

Adrenocortical responses to offspring-directed threats in two open-nesting birds

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ABSTRACT

Dependent young are often easy targets for predators, so for many parent vertebrates, responding to offspring-directed threats is a fundamental part of reproduction. We tested the parental adrenocortical response of the endangered black-capped vireo (*Vireo atricapilla*) and the common white-eyed vireo (*Vireo griseus*) to acute and chronic threats to their offspring. Like many open-nesting birds, our study species experience high offspring mortality. Parents responded behaviorally to a predator decoy or human 1–2 m from their nests, but, in contrast to similar studies of cavity-nesting birds, neither these acute threats nor chronic offspring-directed threats altered plasma corticosterone concentrations of parents. Although parents in this study showed no corticosterone response to offspring-directed threats, they always increased corticosterone concentrations in response to capture. To explain these results, we propose that parents perceive their risk of nest-associated death differently depending on nest type, with cavity-nesting adults perceiving greater risk to themselves than open-nesters that can readily detect and escape from offspring-directed threats. Our results agree with previous studies suggesting that the hypothalamic–pituitary–adrenal axis, a major physiological mechanism for coping with threats to survival, probably plays no role in coping with threats to offspring when risks to parents and offspring are not correlated. We extend that paradigm by demonstrating that nest style may influence how adults perceive the correlation between offspring-directed and self-directed threats.

Keywords:

Chronic stress
Corticosterone
Parental care
Nest predation
Stress response

1. Introduction

Wild animals must sense and respond to a variety of threats and unpredictable disturbances in order to maximize fitness. A common approach to studying this process is to observe (Clinchy et al., 2004), introduce (Cockrem and Silverin, 2002), or remove (Ahola et al., 2006) a potential threat to an animal, and measure subsequent changes in physiology, behavior, or survival. Such studies have revealed much about the mechanisms, benefits, and costs that underlie responses to acute threats to individual survival. However, much less is known about how animals perceive and respond to acute threats to the survival of their offspring.

Parental responses to offspring-directed threats are readily studied in nesting species with extended parental care. In particular, the adrenocortical response to stress, typified by changes in plasma concentrations of the glucocorticoid corticosterone (hereafter "CORT"; Wingfield and Romero, 2001), has been a useful measure of the parental response to offspring-directed threats in birds. For example, Magellanic penguins (*Spheniscus magellanicus*; Fow-

ler, 1999; Walker et al., 2006) and European starlings (*Sturnus vulgaris*; Cyr and Romero, 2007) show altered parental CORT concentrations following acute and chronic threats to nests containing eggs and nestlings. However, threats to offspring were not fully isolated from threats to adults in these studies because the adults could not easily escape, either because they were non-volant (Magellanic penguins, Fowler, 1999; Walker et al., 2006), or because they were nesting in boxes with a single small entrance hole (European starlings, Cyr and Romero, 2007). Additionally, in adult pied flycatchers (*Ficedula hypoleuca*) nesting in boxes, parental CORT increased in response to a decoy of a predator of adults and offspring (weasel, *Mustela vulgaris*), but not in response to a predator of offspring only (woodpecker, *Dendrocopos major*; Silverin, 1998). Thus, to understand the parental response to offspring-directed threats, those threats must be distinct from self-directed threats, either because parents are free to modulate their level of risk (e.g., free-living great tits, *Parus major*, exposed to a predator, Cockrem and Silverin, 2002), or because the threat is offspring-specific (e.g., the offspring-only predator used by Silverin, 1998).

We attempted to isolate the parental response to offspring-directed threats in two passerines nesting in highly exposed open cups, where parents at the nest could easily detect and escape from nest-directed threats. One of our study species, the black-capped vireo (*Vireo atricapilla*), is endangered and has a limited range in

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central North America, while our other study species, the white-eyed vireo (*V. griseus*), is common and widespread. In Experiment 1 we tested the prediction that naturalistic and anthropogenic offspring-directed threats and disturbances lasting several days would result in parents with suppressed baseline and stress-induced CORT, typical of chronic stress in European starlings nesting in boxes (Rich and Romero, 2005; Cyr and Romero, 2007). In Experiment 2 we tested the predictions that naturalistic and anthropogenic offspring-directed threats lasting several minutes would increase plasma CORT in parents, typical of an acute stress response, and alter CORT response of parents to a subsequent self-directed threat (capture and handling; Dallman et al., 1992). This study is distinct from all prior investigations of the parental response to offspring-directed threats because (1) the focal species were volant open-nesters (2) responses to a naturalistic threat were compared to a human threat (3) effects of acute offspring-directed threats were compared to chronic threats, and (4) responses of an endangered species were compared to a common species.

2. Materials and methods

2.1. Study site

Experiments were conducted at Fort Hood, an 88,000-ha military training area in Texas, USA (31.2°N 97.8°W), which supports the largest managed population of black-capped vireos (Cimprich and Kostecke, 2006) and a large sympatric population of white-eyed vireos. We avoided working where training occurred, we rarely detected training activity anywhere in our study site, and we never sampled a bird on the same day that we detected any activity within view of the breeding territory.

2.2. Experiment 1

From 1 May to 5 June 2006, we attempted to create chronically-stressed parent vireos following the protocols of Rich and Romero (2005) as adapted to field conditions by Cyr and Romero (2007) for European starlings. Our disturbance protocol lasted 7–12 days and occurred while adults were incubating eggs or brooding and feeding nestlings, tasks shared about equally between sexes in black-capped vireos (Grzybowski, 1995) and white-eyed vireos (Hopp et al., 1995). We presented four, 30–60 min offspring-directed threats or disturbances (i.e., stressors) at each nest per day, with varying amounts of time between presentations (0.5–3.0 h), and in random order but with the same threat never repeated at the same nest within a day. Threats were either naturalistic decoys of predators on adults and nestlings (2-m rubber snake, plastic American crow (*Corvus brachyrhynchos*) with call playback, and plastic eastern screech owl (*Megascops asio*) with call playback), or anthropogenic disturbances (human sitting or standing, human voice playback, and novel objects, such as a starkly-colored helium balloon).

Predator decoys and novel objects were attached to vegetation 1–2 m from the focal nest at or above the height of the nest. Decoys were placed in an upright, perched position, and oriented toward the nest. During human presence, one person sat or stood and occasionally moved and spoke.

Predator calls and human voice playback were made using battery-powered, handheld digital audio players (Muvo mp3 players, Creative Labs) connected to small (9 × 8 × 5 cm) amplified speakers (Mini Audio Amplifier, RadioShack) placed on the ground below or in vegetation <2 m from the decoy. Ten-second predator calls were played randomly among two 20-s bouts of silence, and were audible no more than 5–10 m from the nest to avoid attracting predators. We used 35–40 min of a radio talk show for human voice playback.

Rich and Romero (2005) showed that a very similar chronic stress protocol caused CORT concentrations to decrease in captive starlings after 8–10 days, and then remain lower than non-chronically-stressed concentrations for at least 27–29 days, when the protocol was discontinued. Likewise, Cyr and Romero (2007) showed that a very similar protocol lasting 9 days caused CORT to decrease in free-living European starlings. We targeted adult vireos for sampling after 7–9 days of stressors in order to reduce the high risk of losing samples because of nest loss. In Experiment 1, no stressors were presented on the day CORT was sampled.

2.3. Experiment 2

From 28 April to 2 July 2007, we sampled CORT in adults randomly assigned to one of four disturbance treatments. One group was presented only with 10–35 min of crow decoy or human presence (as described in Experiment 1) immediately before capture and sampling. A second group was exposed to the chronic disturbance protocol of Experiment 1 for 5–6 days (including the crow and human presentations), and then presented with the crow or human for 10–35 min immediately before capture. A third group was exposed to the chronic disturbance protocol for 5–6 days but received no presentation immediately before capture. A fourth group received no disturbance of any kind before blood sampling. All groups were held for 30 min before a second blood sample was taken to measure the CORT response to a self-directed threat (capture and restraint).

2.4. Disturbance trials and capture of focal animals

On the day before blood sampling, one or two closed, 6 × 2.6-m mist nets were placed 2–10 m from the target nest and perpendicular to flight paths used by adults to access the nest. For crow and control trials, hiding places where one or two observers could continuously monitor the mist nets were also identified. Hiding places were located on the ground below or between leafy shrubs at least 5 m from the target nest, depending on vegetation density and sight lines, with other vegetation arranged to conceal the observer(s). Small speakers were placed near the net(s) and connected to audio cables extending to the hiding place for playing conspecific songs or calls to lure the focal animals to the net(s).

On the day of a crow trial, one or two people quickly entered the territory, opened the mist net(s), turned on the capture playback speakers, placed the crow in vegetation 1–2 m from the nest and directed it toward the nest, placed the crow call speaker under the crow, and started the crow playback. The end of a 0.6-cm nylon rope tied to the crow was then carried to the hiding place. This process lasted 1–3 min, and was usually completed before adults returned from foraging. Adults were exposed to the crow for 10–35 min before capture, and then immediately sampled for blood. Adults almost always stopped feeding nestlings during the crow trial, so after 30–35 min the crow was pulled away using the rope, at which point adults usually fed the nestlings or inspected the nest before flying away and being captured in one of the mist nets.

On the day of a human presence trial, one person entered the territory, opened the mist net(s), turned on the capture playback speakers, and stood 1–2 m from the nest in view of the adults for 10–35 min before blood sampling. Adults usually stopped feeding nestlings after the person approached the nest, but occasionally, especially late in a trial, the person moved around the nest to thwart adults attempting to access it.

The control group was exposed to no experimental disturbance immediately before capture (i.e., an observer simply opened the nets, turned on the speakers, and hid), or was exposed to less than 10 min of crow or human. If no adult was captured 70 min after the start of a trial, the mist nets were

closed and the crow or human removed for at least 2 h before trying the other disturbance trial.

In 2006, logistics occasionally required abandoning the above protocol and setting a net on the same day we sampled adults, but this probably had no effect on CORT (see Section 3). In both years, trials were performed on nests containing nestlings for all black-capped vireo samples and most white-eyed vireo samples (one crow trial and two human trials were performed on white-eyed vireo nests that contained eggs).

2.5. Blood sampling and analysis

Mist nets were monitored continuously and blood samples were acquired within 3 min of capture, so CORT concentration at capture probably reflected response to the preceding disturbance and not to capture (Romero and Romero, 2002; Romero and Reed, 2005). Blood was drawn into 60- μ l heparinized glass capillary tubes after pricking the alar vein with a 26-ga needle. Bleeding was stanchied by applying cotton and pressure. After the first (baseline) sample, birds were placed in an opaque cotton bag for 30 min, and then sampled for stress-induced concentrations before release. Methods were approved by the Institutional Animal Care and Use Committee at Tufts University.

Blood was stored on ice in the field and centrifuged within 24 h, at which time the plasma was removed and frozen until it was returned to Tufts University for assaying. Total plasma CORT concentration was assayed using a standard radioimmunoassay (Wingfield et al., 1992), with interassay variation of 17.6%, intra-assay variation of 3.9%, and a detection limit of 1.34 ng/ml. Due to the small size of both species (8–10 g), and the endangered status of black-capped vireos, legal limitations precluded sampling large numbers of individuals and making comparisons of corticosterone binding globulin (CBG), which is thought to carry CORT to target substrates or bind CORT and make it unavailable to target substrates (Breuner and Orchinik, 2002; Romero, 2002). Thus, we report only total plasma CORT levels.

In both study species, males take part in building the nest, incubating the eggs, and feeding nestlings and fledglings (Graber, 1961; Hopp et al., 1995), and baseline and stress-induced CORT concentrations do not differ between the sexes on our breeding site (unpublished data), so we combined males and females in our analyses. Samples did not violate the assumption of homogeneous variance (all $p > 0.05$ in Levene's test; Zar, 1996). We used a mixed model ANOVA (SAS Institute, 2004) to compare baseline and restraint-induced CORT levels between chronically- and non-chronically-disturbed birds (Experiment 1) and among disturbance treatments (crow, human, and control; Experiment 2). This statistical procedure incorporates individuals from which only a baseline or a 30-min sample was obtained. To verify that our nest-directed threats actually disturbed adults, we made qualitative descriptions of adult behavior during the human and crow presentations. Samples in all groups were distributed over time, but we included day of year as a covariate in 2007 analyses because sampling spanned more than two months. Alpha was set at 0.05.

3. Results

3.1. Behavioral response to crow and human

Adults at the nest flew away when the crow decoy was placed, or when the human approached and stood, at the nest. Adults then usually behaved as they do when a snake (the most common nest predators at our site; Stake and Cimprich, 2003) or predatory bird is near the nest or elsewhere in the territory, by making characteristic alarm

vocalizations at a high rate, and halting feeding and territorial behavior (LKB and TJH, personal observation).

3.2. Experiment 1

CORT concentrations of white-eyed (Fig. 1a) and black-capped vireo (Fig. 1b) parents strongly increased in response to 30 min of capture and restraint (ANOVA, time after capture: $F_{1,23} = 45.5$, $p < 0.0001$ and $F_{1,28} = 33.3$, $p < 0.0001$ for white-eyed and black-capped vireos, respectively). However, contrary to prediction, chronic offspring-directed threats did not alter baseline or stress-induced plasma CORT concentrations in parents of either species (white-eyed vireos, stress treatment: $F_{1,23} = 0.01$, $p = 0.91$; stress treatment \times time after capture: $F_{1,23} = 0.04$, $p = 0.85$; black-capped vireos, stress treatment: $F_{1,28} = 0.001$, $p = 0.95$; stress treatment \times time after capture: $F_{1,28} = 0.02$, $p = 0.90$), compared to undisturbed (control) parents (Fig. 1).

3.3. Experiment 2

The first exposure to the crow decoy or a human at the nest caused no significant increase in CORT compared to control (undisturbed) adults in either white-eyed vireos (ANCOVA with day of year as covariate, overall effect of threat treatment: $F_{2,13} = 1.55$, $p = 0.25$, Fig. 2a) or black-capped vireos (threat treatment: $F_{2,17} = 1.16$, $p = 0.34$, Fig. 2b). CORT increased after 30 min of capture and restraint in both species (time after capture, $F_{1,26} = 23.2$, $p < 0.0001$ and $F_{1,31} = 37.3$, $p < 0.0001$ for white-eyed and black-capped vireos, respectively), but interestingly, this response was lower in adults if

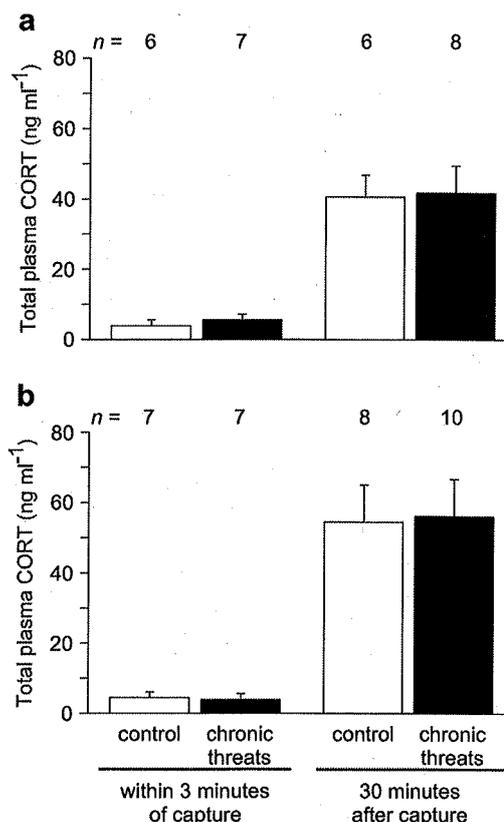


Fig. 1. Corticosterone (CORT) levels in parent vireos within 3 min of capture and after 30 min of restraint. Several days of prior chronic disturbances (filled bars) had no effect on CORT levels compared to undisturbed controls (open bars) in (a) white-eyed and (b) black-capped vireos.

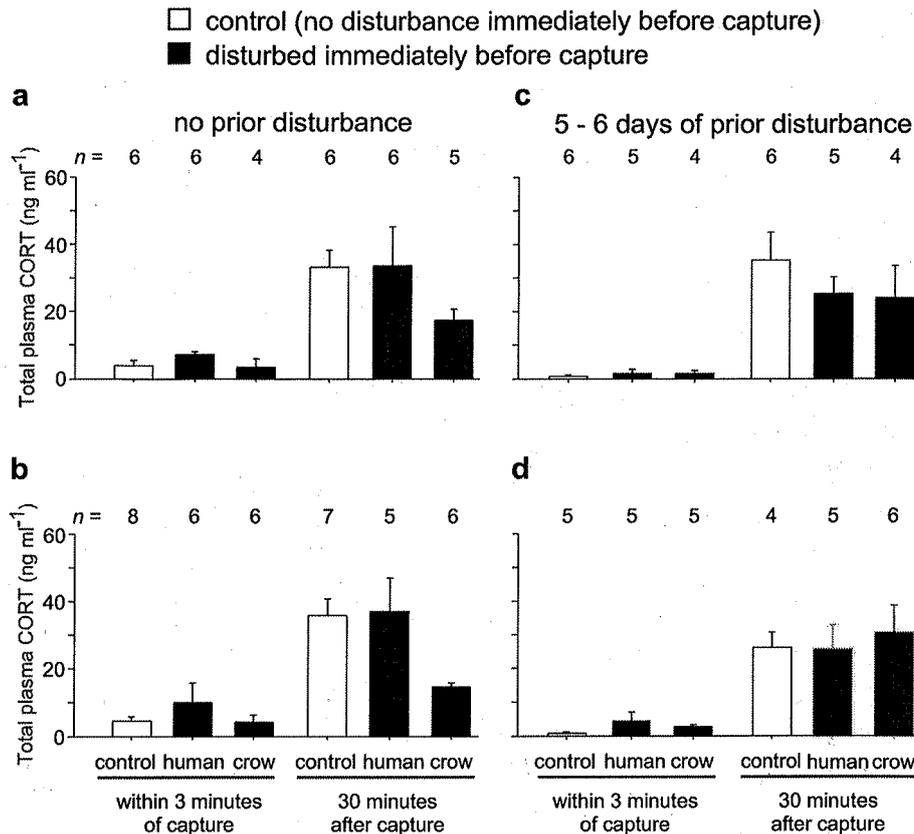


Fig. 2. Corticosterone (CORT) levels in adult vireos within 3 min of capture and after 30 min of restraint following their first exposure (a, white-eyed vireos; b) black-capped vireos) to nest-directed threats (crow or human; filled bars) or no threat (open bars). Same responses after 5–6 days of several nest-directed threats per day (c, white-eyed vireos; d) black-capped vireos).

they were exposed to the crow decoy immediately before capture (Fig. 2a and b). This effect was marginally non-significant in black-capped vireos (interaction term threat treatment \times time after capture: $F_{2,31} = 2.90$, $p = 0.07$; white-eyed vireos: $F_{2,23} = 0.92$, $p = 0.41$), but disappeared after 5–6 days of offspring-directed threats ($F_{2,23} = .23$, $p = 0.80$, Fig. 2d). Although the CORT response of black-capped vireos to their first exposure to human presence (mean \pm SE = 10.1 ± 5.7 ng ml⁻¹, $n = 6$) might appear higher than undisturbed controls (4.6 ± 0.9 ng ml⁻¹, $n = 8$; Fig. 2b), that difference disappears if one individual with a baseline CORT concentration (37.5 ng ml⁻¹) similar to capture stress-induced levels (35.8 ± 5.2 ng ml⁻¹, $n = 7$) is removed (first exposure to human presence excluding that sample, 4.6 ± 1.8 ng ml⁻¹, $n = 5$). The exceptionally-high CORT concentration at the time of capture in this individual might reflect inter-individual variation in sensitivity to humans, or it might indicate that the animal was exposed to an unknown stressor prior to capture.

Adults did not develop sensitivity to offspring-directed disturbances after repeated exposure (i.e., examining "baseline" concentrations before and after 5–6 of disturbance). Baseline CORT was unaltered by the crow disturbance and the human disturbance in both species after 5–6 days of offspring-directed threats (white-eyed vireos, threat treatment: $F_{2,12} = 0.60$, $p = 0.57$, Fig. 2c; black-capped vireos, threat treatment: $F_{2,12} = 2.20$, $p = 0.16$, Fig. 2d).

Finally, similar to Experiment 1, in which we found no effect of 7–10 days of offspring-directed threats on baseline or restraint-induced CORT levels of adults, we found no effect of 5–6 days of offspring-directed threats on CORT (comparing controls before and after 5–6 days of threats, in ANCOVA with day of year as covariate, all $p > 0.10$).

4. Discussion

We attempted to isolate and measure the adrenocortical response of open-nesting birds to threats to their dependent young, and compare that response to threats to adults. Adults responded to capture stress with a large increase in CORT, but acute and chronic offspring-directed threats had little effect on glucocorticoid concentrations. Our findings suggest that in our two study species, functioning of the hypothalamic–pituitary–adrenal (HPA) axis is, at most, only very weakly affected by offspring-directed threats. Nevertheless, our threats appeared to be perceived as threats by parents because normal parental care almost always ceased during presentations and alarm vocalizations and overall activity around the nest increased, very similar to the effect of live natural predators. This contrast between physiological and behavioral responses to a threat to fitness has been found in other vertebrates (e.g., Silverin, 1998; Fletcher and Boonstra, 2006). Thus, although a major effect of increased glucocorticoid concentrations, energy mobilization (Sapolsky et al., 2000), would seem to help sustain the observed behavioral responses of parents, or to help prepare them if the offspring-directed threat became an adult-directed threat, we found no evidence that CORT is involved in permitting or preparing for defense behaviors in parents.

Our results contrast similar studies showing that the HPA axis was altered (either stimulated or suppressed) following short- and long-term exposure to offspring-directed threats. Threats to nest boxes increased CORT in pied flycatchers after several minutes (Silverin, 1998), and decreased CORT in European starlings after several days (Cyr and Romero, 2007). Additionally, male East

African stonechats (*Saxicola torquata axillaris*) nesting in territories shared by a fiscal shrike (*Lanius collaris*), a predator on adults and young which likely acted as a chronic stressor, had higher CORT than stonechat males in territories with no shrike (Scheuerlein et al., 2001). Parent blue tits (*Parus caeruleus*) showed no CORT response to a human 20–30 m away from a nest (Müller et al., 2006), but it is unknown whether parents perceive humans as a threat to their offspring in this species. Based on these studies, a reasonable conclusion would be that threats to nests, and thus threats to reproductive success, elicit CORT responses in parent songbirds. Our data clearly do not fit this pattern, which we suggest results from contrasts in the degree of overlap between offspring- and adult-directed threats.

We propose that nest-directed threats elicit a physiological response (i.e., CORT release) in adults only when they threaten adults and offspring alike, which depends on the type of threat and the ability of adults to escape from it. Previous studies of the adrenocortical response to offspring-directed threats have not fully separated threats to offspring from threats to adults because the nest characteristics of the study species put adults at a higher risk of nest-associated death than the vireos in our study. Stonechats (Scheuerlein et al., 2001) build well-hidden and sometimes domed nests near or on the ground (Greig-Smith, 1982; Scheuerlein and Gwinner, 2006), and pied flycatchers (Silverin, 1998) and starlings (Cyr and Romero, 2007) were nesting in boxes with a single, small, side opening. Thus, detecting and escaping from a nest-directed threat may pose a challenge for adults of those species. Indeed, predators can be highly successful at targeting cavity-nesting adults in their nest: at least half of predation events at the natural cavity nests of marsh tits (*Parus palustris*) resulted in death of an incubating adult inside the nest cavity (Wesolowski, 2002). In contrast, when we threatened active nests of black-capped and white-eyed vireos, parents could easily detect and control their perceived risk relative to the disturbance, because they build cup-shaped pendant nests attached near the tips of thin branches (Graber, 1961; Hopp et al., 1995) that are often flanked by flight lanes and surrounded by a "bubble" of open space.

An alternative explanation may be that a glucocorticoid response to nest-directed threats is unfavorable when threats are common and when redirection of behavior from breeding to survival lowers overall fitness. Nest failure is much more common for black-capped and white-eyed vireos (~50–70%; Cimprich, 2006; Hopp et al., 1995) than for pied flycatchers and European starlings (~10%; Huhta et al., 1998; Collins and DeVos, 1966). Furthermore, vireos at our site commonly re-nest following nest failure, which is possible because of the extended breeding season in Texas (31°N). In contrast, pied flycatchers were studied at 57°N (Silverin, 1998) and European starlings were studied at 42°N (Cyr and Romero, 2007), where breeding seasons are considerably shorter. Therefore, a large CORT response to a nest-directed threat might reduce fitness of a Texas vireo by suppressing successful re-nesting, but in a flycatcher or starling breeding at northern latitudes, a large CORT response might increase fitness by facilitating abandonment of futile re-nesting activities in preparation for molt, migration, or winter. However, one might predict higher CORT responses in low latitude species because they can afford to abandon a breeding attempt when other attempts are possible in the same year (Wingfield and Sapolsky, 2003).

However, several independent lines of evidence support our hypothesis that the ability to modulate risk exposure determines whether birds initiate a CORT response to a nest-directed threat. Incubating Magellanic penguins can see a human approaching but they cannot easily escape because they do not fly, and they increase CORT in response to a human standing nearby (Fowler, 1999; Walker et al., 2006). Free-living great tits (*Parus major*) showed a much smaller CORT response to a stuffed owl (a predator

on tits) than did captive tits trapped with the same owl in an aviary (Cockrem and Silverin, 2002). Longnose killifish (*Fundulus majalis*) exhibited a smaller cortisol response to the sight of a predatory fish in tanks containing vegetative cover than in open tanks (Woodley and Peterson, 2003). Therefore the ability to escape a threat, and not the perception of the threat alone, appears to influence whether the HPA axis is stimulated.

We cannot exclude the possibility that CBG concentrations declined in parents in response to our offspring-directed threats (Breuner et al., 2006), which would increase free CORT concentrations without increasing total CORT. In female European starlings, free CORT, and not total CORT, predicted nest desertion (Love et al., 2004). However, we never observed nest desertion by parents among the dozens of nests we exposed to our chronic disturbance protocol (LKB, unpublished data), so we believe that the types and intensities of disturbances used in this study are unlikely to cause nest abandonment in these species.

Using physiological measures to detect disturbance caused by human activity is a growing practice (Creel et al., 2002; Homan et al., 2003; Romero, 2004; Gobush et al., 2008), and identifying what makes some species at risk of extinction is an important goal of conservation (Owens and Bennett, 2000; Purvis et al., 2000; Reynolds et al., 2005). However, to our knowledge this is the first study to compare the endocrine responses of a free-living endangered animal and its common, sympatric relative to experimental offspring-directed disturbances. If the endangered status of black-capped vireos was due to a special sensitivity of the species to human activity during the breeding season, then we might predict that black-capped vireos would show a greater adrenocortical response than white-eyed vireos to our nest-directed threats, but this was not the case. However, the contrasting sensitivities to nest-directed threats between open-nesters (this study) and cavity-nesters (Silverin 1998; Cyr and Romero 2007) suggest that a nest style that causes a high correlation between offspring- and adult-directed threats may be a risk factor for sensitivity to human activity.

The present study illustrated a contrast in HPA axis function in two free-living birds presented with multiple threats to their offspring contained in open cup nests. Whereas glucocorticoid concentrations in parents increased dramatically following capture, two different threats to offspring caused no such increase, suggesting that adults perceived no risk to themselves from nest-directed threats. Although adults responded behaviorally to offspring-directed threats, this behavioral change was not accompanied by a change in the HPA axis, unlike the behavioral response to other stressors. Thus, a major physiological coping mechanism of vertebrates, the HPA axis, does not appear to be involved in responding to threats to reproduction as it is involved in responding to threats to survival.

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