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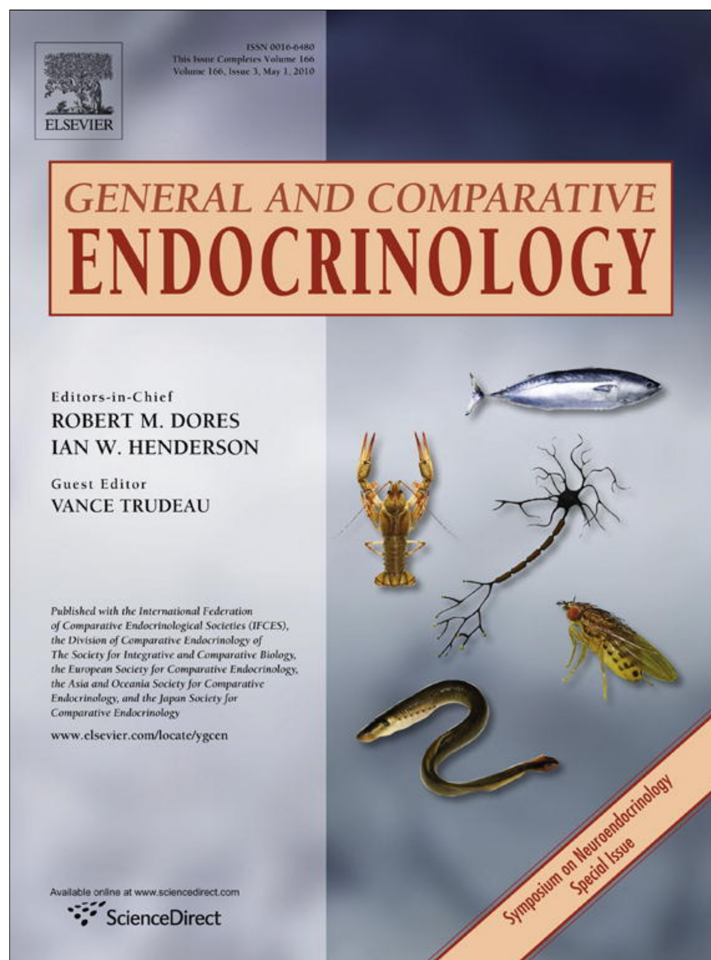

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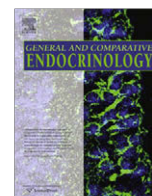

Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story?

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ABSTRACT

Many studies have recently focused on stress as a marker of an animal's well being. Since animals respond to a stressor by increasing their glucocorticoid (GC) levels there has been much interest in measuring these hormones. Fecal GC analyses have been used in a wide range of studies as they are an easily obtained, non-invasive measure of these stress hormones. However, these analyses rest on two major assumptions. First, they assume that fecal GC metabolites reflect free, biologically active levels of GCs in the plasma. Second, they assume that differences in fecal GC metabolite levels among animals are an accurate reflection of their physiological state and thus of their ability to respond to a stressor. We tested these assumptions in a population of free-ranging snowshoe hares (*Lepus americanus*) in the southwestern Yukon, from 2006 to 2008. Both assumptions were verified. Plasma free cortisol levels mirrored bile and fecal cortisol metabolite (FCM) levels, but plasma total cortisol levels did not. Differences in FCM concentrations among hares robustly predicted their response to a hormonal challenge. Hares with higher FCM concentrations showed a greater resistance to the suppression of their free plasma cortisol following a dexamethasone injection and a more marked increase of free plasma cortisol following an ACTH injection. Furthermore, we found that changes in FCM concentrations in autumn and winter over two years reliably tracked changes in plasma free cortisol levels obtained from the hormonal challenge test. These results indicate that both fecal and plasma measures of an animal's stress physiology are concordant: they tell the same story.

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1. Introduction

Understanding the effects of stress hormones is important to the study of natural populations. This knowledge can address questions about how stressors (any environmental perturbation that disrupts homeostasis) affect the survival and reproductive success of free-living animals and broader ones pertaining to management strategies, relocation or reintroduction, habitat disturbance, and population dynamics (Boonstra and Singleton, 1993; Wasser et al., 1997; Creel et al., 2002; Cyr and Romero, 2007; Sheriff et al., 2009a). When encountering a stressor, animals respond by increasing their glucocorticoid (GC) production, and this stress response is primarily mediated by the hypothalamic–pituitary–adrenal (HPA) axis (Owen et al., 2005). A number of techniques have been used to measure GC concentrations and these include sampling blood and feces.

Blood GC concentrations have been used as an index of stress in a wide range of studies (e.g. Boonstra et al., 1998; Hopster et al., 2002; Hackländer et al., 2003; Romero and Reed, 2005; Sheriff et al., 2009a; Kitaysky et al., 2007). Blood sampling can provide not only total GC concentrations but also the amount that is free. GCs are normally tightly bound to a carrier protein, corticosteroid-binding globulin (CBG), and only the free form (5–10% of the total) is biologically active (Rosner, 1990). However, to obtain free levels, it is necessary to measure both the CBG levels and to know the binding coefficients. Serial blood samples during restraint or hormone challenges allow an integrated picture of the responsiveness of an animal and thus an insight into what it has been experiencing in its recent past (Boonstra et al., 1998; Kenagy and Place, 2000; Romero and Romero, 2002; Kitaysky et al., 2007). However, blood sampling is invasive and capture, handling, and bleeding can cause a rapid increase in blood GC concentrations (within 3 min) and bleeding free-ranging animals within 3 min may not be possible (Romero and Romero, 2002). Furthermore, the increase in GC concentrations due to blood sampling may be undesirable, especially in reproductive studies in which increased GC concentrations may negatively affect reproductive fitness (Sheriff et al., 2009a).

An alternative method to assess stress levels in animals is the use of fecal GC metabolite concentrations (see reviews by Wasser et al., 2000; Goymann, 2005; Palme et al., 2005). This method has

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been used to investigate diurnal and seasonal patterns of GC levels, social and dominance interactions, the impacts of habitat degradation, transport stress, predator–prey interactions, the effects of maternal stress on reproduction, maternal effects, and population dynamics (Wasser et al., 1997; Kotrschal et al., 1998; Goymann et al., 1999; Palme et al., 2000; Touma et al., 2003; Bosson et al., 2009; Sheriff et al., 2009a). GCs are metabolized by the liver prior to excretion both through the urine and the feces via the bile (Taylor, 1971; Palme et al., 2005). It is assumed that only the free GCs (i.e. not bound to CBG) are degraded by the liver (Palme et al., 2005). Thus fecal samples provide an integrated hormone profile over time with less interference from acute stressors. The interpretation of fecal assays rest on two critical, untested assumptions: first, that fecal GC metabolites reflect free, biologically active, GC levels in the plasma; and second, that differences in fecal GC metabolite levels among animals are an accurate reflection of their physiological state and thus of their ability to respond to a stressor.

We tested both of these assumptions in a population of free-ranging snowshoe hares in the southwestern, Yukon, from 2006 to 2008. First, in a shot sample of hares we tested the assumption that only free cortisol (the major GC in hares – Boonstra et al., 1998) is metabolized by the liver, whereas CBG bound cortisol passes through the liver. Thus, the cortisol metabolite levels in the bile and feces should directly reflect free plasma cortisol concentrations but not total plasma cortisol concentrations. Second, in a sample of free-ranging hares we compared their fecal cortisol metabolite (FCM) concentration with their ability to respond to standardized hormone challenge (feces were collected prior to the hormone challenge). This hormone challenge consisted of a dexamethasone (Dex) suppression test followed by an adrenocorticotropic hormone (ACTH) stimulation test. Dex acts as an artificial glucocorticoid agonist that mimics endogenous cortisol through feedback inhibition of the HPA axis. In hares exhibiting greater Dex resistance, plasma cortisol levels do not fall as much as in hares where the feedback inhibition is operating normally (Axelrod and Reisine, 1984). The ACTH stimulation tests the responsiveness of the adrenals directly and acts to increase circulating blood cortisol concentrations (Miller and Tyrrell, 1995). Boonstra et al. (1998) has shown (in plasma free cortisol concentrations) that snowshoe hares under chronic stress are more Dex resistant and exhibit an increased response to the ACTH injection. Studies have shown that a Dex or ACTH injection leads to changes in FCM levels and this is part of the standard validation procedure for measuring FCM levels (Touma and Palme, 2005). However, it has not yet been shown that animals with greater FCM concentrations have fundamentally altered responses to these injections (Wasser et al., 2000; Mateo and Cavigelli, 2005; Sheriff et al., 2009b). We tested the assumption that FCM concentrations are indicative of an animal's responsiveness to a stressor (i.e. if an animal was compromised by chronic stress it will not respond as well as if it were not). Thus, hares with greater FCM concentrations should have a reduced response to the Dex suppression test (such that after the Dex injection cortisol levels should be greater) and a greater, prolonged, cortisol production in response to the ACTH stimulation test. To further assess the utility of measuring FCMs relative to plasma cortisol derived from the stress response to a hormonal challenge, we examined the seasonal changes in both measures in free-ranging snowshoe populations subject to different intensities of predation risk (Sheriff et al., 2009a).

2. Methods

2.1. Snowshoe hare natural history

Snowshoe hares are the smallest of the hare species world wide. In the southwestern Yukon, adults weigh 1200–1800 g. They are found throughout Canada and the northern parts of the U.S.A. They are most active at dusk and dawn when they do the majority of their feeding. Hares do not have nests or burrows and remain active throughout the year. In the boreal forest, hares have a 10-year population cycle that impacts the entire ecosystem. Hares can be subject to high predation risk, especially during the decline phase of their population cycle when over 95% die because of predation (Krebs et al., 1995).

2.2. Study area and context

This study was conducted in the boreal forest near the Arctic Institute Base at Kluane Lake in the southwestern, Yukon, Canada (60°57′ N, 138°12′ W). It took place during the hare peak (2006 – 0.92 hares per ha) and the first two years of the decline (2007 – 0.79 hares per ha and 2008 – 0.35 hares per ha – Sheriff et al., 2009a). Our research was approved by the University of British Columbia Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

2.3. Shot sample

To test whether free plasma cortisol levels were correlated to metabolized levels in the bile and feces we collected eleven adult snowshoe hares (9 female and 2 male, shot with a .22 caliber gun) between dusk and dawn (23:30–04:00 h) in July and at dawn (07:00–09:00 h) in October, 2006 (bile could only be collected from 8 individuals). Samples of blood (0.05–0.25 mL via heart puncture, within 3 min) and of bile (0.01–0.025 mL from the gall bladder) were immediately collected. Carcasses were stored in a fridge at 4 °C within 1 h of being shot at the Arctic Institute Base. Fecal samples (all feces within the distal colon) were taken within 8 h of being shot. All samples were frozen at −80 ° C within 1 h of collection at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at −80 °C until analyzed.

2.4. Animal trapping

To assess the relationship between FCM and the stress response to a hormonal challenge 32 adult snowshoe hares (26 females and 6 males) were live-trapped using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) in autumn (October) and winter (February and March) from 2006 to 2008. The traps were set at 22:00 h and checked at 06:00 h and thus hares could only be in the traps for a maximum of 8 h. This is important as the lag between the production of cortisol in the body and the appearance of its metabolites in the feces is between 8 and 12 h (Sheriff et al., 2009b). Thus fecal steroid levels should not reflect the stress of live-trapping. Trapping did not occur on nights that dropped below −20 °C.

Each hare was weighed with a Pesola spring scale (±10 g), its right hind foot (RHF) length measured (in duplicate) as an index of body size, an ear-tag was placed in its right ear (No. 3 Monel tags, National Band and Tag Co., Newport, Kentucky, USA), and its sexual condition assessed (i.e. scrotal or not in males, no females were pregnant at this time). A fecal sample was collected from below the trap prior to the hormone challenge. Hares were then transferred in burlap sacs to a quiet, dimly lit laboratory (heated to 5–10 °C in winter) at the nearby Arctic Institute Base at Kluane Lake. Hares were kept in the burlap sacs and allowed to habituate to the laboratory conditions for 1–2 h prior to the start of the hormone challenge. They were not fed throughout the experiment.

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2.5. Hormone challenge

Each hare was bled five times (0.3 ml per bleed) from an ear artery using 28 gauge needles (0.36 × 13 mm) and heparinised 0.5 ml syringes (Lo-Dose U-100 insulin syringes, Becton Dickinson and Company, New Jersey, USA). The first blood sample (base bleed) was immediately followed by an injection of 0.4 mg/kg of dexamethasone sodium phosphate (Sabex, Quebec, Canada) into an ear vein. The second bleed (Dex bleed) assessed the inhibition response to Dex and occurred 2 h later. It was followed immediately by an intramuscular injection in the thigh of 40 μg/kg of synthetic ACTH (Synacthen Depot, CIBA, Ontario, Canada). The remaining three bleeds assessed the stimulation response to ACTH and occurred 30, 60, and 120 min post-ACTH injection (called the P30, P60, and P120 bleeds, respectively).

Total plasma cortisol was measured in duplicate using a radioimmunoassay (Clinical Assays GammaCoat Cortisol ^{125}I RIA Kit, DiaSorin, Minnesota, USA) with an intra- and inter-assay coefficient of variation of 2.4% and 12.4%. Maximum corticosteroid-binding capacity (MCBC) levels were measured in duplicate using a radioimmunoassay described by Boonstra and Singleton (1993), with an intra- and inter-assay coefficient of variation of 2.6% and 4.9%. MCBC is a measure of the corticosteroid-binding globulin and free cortisol is the portion not bound by this carrier protein. Free cortisol concentrations were calculated using the procedures and binding coefficients outlined in Boonstra et al. (1998).

We assessed the integrated response of each hare to the ACTH stimulation from the time of the ACTH injection to the P120 bleed by calculating the area under the curve over this 2 h period. This is the cumulative measure of the entire response that accounts for both the peak levels as well as the duration, both of which are important in terms of effect on the free cortisol on the body (Dallman and Bhatnagar, 2001). Some statistical packages will calculate this measure (e.g. GraphPad's Prism), though we made our calculations with a simple geometry algorithm.

2.6. Bile and fecal cortisol metabolite assay

We used an enzyme immunoassay (EIA) to measure bile and fecal cortisol metabolite concentration, validated to measure FCM levels in snowshoe hares (Sheriff et al., 2009b). Within 1 h of collection, samples were stored at −80 °C at the Arctic Institute Base. Samples were kept on ice during transport to the University of Toronto (they were still frozen upon arrival) and stored at −80 °C until analyzed.

Fecal samples were freeze dried using a lyophilizer (LabConco, Missouri, USA) for 14–18 h to control for fiber and water content (Wasser et al., 1993) and homogenized with a coffee grinder. We then extracted 0.300 ± 0.05 g of the ground feces with 5 ml of 80% methanol (v/v) for 30 min at 15 000 rpm on a multi-tube vortexer. After centrifugation (15 min at 2500 g) an aliquot of the supernatant was diluted (1:10) with assay buffer and frozen at −80 °C until analysis. Bile samples were already liquid and an extraction step was not performed.

Bile and fecal cortisol metabolite concentrations were measured following the methods outlined by Sheriff et al. (2009b) using the 11-oxoaetiocholanolone-EIA developed by Palme and Möstl (1997). Briefly, 50 μl of extracted samples (in duplicate; bile samples were diluted 1:5 and fecal samples diluted 1:25 with assay buffer) were incubated with 100 μl of biotinylated steroid label (11-oxoaetiocholanolone-3-glucosiduronate-DADOO-biotin) and 100 μl antibody (11-oxoaetiocholanolone-3-HS:BSA raised in rabbits) at 4 °C on a plate shaker overnight. Plates were then washed four times with 0.05% TWEEN 20 (Merck 822184) solution and blotted dry. Into each well 250 μl of streptavidin peroxidase solution (1 μl streptavidin POD, 500 mU/μl [Boehringer 1089153] added to 30 ml assay buffer) was added and plates were incubated on plate shaker for 45 min at 4 °C. Plates were washed and then developed for 45 min at 4 °C on a plate shaker with 250 μl of tetramethybenzedine solution. The enzymatic color reaction was stopped using 50 μl of 2 M sulfuric acid. Absorbance was measured at a wavelength of 450 nm with an automated plate reader (VERSAmax microplate reader, Molecular Devices, Sunnyvale, California). This EIA had an intra- and inter-assay coefficient of variation of 6.3% and 10.3%, respectively.

2.7. Statistical analysis

General linear regressions and *t*-tests were performed using the software package STATISTICA 6. The assumption of normality was tested with Shapiro–Wilks test and the assumption of homogeneity of variances was tested with Levene's test. If these assumptions were not met the data were log-transformed $(x + 1)$ (Quinn and Keough, 2003). We used a *t*-test with a Bonferroni correction to examine the seasonal changes in hares' responsiveness and FCM concentrations (thus significance levels are $P < 0.02$). All other comparisons of the means were considered significant if $P < 0.05$. We found no difference between males and females and the sexes were pooled. All data are expressed as means ± 1 SE.

3. Results

True base (sampled <3 min) plasma free cortisol levels were directly correlated to bile cortisol metabolite levels ($r^2 = 0.53$; $F_{1,5} = 7.68$, $P < 0.05$; Fig. 1) and to FCM levels ($r^2 = 0.59$; $F_{1,8} = 6.63$, $P < 0.05$; Fig. 2). However, true base plasma total cortisol levels were not correlated to bile cortisol metabolites ($r^2 = 0.03$; $F_{1,5} = 1.21$, $P > 0.05$; Fig. 1) nor to FCM levels ($r^2 = 0.26$; $F_{1,8} = 0.16$, $P > 0.05$; Fig. 2). Nominal base (sampled >3 min) were the first bleeds from the hormonal challenge experiment and represent samples of the hares stressed by capture, handling, and transport to the lab. Nominal base plasma free cortisol levels were not correlated to FCM levels ($r^2 = 0.03$; $F_{1,28} = 0.60$, $P > 0.05$; Fig. 3).

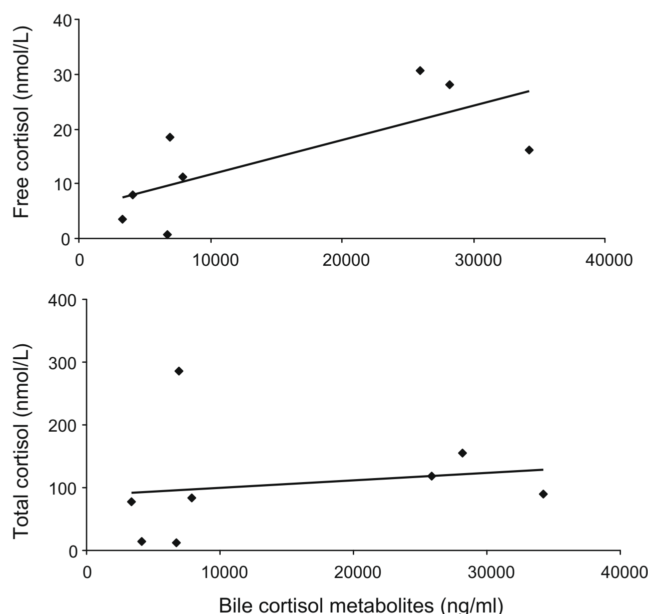

Fig. 1. Bile cortisol metabolite concentrations (ng/ml) in a sample of shot snowshoe hares ($n = 8$) and the relationship to their plasma free cortisol concentrations (nmol/L) ($r^2 = 0.53$; $y = 5.40 + 0.0006x$) and to their total cortisol concentrations (nmol/L) ($r^2 = 0.03$; $y = 87.29 + 0.0012x$).

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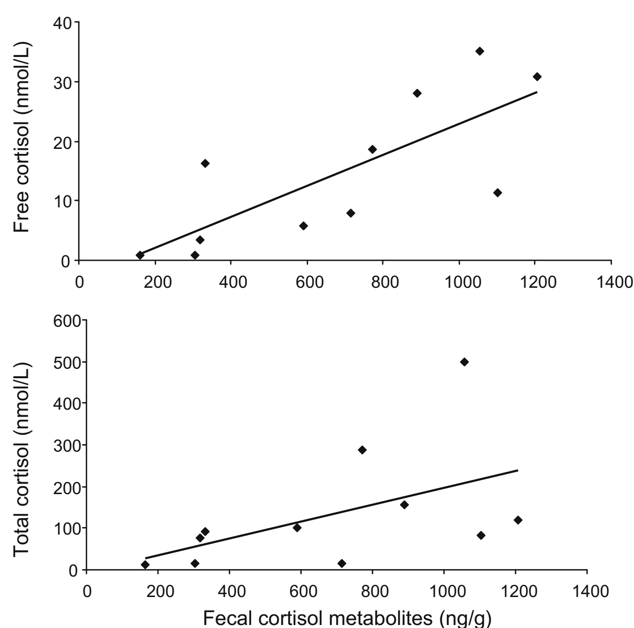

Fig. 2. Fecal cortisol metabolite concentrations (ng/g) in a sample of shot snowshoe hares ($n = 11$) and the relationship to their plasma free cortisol concentrations (nmol/L) ($r^2 = 0.59$; $y = 3.15 + 0.026x$) and to their plasma total cortisol concentrations (nmol/L) ($r^2 = 0.26$; $y = 5.06 + 0.20x$).

FCM levels directly reflected the changes in endogenous plasma free cortisol levels in response to the hormonal challenge. Hares with greater FCM concentrations exhibited a reduced suppression of free cortisol concentrations after the Dex injection ($r^2 = 0.33$; $F_{1,28} = 9.43$, $P < 0.001$; Fig. 4) and an increased, prolonged, production of free cortisol after the ACTH injection ($r^2 = 0.39$; $F_{1,28} = 13.60$, $P < 0.0001$; Fig. 5). There was a strong correlation between the Dex resistance and the ACTH stimulation tests ($r^2 = 0.26$; $F_{1,28} = 10.17$, $P < 0.003$).

Stress levels of hares' increased from autumn 2006 to winter 2007, decreased in autumn 2007, and remained similar from autumn 2007 to winter 2008 as indicated by the Dex suppression test, by the ACTH stimulation test, and by FCM levels (Fig. 6). The Dex injection resulted in a greater suppression of free cortisol (i.e. hares had lower free cortisol levels after the injection) in autumn 2006 than in winter 2007 (by 3-fold; $t_{14} = -4.32$, $P < 0.02$), a reduced suppression in winter 2007 than in autumn 2007 (by 3-fold; $t_{11} = -4.04$, $P < 0.02$), and a similar suppression in autumn 2007 and in winter 2008 ($t_{13} = 0.018$, $P > 0.02$). The ACTH injection resulted in a lower response (i.e. hares had lower free cortisol after the injection) in autumn 2006 than in winter 2007 (by 1.5-fold; $t_{14} = -2.58$, $P < 0.02$), greater response in winter 2007 than in autumn 2007 (by 1.5-fold; $t_{11} = -3.01$, $P < 0.02$), and a similar response in autumn 2007 and winter 2008 ($t_{13} = -2.03$, $P > 0.02$). FCM levels increased from autumn 2006 to winter 2007 (by 1.55-fold; $t_{14} = -3.83$, $P < 0.02$), decreased in autumn 2007 (by 1.5-fold; $t_{11} = -4.97$, $P < 0.02$), and remained similar in winter 2008 ($t_{13} = -0.732$, $P > 0.02$).

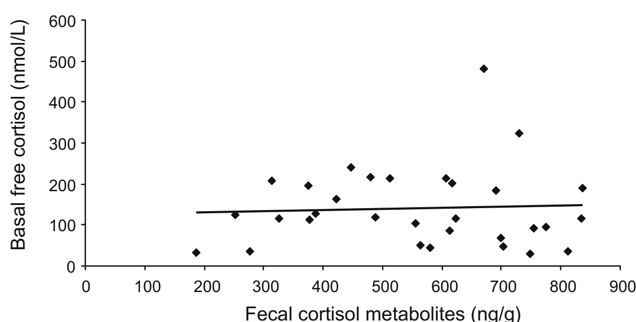

Fig. 3. Fecal cortisol metabolite concentration (ng/g) of snowshoe hares ($n = 32$) and the relationship to their plasma free cortisol concentration (nmol/L) at the nominal base bleed of the hormonal challenge (bled >3 min after capture and handling) ($r^2 = 0.03$; $y = 124.78 + 0.03x$).

4. Discussion

We tested the assumptions that fecal glucocorticoid metabolite concentrations accurately reflect both an animal's free cortisol in the blood and its ability to respond to a stressor and we validated both. Our results showed that bile cortisol metabolite concentrations and FCM concentrations mirrored that of true base plasma free cortisol concentrations when animals were bled within 3 min but not true base plasma total cortisol concentrations (Figs. 1 and 2). FCM concentrations did not reflect nominal base plasma free cortisol concentrations when sampling occurred more than 3 min after capture and handling (Fig. 3). However, the hormonal challenge got around the problem of capture and handling and an animal's FCM levels were indicative of their ability to respond to a the Dex and ACTH injections (Figs. 4 and 5). Furthermore, seasonal changes in FCM concentrations in natural populations were in concordance with those in plasma free cortisol concentrations when hares were hormonally challenged (Fig. 6).

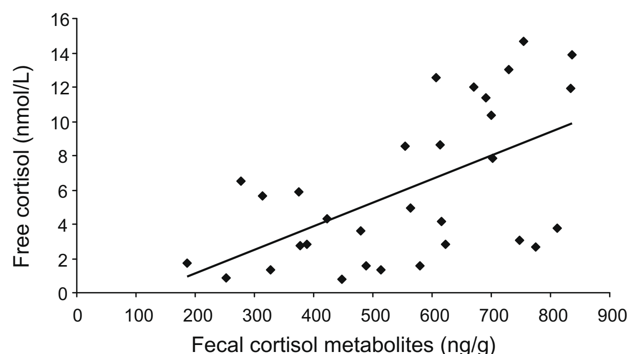

Fig. 4. Dexamethasone resistance in snowshoe hares. Fecal cortisol metabolite concentrations (ng/g) ($n = 32$) at capture and their free plasma cortisol concentrations (nmol/L) 2 h after the dexamethasone injection ($r^2 = 0.33$; $y = -1.65 + 0.014x$).

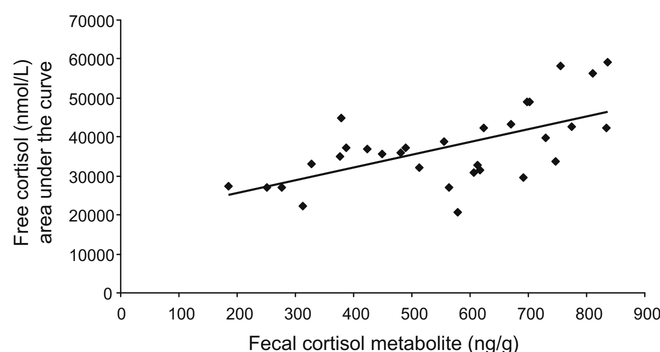

Fig. 5. ACTH stimulation in snowshoe hares. Fecal cortisol metabolite concentrations (ng/g) ($n = 32$) at capture and their integrated plasma free cortisol response (nmol/L) from the time of the ACTH injection to 2 h later, measured cumulatively as the area under the response curve ($r^2 = 0.38$; $y = 19146.00 + 32.72x$). Free cortisol concentrations were measured at 30 min, 60 min, and 120 min after the ACTH injection.

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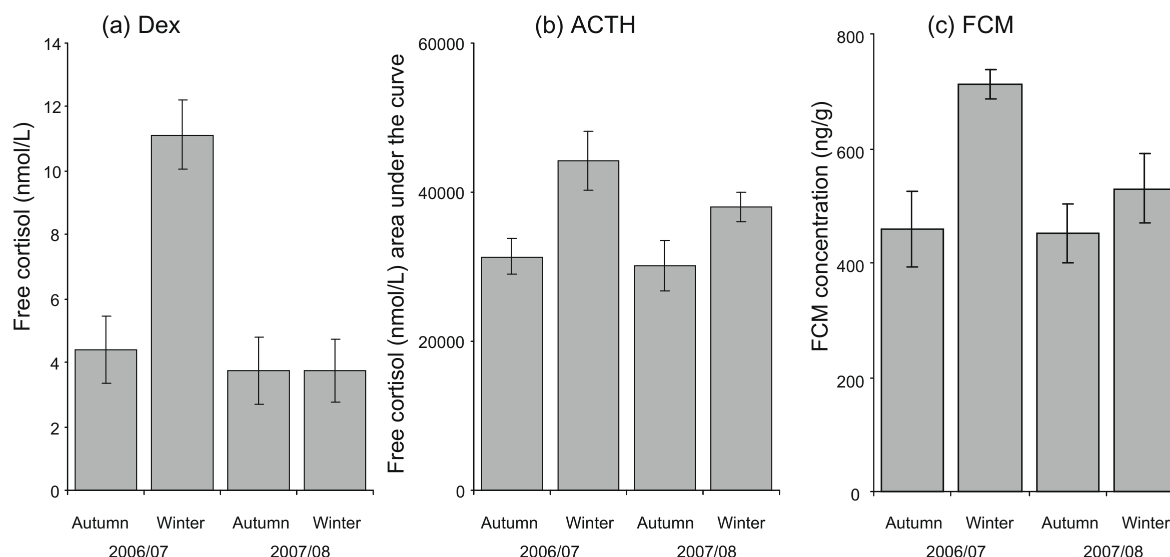

Fig. 6. Seasonal changes in plasma free cortisol (mean ± SE) from snowshoe hares subjected to a dexamethasone (Dex) resistance test (a) and to an adrenocorticotropic hormone (ACTH) stimulation test (b) and the changes in their fecal cortisol metabolite (FCM) concentrations at capture (c). The integrated response to ACTH was measured as the area under the curve. Hares were captured in the autumn ($n = 7$) and winter ($n = 9$) of 2006/07 and in the autumn ($n = 5$) and winter ($n = 11$) of 2007/08.

Studies have shown that fecal GC metabolites are positively correlated to total plasma GCs. Cavigelli (1999) compared fecal and plasma GC concentrations in semifree-ranging lemurs, *Lemur catta*. She found that fecal and plasma GC levels were significantly correlated. Mateo and Cavigelli (2005) found that in Belding's ground squirrels, *Spermophilus beldingi*, fecal GC metabolites were positively correlated with plasma total GC levels (sampled within 3 min of handling) taken 24 h after the fecal samples were collected. However these studies did not determine CBG levels and could not calculate the plasma free cortisol concentrations. It is critical that FCM concentrations reflect free GC concentrations in the blood as only the free, unbound GCs are thought to be biologically active (Rosner, 1990, but see Breuner and Orchinik, 2001). Total GC concentrations do not necessarily reflect free GC concentrations. For example, house sparrows (*Passer domesticus*) show seasonal changes in their baseline and stress-induced total GC concentrations. However, their CBG capacity also varies seasonally, which resulted in similar free GC concentrations across seasons (Breuner and Orchinik, 2001). Conversely, supplementally-fed snowshoe hares had similar total GC concentrations compared with controls. However, they had a significantly greater CBG capacity and this resulted in significantly lower free GC concentrations (Boonstra and Singleton, 1993). Our results show that FCM concentrations reflected the physiological state of an animal – both the true base free GC concentrations and the responsiveness to a stressor. We found that hares with greater FCM concentrations had a greater true base free cortisol concentrations in their blood (Fig. 2) and a greater ability to respond to a stressor (Figs. 4 and 5). Furthermore, our findings show that FCM concentrations reflected a hare's total response to a stressor not just the maximum response (Fig. 4). Since FCMs are not a point sample of an animal's state, but rather an integrated picture of the total amount of free GCs released in response to a stressor, it appears a very powerful method to assess the stress profile of an animal.

The magnitude and duration of free GC release are also equally important and this is often underappreciated in the study of stress physiology – our measure being the area under the response curve after the ACTH injection (Fig. 5). Dallman and Bhatnagar (2001) found that the biological effects of the stress response result from the hormone-receptor interactions over the entire time course of the stress response, not just at the peak of free GC release. For example, in a population of free-living baboons, *Papio anubis*, subordinates had a lower maximal free GC release to stressor than did dominants. However, the subordinates had a much longer duration of free GC response resulting in an overall greater total amount of free GC release (Sapolsky, 1993). This greater free GC exposure was then linked to cardiovascular problems in subordinates (Sapolsky and Share, 1994). Thus, the measurement of the total amount of free GC released, a function of both the maximum and duration of release, is the important variable to measure, not simply the maximum alone. Since FCM assays integrate both the baseline and total free GC release they provide a powerful indicator of the physiological state of an animal.

We have also shown that FCM concentrations can reliably track changes in plasma free cortisol concentrations. Snowshoe hares' free cortisol concentrations fluctuated across the two years of this study and this was mirrored in their FCM concentrations (Fig. 5). These seasonal differences are likely due to the major differences in predation risk during this time (Sheriff et al., 2009a; Sheriff, unpublished data). Very few studies have measured both the plasma GC concentrations and the FCM concentrations at different time points. Wasser et al. (1997) found that the transfer of a captive owl from her usual enclosure to a novel environment at the Department of Animal Health resulted in a comparable response in both serum and fecal corticosterone levels. Mashburn and Atkinson (2004) found that Stellar sea lions exposed to an ACTH challenge had a 3-fold increase in serum cortisol concentrations and an 18-fold increase in FCM concentrations. Thus changes in FCM concentrations are a good indicator of changes in plasma GC concentrations in captive, experimental, and free-ranging animals.

In conclusion, our results support the assumptions that fecal GC metabolites reflect free, biologically active, GC concentrations in the blood and that fecal GC metabolites were an excellent predictor of the responsiveness of an animal to a stressor. Furthermore, we showed that fecal GC metabolite concentrations reliably track changes in free GC concentrations.

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