 52% higher free cortisol concentrations in late than early winter (Fig. 5a).

MCBC levels (Fig. 5b), averaged over the entire hormone challenge, were 32% lower in the winter of 2006/2007 than in the winter of 2007/2008 ($F_{1,46} = 17.56$, $P < 0.0001$) and 35% lower in late winter than in early winter ($F_{1,46} = 24.89$, $P < 0.0001$). MCBC varied significantly over time in response to the hormone challenge in all cases ($F_{1,184} = 63.61$, $P < 0.0001$), and there were interaction effects between time and season ($F_{1,184} = 4.00$, $P < 0.01$). Hares averaged 41% lower MCBC levels in late winter than in early winter (Fig. 5b). Thus, hares in 2007/2008 were better able to handle the hormone challenge than those in 2006/2007, and hares in early winter were better able to handle the hormone challenge than those in late winter as indicated by their higher MCBC levels and lower free cortisol levels.

Energy mobilization during hormone challenge

Glucose levels (Fig. 5c), averaged over the entire hormone challenge, were similar in the winter of 2006/2007 and 2007/2008 ($F_{1,46} = 3.15$, $P = 0.08$), but were significantly higher in late than early winter (by 19%; $F_{1,46} = 12.92$, $P < 0.0001$). Glucose levels varied significantly over time in response to the hormone challenge in all cases ($F_{1,184} = 65.32$, $P < 0.001$), and there were interaction effects between time and year ($F_{1,184} = 3.56$, $P < 0.01$) and time and season ($F_{1,184} = 4.47$, $P < 0.002$). Hares averaged 8% higher glucose levels in the winter of 2006/2007 than in the winter of 2007/2008 and 18% higher glucose levels in late than early winter (Fig. 5c).

Free fatty acid levels (Fig. 5d), averaged over the entire hormone challenge, were similar in the winter of 2006/2007 and 2007/2008 ($F_{1,46} = 0.04$, $P > 0.1$) and in the early and late winter ($F_{1,46} = 0.50$, $P > 0.1$). However, free fatty acids varied significantly over time in response to the hormone challenge in all cases ($F_{1,184} = 348.72$, $P < 0.0001$), and there were interaction effects between time and season ($F_{1,184} = 4.04$, $P < 0.05$). These results are complicated by an interaction effect between time, year, and season ($F_{1,184} = 14.14$, $P < 0.0001$). In the winter of 2006/2007,

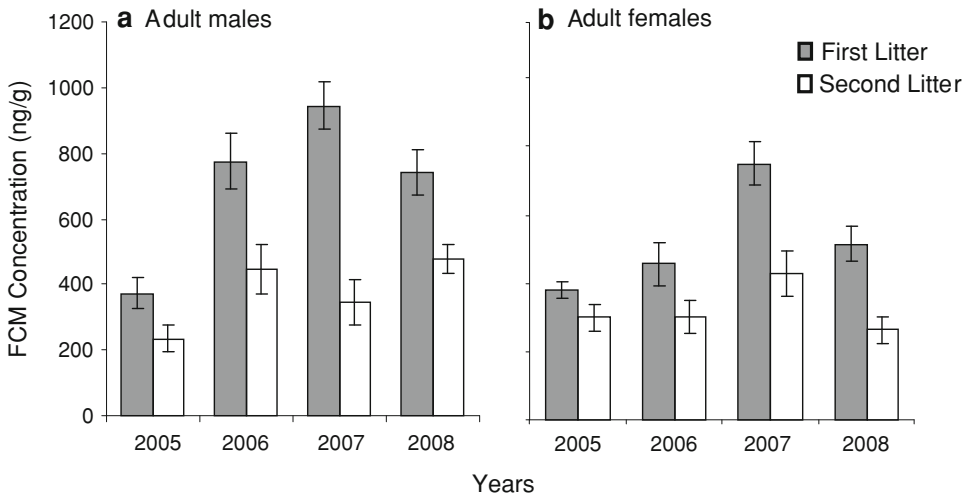

Fig. 4 Fecal cortisol metabolite (FCM) concentrations (mean ± SE) in adult male and adult female feces collected just after the birth of the first and second litters of the breeding season, 2005–2008 from **a** males (n = 2, 10, 10, 10; 5, 10, 9, 10 for the first and second litters, respectively; 2005–2008), and **b** females (n = 4, 10, 8, 10; 5, 10, 10, 8 for the first and second litters, respectively; 2005–2008). See text for statistical comparisons

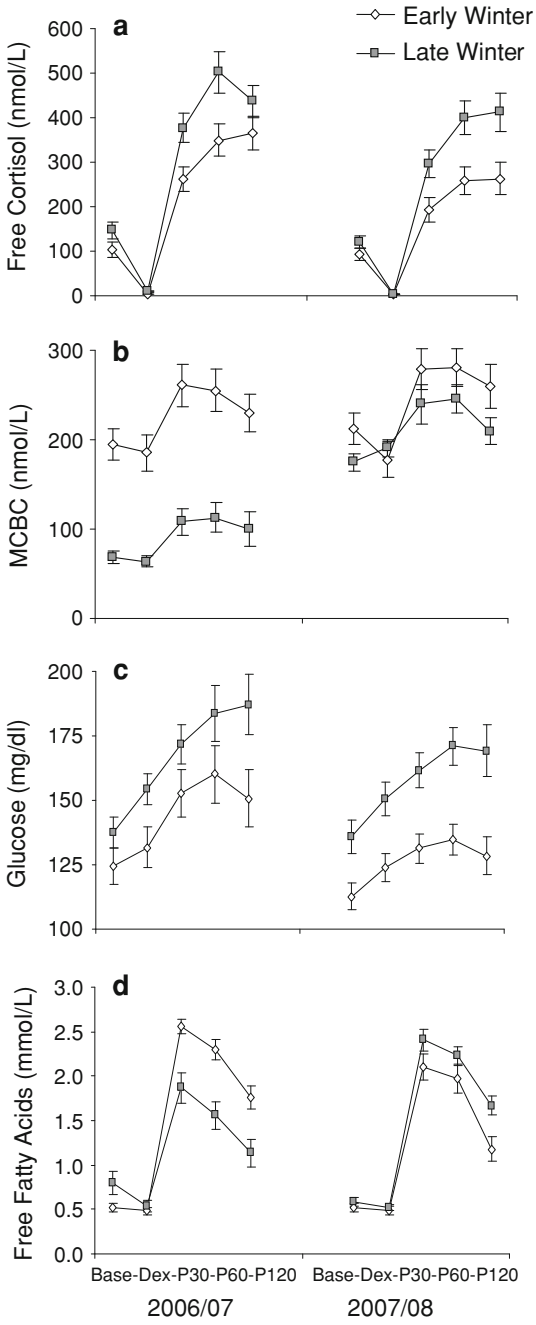

Fig. 5 Responses over time in plasma concentrations (mean ± SE) of **a** free cortisol, **b** maximum corticosteroid-binding capacity (MCBC), **c** glucose, and **d** free fatty acids (FFA) to the hormone challenge in snowshoe hares from early and late winter in 2006/2007 and 2007/2008; (cortisol and MCBC $n = 12, 12; 13, 13$; glucose $n = 12, 10; 10, 11$; FFA $n = 14, 10; 12, 14$ in early and late winter, respectively, from 2006/2007 and 2007/2008). *Base* values at the initial bleed, *DEX* values 2 h after the dexamethasone injection, *P30*, *P60*, and *P120* values 30, 60, and 120 min, respectively, after the adrenocorticotropic hormone (ACTH) injection. See text for statistical comparisons

FFA levels were higher in late winter than in early winter at the nominal base bleed (by 52%) and at the DEX bleed (by 11%), but were lower in response to the ACTH injections (by 43%; Fig. 5d). In 2007/2008, FFA levels were similar in both early and late winter at all five bleeds of the hormone challenge (Fig. 5d). Thus, hares had a greater ability to mobilize glucose in winter 2006/2007 than in winter 2007/2008 and in late than early winter. FFA mobilization was similar between the winters of 2006/2007 and 2007/2008, and was lower in late than early winter in 2006/2007 but not in 2007/2008.

Table 2 Number of major cell types (mean ± SE) of leucocytes in a count of 100 cells per slide (2 slides per hare) and *N*:*L* ratios from snowshoe hares from early and late winter in 2006/2007 and 2007/2008

Leucocytes	Season	2006/2007	2007/2008
Neutrophils	Early winter[a]	55 ± 2 (14)	48 ± 3 (16)[b]
	Late winter	66 ± 1 (13)	61 ± 2 (13)[b]
Lymphocytes	Early winter[a]	33 ± 2	37 ± 3
	Late winter	26 ± 1	26 ± 2
Eosinophils	Early winter[a]	6 ± 1	6 ± 1[b]
	Late winter	2 ± 1	5 ± 1[b]
Monocytes	Early winter	3 ± 0	6 ± 1[b]
	Late winter	4 ± 1	5 ± 1[b]
N:*L* ratios	Early winter	1.8 ± 0.2	1.5 ± 0.2
	Late winter	2.6 ± 0.2	2.8 ± 0.5

Samples were obtained from the nominal base bleed of the hormone challenge. Sample sizes are in parentheses and are given for neutrophils only and are the same for all other cell types. *N*:*L* ratios were not statistically compared since neutrophil and lymphocyte counts were

[a] Significant change from early to late winter

[b] Significant change from winter 2006/2007 to winter 2007/2008

Immunology and body condition

Leukocyte profiles varied by both winter and season (Table 2). In the winter of 2006/2007, neutrophils were 33% higher ($Z = 2.32$, $P < 0.05$), lymphocytes did not change ($Z = -0.41$, $P > 0.1$), and eosinophils and monocytes were 32 and 38% lower, respectively ($Z = -2.30$, $P < 0.05$, and $Z = -3.26$, $P < 0.005$, respectively) than in the winter of 2007/2008. In the late winter, neutrophils were higher (by 23%, $Z = -4.15$, $P < 0.0001$), lymphocytes and eosinophils were lower (by 26%, $Z = 2.92$, $P < 0.005$, and 39%, $Z = 2.76$, $P < 0.01$, respectively), and monocytes did not change ($P > 0.1$) than in the early winter.

Body condition was measured by both the hematocrit values (Fig. 6a) and the mass index (MI; Fig. 6b). Hematocrit values were lower in the winter of 2006/2007 than in the winter of 2007/2008 (by 7%; $Z = -2.34$, $P < 0.05$) and in late winter than in early winter (by 15%; $Z = 3.38$, $P < 0.001$). MI was similar in the winters of 2006/2007 and 2007/2008 ($Z = 0.42$, $P > 0.1$) and tended to be lower in late winter (0.94) than in early winter (0.98) ($Z = 1.81$,

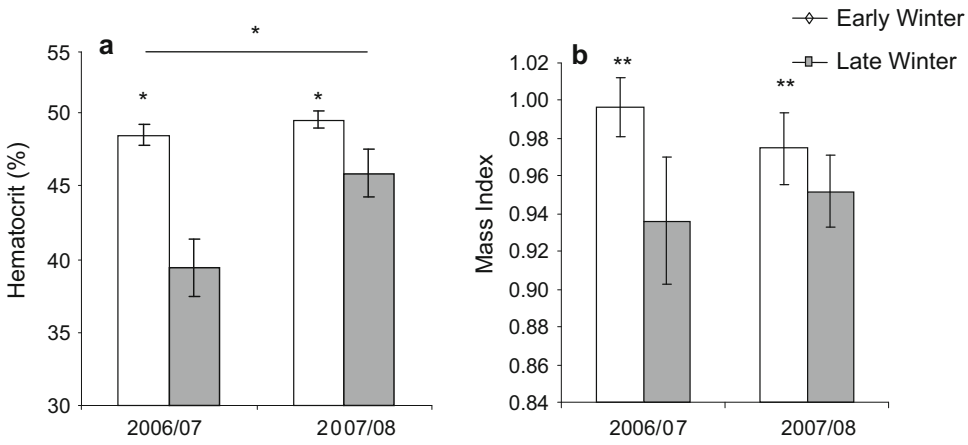

Fig. 6 Changes (mean ± SE) in **a** hematocrit (%) measured as the packed red-blood cell volume and **b** mass index in snowshoe hares from early (n = 14, 13) and late (n = 16, 15) winter in 2006/2007 and 2007/2008, respectively. Hematocrit was measured at the nominal base bleed. The mass index was obtained from measurements at the time of trapping. Hematocrit and mass index were measured on the same hares. Significant differences: *$P < 0.05$, **$P < 0.1$

$P = 0.06$). Thus, hares had a more compromised immune response and poorer body condition during 2006/2007 than during 2007/2008 and in late than early winter.

Discussion

We investigated the pattern of changes in the stress physiology of snowshoe hares during the increase (2005), peak (2006), decline (2007–2008), and low phases (2009) of their population cycle (Fig. 1a). Our findings are consistent with both hypotheses posed in the "Introduction" (findings summarized in Table 3). First, with respect to hypothesis 1, we found that hares were more stressed during the decline phase than at other times of the cycle. They had greater fecal cortisol metabolite (FCM) levels during the decline phase (when the risk of predation was greatest; Fig. 1) than during the increase, peak, and low phases (Figs. 2 and 4). Furthermore, hares had greater nominal base cortisol levels (Fig. 5a), a greater HPA responsiveness (Fig. 5a), a greater *N*:*L* ratio (Table 2), and poorer body condition (Fig. 6) in the first year of the decline (when the number of predators was greatest) than in the second year (when the number of predators was lower). Second, with respect to hypothesis 2, we found that hare stress levels as monitored by cortisol levels and other physiological indices changed within a season in response to changes in predation risk. Hares became more stressed as winter progressed [i.e., in comparison with hares from early winter, those from late winter had greater FCM levels (Fig. 3), greater nominal base cortisol levels (Fig. 5a), a greater HPA responsiveness (Fig. 5a), a greater ability to mobilize glucose (Fig. 5c), a greater *N*:*L* ratio (Table 2), poorer body condition (Fig. 6)]. Within a breeding season, all adult hares had greater FCM levels during the period of first litter than during the period of the second (Fig. 4). Below, we discuss each piece of evidence in turn and ultimately how the risk of predation may have far-reaching effects on both animal populations and communities.

Stress physiology

Fecal cortisol metabolites

Snowshoe hares' FCM concentrations fluctuated both among and within years (Figs. 2, 3 and 4). Among years, we found that FCM concentrations were highest in the first year of the decline (2007) and greater in the winter of 2006/2007 than in the winter of 2007/2008 (Figs. 2 and 3). Thus, hares were more stressed when the number of predators was greatest (Fig. 1). Boonstra et al. (1998a) also found that hares in winter were more stressed during the decline phase when predator numbers were greatest than during the late low phase when they were the lowest. Charbonnel et al. (2008) also found that cyclic populations of water voles, *Arvicola scherman*, were more stressed during the decline phase compared with the peak. Additionally, an increase in the number of predators has been shown to increase glucocorticoid (GC) levels in a number of other non-cyclic free-ranging mammals and birds (Silverin 1997; Hik et al. 2001; Scheuerlein et al. 2001).

We found that FCM concentrations were greater in the late winter than in the early winter (Fig. 3). In a population of free-ranging rabbits, *Oryctolagus cunicullus,* Monclús et al. (2009) found elevated FCM levels in response to increased predator numbers and decreased food availability. In a population of free-ranging song sparrows, *Melospiza melodia,* Clinchy et al. (2004) found that birds with increased predation risk and decreased food availability had the greatest stress levels. We found that as the winter progressed the hare:lynx ratio declined, such that there were half as many hares per lynx in the late winter than in the early winter (Fig. 1b) This estimate is based on known changes in hare density from early to late winter obtained from trapping, and constant or little change in lynx density over this time. Available food resources may also have declined over this period. Sinclair et al. (1982) found that the quality of food (as indicated by more fiber in feces in

Table 3 Overview of predictions and results of the consequences of variation in predation risk on snowshoe hare physiology

Physiological consequences	Predictions			Results		
	Among years	Within winters	Breeding season	Among years	Within winters	Breeding season
Stress physiology						
Fecal cortisol metabolites	Decline > increase, peak and low	Late > early	First > second litter	2007 > 2005, 2006 and 2009	Late > early	First > second litter
Nominal base plasma cortisol	1st > 2nd year of decline	Late > early		2006/2007 > 2007/2008	Late > early	
Dexamethasone resistance	1st > 2nd year of Decline	Late > early		2006/2007 > 2007/2008	Late > early	
ACTH stimulation	1st > 2nd year of Decline	Late > early		2006/2007 > 2007/2008	Late > early	
MCBC	1st < 2nd year of Decline	Late < early		2006/2007 < 2007/2008	Late < early	
Energy mobilization						
Glucose	1st > 2nd year of decline	Late > early		2006/2007 = 2007/2008	Late > early	
Free fatty acids	1st < 2nd year of decline	Late < early		2006/2007 = 2007/2008	Late = early	
Immunology and body condition						
Neutrophil:lymphocyte ratio	1st > 2nd year of decline	Late > early		2006/2007 > 2007/2008	Late > early	
Hematocrit	1st < 2nd year of decline	Late < early		2006/2007 < 2007/2008	Late < early	
Mass index	1st < 2nd year of decline	Late < early		2006/2007 = 2007/2008	Late < early	

Predation risk varied within seasons and among years during the increase (2005), peak (2006), decline (2007–2008) and low (2009) phases of the hare cycle

MCBC maximum corticosteroid-binding capacity, *ACTH* adrenocorticotropic hormone

late winter) decreased during this time, reaching its lowest point in late winter. Reduced food intake has been found to cause an increase in GC levels in many other mammals and birds (Harris et al. 1994; Kitaysky et al. 1999; Ortiz et al. 2001). A consequence of the decrease in food quality from early to late winter may force hares to forage in more risk-prone habitats (Hik 1995; Murray 2002).

Among breeding seasons, we found that adult snowshoe hare FCM concentrations increased from 2005 to 2006 and peaked in 2007 when the number of predators was greatest (Fig. 4). This pattern was more pronounced during the period of the first litter than that of the second litter (Fig. 4). Within a breeding season, adult snowshoe hares had higher FCM concentrations during the period of the first litter than during the second (Fig. 4). Changes during this short time span may be driven both by declines in the risk of predation and by increases in food availability. Boutin et al. (1986) found that predation rates on snowshoe hares decreased from winter to summer. The risk of predation may also decrease due to an increase in the hare:lynx ratio from the first to the second litter, due to the birth of the juvenile hares, and the presence of other prey (snowmelt makes voles accessible, arctic ground squirrels emerge from hibernation, and young from both species are born), though this may be counterbalanced by the birth of predator young. The first litter is also born during the late winter–early spring when the winter snowpack is melting and prior to the flush of new vegetation, whereas the second litter is born during late spring–early summer when new vegetative growth is nearing its peak (Sinclair et al. 1982).

Hormone challenge

In snowshoe hares, fecal cortisol metabolites are an accurate reflection of the state of the stress axis and thus of their average plasma cortisol levels (Sheriff et al. 2010b). In our hormone challenge, we were able to separate baseline cortisol levels and stress-induced levels to determine how the HPA axis changed in response to changes in the risk of predation. Our results echo our FCM findings such that free cortisol concentrations were higher in response to the hormone challenge in the winter of 2006/2007 (when the number of predators was greatest; Fig. 1) than in the winter of 2007/2008 and in late winter (when the hare:lynx ratio was lowest) than in early winter (Fig. 5a). The greater response to the hormone challenge is indicative of the animals being more stressed (Wingfield et al. 1998; Sapolsky et al. 2000; Romero 2004).

The changes in free cortisol were directly tied to changes in the opposite direction in MCBC (Fig. 5b). MCBC is a measure of the capacity of corticosteroid-binding globulin to bind cortisol in the plasma and this protein declines when an animal is chronically stressed. Since only the unbound, free cortisol is active, lower MCBC levels, as seen in 2006/2007 and in the late winter, are indicative of hares being less able to buffer high cortisol concentrations (Sitteri et al. 1982; but see Breuner and Orchinik 2002).

Our results show that snowshoe hares are extremely sensitive to the risk of predation, with the most pronounced indices of stress occurring when the number of predators was greatest. However, though our evidence amongst years

is consistent with the predominant role of predation risk, our seasonal evidence suggests that fluctuating food resources also affect hare stress levels. Notwithstanding these results, as we argued in the "Introduction", nutritional stress alone cannot explain the pattern of hare stress levels among years and particularly during the summer. Although food varies across the cycle (Krebs et al. 2001c), it reaches a nadir during the hare peak and quickly recovers by the first year of the decline. If food was the primary driver, FCM concentrations in 2006 (hare peak) should have been greater than those in 2008, but was in fact lower. Thus, our findings are largely consistent with hypothesis 2 posed in the "Introduction".

Energy expenditure

Glucocorticoids play a key role in sustaining energetic responses to stress. Snowshoe hares had a moderately elevated glucose mobilization in 2006/2007 compared with that in 2007/2008 but a much greater mobilization in late winter than in early winter (Fig. 5c). An increase in glucose mobilization has been found in both free-ranging snowshoe hares and arctic ground squirrels (*Spermophilus parryii plesius*) under duress (Boonstra et al. 1998a; Hik et al. 2001). The increase in glucose mobilization in response to increased predation risk must be extremely costly to hares during the food-scarce winter. Although hares have reduced energy expenditure in winter (Sheriff et al. 2009c), they have minimal winter body reserves (Whittaker and Thomas 1983) and would need to increase foraging (in an already food-reduced winter environment) to compensate for the increased glucose mobilization, further exposing them to predators.

Free fatty acids (FFA) are one of the substrates delivered to the liver during gluconeogenesis. We expected that as glucose mobilization increased the ability to mobilize FFA should decline, and this is what we found in 2006/2007 (Fig. 5d). Snowshoe hares were less able to mobilize FFA in the late winter than in early winter. However, in the early to late winter in 2007/2008, FFA mobilization was the same. Boonstra et al. (1998a) found that food supplementation resulted in elevated FFA levels in hares. Thus, the similar levels in 2007/2008 may be due to the lower snowpack level in the late winter of 2007/2008, allowing hares greater access to food and an increased ability to mobilize FFA. Together, these results provide evidence that the risk of predation affects snowshoe hares' ability to mobilize energy and that this is further affected by food availability.

Immunology and body condition

Chronic high GC levels can act as an immunosuppressant (Munck et al. 1984) and this is normally reflected in lower counts of white blood cells (WBC). However, if an increase in cortisol is associated with an infection (an acute increase in cortisol), WBC counts would be elevated. In an attempt to rectify this potential confusion, we measured snowshoe hares' leukocyte profiles. We found that when the risk of predation was higher in 2006/2007 compared with 2007/2008 and in the late winter compared with the early winter, snowshoe hares' leukocyte profiles shifted into a pattern indicative of a more stressed animal (Table 2). Specifically, neutrophil numbers increased whereas those of lymphocytes, eosinophils and monocytes decreased. Increased *N*:*L* ratios have been shown in a variety of other wild animals subjected to stressors (Baker et al. 1998; Davis 2005; López-Olvera et al. 2005).

Snowshoe hares' hematocrit values were higher in 2007/2008 than in 2006/2007 and in early winter than in late winter (Fig. 6a). High hematocrit values have been linked to better nutritional and health status (Hellgren et al. 1993; Moreno et al. 1998), whereas lower values have been linked to increased predation risk in mammals (Boonstra et al. 1998a; Hik et al. 2001) and birds (Clinchy et al. 2004). Hare mass index (MI) values were similar among years, but lower in late winter than in early winter (Fig. 6b). Although the values in early and late winter were just under a significant difference ($P = 0.06$), they may be biologically relevant (Yoccoz 1991). We conclude that, under high predation risk, hares have a compromised immunology and poorer body condition.

In summary, we have shown that snowshoe hares are highly sensitive to the risk of predation, having greater glucocorticoid levels, a greater ability to mobilize energy, a leukocyte profile indicative of greater stress, and poorer body condition when the number of predators was highest and, within a season, when the hare:lynx ratio was the lowest and the food availability the worst. Coupled with our previous findings that predator-induced maternal stress lowers reproductive fitness (Sheriff et al. 2009a) and compromises their offspring's stress axis into adulthood (Sheriff et al. 2010a), we propose that the low phase of the hare population cycle is the result of the impact of intergenerational, maternally inherited stress originating during the decline due to high predation risk. The population cycle of hares has far-reaching implications for the entire boreal forest. Snowshoe hares are typically the dominant herbivore in the boreal forest, and their cyclic fluctuations have widespread ramifications for the herbs, shrubs, and trees eaten by hares, for the resident and transient predators that eat hares, and for some, but not all, of the other forest herbivores that may compete with hares for food or that serve as alternative prey for predators (Keith 1990; Krebs et al. 2001b). Thus, predation is the predominant organizing process for a significant part of the North American boreal forest ecosystem. It acts both directly through hare mortality and indirectly because of the acute sensitivity of hares to fluctuating predation risk,

producing a pattern of change in hare stress physiology that appears to have long-term intergenerational effects.

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