Toolbox

Within-sample variation in snowshoe hare faecal glucocorticoid metabolite measurements

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Faecal glucocorticoid metabolite (FGM) concentrations are used increasingly as a non-invasive measure to index physiological stress experienced by diverse taxa. However, FGM may not be evenly distributed throughout a faecal mass or faecal pellet group. Moreover, within-sample variation in FGM measurements associated with different sampling and/or processing techniques is rarely reported despite potentially having important implications for inferring stress levels in free-ranging wildlife. Using a captive collection of snowshoe hares (*Lepus americanus*), we (i) assessed repeatability of FGM measurements (i.e. precision) from two processing techniques (measurements derived from dividing whole pellet groups into equal proportions prior to processing [G1], measurements from subsamples derived from thoroughly homogenized whole pellet groups [G2]) and (ii) conducted a power analysis to estimate sample-size requirements for detecting statistically significant differences in FGM concentrations at a population level. Our results indicate that the mean percent coefficient of variation (%CV) for within-sample FGM variation was slightly higher for G1 (%CV = 35, range 13.45–65.37) than for G2 (%CV = 23, range 7.26–47.94), though not statistically significant (two sample *t*-test, $n = 8$, $t = 1.57$, $P = 0.16$). Thus, FGM is relatively evenly distributed within snowshoe hare faecal pellet groups. However, subsampling from homogenized whole pellet groups may be more appropriate when the sampling time frame is less controlled (e.g. multiple defecation events) because a subsample derived from a homogenized whole pellet group might be more representative of the animal's 'average' physiological state compared to FGM concentrations derived from a few haphazardly selected faecal pellets. Power analysis results demonstrated the importance of *a priori* consideration of sample sizes. Relatively small effect sizes (e.g. <20%) may require sampling that is logistically and/or cost prohibitive. Yet for many situations of ecological or conservation interest, treatment effects may be substantial (>25%) and thus moderate sample sizes may be sufficient for testing research hypotheses regarding changes FGM concentrations.

Key words: cortisol, glucocorticoids, lagomorph, non-invasive, power analysis, stress physiology

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Introduction

A major focus of conservation physiology is to identify the causes and consequences of adrenocortical activity (hereafter stress) in wildlife populations and to determine whether the stress responses of individuals scales up to effect population-level health and dynamics. As such, measures of glucocorticoid (GC) hormones (i.e. cortisol or corticosterone depending upon the species) or their metabolites are being used increasingly to index stress in free-ranging and captive wildlife and as a biomarker to infer population-level health (Möstl and Palme, 2002; Millspaugh and Washburn, 2004; Palme *et al.*, 2005; Sheriff *et al.*, 2011a; Goymann, 2012; Dantzer *et al.*, 2014). For example, measures of GCs or their metabolites can provide quantitative information linking individual stress responses to environmental perturbations associated with anthropogenic disturbances (Tarlow and Blumstein, 2007; Creel *et al.*, 2013a), climate change (Romero and Wikelski, 2001, 2010), spatial-temporal variability in food availability (Kitaysky *et al.*, 1999, 2007), the social competitive environment (Bryan *et al.*, 2013, 2014; Creel *et al.*, 2013b) and predation risk (Monclús *et al.*, 2009; Sheriff *et al.*, 2009b). Further, GC concentrations and their metabolites have been linked to variation in reproductive output and differences in population performance in diverse taxa (Creel *et al.*, 1997; Barrett *et al.*, 2002; Kitaysky *et al.*, 2007; Charbonnel *et al.*, 2008; Sheriff *et al.*, 2009b, 2010b; Scarlata *et al.*, 2012). Thus, measures of GCs have tremendous potential to enhance sustainable wildlife management strategies (e.g. conservation initiatives) and to improve health monitoring of both captive and free-ranging populations (Möstl and Palme, 2002; Millspaugh and Washburn, 2004; Palme *et al.*, 2005; Sheriff *et al.*, 2011a; Goymann, 2012; Dantzer *et al.*, 2014).

Although measures of GCs and their metabolites can be obtained from diverse sources (e.g. blood, urine, saliva, hair), faeces may be the most reliable matrix for indexing stress via measures of faecal glucocorticoid metabolite (FGM) concentrations for wild populations (Millspaugh and Washburn, 2004; Palme, 2005; Sheriff *et al.*, 2010a, 2011a; Dantzer *et al.*, 2014). For example, faeces collection is efficient and cost-effective relative to other non-invasive stress hormone sampling techniques (i.e. hair cortisol extraction) (Möstl and Palme, 2002; Millspaugh and Washburn, 2004; Palme *et al.*, 2005; Sheriff *et al.*, 2011a; Goymann, 2012; Dantzer *et al.*, 2014). Sampling faeces can eliminate the potential confounding effects of hormonal habituation due to repeated capture and handling of the same individuals when assessing long-term stress within a population (Cyr and Romero, 2009). Further, FGM concentrations reflect an integrated measure of circulating GC levels, thereby providing an excellent matrix from which long-term stress can be indexed (Harper and Austad, 2000; Sheriff *et al.*, 2010a; Palme, 2012). And while a variety of factors (e.g. abiotic, biotic) affect measured FGM concentrations, these concerns are outlined in the literature and can often be mitigated with appropriate study design and standardized faecal sample processing techniques (Millspaugh and Washburn, 2004; Goymann, 2005, 2012; Touma and Palme, 2005; Sheriff *et al.*, 2011a). However, two important aspect of study design regarding the use of non-invasive sampling of faeces that have received little attention are the repeatability of FGM measurements from a single sample (i.e. precision) and sample-size requirements needed for being able to detect meaningful differences (e.g. 10%, 20% and 40%) in FGM concentrations at a population level.

Snowshoe hares (*Lepus americanus*) are an excellent study species for examining within-sample variation in FGM concentration. First, the use of 11-oxoetiocholanolone enzyme immunoassay (EIA) has been rigorously tested (i.e. dexamethasone suppression test, adrenocorticotropic stimulation test) and found to reliably detect temporal changes in snowshoe hare adrenocortical activity (Sheriff *et al.*, 2009a, 2009b, 2010a, 2011b). Moreover, snowshoe hare plasma GC levels and FGM concentrations are concordant over time (Sheriff *et al.*, 2010a), indicating that snowshoe hare faecal pellets are a suitable non-invasive biological matrix for indexing an individual's stress burden. Research also shows that fresh snowshoe hare pellets represent the physiological state of the individual 8–12 h prior to defecation (Sheriff *et al.*, 2009a), thereby allowing for a sampling frame that can account for the confounding effects of variation in FGM concentrations due to diel hormonal fluctuations associated with the circadian rhythm (Rich and Romero, 2001; Carere *et al.*, 2003; Sheriff *et al.*, 2009a). Further, snowshoe hares typically defecate while foraging, producing variable amounts of faeces in the form of pellets that are easy to subsample because the pellets are not accumulating in a latrine. Thus, using a captive collection of snowshoe hares our objectives were to (i) assess the repeatability of FGM measurements (i.e. precision) from two processing techniques (FGM measurements derived from dividing whole pellet groups into equal proportions prior to processing [G1]; FGM measurements from subsamples derived from thoroughly homogenized whole pellet groups [G2]) and (ii) conduct a power analysis to estimate sample-size requirements to detect differences (e.g. 10%, 20% and 40%) in FGM concentrations at a population level following a perturbation.

Materials and methods

Animals and housing

Eight wild-caught adult snowshoe hares were used for this experiment. Animals were live-captured in the Mt. Baker-Snoqualmie National Forest in Washington, USA (four females, four males) using Tomahawk traps (Tomahawk Live Trap Co., Hazelhurst, WI, USA). Animals were transported to North Carolina State University College of Veterinary Medicine (NCSU CVM) and housed in environmental rooms that replicate weekly 30-year average temperature and photoperiod from their capture location. Daytime and nighttime temperatures in the environmental rooms

ranged between −1°C and −4°C (winter) during the sampling period. Animals were housed individually in wire mesh cages (121.9 cm width × 60.9 cm depth × 73.6 cm height) with a wire mesh floor. The cages included an acrylic hide box (30.5 cm width × 60.9 cm depth × 73.6 cm height) that served as a visual barrier between animals, as well as an edible grass hut to hide in or sit atop. Premium natural adult rabbit food (Sherwood Pet Health, Logan, UT, USA) and water were available ad libitum. Capture, handling, transport, husbandry and sample collection procedures were approved by NCSU Institutional Animal Care and Use Committee (Protocol 14-069-0).

Faecal sample collection

Faecal pellets fell through the wire mesh floor and were collected in a tray underneath each cage. Faecal samples were collected over a 2-day period (24–25 December 2015), resulting in two samples per individual with each sample consisting of the total pellets accumulated (faecal pellet group) over a single 10-h sampling frame. To acquire these samples, the collection tray beneath each animal's cage was cleaned between 2200 h and 2220 h, lined with absorbent paper, and the following morning all faecal pellets were collected for each animal between 0800 h and 0820 h; care was taken to avoid pellets exposed to urine. During collection, we placed pellet samples in pre-labelled plastic bags and stored the samples at −20°C within 10 min of collection. This 10-h sampling frame was selected to mimic a plausible period over which animals may be held in traps during field-based operations, and from which faecal pellets may be collected. As such, each sample likely represents multiple defecation bouts per animal per night (Hodges, 1999).

Experimental approach

All faecal pellet samples were dried for 48 h at 80°C (Möstl et al., 2005). The two samples per individual were randomly assigned to one of two experimental sampling treatment groups. Samples assigned to Group 1 (G1) were first subdivided into five equal portions of intact pellets based on the total dry mass, resulting in five G1 replicates per individual that were separately homogenized using a glass mortar and pestle. For samples assigned to Group 2 (G2), samples were first thoroughly homogenized using a glass mortar and pestle, and subsequently subdivided into five equal portions of powdered faeces based on total dry mass, resulting in five G2 replicates per individual.

FGM extraction and quantification

From thoroughly homogenized G1 and G2 subsamples, we removed a 0.15 mg subsample and placed it in 15 ml centrifuge tube with 5.0 ml of 80% methanol. Next, we shook samples on a hand vortex for 1 min and centrifuged each sample at 2500 g for 15 min (Palme et al., 2013). Following centrifugation, 0.5 ml of supernatant was transferred to microcentrifuge tubes and dried for ~2 h at 80°C (Palme et al.,

2013). Samples were shipped to the Department of Biomedical Sciences, Unit of Physiology, Pathophysiology and Experimental Endocrinology at the University of Veterinary Medicine in Vienna, Austria for EIA analysis. FGM concentrations were quantified in the re-dissolved extracts using an 11-oxoetiocholanolone-EIA (Palme, 2005; Rehnus et al., 2009; Sheriff et al., 2009a).

Data analysis

To quantify within-sample variation in FGM concentrations, we compared the coefficient of variation (CV) for G1 and G2 for each individual using a paired t-test. In addition, to facilitate future sampling efforts we conducted a power analysis to determine the sample size necessary to detect differences of 10%, 20%, 30%, 40% and 50% in FGM based on the percent differences and degree of variation we observed. We fixed statistical power at 0.80 and α at 0.05. All statistical analyses were conducted in R 3.2.4 (R Core Team, 2014).

Results

Within-sample variation in FGM measurements represented by mean %CV was slightly lower for sample processing technique G2 (%CV 23, range 7.26–47.94) compared to G1 (%CV = 35, range 13.45–65.37), although the mean difference was not significant (two sample t-test, $n = 8$, $t = 1.57$, $P = 0.16$; Fig. 1). This EIA had an intra-assay CV of 3.7% and the inter-assay coefficients of variation for high- and low-concentration
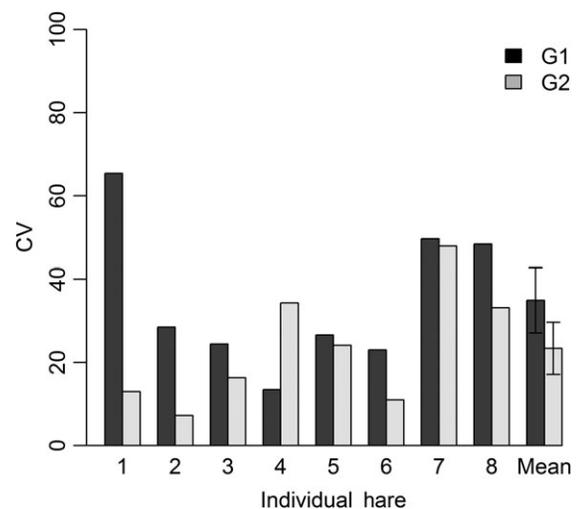

Figure 1: Percent CV in faecal cortisol metabolite concentration based on five repeated measures from each individual hare and overall mean (Mean) of all hares for two sampling techniques. Group 1 (G1) consisted of whole samples of intact pellets that were subsequently subdivided into five equal portions of intact pellets based on the total dry mass; Group 2 (G2), the whole samples were first thoroughly homogenized and subsequently subdivided into five equal portions of powdered faeces based on total dry mass. Standard error indicated with error bars.
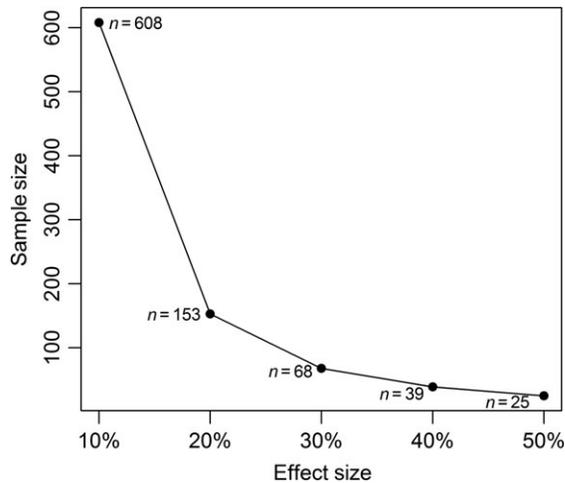
Figure 2: Necessary sample size to detect a difference (10%, 20%, 30%, 40% and 50%) in faecal cortisol metabolite concentrations when whole samples were first thoroughly homogenized and subsequently subdivided into five equal portions of powdered faeces based on total dry mass (G2). Statistical power was fixed at 0.80 and $\alpha = 0.05$.

standards across plates were 8.9% and 14.1%. We based our power analysis on the technique with the slightly lower CV (G2: homogenized complete sample). Based on variance estimates from our experiments, ~600 faecal pellet samples would be necessary to detect a 10% difference in FGM concentrations, whereas an effect of 20% would only require ~150 samples (Fig. 2).

Discussion

Based on our finding of no significant differences in %CV between our two sampling techniques, FGM appears to be relatively evenly distributed throughout snowshoe hare faecal pellet groups. The %CV we calculated for FGM derived from snowshoe hare pellets was higher than %CV calculated in other studies of distribution of steroid metabolites in faeces (Brown *et al.*, 1994; Wasser *et al.*, 1996; Millspaugh and Washburn, 2003), although such differences among studies are not surprising given species-specific and methodological differences (Millspaugh and Washburn, 2003). Although not statistically different, we found a slightly lower %CV for subsamples taken from homogenized whole pellet groups (G2) compared to subsamples comprised of a few intact faecal pellet (G1).

Because our goal was to estimate the precision of FGM estimates from a single sample and to determine sample-size requirements for detecting differences in FGM concentrations among treatments, it is important to consider factors that may contribute to the levels of within-sample variation observed in our study. First, our sampling regime was designed to reflect a time frame (e.g. 10 h) similar to field studies where pellets are collected from individual snowshoe

hares in live traps. Because captive snowshoe hares may defecate several times during this time interval (Hodges, 1999, Lafferty pers. obs), within-sample variability in FGM concentrations may be the result of subsamples that reflect hormonal fluctuations associated with both the circadian rhythm (Rich and Romero, 2001; Carere *et al.*, 2003; Sheriff *et al.*, 2009a) and adrenocortical responses over shorter time periods. Thus, subsampling a few snowshoe hare faecal pellets from the whole pellet group may capture specific points in the diel cycle that contain lower or higher FGM concentrations compared to the average concentration of FGM for the whole faecal pellet group (see Sherrif *et al.*, 2009a). Millspaugh and Washburn (2003) reported similar findings in which analysis of a few selected white-tailed deer (*Odocoileus virginianus*) pellets resulted in greater variability in FGM concentrations compared to subsamples taken from homogenized whole pellet groups. Results from previous studies also suggest that diet as well as the size of the faecal mass produced can influence within-sample variability in FGM concentrations (Wasser *et al.*, 1993; Brown *et al.*, 1994; Sheriff *et al.*, 2009a). Millspaugh and Washburn (2003) posited that low %CV in white-tailed deer faecal pellets compared to other species may be the result of a fairly homogeneous diet and relatively small volume of faeces produced. Although fed a consistent pelleted commercial diet during this study, snowshoe hares produce a large amount of faeces relative to their body weight. Measures of FGM concentration from field-derived samples may result in greater levels of within-sample variation than we found during our controlled laboratory experiment, due to greater exposure to a range of abiotic and biotic environmental conditions and potentially less control over the temporal sampling frame (Wilkening *et al.*, 2016). As such, FGM measures derived from subsamples of faecal pellet groups should be considered sample estimates that represent a range of potential FGM values, not point values without error.

Findings from our study and others indicate that sampling faeces in a systematic manner as well as standardized processing is critical when collecting samples for FGM analysis, particularly for comparative studies (Wilkening *et al.*, 2016). Our work demonstrates that whether subsampling—faeces or collecting and homogenizing whole faecal pellet groups or a whole faecal mass, researchers should determine *a priori* the sampling protocol that will be used throughout a study to avoid introducing a controllable source of variation to the data (e.g. measurement error) that can bias FGM measurements (Jeffcoate, 1981; Millspaugh and Washburn, 2003). And while collecting a subsample of faeces may be preferential in some situations, collecting and homogenizing whole pellets groups or entire faecal masses (e.g. larger volume samples) may be more appropriate when the sampling time frame is less controlled because a subsample derived from a homogenized whole pellet group or whole faecal mass might be more representative of the animal's 'average' psycho-physiological state compared to FGM concentrations derived from a few haphazardly collected pieces of faeces.

Further, we encourage *a priori* consideration of how sample sizes and within-sample variation in FGM will influence the detection of treatment effects. For example, if effect sizes are relatively small (e.g. <20%) the sampling requirement for obtaining sufficient power to detect a statistically significant differences in FGM may be logistically and/or cost prohibitive. However, for many situations of ecological or conservation interest, treatment effects may be quite substantial (e.g. ~25% to >100%) (Hik *et al.*, 2001; Sheriff *et al.*, 2009b, 2011b; Creel *et al.*, 2013a) and can therefore be detected with fewer samples. For example, Sheriff *et al.* (2009b) documented an increase of 214% and 837% in FGM concentrations among female snowshoe hare experimentally exposed to predation risk relative to a control group not exposed to predation risk. In studies of lions (*Panthera leo*), Creel *et al.* (2013a) found 25% differences in FGM concentrations based on land use status and 22% decreases per kilometre with increasing distance from human settlement (Creel *et al.*, 2013a). In such cases with relatively large effect sizes, more moderate sample sizes may be sufficient for testing research hypotheses regarding FGM concentrations.

Although non-invasive measures of stress are being used increasingly to glean insight into the health and well-being of captive and free-roaming wildlife, much remains unknown of the factors that influence measures of FGM concentrations. For example, species-specific insights regarding the linkages among steroid hormone metabolites and diet (Wasser *et al.*, 1993; Stetz *et al.*, 2013), reproductive state (Sapolsky, 1987; Weingrill *et al.*, 2004; Dantzer *et al.*, 2010), body condition (Kitaysky *et al.*, 1999; Macbeth *et al.*, 2012) and circadian rhythmicity (Breuner *et al.*, 1999; Carere *et al.*, 2003; Sheriff *et al.*, 2009a) would enhance the utility of FGM as a tool for wildlife ecology, conservation and management. Nevertheless, non-invasive measures of endocrine activity have tremendous potential to aid our understanding of wildlife responses to environmental change, enhance wildlife health monitoring, and contribute to the conservation of free-ranging wildlife populations (Madliger *et al.*, 2016). With further refinement of sampling methodologies, measures of FGM concentrations will be a powerful non-invasive tool for assessing and mitigating the impacts of environmental stressors on wildlife health at both the individual and population level.

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References

Barrett GM, Shimizu K, Bardi M, Asaba S, Mori A (2002) Endocrine correlates of rank, reproduction, and female-directed aggression in male Japanese macaques (*Macaca fuscata*). *Horm Behav* 42: 85–96.

Breuner CW, Wingfield JC, Romero LM (1999) Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow. *J Exp Zool* 284:334–342.

Brown JL, Wasser SK, Wildt DE, Graham LH (1994) Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol Reprod* 51:776–786.

Bryan HM, Darimont CT, Paquet PC, Wynne-Edwards KE, Smits JE (2013) Stress and reproductive hormones in grizzly bears reflect nutritional benefits and social consequences of a salmon foraging niche. *PLoS One* 8:e80537.

Bryan HM, Darimont CT, Paquet PC, Wynne-Edwards KE, Smits JE (2014) Stress and reproductive hormones reflect inter-specific social and nutritional conditions mediated by resource availability in a bear–salmon system. *Conserv Physiol* 2:cou010.

Carere C, Groothuis T, Möstl E, Daan S, Koolhaas J (2003) Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. *Horm Behav* 43: 540–548.

Charbonnel N, Chaval Y, Berthier K, Deter J, Morand S, Palme R, Cosson JF (2008) Stress and demographic decline: a potential effect mediated by impairment of reproduction and immune function in cyclic vole populations. *Physiol Biochem Zool* 81:63–73.

Creel S, Creel NM, Mills MG, Monfort SL (1997) Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. *Behav Ecol* 8:298–306.

Creel S, Christianson D, Schuette P (2013a) Glucocorticoid stress responses of lions in relationship to group composition, human land use, and proximity to people. *Conserv Physiol* 1:cot021.

Creel S, Dantzer B, Goymann W, Rubenstein DR (2013b) The ecology of stress: effects of the social environment. *Funct Ecol* 27:66–80.

Cyr NE, Romero LM (2009) Identifying hormonal habituation in field studies of stress. *Gen Comp Endocrinol* 161:295–303.

Dantzer B, McAdam AG, Palme R, Fletcher QE, Boutin S, Humphries MM, Boonstra R (2010) Fecal cortisol metabolite levels in free-ranging North American red squirrels: assay validation and the effects of reproductive condition. *Gen Comp Endocrinol* 167: 279–286.

Dantzer B, Fletcher QE, Boonstra R, Sheriff MJ (2014) Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? *Conserv Physiol* 2: cou023.

Goymann W (2005) Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. *Ann NY Acad Sci* 1046:35–53.

Goymann W (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Methods Ecol Evol* 3:757–765.

Harper JM, Austad SN (2000) Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. *Physiol Biochem Zool* 73:12–22.

Hik DS, McCOLL CJ, Boonstra R (2001) Why are Arctic ground squirrels more stressed in the boreal forest than in alpine meadows? *Ecoscience* 8:275–288.

Hodges KE (1999) Proximate factors affecting snowshoe hare movements during a cyclic population low phase. *Ecoscience* 6:487–496.

Jeffcoate SL (1981) *Efficiency and effectiveness in the endocrine laboratory*. Academic Press, NY, USA.

Kitaysky A, Wingfield J, Piatt J (1999) Dynamics of food availability, body condition and physiological stress response in breeding black-legged kittiwakes. *Funct Ecol* 13:577–584.

Kitaysky A, Piatt J, Wingfield J (2007) Stress hormones link food availability and population processes in seabirds. *Mar Ecol Prog Ser* 352:245.

Macbeth BJ, Cattet MR, Obbard ME, Middel K, Janz DM (2012) Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears. *Wildl Soc Bull* 36:747–758.

Madliger CL, Cooke SJ, Crespi EJ, Funk JL, Hultine KR, Hunt KE, Rohr JR, Sinclair BJ, Suski CD, Willis CK (2016) Success stories and emerging themes in conservation physiology. *Conserv Physiol* 4:cov057.

Millspaugh JJ, Washburn BE (2003) Within-sample variation of fecal glucocorticoid measurements. *Gen Comp Endocrinol* 132:21–26.

Millspaugh JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen Comp Endocrinol* 138:189–199.

Monclús R, Palomares F, Tablado Z, Martínez-Fontúrbel A, Palme R (2009) Testing the threat-sensitive predator avoidance hypothesis: physiological responses and predator pressure in wild rabbits. *Oecologia* 158:615–623.

Möstl E, Palme R (2002) Hormones as indicators of stress. *Domest Anim Endocrinol* 23:67–74.

Möstl E, Rettenbacher S, Palme R (2005) Measurement of corticosterone metabolites in birds' droppings: ananalytical approach. *Ann NY Acad Sci* 1046:17–34.

Palme R (2005) Measuring fecal steroids: guidelines for practical application. *Ann NY Acad Sci* 1046:75–80.

Palme R (2012) Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. *Anim Welf* 21:331–337.

Palme R, Rettenbacher S, Touma C, El-Bahr S, Möstl E (2005) Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. *Ann NY Acad Sci* 1040:162–171.

Palme R, Touma C, Arias N, Dominchin M, Lepschy M (2013) Steroid extraction: get the best out of faecal samples. *Wien Tierarztl Monatsschr* 100:238–246.

R Core Team (2014) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. 2013. ISBN 3-900051-07-0.

Rehnus M, Hackländer K, Palme R (2009) A non-invasive method for measuring glucocorticoid metabolites (GCM) in Mountain hares (*Lepus timidus*). *Eur J Wild Res* 55:615–620.

Rich E, Romero L (2001) Daily and photoperiod variations of basal and stress-induced corticosterone concentrations in house sparrows (*Passer domesticus*). *J Comp Physiol B* 171:543–547.

Romero LM, Wikelski M (2001) Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. *Proc Natl Acad Sci USA* 98:7366–7370.

Romero LM, Wikelski M (2010) Stress physiology as a predictor of survival in Galapagos marine iguanas. *Proc Biol Sci* 277: 3157–3162.

Sapolsky RM (1987) Stress, social status, and reproductive physiology in free-living baboons. In Crews D ed. *Psychobiology of Reproductive Behavior: An Evolutionary Perspective*. Prentice Hall, Inc., Englewood Cliffs, NJ, pp. 291–322.

Scarlata CD, Elias BA, Godwin JR, Powell RA, Shepherdson D, Shipley LA, Brown JL (2012) Relationship between fecal hormone concentrations and reproductive success in captive pygmy rabbits (*Brachylagus idahoensis*). *J Mammal* 93:759–770.

Sheriff MJ, Bosson CO, Krebs CJ, Boonstra R (2009a) A non-invasive technique for analyzing fecal cortisol metabolites in snowshoe hares (*Lepus americanus*). *J Comp Physiol B* 179:305–313.

Sheriff MJ, Krebs CJ, Boonstra R (2009b) The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *J Anim Ecol* 78:1249–1258.

Sheriff MJ, Krebs CJ, Boonstra R (2010a) Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story? *Gen Comp Endocrinol* 166:614–619.

Sheriff MJ, Krebs CJ, Boonstra R (2010b) The ghosts of predators past: population cycles and the role of maternal programming under fluctuating predation risk. *Ecology* 91:2983–2994.

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011a) Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* 166:869–887.

Sheriff MJ, Krebs CJ, Boonstra R (2011b) From process to pattern: how fluctuating predation risk impacts the stress axis of snowshoe hares during the 10-year cycle. *Oecologia* 166:593–605.

Stetz J, Hunt K, Kendall KC, Wasser SK (2013) Effects of exposure, diet, and thermoregulation on fecal glucocorticoid measures in wild bears. *PLoS One* 8:e55967.

Tarlow EM, Blumstein DT (2007) Evaluating methods to quantify anthropogenic stressors on wild animals. *Appl Anim Behav Sci* 102: 429–451.

Touma C, Palme R (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann NY Acad Sci* 1046:54–74.

Wasser S, Thomas R, Nair P, Guidry C, Southers J, Lucas J, Wildt D, Monfort S (1993) Effects of dietary fibre on faecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). *J Reprod Fertil* 97:569–574.

Wasser S, Papageorge S, Foley C, Brown J (1996) Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. *Gen Comp Endocrinol* 102:255–262.

Weingrill T, Gray DA, Barrett L, Henzi SP (2004) Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. *Horm Behav* 45: 259–269.

Wilkening JL, Ray C, Varner J (2016) When can we measure stress non-invasively? Postdeposition effects on a fecal stress metric confound a multiregional assessment. *Ecol Evol* 6:502–513.