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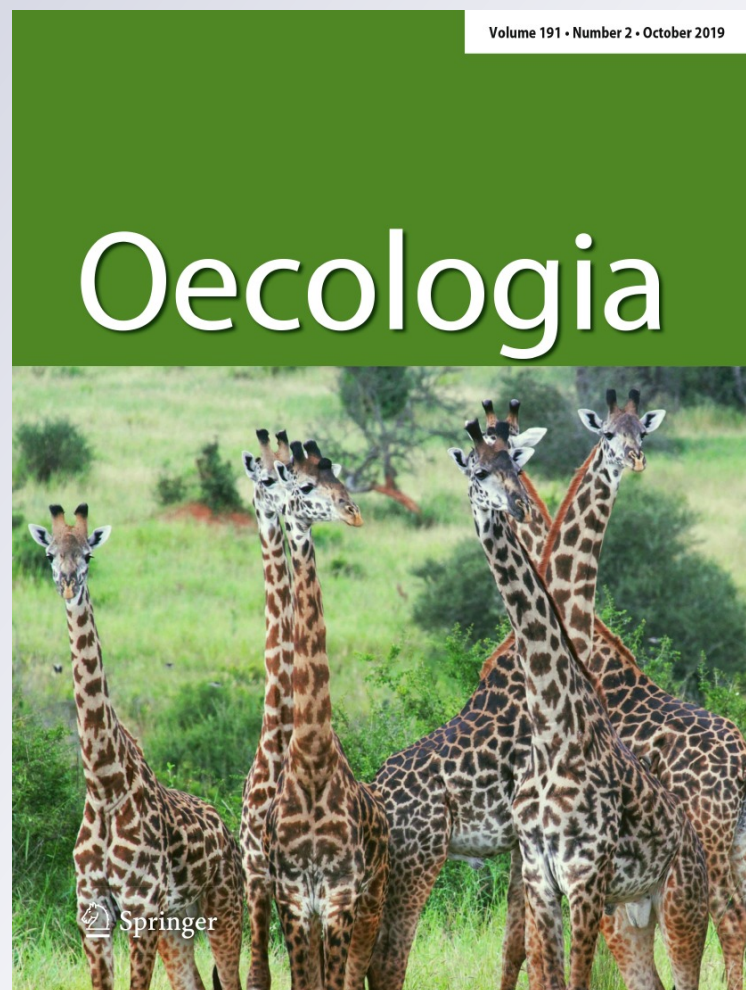
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Experimental increase in predation risk causes a cascading stress response in free-ranging snowshoe hares

Melanie R. Boudreau¹ · Jacob L. Seguin¹ · Rudy Boonstra² · Rupert Palme³ · Stan Boutin⁴ · Charles J. Krebs⁵ · Dennis L. Murray¹

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Abstract

Extensive research confirms that environmental stressors like predation risk can profoundly affect animal condition and physiology. However, there is a lack of experimental research assessing the suite of physiological responses to risk that may arise under realistic field conditions, leaving a fragmented picture of risk-related physiological change and potential downstream consequences on individuals. We increased predation risk in free-ranging snowshoe hares (*Lepus americanus*) during two consecutive summers by simulating natural chases using a model predator and monitored hares intensively via radio-telemetry and physiological assays, including measures designed to assess changes in stress physiology and overall condition. Compared to controls, risk-augmented hares had 25.8% higher free plasma cortisol, 15.9% lower cortisol-binding capacity, a greater neutrophil:lymphocyte skew, and a 10.4% increase in glucose. Despite these changes, intra-annual changes in two distinct condition indices, were unaffected by risk exposure. We infer risk-augmented hares compensated for changes in their stress physiology through either compensatory foraging and/or metabolic changes, which allowed them to have comparable condition to controls. Although differences between controls and risk-augmented hares were consistent each year, both groups had heightened stress measures during the second summer, likely reflecting an increase in natural stressors (i.e., predators) in the environment. We show that increased predation risk in free-ranging animals can profoundly alter stress physiology and that compensatory responses may contribute to limiting effects of such changes on condition. Ultimately, our results also highlight the importance of biologically relevant experimental risk manipulations in the wild as a means of assessing physiological responses to natural stressors.

Keywords *Lepus americanus* · Cortisol · Field experiment · Hormone challenges

Introduction

During recent decades, studies examining how environmental stressors affect organisms have been crucial in revealing complex structure and function of physiological systems (White and Jentsch 2001; Turner 2010). Environmental stressors include a variety of challenges that are imposed on free-ranging animals, including predation risk (e.g., Menge and Sutherland 1976; Preisser et al. 2005; Peckarsky et al. 2008). Increased risk perception can elicit marked changes in prey physiology (e.g., Creel et al. 2007), morphology (e.g., Trussell et al. 1993), behavior (e.g., Lima and Dill 1990), and metabolism (e.g., Van Dievel et al. 2016; Paul et al. 2018), but our current understanding of the linkage between these responses remains largely in its infancy, in part owing to difficulties in robustly assessing effects of stressors in a natural setting. Hence, while prey responses

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to predation risk have been identified as having a strong role in shaping ecological communities (Menge and Sutherland 1976; Preisser et al. 2005), the actual effects of predation risk on prey physiology, how such risk affects condition, and whether prey can compensate for physiological change via other means, remain largely unknown.

Chronic stress has risen to the forefront of our understanding of prey responses to predation risk (Hawlena and Schmitz 2010; Boonstra 2013; Clinchy et al. 2013), and a variety of physiological processes clearly link risk perception by an individual to changes in its behavior or condition, including through increased stress hormones (e.g., Clinchy et al. 2013), increased muscle enzymatic activity (e.g., Strobbe et al. 2010), and altered immune function (e.g., Hinam and St.Clair 2008; Seiter 2011). Such physiological change may further affect prey growth and morphology (e.g., McPeck et al. 2001), metabolic processes (e.g., Thaler et al. 2012), and body composition (e.g., Costello and Michel 2013). Typically, physiological changes related to predation risk are measured using total plasma cortisol (e.g., Eilam et al. 1999; Barcellos et al. 2007), oxidative status and antioxidants (e.g., Slos and Stoks 2008; Janssens and Stoks 2013), immunoglobulins (e.g., Hinam and St.Clair 2008), as well as variation in respiration/heart rate (e.g., Woodley and Peterson 2003), and heat shock proteins (e.g., Kagawa et al. 1999). In field studies, typically only one or two metrics serve to determine physiological effects of predation risk (e.g., Hawlena and Schmitz 2010; Zanette et al. 2014), with the most common metrics often failing to adequately represent the breadth of physiological changes that individuals can undergo following changes in risk perception (e.g., total cortisol; Breuner and Orchinik 2002). In contrast, information provided by multiple physiological measures that collectively assess the overall stress axis and condition of individuals should better reflect the full extent of physiological change (e.g., free plasma cortisol and its cascading effects on glucose, immunology, and hematology or physical structure). It follows that increased use of comprehensive and complementary physiological metrics could elucidate possible compensatory responses to increased risk, if they occur. However, to date there is limited evidence of such compensation (but see Thaler et al. 2012; Van Dievel et al. 2016), largely owing to the fact that a holistic approach is rarely implemented when assessing physiological responses to risk. Doubtless, this type of integrative analysis should become the accepted standard, when possible, if we aim to understand the breadth of risk-related responses in nature (Boonstra et al. 1998; Clinchy et al. 2011).

A further constraint to our understanding of physiological responses to predation risk is that most research has been conducted in the laboratory using artificial stimuli or risk exposure levels that may induce changes that are not reflective of natural responses (Korpimäki and Krebs 1996, Skelly

and Kiesecker 2001; Srivastava et al. 2004). In addition, there are few cases where prey physiological change has been addressed experimentally in the wild, in part because of the logistical difficulties doing so (Korpimäki and Krebs 1996; Srivastava et al. 2004). Therefore, combining robust, comprehensive physiological assessment with more realistic risk manipulation should better elucidate the effects of risk-related responses in natural prey populations.

We conducted a field experiment manipulating predation risk in free-ranging snowshoe hares (*Lepus americanus*) to assess prevalence and intensity of their physiological response in a natural setting. Snowshoe hare populations undergo regular 8–11-year cyclic fluctuations, with up to 100% of proximate causes of deaths during cyclic declines being attributable to predation, mainly from terrestrial carnivores such as lynx (*Lynx canadensis*) and coyotes (*Canis latrans*; Hodges et al. 2001), whose numbers also correspond to hare cycles (Boutin et al. 1995). Hare stress physiology seems to fluctuate with cyclic propensity, with higher free plasma cortisol, lower cortisol-binding capacity, higher glucose levels, and greater variation in leukocyte numbers occurring during cyclic declines (Boonstra et al. 1998; Sheriff et al. 2011). Predation risk also elicits higher fecal cortisol metabolites in captive hares (Sheriff et al. 2009a) and condition indices, such as hematocrit (Boonstra et al. 1998; Sheriff et al. 2011) and physical structure (Hodges et al. 1999; Murray 2002), decline with increased predation pressure or diminished food supply. However, attempts to establish causal links between variation in hare predation risk perception and corresponding changes in stress physiology or condition indices have been inconclusive, largely owing to a correlational approach in past studies (see Boonstra et al. 1998; Boonstra and Singleton 1993; Sheriff et al. 2011). We, therefore, collectively examine the breadth of potential stress responses and changes in condition by experimentally increasing risk in free-ranging hares. As changes in risk-related physiological metrics may be inconsistent across species (e.g., Zanette et al. 2014), we provide hare-specific predictions in Table 1.

Table 1 Predicted outcomes for stress and condition-related metrics for snowshoe hares under risk augmentation compared to controls

Metric	Index of	Predicted outcome under increased risk
Fecal cortisol metabolites	Stress	Increase
Free plasma cortisol	Stress	Increase
Cortisol-binding capacity	Stress	Decrease
Leukocyte ratios	Stress	Increased skew
Glucose	Stress	Increase
Hematocrit	Condition	Decrease
Structural Index	Condition	Decrease

Methods

Study site

Our study was conducted near Kluane Lake in southwestern Yukon, Canada (61°58'N, 138°12'W) on two study areas located ~5 km apart (hereafter referred to as “eastern” and “western” sites). The study region is within the rain shadow of the St. Elias Mountains, receiving < 30 cm of precipitation a year, mostly as rain during summer (Environment Canada 2017). Summer (June–August) and winter (November–February) temperature average 12 °C and –17 °C, respectively (Environment Canada 2017). The region is dominated by white spruce (*Picea glauca*), with a mixed understory of gray willow (*Salix glauca*), bog birch (*Betula glandulosa*), soapberry (*Shepherdia canadensis*) and other herbaceous plants (Krebs et al. 2001). Local land use is focused around mining and recreation (Krebs et al. 2001); however, anthropogenic disturbance at our sites was minimal. Our study coincided with the increase-peak phase of the snowshoe hare cycle in the Kluane region, and during our 2-year study (2015–2016) hare densities increased 1.85-fold, reaching a maximum of 1.82 ± 0.35 hares/ha in fall 2016 (Krebs et al. 2018). Concurrently, predator population indices, based on snow tracking, increased 1.04-fold (average for coyote and lynx; CEMP 2017).

Live trapping

From April to October 2015 and 2016, we live-captured snowshoe hares at each site (Tomahawk Live Trap Co., Tomahawk, WI, USA). Live trapping occurred during either the last 2 weeks of each month or until all relevant data had been collected, whichever came first (mean = 6 nights/month). We pre-baited traps for 3 days with apple and a mixture containing rabbit chow, oats, strawberry jam, and molasses. Live-traps were set and checked overnight on an 8-h schedule. Given the 8–12 h transit time for fecal steroids in snowshoe hares (Sheriff et al. 2009a), our trapping protocol avoided collection of feces that could reflect capture stress. Hares were ear-tagged at first capture (National Band and Tag Co., Newport, KY, USA) and at each capture were weighed, sexed, assessed for reproductive condition (males: scrotal/non-scrotal; females: pregnancy/lactation/non-pregnancy; Keith et al. 1968) and a right hind foot measure was taken. Live trapping and handling procedures followed established guidelines (Sikes et al. 2011) and were approved by the Trent University Animal Care Committee (protocol 23373).

Experimental manipulation

Each year, adult hares (> 1000 g) that were caught > 2 times during the initial live-trapping session were equipped with radio collars with mortality sensors (Wildlife Materials, Murphysboro, IL, USA and Telemetry Solutions, Concord, CA, USA) and attached GPS units (Gypsy 5, Technosmart, Rome, Italy). After collaring, we gathered hare location information hourly via VHF triangulation over several days (2015) or using GPS telemetry (2016; Gypsy 5, Technosmart, Guidonia, Rome, Italy). For both years, we used a minimum of 30 locations from a 48-h period to create minimum convex polygons which reflected established home ranges. Polygons were used to assign individuals to experimental groups, with animals located in the same general area receiving a common treatment (i.e., either control or predator-exposed). Site proximity makes it reasonable to expect that all study animals were exposed to similar baseline predation risk, and ancillary analysis revealed similar habitat characteristics in hare home ranges across treatment groups (M. Boudreau, unpubl.).

Hares were exposed to simulated predation attempts using a trained domestic dog (*Canis familiaris*) as a model for coyotes (see Sheriff et al. 2009a, b). Coyotes are an important predator of hares in our area (O'Donoghue et al. 1998), and our dog (an Australian cattle dog) was comparable to a coyote in terms of size and morphology. During June–September (2015) and May–September (2016), hares in the treatment group were chased with the dog (off leash) on random days/times three times per week. Due to logistical and safety constraints, we conducted chases only during daylight hours. While this did not allow us to directly match predator activity (which occurs throughout a 24-h period; E. Studd unpubl.), due to prolonged daylight during the summer months, which in our study area spanned 03:30 to 22:00 in June and 06:30 to 19:00 in September, we were able to minimize chase bias across a 24-h period. As treatment groups were only spatially separated by 200–300 m on each study site, we used telemetry to estimate control hare proximity to risk-augmented individuals and ensure no overlap of our treatment groups prior to chase initiation. In cases when risk-augmented and control hares were in close proximity (i.e., < 100 m; this occurred 15 and 4 times in 2015 and 2016, respectively), a second attempt was tried when animals were again spatially separated. Working in teams of two, one individual used telemetry to locate and approach the target hare within 20 m, while the other directed the dog to the hare. On average, chases lasted ~20–60 s until a hare response was logged via VHF telemetry (i.e., change in signal strength or direction, indicating movement of the hare) and the dog was then retrieved. The dog never successfully captured or made physical contact with a hare ($N = 1800$ chases). Individual hares received, on average, 32.4 (range

15–43) chases during June–September 2015 and 45.8 (range 30–59) chases during May–September 2016.

Stress metrics

Fecal cortisol metabolites

We obtained hare fecal samples by collecting pellets deposited beneath live-traps by recently captured animals. Fecal samples were not contaminated by urine and used to index free endogenous plasma cortisol levels (Sheriff et al. 2010). During initial collaring (i.e., prior to the initiation of risk exposure; May 2015 and April 2016), the first fecal sample collected for each hare was taken as a measure of pre-chase cortisol levels. During months when chases occurred (i.e., June–September 2015 and May–September 2016), we collected > 20 fecal samples from recaptured individuals per treatment group, per month. Samples were stored at -20°C within 2 h of collection, lyophilized for a minimum of 12 h (see Sheriff et al. 2009a; LabConco, Kansas City, MO, USA) and then homogenized using liquid nitrogen and mortar and pestle. We then extracted hormone metabolites from 50.0 ± 2.0 mg of ground feces using 0.5 ml of 80% methanol (v/v) for 30 min at 1450 rpm on a multi-tube vortexer. After centrifugation for 15 min at 2500 g (Eppendorf Centrifuge 5810 R, Mississauga, ON, Canada), the supernatant was stored at -80°C . Fecal cortisol metabolite concentrations were measured using an 11-oxoetiocholanolone enzyme immunoassay protocol developed by Palme and Möstl (1997) and validated for snowshoe hares by Sheriff et al. (2009a). As samples were analyzed in the fall of the associated year to prevent sample degradation, a quality control created from pooled sample extract ($n = 50$) was included to ensure comparability. Intra- and inter-assay variability was < 11% and < 15%, respectively.

Hormone challenges

Fecal cortisol metabolites should be indicative of risk exposure over the course of the experimental manipulation; however, this metric can be influenced by a variety of factors (Goymann 2012), and as such it may be less accurate for measuring adrenocortical reactivity compared to alternative methods; hormone challenges allow for an integrated picture of an individual's physiological past while over-riding capture and handling stress. We used hormone challenges to infer cumulative stress of hares through an integrated time period (Boonstra et al. 1998; Sheriff et al. 2011). In October, 1 week after chases had ceased, and when hares were in a post-reproductive state, males ($n = 22$) and females ($n = 16$) from each treatment group were subjected to a hormone challenge ($n = 10$ and $n = 12$ hares from each treatment group for 2015 and

2016, respectively; protocol from Boonstra et al. 1998). Upon capture, we transported hares to a nearby laboratory where they were acclimated for 2 h (per Boonstra et al. 1998). Each individual was bled 5 times (0.5 ml for the first bleed and 0.3 ml thereafter) from an ear artery using a heparinized syringe with 29G $\frac{1}{2}$ in needle (Ideal U-40 Insulin Syringes). The first blood sample (initial bleed) was followed by a 0.4 mg/kg injection of dexamethasone sodium phosphate (i.e., Dexamethasone; Vetoquinol Inc., Lavaltrie, Quebec, Canada; Boonstra et al. 1998) into an ear vein. The dexamethasone bleed occurred 2 h later and assessed feedback inhibition of cortisol secretion in response to dexamethasone. It was followed immediately by an intramuscular thigh injection of 40 $\mu\text{g}/\text{kg}$ of synthetic adrenocorticotrophic hormone (ACTH; Cortrosyn, Amphistar Pharmaceuticals Inc., Rancho Cucamonga, CA, USA). The three remaining bleeds assessed the capacity of adrenals to respond to ACTH and occurred 30, 60 and 120 min post-ACTH injection (P30, P60 and P120 bleeds, respectively). We centrifuged blood samples at 8800 g for 10 min (Micro Centrifuge 5413, Eppendorf, Mississauga, ON, Canada) and the plasma was frozen at -80°C .

For each bleed, we measured glucose, total cortisol, and maximum corticosteroid-binding capacity (i.e., the extent to which there can be bound cortisol in the blood). Increased glucose levels throughout the challenge may indicate increased capacity for liver gluconeogenesis due to chronic stress (Miller and Tyrrell 1995; Boonstra et al. 1998). Glucose was measured within 5 min of sample collection (Free-Style Glucometer, Abbott Diabetes Care, Mississauga, ON, Canada). Total plasma cortisol and cortisol-binding capacity were measured in duplicate using a radioimmunoassay with total plasma cortisol measured using an I^{125} RIA Kit (MP Biomedical ImmuChem-Coated Tube Cortisol I^{125} RIA Kit, Orangeburg, NY, USA) and binding capacity measured using an H^3 protocol (Boonstra and Singleton 1993). To ensure comparability, a quality control created from pooled plasma was included in all assays; intra- and inter-assay variability was < 13% and < 10%, respectively. Free cortisol concentrations were calculated according to Boonstra et al. (1998).

Increased liver gluconeogenesis should be achieved at the cost of peripheral tissues, leading to declines in condition that should be indicated by lower structural mass relative to size (Boonstra et al. 1998; Silverman and Sternberg 2012). Changes in condition should, therefore, also be reflected in other condition indices (i.e., low hematocrit levels; Franzmann and Leresche 1978; Hellegren et al. 1993). We used our measure of mass relative to the right hind foot as our structural condition index (Murray 2002), and for the first blood sample (prior to centrifugation), we measured

hematocrit levels in duplicate and averaged values for our physiological condition index.

Hares under chronic stress have higher neutrophil to lymphocyte (N:L) ratios, although similar ratios may be achieved through infections (Sheriff et al. 2011). Accordingly, both eosinophil to lymphocyte (associated with parasite load; E:L) and monocyte (associated with bacterial infection; M:L) ratios should differentiate stress from infection. We created smears from the initial blood sample, which were then fixed in methanol and dried and stained using a modified Wright stain technique (Diff-Quick, Dade International, FL, USA). The first 100 leukocytes were recorded for each smear and then averaged for each hare (Sheriff et al. 2011).

Statistical analysis

For all fecal cortisol metabolite (FCM) data, samples which potentially captured trapping stress (i.e., were taken past the 8-h fecal transit time; $n = 30$) or hare injury ($n = 4$) were excluded. To examine the site effects, FCM values from different sites obtained for the same treatment group day were analyzed using a (treatment \times year) linear mixed model with month nested within year. As there was no effect of the site on FCM values ($F_{1,36} = 0.39$, $P = 0.54$), site was excluded from subsequent FCM analyses. Pre-chase samples were used to see if groups differed significantly before the risk exposure treatment was initiated. Pre-chase and chase FCM were analyzed using a (year \times treatment \times sex) linear mixed model with the chase FCM model containing month nested within year.

We examined male hare structural condition using the residuals of a Model I regression between average monthly mass and the right hind foot measure (controls $n = 22$ and risk-augmented $n = 30$); females were excluded because pregnancy confounded their mass measurements (Murray 2002). As hares came from separate sites, we tested for an effect of site on structural condition and as there was no effect of site (linear mixed model: $P = 0.81$), data were pooled. Structural condition was examined using a (year \times treatment \times month) linear mixed model. All linear mixed models had hare ID as a random factor to account for multiple samples from the same individual.

Area under the curve (AUC) was calculated for total cortisol values of the individuals in each treatment group using the equation:

$$\text{AUC} = (30 * (\text{P30} + \text{P60})/2 + 30 * (\text{P60} + \text{P120})/2 - (60 * \text{P30})) - (\text{P120} - \text{Dex}),$$

with Dex, P30, P60 and P120 referring to the total cortisol value for the corresponding Dexamethasone and post-ACTH bleeds (Delehanty and Boonstra 2011). Comparison of AUC can be used to examine the possibility that higher amounts

of plasma cortisol between treatment groups were related to increased sensitivity of adrenals to ACTH or increased capacity of adrenals to produce cortisol (Delehanty and Boonstra 2011). A multivariate ANOVA (MANOVA) was used to examine leukocyte ratios and a separate MANOVA was used to assess responses of blood components (free plasma cortisol, cortisol-binding capacity, and glucose). Significance was examined using Pillai's trace which is more robust to unbalanced datasets (Scheiner 2001). If a significant result was detected, separate univariate ANOVAs were performed. Each leukocyte ratio was examined using an ANOVA, while univariate analyses of free plasma cortisol, cortisol-binding capacity, and glucose were conducted using a repeated-measures ANOVA, with "time" being the response over the five bleeds, which was added as a within-subject factor (e.g., Boonstra et al. 1998). Due to the non-independence between bleeds, a conservative Greenhouse–Geisser epsilon correction was applied (e.g., Boonstra et al. 1998). An ANOVA was used to examine AUC and hematocrit values. All MANOVAs and ANOVAs included year \times treatment \times sex as main effects. For all hormone challenge data, only 6 hares from the risk-augmented group in 2016 came from the western site, all other hares were located on the eastern site; visual examination of means and standard errors indicated no significant effects of site across metrics.

We used a log-rank test (Harrington and Fleming 1982) to compare survival between treatment groups. Fecal cortisol metabolite data and leukocyte ratios were log-transformed, and area under the curve and structural condition values were square-root transformed to meet the assumption of normality. Comparisons of the means were considered significant if $P < 0.05$, and values of $0.05 < P < 0.10$ were considered marginally significant. Effect sizes were calculated as eta-squared (Olejnik and Algina 2003). All ANOVA analyses were conducted using a type III sum of squares and all analyses were performed in R v. 1.0.143 (R Core Team 2013).

Results

In total, we monitored hare physiology in 56 and 53 individuals during 2015 and 2016, respectively. In 2015, we had 15 (8 female; 7 male) and 17 (9 female; 8 male) controls on the eastern and western sites, respectively, and in 2016 we had 24 controls (15 female; 9 male) on the eastern site. In 2015, we exposed 24 hares to predator chases (10 female; 14 male) on the eastern site, and in 2016, 15 (7 female; 8 male) and 14 (9 female; 5 male) hares on the eastern and western sites, respectively. Twenty-one hares were killed by predators during the study (2015: 7 controls and 5 risk-augmented; 2016: 4 controls and 5 risk-augmented), and 30-day

survival probabilities were comparable between treatment groups [treated: 0.95 ± 0.15 (95% CI); control: 0.94 ± 0.17 ; Log-rank test: $\chi^2 = 0.8$, $P = 0.38$], suggesting that our experimental treatment did not have a direct effect on hare survival and thus was appropriately considered as strictly sublethal.

Fecal cortisol metabolites (FCM)

Pre-chase fecal cortisol metabolite levels did not differ between treatments or years (Table 2), indicating that individual hares had similar starting conditions prior to risk exposure (Fig. 1a). Males had, on average, $18.7 \pm 1.3\%$ (mean \pm SE) more FCM compared to females before chases began (Table 2), and this was different between years for each sex, with males having $29.1 \pm 2.3\%$ and $3.4 \pm 1.6\%$ more FCM compared to females in May 2015 and April 2016, respectively (Table 2). During the chase period, overall FCM did not increase due to increased risk exposure (Table 2, Fig. 1b). Fecal cortisol metabolites were also comparable between sexes (Table 2). However, overall FCM levels were marginally higher ($7.1 \pm 1.4\%$) in 2016 compared to 2015 (Table 2).

Plasma cortisol and adrenal function (AUC)

Overall, blood components were affected both by the predation risk treatment (MANOVA: $F_{1,175} = 10.60$, $P < 0.001$) and by year ($F_{1,175} = 126.32$, $P < 0.001$), as well as a significant time \times treatment interaction ($F_{1,175} = 2.68$, $P = 0.002$). We found that free plasma cortisol concentrations for risk-augmented hares were, on average, $25.8 \pm 7.6\%$ higher compared to controls over the course of the challenge (Table 3; Fig. 2a). Free cortisol concentrations changed appropriately over the course of the hormone challenge (i.e., decrease in response to Dexamethasone and increase in response to ACTH; see Table 3 (time), Fig. 2a). We also noted inter-annual variability, with overall free cortisol levels being

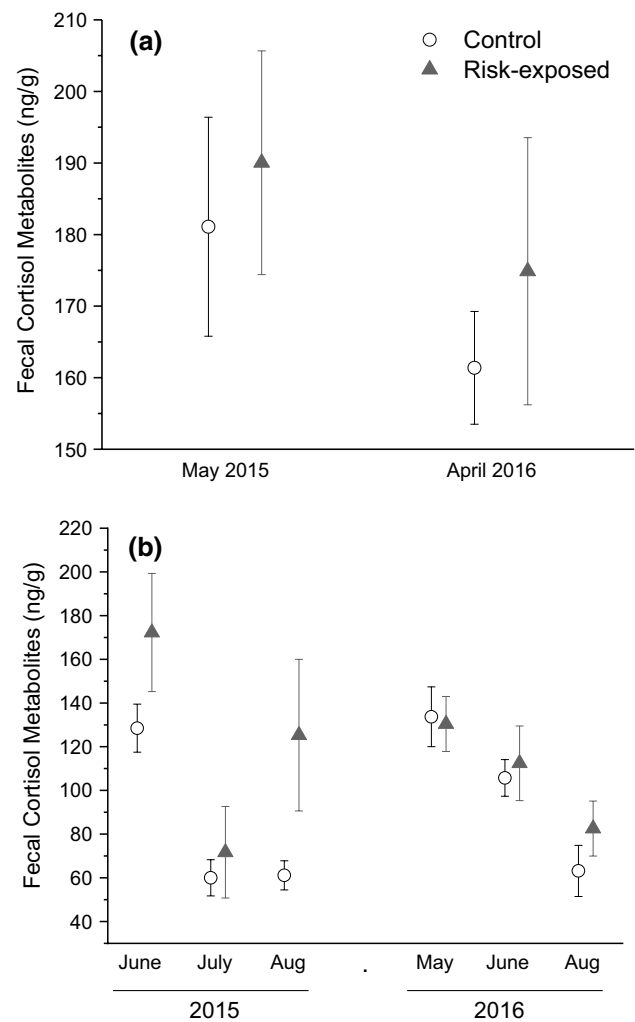


Fig. 1 Fecal cortisol metabolite concentrations (mean \pm SE) of snowshoe hares exposed to predation risk versus controls; **a** before and **b** during the predator chase period

Table 2 Effects of experimental increase in predation risk exposure (treatment) on snowshoe hare fecal cortisol

	Pre-chase period fecal				Chase period fecal			
	η^2	df	F	P	η^2	df	F	P
Treatment	0.001	1, 56	0.34	0.560	0.001	1, 165	0.09	0.765
Year	0.002	1, 56	0.38	0.536	0.200	1, 176	3.04	0.083
Sex	0.038	1, 56	7.72	0.006	0.005	1, 169	0.01	0.916
Treatment \times year	0.001	1, 56	0.72	0.601	0.019	3, 174	0.01	0.934
Treatment \times sex	< 0.001	1, 56	0.08	0.785	0.014	3, 182	0.01	0.908
Year \times sex	0.028	1, 56	5.69	0.018	0.021	3, 173	0.07	0.785
Treatment \times year \times sex	< 0.001	1, 56	0.98	0.977	0.033	3, 169	0.14	0.710

F values, P values, effect sizes, and degrees of freedom are for the univariate analyses for fecal samples taken before chases were conducted (May 2015 and April 2016) and during the chase period (June–September 2015 and May–September 2016)

Bold values indicate the significant effect

Table 3 Effects of experimental increase in predation risk exposure (treatment) on glucose, maximum cortisol-binding capacity and free plasma cortisol in snowshoe hares

	Glucose				MCBC				Free plasma cortisol			
	η^2	<i>df</i>	<i>F</i>	<i>P</i>	η^2	<i>df</i>	<i>F</i>	<i>P</i>	η^2	<i>df</i>	<i>F</i>	<i>P</i>
Treatment	0.068	1	4.47	0.04	1.4e–5	1	0.01	0.93	0.007	1	1.93	0.17
Year	0.243	1	19.63	<0.001	0.224	1	11.43	0.001	0.541	1	145.53	<0.001
Treatment×year	0.0003	1	0.02	0.88	0.004	1	0.22	0.64	0.004	1	1.10	0.31
Subject (time)		40				35				35		
Time	0.410	4	80.51	<0.001	0.544	4	39.50	<0.001	0.255	4	49.25	<0.001
Time×treatment	0.009	4	1.07	0.36	0.187	4	13.59	<0.001	0.055	4	2.77	0.03
Time×year	0.109	4	14.08	<0.001	0.020	4	1.47	0.22	0.181	4	34.91	<0.001
Time×treatment×year	0.0007	4	0.08	0.97	0.020	4	1.48	0.22	0.005	4	1.02	0.40
Error		160				140				140		

F values, *P* values, effect sizes, and degrees of freedom are for the univariate analyses for each physiological measure

Bold values indicate the significant effect

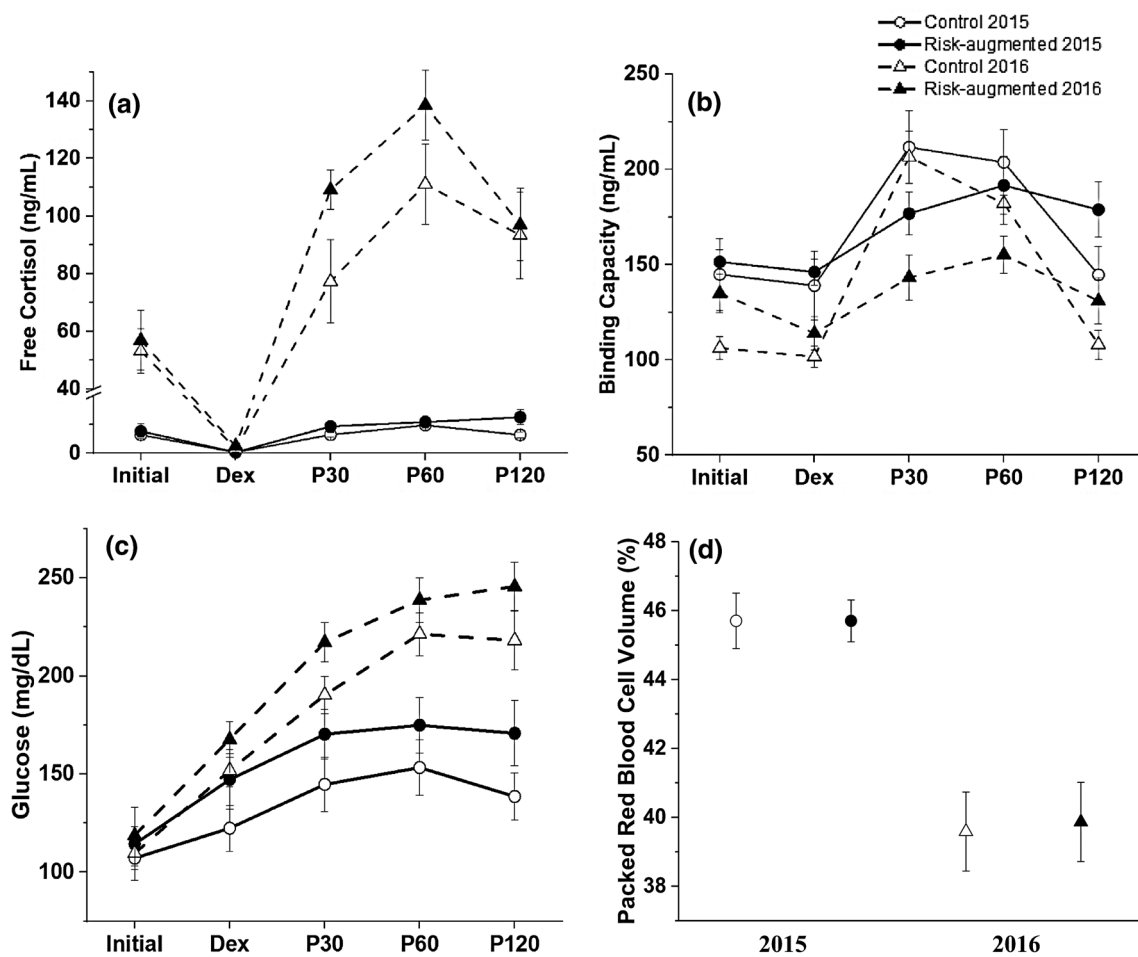


Fig. 2 Plasma concentrations (mean \pm SE) of snowshoe hares subjected to hormone challenges following exposure to predation risk versus controls in the wild. The x-axis refers to an initial bleed, after injection of dexamethasone (Dex) as well as blood collections

at $t=30, 60$, and 120 min post-ACTH injection. We report: **a** free plasma cortisol, **b** maximum corticosteroid-binding capacity, **c** glucose levels, and **d** hematocrit levels

Table 4 Effects of experimental increase in predation risk exposure (Treatment) on the area under the response curve (AUC) for total cortisol and hematocrit in snowshoe hares

	AUC				Hematocrit			
	η^2	df	F	P	η^2	df	F	P
Treatment	0.001	1	0.15	0.701	0.001	1	0.03	0.859
Year	0.993	1	119.22	< 0.001	0.940	1	32.23	< 0.001
Sex	0.006	1	0.66	0.421	0.059	1	2.01	0.164
Treatment \times year	< 0.001	1	0.04	0.849	< 0.001	1	0.01	0.930
Error		36				39		

F values, P values, effect sizes, and degrees of freedom are given

Bold values indicate the significant effect

Table 5 Effects of experimental increase in predation risk exposure (treatment) on neutrophil to lymphocyte ratios (N:L), monocyte to lymphocyte ratios (M:L), and eosinophil to lymphocyte ratios (E:L) in snowshoe hares

	N:L				M:L				E:L			
	η^2	df	F	P	η^2	df	F	P	η^2	df	F	P
Treatment	0.283	1	15.79	< 0.001	0.028	1	1.15	0.289	0.007	1	0.30	0.586
Year	0.552	1	49.27	< 0.001	0.053	1	2.22	0.144	0.549	1	48.61	< 0.001
Treatment \times year	< 0.001	1	0.0003	0.985	0.054	1	2.30	0.137	0.085	1	3.70	0.062
Error		40				40				40		

F values, P values, effect sizes, and degrees of freedom are for the univariate analyses for leukocyte ratios

Bold values indicate the significant effect

90.4 \pm 5.3% higher in 2016 compared to 2015 (Table 3, Fig. 2a).

Free cortisol concentrations were influenced by cortisol-binding capacity, and risk-augmented hares had, on average, a 15.9 \pm 13.5% lower cortisol-binding capacity compared to controls over the course of the challenge (Table 3, Fig. 2b). Cortisol-binding capacity varied appropriately over the course of the hormone challenge (i.e., no response dexamethasone and increase post-ACTH; Table 3 (Time), Fig. 2b). We also noted a 19.0 \pm 6.9% lower cortisol-binding capacity in 2015 compared to 2016 (Table 3, Fig. 2b). Plasma-free cortisol differed between the treatment groups (Fig. 2a), although AUC scores were comparable, indicating that adrenal sensitivity and capacity were unaffected by the experiment (Table 4). This implied that changes in negative feedback mechanisms were likely driving observed differences in free plasma cortisol. However, AUC values were 58.9 \pm 6.6% higher in 2016 (Table 4), indicating increase in either ACTH sensitivity or cortisol production capacity of the adrenals.

Immunology

Leukocyte ratios were affected by both risk treatment (MANOVA: $F_{1,40}=6.18$, $P=0.002$) and year ($F_{1,40}=21.67$, $P<0.001$), with univariate analyses revealing that N:L ratios varied by both treatment and year, as evidenced by an increased skew from increased risk exposure through time (Table 5; Fig. 3). However, there were no corresponding effects of higher risk exposure on M:L or E:L ratios

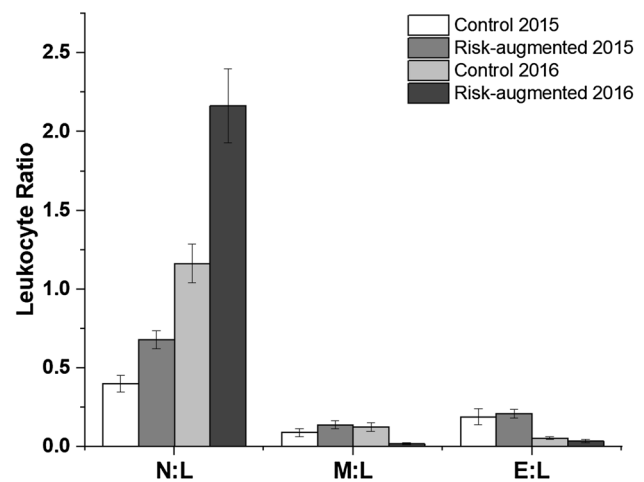


Fig. 3 Leukocyte ratios (mean \pm SE) (neutrophil: lymphocyte (N: L), monocyte: lymphocyte [M: L], and eosinophil: lymphocyte (E: L)) of snowshoe hares exposed to predation risk versus controls

(Table 5; Fig. 3). Thus, the increase in neutrophils relative to lymphocytes is directly related to the stress of increased risk exposure rather than infection. There was no annual change in monocytes (Table 5), although we did observe a 75.2 \pm 0.3% decrease in the E:L ratio between years (Table 5; Fig. 3). While there was annual variation in the eosinophil to leukocyte ratio (which is related to parasite load), their change contrasted to the pattern of change for N:L ratios, implying that the N:L variation was not due to parasitic infection.

Glucose mobilization and condition

On average, risk-augmented hares had $10.4 \pm 7.7\%$ more glucose (Table 3, Fig. 2c). This effect was consistent across years, with hares on average mobilizing $22.6 \pm 7.2\%$ more glucose in 2016 compared to 2015 (Table 3, Fig. 2c). Glucose levels increased over the course of the challenge (i.e., both hormones increased glucose; Table 3, Fig. 2c). The time \times year interaction (Table 3) highlighted inter-annual variation in the magnitude of the glucose response (Fig. 2c).

Hematocrit levels were unaffected by increased risk exposure (Table 4) and declined from 2015 to 2016 ($13.0 \pm 1.0\%$ lower; Table 4; Fig. 2d), indicating yearly changes in physiological condition. Male hare structural condition was not affected by risk augmentation ($\eta^2 = 0.011$, $F_{1,103} = 0.13$, $P = 0.92$); however, monthly increases in structural condition were observed during both summers ($\eta^2 = 0.837$, $F_{1,103} = 9.20$, $P = 0.003$; Fig. 4).

Discussion

We found that following exposure to experimental increase in predation risk, snowshoe hares exhibited marked hormonal, energetic, and immunological changes that were indicative of a markedly heightened stress response. Treatment effects on hare stress physiology intensified during the second summer; however, increase in free plasma cortisol did not translate to intra-annual changes in either condition index, likely implying that hares compensate for heightened stress through behavioral or metabolic responses. This study extends previous work (e.g., Hik et al. 2001; Clinchy et al. 2011; Seiter 2011) by including a suite of physiological metrics that more fully assess the stress responses of prospective prey in field conditions, and our results establish a basis for

more detailed investigation into the suite of potential physiological responses to high stress and how individuals may compensate for higher risk to avoid compromised condition.

For vertebrates, the stress axis is catabolic and energetically demanding, meaning that continuous environmental challenge increases free plasma cortisol, which can influence downstream resource allocation to immunity, energy stores, and overall condition (Boonstra et al. 1998; Liesenjohn et al. 2013; Zanette et al. 2014). Taken individually, risk-augmented hares exhibited responses that are characteristic of those reported previously, including elevated free plasma cortisol (e.g., Clinchy et al. 2013), lower cortisol-binding capacity (e.g., Breuner et al. 2006), skewed immunological profiles (e.g., Hinam and St. Clair 2008), and higher glucose levels (e.g., Clinchy et al. 2013; Boonstra et al. 1998). These metrics provide an indication that hare stress physiology was affected by predation risk, although it is noteworthy that the free plasma cortisol metric in particular is an especially powerful indicator of stress capacity as it is crucial in stress axis feedback mechanisms (Boonstra et al. 1998) and has a strong relationship with steroid-binding proteins (i.e., corticosteroid-binding globulins; see Breuner and Orchinik 2002). Extended free plasma cortisol exposure has long-term influence on a variety of processes due to energy reallocation for stress axis support (Boonstra et al. 1998; Silverman and Sternberg 2012), and downstream consequences include alterations to other physiological processes, such as immune system function (e.g., Fast et al. 2008) and skeletal mass (Silverman and Sternberg 2012). Accordingly, elevated free plasma cortisol should portend a variety of risk responses, and thus lack of ancillary responses, such as that seen with our condition indices, may indicate either that risk exposure was not sufficiently strong or prolonged to enact downstream consequences, or that compensatory responses to stress were activated.

Compensatory responses to increased risk are not uncommon and may be enacted via a behavioral or metabolic mechanism. For example, kangaroo rats (*Dipodomys merriami*; Daly et al. 1992) and snowshoe hares (Griffin et al. 2005) both lower their activity in association with increased risk on moonlit nights, while grasshoppers (*Ageneotettix deorum*; Oedekoven and Joern 2000) feed on higher quality plants when under higher risk of predation. Our comprehensive physiological assessment revealed that hematocrit, which is normally considered a general physiological condition index (Franzmann and Leresche 1978; Hellegren et al. 1993; Boonstra et al. 1998), was unaffected by increased risk exposure. Likewise, structural condition increased throughout the summer months irrespective of the experimental treatment. It is evident that condition indices should show deterioration when the organism is in a sustained catabolic state (Silverman and Sternberg 2012); however, as prey often exhibit plasticity in their responses to risk perception, it is plausible

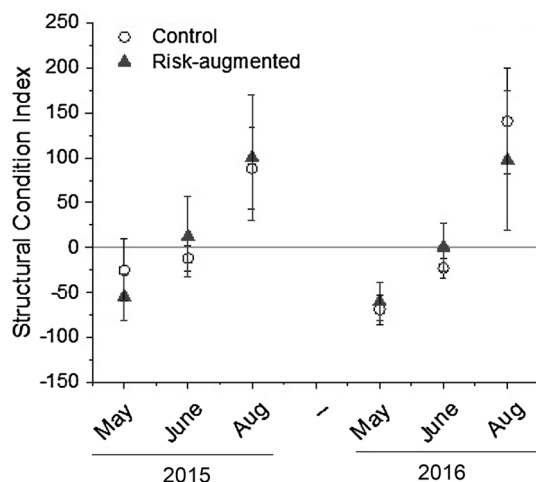


Fig. 4 Male structural condition (mean \pm SE) for snowshoe hares exposed to predation risk versus controls

that risk exposure elicited increases in food intake or food selectivity of hares (e.g., Schmitt and Holbrook 1985). Likewise, risk exposure may have prompted reduced activity (e.g., Griffin et al. 2005) or the use of specific habitats associated with lower risk (e.g., Sweitzer and Berger 1992) in an effort to mitigate risk-related physiological costs. Thus, it is entirely plausible that compensatory behavioral responses contribute to maintenance of hare condition under high risk.

Alternatively, similar, presumed compensatory, responses have been observed in a set of studies involving metabolic responses to risk. For example, Thaler et al. (2012) found that hornworms (*Manduca sexta*) increased assimilation efficiency relative to risk in the environment, whereas Van Dievel et al. (2016) found that damselflies (*Enallagma cyathigerum*) altered metabolic rates and energy stores in the face of risk alteration; both species expressed these responses through increased structural size, implying that risk exposure may elicit metabolic alterations. However, it is notable that the above studies were conducted under laboratory conditions, where risk manipulation often lacks an analogous stimulus intensity and experimental context to that expected in a natural setting (e.g., Korpimäki and Krebs 1996; Hossie et al. 2017). In our study, risk-augmented hares could have invoked a variety of behavioral and/or metabolic responses to treatment and it seems plausible that these responses could be more pronounced during summer, when food and cover are abundant and predation-related mortality tends to decline (Sinclair et al. 1988; Hodges et al. 2001). Indeed, under such conditions hares may experience increased plasticity, allowing them to adjust their behavior or metabolism in response to heightened risk. In contrast, during winter, food resources are more limited (Sinclair et al. 1988), predation rates (and presumably risk perception) are higher (Hodges et al. 2001), and thus hares may be especially constrained in their ability to mount compensatory responses to risk augmentation. It follows that further research should address whether increased risk exposure elicits compensatory responses in prey and whether such responses vary seasonally.

The observed yearly change in hormonal, energetic, and immunological responses corresponded to observed changes in local predator density (Krebs et al. 2018). While temporal variation in physiological responses may have reflected changes in the risk environment for hares, observed intra-annual compensatory responses on hare condition indices did not occur for yearly changes in hematocrit, a primary indicator of condition (Franzmann and Leresche 1978; Hellegren et al. 1993; Boonstra et al. 1998). Similar yearly variation in physiological stress responses have been observed in other hare studies (e.g., Boonstra et al. 1998), with food addition or risk removal alone failing to fully overcome yearly variability in hematocrit (see Boonstra et al. 1998). In contrast, we found no yearly differences in structural

condition, a metric which has been shown to fluctuate with alterations in food resources and risk (Hodges et al. 1999; Murray 2002). Although both physiological and structural condition indices should be influenced by high free plasma cortisol levels (Silverman and Sternberg 2012), there is evidence that these measures are not directly correlated (e.g., Dawson and Bortolotti 1997) and that specific responses may vary across taxa. Further study of exactly how physiological and structural conditions are related and how they vary through temporally dynamic environments will contribute to our understanding of these measures.

Finally, there is a general consensus that the proper calibration of levels of risk exposure in studies such as ours represents a major challenge in understanding non-consumptive effects of predators on prey (see Korpimäki and Krebs 1996; Peers et al. 2018). In our study, free-ranging hares experienced increased stress from predation risk associated with both experimental and natural sources (e.g., variation in natural predation or food quality/quantity, Hodges et al. 2001; winter conditions, Sheriff et al. 2009b). It is notable that in our study the magnitude of the observed treatment response for each physiological metric was substantially less than its yearly variation, implying that our treatment level likely was biologically realistic because it captured a physiological response that was within the bounds of what hares experience in the real world. As such, our risk exposure treatment was sufficient to elicit a realistic physiological response under natural conditions. More broadly, we show that risk exposure experiments in free-ranging animals can provide robust insight into risk-related responses despite longstanding concerns over the realism of such treatments when deployed in an experimental context (e.g., see Hossie et al. 2017; Peers et al. 2018). Our ability to capture a biologically relevant response should thus serve as a guide for future risk augmentation experiments.

Future research striving to better understand animal responses to environmental stressors should redouble efforts to incorporate comprehensive physiological approaches using contextually relevant experimental manipulations. Indeed, these approaches may be crucial in helping develop a mechanistic understanding of how physiological changes translate to downstream behavioral, demographic, and population-level responses. These efforts may be especially important in taxa that experience little natural variation in environmental stressors through space and time (e.g., Rollins-Smith 2017) and, therefore, are particularly vulnerable to environmental change. Therefore, implementation of comprehensive, realistic field assessments complemented with a suite of physiological responses to environmental stressors should play a foundational role in understanding the cascade of potential responses to environmental stress, and how they may help elicit population- and ecosystem-level responses.

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Author contribution statement MRB, JLS and DLM conceived and designed the experiments. MRB and JLS performed the field experiments while MRB, JLS, RB and RP performed the laboratory work. MRB analyzed the data and MRB and DLM wrote the manuscript; all authors provided editorial advice.

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