

Multiple measures elucidate glucocorticoid responses to environmental variation in predation threat

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Abstract Predator-induced changes in the glucocorticoid responses of prey have been proposed to mediate indirect predator effects on prey demography. Ambiguities exist, however, as to whether differences in predation threat in the environment at large affect the mean glucocorticoid response in wild birds and mammals, and whether this is likely to affect reproduction. Most studies to date that have examined glucocorticoid responses to environmental variation in predation threat have evaluated just one of the several potential measures of the glucocorticoid response, and this may be the source of many ambiguities. We evaluated multiple measures of the glucocorticoid response [plasma total CORTicosterone, corticosteroid binding globulin (CBG) and free CORT] in male and female song sparrows (*Melospiza melodia*) sampled at locations

differing in predation threat in the environment at large, where we have previously reported reproductive differences suggestive of indirect predator effects. Total CORT varied markedly with predation threat in males but not females whereas the opposite was true for CBG, and both sexes demonstrated the same moderately significant free CORT response. Considering all three indices, a glucocorticoid response to environmental variation in predation threat was evident in both sexes, whereas there were ambiguities considering each index singly. We conclude that collecting multiple physiological measures and conducting multivariate analyses may provide a preferable means of assessing glucocorticoid responses to environmental variation in predation threat, and so help clarify whether such glucocorticoid changes affect reproduction in wild birds and mammals.

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Introduction

Predator-induced changes in the glucocorticoid responses of prey represent a potential mechanism mediating indirect predator effects on prey demography. Predator effects on prey demography have traditionally been ascribed solely to direct killing. Indirect predator effects on prey demography include any effect on prey demography that does not involve direct killing, such as a negative effect on the birth rate or an increase in deaths from other causes (Creel et al. 2007; Sheriff et al. 2009). Though theory has long suggested that predator-induced changes in prey physiology and behavior could scale-up to affect prey demography

(e.g., McNamara and Houston 1987), there remain few studies that have demonstrated this in birds and mammals (Creel and Christianson 2008; Lima 2009). Negative effects on the birth rate provide clearer evidence of indirect predator effects because diagnosing the cause of death of wild animals is often problematic (Creel et al. 2007; Creel and Christianson 2008). Glucocorticoid changes represent a potential link between predation threat and prey reproduction because (1) numerous studies have shown that the acute presentation in close proximity to the prey of a live predator, predator model or predator cues, typically induces an immediate glucocorticoid response (e.g., Canoine et al. 2002; Cockrem and Silverin 2002; Campeau et al. 2008; Sheriff et al. 2009), and (2) changes in glucocorticoid exposure may inhibit reproduction (Preisser 2009; Rodrigues et al. 2009; Sheriff et al. 2009).

Whether differences in predation threat in the environment at large affect the mean glucocorticoid response in wild birds and mammals is open to question (Creel et al. 2009). Though they provide useful information on cause and effect, extrapolating from predator presentation experiments to longer time and larger spatial scales is potentially problematic. Conceivably, where predators are more abundant, close encounters with them may be more frequent, and at any given time, prey might then be expected to be demonstrating an immediate glucocorticoid response. Creel et al. (2009), however, have argued that such an effect need not be expected if animals learn to avoid close encounters in environments where predators are abundant—and so demonstrate a behavioral change in lieu of a physiological one.

To date, only a handful of studies on wild birds and mammals have evaluated whether differences in predation threat in the environment at large affect mean glucocorticoid responses. In a recent review, Creel et al. (2009) identified two studies on birds (Scheuerlein et al. 2001; Clinchy et al. 2004) and three more on mammals (their study; Boonstra et al. 1998; Sheriff et al. 2009), that measured between-population differences in glucocorticoid responses in prey populations demonstrating reproductive differences potentially due to differences in predation threat. Though they did not include data on reproduction as did those reviewed by Creel et al. (2009), three additional studies on mammals have measured between-population differences in glucocorticoid responses in relation to environmental variation in predation threat (Hik et al. 2001; Mateo 2007; Monclús et al. 2009). Creel et al. (2009) found no difference in fecal glucocorticoid metabolites between elk (*Cervus elaphus*) populations differing in predation threat, and suggested that there was no clear pattern in the previous studies they reviewed. Scheuerlein et al. (2001), for example, found a significant effect of predation threat on total glucocorticoid levels in male, but

not female, tropical stonechats (*Saxicola torquata axillaris*).

Just as animals may demonstrate a behavioral change in lieu of a physiological change (Creel et al. 2009), they may also demonstrate one physiological change in lieu of another. The same physiological effect might be accomplished by varying any or all of the total amount of glucocorticoid hormone (total CORT) released into the blood, the circulating amount of corticosteroid binding globulins (CBG), or the number of receptor sites in target tissues (Kudielka and Kirschbaum 2005; Rodrigues et al. 2009). Some of the ambiguities identified by Creel et al. (2009) could stem from the fact that most studies (6 of 8) to date, that have examined glucocorticoid responses in relation to variation in predation threat in the environment at large, have evaluated just one of the several potential measures of the glucocorticoid response. Sex differences in which measure of the glucocorticoid response (i.e. total CORT, CBG or receptor number) is more strongly affected by a stressor have been reported in many studies on humans and laboratory rodents (Kudielka and Kirschbaum 2005), and such a sex difference might explain the ambiguity in, for example, Scheuerlein et al.'s (2001) results.

We evaluated between-population differences in glucocorticoid responses in male and female song sparrows (*Melospiza melodia*) in the context of a long-term study in which we have previously documented between-population differences in both predation threat (predator abundance, nest predation rate; Zanette et al. 2006a) and reproduction (clutch size; Zanette et al. 2006b; partial brood loss, annual reproductive success; Zanette et al. 2006a), and have experimentally demonstrated indirect predator effects on clutch size (Travers et al. 2010). In the context of this long-term study, Clinchy et al. (2004) reported between-population differences in total CORT in male sparrows, paralleling Scheuerlein et al.'s (2001) results. In this paper, we evaluate the levels of plasma total CORT, CBG and free CORT, measured in both male and female sparrows. Our hypothesis was that both sexes respond to environmental variation in predation threat, but because they may differ in which measure of the glucocorticoid response (i.e., total CORT or CBG) is more strongly affected, collecting multiple physiological measures would prove essential to accurately evaluating their glucocorticoid responses to environmental variation in predation threat.

Materials and methods

Study design

The long-term study that this work was a part of contrasts multiple high predator pressure (HPP) locations just

outside of Victoria, BC, Canada, on the Vancouver Island ‘mainland’ (31,284 km²), with low predator pressure (LPP) locations situated on several small (<0.2 km²), coastal islands <2 km offshore. Zanette et al. (2003, 2006a, b) provide exhaustive documentation regarding the lower abundance of predators, lower nest predation rates, lower rate of partial clutch or brood loss, and higher survival of adult sparrows on these small coastal islands as compared to the adjacent ‘mainland’. Clinchy et al. (2004) reported that, among parental males sampled during chick-rearing, those at the HPP locations demonstrated higher ‘baseline’ (<3 min post-capture) plasma total CORT levels than those at the LPP locations, as well as higher restraint-induced (30 min post-capture) plasma total CORT levels. Travers et al. (2010) demonstrated experimentally that the higher rate of nest predation experienced at HPP locations may be expected to affect a broad array of physiological measures in breeding females. Groves (2009) reported significant differences in parental nest attendance during chick-rearing between the HPP and LPP locations, based on continuous video records from 116 nests.

There are no significant differences in territory size (i.e., density) or habitat structure between the HPP and LPP locations (Clinchy et al. 2004; Zanette et al. 2006a, b), nor are individuals at these locations isolated from one another as many birds move between them during the non-breeding season (Reid et al. 2006). Clinchy et al. (2004) reported that parental males at the HPP and LPP locations demonstrated comparable physiological responses to food supplementation, consistent with there being little difference between the HPP and LPP locations in the availability of naturally-occurring food.

Sampling

Clinchy et al. (2004) captured and collected blood from fathers on day 6 of the 11-day chick-rearing period. Though both parents feed the chicks, the mother alone broods them. Consequently, to compare the responses of both fathers and mothers, we were constrained to evaluating only baseline glucocorticoid responses. Video data collected as part of this long-term project suggests sparrows subjected to a 30-min capture–restraint protocol, such as employed by Clinchy et al., may take many hours or even an entire day to recuperate and resume parental activities. Whereas the absence of the father for such an extended period might result in his chicks being underfed, the absence of the mother is likely to be fatal to the chicks if she is not there to brood them at night.

In this study, we captured and collected blood from both fathers and mothers on day 6 of the chick-rearing period, taking several steps to avoid causing undue distress to either the parents or chicks. In addition to collecting only

baseline bleeds, to minimize the time in hand and distress to the parent, we also strove to minimize the amount of blood collected (150 µl). Mothers were captured between 0800 and 1600 hours (mean ± SE = 1200 hours ± 15 min), which gave even those captured later in the day several hours to recuperate and so resume brooding before dark. Every adult was given one or two drops of fruit juice immediately before being released, to aid their recuperation and resumption of parental activities. Nests were continuously monitored after the release of the parents, and chicks were hand fed ad libitum Hagen Living World Egg Biscuit (Rolf C. Hagen, Baie d’Urfé, PQ, Canada) once per hour, until the parents were seen to be returning regularly to the nest.

Collecting blood on a specific day during the chick-rearing period experimentally controls for the variation in total CORT and CBG with stage (e.g., incubation vs chick-rearing) in the 25-day nesting cycle, that is known to exist in song sparrows (Wingfield 1984) and other species (Love et al. 2004). Birds were captured using mist-nets and blood was collected from the brachial vein <3 min after they hit the net. Blood was stored on ice for transport to the laboratory. All samples were centrifuged and plasma was extracted and frozen at –20°C, within 8 h.

Samples were collected over three breeding seasons. We collected blood from 45, 25 and 29 fathers and 53, 40 and 33 mothers in the first, second and third breeding seasons, respectively. In many cases (38, 18 and 21 in the first, second and third breeding seasons, respectively), we succeeded in collecting blood from both the father and mother at the same nest, on the same day.

Measurement of the glucocorticoid response

As noted in the “Introduction”, any or all of the level of total CORT, CBG, or number of receptor sites in target tissues may affect the glucocorticoid response (Kudielka and Kirschbaum 2005; Rodrigues et al. 2009). We did not evaluate receptor sites because this entails killing the subject.

Total CORT concentrations were measured by radioimmunoassay of 5- to 20-µl samples of plasma following the methods described by Clinchy et al. (2004) and Newman et al. (2008). CBG binding capacity was determined following the methods described in Hammond and Lähteenmäki (1983) and Charlier et al. (2009). Briefly, plasma samples were diluted 1/100 and incubated 30 min at 20°C with a dextran-coated charcoal (DCC) suspension. The DCC was then precipitated by centrifugation and an aliquot of the samples was added into duplicate tubes containing 1 pmol of 1,2-³H CORT (specific activity: 50 Ci/mmol; ARC, St Louis, MO, USA), and 1 tube containing 0.5 µM of cold CORT to determine non-specific binding. After 1 h

incubation at 20°C, the tubes were put in an ice-water bath for 30 min. Next, ice-cold DCC was added, the reaction was incubated for 10 min, and the tubes were centrifuged at 2,800g at 4°C for 5 min. The supernatants were transferred to scintillation vials and radioactivity was measured by standard liquid scintillation spectroscopy using a Beckman-Coulter LS6000 K.

Of the total circulating CORT (total CORT), most is bound to CBG, whereas only a small fraction circulates as unbound, or ‘free’, CORT. The ‘free hormone’ hypothesis suggests that because only free (unbound) hormones are able to enter cells and activate intracellular or membrane receptors, and are thus biologically active, the magnitude of the effect that the glucocorticoid response has on the body is determined by the circulating level of free CORT. Though some evidence supports the free hormone hypothesis, other results suggest the role of CBG in not just to ‘mop-up’ free hormone but also to deliver bound hormones to target tissues (Breuner and Orchinik 2002; Khosla 2006; Petersen et al. 2006). Given the hypothesized importance of free CORT, we calculated the level of free CORT in the plasma of each individual using the free CORT index (FCI), defined as $(\text{total CORT} \times 100)/\text{CBG}$, with both total CORT and CBG in molar units (Hamrahian et al. 2004; Davidson et al. 2006). The FCI has been shown to be highly correlated with direct measurements of free CORT in humans (le Roux et al. 2002). The results of the statistical analyses described below were the same, whether we used the FCI, or the more complicated algorithm suggested by Breuner and Orchinik (2002) together with any of the affinity estimates reported in Breuner et al. (2006, their Table 2).

Statistical analyses

We report the results from three sets of analyses that are progressively more restrictive as to which individuals are included. Because of occasional sample loss, we were sometimes only able to assay either total CORT or CBG in an individual, and because of the logistical difficulty, we were sometimes only able to catch either the father or the mother at a nest. In the first set of analyses, we utilized all the available data from all individuals to evaluate predator pressure (HPP vs. LPP), sex (male vs. female) and year effects on total CORT, CBG and free CORT (FCI), respectively, using three-way ANOVAs. Next, we included only those individuals in which we successfully assayed both total CORT and CBG and conducted a MANOVA on both measures, evaluating predator pressure (HPP vs. LPP), sex (male vs. female) and year effects, with nest identity included as a random effect. Finally, in the third set of analyses, we compared the father and mother caught at the same nest on the same

day. This last set of analyses is the most rigorous because comparing the father and mother at the same nest controls for numerous potentially confounding sources of variation between them, such as sampling date, brood size, territory quality and prior reproductive effort (since song sparrows rarely switch mates between nesting attempts within a season; Smith et al. 2006). We utilized the values for the father and mother from the same nest as the repeated measure in a set of repeated-measures two-way ANOVAs evaluating predator pressure (HPP vs. LPP) and year effects on, total CORT, CBG and free CORT (FCI), respectively.

Prior to analysis all data were Box–Cox transformed (Krebs 1999) and tested for normality and homogeneity of variances. In each ANOVA, we tested all main effects and possible interactions. Degrees of freedom vary because of occasional sample loss. In “Results”, we only report *F* values for those terms that were significant or where non-significant terms are relevant to the “Discussion”. The descriptive statistics reported are means \pm SE.

Results

Considering all the available data from all individuals, the mean level of total CORT measured in males at high predator pressure locations was much higher than the mean among males at LPP locations, whereas the mean among females did not vary with predator pressure (Fig. 1a). This contrast between the sexes in their total CORT response to predator pressure was significant (predator pressure by sex interaction $F_{1,211} = 6.3$, $P = 0.013$) and evident in all 3 years (three-way interaction $F_{1,211} = 0.1$, $P = 0.887$). The contrast between the sexes in their total CORT response to predator pressure was mirrored by an inverse contrast in their CBG response to predator pressure. Whereas male CBG levels did not vary markedly with predator pressure, females at high predator pressure locations had much lower CBG levels than females at LPP locations (Fig. 1b). As with total CORT, the contrast between the sexes in their CBG response to predator pressure was significant (predator pressure by sex interaction $F_{1,119} = 6.2$, $P = 0.014$) and evident in all years (three-way interaction $F_{1,119} = 0.8$, $P = 0.387$). Higher total CORT or lower CBG will result in an increase in free CORT because the former increases the numerator and the latter decreases the denominator in the equation used to calculate free CORT $[(\text{total CORT} \times 100)/\text{CBG}]$. Correspondingly, the higher total CORT in males (Fig. 1a) and lower CBG in females (Fig. 1b) resulted in mean free CORT being significantly higher at high predator pressure locations, independent of sex (Fig. 2; predator pressure $F_{1,114} = 5.4$, $P = 0.022$; predator pressure by sex

Fig. 1 Plasma total CORTicosterone concentrations (a) and corticosteroid binding globulin (CBG) binding capacities (b) in parental male (closed circles connected by solid line) and female (open circles connected by dashed line) song sparrows (*Melospiza melodia*) on day 6 of the chick-rearing period, at low- and high predator pressure locations. Symbols show means \pm SE

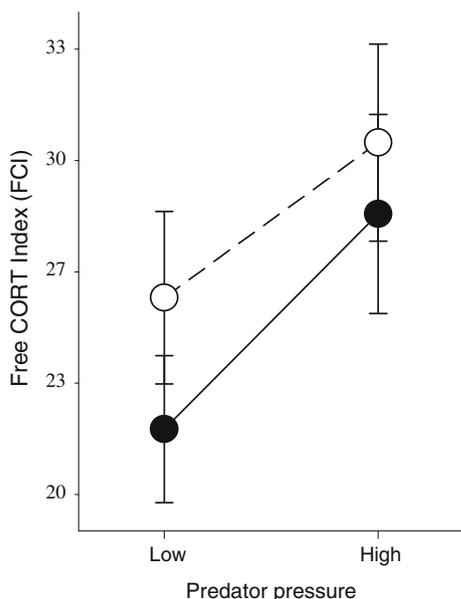
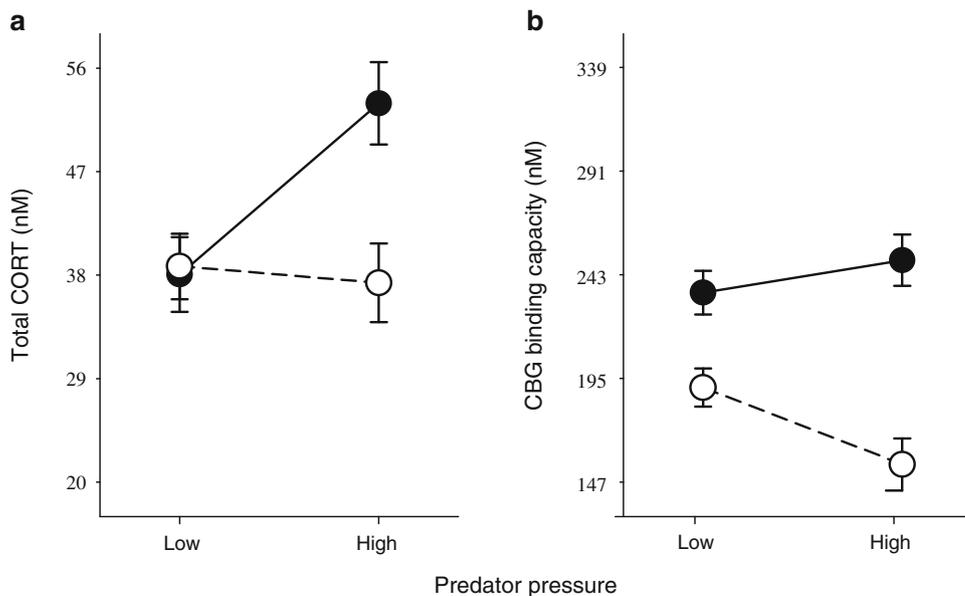


Fig. 2 Free CORTicosterone index (FCI) values in parental males (closed circles connected by solid line) and females (open circles connected by dashed line) on day 6 of the chick-rearing period, at low- and high predator pressure locations. Symbols show means \pm SE

interaction $F_{1,114} = 0.2$, $P = 0.663$; three-way interaction $F_{1,114} = 0.6$, $P = 0.462$).

Considering just those individuals in which we assayed both total CORT and CBG, there was a significant predator pressure by sex interaction ($F_{2,32} = 4.6$, $P = 0.018$) in our MANOVA of both variables, that was evident in all years (three-way interaction $F_{2,32} = 0.6$, $P = 0.562$).

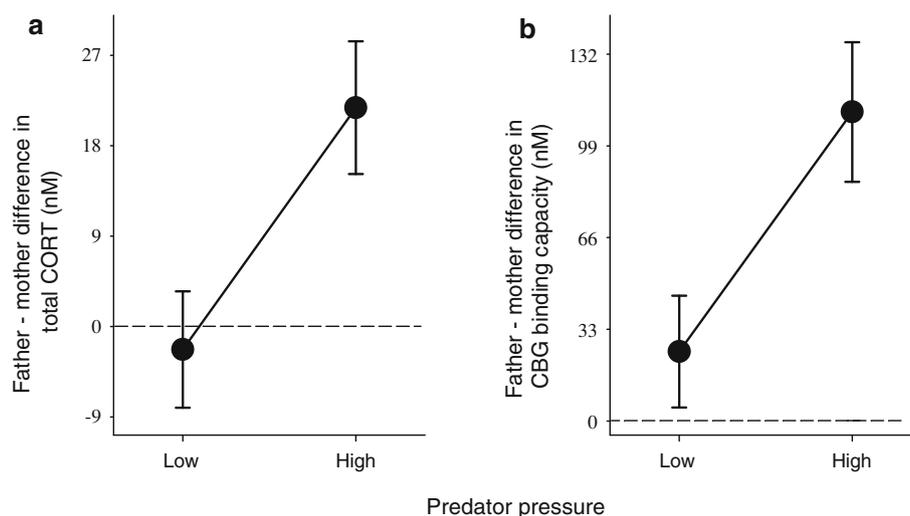
Our most rigorous analyses, comparing the father and mother sampled at the same nest on the same day, revealed the same patterns seen in the less restrictive analyses, and

so verified that the patterns seen were not due to confounding variation in sampling date, brood size, territory quality or prior reproductive effort. Total CORT levels differed markedly between the father and mother at the same nest at higher predator pressure locations, whereas they differed little at LPP locations [Fig. 3a; predator pressure by repeated measure (sex) interaction $F_{1,69} = 7.1$, $P = 0.010$; three-way interaction $F_{1,69} = 1.6$, $P = 0.208$]. Similarly, CBG differed dramatically between the father and mother at the same nest at high predator pressure locations, and differed much less at LPP locations [Fig. 3b; predator pressure by repeated measure (sex) interaction $F_{1,33} = 7.3$, $P = 0.011$; three-way interaction $F_{1,33} = 0.2$, $P = 0.679$]. Consistent with there being no contrast between the sexes in the effect of predator pressure on free CORT (Fig. 2), free CORT levels differed little between the father and mother at the same nest at either high or LPP locations [predator pressure by repeated measure (sex) interaction $F_{1,32} = 0.1$, $P = 0.775$; three-way interaction $F_{1,32} = 0.3$, $P = 0.583$; HPP = -1.6 ± 6.1 father–mother difference in FCI, LPP = -3.9 ± 5.0].

Discussion

Parental male song sparrows had higher total CORT levels at high predator pressure locations (Fig. 1a), as previously reported by Clinchy et al. (2004). Female total CORT levels did not, in contrast, differ markedly between high and LPP locations (Fig. 1a). A total CORT response to environmental variation in predation threat in males, but not females, is what Scheuerlein et al. (2001) found in tropical stonechats. Our evaluation of CBG and free

Fig. 3 Difference in plasma total CORTicosterone concentration (**a**) and corticosteroid binding globulin (CBG) binding capacity (**b**) between the father and mother caught at the same nest on the same day caring for day 6 chicks, at low- and high predator pressure locations. *Panels* illustrate the value for the father minus the value for the mother. The *horizontal dashed lines* indicate no difference between the father and mother. *Symbols* show means \pm SE



CORT, in addition to total CORT, illustrated that female sparrows did in fact demonstrate a glucocorticoid response to environmental variation in predation threat (Fig. 1b), and in the case of free CORT, showed a response parallel to that in males (Fig. 2). Environmental variation in predation threat (HPP vs. LPP) determined whether the father and mother caught at the same nest on the same day did or did not differ markedly in total CORT (Fig. 3a) or CBG (Fig. 3b), demonstrating that other factors were unlikely to explain the between-population differences identified in the mean glucocorticoid responses of males and females (Fig. 1).

The reason why the sexes differed in their glucocorticoid response to environmental variation in predation threat probably involves the interplay between CORT, CBG, progesterone and testosterone. Deviche et al. (2001) showed that, in songbirds, CBG is most likely to bind with progesterone, then CORT, and then testosterone. Progesterone may facilitate parental behavior (Charlier et al. 2009) and testosterone may inhibit it (Soma 2006), though the function of both hormones in wild birds, and particularly progesterone, remains open to question. On day 6 of chick-rearing, both sexes spend the bulk of their time feeding their young (Groves 2009)—the most salient difference in their parental behaviors being that mothers alone brood the chicks. Mothers at high predator pressure locations spend significantly more time at the nest (Groves 2009), presumably to better defend it against predators (e.g., Tewksbury et al. 2002), and increasing their free progesterone by lowering their CBG (Fig. 1b) could conceivably facilitate this (Charlier et al. 2009). In males, lowering their CBG would increase their free testosterone as well as their free CORT, and fathers may conceivably keep their CBG constant (Fig. 1b) to avoid having testosterone inhibit their parental behavior (Soma

2006). Both sexes demonstrated higher free CORT at high predator pressure locations (Fig. 2) presumably to better cope with the higher predation threat, and the different pathways by which they achieved this (i.e. higher total CORT in males, Fig. 1a, and lower CBG in females, Fig. 1b) are consistent with their not compromising their parental duties to do so. We readily acknowledge that this interpretation rests on many untested assumptions about the endocrinology of wild birds but suggest that it does help point to productive avenues for future studies.

Measuring both total CORT and CBG revealed that both males and females demonstrated a glucocorticoid response to environmental variation in predation threat whereas measuring either singly would have led to an ambiguous result. Even if one measures both total CORT and CBG and finds no effect, this is not sufficient to conclude there is no glucocorticoid response, since the number of receptor sites in target tissues may also mediate the glucocorticoid response (e.g., Breuner et al. 2003). Measuring the number of receptor sites entails destructive sampling and so may often be infeasible. An alternative is to measure 'downstream' physiological changes likely to result from a glucocorticoid response (Boonstra et al. 1998). Several of the studies to date that have reported between-population differences in either total CORT or CBG, in response to environmental variation in predation threat, have also reported effects on haematocrit, white blood cell differentials, plasma-free fatty acids, plasma glucose and body condition (Boonstra et al. 1998; Hik et al. 2001; Scheuerlein et al. 2001; Clinchy et al. 2004). Travers et al. (2010) measured multiple physiological variables (15 in total) and used multivariate statistics (e.g., discriminant function analysis) to evaluate the effects on breeding female sparrows of suffering frequent nest predation. We suggest that, at

present, such an approach provides the best means of establishing if environmental variation in predation threat affects the mean glucocorticoid response in wild birds and mammals, given that different individuals may demonstrate one physiological pathway in lieu of another (Fig. 1), for reasons (e.g., the interplay between CBG and other hormones) that are currently difficult to predict.

Our results reveal a link between predation threat in the environment at large and glucocorticoid responses in both males and females. For this to be of relevance to demography further requires demonstrating a link between such glucocorticoid responses and effects on reproduction. To our knowledge, this has not been demonstrated definitively in any wild bird or mammal, though the results from two recent experiments (Sheriff et al. 2009; Travers et al. 2010) provide strong support for such a link. Sheriff et al. (2009) reported both correlational results linking predation threat to glucocorticoid responses and reproduction in wild snowshoe hares (*Lepus americanus*), and experimental results demonstrating that captive female hares exposed to a live predator (a trained dog) for 2 min every other day had elevated fecal glucocorticoid levels and were less likely to give birth. Our results, in conjunction with results from previous papers (Zanette et al. 2003, 2006a, b), provide correlational evidence of links between predation threat, glucocorticoid responses and reproduction in wild song sparrows. Travers et al. (2010), working on wild sparrows at our long-term study sites, compared females subject to either frequent experimental nest predation or no nest predation, and found significant effects on both an array of physiological measures (including total CORT and CBG) and the size of subsequent clutches. Both experimental studies may be faulted, Sheriff et al. because theirs was on captive hares, and Travers et al. because the physiological changes observed may have resulted from either predation threat or more frequent egg-laying. The definitive experiment linking predator-induced glucocorticoid changes to effects on reproduction in wild birds and mammals thus remains to be conducted. Nonetheless, we suggest it appears increasingly probable that predator-induced glucocorticoid responses do mediate indirect predator effects on demography, in at least some birds and mammals.

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