Population Declines in the Snowshoe Hare and the Role of Stress

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Every 10 years snowshoe hare populations across the boreal forest of North America go through a population cycle, culminating in a decline lasting 4 or more years. We tested the hypothesis that snowshoe hares during the decline are in poor condition and less able to respond to challenges in their environment by examining the stress response of male hares. Three groups from February and May, 1991 (the second year of the hare decline in the Yukon), were compared: baseline hares were collected to obtain resting hormone levels; control hares were wild animals caught at randomly placed sites; and fed hares were wild animals caught on supplementary fed areas. The latter two groups were sequentially bled to examine their response to dexamethasone (DEX) followed by adrenocorticotrophic hormone (ACTH). Trapping and handling were stressful to the experimental hares as the initial blood levels of total and free cortisol levels were higher (especially in controls), testosterone levels were lower, and glucose levels were higher in experimental hares than in baseline hares. Control and fed hares showed similar total and free cortisol responses, falling to low levels after the DEX injection and increasing rapidly in response to the ACTH injection. However, control hares were in worse condition than fed hares as indicated by the higher free cortisol levels and lower maximum corticosteroid-binding capacity (MCBC) in control hares. In addition, though testosterone levels fell in both groups in response to DEX, only the fed hares showed a large, transitory increase 30 min after the ACTH injection. An unexpected finding was a dramatic increase in MCBC levels 30 min after the ACTH injection in both experimental groups, but it was more pronounced in the fed group. We conclude that the pituitary-adrenocortical feedback system in hares from declining populations is operating normally and that they should be able to cope with acute, short-term stressors, but that they are in poor condition and are exposed to higher levels of free cortisol than fed hares in good condition. © 1993 Academic Press, Inc.

Snowshoe hare populations (*Lepus americanus*) show regular 9- to 10-year cycles in population numbers throughout the boreal forest of North America and these have been going on for at least 200 years (Elton and Nicholson, 1942; Keith, 1963; Sinclair *et al.*, 1993). Two major, related hypotheses have been proposed to explain these cycles. Keith (1983, 1987) proposed that population declines in hares were caused by food shortage acting either directly through starvation or indirectly by increasing susceptibility of malnourished hares to predators. Evidence in support of this hypothesis comes from studies on hare dynamics in Alberta (Keith and Windberg,

1978; Keith *et al.*, 1984; Pease *et al.*, 1979). Krebs *et al.* (1986) and Sinclair *et al.* (1988) proposed the alternative hypothesis that predators alone were necessary and sufficient to cause population declines. Evidence in support of this hypothesis comes from work at Kluane Lake, Yukon (Boutin *et al.*, 1986; Krebs *et al.*, 1986; Trostel *et al.*, 1987). However, Smith *et al.* (1988) concluded that the results from the Yukon were also compatible with the hypothesis that food shortages predisposed hares to die from predation. They argued that although these hares did not experience an absolute food shortage in winter at peak densities, they may have experienced a rel-

ative food shortage because they had to forage further from cover to find food and thus experience greater predation risk.

We tested the hypothesis that snowshoe hares during a decline are in poor condition. The hypothalamo–pituitary–adrenocortical feedback system is crucial in permitting the animals to deal effectively with environmental challenges. Stress can be defined as the set of responses by mammals to potentially harmful environmental changes (Selye, 1946; Lee and McDonald, 1985). The direct effect on this endocrine system is an increased production of glucocorticoids, such as cortisol. These play a central role in allowing animals to adapt to acute challenges by stimulation of hepatic gluconeogenesis, inhibition of glucose uptake by peripheral tissues, suppression of inflammatory responses, suppression of immune reactions, and inhibition of secretion of several hormones and neuropeptides (Munck *et al.*, 1984). However, for hares during declines the necessities of finding food and cover and of avoiding predators are chronic in nature and may reduce the sensitivity of the hypothalamo–pituitary–adrenocortical feedback system to any specific challenge (Kalin *et al.*, 1981; Lee and McDonald, 1985). In this study we used the response to a standardized challenge as an index of the “quality” of hares by comparing wild-caught control hares with wild-caught hares from a supplementally fed population.

We used two methods to assess hare stress response: the dexamethasone suppression test and the ACTH stimulation test. The dexamethasone suppression test is an effective method to assess whether the pituitary–adrenal feedback system is operating normally. It has been used on two species of Australian marsupials to show that during the breeding season, total male mortality is directly related to the failure of the glucocorticoid feedback system (Bradley, 1990; McDonald *et al.*, 1986). The ACTH test has been used to probe the responsive-

ness of adrenals directly (e.g., Sapolsky, 1983; McDonald *et al.*, 1986; Harlow *et al.*, 1992) and in chronically stressed animals to determine whether the adrenocortical response is diminished. Two *in vitro* studies on snowshoe hares have already indicated that the primary glucocorticoid is cortisol (Feist, 1980; Fevold and Drummond, 1976). We examined hares both in late winter (February), when the cumulative effects of food shortage and cold temperature should be most pronounced, and again at the onset of spring (May), when overwinter survivors now had to cope with the additional effects of reproduction. If control hares are in poor condition relative to fed hares, we predict that this should be reflected in a slower and less intense response to dexamethasone and to ACTH in control hares.

Study Site and the Hare Population Cycle

The study was conducted near the Arctic Institute Base at Kluane Lake in the southern Yukon, Canada (Krebs *et al.*, 1992). It is the site of a 10-year study into the community dynamics of the vertebrates in the boreal forest. As part of this research, snowshoe hare populations have been live-trapped at least four times per year since 1986 (Krebs *et al.*, 1992). Hares reached a peak in numbers in the summer of 1989 and started declining in 1990. Our study was conducted during the second winter of the decline, with numbers falling 26.4% from April, 1990 to April, 1991, on control grids (from 1.25/ha to 0.92/ha) (Krebs *et al.*, personal communication). Estimates of overwinter loss from 1 October, 1990, to 1 April, 1991, were even more dramatic, with 90% of radiocollared hares dying, largely through predation, and 42% of the entire population disappearing. Thus the winter of 1990–1991 represented a period of significant decline for the hare population.

Finally, to understand the sequence of changes in hormone levels it is necessary to appreciate the yearly reproductive cycle of

hares. Females have a gestation period of 35 days and postpartum estrus. In the Yukon, they typically have three litters in reasonable synchrony, with the first estrus period being in mid-April, the second in mid-late May, and the last occurring in late June to early July (Hik and O'Donoghue, personal communication). Virtually all males are scrotal by the end of February and thus all males were in reproductive condition throughout our study (Hik, personal communication).

METHODS AND MATERIALS

Experimental animals. Sampling was conducted in 1991 in late winter (February 22–March 3) and in early spring (3–11 May). Blood was obtained from three groups of animals:

(i) Baseline hares were a random sample of hares collected between 0400 and 0930 hr near the Alaska Highway by a gun shot (22 caliber) to the neck and bled by heart puncture immediately (within 2–3 min). They were collected to obtain estimates of blood parameters from hares without the stress of capture and handling involved in live-trapping. We immediately filled hematocrit tubes and obtained blood smears, blood glucose levels, and hemoglobin levels. The remaining blood was stored in heparinized tubes, kept cool, and centrifuged on our return to the laboratory.

(ii) Control hares were a random sample of hares caught in the forest adjacent to the Alaska Highway by live-trapping. All trapping sites were at least 1 km from the nearest experimental trapping grid.

(iii) Fed hares were a random sample of hares captured on two experimental grids (1 km²) to which rabbit chow (approximately 300 kg with 16% protein) was distributed every 5–6 days since 1987.

To capture control and fed hares, live-traps were prebaited with alfalfa cubes for 2–3 days before being set. The general trapping methods used can be found in Krebs *et al.* (1986). All traps were set between 2300 and 2400 hr and checked between 0600 and 0700 hr. Animals were immediately sexed and, except for three females retained in the first trapping session in February, only males were taken to the laboratory; all females were released. Only males were bled to avoid the complicating effects of reproduction on hormone levels in females (Mulay *et al.*, 1973; Caillol *et al.*, 1990). The animals were transferred to burlap bags and taken to a quiet, dimly lit laboratory heated to 5° in February and 10° in May. In an attempt to try to standardize the conditions to which the hares were exposed prior to the experiment, they were allowed to habituate to these laboratory conditions for about 3 hr. No more than six hares were processed in any 1 day.

At the end of the experiment, fed hares were released at their site of capture between 1600 and 1700 hr; two hares in both February and March were fitted with radiocollars to determine their post-treatment survival. Control hares were killed by cervical dislocation. All hares were weighed on Pesola scales (± 10 g) and the right hindfoot was measured (mm). Both baseline and control hares were autopsied and an index of condition, fat in the tibia, was obtained by extracting the bone marrow, weighing immediately, and then again after it had been oven dried for 24 hr at 80°. Percentage fat was calculated by dividing dry weight by wet weight and multiplying by 100.

Injections. Each hare was bled five times from an ear artery. Blood from the first sample (0.5 ml) was used to obtain baseline estimates of blood parameters. This was immediately followed with an injection of 0.4 mg/kg of dexamethasone sodium phosphate (Sabex, Montreal, Canada) into an ear vein. The second bleed (0.3 ml) occurred 2 hr later, followed immediately by an intramuscular injection in the thigh of 4 IU of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada). The remaining bleeds (all 0.3 ml) were conducted at 30, 60, and 120 min post-ACTH injection. The amounts of the dexamethasone and ACTH used were within the range found to give optimal response by others and from a preliminary test of the protocol carried out in January, 1991, on a laboratory colony (dexamethasone: Hamilton and Weeks, 1985; Kant *et al.*, 1988; ACTH: Hamilton and Weeks, 1985; Fenske *et al.*, 1981; Smith and Bubenik, 1990).

Hematology. Blood was collected in heparinized 0.5-ml 28-gauge syringes (Lo-Dose U-100 insulin syringes, B-D) and in 75- μ l microhematocrit tubes. All glucose concentrations were measured after every bleed. Glucose concentration (mg/dl) was determined with a glucose oxidase/peroxidase reaction (Accu-chek III, Mannheim-Boehringer, Mannheim, Germany). We compared the accuracy of the device against control solutions and values were within 10%. A separate paper will give the results of the hematology (blood smears, hemoglobin concentrations, and packed cell volume) of initial blood samples of these hares (Singleton and Boonstra, in preparation). The remaining blood was centrifuged at 8800g for 5 min in an Eppendorf Micro Centrifuge. The separated plasma was then frozen at -20° , transported to Toronto, and stored at -70° until analysis.

Cortisol assay. We measured total plasma cortisol by the radioimmunoassay method of Etches (1976), as modified by Boonstra and Boag (1992), using anti-cortisol antibody A-155 obtained from Western Chemical (Fort Collins, CO). This antibody has previously been used in studies by Harlow *et al.* (1992, and papers cited therein) and shows the following cross-reactivities: corticosterone, 1.4%, cortisone, 1.6%; deoxycorticosterone, 1.3%; progesterone, 1.0%; and estradiol, estriol, and estrone, $<0.1\%$. All reagents

were redistilled. The following modifications were made to the protocol: 40 μ l double-distilled water and 20 μ l NH_4OH (to saponify triglycerides) were added to duplicate 10- μ l samples of plasma. Two milliliters of dichloromethane (Fisher) was added to the washed plasma, vortexed, and centrifuged at 1000 rpm, and the aqueous layer was aspirated. One 400- μ l sample of the dichloromethane extraction mixture was removed, transferred to a new tube, and dried in a 37° water bath under nitrogen, and 300 μ l of phosphate buffer (pH 7) was added and allowed to equilibrate at room temperature for 1 hr. Blank values of charcoal-stripped plasma and of solvent did not differ significantly from zero. The rest of the assay was similar to that of Etches (1976).

This assay was validated for hare plasma by two methods, parallelism and quantitative recovery. To assess parallelism a twofold dilution series in triplicate of hare plasma (from 0.125 to 10 μ l plasma) and of cortisol in buffer (20 ng/ml) was constructed and extracted and this was then compared with the unextracted standard curve. To analyze these data the amount of binding at each point was expressed as a percentage of control binding and logit-transformed. These values were then regressed against the logarithm of the dilution. We tested for parallelism by assessing the equality of slopes through an analysis of covariance (ANCOVA) with the program SUPERANOVA (Gagnon *et al.*, 1990). The lines produced from the plasma dilution series, the extracted buffer series, and the unextracted buffer standard curve were all parallel ($F(2,16) = 0.82$, $P = 0.46$). Quantitative recovery was assessed by adding known amounts (range 60–1000 pg/10 μ l) of cortisol in duplicate to charcoal-stripped plasma and then examining recovery after extraction. The regression of the cortisol added versus that recovered resulted in a line with a slope of 1.00 and a correlation coefficient of 0.97. The assay was sensitive to 10 pg/10 μ l. The mean recovery of [1,2,6,7- ^3H]cortisol (Amersham) added to plasma was 99% (SEM 0.78) (range 95.2–103.4). The intra- and interassay coefficients of variation were 10 and 16%, respectively. This variation is comparable to that reported by Etches (1976).

Corticosteroid-binding globulin concentration. Plasma corticosteroid-binding globulin (CBG) was measured as the maximum corticosteroid-binding capacity (MCBC) using [1,2,6,7- ^3H]cortisol, diluted in nonradioactive cortisol to known specific activity 5- to 20-fold in excess of the expected capacity (see McDonald *et al.*, 1981 for details of method). We allowed for the contribution of the endogenous cortisol in the sample. The high-affinity fraction in the 10- μ l sample of plasma, diluted in 0.5 ml phosphate buffer, was measured by liquid scintillation after separation from the free and albumin-bound fractions with dextran-coated charcoal (Tan and Mulrow, 1975). The high-affinity bound cortisol was then calculated knowing the specific activity and the radioactivity in the bound

fraction. We calculated the amount of free cortisol present using the CBG-binding constant of $4 \times 10^7 \text{ M}^{-1}$ determined for the laboratory rabbit (Westphal, 1967) and the binding equation developed by Tait and Burstein (1964) (program provided by I. McDonald personal communication).

Androgen assay. Plasma testosterone plus dihydrotestosterone was measured by radioimmunoassay using diethyl ether extracts of plasma. The antibody (P43/11) was produced by Croze and Etches (1980) and showed a major cross-reaction with dihydrotestosterone (62%) relative to testosterone. The protocol for the radioimmunoassay of testosterone was based on that of Abraham *et al.* (1971) with double diethyl ether extraction of plasma samples. Each plasma sample (50 μ l) was treated with 20 μ l NH_4OH prior to extraction. Blank values of charcoal-stripped plasma and of solvent did not differ significantly from zero.

This assay was validated for hare plasma by two methods, parallelism and quantitative recovery, using the same methods as outlined for cortisol. For parallelism, the plasma dilution series ranged from 1.56 to 200 μ l and the buffer dilution series used a concentration of 20 ng/ml. The lines produced from the plasma dilution series, the extracted buffer series, and the unextracted buffer standard curve were all parallel ($F(2,15) = 0.85$, $P = 0.45$). Quantitative recovery was assessed by adding known amounts (range 50–1000 pg/50 μ l) of cortisol in duplicate to charcoal-stripped plasma and then examining recovery after extraction. The regression of the cortisol added versus that recovered resulted in a line with a slope of 0.99 and a correlation coefficient of 0.94. The assay was sensitive to 10 pg/50 μ l plasma. The mean recovery of [1,2,6,7- ^3H]testosterone (Amersham) added to plasma was 96.5% (SEM 0.7) (range 92–102%). Validation of this assay was the same as for the cortisol assay. The intra- and interassay coefficients of variation were 5 and 6%, respectively.

Statistical analysis. Data are expressed as means $\pm$ SEM. We used Cochran's test (Winer, 1971) as recommended by Day and Quinn (1989) to test for homogeneity of variance. We found that the basic demographic and autopsy data satisfied this criterion, and hence we used no transformations. The hormone data for the repeated-measures analysis and the glucose data did not, and we used the $\log(x + 1)$ transformation to make the variances homogeneous. All ANOVAs were performed using SUPERANOVA (Gagnon *et al.*, 1990). A repeated-measures ANOVA was carried out on the hares challenged with the hormones. In a repeated-measures design, the values recorded from the same animal will be correlated with each other and thus are not independent as assumed by ANOVA. To compensate for this, we used the conservative Greenhouse-Geisser epsilon to adjust the degrees of freedom before calculating the probabilities as recommended by Keppel (1982). We used the

Tukey-Kramer multiple comparison post-hoc test to examine the significance of main effects.

RESULTS

Summary of Basic Data

Table 1 gives the summary of the basic data collected on the hares. We carried out a two-way ANOVA on body weight and found no difference among treatments (baseline, control, or fed hares) nor any interaction between treatment and month. However, hares in February weighed almost 200 g more (1550.1 ± 19.1) than those in May (1355.1 ± 21.4) ($F(1,64) = 44.58$, $P < 0.0001$). We carried out a similar analysis on right hindfoot length, a measure which should not be condition-dependent as weight may be. We found a significant interaction between treatment and month ($F(2,64) = 6.11$, $P < 0.01$), with hares for both the baseline and fed samples being slightly smaller in May than in February, whereas control hares were larger in May than in February. Percentage fat could only be compared between baseline and control hares as fed hares were released back onto the live-trapping grid. We carried out a two-way ANOVA and found that there were no differences between the two treatments nor any interaction between treatment and month. However, February hares had fat levels that were over three times higher

than those in May ($F(1,41) = 256.21$, $P < 0.0001$) (65.34 ± 1.95 versus 19.20 ± 2.16 , respectively). Thus, male hares showed a major drop in weight from February to May and, for the baseline and control hares, a loss of condition, but no consistent change in an index of body size, right hindfoot length.

Plasma Cortisol Levels

A two-way ANOVA on treatment and month for transformed initial plasma values indicated no interaction effects for either of these factors for cortisol, MCBC, or free cortisol (Table 2). Since February was significantly lower than May ($P < 0.0001$) for each one of these plasma levels, we present the data separately by month (Fig. 1). Cortisol levels for baseline hares were significantly lower ($P < 0.0001$) than those for either control or fed hares, which had levels similar to each other. MCBC levels were similar in all three treatments. Free cortisol levels were significantly different ($P < 0.0001$) in each of the treatments as follows: baseline < fed < control. Thus, the stress of trapping and holding in the laboratory resulted in a major increase in cortisol levels (total and free), but not in MCBC levels, and this effect was more pronounced in control than in fed hares.

The repeated-measures ANOVA indicated that averaged over the entire experi-

TABLE 1
SUMMARY OF BASIC DATA ON ANIMALS USED IN THE STUDY (MEANS $\pm$ SEM)

	Baseline	Control	Fed
February			
Sample size*	16	9	10
Weight (g)	1532.0 ± 22.1 (15)	1534.4 ± 35.4	1591.5 ± 46.1
Right hindfoot (mm)	137.8 ± 1.3	132.9 ± 1.4	134.7 ± 1.2 (9)
Percentage of fat	65.9 ± 3.0 (14)	64.4 ± 2.1	
May			
Sample size	14	12	10
Weight	1325.0 ± 35.4	1392.9 ± 37.3	1352.0 ± 38.8
Right hindfoot	135.5 ± 1.4	139.3 ± 1.3	131.6 ± 1.5
Percentage of fat	23.3 ± 3.3 (13)	14.8 ± 2.2	

* Measurements were not made on some individuals and the lower sample sizes are indicated in parentheses.

TABLE 2
TWO-WAY ANOVAS TESTING DIFFERENCES IN
TREATMENT (T) (BASELINE, CONTROL, AND FED)
AND MONTH (M) (FEBRUARY VS MAY) IN INITIAL
HORMONE VALUES OF SNOWSHOE HARES

Source	df	MS	F	P*
Cortisol				
Treatment	2	3.84	88.22	.0001
Month	1	1.22	27.96	.0001
T × M	2	.04	1.00	
Error	65	.04		
MCBC				
Treatment	2	.05	.14	
Month	1	.40	15.80	.0001
T × M	2	.01	.45	
Error	65	.03		
Free cortisol				
Treatment	2	5.05	111.19	.0001
Month	1	.86	18.80	.0001
T × M	2	.05	1.00	
Error	65	.04		
Testosterone				
Treatment	2	.93	12.83	.0001
Month	1	.33	4.56	.04
T × M	2	.32	4.50	.02
Error	65	.07		
Glucose^a				
Treatment	2	.21	10.31	.0001
Month	1	.001	.05	
T × M	2	.008	.38	
Error	59	.02		

^a Sample sizes were lower because values were not obtained in some males.

* All absent probability values are nonsignificant.

ment (after the variance owing to within-subjects effects had been removed) there was still a significant effect of treatment and of month (Table 3) (interaction effects between treatment and month were not significant). Fed hares had significantly higher MCBC levels and lower free cortisol levels, but similar total cortisol levels, than control hares (Table 4). Thus, fed hares appeared to be better able to handle the stress of handling and of the experimental manipulation than control hares. May hares had significantly higher cortisol, MCBC, and free cortisol levels than February hares (Table 4).

The within-subjects effects examining the responses to the injections showed the same thing for each of the three analyses: that changes in total cortisol, MCBC, and free cortisol were highly significant over the course of the experiment; that the interactions between both treatment and these levels, and month and these levels were also significant; and that the three-way interaction among the levels, treatment, and month were not significant (Table 3). Because of the significance of the interaction effects, we could not pool sums of squares to get an overall mean square with which to test the significance of the average changes in hormone/protein levels over the experiment. Nevertheless, from Fig. 2 and the size of the *F* values for the main effects in Table 3, it is clear that there are biological

FIG. 1. Initial plasma concentrations (means $\pm$ SEM) of total cortisol, MCBC, and free cortisol in male snowshoe hares in the late winter and spring of the second year of the cyclic decline, 1991.

TABLE 3
REPEATED-MEASURES ANOVA TESTING
DIFFERENCES IN TREATMENT (T) (CONTROL VS FED)
AND MONTH (M) (FEBRUARY VS MAY) OVER TIME
IN RESPONSE TO THE HORMONAL CHALLENGE

Source	df	MS	F	P*
Cortisol				
Treatment	1	.20	2.37	
Month	1	1.24	14.96	.0005
T × M	1	.11	1.26	
Subject (group)	35	.08		
Time	4	20.77	470.31	.0001
Time × T	4	.15	3.35	.05
Time × M	4	.29	6.52	.0045
Time × T × M	4	.03	.68	
Error	140	.04		
MCBC				
Treatment	1	.37	4.69	.04
Month	1	.42	5.38	.03
T × M	1	.02	.28	
Subject (group)	35	.08		
Time	4	.23	101.33	.0001
Time × T	4	.02	8.03	.0002
Time × M	4	.04	16.59	.0001
Time × T × M	4	.002	.82	
Error	140	.002		
Free cortisol				
Treatment	1	.40	4.71	.04
Month	1	.80	9.35	.004
T × M	1	.21	2.42	
Subject (group)	35	.09		
Time	4	22.81	547.10	.0001
Time × T	4	.13	3.18	.05
Time × M	4	.16	3.96	.02
Time × T × M	4	.02	.41	
Error	140	.04		
Testosterone				
Treatment	1	.21	3.2	.08
Month	1	.01	.18	
T × M	1	.08	1.26	
Subject (group)	35	.06		
Time	4	.22	8.03	.0001
Time × T	4	.09	3.35	.02
Time × M	4	.02	.77	
Time × T × M	4	.08	2.90	.04
Error	140	.03		
Glucose				
Treatment	1	.03	.33	
Month	1	.004	.05	
T × M	1	.03	.38	
Subject (group)	33	.08		
Time	4	.18	34.49	.0001
Time × T	4	.005	.88	
Time × M	4	.008	1.57	
Time × T × M	4	.001	.18	
Error	132	.005		

Note. The probabilities of the within-subject (time) analysis were adjusted using the Greenhouse-Geisser estimate.

* All absent probability values are nonsignificant. The treatment value for testosterone is just above significance and is also given.

TABLE 4
THE AVERAGE EFFECTS (MEANS ± SEM—nM) OF
TREATMENT (CONTROL VS FED) AND MONTH
(FEBRUARY AND MAY) IN EXPERIMENTAL HARES

	Factor	
	Treatment	
	Control (N = 95)	Fed (N = 100)
Cortisol	491.51 ± 35.12	462.98 ± 31.23
MCBC	172.48 ± 5.44	209.64 ± 7.46*
Free cortisol	211.29 ± 16.42	183.44 ± 13.18*
	Month	
	February (N = 90)	May (N = 105)
Cortisol	437.75 ± 34.05	510.42 ± 31.98***
MCBC	174.25 ± 7.09	206.36 ± 6.28*
Free cortisol	182.37 ± 15.13	209.56 ± 14.50**

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

changes in these levels independent of the interaction effects of treatment and month.

We focus first on the interaction effects between the levels and treatment and make three major points (Fig. 2). First, both control and fed hares responded dramatically and similarly to the dexamethasone injection, with average total cortisol levels fall-

FIG. 2. Responses over time in plasma concentrations (means ± SEM) of total cortisol, MCBC, and free cortisol in male snowshoe hares from control and fed areas. BASE levels indicate initial values, DEX indicates values 2 hr after dexamethasone injection, and POST-30, POST-60, and POST-120 indicate values 30, 60, and 120 min after ACTH injection.

ing from 283.04 ± 22.90 to only 23.41 ± 5.68 nM 2 hr later, and free cortisol levels falling from 105.60 ± 9.73 to 6.75 ± 1.98 nM, respectively. Average MCBC levels showed little change over this interval, falling only from 164.16 ± 10.07 to 155.10 ± 8.76 nM, respectively.

Second, the ACTH injection had equally dramatic effects in both control and fed hares, but this time on all levels (Fig. 2). At 30 min after the ACTH injection, the total cortisol levels increased to 644.96 ± 33.07 nM, MCBC levels to 222.60 ± 10.67 nM, and free cortisol to 262.42 ± 16.68 nM. Free cortisol levels remained consistently higher than the MCBC levels for the remainder of the experiment.

Third, an extremely interesting finding to highlight is that MCBC also showed a rapid increase in response to the ACTH injection, particularly in the fed hares, and thus buffered these hares to some extent from the high cortisol levels. In the fed hares, MCBC increased 57% from 161.02 ± 13.72 nM at the dexamethasone bleed to 252.74 ± 15.03 nM 30 min after the ACTH injection compared with 28% in the control hares, 148.87 ± 10.93 to 190.86 ± 11.56 nM, respectively. By 120 min post-ACTH injection, MCBC levels had started to fall in both control and fed hares.

Figure 3 shows the interaction in the changes in hormone/MCBC levels as a function of month. May animals tended to have higher total and free cortisol levels initially and at post-30 and post-60 min after ACTH injection, although not at the dexamethasone and post-120-min bleeds. MCBC levels also tended to be higher in May-caught hares, but these differences were minimal at post-30 and post-60 min. Thus in general, although the May animals had higher total cortisol levels, this was compensated to some extent by higher MCBC levels; however, these differences were not consistent across the various bleeds.

FIG. 3. Responses over time in plasma concentrations (means $\pm$ SEM) of total cortisol, MCBC, and free cortisol in male snowshoe hares from February and May, 1991. See the legend to Fig. 2 for additional details.

Plasma Testosterone Levels

A two-way ANOVA on transformed initial plasma values indicated a significant interaction between treatment and month (Table 2). Thus the appropriate error mean square became the interaction mean square in the analysis (Sokal and Rohlf 1981), with the result that neither treatment nor month was significant. Figure 4 shows that the baseline levels of shot hares were approximately two to four times those of either control or fed hares and that this effect was

FIG. 4. Initial plasma concentrations (means $\pm$ SEM) of testosterone in male snowshoe hares in the late winter and spring of the second year of the cyclic decline, 1991.

more pronounced in May than in February. Overall, testosterone levels were inversely related to free cortisol levels ($r = -0.37$, $F(1,69) = 11.1$, $P < 0.001$). Thus, trapping and handling hares had a rapid, depressing effect on testosterone levels.

The repeated-measures ANOVA indicated that averaged over the entire experiment there was no difference between February and May testosterone levels, although the difference between control hares (6.37 ± 0.63) and fed hares (8.41 ± 0.93) approached significance (Table 3). It also indicated that over time there were significant changes in testosterone levels and that there was a significant interaction between time and treatment. However, because there was a significant interaction among time, treatment, and month, the mean square for this term becomes the proper error term and the previous significant values drop out. Figure 5 shows that in all groups, testosterone levels fell after the dexamethasone injection (mean base = 8.19 ± 1.57 ; mean dexamethasone = 4.59 ± 0.67) and this difference is significant (by the method of contrasts (Snedecor and

Cochran, 1989), $F = 10.69$, $P < 0.003$). In response to the ACTH, a major pulse in testosterone levels 30 min after injection occurred in fed hares which averaged 15.31 ± 2.01 compared with 6.71 ± 1.03 in controls, after which levels returned to baseline values.

Plasma Glucose Levels

The ability to mobilize glucose rapidly in response to an environmental challenge is crucial to survival. We compared the initial glucose levels of the baseline hares with those from the control and fed hares in both February and May with a two-way ANOVA on transformed values. Baseline hares (89.48 ± 6.58 mg/dl) had significantly lower values than either control (128.74 ± 12.62 mg/dl) or fed hares (120.63 ± 3.49 mg/dl), which were not different from each other. Thus, in response to trapping and handling, the experimental hares mobilized glucose. We examined for differences in ability of the experimental hares to mobilize glucose with a repeated-measures ANOVA. We were unable to obtain a glucose value in one hare during a post-60 min bleed and therefore the degrees of freedom were less than those in the other similar analyses. There were no significant difference between control and fed hares nor between February and May. However, there was a significant increase in glucose levels over time (Table 3). To examine how glucose levels changed as a function of stress of handling and as a function of the sequential injection of DEX and ACTH, both of which mobilize glucose, we used the baseline levels of the shot hares as an estimate of resting glucose levels, calculated the average length of time from removal from the trap to first bleed in the laboratory for the experimental hares (211.5 ± 10.3 min), and related this to changes in glucose levels (ideally the length of time spent in the trap prior to trap removal may have been a bet-

FIG. 5. Responses over time in plasma testosterone concentrations (means $\pm$ SEM) in male snowshoe hares from February and May, 1991. See the legend to Fig. 2 for additional details.

ter measure of time, but we did not know this). Figure 6 shows that glucose levels increased in an almost linear fashion with time over the course of the experiment ($r^2 = 0.92$, $F(1,4) = 49.57$, $P < 0.002$), although clearly the hares were not simply responding to the amount of time subsequent to the removal from the trap, but were also directly responding to the injection of the two hormones. The important point is that the hares during this decline year were still able to mobilize increasing levels of glucose for up to 8 hr.

Survival of Radiocollared and Tagged Fed Hares

Of the two fed hares radiocollared and released in February after the experimental treatment, one survived for more than 287 days and the other for at least 34 days, when radio contact was lost. Two of the hares that were only eartagged lived a minimum of another 28 days (determined from capture in traps). Thus at least 4 of 10 fed hares in February lived a minimum of another 4 weeks subsequent to the experimental treatment. Of the two collared in May, one survived for 175 days and the other for 28 days. Three of the hares that were only eartagged lived a minimum of an-

other 77 days. Thus at least 5 of 9 fed hares in May lived a minimum of another 4 weeks. Thus the experimental treatment was not severe enough to compromise survival in fed hares over the short term.

DISCUSSION

Our study has demonstrated that snowshoe hares during the second year of a population decline exhibit normal responses to dexamethasone and to ACTH and thus their feedback mechanisms are intact and operative. Both control and fed hares in February and May showed an almost total suppression of cortisol production after the dexamethasone injection (Figs. 2 and 3) and it is reasonable to conclude that this is mediated by a suppression of ACTH secretion. Both groups then showed a similar, rapid response to the ACTH injection (Figs. 2 and 3). Finally, both groups showed a similar mobilization of glucose (Table 2 and Fig. 6). Thus, unlike the situation found in some Australian small mammals in which an imbalance in the ratio of corticosterone to MCBC results in high levels of free corticosterone and total male mortality (Bradley *et al.*, 1980; McDonald *et al.*, 1981; Bradley, 1987), snowshoe hares are not likely to be dying because of a similar stress response. We therefore conclude that any differences in condition that occur between control and fed hares are not sufficient to affect the normal pituitary-adrenocortical stress response which could directly affect survival.

There are three caveats that must first be addressed in interpreting the data. First, the baseline hares were collected over a 5-hr period in the early morning and since cortisol levels are known show circadian rhythms (see references in Dallman *et al.*, 1990), the levels in the animals collected at the extremes would be expected to show greater variance than if they had all been collected at 0600 (which is impossible under field conditions). Snowshoe hares are ac-

FIG. 6. Changes in glucose levels (means $\pm$ SEM) of male snowshoe hares over time. BASELINE levels indicate values of shot hares and the other values indicate means obtained from the experimental hares. See the legend to Fig. 2 for additional details.

tive during the night and since the highest levels of cortisol typically are reached at the onset of the active period, cortisol levels at the time of our collection should be approaching their nadir. To reduce the potential that higher variance was caused by sampling baseline hares over a 5.5-hr period, we carried out an analysis in which all animals shot before 0600 or after 0900 were removed, thus leaving only animals collected in a 3-hr window. This procedure eliminated five and six baseline males from February and May, respectively. The analysis resulted in the same general patterns and significant differences as were seen when all data points were included. Thus, the means of the baseline hares for total cortisol, free cortisol, testosterone, and glucose (Table 2) were significantly different from initial values obtained for control and fed hares and this was not an artifact introduced by the range of times of collection.

Second, Table 1 indicates that all hares showed similar losses in weight from February to May and this may superficially lead to the conclusion that control and fed hares were identical in quality and were experiencing similar food conditions during the winter of 1991. Baseline hares were approximately 14% lighter in May than in February, controls 9%, and, surprisingly, fed animals 15% (Table 1). These weight losses are echoed by sharp declines in fat content of the bone marrow (64% in baseline animals and 77% in controls) (Table 1). There are two reasons why similar weight losses in control and fed hares do not necessarily reflect exposure to similar environmental conditions. First, weight loss in overwintering hares appears to be an aspect of the biology of this species which occurs every year prior to and after peak numbers (Keith, 1987) and is probably related more to a species adaptation to winter. For example, there may be advantages in having heavier body weight over winter as insurance against low food periods and this excess weight is then lost at higher population

densities during the competition among males for access to females in spring, something which would go on in both control and fed populations. Second, McNamara and Houston (1987) have elegantly shown that animals that can control both their probability of starvation and of predation through their behavior essentially have to trade off one for the other. In the face of a decreased food supply, relative numbers of deaths from starvation may in fact decline as control hares try to compensate by foraging further from cover and increasing predation risk (hence the high rates of loss of radio-collared hares to predation on control areas). Fed hares may not have to increase their predation risk because of the ready access to food. Consequently, we may see no difference in weight loss between the hares from the fed and control areas, but control hares are exposing themselves to much greater danger than fed hares.

Third, there may have been a systematic bias in the length of time the control hares were in the traps before removal to the laboratory relative to fed hares. If control hares were more hungry than fed hares, they may have entered the traps earlier and been in them longer than fed hares, thus spending a longer time having the stress response. We do not believe this bias occurred for three reasons, all of which are based on tracking and radiotelemetry data on trap entry collected by two students working on other aspects of snowshoe hare biology at Kluane (D. Hik and J. Boulanger, personal communication—University of British Columbia). First, Hik set a limited number of traps in the late evening in winter to capture animals for tracking and was always able to capture animals readily (within 40–60 min) independent of whether the animals occurred on control or fed sites. Second, hares show two major bouts of activity (based on radiotelemetry data) in the evening independent of treatment, being very active early in the evening and less active later on. Third, hares on all

trapping grids regularly checked traps for food (based on tracking information with spool-and-line threads—Hik) so there is no reason to expect that hares on control grids would enter traps earlier than those on fed grids. Thus, we do not think there is any systematic bias against control hares which could have affected our results.

There are three things which indicate that control hares were in worse condition than fed hares and that control hares were more likely to be affected by the deleterious effects of a stressful environment. First, trapping and handling had a greater impact on control than on fed hares, resulting in higher initial levels of free cortisol in controls, particularly in May (the height of the breeding season) (Fig. 1, Table 2). Second, MCBC levels over the course of the experiment were significantly lower in control than in fed hares and this had the direct consequence that free cortisol levels were higher in control hares (Fig. 2, Table 4). The difference in MCBC levels is particularly pronounced 30 min after the ACTH injection, with fed animals having MCBC levels 32% higher than those of control animals (Fig. 2). Since it is the free cortisol which is believed to be the biologically active form (Rosner, 1990), the higher levels in control animals may be more detrimental. Sapolsky *et al.* (1987) hypothesized that repeated exposure to chronic stressors results in high levels of glucocorticoids which then damage regulatory centers in the hippocampus, accentuate the rate of aging, and compromise the animal's ability to maintain homeostasis. Thus, this is one mechanism by which the external environment can permanently affect an animal's ability to cope with challenges. We may be observing one aspect of that damage, i.e., higher free cortisol levels in response to trapping and to the experiment, which may be indicative of their responses to normal stressors experienced in the wild and which fed hares do not experience because of ready access to high quality food. Keith

(1987) also found evidence that winter nutrition is the pathway through which the pituitary–adrenocortical system is affected. He found that hares that lost the most weight in late winter also had the largest adrenal glands, but that the latter was not related to population density. Thus, poor nutrition and all that it encompasses (competition for foraging sites, increased exposure to the risk of predation because of the need to forage further from cover, etc.) is a chronic stressor. However, given the low levels of free cortisol of baseline hares and the rapid responses in hormone levels in control hares to both dexamethasone and ACTH, we conclude, that up to this point in the cycle, hares are not sufficiently compromised in their ability to deal with challenges.

Third, 30 min after the ACTH injections, testosterone levels increased dramatically in fed animals relative to those in control animals so that their levels were over 2 times those of controls (Fig. 5). In some respects this dichotomy in response is similar to that observed by Sapolsky (1985) when he compared the response of dominant and subordinate olive baboons (*Papio anubis*) to the stress of immobilization. For the first hour after immobilization, testosterone levels rose in dominant baboons but fell in subordinates. However, in a subsequent study on new animals in which he successively injected baboons with a variety of hormones culminating with an ACTH injection, he found no response in testosterone levels. However, the previous injections may have compromised the ability of the animals to respond. He attributed rise in testosterone levels in dominants following immobilization to an adaptive response derived in part from the sense of control and predictability that these animals have at the top of the dominance hierarchy (Sapolsky, 1990). Mann and Orr (1990) have also reported a rise in testosterone concentrations in rats 1 hr after restraint stress, but which then fell below control levels after 3

hr of restraint. It is unlikely that our fed hares or Mann and Orr's rats were all dominant animals. Rather, we suggest that the transient testosterone pulse seen in hares and rats is a function of good nutrition. This may also explain the testosterone response of the dominant baboons, as these individuals obtain better than average nutrition, both by finding their own and by displacing subordinates from food the latter have found (Sapolsky, 1990).

The transient, rapid rise in MCBC levels in response to the ACTH injection (within 30 min) has not been documented before to our knowledge (Figs. 2 and 3) (Westphal, 1971, 1986). This in part may be because corticosteroid-binding globulin levels often are not measured (e.g., Hamilton and Weeks, 1985; Fenske *et al.*, 1981; Smith and Bubenik, 1990; Sapolsky, 1985) or because the protocols used are such that the first blood sample after the ACTH injection did not occur until 2 or more hr had elapsed (e.g., McDonald *et al.*, 1986; Bradley, 1990). In general, high corticosteroid levels produced through certain disease states (Savu *et al.*, 1981; Schlechte and Hamilton, 1987; Frairia *et al.*, 1988), through external stressors (Kattesh *et al.*, 1980), or directly through glucocorticoid injections (Gala and Westphal, 1966; Feldman *et al.*, 1979; Levin *et al.*, 1987; Dallman *et al.*, 1989) cause a decrease in CBG levels. In our study the rapid increase in MCBC lasted for about 60 min and was condition-dependent, with fed hares showing the greatest response (Fig. 2). The liver is the main site of production of CBG in rabbits (Seralini *et al.*, 1990) and it seems unlikely that the rapid increases we observed were the direct result of increased production. Rather, we speculate that the liver may also be a storage site for CBG which can then be rapidly released subsequent to an acute stressor. Since CBG binds cortisol strongly and the half life of CBG is long (13 hr in rabbits; Seralini *et al.*, 1989) relative to cortisol (very little cortisol remained in circulation 2

hr after the DEX injection, Fig. 2), high levels of CBG would serve to maintain cortisol levels at higher values longer than they otherwise might have been. This may serve to keep the animal in a state of readiness subsequent to the stressor.

Reproductive function in male mammals can be inhibited by a variety of stressors (Levine *et al.*, 1989) and these can act through a variety of mediating mechanisms, such as glucocorticoids, β -endorphins, and prolactin (see references cited in Sapolsky, 1985, and in Orr and Mann, 1992). We have found two lines of evidence that high levels of glucocorticoids, produced either through the psychogenic stressor of trapping and handling or through dexamethasone, are associated with suppression of testosterone levels. The trapping and handling procedures were clearly stressful as initial free cortisol levels were significantly higher in both experimental hares (control > fed) than in baseline hares (Fig. 1) and these levels were inversely correlated to testosterone levels (Fig. 4). The effect of the inhibition was more pronounced in May than in February because baseline levels in May were two times those in February (Fig. 4). Our May sampling period occurred just prior to breeding for the second litter and testosterone levels should consequently also have been high. The stress of trapping and handling was thus all the more apparent.

The dexamethasone suppression of plasma testosterone levels (Fig. 5) is a direct indication of the negative effect of glucocorticoids on reproductive function. This effect has also been observed in three marsupial species (*Didelphus virginiana*, Vinson and Renfree, 1975; *Antechinus swainsonii*, McDonald *et al.*, 1986; and *Phascogale calura*, Bradley, 1990, in the olive baboon (Sapolsky, 1985), and in the laboratory rat (Bambino and Hsueh, 1981). Dexamethasone may be acting through an inhibition of testicular sensitivity to luteinizing hormone (Bambino and Hsueh, 1981; Sa-

polsky, 1985) and/or through suppression of luteinizing hormone secretion from the pituitary (Thibier and Rolland, 1976).

We carried out the experiment in both February and May because we predicted that any differences between control and fed hares would be exacerbated in May, both because the males now had to deal with the additional challenges of reproduction and because those males surviving to May had experienced two more months of winter (snow melt typically occurs in the first week of May in this area). There are indications that males were indeed in worse condition in May. The body weight loss and fat content decline of the marrow may be reflections of this (Table 1). In addition, the initial levels of total and free cortisol and of MCBC were significantly higher in May than in February (Fig. 1). In spite of this evidence, there was no interaction among treatment, month, and sequence of injection times for either glucose or the total and free cortisol and MCBC (Table 3), and the responses of February and May animals were very similar to both dexamethasone and ACTH. Thus, we conclude that May animals still had a feedback system which responded normally.

The stress of handling and trapping was indicated by higher glucose levels in experimental hares than in baseline hares (Fig. 6). Green and Larson (1938) proposed that "shock disease" owing to severe hypoglycemia caused the hare decline and that the stress of trapping or captivity could precipitate it. Chitty (1959) rejected their argument proposing that the deaths they observed were artifacts induced by their handling techniques. We found no evidence of hypoglycemic shock, although very clearly our handling procedure put the animals in a very difficult position (free cortisol levels exceeding MCBC levels after ACTH injection) and, were it to continue for much longer, could have compromised their survival because of immunosuppression and anti-inflammatory effects. However, the

experimental treatment was not severe enough to endanger short-term survival as both the radiocollared fed hares in February and May survived for at least 28 days. We have no evidence indicating how live-trapping by itself affects the hormone levels of hares, although we expect that hares probably respond rapidly to it in terms of higher cortisol levels. Repeated capture and release to obtain demographic information may depress survival because of the cumulative experience of repeated bouts of high free cortisol levels.

In conclusion, the pituitary-adrenocortical feedback system in hares from the second year of a declining population is operating normally and they should be able to cope with acute, short-term stressors. However, decline hares are in poor condition and are exposed to higher levels of free cortisol when challenged than good-condition fed hares. Does the hypothalamo-pituitary-adrenocortical axis play any role in generating population cycles in hares? We suggest it probably does not cause the decline which appears related to an interaction between food and predation, but that it may play a role in prolonging the low phase. One of the enigmas of the cycle is the very poor survival of juveniles even after the population has declined to low numbers and recovery does not occur until after juvenile survival improves (Krebs *et al.*, 1986; Keith, 1987). It is possible that the stressors to which the females are exposed during the decline (low food availability, high risk of predation, competition for access to cover near food) leave an indelible imprint on them (*sensu* Sapolsky *et al.*, 1987) which then translates into lower reproductive fitness. In the laboratory, stressors occurring prior to (Christian and LeMunyan 1958) and during pregnancy (e.g., Rhees and Fleming, 1981; Crump and Chevins, 1989; Takahashi *et al.*, 1990) may have long-term, irreversible effects on progeny behavior and reproductive performance and these effects can last several

generations (Pollard, 1986). In the field, stressors acting through poor maternal condition have negative consequences for offspring fitness (e.g., Albon *et al.*, 1987; Fryxell *et al.*, 1991) and this may also last for several generations (Mech *et al.*, 1991). From a study on fluctuating meadow vole populations, Mihok and Boonstra (1992) found that decline-phase females had very low reproductive performance in the benign conditions of the laboratory, relative to increase-phase females. Thus, the conditions to which the hares are exposed during the decline phase may have permanent detrimental effects on the hypothalamic-pituitary-adrenal axis and have long-term population consequences through hare reproduction and offspring fitness, leading to a delay in recovery from low numbers.

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