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Source: *Physiological and Biochemical Zoology*, Vol. 86, No. 2 (March/April 2013), pp. 279-284
Published by: The University of Chicago Press
Stable URL: http://www.jstor.org/stable/10.1086/668893
Accessed: 25/03/2013 09:55

TECHNICAL COMMENT

An Experimental Test of the Capture-Restraint Protocol for Estimating the Acute Stress Response

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Accepted 10/30/2012; Electronically Published 1/10/2013

ABSTRACT

Stress-induced increases in glucocorticoids (GCs) modulate behavior and are key in directing energy reserves. The capture-restraint protocol was developed to experimentally stimulate and quantify the magnitude of the acute stress response by comparing baseline GC levels with those collected after restraining a subject for a period of time, typically 30 min. This protocol has been used extensively in the field and lab, yet few studies have investigated whether it parallels hypothalamic-pituitary-adrenal (HPA) activation under natural acute stressors. We examined the hypothesis that acute stress from the capture-restraint protocol accurately mimics the adrenocortical response induced by a natural acute stressor. Using wild-caught rock pigeons *Columba livia* in a repeated-measures design, we compared plasma corticosterone (CORT) concentrations at baseline, after exposure to acute capture-restraint (30 min in a cloth bag), after tethering in a harness (30 min), and after a real but nonlethal attack by a predatory raptor. As found in previous studies, the capture-restraint treatment significantly increased CORT levels of pigeons compared with baseline. However, we also found that when pigeons were exposed to an attack by a raptor, their CORT levels were more than twice as high compared with the capture-restraint treatment. Our results provide evidence that an authentic acute stressor can activate the HPA axis to a greater extent than the capture-restraint protocol and also suggest that real predation attempts can have a significant effect on acute stress levels of wild birds.

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Physiological and Biochemical Zoology 86(2):279–284. 2013. 1522-2152/2013/8602-2075$15.00. DOI: 10.1086/668893

Introduction

Acute stress activates the hypothalamic-pituitary-adrenal (HPA) axis and results in increased circulating glucocorticoid (GC) levels. Elevated GCs modulate metabolism, behavior, immune function, and energy allocation (Romero 2004; Martin 2009), and the resulting physiological and behavioral responses are major factors influencing an organism's survival and reproduction in the wild (Wingfield et al. 1997; Romero and Wikelski 2001; Sheriff et al. 2009; Koren et al. 2011). However, measuring the physiological response to acute stress in the wild has been limited by the inability to realistically simulate acute stressors such as predation threat.

In many animal model systems, the acute stress response has been measured by quantifying corticosterone (CORT) concentrations using the standardized "capture-restraint protocol" (Wingfield et al. 1992, 1994). The capture-restraint protocol compares plasma CORT levels collected at baseline with those collected after a standard period of acute restraint, typically 30 min in a cloth bag (Wingfield et al. 1992, 1994). This method allows quantification of HPA axis activation in response to an acute stressor. Capture-restraint has been used in many avian stress studies (e.g., Astheimer et al. 1994; Kitaysky et al. 1999; Scheuerlein et al. 2001; Canoine et al. 2002; Love et al. 2004; Newman et al. 2008; Sheldon et al. 2008; Clinchy et al. 2011) and is commonly applied across taxa (e.g., mammals [Figueiredo et al. 2003; Romero et al. 2008], fish [Baer and Thomas 1990], and reptiles [Jessop et al. 2002, 2004; Berger et al. 2007; Palacios et al. 2012]).

In the only evaluation of the capture-restraint protocol, Canoine et al. (2002) used captive-raised European stonechats *Saxicola torquata rubicola* to compare CORT levels in one set of birds after a 30-min restraint in a cloth bag with CORT levels in different sets of birds that were either housed in a novel cage or housed in a novel cage and simultaneously exposed to a blind, tame owl (predator). Owl exposure increased CORT levels more than the 30-min restraint; however, the CORT levels in birds exposed to the owl were not different from the CORT levels in birds kept in the novel cage without exposure to the owl (Canoine et al. 2002), making the distinction between exposure to the novel cage or the predator unclear. One possibility is that because the owl was tame and did not directly attack the birds, it may not have accurately elicited a predator response from the subjects. Alternatively, captive-raised birds may respond differently to stress treatments than wild birds. Indeed, Cockrem and Silverin (2002) reported that wild free-living great tits *Parus major* exposed to a stuffed predator did not increase CORT levels. However wild-caught captive great tits did increase CORT levels in response to a stuffed predator, suggesting that in wild birds, the ability to escape may play a role in the HPA axis response to a stressor.

To examine the hypothesis that the capture-restraint protocol accurately mimics exposure to an acute stressor, such as an encounter with a natural predator, we compared baseline plasma CORT levels of wild-caught rock pigeons *Columba livia* with CORT levels 30 min after exposure to (1) the capture-restraint protocol and (2) acute exposure to an authentic predation attempt by a raptor. We predicted that stress-induced CORT levels would not differ between these two acute stressors. To our knowledge, we provide the first field study comparing the acute stress response from the capture-restraint protocol with a realistic natural acute threat.

Methods

Study Species

Nonbreeding rock pigeons *Columba livia* ("pigeons" hereafter) were captured in Toronto, Ontario, in early September 2011 and transferred to the study site near Sparta, Ontario (42°7′N, 81°2′W). At this study site, migratory raptors were captured for banding purposes using the pigeons as lures from September to December 2011 under permits from the Ontario Ministry of Natural Resources and Environment Canada. As part of the raptor banding process, pigeons (431 ± 10.2 g) are placed in a protective leather harness and tethered to a lead line. With the ability to move freely on the lead line, the pigeons bait migrant raptors into a bow net that is triggered by the observer, or the raptors fly into a nearby mist net. The pigeons ($n = 20$) were maintained in a large open-air outdoor aviary (2.5 m × 2.5 m × 2.5 m) until experimental trials commenced. In three separate groups (S1: $n = 6$; S2: $n = 7$; S3: $n = 7$), subjects were then transferred to a smaller open-air outdoor enclosure (1 m × 1 m × 0.75 m) to facilitate rapid capture. However, in the smaller enclosure, they were still free to move and fly as in the larger aviary. Experimental trials for each group required 3–4 wk. Subjects were acclimated to the smaller cage for a 7-d period before blood sampling. In the cages, pigeons had access to food and water ad lib. and were monitored daily to ensure conditions were in accordance with the Canadian Council on Animal Care. Body mass was measured regularly throughout the experimental period, and we detected no significant change in body mass over time. Colored leg bands or a combination of plumage characteristics and iris color allowed for individual identification.

Blood Collection

We collected five blood samples per individual, separated by 2–9 d, over the course of a 3–4-wk period. Blood collection was divided across four treatments (described in detail below): baseline (two samples collected 7 d apart), acute restraint in a cloth bag, harness only, and harness + predator. Blood (~150 μL) was collected from the brachial vein into heparinized microhematocrit tubes after puncture with a 26-gauge needle (Hoysak and Weatherhead 1991). Blood samples were kept on ice while in the field and centrifuged within 2–6 h to separate the plasma, and plasma samples were stored at −20°C until analysis.

Experimental Treatments

Baseline and Acute Restraint. One week after pigeons were transferred to the smaller enclosure, baseline blood samples were collected within 3 min of opening the cage door (Romero and Romero 2002; Romero and Reed 2005). Following this baseline sample, pigeons were restrained in a well-ventilated cloth bag for 30 min, after which blood was again collected to replicate the capture-restraint protocol (Wingfield et al. 1992, 1994). In week 2, a second baseline sample was collected from each pigeon to ensure there was not a systematic increase with time or as a result of the week 1 capture-restraint testing. There were no differences in CORT levels between the first and second baseline samples ($t_{19} = 0.91$, $P = 0.37$) and no effects of sampling time (between 1.02 and 2.52 min: $F_{1,19} = 0.14$, $P = 0.71$). Therefore, within an individual we pooled the data for two baseline samples (mean ± SE baseline CORT levels: 3.31 ± 0.37 ng/mL).

Harness Only. In week 2, following the second baseline blood collection, the pigeons were placed in a protective leather harness, attached to a lead line, and gently raised in the air (height of ~0.1–0.3 m) three times over a period of 30 min. This was the same procedure that was used to bait raptors except we conducted these trials when no raptors were seen in the area. The harness allowed for full mobility of the wings and legs while protecting the pigeon from injury during a raptor encounter. We included this harness treatment to separate the potential effects of the harness from the effects of predator exposure on CORT levels. Blood was collected after 30 min of harness treatment.

Harness + Predator. During week 3, the pigeons were again placed in the harness but then exposed to a migratory raptor. To avoid acute activation of the HPA axis before raptor exposure, we did not collect a baseline sample during week 3. Following standard raptor-trapping protocol, we used pigeons to lure raptors that were captured in either a bow net or a mist net. If caught in a bow net, all raptors had direct contact with the pigeon ($n = 11$ pigeons), whereas if caught in a mist net, raptors were within 0.1–2.0 m of the pigeon ($n = 7$ pigeons). Captured raptors were carefully removed, and pigeons were then left undisturbed in a small shelter to avoid further attacks until blood was collected 30 min after predator exposure. The harness provided protection from direct contact with the predator, and none of the pigeons showed visible signs of injury or bleeding. Five species of raptors were involved in the harness + predator treatments: sharp-shinned hawk *Accipiter striata* ($n = 1$), Cooper's hawk *Accipiter cooperii* ($n = 7$), northern goshawk *Accipiter gentilis* ($n = 6$), red-tailed hawk

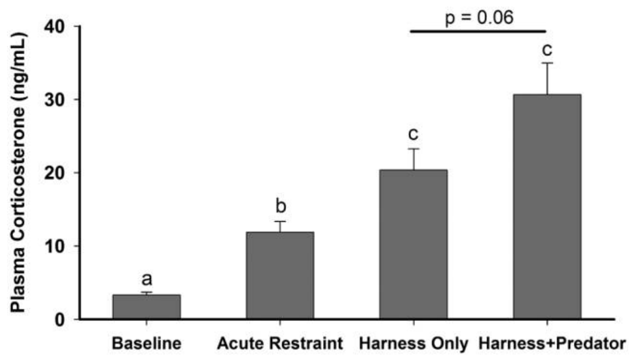

Figure 1. Comparison of baseline samples ($n = 20$) with the effects of the traditional capture-restraint protocol ($n = 20$), harness only ($n = 20$), and acute harness + predator ($n = 18$) exposures on plasma CORT response in nonbreeding rock pigeons *Columba livia*. Different letters represent significant differences among groups.

Buteo jamaicensis ($n = 3$), and northern harrier *Circus cyaneus* ($n = 1$).

Plasma CORT Analysis

To measure CORT, we used a sensitive and specific double antibody ^{125}I RIA (ImmuChem 07-120103; MP Biomedicals, Orangeburg, NY) that was modified for avian plasma (Washburn et al. 2002; Newman et al. 2008). Each plasma sample was measured in duplicate. Briefly, manufacturer's directions were followed except that all reagents were halved and plasma was diluted 1 : 25 (10 μL plasma + 240 μL assay buffer).

We validated this assay for pigeon plasma. Using a plasma pool, we examined recovery of 24 pg of exogenous CORT added to plasma samples ($n = 6$ replicates). CORT concentrations in these samples were compared with concentrations from plasma samples from the same pool without exogenous steroid added ($n = 6$ replicates). Recovery of CORT from pigeon plasma was 106.1% (similar to previous reports on avian plasma; Newman and Soma 2011). We also examined parallelism between the standard curve and serially diluted unextracted plasma and found that the two lines were parallel (ANCOVA, $r = 0.99$, no significant interaction, $F_{1,12} = 1.58$, $P = 0.24$).

Statistical Methods

We used a mixed-design ANOVA to examine the effect of treatment (acute restraint, harness only, harness + predator) on plasma CORT concentrations where subject identity was included as a random effect. The effects of time on the harness before raptor attack, raptor species, raptor contact (direct vs. indirect), body mass, and time in captivity (groups S1–S3) on plasma CORT concentrations were also analyzed using one-way ANOVA. Significant effects were explored using Tukey's honest significant difference tests. All statistical analyses were conducted at $\alpha = 0.05$. CORT concentrations were log transformed to reduce heteroscedasticity, and statistical analyses were performed using the R statistical package (ver. 2.14.1; R Development Core Team 2011). Data are presented as means ± SE.

Results

There was a significant effect of treatment on plasma CORT levels where CORT concentrations were significantly higher than baseline in the acute restraint, harness only, and harness + predator treatments ($F_{3,75} = 97.94$, $P < 0.001$; fig. 1). Importantly, the magnitude of the increase differed across treatments (table 1). The harness only and harness + predator exposure both had significantly higher plasma CORT values than the acute restraint treatment (table 1; fig. 1). Although the harness + predator exposure had the highest mean plasma CORT values, the post hoc P value when compared with the harness-only treatment was 0.06 (table 1; fig. 1).

Whether the attack involved a direct hit on a pigeon or the raptor was captured in a mist net beside the pigeon (see "Methods"), there was no effect on CORT response ($F_{1,10} < 0.001$, $P = 0.99$). Also, the amount of time a pigeon was on the harness before a predator attack (6–125 min) was not related to CORT levels 30 min following the predator attack ($F_{1,17} = 0.01$, $P = 0.92$). Furthermore, there was no evidence that the type of raptor species attacking the pigeon had an effect on plasma CORT levels ($F_{4,10} = 0.18$, $P = 0.94$), although given the rarity of some raptor species, the statistical power was too low to accurately test for a species effect. Across all treatments, variation in pigeon body mass (g) did not have a significant effect on plasma CORT ($F_{1,49} = 0.90$, $P = 0.34$), and individual body mass did not change significantly over the course of the study ($F_{1,11} = 1.53$, $P = 0.24$).

Time in captivity before the 3–4-wk experimentation period had a significant effect on baseline CORT levels ($F_{1,19} = 3.52$, $P = 0.04$) but not on acute stress-induced levels of CORT. Post hoc tests revealed that the third set of individuals (S3) had higher baseline CORT concentrations than either the S1 or S2 sets. In contrast, time spent in captivity did not affect stress-induced levels of CORT (acute restraint: $F_{1,19} = 0.75$, $P =$

Table 1: Post hoc comparison (Tukey's honest significant difference) summary of plasma CORT response across experimental treatments

Treatment comparison	z	P
Baseline:		
Acute restraint	8.84	<.001
Harness only	12.50	<.001
Harness + predator	14.84	<.001
Acute restraint:		
Harness + predator	5.55	<.001
Harness only	3.17	.008
Harness + predator:		
Harness only	2.48	.062

Note. $n = 4$ treatments; $n = 98$ observations.

0.49; harness only: $F_{1,19} = 2.29$, $P = 0.13$; harness + predator: $F_{1,17} = 1.11$, $P = 0.36$).

Discussion

Due to the often lethal nature of acute stressors such as predator attacks, simulating natural acute stressors in free-living vertebrates has been a major challenge in the wild. The capture-restraint protocol was developed to stimulate, in a standardized fashion, maximal activation of the HPA axis in birds and thus represent exposure to acute stress (Wingfield et al. 1992, 1994). Our results demonstrate that exposure of wild-caught birds to tethering in a harness or a natural acute stressor produced a greater CORT response than the capture-restraint protocol (table 1; fig. 1) and suggest that the magnitude of HPA axis activation may depend on the nature of the stimulus. Interestingly, in different contexts, plasticity of the HPA axis has been demonstrated previously. For example, using novel stimuli (e.g., loud music, cage shaking) Rich and Romero (2005) demonstrated that the magnitude of adrenocortical response varies with the type of stressor and is different from 30-min restraint. Further, Cockrem and Silverin (2002) demonstrated that the ability of the individual to escape from a potential predator also affected the stress response.

One criticism of our study design might be that we were simulating an actual predation event and that the CORT levels we measured were not realistic because birds would have died under "normal" circumstances. However, predation attempts on birds are common occurrences, and raptors often hit but fail to capture prey. In addition, in several instances in our study, the raptors did not hit the pigeon but instead flew into a mist nest less than 2 m away. The CORT levels of pigeons in these cases were not significantly different from those in cases where the raptor made contact with the pigeon. Given both these observations, we believe our study design was effective in simulating a natural predation attempt.

Interestingly, although acute restraint significantly increased CORT levels, the increase was not as great as when exposed to the harness-only or harness + predator treatment. Furthermore, CORT levels after exposure to harness + predator tended to be even higher than CORT levels after exposure to harness only (table 1; fig. 1). While it is possible that handling the birds during transfer between aviaries somewhat habituated their response to capture-restraint if compared with naive individuals, our results imply that pigeons have the ability to physiologically differentiate and possibly behaviorally alter their response to a variety of stressful stimuli. Plasticity of the acute stress response has been suggested in previous studies on birds (Canoine et al. 2002; Blas et al. 2007; Clinchy et al. 2011) as well as other taxa (Bateson and Bradshaw 1997; Figueiredo et al. 2003; Berger et al. 2007; Romero et al. 2008). In theory, the ability to alter the adrenocortical response based on the type of stressor or duration of exposure provides a significant advantage for survival in the wild. It allows an organism to activate fully the HPA axis and commit to a "fight-or-flight" response or to suppress this response and avoid deleterious effects associated with prolonged HPA activation (Sapolsky et al. 2000).

It is worth noting that unlike the strict 30-min time line between initial HPA axis activation (baseline blood sample collection) and the second blood sample collection after either restraint in a cloth bag or harness-only treatment, the time an individual was in the harness before a predator attack differed among individuals. Nonetheless, time on the harness before a predator attack was not related to CORT levels 30 min after a predator attack, and the 30 min between activation of the HPA axis by the predator and blood sample collection was consistent with the previous treatments.

Canoine et al. (2002) had a similar goal in evaluating the validity of the capture-restraint protocol; however, they found that the CORT levels of stonechats were similar between the cage-only treatment and the cage + predator treatment. It is possible that the lab-raised captive stonechats used as subjects in their experiment and the blind, tame owl used as the treatment did not accurately represent wild prey and predators in their natural environment. While we also found that our analogous control (harness only) significantly elevated CORT levels compared with baseline, there was a much greater difference between baseline and the authentic predation attempts versus the harness-only control (fig. 1).

Several avian species have been shown to possess variation in plasma CORT levels across seasons, physiological state, gender, and latitude (Wingfield et al. 1992, 1994, 1995; Astheimer et al. 1994; Romero et al. 1998; Boonstra 2004). However, rock pigeons do not alter their stress response or their baseline plasma CORT levels between seasons, nor does the acute stress response vary throughout the year (Romero and Wingfield 2001). Also, recent research has demonstrated a lack of sexual variation in terms of CORT release in rock pigeons (Constantini 2010). Thus, it is unlikely that in our sampling time line sex and seasonality had any confounding effects on our results.

Nonetheless, our results suggest that time in captivity may have had effects on the adrenocortical system (Westerhof et al. 1994; Dickens et al. 2009). Pigeons were housed in either a large aviary or the smaller enclosure for a maximum of 76 d; however, because the pigeons were split into three smaller subject sets of six or seven individuals (S1–S3), the time spent in captivity before the 3–4-wk treatment period differed among the three subject sets ($S1 < S2 < S3$). Although baseline CORT from pigeons in S3 was higher than that in S1 and S2, the stress-induced CORT levels for acute restraint, harness only, and harness + predator were not related to time in captivity. It is possible that short periods of inclement weather in November altered baseline CORT in the third set of individuals or that the social composition of the third set was skewed to males or females, possibly explaining why subjects in the third group may have had elevated baseline CORT levels compared with subjects sampled in late September.

Our data suggest that while the capture-restraint protocol is effective at activating the HPA axis and provides valuable information on the sensitivity of the HPA axis, it does not accurately reflect HPA axis activation in response to a natural life-threatening acute stressor. Avian studies have confirmed that a variety of stressors—such as inclement weather (Wingfield et al. 1983), stress during development (Constantini 2010), and conspecific confrontations (Carere et al. 2003)—also activate the HPA axis and produce a CORT response similar to that of the capture-restraint protocol. In contrast, we present evidence that acute stress from natural predation threat results in higher CORT levels than the capture-restraint protocol. These data suggest it will be important to quantify variation in the stress response within and among species when investigating a wide range of questions ranging from predator-prey dynamics to the long-term effects of climate change and habitat loss on individual physiology.

Acknowledgments

We thank P. Hunter (Ontario Ministry of Natural Resources) and C. Crocker for advice with permits and field site access; G. Prieksaitis for obtaining study subjects; A. Hurst, D. Fowler, C. Fowler, R. Hubert, and R. Chantler for assisting with fieldwork; and N. Bernier for sharing equipment and providing valuable comments on the manuscript. We are grateful to G. S. Betini and D. T. Flockhart for their advice. This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (to D.R.N.).

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